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Neonicotinoid-contaminated diet causes behavior changes in forager honey bees (*Apis mellifera*) that may reduce colony survival during late fall

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ORIGINAL RESEARCH ARTICLE

Neonicotinoid-contaminated diet causes behavior changes in forager honey bees (*Apis mellifera*) that may reduce colony survival during late fall

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Neonicotinoid insecticides are often detected in pollen and nectar, which are the main food resources for honey bees. Sub-lethal and chronic exposure to these insecticides may have a prolonged effect on honey bees who are solely dependent on the stored food inside of the hive during cold weather. Our study investigated the effects of sub-lethal concentrations of imidacloprid on late fall forager honey bees' behavior by accessing their activity levels and walking performance after being fed *ad libitum* with six different concentrations (2—125 μg/kg) of imidacloprid-dosed syrup for up to 48 h in the laboratory. Numbers of honey bees that walked after being released into a UV light illuminated tunnel decreased significantly as dosages of neonicotinoid in their diet increased. However, their walking speeds were not significantly affected by imidacloprid. The behavioral changes we observed in honey bees chronically exposed to neonicotinoid via diet could negatively affect individual honey bee performance of hive duties and consequently, colony survival during cold weather.

Keywords: imidacloprid; *Apis mellifera*; behavior; contaminated diet; cold weather

Introduction

Honey bees (*Apis mellifera*) are extremely sensitive to pesticide stressors, especially during cold weather (Kiljanek et al., 2016; Straub et al., 2015), because they possess a limited number of detoxification enzymes compared to other insects (Claudianos et al., 2006). Recently, concern has been raised over the possible effects of neonicotinoid pesticides on the health of honey bee colonies during late fall and winter (Kiljanek et al., 2016; Lu et al., 2014; Woodcock et al., 2017). Neonicotinoid insecticides have similar chemical structures to nicotine, which causes both behavioral stimulation and anxiety in humans and animals (Picciotto et al., 2002). Like nicotine, neonicotinoids may be addictive because honey bees have been shown to prefer foods containing imidacloprid even though they cannot taste neonicotinoids; they may be attracted to the stimulant effects (Kessler et al., 2015). However, exposure to sub-lethal levels of neonicotinoids can disrupt honey bee behavior (Medrzycki et al., 2003), impair their social activities (Gill et al., 2012; Laurino et al., 2011), learning ability (Palmer et al., 2013), walking speed (Charreton et al., 2015; Tosi & Nieh, 2017), navigation (Fischer et al., 2014), thermoregulation (Crall et al., 2018; Tosi et al., 2016), motor activity, and attraction to light (Lambin et al., 2001).

Imidacloprid, a neonicotinoid insecticide detected in nectar (e.g. Dively & Kamel 2012; Heller et al., 2020; Pohorecka et al., 2012), pollen (e.g. El-Hady et al., 2019; Jiang et al., 2018; Mullin et al., 2010), honey (e.g. Van Der Zee et al., 2015), honey bees (e.g. Hadi et al., 2019; Tsvetkov et al., 2017), and beebread (e.g. Gooley et al., 2018; Van Der Zee et al., 2015), is commonly applied as a seed coating to agricultural crops for systemic uptake. As temperatures cool in the fall and available floral resource decreases, honey bee colonies could be exposed to imidacloprid chronically if stored food is contaminated; exposure during winter has been estimated as 1.1 (range of 0.2 – 6.2) ng/bee via stored honey and 4.2 ng/bee via stored pollen (Codling et al., 2016). Colonies subjected to chronic exposure to neonicotinoids showed increased honey bee mortality and symptoms of queen-lessness (Tsvetkov et al., 2017), and those exposed in late fall showed decreased overwintering survival in the field (Lu et al., 2014; Wood et al., 2019).This could be due to a disruption of individual honey bee behavior leading to decreased colony fitness going into the winter because any stressors that affect behavior and health at that time could potentially inhibit individual bees' activity and negatively affect colony survival (Genersch et al., 2010; Straub et al., 2015).

Few studies have examined the effect of the neonicotinoid on forager honey bee's behavioral performance (i.e. activity levels, walking behavior, and walking speed) at a cool ambient temperature where their sensitivity to pesticides is potentially increased (Wood et al., 2019). We collected late fall honey bee foragers and fed them with syrup that was dosed with a range of sub-

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lethal concentrations of imidacloprid under laboratory conditions to study changes to their activity levels and walking performance at cool ambient temperature (less than 25 °C). Our study will increase the understanding of the relationship between neonicotinoid-contaminated food and honey bee colony fitness during late fall.

Materials and methods

Honey bee hives and collection

We collected the forager honey bees on 29 October 2018 from four healthy hives that descended from a locally captured swarm. The queens in each hive were sisters and the hives were established in April 2017. Each of our experimental hives consisted of two ten-frame deep boxes and two ten-frame honey super boxes. The hives were located 2.6 km NW of Southern Illinois University Carbondale, Illinois, United States. We captured the returning honey bees at the entrance of each hive and kept them individually in a clear cup-shaped cage (approximately 5.5 cm in diameter and 4 cm in height) with a removable base, multiple ventilation holes, and one feeding device (Williams et al., 2013). Each individual honey bee was fed with untreated commercial liquid bee feed (henceforth syrup; sucrose solution with spearmint and lemongrass oils) (Harvest Lane Honey, Utah, United States) immediately after being caged and maintained in a semi-controlled cool room (temperature 20 °C, relative humidity 50%, natural lights) for 24 h to allow acclimation prior to neonicotinoid treatments. Each treatment group consisted of 15 individually maintained honey bees.

Honey bee exposure to dietary neonicotinoid

Honey bee laboratory maintenance and dosing procedures followed the standard methods for adult *A. mellifera* research described by Williams et al. (2013), Medrzyckiet al. (2013), and Suchail et al. (2004). Imidacloprid standard was obtained as a solution in acetonitrile (10 μg/mL, AccuStandards, CT, USA) and added into the syrup at the following concentrations: 125, 50, 20, 10, 5, 2, and 0 (control) μg/L (i.e.87.6, 35.4, 14.1, 6.94, 3.53, 1.43, and 0 μg/kg), which spans both the environmentally-realistic concentrations of imidacloprid in nectar (4.7 – 16 μg/kg, Dively & Kamel 2012) and pollen (1.61 – 206 μg/kg, Jiang et al., 2018; Mullin et al., 2010). Two were within the range of reported 24 h oral LD50 in honey bees (25.3 – 540 μg/kg, Fairbrother et al., 2014). We acknowledge that in the field honey bee exposure to imidacloprid is dependent upon location, landscape composition, floral diversity and availability, time of year, and honey bee choice of forage; thus, honey bees can feasibility be exposed from zero up to the levels of neonicotinoids reported in nectar and pollen and possibly beyond. The final concentration of acetonitrile in the syrup solutions for the control and treatment groups was 0.1% (v/v). We randomly assigned the individual honey bees to different treatment groups. After the initial 24 h of acclimation in cages with untreated syrup, we provided each honey bee with its assigned imidacloprid-dosed syrup treatment up to 48 h.

Activity levels

We performed the experiment in October 2018. All experiments were operated blinded (the observer, ZCG, did not know whether the honey bees received contaminated syrup or not). We started to observe and record the caged honey bees' activities after they were fed with imidacloprid-dosed syrup for 24 h *ad libitum*. Each individual honey bee's activity level was recorded at the beginning of every hour for a 10-h period (total of 10 observation time points) from USCST 8:30 to 17:30 (sunrise was at 7:21 and sunset was at 17:59). Four different behavioral categories related to honey bees' activities were modified from Medrzycki et al. (2013). Activity levels were given scores from 1 to 4: 1-rest (no movement); 2-walk (constantly walking without any wing movement); 3-buzz (wing is moving but not walking); 4-excited (buzzing and walking). An activity score was recorded for each individual honey bee at each of the 10 observation time points. Each honey bee's activity scores were tallied for a total activity level. We used Spearman's rank-order correlation to assess the relationship between imidacloprid concentrations and the honey bees' total activity levels (IBM SPSS statistic 25, IBM Corporation, Armonk, NY, USA). Each honey bee was continuously fed with the assigned imidacloprid-dosed syrup during the behavioral observation experiment.

Walking performance

We measured honey bees' walking performance after being fed with different concentrations of imidacloprid-dosed syrup for 48 h *ad libitum* in a tunnel (50 cm high, 50 cm wide, and 230 cm long; Figure 1) (Ikeno et al., 2013). Three sides of the tunnel were constructed of medium-density-fiberboard (painted white) and the remaining side of the tunnel was constructed of Plexiglas (clear acrylic sheet) for observation. The entrance and end of the tunnel were sealed with a mesh screen to allow ventilation. A UV light (26 Watt, Reptizoo, Guangdong, China) was installed and used as an attractant on the tunnel's ceiling at 220 cm from the entrance. There was no other light source in the room during the experiment. We released the individual honey bee at the start point (10 cm into the end of the tunnel opposite the UV light; Figure 1). Honey bees that did not move in two minutes or walked away from the UV light were recorded as negative responses. Honey bees that flew or walked towards the UV light were recorded as positive responses and their time of

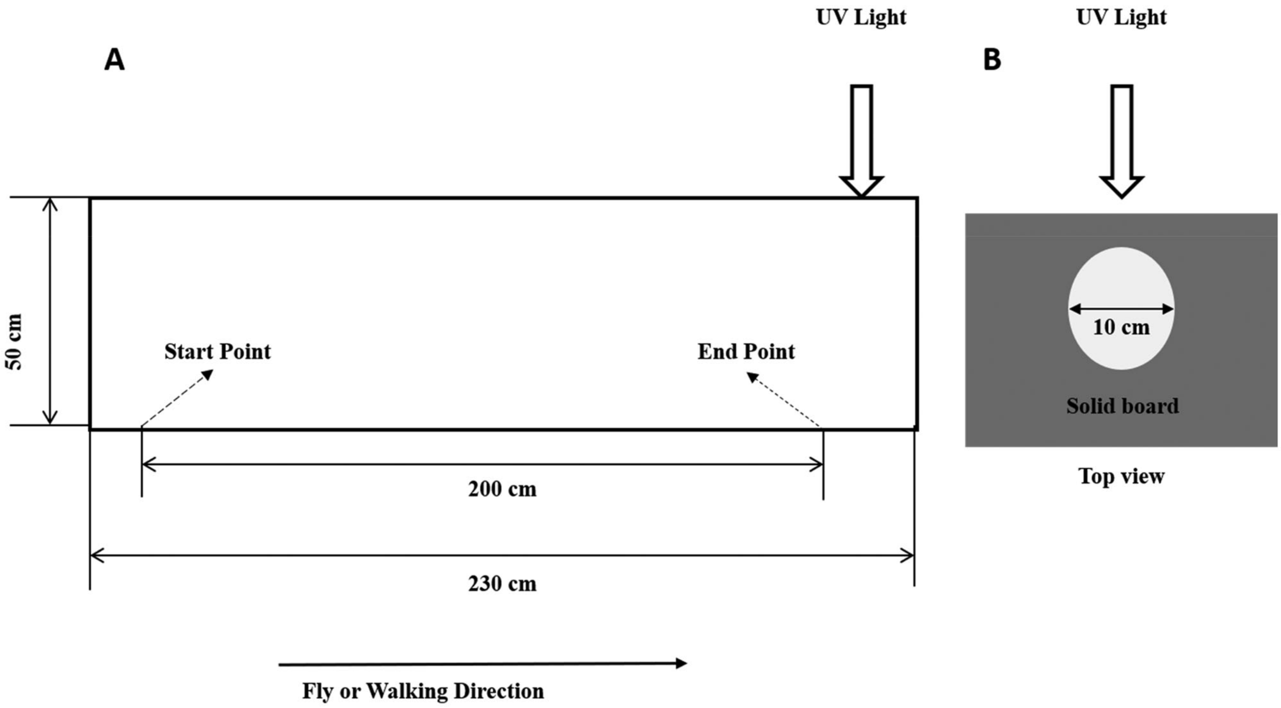

Figure 1. (A) Side view of the tunnel; (B) top view of the UV light.

flight or walk through 200 cm distance in the tunnel were recorded.

We used logistic regression to test if honey bees' walking behavior (i.e. whether walked or not) after the release was affected as the concentrations of imidacloprid increased in their diet. Honey bees that did not move within 2 min were counted as no-movement. Honey bees that walked, flew, or walked away from the UV light were all counted as a movement. For honey bees that completed the walking speed measurement, we used linear regression to test if their walking speed was affected as the concentrations of imidacloprid increased in their diet. The value of concentrations of imidacloprid in honey bees' diet was Log_{10} transformed. Honey bees that walked away from the UV light, flew, or did not continuously walk to the terminal line were excluded from the linear regression statistical analysis.

Results

Mortality rate

A total of 105 honey bees were captured, maintained, and treated individually for the tests. Three honey bees died during the first 24 h of oral treatment prior to the behavioral observation test and another three honey bees died during the second 24 h of oral treatment prior to the walking performance measurement in the tunnel (Table1). The deaths appeared to have been the result of honey bees accidentally becoming covered by syrup from their feeder. No honey bees died from the ingestion of imidacloprid-dosed syrup during the study.

Activity levels

After being exposed to the neonicotinoid contaminated diet for 24 h, honey bees were significantly less active as the concentrations of imidacloprid in the syrup increased ($r_s = -0.295$, $n = 103$, $p = 0.003$). On average across all experimental groups out of the 10 observation time points, 31 ± 24.8% to 63 ± 21.5% (Mean ± SD %) of honey bees were resting, 15 ± 21.5% to 30 ± 21.8% of honey bees were walking, 4 ± 6.5% to 12 ± 8.1% of honey bees were buzzing, and 13 ± 8.8% to 37 ± 6.5% of honey bees were excited (walking and buzzing) (Figure 2). Number of honey bees that were tested during the experiment and used in the statistical analysis were listed in Table 1.

Walking performance

At least 50% of honey bees from each treatment group moved after being released into the tunnel (Figure 3) and the number of moving honey bees significantly decreased as the concentrations of imidacloprid in their diet increased ($\chi^2 = 5.702$, $df = 1$, $p = 0.017$). The Hosmer-Lemeshow goodness-of-fit test was non-significant ($\chi^2 = 6.153$, $df = 5$, $p = 0.292$), indicating an adequate fit of the logistic regression model.Honey bees that continuously walked after being released into the tunnel took approximately 60 ± 29.1 s to 91 ± 31.8 s (Mean ± SD) to travel the 200 cm distance between the start point and end point (Table 2). However, the walking speed of honey bees among different treatment groups was not statistically significant ($F_{1,\ 60} = 1.231$, $p = 0.272$).

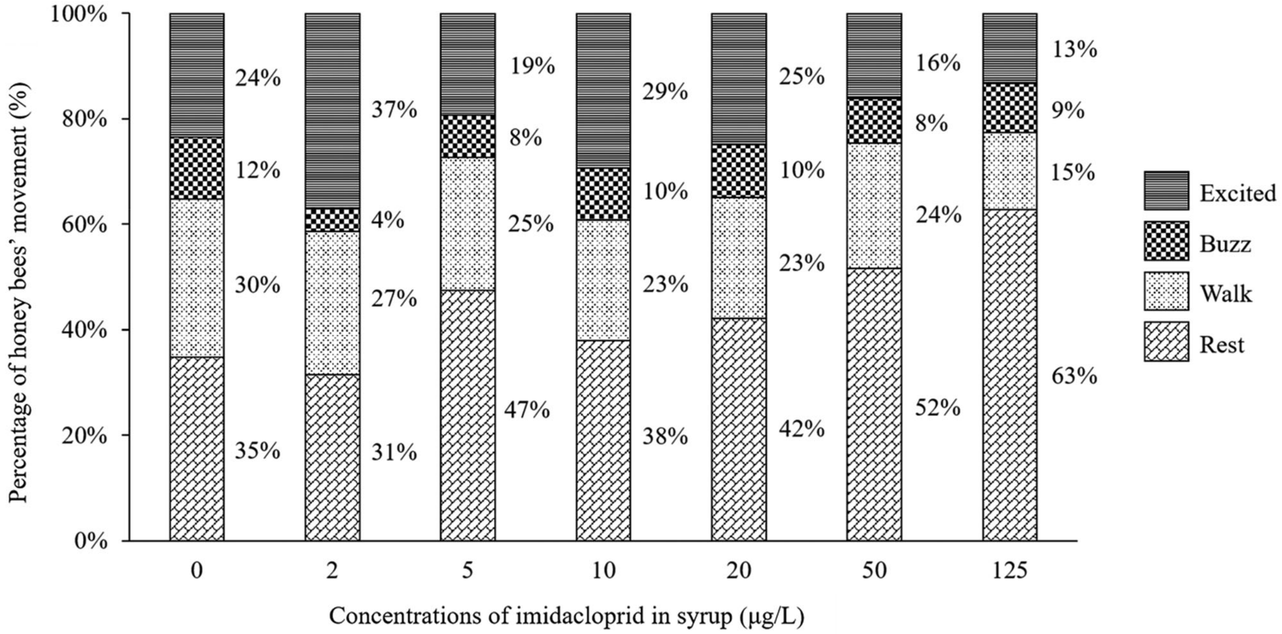

Figure 2. Percentage of honey bees' movement (%) in each treatment group out of the 10 behavioral observation time points each spaced one hour apart. Honey bees were significantly less active as the concentrations of imidacloprid in the syrup increased (r_s = –0.295, $n = 103$, $p = 0.003$).

Table 1. Numbers of bees that were used in the experiment and statistical analysis.

Treatment group (ppb)	# Treated honey bees		# Tested Honey bees		# Statistical analysis	
	Replicates	Dead	activity levels	Walking performance	Activity levels	Walking performance
0	15	0	15	15	15	15
2	15	1	14	14	14	14
5	15	1	15	14	15	14
10	15	0	15	15	15	15
20	15	1	14	14	14	14
50	15	2	14	13	13	13
125	15	1	15	14	15	14
Total	105	6	102	99	101	99

Note: Honey bee's behavioral performance was evaluated after consuming the imidacloprid-dosed syrup for 24 h. Honey bee's movement performance was evaluated after consuming the imidacloprid-dosed syrup for 48 h.

Discussion

During late fall, colony reproduction slows down and the number of worker bees is reduced to reserve food for days without fresh pollen flow (Mattila & Otis, 2006). At this time, any stressors that impact an individual honey bee's behavior and performance could potentially reduce colony fitness prior to overwintering. We found that the behavioral effects of imidacloprid in fall-collected forager honey bees differed significantly by dosage. Changes in behavioral performance after honey bees were exposed to imidacloprid up to 48 h occurred in all dosed groups compared to the control group. In general, honey bees that received higher doses of imidacloprid (50 and 125 μg/L) spent more time motionless and fewer walked after being released into the tunnel compared to those that received lower concentrations ($2 - 20$ μg/L) of imidacloprid in the diet. After 24 h of exposure to a range of sub-lethal doses of imidacloprid, honey bees' activity levels were significantly decreased as the concentrations of imidacloprid increased in their diet. We did not observe any statistically significant effect of imidacloprid on honey bees' walking speed after 48 h of imidacloprid exposure. However, the behavioral and walking performance differed between dosed and undosed individual honey bees in ways that could affect colony survival.

In our study, dosed honey bees had very low activity (resting) overall throughout the day, but their movement appeared over-stimulated (running and buzzing erratically) when they choose to move. In contrast, undosed honey bees had evenly distributed activity levels throughout the day and they walked consistently and calmly (circling in the cage in the same direction). Similar behavioral changes after honey bees exposed to sub-lethal doses (100 and 500 μg/L) of imidacloprid were also observed by Medrzycki et al. (2003). Reduced activity levels may result in less efficient bees in the hives, which is likely to contribute to poor colony

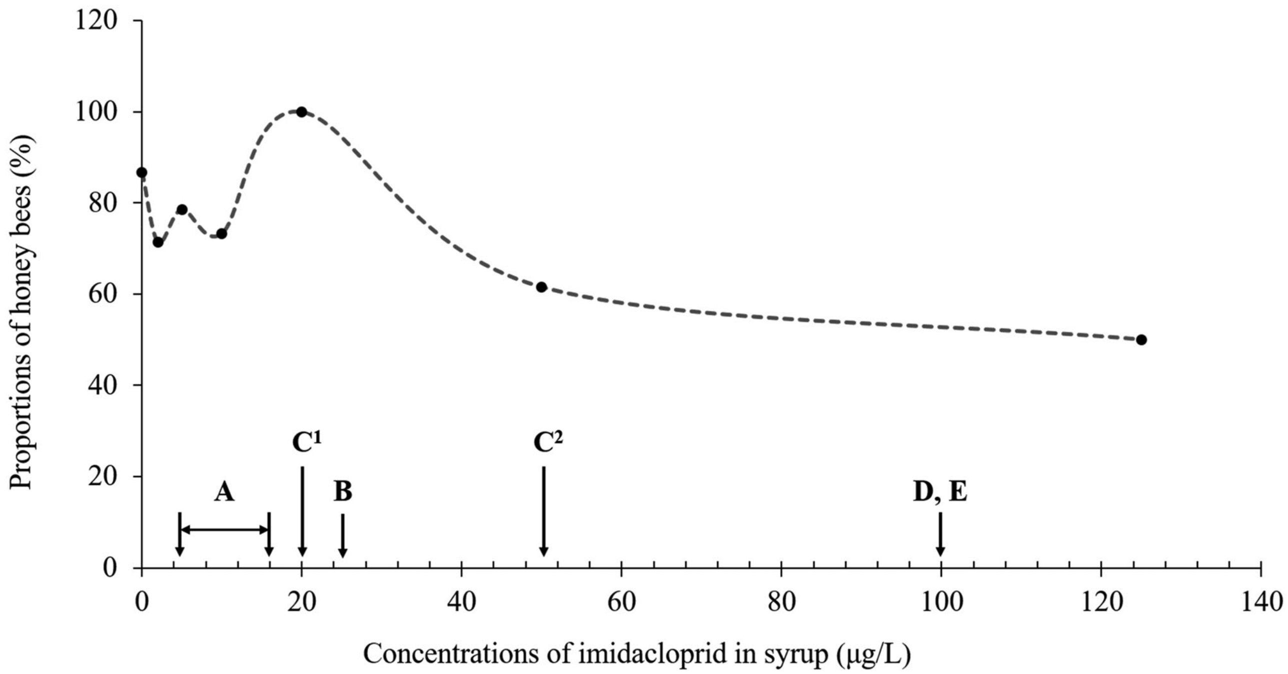

Figure 3. Proportions of honey bees (%) that moved in each treatment group after being released into the tunnel. Honey bees' walking behavior differed by treatment group ($\chi^2 = 5.702$, $df = 1$, $p = 0.017$). **(A)** field-realistic concentrations of imidacloprid that were detected in nectar (Dively and Kamel 2012); **(B)** lowest 24 h oral LD50 value in honey bees that was reported (Fairbrother et al., 2014); **(C^1, C^2)** early symptoms of poisoning in honey bee adult including hyperactivity and tremors (Suchail et al., 2013); **(D)** depressed activity level and disrupted communication between individual honey bees (Medrzycki et al., 2013); **(E)** decreased foraging activity within 2 h after ingestion (Bortolotti et al., 2003).

Table 2. Walking speed (cm/sec) of honey bees after being dosed with a series of concentration of imidacloprid for 48 h.

Treatment	# Honey bees		Walking speed (cm/sec)			
(μg/L)	Tested	Statistical analysis	Min	Max	Mean	SD
0	15	6	2.6	3.9	3.3	0.5
2	14	8	1.6	4.2	2.9	0.9
5	14	7	2.1	6.9	4.2	2.2
10	15	7	1.6	4.3	2.7	0.9
20	14	13	1.4	4.2	2.9	0.9
50	13	6	1.6	2.9	2.4	0.6
125	14	6	1.5	3.3	2.6	0.8
Total	99	53	—	—	—	—

Note: No statistically significant difference was observed ($F_{1,60} = 1.231$, $p = 0.272$).

health and increase their sensitivity to the cold weather (Lu et al., 2014; Straub et al., 2015). When the air temperature drops below 18 °C, honey bees begin to cluster together in the hive to keep themselves and the brood area warm at an average temperature of 27 °C (Stabentheiner et al., 2003). Honey bees near the center of the cluster vibrate their wing muscles to generate heat while honey bees on the outside layers remain still as insulation (Southwick & Heldmaier 1987). The decreased activity levels displayed by the honey bees fed imidacloprid in our study could disrupt this heat-generating behavior and cause a heat deficiency within a cluster. Honey bees in different layers of the cluster rotate their positions to access food stored in the comb (Sumpter & Broomhead 2000). With inadequate heat, honey bee clusters may not be able to move or spread out to reach food at adjacent comb and eventually die from starvation (Owens 1971). Because the clearance of imidacloprid in honey bees is about 4–6 h (Suchail et al., 2004) and the effect of imidacloprid on behavior appears to last up to 24 h (Bortolotti et al., 2003), they may not have enough time to completely clear the ingested neonicotinoids from their systems or recover before the next feeding occurs. Thus, chronic exposure of hives to imidacloprid contaminated food stores during cold weather has the potential to negatively affect colony survival and re-establishment in early spring (Lu et al., 2014). Further studies on the behavioral changes in groups of honey bees (more than 100 individual bees) under cool-temperature environmental conditions are needed to investigate how imidacloprid affects the cluster's behavior.

Our results were consistent with previous studies (Crall et al., 2018; Lambin et al., 2001; Williamson et al.,

2014) where imidacloprid exposure leads to either hyperactivity or immobility depending on doses. In our study, sub-lethal concentrations of imidacloprid influenced honey bees' walking behavior. After release into the tunnel, most of the control honey bees walked towards the UV light immediately, a large proportion of honey bees that received high concentrations of imidacloprid (50 and 125 μg/L) did not move, and similar proportions of honey bees in different treatment groups that received field-realistic concentrations of imidacloprid ($2 - 10\,\mu$g/L) moved. However, we noticed that a higher proportion of bees that received field-realistic concentration of neonicotinoid at 20 μg/L walked toward the UV light after being released into the tunnel compared to the control bees. We suspect that the field-realistic concentrations of neonicotinoid insecticide exposure may have agitated the honey bees and caused over-excitement behavior (Kimura-Kuroda et al., 2012; Picciotto et al., 2002). The hyperactive behavior observed at the low concentrations of imidacloprid exposure could indicate the effect of nicotinic activation, whereas the hypoactive behavior observed at the high concentrations could be caused by some non-specific toxic effects (Lambin et al., 2001).

We did not observe any significant effect of imidacloprid dose on honey bees' walking speed. However, honey bees in our study that were exposed to high concentrations (50 and 125 μg/L) of imidacloprid and completed the walking test showed very inconsistent behavior during the measurement. They either struggled walking towards the end of the tunnel or appeared over-stimulated and not able to walk straight compared to the control and low dosed bees. Similar results were found by Williamson et al. (2014) and Charreton et al. (2015), which did not observe any significant effect of imidacloprid (2.56 μg/L) in honey bee workers' walking speed after 24 h exposure. Sub-lethal concentrations of the other neonicotinoids (i.e. thiamethoxam and acetamiprid) were also found to impair honey bee workers' motor activity but not their walking speed (Charreton et al., 2015; Hassani et al., 2008; Tosi & Nieh, 2017). Efficient walking behavior of honey bees is required to fulfill in-hive tasks, including nursing, building wax cells, cleaning, and maintaining proper temperature around the brood (Charreton et al., 2015). During cold weather, the previously stored food in the hive is for honey bees the sole diet until the floral resources become available again. The energy of honey bees that consumed neonicotinoid contaminated stored food inside the hive may be wasted on the erratic activity instead of maintaining cluster temperature or performing hive duties, which could contribute to colony failure. The behavior of imidacloprid-dosed individual honey bees observed in our study could result in decreased work and energy efficiency and potentially reduce colony fitness during late fall. However, it was not within our study scope to assess the relationship between honey bees' walking performance and their work efficiency.

Chronic exposure to neonicotinoids in late fall appears to decrease colony survival (Lu et al., 2014; Wood et al., 2019). Our study helps to increase understanding of how chronic exposure to neonicotinoids during late fall impacts individual honey bees. Our results showed that different sub-lethal concentrations of imidacloprid altered individual forager honey bees' behavior at cool temperatures in ways that might impact their performance of hive duties (e.g. brood rearing, foraging, thermoregulation). We acknowledge that in field studies, such consequences may be difficult to observe at similar concentrations because of the complexity of bee hives and their ability to buffer the stress through eusocial behavior. Thus, studies of individual honey bees, such as ours, in addition to field studies on both individual honey bees and colonies are needed to help fully understand the complex and potentially subtill impacts of neonicotinoids.

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