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Traces of a Neonicotinoid Induce Precocious Foraging and Reduce Foraging Performance in Honey Bees

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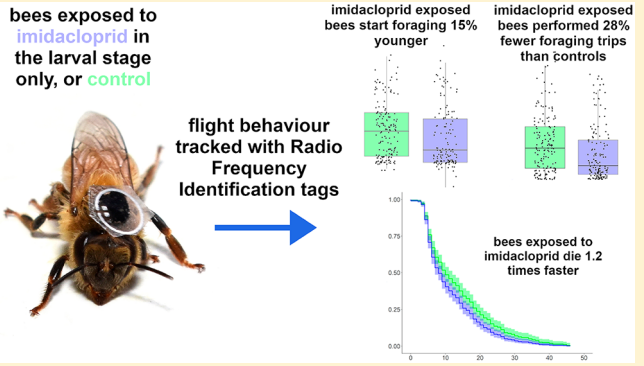
Supporting Information

ABSTRACT: There is increasing worldwide concern about the impacts of pesticide residues on honey bees and bee colony survival, but how sublethal effects of pesticides on bees might cause colony failure remains highly controversial, with field data giving very mixed results. To explore how trace levels of the neonicotinoid pesticide imidacloprid impacted colony foraging performance, we equipped bees with RFID tags that allowed us to track their lifetime flight behavior. One group of bees was exposed to a trace concentration (5 μg/kg, ppb) of imidacloprid in sugar syrup while in the larval stage. The imidacloprid residues caused bees to start foraging when younger as adults and perform fewer orientation flights, and reduced their lifetime foraging flights by 28%. The magnitude of the effects of a trace imidacloprid concentration delivered only during larval stage highlights the severity of pesticide residues for bee foraging performance. Our data suggest that neonicotinoids could impact colony function by imbalancing the normal age based division of labor in a colony and reducing foraging efficiency. Understanding this mechanism will help the development of interventions to safeguard bee colony health.



INTRODUCTION

There is global concern about the impacts of neonicotinoid pesticide residues on honey bee health and honey bee colony survival.[1−8] Many deleterious impacts of neonicotinoid residues on bees have now been reported, and some studies have linked sublethal pesticide exposures to increased colony failure in honey bees and bumblebees.[1,3,5,6,8,9] Different mechanisms have been proposed to explain how pesticide residues cause the rapid failure of a bee colony.[10−14] The issue is complex because the social dynamics of a bee colony are such that stressors at an individual level do not simply translate to outcomes for a colony.[1,11,15] One influential model has argued that a chronic stressor causing a premature death of foragers could cause a dramatic colony failure through the progressive recruitment of younger and younger bees to the foraging force (precocious foragers).[11] Here, we examined how traces (5 μg/kg, ppb) of the neonicotinoid imidacloprid influenced foraging performance and foraging onset in honey bees to explore possible mechanisms of pesticide-related colony failure.

In honey bee colonies, foragers are typically the older adult bees, but a loss of foragers is usually compensated by young bees starting foraging earlier in their adult life.[16−19] This is an adaptive response to rapidly restore the colony's foraging force, but new theories predict that this mechanism could accelerate colony failure in the face of chronic stress. If workers that start foraging precociously are more sensitive to the stressors and less effective at foraging, they will also die prematurely, ultimately driving a population collapse.[3,11,15] This mechanism for bee colony failure is appealing since it is simple, and precocious foraging is known to be induced by nutritional and pathogenic stresses of individual bees[20−22] and forager losses from the colony.[11,23] We do not yet know how neonicotinoid contaminants influence the foraging onset in honey bees.

Neonicotinoid pesticides are found in the dust drift during crop sowing[24,25] in the pollen, nectar, and guttation drops of many flowering crops[3,7,34−38,26−33] and nearby wildflowers.[39] The impacts of neonicotinoids on pollinating insects are subject to intensive scrutiny.[1,4,8,15,40,41] Four different neonicotinoids, thiametoxam, imidacloprid, clothianidin, and thiacloprid have been shown to decrease the homing success of honey bees and hence the survival of foragers[1,42,43] (probably by affecting the learning and memory functions[42,44−50]). Even so, field assessments of the effects of these pesticides on whole bee colonies have often given conflicting results with some studies documenting no effects or highly variable effects in different environments.[4,5,8,40,51]

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Here, we assessed in detail how traces of the neonicotinoid imidacloprid influenced lifetime foraging performance of honey bees to give clarity on the mechanisms by which pesticides could affect bee colonies. To document the effects of imidacloprid on the lifetime forager performance of individual bees, we used miniature Radio-Frequency Identification (RFID) transponders, or "tags", fitted on the thorax of bees that were detected at the entrance of the hive as bees come and go.[11,15] A difficulty commonly faced with this method is that young bees perform a series of nonforaging flights known as orientation flights during which they learn how to navigate before they become foragers.[52−55] This can obscure the point at which a bee transitions to foraging. Previous studies have used the age at the first exit of a bee as a proxy for the age at onset of foraging[15] or used a single flight-time threshold to estimate the point at which bees are likely to have transitioned from orienting to foraging.[11,15] Here, we developed a new analysis method to estimate from the flight pattern data the likely onset of foraging for each individual.

Using this approach, we studied how larval exposure to a trace level of imidacloprid influenced foraging performance in adult bees. Bees were exposed as larvae when colonies of European honey bees were fed 20 kg of a 5 μg/kg (ppb) imidacloprid sugar syrup for 6 weeks. Control colonies received sugar syrup only. When the bees emerged as adults, they were tagged with RFID transponders, and tagged bees from treated and control colonies were introduced together into a full-size bee commercial colony in a standard hive equipped with an RFID reader. We then analyzed the flights patterns and history of the bees to measure the number and duration of orientation flights, the age at which individual bees performed their first foraging flight, the number and duration of their foraging flights, and their longevity.

MATERIAL AND METHODS

Imidacloprid Exposure. To expose colonies to field-relevant concentrations of imidacloprid, we followed a protocol previously published several times.[5,51,56,57] In brief, a 50:50 sugar to water mix was heated to 60 °C to facilitate sugar dissolution and left aside to cool down before it was fed to the colonies in the control group. On the days of treatment, one milligram of pure analytical-grade imidacloprid (Imidacloprid PESTANAL, CAS # 138261−41−3) was dissolved in 100 mL of distilled water using a magnetic mixing bar at ambient temperature (24 °C) to obtain a stock solution of imidacloprid at 10 ppm. Using a pipet, 0.5 mL of the stock solution was then added to 99.5 mL of distilled water, and the mixture was agitated using a magnetic mixing bar. To prepare the syrup, 500 g of sugar was dissolved in 400 mL of distilled water and heated to 60 °C and left aside to cool down. Once the syrup was at ambient temperature, the imidacloprid solution was added to the syrup to obtain a 50:50 sugar to water mix with 5 μg/kg (ppb) imidacloprid, and this mixture was fed to the colonies. Colonies were fed a total of 20 kg of control or imidacloprid sugar syrup over 6 weeks, as in previous experiments,[5,51] in a black plastic in-hive frame feeder to keep the syrup in the dark. Bee colonies commonly collect between 1 and 2 kg of nectar during nectar flows at the beginning of the summer, so this amount only represents a small fraction of what colonies might gather and process over such a time period.[58−60] All the syrup was consumed by the bees within the 6 weeks. This concentration corresponds to residues of imidacloprid treatments, sometimes referred to as "trace" amounts at concentrations found in plants, bees, and hive products.[3,7,26,27,29,61−63] Imidacloprid is found at average concentrations of 10 μg/kg in sunflowers[26] and 2.1 μg/kg in maize pollen,[27] and on cotton crops, its concentration ranges between 1.6 to 64.6 μg/kg in the pollen and between 0 and 1.8 μg/kg in the nectar.[64] Chronic exposure to imidacloprid can occur within a hive through consumption of stored contaminated nectar (honey) and pollen (called bee bread). A worldwide survey of neonicotinoids in honey found imidacloprid in more than half the samples analyzed, at an average concentration of 0.35 μg/kg with a maximum concentration of 6.3 μg/kg.[62] Imidacloprid was most often found in combination with at least one other neonicotinoid.[65] In France, average concentrations of 0.7 μg/kg in honey, 0.9 μg/kg in pollen, and 1.2 μg/kg in honey bee workers were found before the 2013 restrictions on outdoor neonicotinoid use.[29,63] In a previous experiment, where bee colonies were also fed 20 kg of 5 μg/kg imidacloprid syrup over 6 weeks, the average concentration of imidacloprid and imidacloprid metabolites in bees was 0.62 μg/kg.[66] The concentration we use here to feed the colonies lies well within the range of exposure that managed bee colonies are exposed to when collecting pollen and nectar from treated crops.

Hives. On the 22nd of November 2017, at the beginning of the Australian summer, six colonies were established outdoors with full access to the outside from packages containing 1.5 kg of bees. The hives were located at Macquarie University (33°46′07.2″S, 151°06′46.8″E, New South Wales, Australia). Bees were given wax foundations in standard "deep Langstroth" hives containing seven frames and an in-hive frame feeder, and they were fed 4 L of sucrose syrup over 3 weeks on establishment. The four colonies with the largest bee population were selected and randomly assigned to the control or imidacloprid group. Hives were kept 4 m apart and decorated with regular patterns to help minimize worker drift. Entrance reducers were used to avoid robbing (no robbing was observed during the experiment). After 3 weeks of treatment, all the frames were full of capped honey stores and a top box was added to allow bees to keep ingesting and storing the syrup.

Radio-Frequency Identification Setup. One standard 8-frame single box "deep Langstroth" hive containing a fully established colony was placed indoors at Macquarie University (Sydney, New South Wales, Australia). The entrance was modified to funnel all the bees returning to the hive in one direction and all the departing bees in the other (Figure S8). The tunnels leading in and out of the hive were equipped with four RFID antennae (two on the way in and two on the way out to allow sufficient traffic for a full-size bee colony) connected to an RFID reader (Invengo XC-RF807) previously used in bee experiments.[11,67−69] Bees had full access to the outside and no access to the inside of the building, were not fed, and could only forage outside. The lid of the hive was made of an escape board (a board equipped with a narrow funnel entrance leading the bees to the bottom box) to allow us to add tagged bees without disturbing the hive.

Bee Eclosions and tagging. To verify reliability, this RFID setup was tested before the beginning of the experiments on a pretest group of 80 newly emerged bees (less than 10 h old). These bees were obtained by placing frames covered in capped brood in an incubator at 34 °C and 37% humidity overnight, and they were tagged with Radio Frequency Identification tags (RFID; Invengo Technology) which were

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Environ. Sci. Technol. 2019, 53, 8252−8261

glued to their thoraxes. The tagged bees were introduced into the hive by placing them in a vial above the escape board. The entrance board (Figure S3) was observed for 2 h a day for 11 days. Bees were observed walking in the right direction at least 93% of the time. A few bees were collected in a box placed under the entrance of the hives and seemed unable to fly possibly due to the presence of glue on their wings. No misdetections of the tags by the antennae were observed.

Between the 13 and the 15th of April 2018, one frame covered in brood was taken each day from each of the four hives treated with imidacloprid or control syrup and placed in the incubator. Bees were obtained, tagged, and introduced into the hives as described for the pretest group. All bees were successfully introduced into the hive.

Trip Classification. Bees exit the hive to perform various operations at the entrance of the hive and to perform orientation and foraging flights. We excluded all flights that were less than a minute from our data set, as they most likely recorded bees that were sometimes observed fanning under the RFID antennae. Entrances and exits of each tagged bee were collected continuously until the last bee was not seen for 5 consecutive sunny days. To classify flights as orientation or foraging flights, we analyzed daily flight patterns of individual bees of an independent data set. Twenty-nine bees from this pretest group that performed at least two flights outside the hive were used to develop a way of classifying flights as orientation flights or foraging flights. To do so, the daily flights of these bees were plotted (see File S2 for examples) and one observer was asked to indicate when there was a notable change in flight pattern when the number and duration of flights seemed to increase, based on descriptions of the orientation flights from the literature.[52,70] The days before the change in flight pattern were classified as days of orientation flights and the days after the change in pattern as days of foraging flights. The daily number of flights, total flight duration, minimum flight duration, maximum flight duration, average flight duration, and the time of first and last flights since sunset were then compared for the putative orientation and foraging periods. The total flight duration and the time of first flight were found to be the most discriminating variables between the two periods (Figure S2, Files S2 and S3). We performed a linear discriminant analysis with the function "lda" from the Mass v7.3−49 package for R[71] using daily total flight duration and time of first flight as discriminant variables and found that days of orientation flights were accurately classified 79.3% of the time and days of foraging flights were accurately classified 97.3% of the time when using these two variables alone (Files S2 and S3).

We then used these two variables to estimate the days of first foraging flights for the bees in the experimental data set. Specifically, we measured the daily total flight durations and the times of first flight for each bee in the control group and the imidacloprid group and considered that bees started foraging on the first day when they performed their first trip before 5 h after sunrise and spent more than 20 min outside the hive in total (File S1).

Statistical Analyses. All statistical analyses were conducted under R v3.4.3.

The data obtained were not normally distributed, so a Mann−Whitney test was used to compare the age at onset of foraging. For both orientation and foraging flights, the number of flights, total duration of flights in lifetime, and average duration of flights were compared with the function "wilcox.test" of "R base".[72]

A Cox proportional hazards regression model was used to compare the mortality rates of bees in the two treatment groups with the "coxph" function of the "survival" v2.41−3 package for R,[73] and the survival curves were drawn using the "survminer" v0.4.3 package for R.[74]

R scripts and data with additional detail and a polynomial model of the age to number of foraging flights relationship are provided as Supporting Information (Figures S1 and S2, Files S1−S3), results for each colony are explicitly shown in File S4 and information about colony origin is also available in the data set (File S1).

RESULTS

Two hives were fed with 20 kg of control or 5 ppb imidacloprid sugar syrup and two control hives were fed with 20 kg of sugar syrup free of neonicotinoids over 6 weeks. At the end of the treatment, frames of capped brood from these hives were placed in an incubator at 35 °C for 12 h overnight. About 295 bees from each hive were equipped with RFID tags at their emergence. All the bees were placed together in a hive with a modified entrance equipped with RFID readers. The exits and entries of individual bees were continuously recorded until the last tagged bee died. A total of 294 bees from two imidacloprid treated hives and a total of 260 bees from two control hives performed at least one trip of more than a minute and were kept for the experiment. The global flight patterns of these bees matched expectations from previous work on bee activity,[59,60,75−77] i.e., more foragers left the hive in the morning and more returned in the evening (Figure 1); orientation flights lasted 2.88 min ± 0.05 (mean ± standard error) and foraging flights lasted 21.55 min ± 0.27, and bees in the control group started foraging 9.24 days ± 4.46 after emergence.

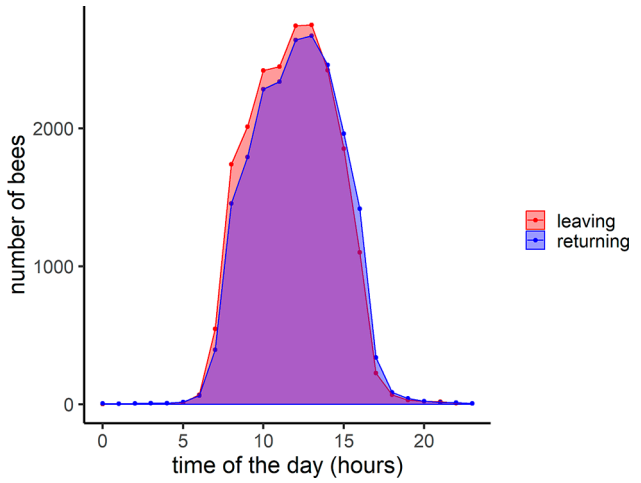

Figure 1. Number of bees departing from (transparent red) and returning to (transparent blue) the hive during the day. Light purple areas show times of day where more bees depart than return to the nest.

Using a previous, independent data set, we determined that the total flight duration and the time of first flight were sufficient to identify days of orientation flights 79.3% of the time and days of foraging flights 97.3% of the time (Figure S2, Files S2 and S3). Bees from the imidacloprid experiment were

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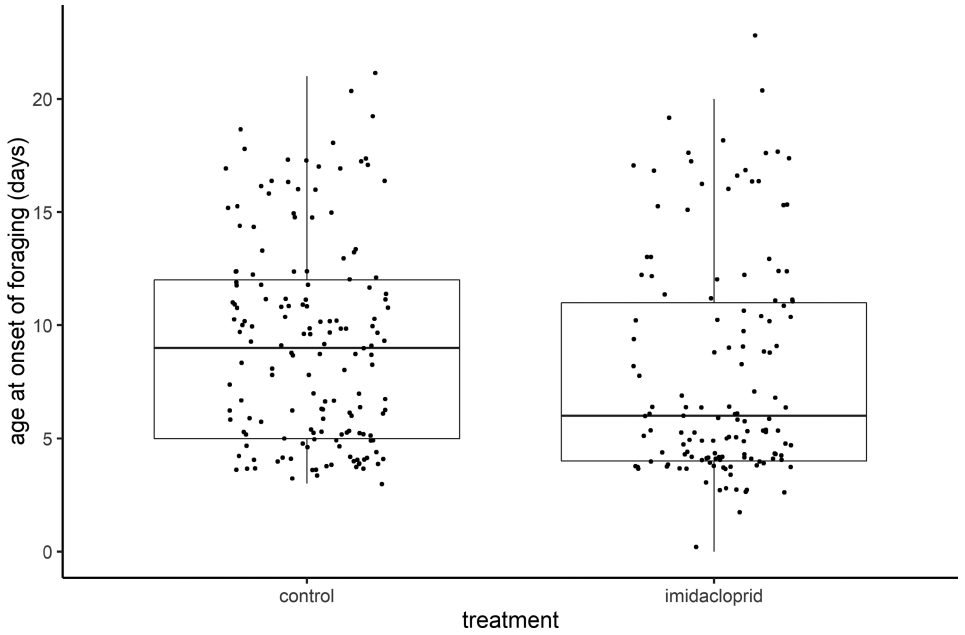

Figure 2. Age at onset of foraging in bees from hives treated with imidacloprid or control syrup. Bees in the imidacloprid group (n = 143) started foraging significantly earlier than bees in the control group (n = 161).

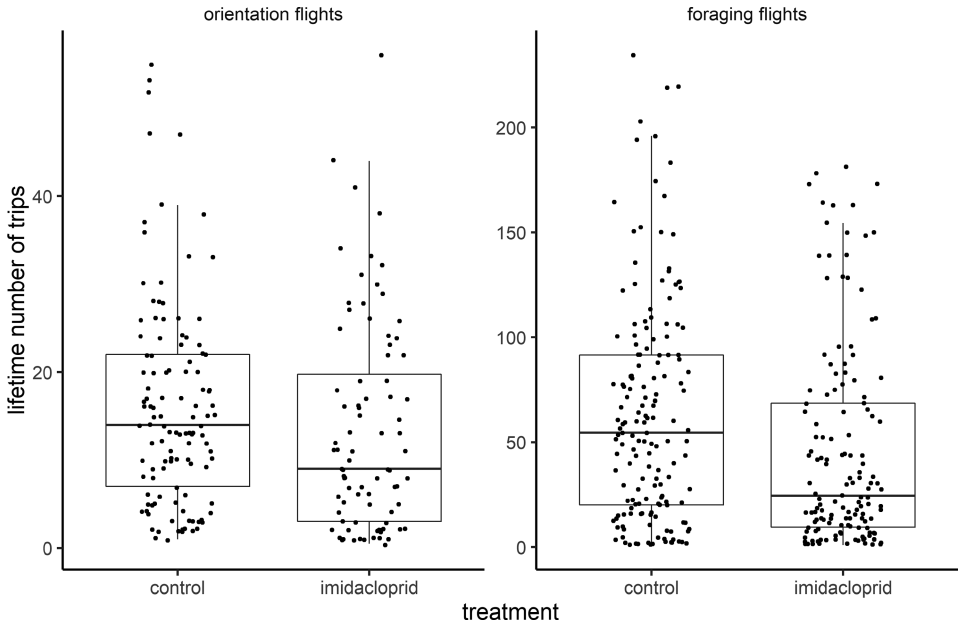

Figure 3. Number of orientation flights performed by bees from the control (n = 117) and imidacloprid (n = 83) groups, and number of foraging flights performed by bees from the control (n = 161) and imidacloprid (n = 143) groups in their lifetime (some bees performed no full days of orientation flights, and no orientation flights were thus counted for them; see Figure 6). Bees exposed to imidacloprid during their larval stage performed significantly fewer orientation and foraging flights during their lifetime. The remainder of the bees died before performing their first foraging flight (n = 100 in the control group, n = 160 in the imidacloprid group) and are not shown on the figures; these data are available in File S1.

considered to have become foragers on the first day when they started foraging within 5 h after sunrise and spent more than 20 min outside the hive in total. Foraging flights matching these characteristics were performed by 73.5% of the tagged bees in the control group and 58.7% of those in the imidacloprid group.

The bees that were exposed to imidacloprid during their larval stage transitioned from orientation to foraging flights, on average, at an age 1.38 days younger than that of bees from the control group (Mann−Whitney test, W = 13251, p-value = 0.001146) (Figure 2). Bees in the imidacloprid group performed, on average, 2.71 less orientation flights than those in the control group (imidacloprid: 13.26 ± 1.3 (mean ± standard error), control: 15.97 ± 1.01, Mann−Whitney test, W = 5780.5, p-value = 0.0331, Figure 3) and spent, on average, 8.13 less minutes performing orientation flights in their lifetime than did the bees in the control group (imidacloprid: 34.39 ± 2.79, control: 42.52 ± 2.12, Mann−Whitney test, W = 5933, p-value = 0.01226, Figure 4), but the average duration of their orientation flights was not statistically different from that of

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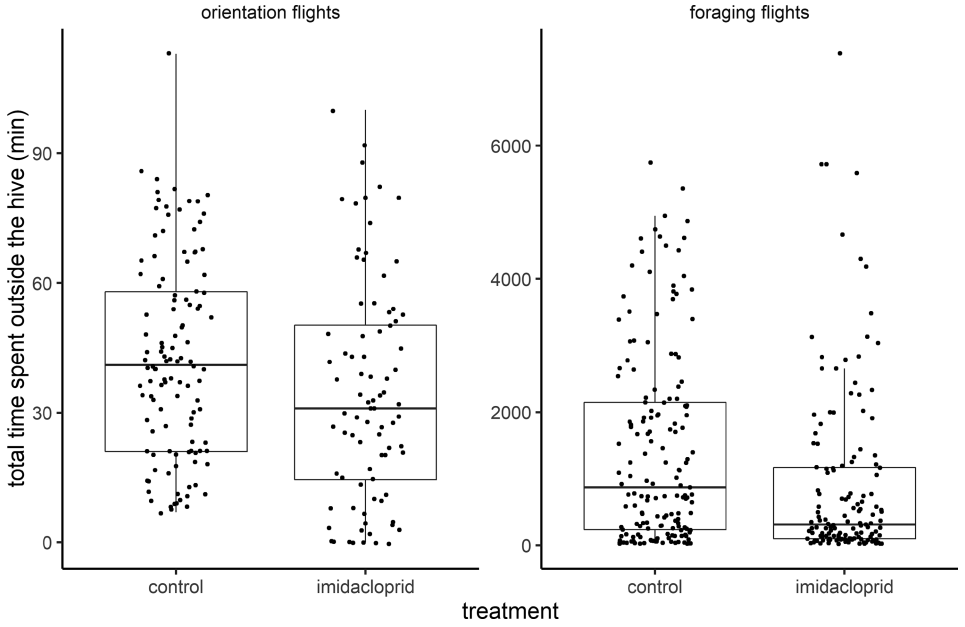

Figure 4. Total time spent performing orientation flights outside the hive by bees from the control (n = 117) and imidacloprid (n = 83) groups, and total time performing foraging flights by bees from the control (n = 161) and imidacloprid (n = 143) groups in their lifetime (some bees performed no full days of orientation flights, and no orientation flights were thus counted for them; see Figure 6). Bees exposed to imidacloprid during their larval stage spent significantly less time out of the hive during their lifetimes for both orientation and foraging flights. The remainder of the bees died before performing their first foraging flight (n = 100 in the control group, n = 160 in the imidacloprid group) and are not shown on the figures; these data are available in the S1 File.

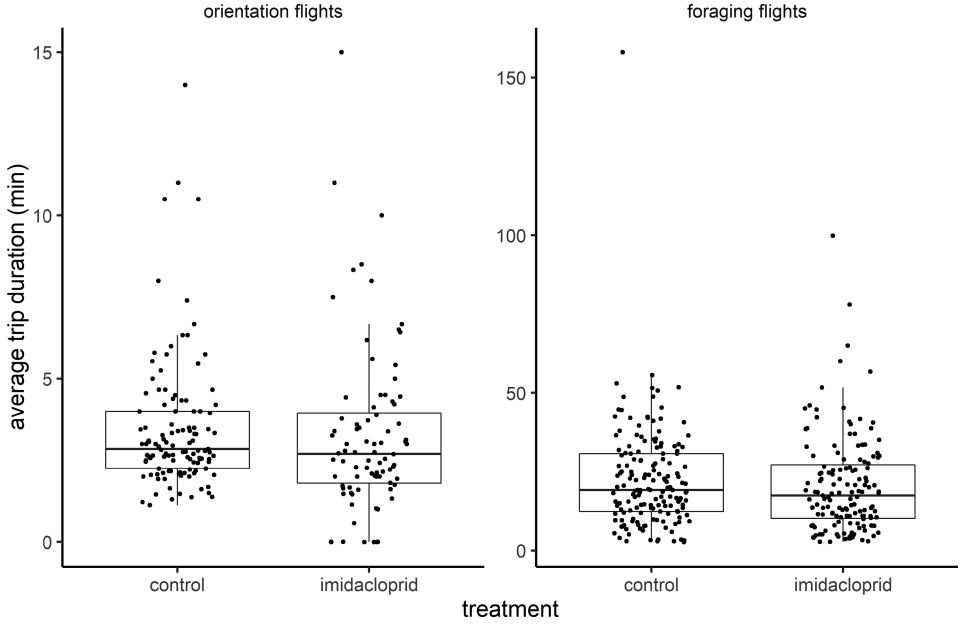

Figure 5. Average trip durations of orientation flights, performed by bees from the control (n = 117) and imidacloprid (n = 83) groups, and of foraging flights, performed by bees from the control (n = 161) and imidacloprid (n = 143) groups in their lifetime (some bees performed no full day, of orientation flights, and no orientation flights were thus counted for them; see Figure 6). There were no significant differences between treatment groups. The remainder of the bees died before performing their first foraging flight (n = 100 in the control group, n = 160 in the imidacloprid group) and are not shown on the figures; these data are available in the S1 File.

bees from the control group (imidacloprid: 3.27 ± 0.28, control: 3.47 ± 0.19, Mann−Whitney test, W = 5533, p-value = 0.1283, Figure 5).

Bees that started foraging at a younger age performed fewer foraging flights in their lifetime (Figure 6), which could explain why bees exposed to imidacloprid during their larval stage performed, on average, 17.86 less foraging flights than those in the control group (imidacloprid: 45.86 ± 4.06, control: 63.72 ± 4.15, Mann−Whitney test, W = 14127, p-value = 0.0006, Figure 3) and spent, on average, 527.33 less minutes foraging in their lifetime than did those in the control group (imidacloprid: 910.28 ± 111.26, control: 1437.61 ± 114.62, Mann−Whitney test, W = 14438, p-value = 0.0001308, Figure 4). Bees in the imidacloprid group tended to perform shorter

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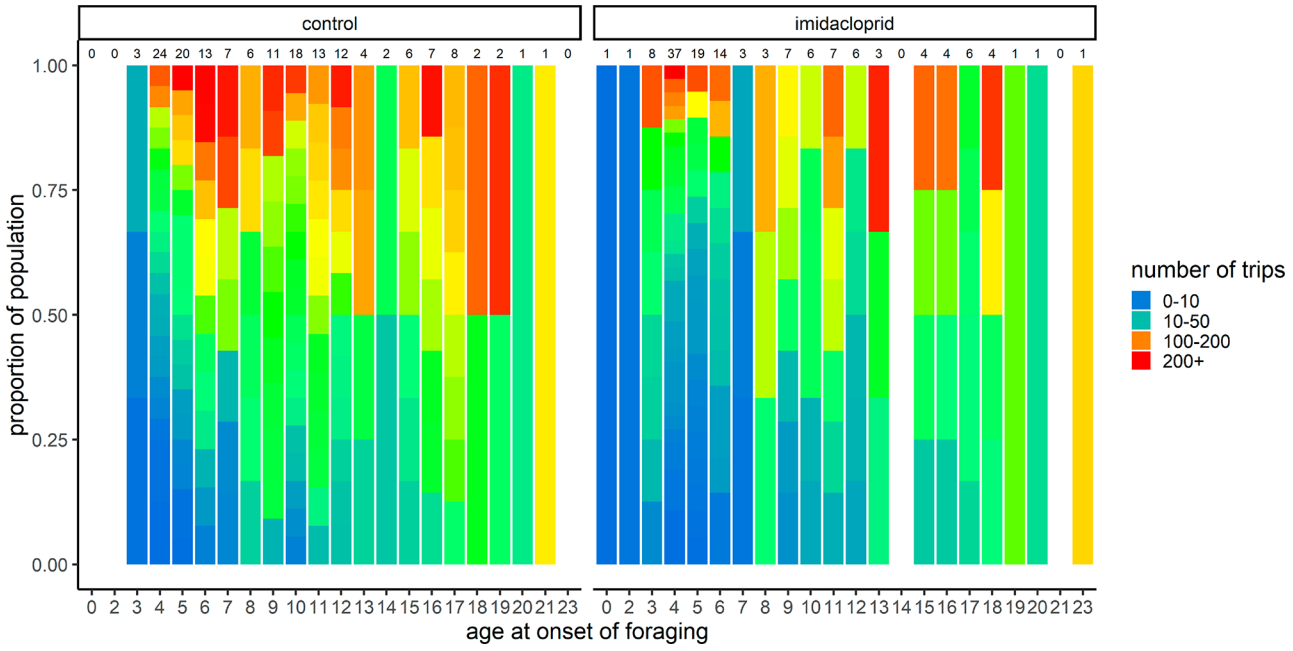

Figure 6. Proportion of the bee population that performed a certain number of foraging flights by the age at which they started foraging. Numbers at the bottom of the bars indicate the age, and those at the top indicate the sample size for each age. The sample size corresponds to bees that survived the orientation phase and then performed at least 1 day of foraging flights. Ages at which no bees started foraging are not represented (day 1, day 22).

foraging flights, but this was not statistically significant (imidacloprid: 20.53 ± 1.29, control: 22.68 ± 1.30, Mann−Whitney test, W = 12826, p-value = 0.08596, Figure 5). Mortality in the imidacloprid treated group was also higher than that in the control group (median survival [0.95% confidence interval]: control: 10 [9−12], imidacloprid: 8 [7−9]; β coefficient imidacloprid = 0.226, hazard ratio = 1.254, standard error = 0.085, z = 2.814, p = 0.00489) (Figure 7).

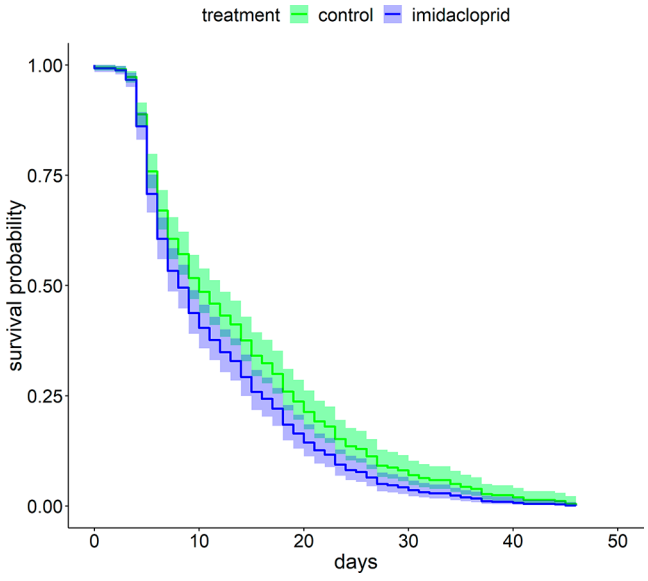

Figure 7. Survival probability of individual honey bees exposed as larvae in the imidacloprid (blue) and control (green) treatments. Adult bees exposed to imidacloprid during their larval stage died significantly faster than bees in the control group.

DISCUSSION

We found that larval exposure to imidacloprid, at a concentration comparable to the residues found in the nectar of treated crops, had dramatic consequences for adult foraging performance. Imidacloprid exposure accelerated foraging onset by 15% from 9.24 days in control bees to 7.8 days, on average, in imidacloprid exposed bees. In addition, imidacloprid exposed bees performed fewer orientation flights (Figures 2 and 3). Imidacloprid exposed bees performed, on average, 28% fewer foraging flights than those in the control group (Figures 3 and 6), a reduction similar to that found for the neonicotinoid thiacloprid.[43] These changes in the age at onset of foraging and foraging performances of bees were similar in magnitude to those in a drastic manipulation involving the removal of the entire population of foragers from a hive.[11]

Here, we successfully differentiated orientation flights from foraging flights by analyzing changes in the daily flight patterns of individual bees (Figures S2 and S3, Files S1−S3). This solves a limitation faced by previous studies that estimated likely foraging onset on the basis of flight-time thresholds derived from the existing literature.[11,52−54,77,78] We believe this is an important methodological advance since orientation is a crucial phase for all nesting insects that need to navigate back to a stationary nest.[79] Our analysis method of the RFID data revealed that imidacloprid reduced the number of orientation flights. Since these bees had less time to learn their environment prior to starting foraging, fewer imidacloprid treated bees successfully transitioned to become foragers (58.7%) than those in the control group (73.5%).

An early onset of foraging reduced the life expectancy and foraging efficiency of honey bees. Irrespective of treatment, most of the bees performing foraging flights within their first 5 days after eclosion performed less than 15 foraging flights in their lifetime, while others performed more than a hundred

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(Figure 6). Larval imidacloprid exposure significantly accelerated foraging onset (Figures 2 and 6). This suggests that imidacloprid residues, fed to colonies at a concentration of 5 μg/kg, have the potential to cause rapid failures of bee colonies by imbalancing the age-based division of labor in a colony and progressively reducing the effectiveness of the foraging force by increasing the population of precocious foragers within it.[11]

It had already been proposed that neonicotinoids could affect the age-based division of labor in bee colonies.[1,15] Our data now show the extent to which imidacloprid treatment accelerates the phase during which bees perform orientation flights, the consequences of this for successful foraging onset, and the reduced performance of foragers. Our data might predict severe consequences for colonies from even trace imidacloprid residues, but we recognize that in field studies, such consequences have not always been documented at similar concentrations.[4,5,8,51] This may be because the social dynamics of a bee hive are complex and nonlinear and incorporate a capacity to "buffer" stress to a degree, with little measurable consequence to colony performance.[15] Models and empirical data have emphasized how colony outcomes are influenced by multiple factors, including season, resource base, and colony size.[10,11,87−89,60,80−86] Further, we are now beginning to realize how different stressors can interact to have synergistic consequences for colony outcomes.[7,77,90−93] Even so, a better understanding of the processes by which pesticides impact colony function will allow us to improve current models and make better recommendations for colony interventions. From this perspective, it is interesting that two non-neonicotinoid pesticide blends that delayed rather than accelerated the age at onset of foraging increased the survival rates of these bees.[77] Pesticides are often applied as blends on crops, and bees are commonly and simultaneously exposed to a variety of fungicide, insecticide, and miticide combinations.[8,77,93] These findings suggest that it may be possible to develop pesticide combinations that are less likely to have negative synergistic or additive effects on bee colonies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02452.

Data set with flights inferior to a minute removed and R script (ZIP)

Examples of a typical orientation to foraging flight change patterns (ZIP)

Data set and R script for the discriminant analysis, PCA and classification of orientation and foraging flights (ZIP)

Number, average duration, and total time spent on orientation and foraging flights by bees with information about their colonies of origin (ZIP)

Number of flights per age for each forager (PDF)

Flight characteristics used to classify orientation and foraging flights (PDF)

Entrance of the hive equipped modified to fit four RFID antennae (PDF)

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Author Contributions

T.C., A.B.B., and X.W. conceived and planned the experiments. T.C. and X.W. carried out the experiments. T.C., A.B.B., and W.G.M. contributed to the interpretation of the results. T.C. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Notes

The authors declare no competing financial interest.

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