



# The Neonicotinoid Imidacloprid Impairs Learning, Locomotor Activity Levels, and Sucrose Solution Consumption in Bumblebees (*Bombus terrestris*)

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**Abstract:** Bumblebees carry out the complex task of foraging to provide for their colonies. They also conduct pollination, an ecosystem service of high importance to both wild plants and entomophilous crops. Insecticides can alter different aspects of bumblebee foraging behavior, including the motivation to leave the hive, finding the right flowers, handling flowers, and the ability to return to the colony. In the present study, we assessed how the neonicotinoid imidacloprid affects bumblebees' foraging behavior after exposure to four different treatment levels, including field-realistic concentrations (0 [control], 1, 10, and 100 µg/L), through sucrose solution over 9 days. We observed the behavior of several free-flying bumblebees simultaneously foraging on artificial flowers in a flight arena to register the bees' complex behavior postexposure. To conduct a detailed assessment of how insecticides affect bumblebee locomotor behavior, we used video cameras and analyzed the recordings using computer vision. We found that imidacloprid impaired learning and locomotor activity level when the bumblebees foraged on artificial flowers. We also found that imidacloprid exposure reduced sucrose solution intake and storage. By using automated analyses of video recordings of bumblebee behavior, we identified sublethal effects of imidacloprid exposure at field-realistic doses. Specifically, we observed negative impacts on consumption of sucrose solution as well as on learning and locomotor activity level. Our results highlight the need for more multimodal approaches when assessing the sublethal effects of insecticides and plant protection products in general. *Environ Toxicol Chem* 2023;42:1337–1345. © 2023 SETAC

**Keywords:** Pollinator; Behavior; Movement; Pesticides; Plant protection products; Object detection; Imidacloprid; Bumblebees

## INTRODUCTION

The ongoing decline in abundance and diversity of pollinators is caused by several factors, including habitat fragmentation and loss, overgrazing, climate change, and exposure to plant protection products (for a full review, see Potts et al., 2010; Wagner et al., 2021). These stressors have all intensified over the last decades, particularly the use of plant protection products (DiBartolomeis et al., 2019; Douglas & Tooker, 2015; Goulson et al., 2018; Tilman et al., 2002).

Neonicotinoids are a group of broad-spectrum insecticides that have been extensively used globally over the last three decades, primarily applied as seed dressing (DiBartolomeis et al., 2019; Goulson et al., 2018). Like most insecticides, neonicotinoids affect the nervous system of insects. They act as an agonist on the nicotinic acetylcholine receptors, which are located in the synaptic neophile regions (mushroom bodies) of the insects' central nervous system (Jeschke et al., 2013). By inducing the same agonistic activation of receptors as the natural neurotransmitter acetylcholine, neonicotinoids cause an inward current that generates action potentials (Jeschke et al., 2013). Because of their ability to disrupt critical neural pathways, also in nontarget organisms, neonicotinoids are considered a significant factor contributing to the decline in pollinators (Alkassab & Kirchner, 2017; Woodcock et al., 2016).

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Bees can learn to associate an odor with a sucrose solution reward and therefore extend their proboscis when they smell such a stimulus (the olfactory proboscis extension reflex [PER] method; Bitterman et al., 1983). The PER method is a useful tool for testing the effects of stressors on learning and memory in bees (Stanley et al., 2015). However, bees used in PER experiments are harnessed and only allowed to move their antennae and mouthparts. The harnessed bees may thus behave differently from freely moving bees (Ayestaran et al., 2010; Mujagic & Erber, 2009). Because foraging is a complex task, including a wide range of behaviors, it is important to also study the effects of neonicotinoids on learning and memory in freely moving bees. In addition, social interaction is an important aspect of free foraging and associative learning in bees (Dawson et al., 2013; Kawaguchi et al., 2007; Slaa et al., 2003; Worden & Papaj, 2005).

Assessing the effects of neonicotinoids on bee behavior and learning is a crucial step in understanding the impact these products might have on bee populations. Although most studies have focused on the effects of exposure to odor stimuli (Muth et al., 2019), visual stimuli have also been used in bumblebees (Lämsä et al., 2018; Muth & Leonard, 2019; Phelps et al., 2018) and honeybees (Ludicke & Nieh, 2020). However, there is a lack of multimodality studies that assess several stimuli and include social interactions. For a more comprehensive risk assessment of insecticides' effects on bees, it is important to include multiple stimuli because bees are exposed to various stimuli while foraging.

Several studies have assessed whether neonicotinoids affect bees' locomotor activity level by focusing on how one or two bees move inside a small space, where they are unable to fly or move freely (Crall et al., 2018; Lambin et al., 2001; Medrzycki et al., 2003; Muth et al., 2020; Sánchez-Bayo et al., 2017; Teeters et al., 2012; Tosi & Nieh, 2017). Flight activity has also been assessed by counting the number of individuals inside a 1 × 1-m square once a day in a semifield environment (Dietzsch et al., 2019). Although studies that confine bees to small spaces can provide precise measurements of locomotor activity, free flying outside the hive to forage is a significant part of a bee's life, and negative effects on learning and memory are expected to have a strong impact in this context. Siviter et al. (2018) identified the lack of studies on free-moving bees to be a major knowledge gap.

The goal of the present study was to assess the effects of neonicotinoids on bumblebees' foraging behavior in a more ecologically realistic setting. Therefore, we conducted experiments using several free-flying bumblebees simultaneously in a flight arena and exposed them to both visual and olfactory stimuli in a multimodality testing regime. By allowing several bumblebees to forage simultaneously, individual bees are able to learn from their nestmates (Alem et al., 2016; Loukola et al., 2017). To maximize the number of registered responses, we used automatic analyses of video recordings of the bees' behavior. Our focal insecticide was the neonicotinoid imidacloprid (CAS number: 138261-41-3). Although imidacloprid has been banned for agricultural use in the European Union since 2018, it is still widely used globally; and neonicotinoids can

remain in the soil for several years after application (Hladik et al., 2017; Thompson et al., 2020, Table 2; Woodcock et al., 2018).

## MATERIALS AND METHODS

### Bumblebees

Colonies of *Bombus terrestris* with approximately 80–100 workers each were obtained from a continuous mass rearing program (Natupol Beehive; Koppert, Berkel en Rodenris, The Netherlands). The bumblebee colonies were housed in standard plastic nest-boxes (25.4 × 22.9 × 12.7 cm) covered by a cardboard box for the duration of our study. The bumblebees stayed in the nest-box except when participating in experiments in the flight arena. The bumblebees were kept in a temperature- and humidity-controlled environment, at approximately 28 °C and 55% relative humidity and subjected to a feeding regime where both sucrose solution (Attractor: fructose/glucose/saccharose solution, 1.27 kg L<sup>-1</sup>; Koppert) and pollen (acquired from *Bombus*, Norway) were restricted for 24 h prior to testing. Before testing, the bumblebees were fed ad libitum with pollen and sucrose solution without imidacloprid.

### Treatment

Colonies were randomly assigned to one of four different treatment levels: 0 (control), 1, 10, and 100 µg/imidacloprid (PESTANAL, analytic standard, purity [high-performance liquid chromatography area] ≥98.0%), with four colonies receiving each treatment level. The sugar concentration was equal across treatment levels because we added the same amount of distilled water to all treatment levels, including the control. Based on environmental concentrations of imidacloprid residuals in nectar found in previous studies, 1 and 10 µg/L represent the lower and higher ends of the field-realistic exposure range, respectively, while 100 µg/L represents an extreme exposure to imidacloprid (Byrne et al., 2014; Cresswell, 2011; Krischik et al., 2007; Schmuck et al., 2001; Siviter et al., 2018; Stoner & Eitzer, 2012).

Pure imidacloprid (Sigma-Aldrich) was dissolved in distilled water and added to the sucrose solution using a dilution scheme which was well below the solubility of imidacloprid (610 mg/L) at all time points. The bumblebees were orally exposed to imidacloprid for 9 days through sucrose solution from a standard feeding bag equipped with the hives, placed under the hives inside the hive box. Imidacloprid in concentrations <70 mg/L<sup>-1</sup> stored at room temperature (21 ± 1 °C) has been shown not to degrade during a period of 22 days (Tišler et al., 2009), and previous studies, under the same testing regime, have indicated good compliance in the comparison of nominal to measured exposure in sucrose solution (Aarønes et al., 2021).

### Experimental procedure

A flight arena with dimensions of 130 × 100 × 35 cm was used to conduct the behavioral experiments (Figure 1A). The

arena was custom-made and covered with a transparent Plexiglas® lid. The walls and floor of the arena were lined with white plastic plates, which were sanded to minimize reflections that might interfere with the detection of bees. Artificial flowers (Figure 1B), measuring 24 × 24 mm with a foot 12.5 mm diameter, 40 mm height, and a base of 24 × 24 mm, were placed inside the flight arena (Raine et al., 2006).

Each colony was given access to the flight arena twice; for 1 h of pre-exposure *training* and for 2 h of postexposure *testing*. During the preexposure training, 18 bicolored blue and yellow flowers, each filled with approximately 1.5 ml of sucrose solution, were placed in the flight arena to familiarize the bumblebees with the artificial flowers and to associate the colors and odors of the artificial flowers with reward. After training, the bumblebees were exposed to imidacloprid via sucrose solution for 9 days. On day 9, postexposure testing was conducted for 2 h. During postexposure testing, the 18 bicolored flowers were replaced by nine blue (rewarding) and nine yellow (unrewarding) flowers in the flight arena, each containing approximately 1.5 ml sucrose solution or tap water, respectively. The artificial flowers were washed with 30% ethanol in water solution between each round of testing in the flight arena to remove olfactory cues. Bumblebees were not allowed to return to the hive during postexposure testing. When 15 bumblebees had entered the flight arena or 1 hour had elapsed, whichever occurred first, the hive opening was closed to prevent additional bumblebees from entering the flight arena.

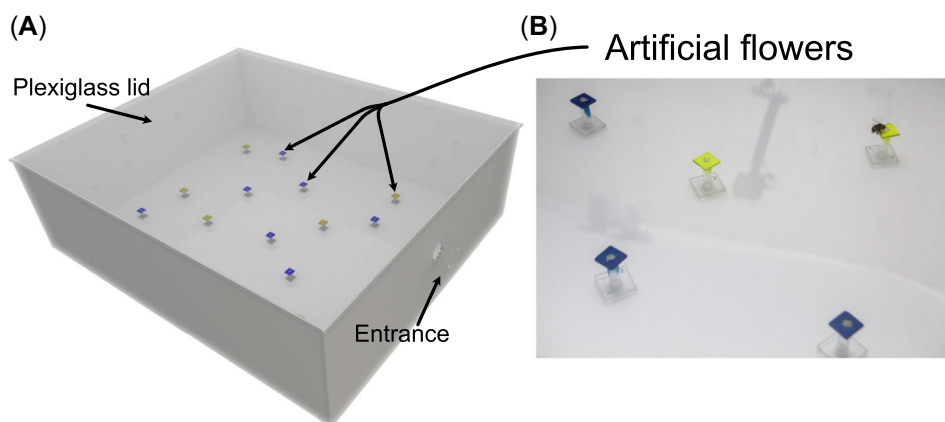
The flight behavior of the bees in the flight arena was recorded using two cameras (GoPro Hero 5 Black), set to ISO 3000, 60 frames per second, and a resolution of 2704 × 1520 pixels with the “linear” lens setting. Each camera was mounted on cylinders centered on each long side of the flight arena, enabling the cameras to record the entire flight arena from two sides. The starting and stopping of the recordings were controlled by a remote control (GoPro smart Remote) connected to the cameras' Wi-Fi. The remote control enabled synchronized filming and subsequent simultaneous analysis of recordings of the bumblebees from two different angles.

At the end of the 9-day exposure period and testing, the remaining bumblebees in the hives were euthanized by freezing the hives for a minimum of 48 h in a −20 °C freezer. The total amount of sucrose solution consumed during the exposure period was calculated by weighing the sucrose solution bag at the start and end of the exposure period.

The bumblebees filled the honeypots inside the hive with honey they produced from the sucrose solution, which was provided to them both pre- and postexposure. The number of honeypots within each hive was counted, and each honeypot was recorded as containing nectar or empty. Because honeypots vary in shape and size and the number of honeypots per hive is high, we did not measure the volume of honey in each honeypot.

### Flower visits analysis

We developed a computer program for automated behavioral tracking of several bumblebees foraging at the same time using the video recordings based on the OpenCV computer vision library. The program enabled us to detect bumblebees on individual artificial flowers and track individual bumblebees' motion (Supporting Information, Automatic flower visit detection). The source code is available on GitHub (github.com/henriasv/bumblebee-tracker-2). The program allowed for computationally efficient bumblebee detection using a high-end graphics processing unit (NVIDIA P100). Furthermore, the number of visits to flowers of each color could be extracted, with each colony as an experimental unit. Bumblebees were regarded as having no identity; that is, they were indistinguishable. Thus, when designing the criteria for flower visits, the focus was on the flowers rather than the bees, and the detected property was whether some bumblebee(s) visited a given flower in a given frame of the video. We counted a visit to a rewarding flower (blue) as a success and a visit to an unrewarding flower (yellow) as a failure. We defined a flower visit as frames where both video streams simultaneously showed the bumblebee's center inside the border of a flower, in practice meaning that the bumblebee was standing on a



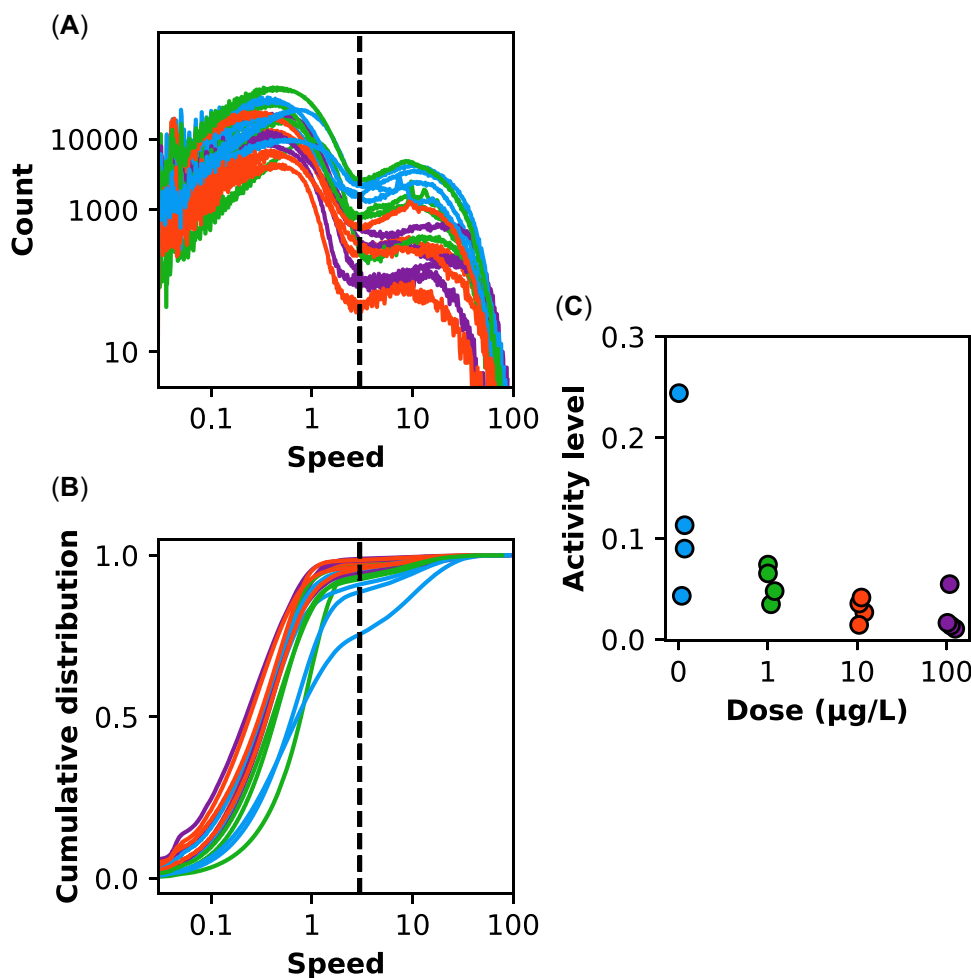
**FIGURE 1:** Overview of the experimental setup and artificial flowers. **(A)** Overview of the flying arena (130 × 100 × 35 cm) used in the experimental setup, showing artificial flowers as squares in blue and yellow and the entrance on one of the sides. **(B)** Detail picture of the flying arena during training. The picture shows two yellow (unrewarding) and three blue (rewarding) artificial flowers. The circular marks in the center of the flower contain an Eppendorf tube that allows for a small amount of sucrose solution to be placed in the flower. A *Bombus terrestris* worker is visiting one of the yellow (unrewarding) flowers. Photo: Simen Kjellin.

flower. We regarded standing on a flower as a measurement of selecting a flower because a bumblebee can detect nectar via chemosensors in its feet or via extending the proboscis (de Brito Sanchez et al., 2008). The inclusion of two parallel video streams in the definition of flower visit detection eliminated false visits. To exclude instances where a bumblebee was passing above a flower instead of landing, we set the criterion for a flower visit to be the detection of a bumblebee on more than half of the video frames in a 2-s time interval.

### Locomotor activity level

Locomotor activity levels (the proportion of time that animals spent active) were measured by constructing trajectory fragments using the Python package Trackpy 0.3.3 (Allan et al., 2014) on the position data obtained from our custom-developed software. A trajectory fragment is a continuous sequence of positions for the same bee for some time but not through the whole experiment. Trajectory fragments enable the investigation of short-term trajectory properties, such as speed. A fragment ends when the

Trackpy's algorithm fails to identify the same bee between consecutive frames, either because it crosses another bee or because it goes out of sight, for example, under a flower. All speeds of bees were extracted from the trajectory fragments and presented as a histogram over the measured speeds. Each speed measurement in the trajectory fragment is computed on a frame-to-frame basis, and thus the length of the trajectory fragments or any systematic difference in the average time/length of trajectory fragments at different flying speeds does not introduce bias. As expected from previous studies on animal locomotor activity level and trajectory data (Edelhoff et al., 2016), we observed a clear difference between fast-moving animals and slow-moving animals. Thus, a threshold value on the speed distribution was used to distinguish slow (passive) and fast (active) movement. We chose the threshold value as the speed at which the movement plateaued and defined it by visual inspection of the data (Figure 2A). We chose the same threshold value of three pixels per 1/60 second for all experiments because the speed at which the movement plateaued always appeared approximately at the same value. This speed corresponds approximately to 5 cm/s but



**FIGURE 2:** Locomotor activity. (A) Speed histogram showing each colony as a line, with each color representing a treatment level: blue = control; green = 1  $\mu\text{g/L}$ ; red = 10  $\mu\text{g/L}$ ; purple = 100  $\mu\text{g/L}$ . The dashed line is the threshold value on the speed distribution separating slow (passive) and fast (active) movement. (B) Cumulative speed distributions made from the data shown in (A). For each line in (B), the cumulative distribution value at the intersection with the dashed line represents the proportion of time in slow movement, and thus the locomotor activity level is 1 subtracted by this number. (C) Locomotor activity level (proportion of time in flight).

will depend slightly on the position of the bee in the flying arena because the video streams are two-dimensional, and we do not perform three-dimensional reconstruction of the bee positions. We defined the colony's *locomotor activity level* as the proportion of the speed measurements that are above the threshold. This is measured from the cumulative distribution of the speeds (Figure 2B).

### Statistical analysis

The statistical analysis was performed using R statistical software (2022). The significance level for all tests was set to  $p < 0.05$ . To test for a relationship between the response variable and treatment, the Jonckheere-Terpstra test was used (Jonckheere, 1954). This test examines whether there is a statistically significant trend in the data when the expected order of response treatments has been asserted a priori. To assess the statistical significance of the trend at specific treatment levels, we used the Shirley-Williams trend test (Shirley, 1977; Williams, 1986), along with significance tables from Williams (1972), which specifies  $t$  values for significance levels of 0.05, 0.025, 0.01, and 0.005. The Jonckheere-Terpstra test and Shirley-Williams test are recommended by the Organisation for Economic Co-operation and Development (2006) for nonparametric, trend-based hypothesis testing with continuous data.

Several endpoints were measured in the present study: the total number of visits to all flowers, the number of visits specifically to blue flowers (rewarding), the locomotor activity level, the proportion of empty honeypots, and the mass of sucrose solution consumed after the treatment was added to the sucrose solution bag.

## RESULTS

### Effect of imidacloprid exposure on flower visits

The total number of visits to flowers of both colors did not differ significantly between treatments overall (Jonckheere-Terpstra test,  $p = 0.137$ ; Figure 3B).

The number of flower visits made by bumblebees ranged from 1 to 65 visits for all colonies combined, with the control group having the highest range of visits (6–65) and the colonies exposed to 1  $\mu\text{g/L}$ , 10  $\mu\text{g/L}$ , and 100  $\mu\text{g/L}$  having ranges of 4–31, 1–12, and 3–39 visits, respectively (Figure 3). The trend analysis showed a statistically significant decreasing trend in the number of visits to blue (rewarding) flowers with increasing imidacloprid exposure (Figure 3A). Specifically, the Jonckheere-Terpstra test indicated a negative relationship between imidacloprid exposure and visits to blue flowers, with a  $p$  value of 0.012. This trend was statistically significant for the 10- $\mu\text{g/L}$  treatment level and higher (Shirley-Williams test,  $p \approx 0.025$ ; Figure 3A).

### Effect on locomotor activity level

The trend analysis showed a statistically significant decreasing trend in the locomotor activity level with increasing imidacloprid treatment (Jonckheere-Terpstra test,  $p < 0.001$ ; Figure 2C). This trend was statistically significant for the 10- and 100- $\mu\text{g/L}$  treatment levels (Shirley-Williams test,  $p < 0.05$  and  $p < 0.01$ , respectively).

### Effect on nectar storing (honeypots)

The trend analysis showed a statistically significant decreasing trend in the proportion of empty honeypots with increasing imidacloprid treatment (Jonckheere-Terpstra test,  $p < 0.01$ ; Figure 4B). This trend was statistically significant for all treatment levels (Shirley-Williams test,  $p < 0.025$ ).

### Effect on sucrose solution consumption

The trend analysis showed a statistically significant decreasing trend in sucrose solution consumption with increasing imidacloprid exposure (Jonckheere-Terpstra test,  $p < 0.001$ ; Figure 4A). This trend was statistically significant for treatment levels 10 and 100  $\mu\text{g/L}$  (Shirley-Williams test,  $p < 0.05$  and  $p < 0.01$ , respectively).

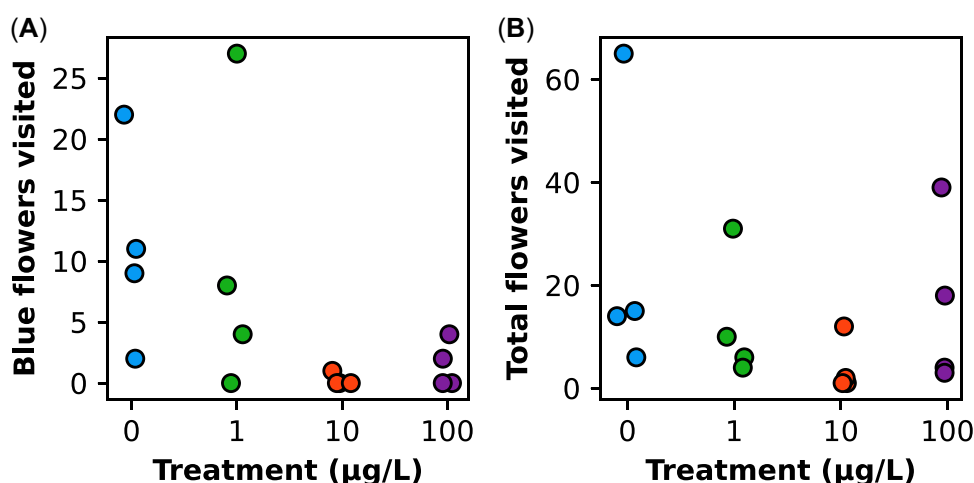
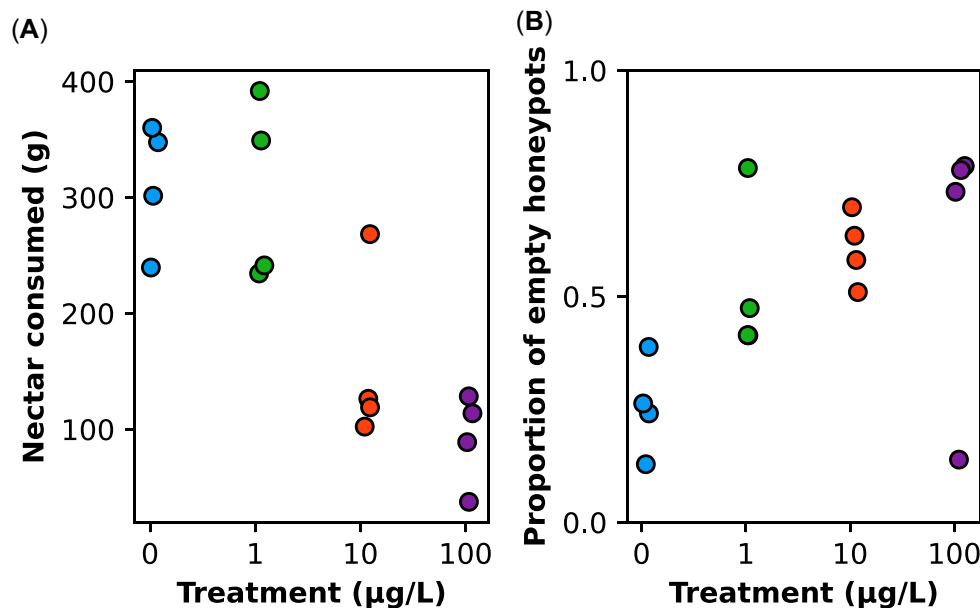


FIGURE 3: Number of visits to flowers. (A) Visits to blue flowers (rewarding) and (B) total number of visits to flowers of any color.



**FIGURE 4:** Sucrose solution consumed and proportion of empty honeypots. (A) Amount of sucrose solution consumed after the treatment was added. (B) Proportion of empty honeypots after the exposure period.

## DISCUSSION

We have shown that chronic exposure to imidacloprid, at field-realistic concentrations, impairs several aspects of bumblebee behavior, including learning, locomotor activity level, and feeding. As far as we know, ours is the first study that assesses behavioral changes after neonicotinoid exposure in multiple bumblebees flying at the same time and thus includes social interaction. Our study highlights the potential for automated analysis in behavior toxicology and ecology in invertebrates. Moreover, we have shown that automatic analysis can document the multitude of behaviors that can be affected when bees are exposed to insecticides.

### Flower visits

Contrary to the findings of other studies (Lämsä et al., 2018; Morandin & Winston, 2003; Muth & Leonard, 2019; Phelps et al., 2018), we did not observe any effect on the total number of flowers visited, meaning that the foraging motivation did not differ between treatment groups. This suggests that the bumblebees learned to forage on artificial flowers during the training period and that their long-term memory was not affected by the exposure (Chittka, 1998; Wright et al., 2015).

Exposure to imidacloprid reduced the number of rewarding flowers visited in the high-end field-realistic treatment (10 µg/L) compared to the control, indicating that imidacloprid impairs olfactory and/or visual learning. Our findings are consistent with studies on learning in harnessed bees that assessed the PER (Decourtye et al., 2003; Stanley et al., 2015).

We found that exposure to imidacloprid had adverse effects on learning when bumblebees were subject to a combination of olfactory and visual stimuli, in contrast to studies that assessed visual stimuli only and that did not find any effect on

learning (Colin et al., 2020; Lämsä et al., 2018; Ludicke & Nieh, 2020; but see Muth et al., 2019; Phelps et al., 2018). However, our findings align with studies on both olfactory and visual stimuli, showing that olfactory learning is impaired by imidacloprid exposure (Muth et al., 2019). This suggests that olfactory learning was affected in our study, while visual learning was not.

### Locomotor activity level

Locomotor activity level of bumblebees decreased as the concentration of imidacloprid increased to the high-end field-realistic concentrations of 10 µg/L and higher. Our study differs from previous research in both observation time (2 h) and methodology. Although a direct comparison of findings is difficult, our findings align with other studies demonstrating that imidacloprid reduces flight distance and duration in bumblebees in a flight mill (Kenna et al., 2019). Other studies have shown that imidacloprid exposure can increase the movement speed of worker bumblebees inside the nest (Crall et al., 2018), both increase and decrease activity level in an open field-like apparatus (Lambin et al., 2001), and reduce distance moved inside Petri dishes (Teeters et al., 2012; Williamson et al., 2014). Because foraging requires a diverse range of behaviors, from handling flowers to relocating to the most rewarding flower patches, our study including social interactions and free-flying bumblebees may provide a broader perspective on how imidacloprid can affect bumblebees during foraging.

### Sucrose solution intake and storage

The reduction in sucrose solution intake observed at and above the high-end field-realistic concentration, along with

the high proportion of empty honeypots across all exposed groups, suggests that the bumblebees emptied the honeypots but did not refill the honeypots. One possibility is that the bumblebees consumed the stored sucrose solution in the honeypots rather than the sucrose solution, containing imidacloprid, provided in the bag. However, there is no evidence that bumblebees can smell or taste neonicotinoids (Kessler et al., 2015; Muth et al., 2020), and research on potential postingestive feedback did not find evidence that bumblebees formed negative associations between sugar solutions containing imidacloprid and postingestive effects (Muth et al., 2020). Alternatively, pesticide-induced death or lethargy, shown by lower locomotor activity level in bees in the exposed groups, may have influenced the workers' ability to conduct hive tasks, such as filling the honeypots. Previous studies have shown that neonicotinoids indeed influence workers' ability to conduct hive tasks (Crall et al., 2018).

Effects on feeding motivation in bees exposed to neonicotinoids are well documented, and our findings are in line with other studies (Cresswell et al., 2012; Laycock et al., 2014; Muth et al., 2020; Scholer & Krischik, 2014). Whether the feeding motivation is a result of the bumblebees' lower activity level or the lower activity level is a result of the feeding motivation is beyond the scope of the present study. However, previous studies have shown that imidacloprid suppresses feeding activity by decreasing the bees' metabolic rate (Contreras & Bradley, 2010; Hatjina et al., 2013), suggesting that the reduction in metabolic rate causes both the lower activity level and the reduced feeding motivation.

## CONCLUSIONS

We have identified adverse effects on learning, locomotion, and feeding behavior of bumblebees exposed to field-realistic concentrations of the neonicotinoid imidacloprid. Using innovative approaches, including video recordings, machine learning, and automated analyses of videos, we have shown that it is possible to implement more ecologically relevant methods in toxicity testing, even with limited resources. Our findings underscore the importance of comprehending the complexity of foraging behaviors when assessing the impact of pesticides on bees and other non-target organisms.

**Supporting Information**—The Supporting Information is available on the Wiley Online Library at <https://doi.org/10.1002/etc.5611>.

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**Disclaimer**—The authors declare no conflict of interest.

**Author Contributions Statement**—**Julie Sørli Paus-Knudsen**: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Visualization; Writing—original draft; Writing—review & editing. **Henrik Andersen Sveinsson**: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Visualization; Writing—review & editing. **Anders Nielsen**: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Validation; Writing—review & editing. **Katrine Borgå**: Conceptualization; Methodology; Supervision; Validation; Writing—review & editing. **Merete Grung**: Supervision; Validation; Writing—review & editing.



This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at <https://zenodo.org/record/7693808>. Learn more about the Open Practices badges from the Center For Open Science: <https://osf.io/tvyxz/wiki>.

**Data Availability Statement**—Tracking data and data analyses from the bee tracking algorithm are available on Zenodo (<https://zenodo.org/record/7693808>).

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