UNVIERSITY OF CALIFORNIA, SAN DIEGO

Sublethal doses of the pesticide imidacloprid alter honey bee (*Apis mellifera*) response threshold and navigation, potentially affecting colony health

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

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in

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by

Daren Eiri

Committee in charge:

Professor James Nieh, Chair
Professor David Holway
Professor David Woodruff

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

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Chair

University of California, San Diego

2011

DEDICATION

To my parents, for encouraging me to study at UC San Diego instead of studying at a local college, for financially assisting my education when I needed it, and supporting my decisions.

To old friends, and new friends, for good memories and your support throughout the years.

EPIGRAPH

"If ... we have concluded that we are being asked to take senseless and frightening risks, then we should no longer accept the counsel of those who tell us that we must fill our world with poisonous chemicals; we should look about and see what other course is open to us"

Rachel Carson, Silent Spring, 1962

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ABSTRACT OF THE THESIS

Sublethal doses of the pesticide imidacloprid alter honey bee (*Apis mellifera*) response threshold and navigation, potentially affecting colony health

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Daren Eiri

Master of Science in Biology

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Professor James Nieh, Chair

Much attention on honey bee declines has focused on the sublethal effects the pesticide, imidacloprid, has on honey bee behavior. How it affects individual foragers and their preference for nectar or their ability to navigate to communicated food sources is unknown. We use the proboscis extension reflex (PER) assay to test an individual's response threshold. Bees treated with the pesticide have higher response thresholds and respond less often to high concentrations of sucrose than control bees. Preliminary trials also show negative effects of a forager's ability to communicate to other nest mates for sweet sugar resources. In a separate experiment, using tunnels to provide optic flow,

preliminary data suggest that bees treated with sublethal doses of imidacloprid travel shorter distances than control bees to a trained location. The increased preference for sweeter sucrose concentrations, reduced communication performance, and navigational inefficiency may contribute to a colony's decline.

Chapter 1:

The pesticide imidacloprid alter response threshold and sucrose preference

Introduction

Although managed honey bee (*Apis mellifera)* colonies have been steadily in decline since the 1940's (Pettis and Delaplane 2010), beekeepers have recently reported unusual colony losses, including hive losses up to 30-90 percent (USDA 2009). Many of these losses are related to a syndrome described as colony collapse disorder (CCD). CCD is being investigated by many researchers and has been attributed, in part, to parasites carrying diseases and pathogens (Gross 2009). However, CCD is only one type of disorder that has contributed to honey bee declines, a problem that has multiple sources (vanEngelsdorp et al. 2008).

For example, pesticides are found inside bee colonies. Entombed pollen (encapsulated with propolis), is associated with colony mortality and contains more pesticide than normal pollen (vanEngelsdorp et al. 2009). Pesticides, even at sublethal doses, may elevate the risk of individual (Toth 2008) and colony death, compromise the honey bee immune system (vanEngelsdorp et al. 2008), and contribute to honey bee population decline. Researchers have therefore become increasingly interested in the role pesticides play in colony health.

Much research has focused on the sublethal effects the pesticide imidacloprid has on honey bee behavior. Imidacloprid, belonging to a class of pesticides called neonicotinoids, is one of the most widely used insecticides in the world (Rortais et al. 2005) and the 6th most commonly used insecticide in California, where many bee-pollinated crops are grown (Johnson et al. 2010b). The pesticide targets nicotinic acetylcholine receptors, blocking signals induced by acetylcholine (Matsuda et al. 2001).

Research has shown that imidacloprid interferes with memory formation in honey bees (Decourtye et al. 2004a). It has also been shown that this pesticide reduces foraging rates (Decourtye et al. 2004b, Ramirez-Romero et al. 2005), increases the time delay in a forager's visit to a food source (Yang et al. 2008) and impairs associative learning in honey bees (Decourtye et al. 2003, Decourtye et al. 2004a, Decourtye et al. 2004b).

The studies investigating the effects imidacloprid has on associative learning in honey bees are done by conditioning the proboscis extension reflex (PER). An extension of the proboscis (tongue) is elicited when stimulating the antennae with sucrose, a natural behavior that occurs when foragers search and find nectar sources.

This commonly used bioassay has also been used to assess a bee's perception of sugar (Page et al. 1998). By stimulating a bee's antennae with an ascending sucrose concentration gradient, its response threshold, or the concentration at which the bee first responds to, can be determined (Marshall 1935). An individual's response threshold can indicate its current foraging task. Bees with low response thresholds typically become pollen and water foragers, with higher response thresholds corresponding to nectar foragers (Page and Fondrk 1995, Pankiw and Page 2000).

This study aims to understand of how sublethal doses of imidacloprid affect the foraging preferences of a honey bee colony -- does the pesticide alter an individual's response threshold? A change in a bee's response threshold may alter their tasks, foraging behavior, and the willingness of foragers to collect nectars with different nectar concentrations, affecting the food flow to the nest. These disruptions within a colony will decrease the probability of surviving the winter season, when most colonies are at their weakest (Seeley 1996). We predicted that imidacloprid would affect the response

threshold of individual foragers and result in a reduction in their willingness to feed on nectar resources.

Previous studies suggest a decrease in hive activity from imidacloprid exposure may be to due the decreased effectiveness of recruitment activity (Decourtye et al. 2004b). To quantify how their willingness to forage decreases, dance behavior of returning bees was recorded in a separate experiment. We hypothesized that bees treated with imidacloprid would reduce the number of dance circuits performed and decrease in the number of visits to the feeder.

Materials and Methods

Response Threshold

This study was conducted at the UC San Diego Biological Field Station between February and October 2010, and February and April 2011. Honey bees were trained to feed at a feeder containing 2.0M sucrose (56% w/w) or pollen at least 1.5 m from the colony entrance. Foragers that landed and fed on the sucrose feeder were identified as nectar foragers and were captured immediately to ensure consistent responsiveness among bees during trials (Mujagic and Erber 2009). Pollen foragers were identified by allowing bees to forage for pollen freely and were also captured individually.

After bees were captured, each bee was fed 7μL of the control (2.0M sucrose), or one of two imidacloprid treatments (0.21 ng/bee, 24 ppb; 2.16 ng/bee, 240 ppb). The oral LD_{50} value ranges reportedly from 3.7 ng to 81 ng per bee (Cole 1990, Nauen et al. 2001).

The bees were harnessed into stainless steel tubes and were placed inside an incubator (30°C, 70% humidity) for one hour to allow pesticide absorption. No further pesticide was provided during the trial. The PER for each individual was tested by stimulating the antennae simultaneously with an ascending sucrose concentration of 0, 0.1, 0.3, 1, 3, 10, 30, 50% (w/w) (Mujagic and Erber 2009, Fig. 1). Sucrose solutions were prepared and contained no pesticide. The duration of stimulation was three seconds, and the inter-trial between testing different concentrations was 2 minutes (Page et al., 1998). For each trial, 7-15 bees were tested, with half of the group treated with the control solution and the other with one of the imidacloprid-laced sucrose solutions. Only proboscis extensions that were fully extended were recorded as a response. Slight

extensions that resulted in little movement of the proboscis were not recorded as a response. A low response threshold corresponds to a high PER response and a high response threshold corresponds to a low PER response.

Free-flying Foraging

To test the effect of imidacloprid on unharnessed bees, bees were trained to a feeder located at the colony entrance with 50% (w/w) sucrose solution in November and December 2010. Bees were uniquely labeled with paint on the thorax, with an observer outside recording foraging behavior at the feeder and an observer near the observation hive recording recruitment behavior. Labeled bees that were consistent in their foraging (number of visits at feeder) and recruitment (number of dance circuits) activity were captured in individual vials. They were then fed 33μL of the control (2.0M sucrose) or treatment (2.16 ng imidacloprid/bee, 48 ppb). Bees were incubated for one hour and then released. The next day, the number of dance circuits per nest visit and number of times a bee accepted the sucrose solution was recorded for 50, 30, 10, and 3% (w/w) sucrose, with 25-minute intervals.

Statistics and analysis

We calculated the mean response threshold by taking the first sucrose concentration at which individual bees responded to for each treatment. Bees that did not respond to any sucrose concentration were not included in the calculation.

Estimation of intake energy of a colony was based on data collected by Seeley (1995). Hives were located in natural environments for 6 days total in May and June

1989, and artificial food sources were provided due to a lack of abundant natural nectar sources. The number of loads for sugar concentrations from 0-60% (w/w) was estimated and converted into joules using standard methodologies (Kearns and Inouye 1993, Bubnik 1995). The estimated reduction of intake energy was based on PER response data collected during this study.

Repeated measures ANOVA was used to compare the PER response of control bees to 0.21 ng and 2.16 ng/bee separately. The treatment was nested within each bee, and was designated as a random effect. Significant effects for the main effect of sucrose concentration or treatment, or the interaction between the two, were further analyzed using the *post-hoc* Tukey HSD analysis. Analysis of response thresholds was done through ANOVA, followed by *post-hoc* Tukey HSD (JMP v9.0 statistical software). Bees that did not elicit a PER response were not included in the analysis.

A

B

Figure 1 – 1: Honey bee in stainless steel apparatus for PER assay. **(A)** When antennae are not stimulated, the proboscis remains inactive until antennae are stimulated with sucrose **(B)** and the bee extends its proboscis.

Results

Response Threshold

For pollen foragers, 209 individuals were tested. Repeated measures ANOVA indicated an insignificant overall effect in PER response due to the treatments used (Fig. 1 – 2a; $F_{2,1659} = 2.27, P = 0.1042$). Between individual bees, there was a significant difference in treatment ($F_{237,1659} = 5.90, P < 0.0001$). A significant difference in PER response from antennae stimulation using different concentrations of sucrose was also found ($F_{7,1659} = 124.31, P < 0.0001$). The interaction between the antennal response from each concentration of sucrose and which treatment the bee received was significant ($F_{14,1659} = 2.47, P = 0.0019$), and a *post-hoc* Tukey HSD analysis showed significant differences in PER response for each sucrose concentration between the control and 2.16 ng imidacloprid-treated bees ($Q = 3.64, P < 0.05$). The highest sucrose concentration at which the control and 0.21 ng imidacloprid-treated bees had significantly different responses was at 10%.

For nectar foragers, 314 individuals were tested. Repeated measures ANOVA indicated a significant overall effect in PER response due to the treatments used (Fig. 1 – 2b; $F_{2,3759} = 6.76, P = 0.0012$). Between individual bees, there was a significant difference in treatment ($F_{537,3759} = 7.47, P < 0.0001$). A significant difference in PER response from antennae stimulation using different concentrations of sucrose was also found ($F_{7,3759} = 162.74, P < 0.0001$). The interaction between the antennal response from each concentration of sucrose and which treatment the bee received was ($F_{14,3759} = 8.85, P < 0.0001$), and a *post-hoc* Tukey HSD analysis showed significant differences in PER

response for each sucrose concentration due to treatment used ($Q = 3.64$, $P < 0.05$). At the highest sucrose concentration (50%), only 54% and 25% of bees that received a dose of 0.21 and 2.16 ng imidacloprid, respectively, responded, compared to the 70% response from control bees.

The response thresholds of imidacloprid-fed bees were higher, compared to the control group. Pollen foragers dosed with 2.16 ng have a significantly higher response threshold than control bees (Fig. 1 – 3a; $F_{2,239} = 15.43$, $P < 0.001$; Tukey HSD, $Q = 2.36$, $P < 0.05$). The mean response threshold was 5.6% for sucrose bees, and 18.1% for bees dosed with 2.16 ng imidacloprid. Nectar foragers that fed the 0.21 ng dose increased in their response thresholds (1 – 3b; $F_{2,243} = 7.06$, $P = 0.001$; Tukey HSD, $Q = 2.35$, $P < 0.05$). The response threshold for bees dosed with 2.16 ng also significantly increased. The mean response threshold for sucrose-treated bees was 11%, and 18.8% and 19.1% for 0.12 and 2.16 ng bees, respectively.

Estimates, based on Seeley (1995), suggest that at the lowest concentration of imidacloprid, there is a 30% overall reduction in intake energy (J) due to reduced PER response. With the highest concentration, a 67% reduction in intake energy is estimated (Fig. 1 – 4).

Free-flying foraging

Preliminary data suggest that imidacloprid affects a bee's recruitment and foraging behavior. There was a significant decrease in dancing activity for 30% concentration (Fig. 1 – 5a; $F_{1,7} = 6.0$, $P = 0.044$) for the corrected number of dance circuits (the change in the number of dance circuits before capture subtracted by the

number of dance circuits after the control or pesticide treatment). There was no significant effect of pesticide at any other concentration. Pesticide-treated bees also accepted 30% sucrose significantly less often than control bees (Fig. 1 – 5b; $F_{1,7} = 6.70$, $P = 0.041$).

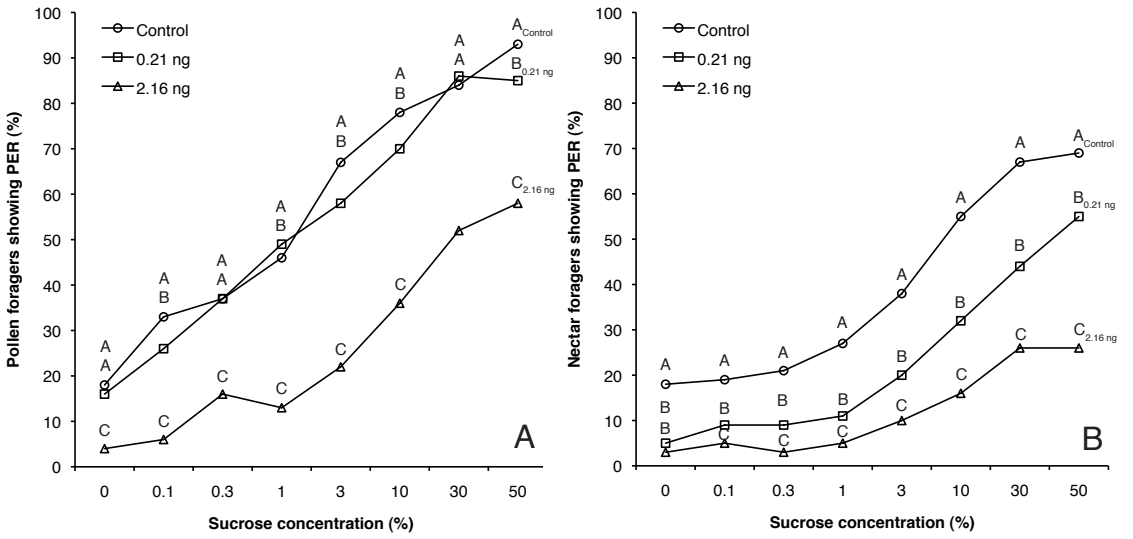

Figure 1 – 2: Percent of bees showing PER response for each sucrose concentration. Different letters indicate significant differences in PER response within each sucrose concentration. (A) Pollen foragers: $n_{Control} = 100$, $n_{0.21\ ng} = 73$, $n_{2.16\ ng} = 67$, with one colony. (B) Nectar foragers: $n_{Control} = 197$, $n_{0.21\ ng} = 184$, $n_{2.16\ ng} = 159$, with two colonies.

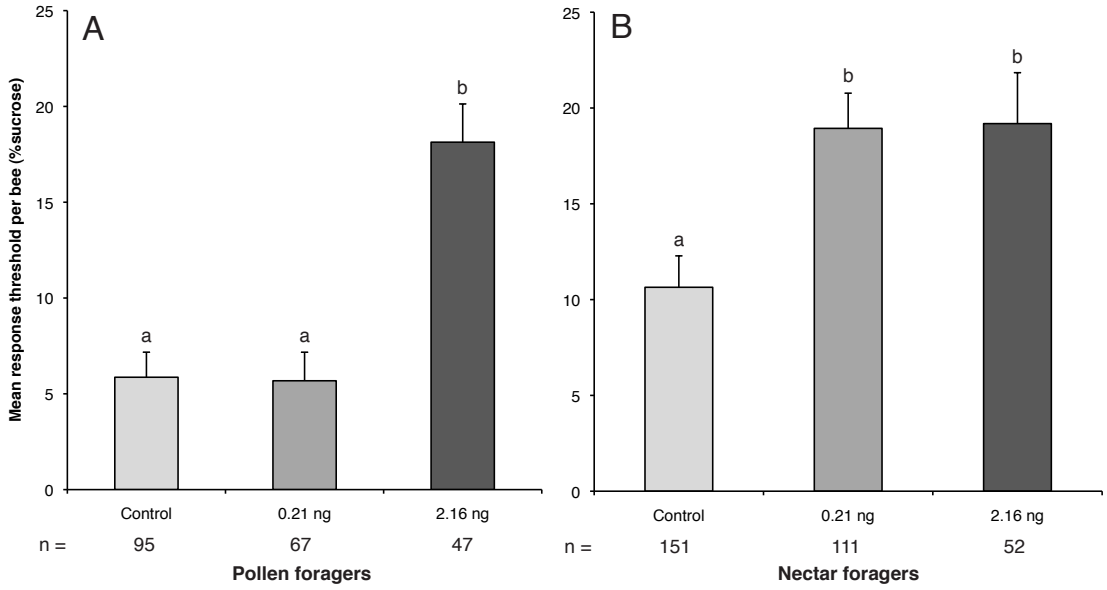

Figure 1 – 3: Mean response thresholds of (A) pollen and (B) nectar foragers. Different letters indicate significant differences.

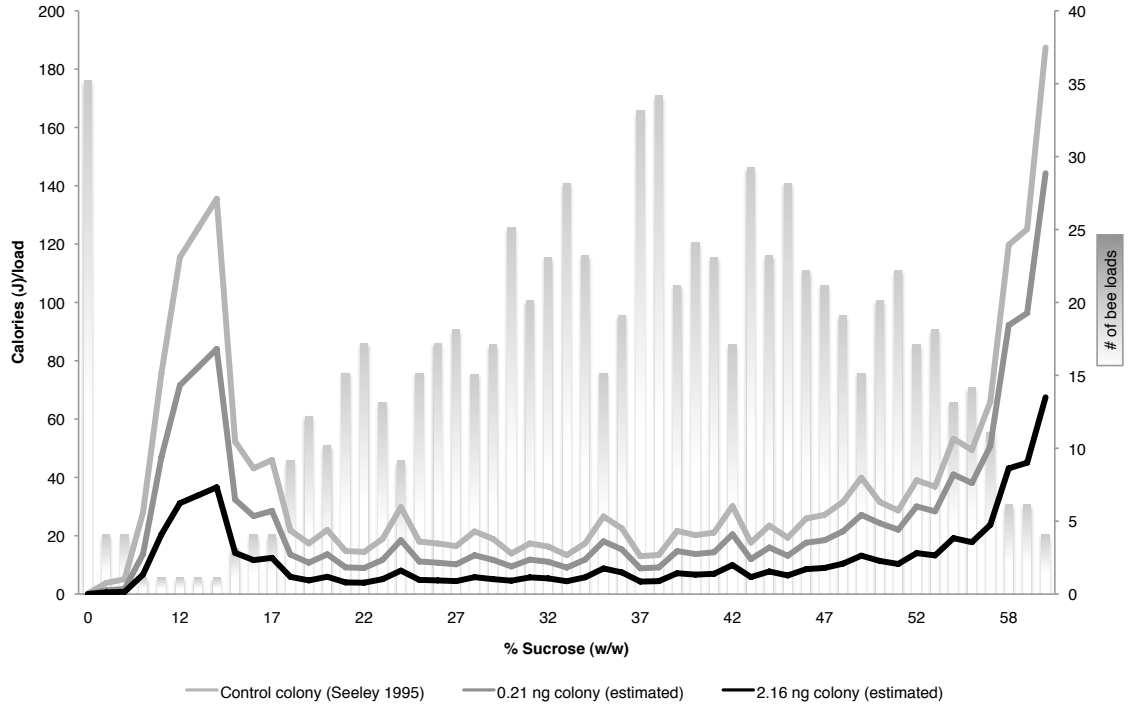

Figure 1 – 4: Caloric intake/load of honey bee foragers, based on Seeley (1995), Figure 2.12.

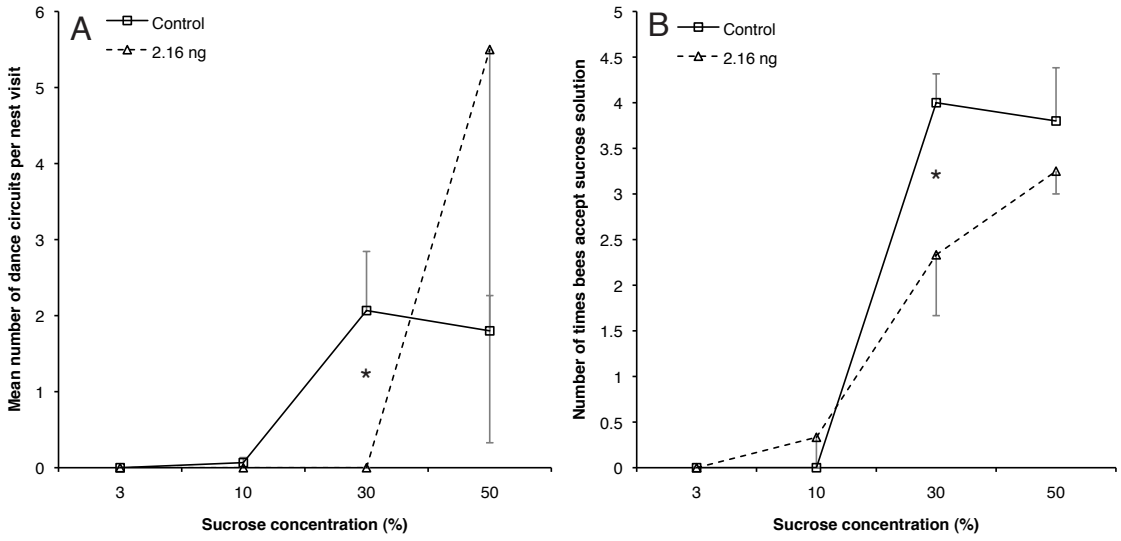

Figure 1 – 5: Communication and foraging activity are reduced when dosed with 2.16 ng of imidacloprid (48 ppb), with SE bars. (A) Dance circuits and (B) number of times fed significantly decreased at 30% sucrose.

Discussion

Bees that were treated with the control treatment exhibited similar response thresholds shown in previous studies (Pankiw and Page 2000, Scheiner et al. 2004). Foragers that were treated with imidacloprid had an increased response threshold, on average responding only at significantly higher sucrose concentrations than the control group. There was also a significant decrease in the response of bees treated with the pesticide to each sucrose concentration. The exception with both of these results is the response threshold of pollen foragers dosed with 0.21 ng. The highest sucrose concentration with a significant difference in pollen foragers' PER response was at 10%. Research is needed to investigate how imidacloprid affects different workers on a molecular and physiological level. We also found a reduction in dance and feeding activity through our semi-field test.

In this study, bees that were fed imidacloprid were given a dose similar to what they may encounter in the field over time. Rortais et al. (2005) estimated that some bees may possibly be exposed to 0.2 ng of imidacloprid after seven days of feeding on pollen collected by other nest mates in crop areas treated with imidacloprid. Additional studies have also reported sublethal effects at similar dose levels (Guez 2001, Decourtye et al. 2004a, Decourtye et al. 2004b, Bonmatin et al. 2005, Yang et al. 2008). However, other studies report no adverse effects on colony health (Schmuck et al. 2001, Nguyen et al. 2009) These conflicting studies may be due to several factors, including colony variation to imidacloprid sensitivity (Suchail et al. 2001), possible exacerbation of other factors such as diseases and starvation (Cresswell 2011), and the selective use of scientific literature (Maxim and van der Sluijs 2007). The use of different methods to quantify

behavioral changes also contributes to these authors arriving at different conclusions (Eiri, in prep.).

The response threshold may be indicative of a bee's perception of sucrose quality. Pankiw et al. (2001) suggests that the modulation of response thresholds may affect foraging decisions for individuals and the colony. A forager's perception of food source profitability has been shown to change the recruitment behavior, with a positive increase of dance probability to increasing sucrose concentrations imbibed (Waddington and Kirchner 1992). According to these results, PER significantly decreased at all sucrose concentrations following treatment at all doses of imidacloprid, compared to the control (with the exception of 0.21 ng for pollen foragers). Extrapolated to natural foraging, this should therefore result in less nectar entering the colony (Fig. 1 - 5). With respect to changes in the minimum concentration of sucrose solution that foragers were willing to feed on, 0.21 ng/bee increased the response threshold by 1.7 fold for nectar foragers. Similarly, at 2.16 ng/bee, response threshold increased by 1.8 fold for nectar foragers. While these results should be used with care, as laboratory studies may not correlate to field conditions, our semi-field experiments still suggest a reduction of high quality nectar intake, limiting a colony's nectar stores needed to survive the winter months (Seeley 1995). It may be suggested that the results of the PER experiment may be due to habituation of bees during sequential sucrose antennal stimulation. However, this is unlikely as previous experiments have shown that sequential and non-sequential treatments look identical (Pankiw and Page 1999). Other authors have also concluded that imidacloprid does not affect their motor activity (Decourtye et al. 2004a).

The largest effect should be on the balance between foraging for nectar and other resources. Bees with low response thresholds typically become pollen and water foragers (Page and Fondrk 1995, Pankiw and Page 2000). Thus, pollen foragers whose response thresholds are pesticide-elevated should switch to become nectar foragers. Honey bee colonies typically hold small reserves of pollen, making them very sensitive to environmental changes (Pernal and Currie 2001). Any further reduction in pollen collection could affect the production of brood and bees, thus affecting the health of the colony. In addition, pollen foragers have low response thresholds and thus are more responsive to important chemical stimuli, such as brood pheromone, which modulates a colony's response to collect pollen (Pankiw et al. 1998). If pollen foragers have increased response thresholds due to pesticide exposure, they may become less responsive to brood pheromone and would result in a pollen deficit for the colony. While there is evidence that suggests response thresholds correlate to sensitivity of brood pheromone, it is unclear if such changes alter pollen foraging behavior (Pankiw et al. 1998, Scheiner et al. 2004). One study reported that chronic feeding of imidacloprid increased pollen foraging activity (Faucon et al. 2005). However, the authors stated that their method for quantifying this variable was semi-quantitative and its results should be used with care. The specific dose each bee fed for each concentration for this study is also unknown.

A reduction of water foragers would also reduce the fitness of the colony. A colony's need for water will vary, depending on the number of brood and the temperature of the colony (Seeley 1995). Water is used to produce liquid food for brood when there are many larvae to feed, and to regulate the temperature inside the colony through

evaporative cooling. A colony that is unable to perform these tasks could be harmful to the health of the colony.

Our preliminary results show that at low doses, recruitment behavior is reduced. Similar effects were observed by Kirchner (1999), noting that bees were trembling at 20 ppb. This provides additional evidence, from studies resulting in reduced foraging activity, that imidacloprid decreases recruitment activity of the hive.

The results of the response threshold and free-flying foraging experiments are similar – bees treated with imidacloprid perceive high concentrations of sucrose as rewarding less often. However, the free-flying foraging experiment tested bees twenty-four hours after treatment with effects still occurring, illustrating possible long-term effects of imidacloprid from a single dose.

These results provide further insight to the continuing debate on the sublethal effects imidacloprid has on this beneficial agricultural insect. With an increased response threshold, nectar foragers may become more selective about what they collect. Further, task allocation within a colony may be negatively altered, reducing the fitness of a colony. While the outcomes from these experiments contradict other published studies, there are additional studies that report similar findings. Further studies should investigate in the field how the pesticide affects individual foragers by recording the flight frequency of pollen foragers before and after treatment, as well as nectar concentrations collected by nectar foragers. Multiple colonies should be used to account for variation of colony health and food storage.

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Chapter 2:

Sublethal effects of imidacloprid on honey bee search distance estimation

Introduction

Modern agriculture has become dependent on the pollinating services provided by honey bees (*Apis mellifera*). In the United States, their value as commercial pollinators is estimated to be $15 billion annually (Morse and Calderone 2000). Although the number of managed honey bee colonies has been in decline since the 1940s, the recent decline of colony losses have been unusually large (Pettis and Delaplane 2010). These loses have been contributed to a poorly understood phenomenon described as colony collapse disorder (CCD). While the cause of CCD is still being elucidated, many other factors are contributing to the decline of honey bee colonies (vanEngelsdorp et al. 2008).

In the past, pesticide exposure from organophosphates and pyrethroids, among others, were thought to be responsible for most honey bee losses from 1966-1979 (Atkins 1992). Once again, the application of pesticides on agricultural crops and the effects pesticide exposure has on honey bee foraging behavior and colony health has become the focus for numerous studies. Pesticides, even at sublethal doses, may elevate the risk of individual (Toth 2008) and colony death, compromise its immune system (vanEngelsdorp et al. 2008), and contribute to the honey bee population decline. It has also been reported that colony mortality is associated with entombed pollen that contains more pesticide than normal pollen (vanEngelsdorp et al. 2009).

Neonicotinoids, a class a pesticides currently used on many bee-pollinated crops, (Johnson et al. 2010b), has become the focus of many studies looking at the sublethal behavioral effects on honey bees. Imidacloprid is the 6th most commonly used neonicotinoid in California (Johnson et al. 2010b), and is one of the most commonly used in the world (Rortais et al. 2005). The pesticide specifically acts upon nicotinic

acetylcholine receptors in the insect brain, which may affect and interfere with nerve impulse transmission and the formation of memories (Matsuda et al. 2001, Decourtye et al. 2004a). Imidacloprid may decrease the spatial precision of waggle dance communication, and may therefore affect a forager's ability to navigate to a communicated food source (Kirchner 1999). Studies show that it also affects foraging rates (Ramirez-Romero et al. 2005, Yang et al. 2008) and associative learning (Decourtye et al. 2004a, Decourtye et al. 2004b).

Much research has shown that honey bees are able to communicate the distance and location of a discovered food source to their nestmates through the "waggle dance" (von Frisch 1993). Recent studies have determined that bees are able to communicate the distance of a food source through a visually-mediated "odometer," using optic flow (Srinivasan et al. 1996, Srinivasan et al. 2000).

Here, we examine how the pesticide, imidacloprid, affects a honey bee forager's ability to navigate. Are bees able to estimate or remember the distance flown to a food source? The alteration of a honey bee forager's "odometer" could lead to incorrect estimates of distances being travelled to and from a communicated food source, risking the individual of potentially becoming lost and never locating the food source or returning to the colony.

Materials and Methods

Two tunnels were constructed in which bees were trained to fly inside and forage for a food source, which varied in sucrose concentration, depending on the number of foragers visiting. Experiments took place at the Biological Field Station at the University of California, San Diego, between August 2009 and November 2009, and again between May 2010 and June 2010. Each tunnel was 7.2 m long, covered with mesh, and lined with black and white stripes (0.048 m wide), oriented perpendicular to the axis of the tunnel to increase the image motion the bee experiences (after Srinivasan et al. 1997). Only one tunnel was used at a time, with the other covered in plastic to avoid any scent marking. Tunnels were located in an open field where bees have no visual landmarks in their field of view inside the tunnel.

Two experiments were conducted to assess how sublethal doses of imidacloprid affect optic flow in honey bees. The first experiment consisted of training bees to the middle of the tunnel (3.60 m) with a width of 0.21 m. Testing of bees occurred in the second, clean tunnel, also with a width of 0.21 m. Remarkably, a flight within a 6 m tunnel with this width is equivalent to a flight of 93 m outdoors (Srinivasan et al. 2000). In the second experiment, bees were trained to forage 200 cm inside a narrow tunnel, measuring 0.12 m wide. Testing bees that were trained inside the 12 cm tunnel occurred in a clean tunnel, measuring 0.21 m wide.

For both experiments, once bees were trained to their desired location, 10-20 bees were marked on their abdomen using Tester's paint and were able to visit the feeder for two hours to allow reinforcement of the distance being travelled. Bees were then captured individually in plastic vials and fed either a control (7μL 2.0M sucrose) or the

imidacloprid treatment (2.16 ng/bee, 240 ppb). They were then held for one hour in an incubator (30°C, 70% humidity) to allow pesticide absorption and then released where they were captured.

Approximately 24 hours later, bees were allowed to forage inside the tunnel again for an hour. To test search distance estimation, the entrance was closed off and any foragers inside the tunnel were removed. The plastic cover was removed from the clean tunnel and only one bee was allowed to enter at a time. We measured the positions of the first three turns following standard methodology (Srinivasan et al. 1997).

The average of the first three turns provided an estimate of the mean search position. Analysis of search turns between control and imidacloprid-treated bees was done using Student's *t*-test (JMP v9.0 statistical software).

Results

Pesticide-treated bees that were trained inside the 0.21 m tunnel and then tested in the same tunnel width, searched for food at significantly shorter distances than control bees (Fig. 2 – 1; *t*-test: $t = 2.02$, df = 105, $P = 0.0456$). The average distance of the first three turns for bees treated with 10x imidacloprid was 3.70 m, compared to the control group, with an average distance of 4.1 m. However, there was no difference between treated and untreated bees that were trained in the 0.12 m tunnel and searched for the food source inside the wider, 0.21 m wide tunnel (*t*-test: $t = 0.46$, df = 69, $P = 0.32$).

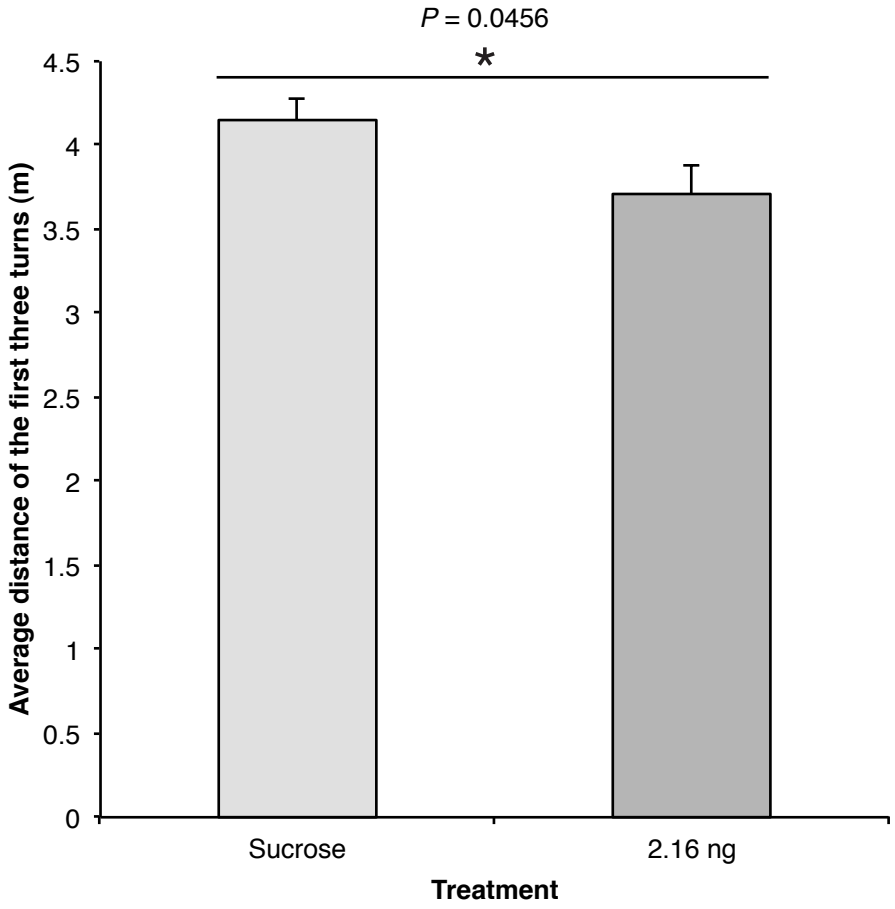

Fig. 2 – 1: Bees treated with imidacloprid search for food at shorter distances than sucrose-treated bees.

Discussion

For the first experiment with the same tunnel widths, results indicate that bees treated with imidacloprid search at shorter distances, on average, than the control group. There was no difference in the average search distance estimation between treated and untreated bees that were tested in the 21 cm tunnel after being trained to forage inside the 12 cm tunnel.

With bees receiving more optic flow in the narrow, 12 cm tunnel, bees should travel farther inside in the wider 21 cm tunnel, where less optic flow is visualized. Bees are in fact travelling farther inside the wider tunnel, but there is no effect on search distance estimation due to the treatment. While the mean search distance estimation is not significantly different between the two groups, pesticide-treated bees are similarly searching closer to the entrance as in the first experiment. Bees are trained to feed at 200 cm inside the narrow tunnel, and on average, searched at 266 and 272 cm for imidacloprid and sucrose-treated bees, respectively.

It is possible that data collection methods used for these trials were not sufficient to capture an individual's true searching behavior, reducing statistical power. Although standard methodologies were used (Srinivasan et al. 1997), a forager entering inside the clean test tunnel would not exhibit searching behavior immediately. The first few turns of a forager were near the entrance of the tunnel, far from the distance where they were trained to search for food. Their tendency of turning at the entrance of the tunnel may be due to the unfamiliarity of foraging inside a clean, unused tunnel, where pheromones may not be present (von Frisch 1993). In addition to this possible confusion, based on personal observations, the closure of the tunnel entrance once a forager flew inside (to exclude

other foragers from entering) also elicited behaviors of making multiple U-turns near the entrance. Thus, an assumption was made – search distance estimation was not recorded until the forager travelled further inside the tunnel, where it was expected to search for the food source.

When testing a bee's searching behavior in the same tunnel width as the training tunnel, the mean search distance is slightly significantly less for bees treated with the pesticide. Incorrectly estimating the distance travelled to a communicated food source could result in bees becoming lost, or expending more energy per foraging trip. Bees that are treated with sublethal doses of pesticide may potentially bring in less nectar to the colony (Eiri and Nieh 2010). The result of bees becoming less efficient foragers may further increase the reduction of nectar availability of a colony, ultimately reducing the fitness of the colony.

These experiments will be repeated again in the future. New criteria will be used to measure a forager's searching behavior, based on previous studies (Eckles, in prep.). Only bees with a steady fight speed with a consistent flight path, and little or no reaction to tunnel closing after bee enters will be used as subjects.

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Chapter 3:

A review of environmental exposure and sublethal effects of imidacloprid on honey bees (*Apis mellifera*)

Introduction

The use of chemical insecticides in agriculture has increased dramatically in the past few decades. In particular, neonicotinoids have become the fastest-growing class of pesticides and now represent 17% of the global insecticide market (Jeschke and Nauen 2008). The growth and popularity of this class of insecticides is due to its specificity and relative safety compared to less targeted, non-specific insecticides. Neonicotinoids act upon the nicotinic acetylcholine receptors in insects and interfere with nerve impulse transmission (Matsuda et al. 2001). As a systemic insecticide, neonicotinoids are taken up by all tissues of the developing plant. They are commonly used as a seed dressing or applied directly into soil in order to reduce the risk of killing non-target organisms that can be more exposed to pesticide if it were sprayed (Croft 1990, Cresswell 2011).

Although systemic insecticides have reduced exposure to non-target organisms, there are still concerns over neonicotinoids found in pollen and nectar, which are collected by insects such as bees. The pesticide, imidacloprid, has received much attention due to the possible relationship between the decline of honey bees and the use of the pesticides at nearby crops, especially in Europe (Bonmatin et al. 2003). Despite this, imidacloprid was the sixth most commonly used insecticide in California (Johnson et al. 2010b). Much research has focused on the sublethal effects of imidacloprid on honey bees and how much imidacloprid is absorbed in treated plants (Maus et al. 2003, Desneux et al. 2006). However, there are no recent reviews of the latest sublethal studies in honey bees (*Apis mellifera*), how these effects relate to concentrations of imidacloprid found in plants, the typical doses that a bee may receive, or what is found in bee-stored pollen and nectar inside the nest.

The goals of this paper are to synthesize this information by addressing (1) the sources of imidacloprid in the environment, (2) how sublethal doses affect the behavior of honey bees, and (3) how bees may be exposed to imidacloprid. Specifically, how does imidacloprid affect foraging activity, communication, learning, and motivation at concentrations found in the field? I will also discuss the consequences of decreased efficiency in honey bee foraging due to imidacloprid exposure and make recommendations for future studies and research directions.

Environmental Exposure

Flower Pollen

Studies that analyze the amount of imidacloprid found in treated plants (e.g. maize, sunflower) vary greatly in their methods, which have contributed to a wide range of reported imidacloprid concentrations (Table 1). This may be due, in part, to how samples are prepared or collected and the methods of detecting imidacloprid, resulting in the variation of limit of detection (LOD). Studies that did not report their residue concentrations in mg kg^{-1} are converted, wherever possible, in this review.

Scientists employed by Bayer Corporation, the manufacturer of imidacloprid, concluded that there were no detectable residues in field-tested flower pollen or nectar in imidacloprid seed-treated plants (Schmuck et al. 2001). However, their LOD was, in some cases, a magnitude greater than reported values found in independent studies (Table 1(Bonmatin et al. 2003, Laurent and Rathahao 2003). These studies reported that in flower pollen, concentrations of imidacloprid ranged from 0.001-0.036 mg kg^{-1}. The upper end of this range may be unusually high, however, due to the 30% higher-than-

recommended application rate for seed treatment in one of the studies (Laurent and Rathahao 2003). The author did not state the reason for this increased application rate.

Bee-collected pollen

The average of mean residue concentration of imidacloprid found in bee-collected pollen, using pollen traps at the entrance of the colonies, is 0.0018 mg kg^{-1}, which is within the range of concentrations found in flower pollen (Table 1). This excludes an American study done by Mullin et al. (2010) because the LOD for imidacloprid metabolites was 0.025 mg kg^{-1}, which is higher than most studies. The mean also excludes a Spanish study done by Bernal et al. (2010), which did not detect any imidacloprid or its metabolites above 0.0004 mg kg^{-1}. Interestingly, a similar three-year study conducted in France found imidacloprid to be the most commonly present insecticide in samples of pollen loads, honey, and honey bees (Chauzat et al. 2009). Why this study found a large proportion of samples contaminated with imidacloprid and Bernal et al. and Mullin et al. found few or none is unclear.

It is possible that the method used to collect pollen samples has an effect. Bernal et al. and Mullin et al. collected pollen from pollen cells inside each colony. The concentration of pesticide in such pollen may be less than pollen collected from bee traps if the cell also contains pollen from uncontaminated sources (Cresswell 2011). The French study used a different technique – collecting pollen from bees directly using pollen traps located outside the colony entrance. Using this method, the authors detected a large proportion of imidacloprid present in samples. Despite the difference in methods, both studies are useful in understanding how residues of imidacloprid are found in pollen

carried on the legs of bees (contact toxicity) and what developing bees are feeding on (oral toxicity).

If the exposure of an individual pollen forager to imidacloprid is of interest, then the pollen trap method is useful. If one is concerned with the level of pesticide ingested by bees inside the nest, particularly larvae, then analyzing pollen from pollen cells is appropriate. One study concluded that treatment colonies filled with combs known to have pesticide residues present, including imidacloprid, delayed development of brood and decreased adult bee life by four days (Wu et al. 2011). Additional studies focusing on how imidacloprid affects larval development using concentrations of the insecticide at concentrations found in flower pollen would be beneficial. Such concentrations may give a more accurate estimate of maximal honey bee exposure than using concentrations found in bee-collected pollen, because of the possibility of contaminated pollen mixing with uncontaminated pollen (Rortais et al. 2005).

Nectar and honey

One study analyzed residues in nectar in the field, detecting no residues above 0.015 mg kg^{-1} (Schmuck et al. 2001). However, this study's LOD is unusually high, given that other studies have found positive results of imidacloprid in honey at significantly lower levels (Chauzat et al. 2009, Nguyen et al. 2009). To date, only one study has looked at the foraging behavior of honey bees using imidacloprid concentrations relevant to what is found in honey or nectar residues (Table 1; < 0.0007 mg kg^{-1}). This study found no negative effects on their behavior, adult population level, capped brood area, frames of brood after wintering, and other parameters (Faucon et al. 2005).

More studies on imidacloprid residues in floral nectar would be useful. These studies would give a better estimate on the amount of imidacloprid a bee ingests over her lifetime, allowing future studies to use a dose or concentration of the pesticide that is more reflective of the possible maximal exposure.

Water

Water is another resource collected by honey bees, and few studies examining the effect of pesticide residues in water collected by honey bees exist. Water is used to produce liquid food for brood, and to regulate the temperature inside the colony through evaporative cooling (Seeley 1995). The US EPA reviewed imidacloprid and reported that it is highly soluble in water and mobile (USEPA 1993). If soils are irrigated with water containing imidacloprid (a method of applying this pesticide), water-collecting bees may be exposed. One study suggests that imidacloprid residues exist in rural, urban and suburban sources where bees forage (Johnson et al. 2010a). Concentrations of imidacloprid in these water sources are within similar ranges of nectar and pollen. Future studies focusing on the foraging behavior of honey bees on water sources containing imidacloprid would be useful. The presence of standing water near bee-visited crops and an analysis of residues found in those sources would also be beneficial.

Sublethal Effects

Foraging Activity

Numerous studies have shown that imidacloprid affects honey bee foraging activity at a trained feeder with sublethal concentration. These studies chronically fed

honey bees to a sucrose feeder laced with imidacloprid between 0.002- 1.0 mg kg^{-1}, which is within range of the mean concentration of imidacloprid found in various matrices (Table 1). Decourtye et al. (2004b) noted that the number of visits to a feeder is reduced after switching the food source from sucrose-only to sucrose laced with imidacloprid. Even after removing the imidacloprid solution, feeding activity did not return to its original level. Other studies, similarly designed, also show similar results. Yang et al. reported a time delay in an individual's foraging activity at a feeder containing imidacloprid: increasing the concentration of imidacloprid further emphasized this behavior (Yang et al. 2008). Another study showed that bees who fed on imidacloprid did not return to a trained location until 24 hours after their release (Bortolotti et al. 2003).

Schmuck et al. (2001) found no difference in feeder activity with imidacloprid concentrations up to 0.02 mg kg^{-1} in trials with a bee colony inside a tunnel enclosure. The number of honey bees on the tunnel roof were counted, in addition to those feeding on the feeder, to determine if imidacloprid caused disorientation or repelled bees from feeding. However, it is unclear how feeding activity changed on the feeder itself because of the method used to count honey bees, and the proportion of bees on the feeder and on the tunnel walls is not reported.

Faucon et al. (2005) found that bee activity at the colony did not differ between colonies fed imidacloprid or sucrose solution. Their finding contradicts the results of Decourtye et al. (2004b). Two main differences in their methods may have contributed to their different conclusions: (1) how bee activity was measured and (2) how bees were orally fed imidacloprid. In Faucon et al., the activity of bees outside the hive was

measured by visually counting bees entering the colony for one minute per day, for each colony in the apiary. Bees were fed imidacloprid-laced sucrose inside the colony using a crown board feeder. Decourtye et al. used an electronic bee counter (BeeSCAN) that counted the number of individuals entering and leaving the hive for one to two hours per trial day. Instead of placing the feeder at the colony, bees were trained to feed 1.5 m away from the colony. When the feeder was switched from sucrose to sucrose laced with imidacloprid, foraging activity at the feeder decreased. Consequently, the number of bees entering and leaving decreased. Since bees were trained to a specific location that was switched with the pesticide solution, Decourtye et al. were able to detect a change in bee activity. In Faucon et al., bees that were feeding directly from the feeder, and their behaviors, were not observed. Bees that were foraging for natural sources (those that were observed), and thus not directly feeding on the provided pesticide solution, must have been either minimally or unaffected for the authors to notice any visual difference in bee activity. Hive activity also fluctuates during the day. Thus, differences in the amount of time spent observing the activity, and at what time of day, may have resulted in different conclusions (Seeley 1995).

Future studies should investigate how pollen foraging changes because of imidacloprid exposure. The collection of pollen is critical for the proper brood development in the colony (Seeley 1995). Faucon et al.'s study provided results suggesting that pollen foraging activity increases due to imidacloprid, but the author notes that the results should be used with care due to its semi-quantitative measurement.

Communication

Communication plays a critical role in the efficiency and fitness of a honey bee colony. A decrease in communication efficiency may result in less pollen or nectar being collected, resources that are important in developing brood and surviving the winter (Seeley 1995). A waggle dance or dance circuit is performed by honey bees coming back from a food source. This activity recruits nestmates to forage for the food source by communicating its distance and direction (von Frisch 1993). Unfortunately, few published studies have examined the effects of imidacloprid on honey bee communication. However, one study showed chronically feeding bees at 0.020 mg kg^{-1} imidacloprid caused the foragers to begin trembling (Kirchner 1999). This may decrease foraging because foragers tremble dance, but do not waggle dance to recruit nestmates. The details of this study are unknown, but other studies that have reported a decrease in foraging activity at a food source also suggest that this is due to the reduced waggle dancing inside the hive (Decourtye et al. 2004b). Future studies should determine whether sublethal exposure to imidacloprid significantly affects honey bee recruitment communication.

Learning and Memory

The proboscis extension reflex (PER) assay has been previously used to assess how pesticides affect honey bee learning (Bitterman et al. 1983, Taylor et al. 1987, Sandoz et al. 1995). In this assay, bees are conditioned to respond to a conditioned stimulus (i.e., an odor) when simultaneously presented with an unconditioned stimulus

(i.e., sugar solution). The unconditioned stimulus elicits the unconditioned response (the bee extends its proboscis and feeds on the sugar solution). With learning, the conditioned stimulus can elicit the unconditioned stimulus on its own. This laboratory assay can be used to study how bee learning of natural nectar and pollen sources is affected by pesticides (Pham-Delégue et al. 2002).

In Decourtye et al. (2003), colonies were fed imidacloprid-laced solution until eggs developed into adults and were 14-15 days old. Thus, bees were fed contaminated solution throughout their development process. Learning performance decreased for bees treated with imidacloprid, which were more sensitive (affected by lower doses) in the summer time. During winter, reduced learning occurred at 0.048 mg kg^{-1}, while in the summer, are affected at 0.012 mg kg^{-1}. This showed that bees are more sensitive to small amounts of imidacloprid during the summer, when they are most actively foraging. The authors reasoned that a poor pollen diet, consisting of stored pollen and a narrow range of natural pollen sources during the winter months, contributed to their increased summer sensitivity.

Additional studies by Decourtye et al. (2004a) showed that after 15 minutes or one hour, a single high dose of imidacloprid (12 ng/bee) decreased medium-term olfactory memory acquisition. Since short-term (3 min) and long-term (24 hours) memory was not affected, the author concluded that memory formation was impaired, rather than retrieval. Extrapolated to natural foraging, this suggests that bees may be less able to learn the route to a food source, or possibly its location. However, these effects were found at doses of 12 ng per bee, which is not a level that bees could be exposed to in a short period from pollen or nectar collected from plants treated with typical levels of

imidacloprid (Rortais et al. 2005). The long terms effects of the accumulation of insecticides in a colony and the repeated feeding of minute doses are unclear.

Imidacloprid has been shown to affect *A. mellifera* subspecies differently (Suchail et al. 2000). *A. m. caucasica* is more sensitive to imidacloprid than *A. m. mellifera* when comparing their LD_{50} contact toxicity. Investigating how different *Apis* species are affected, particularly in how sublethal doses affect their memory and learning, would be important. *Apis cerana,* an important pollinator in Asian agriculture that is also sensitive to pesticide exposure, has not been studied as extensively as *A. mellifera* and should receive more attention in future toxicity studies.(Suchail et al. 2000)

Discussion

The lowest concentration of imidacloprid that caused an observable effect in a sublethal study is 0.006 mg kg^{-1} (Colin et al. 2004). Relatively few studies found samples with a mean imidacloprid residue concentration at 0.006 mg kg^{-1} or more (Table 1). Furthermore, in most residue studies that detected the presence of imidacloprid, only a small number of residue samples were positive. The LOD for most studies were also low enough to detect minute concentrations of the insecticide. However, the amount of imidacloprid received by each bee during a single collecting trip (the dosage) is highly relevant and should be considered, particularly because dosage is a crucial piece of information in toxicity research. Studies should also include data on pesticide dose per bee. Many studies that chronically feed bees imidacloprid-laced sucrose solution lack the specific dose that each bee received, and within the studies included in this review, few estimate the total dose each bee consumed during trials. Acute toxicity studies range from 0.1-12 ng per bee (Guez et al. 2003, Decourtye et al. 2004a, Decourtye et al. 2004b). This range falls within the maximal exposure in Rortais et al., where bees could be exposed to 0.3-4.3 ng per bee if colonies were fully dependent on crops that were treated with imidacloprid for pollen and nectar resources (Rortais et al. 2005). Remarkably, in seven days, an individual nectar forager may ingest 1.1-4.3 ng of imidacloprid. How specific doses affect bee behavior, and how it changes as it accumulates in a bee's body will give a more detailed understanding, at an individual level, on the sublethal effects of imidacloprid.

How long-term exposure to small doses of imidacloprid affect bee larval development and its adult life should still be considered. Few studies address this issue

(Decourtye et al. 2003, Wu et al. 2011). Since larvae develop while suspended in their food sources, the contamination of those food sources may affect larvae differently due to this contact, even at very low doses of imidacloprid (Haydak 1970, Rortais et al. 2005).

Mounting evidence in short-term studies report that bee foraging and learning are affected when exposed to imidacloprid, which may result in a colony collecting fewer resources that are required for its fitness. Future investigations which correlate how honey bee behavior changes over months, and the maximal concentrations found in the field may provide additional evidence of decreased colony performance due to pesticide exposure. A three-year study found no statistical relationship between colony health (brood abundance, honey and pollen stores, and colony mortality) and imidacloprid residues present inside the colony (Chauzat et al. 2009). Unfortunately, this study does not observe bee behavior in detail, and it did not actively treat colonies with imidacloprid treated solutions as in sublethal studies. Thus, it would be beneficial to examine the same parameters of colony health, with greater emphasis on studying bee behavior while being fed imidacloprid. One study designed in this method found no effects of imidacloprid on colony activity, amount of honey stores, comb area, and other colony-wide parameters over 39 days of chronic exposure (Schmuck et al. 2001), but it has received criticism for its significant lack of scientific rigor (Maxim and van der Sluijs 2007). Finally, there is growing awareness that pollinator declines affect not only honey bees (Kremen et al. 2002). Research on the sublethal effects of imidacloprid on other bees, particularly native bees, would help obtain a better understanding of the overall effect of imidacloprid on bee pollinators.

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Table 3 – 1: Residue concentrations of imidacloprid in various matrices from recent publications.

Reference	Matrix	Range of concentration (mg kg^{-1})	Mean concentration (mg kg^{-1})	Total sample size	Positive samples
Shmuck 2001	Flower pollen (greenhouse)	0.0033	NR	22	NR
Shmuck 2001	Flower pollen (field)	ND, LOD 0.0015	ND	NR	NR
Bonmatin 2003	Flower pollen (field)	0.001-0.011	0.003	24	~14
Laurent 2003	Flower pollen (field)	0.0005-0.036	0.013	13	NR
Bonmatin 2005	Bee pollen	<0.0001-<0.010	0.0006	11	11
Chauzat 2006	Bee pollen	0.0002-0.0057	0.0012	81	40
Chauzat 2006	Bee pollen	0.001-0.0057	0.0046	81	11
Chauzat 2009	Bee pollen	NR	0.0009	185	106
Bernal 2010	Bee pollen	ND, LOD 0.0004	ND	845	0
Mullen 2010	Bee Pollen (metabolites)	0.152-0.554	0.201	350	2
Shmuck 2001	Nectar (greenhouse)	0.0019	NR	22	NR
Shmuck 2001	Nectar (field)	ND, LOD 0.015	ND	NR	NR
Chauzat 2009	Honey	NR	0.0007	239	29.7
Nguyen 2009	Honey	0.00005-0.0005	0.0003	48	4
Nguyen 2009	Beeswax	ND, LOD 0.0005	ND	ND	ND
Mullin 2010	Beeswax	0.0024-0.0136	0.002	208	2
Johnson 2010	Water (urban, suburban)	0.007-0.131	0.062	108	9
Johnson 2010	Water (urban, suburban)	0.0002-0.0003	0.0001	108	13
Shmuck 2001	Treated soil, 1-2 years later	< 0.006-0.0178	NR	20	NR
Bonmatin 2003	Treated soil, 1-2 years after	> 0.001	0.006	33	~26
Bonmatin 2003	Treated soil	NR	0.012	74	NR

ND = Not detected
LOD = Limit of detection
NR = Not reported
* Mean concentration of imidacloprid residue detected is greater than the lowest concentration of imidacloprid used in a study (Colin et al. 2004)

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