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Honey Bees' Behavior Is Impaired by Chronic Exposure to the Neonicotinoid Thiacloprid in the Field

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Supporting Information

ABSTRACT: The decline of pollinators worldwide is of growing concern and has been related to the use of plant-protecting chemicals. Most studies have focused on three neonicotinoid insecticides (clothianidin, imidacloprid, and thiamethoxam) currently subject to a moratorium in the EU. Here, we focus on thiacloprid, a widely used cyano-substituted neonicotinoid thought to be less toxic to honey bees and of which use has increased in the last years. Honey bees (*Apis mellifera carnica*) were exposed chronically to thiacloprid in the field for several weeks at a sublethal concentration. Foraging behavior, homing success, navigation performance, and social communication were impaired, and thiacloprid residue levels increased both in the foragers and the nest mates over time. The effects observed in the field were not due to a repellent taste of the substance. For the first time, we present the necessary data for the risk evaluation of thiacloprid taken up chronically by honey bees in field conditions.

INTRODUCTION

Bees, including honey bees, bumble bees, and solitary bees, represent the most prominent group of pollinators worldwide and contribute largely to agriculture because 35% of the food crop production depends on them.[1] The recent loss of pollinator populations can be attributed to multiple factors such as habitat loss and fragmentation, colony management, bee pests and parasites, and additional environmental and anthropogenic elements. Doubtlessly, the use of pesticides for crop protection contributes to the loss of pollinator abundance, both at the species level and for the quantity of a particular species.[2−4] It has also become evident that neonicotinoids (and other insecticides like fipronil) play a crucial role as the promoters of pathogen and parasite infections that effectively drive colony losses.[5−7] Thanks to their systemic properties, neonicotinoids are present in the pollen and nectar and will thus be continuously collected by pollinators for as long as flowering persists. They are agonists of nicotinic acetylcholine receptors (nAChR), which are normally activated by the neurotransmitter acetylcholine.[8] Nicotinic synaptic transmission is a major component of neural integration in the circuits related to sensory integration and functions related to the mushroom bodies, mediating multisensory integration, learning, and memory formation.[9,10] Neonicotinoids negatively affect the mushroom bodies' physiology[11] and function[12] in honey bees. It was already proven that neonicotinoids compromise olfactory learning[13] as well as the ability of worker bees to forage and to communicate.[14−17] The toxicity of sublethal doses is also expected to be reinforced over time.[18,19] However, a detailed analysis of the chronic exposure to thiacloprid on foraging, navigation, and social communication is lacking.

The cyano-substituted neonicotinoid thiacloprid is declared less toxic to bees than nitro-substituted compounds like imidacloprid and thiamethoxam.[20−23] The formulations based on thiacloprid are registered and sold in more than 70 countries worldwide[24] and act against sucking and chewing pest insects that target more than 50 crops.[25,26] The formulations based on thiacloprid are used in the field for spraying treatments at application rates much higher than for the three neonicotinoids suspended in Europe.[21,27] These formulations are allowed to be sprayed during flowering because less damage to pollinators is expected. Thiacloprid is also used in a maize seed treatment since the withdrawal of clothianidin and thiamethoxam on maize across Europe in 2013.

Toxicity tests performed by the company at the time before releasing thiacloprid on the market evaluated only the short-term and lethal effects on worker honey bees. In contrast to acute effects, no standardized protocol exists for measuring chronic effects on individual bees under seminatural conditions.[23] The value of tests on single animals has been questioned because a whole colony may be more robust to

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pesticide exposure.[29] However, honey bees are acting as single animals during foraging; they need to adjust their behavior to the changing availability of food sources, return to the colony for survival, deliver the collected food, and communicate with other foragers. Therefore, testing single foraging honey bees represents best conditions faced by honey-bee foragers and other insect pollinators in nature. A few lab studies have shown that chronic exposure to sublethal doses of thiacloprid affects honey bees' sensitivity to the gut pathogen *Nosema cerenae*,[30−32] and a field study has shown that navigation is compromised when thiacloprid was given as a single acute dose.[33] Chronic and sublethal exposure to the substance is the most likely exposure scenario in the field,[26,34] but no field study to our knowledge has yet uncovered any specific behavioral effect of such condition of exposure. In our experiments, honey-bee foragers were exposed chronically for several weeks in the field to a concentration similar or lower to those used in previous chronic-exposure studies with thiacloprid.[30−32] The concentration of thiacloprid in the contaminated sucrose solutions was 5.4 ng/μL, whereas the concentration of thiacloprid in the Calypso formulation directly sprayed on plants and flowers at a distance of 30 to 40 cm is 150 ng/μL.

Because most of the collected sucrose solution will be deposited by the forager inside the hive, and only a small proportion will be taken up and metabolized by the bee during its return flight from the feeder to the hive; only a small amount of thiacloprid will reach the brain and interfere with nicotinic synaptic transmission.

We found that a chronic exposure to thiacloprid significantly impaired honey bees' foraging behavior, communication, and navigation. The substance increased in the foragers over time, also affecting the animals indirectly exposed in the colony. We found no avoidance of our preference to the substance, supporting the idea that a neural impairment was responsible for affecting the honey bees' abilities to forage, communicate, and navigate rather than a repelling effect.

MATERIALS AND METHODS

Preparation of the Solutions. Stock solution: 10 mg of thiacloprid ([3-[(6-chloro-3-pyridinyl) methyl]-2-thiazolidinylidene] cyanamide, Sigma-Aldrich Pestanal) diluted in 1 mL of acetone (≥99.9%, Sigma-Aldrich) plus 39 mL of distilled water leading to a concentration of 0.25 g/L. Acetone was chosen as the solvent following the EPPO guidelines.[35] The final concentration of acetone (0.05%) in the contaminated sucrose solutions was shown to not have an effect on honey bee navigation.[33] The thiacloprid sucrose solutions used in the field (0.02 mM, 4.5 ppm) as well as for the taste and choice experiments (0.025 mM, 5 ppm) were freshly made every morning from the stock solution. The concentration of thiacloprid at the treated feeder was always the same regardless of the sucrose-solution concentration. The concentration of the solutions used were confirmed by liquid chromatography−tandem mass spectrometry (LC−MS/MS; see Methods S1).

Field Experimental Design. The experimental area is a highly structured agricultural landscape (trees and bushes, pathways, creek, grass fields, etc.) nearby Großseelheim, Germany. A total of two colonies housed in two observation hives (West Seip, Bienenzuchtgerätefabrik) were put up on two opposite sides of a cabin at the western border of the experimental area (50°48′51.9″ N). Each colony of *Apis mellifera carnica* was equipped with one comb of sealed brood plus newborn bees and one comb of food (Deutsch Normal Mass combs) originating from the same honey-bee colony. The queens were kindly provided by the Bieneninstitut Kirchhain; they derived from selected breeder colonies of the carnica breeding population of the institute. They were open-mated and 1 year old. Sister queens were used in an attempt to keep the genetic difference among the honey-bee individuals from each colony at a low level.

Training to the Feeders. A pair of feeders (F1 and F2) were established 350 m northeast and 340 m southeast, respectively, and were separated by an angle of 90° as seen from the cabin. The release site (RS) was located 780 m east of the cabin. A group of foragers from each of the two colonies was trained to its respective feeder and marked individually with number tags. The origin of each newly marked bee from the two colonies was controlled at the respective hive entrance. In experiment 1, one group of bees (treated group) foraged during 19 days on a sucrose solution containing thiacloprid (4.5 ppm), and the other group (control group) foraged over the same time at a feeder containing only sucrose solution. In experiment 2, the control hive became the treated hive, and the treated hive was removed and replaced by a new control hive. The feeders' locations were exchanged between experiments 1 and 2 to exclude any possible landscape effect related to the feeders' position. In experiment 2, the two groups of foragers were feeding at their respective feeder during 29 days. Each feeder was placed in a little wooden box to allow the counting of the entrances and exits of foragers with a retroreflective sensor (Baumer GmbH). The total number and the identity of bees visiting their feeder throughout each day was known as well as the amount of sucrose solution consumed at both feeders. The concentration of the sucrose solution at each feeder was adjusted during the day to regulate the traffic at the feeder (25−40 bees) following the evaluation by the experimenter of the number of trained foragers visiting the feeder. Dance recruitment was induced 19 times on 19 different days (time: 1500−1700 h) by first halving the sucrose concentration at both feeders for 1 h and then increasing it 2-fold for another hour.

Homing Experiment. Colonies were settled in the field for at least a week before the homing experiments started. After a certain number of days foraging at the feeders, single bees were caught on their departure at their respective feeder and transferred into a glass vial after they had freely drunk either a 1 M sucrose solution (control bees) or a 1 M sucrose solution containing 4.5 ppm thiacloprid (treated bees). They were kept in the dark for 45 min while they were transported to the release site. Next, a transponder was fixed to the thorax, and the bee was released (time: 1100−1700 h, temperature: 17−30 °C, wind <15 km/h). No release was made when the sky was evaluated too cloudy or totally overcast, nor when it was raining. Care was taken that the number of control and treated bees released every day were evenly distributed, and it was ensured that each bee was released only once. The radar was shut down not before 120 min after the last bee was released if the bee was not yet back to its hive. Because none of the bees that did not return to the hive after being released were seen at the feeder or at the hive entrance on the same or the following days, we assume that they died in the field.

The method used for tracking bees with a harmonic radar system has been described before.[36−38] The transponders were built by ourselves following the procedure from Riley et al. (1996); their attachment and carrying by the bees do not alter honey bees' flight behavior.[39,40] The flights of the released bees

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carrying a transponder were monitored using the radar system over a distance of up to a 900 m radius and at a temporal resolution of 1/3 Hz.[37]

Electric-Field Recordings. The electric fields emitted by dancing bees[41] consist of low-frequency (movements of the abdomen, 16 Hz on average) and high-frequency (buzzing of the wings, 230 Hz) components synchronization, leading to an average of three to seven electric pulses per waggle. The distance from the hive to a feeding site is encoded in the number of waggle runs, and 1 s is known to represent a distance of about 1 km.[42] The feeders were located 350 m northeast (F1) and 340 southeast (F2) of the hives, and because very few natural food sources existed in the experimental area and none of them were present at the same distance as the feeders, the distinction between dances from trained and untrained foraging bees was possible. Electric-field measurements were performed at the same time on both sides of the lower comb in the control and treated hives using four copper wires with a silver coating positioned in the dance area (12 cm^2 covered), connected on each side to a stereo amplifier (USB Soundbox 7.1, Conrad electronics SE) with a sample rate of 44.1 kHz. Each amplifier was connected to a laptop, and the software Presonus Studio One (version 2.4) was used for saving the data as WAV files. We recorded, in total, 340 h of electric fields on 32 different days (average of 2.67 h per day).

Thiacloprid Residues Analysis. Bees were caught at their feeder after foraging for a certain number of days and after they had filled their crop with a 1 M sucrose solution (contaminated or not). They were then kept in the dark for 45 min before being killed by chilling and put into a −20 °C deep-freezer. We also collected unmarked forager bees at the entrance of the treated and control hives when flying out on a foraging trip to assess the in-hive contamination of foragers not visiting the feeders but exposed indirectly to thiacloprid inside the hive via the stored food. See Methods S1 for details about the residue analysis by LC−MS/MS.

Repellent Effect. *Proboscis Extension Response Experiment.* The proboscis extension response (PER) experiment was used to sample hungry bees' sensitivity to varying concentrations of sucrose[43,44] with or without thiacloprid (5 ppm). Honey bees were captured at 1400 h when leaving the hive, immobilized by chilling, and mounted in small brass tubes that restrained body movements but allowed the antennae and the mouthparts to move freely.[43] A total of 1 h later, they were tested in the laboratory by the touching of both antennae with a droplet of ascending concentrations of sugar concentrations (dry sugar diluted in tap water +0.05% acetone, 0.1%, 0.3%, 1%, 3%, 10%, 30%, and 50%, w/v). Only the bees that showed a PER for the 50% sugar concentration were considered as the nonresponders (control: 1/74, treated: 3/74) were considered physically unable to extend their proboscis.

Choice Experiment. In May, a group of bees was trained to a training and feeding platform located about 30 m from the hive. The platform was composed of a yellow background and 10 blue squares randomly distributed and containing a mini-feeder from which the bees could freely drink a 1 M sucrose solution. The test platform contained only six mini-feeders. During the testing of single bees, three feeders contained 8 μL of a 1 M control sucrose solution each, and the other three contained 8 μL of a 1 M sucrose solution with thiacloprid (5 ppm) each. The positions of the control and treated mini-feeders were randomly allocated on the platform. The number of feeders drunk and the time a bee took to drink at each of the six feeders was recorded. At the end of the test, the bee was killed, and the same test was repeated with a new naive bee.

Flight Tracks and Statistical Analysis. From the x and y coordinates collected by the radar, the length and duration of the flight from the first to the last signal was calculated. The x and y coordinates were fitted into a Google Map scaled in meters using CorelDraw.X5. The criteria used to categorize the different flight parameters were arbitrarily defined. A "vector flight" was considered as such when fitting into an angle of 45° as seen from the release site (±22.5° each side of the feeder-hive vector direction; F1: 313°, F2: 222°) and had a minimal length of 200 m. The angle of a vector component is the angle between the crossing point of the vector track with the 200 m circle around the release and the direction toward the north. The criterion "pass close to F" and "Return to RS" was attributed, respectively, to bees getting closer than 100 m from their feeder or from the release site during their flight.

The electric field data were transformed to SMR files, preliminary filtered in Spike 2 (version 8.03) and further analyzed using custom-made programs written in Visual Basic 2013 (Microsoft). An amount of 6 ± 2 waggles per run (about 360 ± 120 m) was used as a criteria to select the dances indicating the location of the feeders. If the number of waggles per run was exceeding this range, the waggle runs were attributed to the "other bees" group.

For the statistical analysis of the data, we used R and Prism 5 and 6. The normality of the data was tested using the D'Agostino−Pearson omnibus test. If the data were normally distributed, we used a paired−unpaired *t*-test or an analysis of variances with Tukey's posthoc tests. Otherwise, nonparametric tests were performed (Mann−Whitney test and Wilcoxon signed rank test). The Fisher's's exact test was used to compare proportions. For the PER data, we performed a mixed-effects logistic regression in R (lme4 package) with "Bee" and "Date" as random effects to account for the difference between individuals and the date. This was followed by overall likelihood ratio tests and Tukey's posthoc tests (multcomb package). The Wheeler−Watson test was used to calculate the angular distribution of the vector components. The survival analysis was conducted using censored Kaplan−Meier log-rank in R, and the influence of multiple variables was investigated using a Cox-regression model. The numbers of bees tested for each experiment and the test groups are indicated in the legends of the figures and in the text.

RESULTS

Compromising of Honey Bees' Foraging Behavior and Dance Communication by Chronic Exposure to Thiacloprid. The total foraging span of honey bees foraging at the control feeder was significantly longer than the foraging span of honey bees foraging at the treated feeder (Table 1, Kruskal−Wallis, $P < 0.0001$). Control bees foraged at their feeder an average of 0.78 days longer than treated bees ("total", Table 1). The significance was different between the groups according to the experiment (see Table 1).

Sucrose consumption at the control and treated feeder was significantly different in both experiments (Paired *t* test, $P < 0.0001$). Control bees consumed 1.7 times more sugar solution per day than treated bees (Table S1). The average amount of thiacloprid collected per bee and per day at the treated feeder was estimated at 12118 ± 900 ng in experiment 1 and 10990 ± 833 ng in experiment 2 (Table S1). Treated bees performed on average 1.8 times and 1.4 times fewer foraging trips per day

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Table 1. Foraging Span in Days of the Trained Honey Bees at the Control or Treated Feeder

	experiment 1	experiment 2	total[b]
control	5.21 ± 0.32 (n = 67)[c] [a]	4.19 ± 0.24 (n = 72) [a]	4.68 ± 0.20 (n = 139)
treated	4.7 ± 0.22 (n = 79) [a]	3.34 ± 0.14 (n = 111)[c] [b]	3.91 ± 0.13 (n = 190)

[a]Numbers shown are means (days foraging) ± SEM. [b]Mann−Whitney, $P < 0.01$. [c]The control group in experiment 1 and the treated group in experiment 2 correspond to the same colony, as the control colony in experiment 1 became the treated colony in experiment 2 and continued to forage at the same feeder (F1). [d]Different letters indicate significant differences (post-hoc tests with Bonferroni correction): a and b (exp 2), $P < 0.05$; a and b (treated), $P < 0.001$; a and b (F1), $P < 0.001$.

than the control bees in experiment 1 and 2, respectively. On one trip, we estimate that a bee collected, on average, 216 ng of thiacloprid (40 μL of solution). The total amount of thiacloprid metabolized by a bee per day during the return flights to the hive ranges between 141 and 212 ng (Table S1). This calculation is based on the data related by Rortais et al.[45] that a bee needs 8−12 mg of sugar per hour to fly[45,46] and on our measurements (treated bees collected on average 1 M sucrose solution and flew on average 2 min from the feeder to the hive).

The reduced sugar consumption is linked to a reduced visitation rates of foragers at the contaminated feeder. Indeed, treated bees visited their feeder significantly less frequently than the control bees, and higher sucrose concentrations were needed at the contaminated feeder to keep the bees visiting the feeder (Figure 1 a). The median sucrose concentration used for

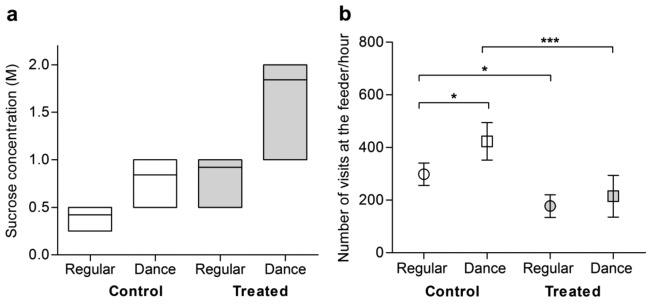

Figure 1. Required sucrose concentrations and foraging activity at the control and treated feeders. (a) Sucrose concentrations used to keep a similar number of foragers coming regularly to the control and treated feeders and to induce dances. Lower sucrose concentrations were required for control bees than for treated bees. (b) Mean (±95% confidence limits) number of visits per hour recorded on the same days (n = 19) at both feeders during regular foraging (circles) and during dance induction (squares). The foraging behavior of the treated bees (filled marks) as well as their ability to recruit new untrained foragers are significantly reduced (ANOVA, $F_{3,72} = 14.01$, $P < 0.0001$ and Tukey post-hoc tests). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

regular foraging was 0.5 M at the control feeder and 1 M at the treated feeder. Recruitment of foragers via the waggle dance was induced by raising the sucrose concentration at the feeder.[42] First, the sucrose concentration at both feeders was reduced to half of the current concentration for 1 h, and then it was increased 2-fold for another hour. Sucrose concentrations as high as 2 M during dance induction did not significantly increase the traffic at the treated feeder (ANOVA, $F_{3,72} = 14.01$, $P < 0.0001$), whereas a median concentration of 1 M increased significantly the number of visits at the control feeder ($p < 0.05$; Figure 1b).

Reduced recruitment at the feeder could indicate less waggle dances or compromised dance performance. Therefore, we monitored and estimated the number of waggle runs performed by the dancing bees in both colonies, taking advantage of the fact that waggle dances can be measured by the temporal modulation of the electrostatic field emanating from the dancing bee.[41] The number of waggles performed by the bees trained to the control feeder was significantly higher than those of the bees trained to the contaminated feeder (Figure 2;

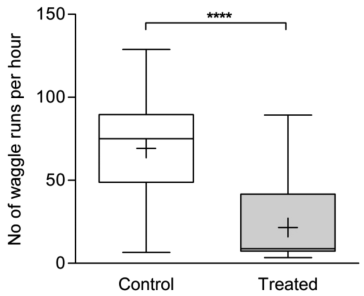

Figure 2. Number of waggles runs performed by the trained bees from the control and the treated feeders. The number of waggles runs per hour was obtained from electrostatic-field recordings performed on the same days in both hives (n days =32). The mean number of waggles runs per hour is represented with a cross in the box-plots, and it was found to be significantly higher for the bees foraging at the control feeder than for the bees foraging at the contaminated feeder (Wilcoxon signed rank test, $p < 0.0001$).

Wilcoxon signed rank test, $p < 0.0001$) although the sucrose concentration during dance induction was higher at the contaminated feeder (Figure 1a). Indeed, honey bees foraging at the control feeder performed on average 3.2 times more waggles per hour than honey bees foraging at the treated feeder. The reduced dance activity of treated bees explains the lower foraging activity at the contaminated feeder.

We also differentiated dances for feeders and dances to unknown natural food sources on the basis of the number of waggle runs as indicators of distance to the respective food source.[41,42] We found significantly lower dance activity advertising for natural food sources in the treated colony (Figure S1), indicating that the accumulation of thiacloprid inside the colony also affected bees that did not forage at the contaminated feeder but were feeding on contaminated stored food.

No Repellent Effect of Thiacloprid. One explanation for lower foraging activity found in treated bees could be an aversive taste of the substance in contaminated sucrose solution. In the laboratory experiment, we tested the proboscis extension response of hungry foragers to water and seven different sucrose concentrations (0.1%, 0.3%, 1%, 3%, 10%, 30%, and 50% w/v) with or without thiacloprid (5 ppm; Figure 3). No difference was found in the PER of bees stimulated either with the control sucrose solutions or with the contaminated sucrose solutions (logistic regression with random effects "Bee" and "Date"; sugar concentration ×

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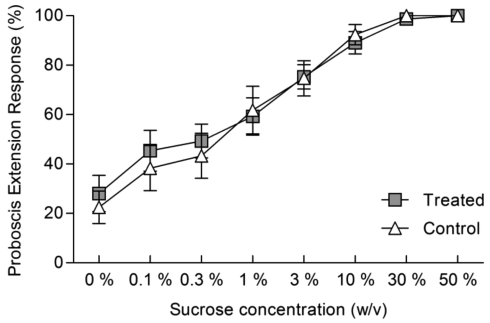

Figure 3. Proboscis extension response (PER) to different sucrose concentrations with (treated) or without (control) 5 ppm thiacloprid. $N_{control}$ = 73. $N_{treated}$ = 71. No difference was found between the two groups (logistic regression with random effects; sugar concentration × treatment: χ_6^2 = 2.5224, P = 0.866).

treatment: χ_6^2 = 2.5224, P = 0.866). The results of the Tukey's posthoc tests between the control and treated groups for each of the different sucrose concentrations tested can be found in Table S2.

In the free-flight experiment, 45 bees had to choose between feeders containing a 1 M sucrose solution with or without thiacloprid (5 ppm). No significant difference was found in the visitation rate of the bees to the control (64%) and contaminated (65%) feeders (n = 135 feeders, Fisher's exact test, P = 0.8989). The average (±SEM) drinking time per bee and feeder was 6.88 ± 0.27 s at the control feeders and 7.37 ± 0.36 s at the contaminated feeders (no significant difference, Mann−Whitney, P = 0.5578). These results rule out the possibility that thiacloprid has a repellent taste for honey bees.

Increase of Thiacloprid Residue Levels in Foragers. The amount of thiacloprid in bees foraging at the contaminated feeders in experiments 1 and 2 was analyzed by LC−MS/MS (Methods S1). Figure 4 shows how it accumulated in different

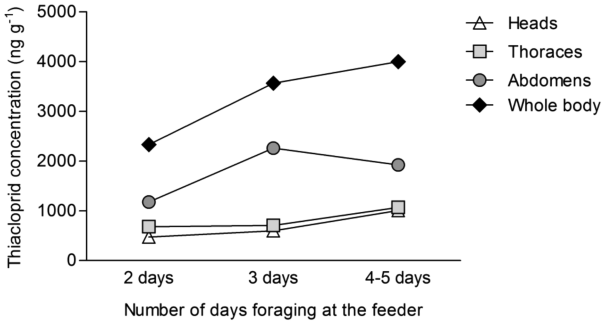

Figure 4. Accumulation of thiacloprid residue in heads, thoraces, abdomens, and in the whole body (representing the sum of the measurements) of honey bees foraging at the contaminated feeder over time. Honey-bee foragers were collected at the end of 2, 3, or 4 days of foraging after they had filled their crop at the feeder containing thiacloprid (4.5 ppm). A total of 10 bees per foraging group were used.

body parts over time. The amount of thiacloprid residues found in bees can be seen as the status of intoxication at the moment a bee is released with a transponder after foraging chronically during 2, 3, or 4 days at the contaminated feeder.

The length of exposure of the foragers at the contaminated feeder, as well as the amount of thiacloprid collected, is related to the amount of residues found in the bees (Figure 4 and Table S3). The more foraging trips honey bees performed to the treated feeder in a certain number of days, the higher the cumulated amount of contaminated sucrose solution collected and the higher the amount of thiacloprid residue found in the bees. Only a fraction of the cumulated total amount of thiacloprid collected by the bees at the feeder will be metabolized, and most of this uptake will happen during their return flights from the feeder to the hive. This fraction was found very close to the amount of thiacloprid residues found in bees after a defined number of days foraging at the contaminated feeder (Table S3).

In-hive contamination was assessed by collecting unmarked forager bees at the entrance of the treated hive when flying out on foraging trip. Thiacloprid was found in these bees but at much lower amounts than in the foragers trained to the contaminated feeder (Table S3). Indeed, these foragers did not visit the contaminated feeder, but they were exposed to thiacloprid inside the hive via the food collected and stored by the foragers visiting the contaminated feeder. Because their waggle dance activity was significantly reduced (Figure S1), even these low levels of thiacloprid impaired social communication.

Impairment of Honey Bees' Homing Success and Navigation Performance. Navigation requires the integration of multisensory cues and the retrieval of appropriate memory about the landscape structure. We tested the navigation abilities of the bees trained to feeder 1 and 2 during experiments 1 and 2. We found that treated bees returned to their hive at a significantly lower proportion than control bees (Figure 5; homing success: control 91.76% and treated 76%;

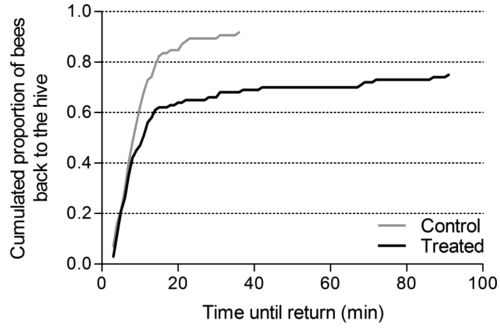

Figure 5. Probability of homing success as a function of time until return. Treated honey bees returned to their hive at a significantly lower proportion than control bees ($n_{treated}$ = 100, 76% return; $n_{control}$ = 85, 91.76% return; Fisher's exact test, P < 0.01). The origin of the temporal axis represents the moment of release.

Fisher's exact test, P < 0.01). Based on the crop-emptying measurements by Fournier et al.,[47] we calculated that the foragers released with a transponder could have assimilated in 45 min up to 7 μL and, thus, 38 ng thiacloprid in addition of the residues already assimilated over n days foraging at the feeder. This value is a higher estimate because the amount of assimilated sucrose during the 45 min waiting time may well be much lower depending on the activity of the waiting bee.[48] In any case, the partial acute treatment component involved in the navigation experiments adds to the chronic effect.

A survival analysis was conducted on the data, and a significant influence of thiacloprid on honey bee homing success was found (Kaplan−Meier log-rank test, χ_1^2 = 12.9, P < 0.001). For the survival analysis, a flight duration of 120 min was settled for bees that flew out of the radar range and did not

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Table 2. Summary of the Cox Regression Model

	model 1				model 2			
variables	regression coefficient	exp (coef)[c]	Z	P	regression coefficient	exp (coef)[c]	Z	P
treatment	−0.577213	0.561461	−3.408	**0.000656**	−0.5866	0.5562	−3.505	**0.000456**
experiment	−0.372878	0.688749	−1.563	0.117983	−0.2864	0.7510	−1.745	0.080899
time foraging[d]	−0.035163	0.965448	−0.674	0.500248				
time exposure[e]	−0.013654	0.986439	−0.838	0.402182				
temperature	−0.007925	0.992106	−0.238	0.811991				
time before flying	0.017345	1.017496	1.133	0.257266				
	R square: 0.091 (max possible = 0.999), likelihood ratio test: 17.71 on 6 df, P = 0.007007				R square: 0.08 (max possible = 0.999), likelihood ratio test: 15.52 on 2 df, P = 0.0004268			

[a]A backward selection on the AIC was performed on Model 1 to obtain Model 2. [b]Values in bold indicate significant differences. [c]exp (coef) = hazard ratio. [d]Time foraging is the time in days during which a bee is foraging at its feeder before being released. [e]Time exposure is the time in days from the first day of the experiment until the day the bee is released.

come back within the radar range or to the hive during this time. The flight duration of all other bees was the flight time in minutes from the release site to the hive or from the release site to a point inside of the radar range where the signal was lost. The influence of multiple variables was tested in a Cox regression model (Table 2). The variable "treatment" shows a significant negative effect on honey-bee survival. The hazard rate of the treated bees, representing the likelihood of returning to the hive, is almost half the hazard rate of the control bees. The period during which the experiment was performed ("experiment") and the number of days a bee foraged at its feeder before being released ("time foraging"), as well as the number of days from the first day of the experiment until a bee was released ("time exposure"), had no significant effect on honey-bee homing abilities. The duration of the exposure had no effect, possibly because 45% of the treated bees individually released foraged at the contaminated feeder for less than 3 days. The temperature at the release time did not seem to play a role in the ability of honey bees to come back to their hive. At their release, 76.5% of the control honey bees and 61% of the treated honey bees waited for a short time at the release site before starting to fly. This waiting time ("time before flying") was not different between the control and the treated bees (mean ± SEM control = 3.17 ± 0.33 min; treated = 4.53 ± 0.69 min; Mann−Whitney, P = 0.5067) and had no influence on the homing success (Table 2).

During the flight, nine pauses were recorded in the control group and 24 in the treated group with a maximum of three pauses per bee (Table S5). The probability of making a pause during the return flight to the hive was not found to be significantly different between the control (13%) and treated groups (24%, Fisher's exact test, P = 0.0617). However, the mean (±SEM) pause duration was higher for the treated bees (20.13 ± 5.28 min) than for the control bees (5.29 ± 2.12) but not significantly different between the two groups (Mann−Whitney, P = 0.0974), possibly because of the limited number of cases and the large variance. The duration of the pause was deleted from the total flight duration to calculate an accurate flight speed (Tables S4 and S5). The total flight duration including pauses was, however, considered for every other analysis. If we take out the duration of the pauses from the total flight duration of the concerned bees and run the survival analysis again, the variable "treatment" remains significant (Kaplan−Meier log-rank test, χ_1^2 = 8.8, P < 0.01; Cox regression model 1: P = 0.00435), and none of the other variables tested before become significant.

Among the bees returning to their respective hives, no significant difference was found between the flight duration of control and treated bees (Table S4, median control = 7.8 min, treated = 7.4 min; Mann−Whitney, P = 0.5741), and no significant difference was found in the distance flown (median control = 2032 m, treated = 1908 m, Mann−Whitney, P = 0.4778). However, the treated bees flew significantly slower than the control bees (Table S4, mean ± SEM; speed of treated = 4.32 ± 0.13 m/s, control = 4.78 ± 0.15 m/s; unpaired t test, P < 0. 05). In a catch-and-release situation like in the test performed here, bees usually fly first along a vector they would have taken if they were departing from the feeder in direction to the hive (vector flight).[49] Next, they usually search for some time before flying back to the hive rather straightly. The proportion of vector flights performed did not differ between the control (n = 55, 71%) and the treated (n = 57, 76%) bees that returned to their hive (Fisher's exact test = 0.4703). There was a difference in the duration of the vector component between the control bees in experiments 1 and 2 (P < 0.05). Also, control bees from experiment 2 flew the vector component faster than control bees from experiment 1 and treated bees from experiment 2 (P < 0.01 and P < 0.05, respectively). Because these bees foraged at different feeding locations, the effect indicates a site-specific component. Therefore, we compared the parameters of the flights of control and treated bees separately for the two training sites and found no differences with respect to the duration, length, and the spatial distribution of the vector component (Table S5). The homing flight was considered as the flight component from the end of the vector to the hive. No difference was found in the length, duration, or speed of the homing flight between control and treated bees (Table S5). However, we found that more control bees returned less than 100 m from their release site at least once during their search flight (Fisher's exact test, P < 0.05), indicating their ability to remember where they were released and use this location to start over the homing flight. Also, significantly more control bees flew less than 100 m close to their feeder (Fisher's exact test, P < 0.01) before heading to the hive, indicating the use of known landmarks for a successful homing. Indeed, all of the bees that passed close to their feeder flew directly back to the hive from the feeder.

The bees that did not return to the hive performed different kinds of flight trajectories before getting lost (Figure 6). None of the control bees got lost out of the radar range, whereas nine treated bees out of 20 were lost bees in experiment 2 and flew in the opposite direction of the hive, left the radar range, and did not return within the range or to the hive. Interestingly,

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Figure 6. Flight trajectories of the nonreturning bees. Map data provided by Google Earth and GeoBasis, DE BKG. The figures show the flight trajectories of individual bees, each in a different color within a group (a–d). The trained route of the bees released at the release site (RS) is represented with a red line between the hive (H) and the feeders (F1 and F2). In experiment 1, F1 was the feeder of the control bees and F2 the feeder of the treated bees. In experiment 2, the situation was reversed (F1: treated bees, F2: control bees). The circle (black dashed line) represents the edge of the radar range (900 m from the radar). Bees leaving the radar range and then returning into it are marked with a black arrow directed to the east (leaving the range) or to the west (returning into the radar range), respectively. A square at the beginning of each flight track marks the first radar signal, and the triangle at the end of the flight marks the last radar signal. See Table S4 for the number of bees lost within each group.

some treated bees (Figure 6c) terminated their flights at the end of the vector component. These bees did not initiate search flights or homing flights and did not arrive at the hive.

DISCUSSION

Our study documents important sublethal effects of a low concentration (4.5 ppm) of thiacloprid taken up chronically by foraging bees. We found that higher-order functions like navigation according to a learned landscape memory, motivation to forage, and to communicate in a social context were compromised.

Honey bees visiting a feeder containing thiacloprid foraged over shorter periods of time, probably because they died earlier than the control bees. This result is not surprising because a 10 day exposure to a sublethal concentration of another neonicotinoid, thiamethoxam, reduced honey bees' life span by 41%.[50] Exposure to pesticide residues in the brood comb was also shown to shorten adult longevity.[51] Overexpression of the vitellogenin transcript in the honey-bee brains could be one of the molecular indicators for the alteration in foraging activity and accelerated aging upon neonicotinoid exposure.[6] Previous studies also demonstrated a reduced foraging activity of honey bees on sucrose solutions contaminated with thiacloprid,[52] imidacloprid,[15,53,54] or clothianidin.[14] These effects could be explained by a prolonged stay inside the hive before returning to the feeder.[14] We found that if occurring, a prolonged stay inside the hive was not used for dance communication because dance activity was highly affected by a chronic uptake of thiacloprid, as was already shown with imidacloprid.[15]

We tried to compensate for the reduced foraging activity by increasing the sucrose concentration at the contaminated feeder, but the reduced dance activity could not be totally compensated for even though very high sucrose concentrations were applied during the dance-induction periods. Thiacloprid increased the minimum sucrose concentration that honey-bee foragers are willing to gather at the feeder, as was found for imidacloprid.[15] Because increasing sucrose concentration could partially compensate for the reduced foraging activity observed at the contaminated feeder, it is most likely that thiacloprid did not alter the sensory or motor components of foraging but rather the motivation to forage. The results on dance performance point in the same direction. Pollination would be disturbed because of a reduced visitation of the flower by bees,[28] leading to fewer flowers pollinated and thus reduced yields for farmers. In addition, honey-bee colonies may suffer from a reduced food inflow, making them more susceptible to other disturbances (weather conditions, additional pesticides intoxication, parasites, and pathogens).

Several studies reported low toxicity of thiacloprid.[20,55] Laurino et al.[55] reported that the acute uptake of thiacloprid (144 ppm) appeared to not be dangerous unless the honey

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bees were starved. It was thus suggested that thiacloprid acts as a repellent, leading to reduced uptake and thus to lower toxicity. Here, we disprove this hypothesis, documenting that thiacloprid does not have a repellent effect on honey bees. Furthermore, we show drastic effects on honey-bee behavior for a concentration 32 times lower than the one used by Laurino et al. The results of our field study, especially on the impairment of the foraging behavior and social communication, cannot be related to an avoidance of the substance, corroborate recent findings with other neonicotinoids.[56]

Chronic exposure to thiacloprid leads to an accumulation over time in both the honey bee foraging at the contaminated feeder as well as in bees of the same colony via a contamination of the stored food. The estimated amount of thiacloprid metabolized by a foraging honey bee can be estimated by the energy supply necessary to perform the return trips from the feeder to the hive, assuming that all energy for the return flight is taken up from the collected sucrose solution. Applying a concentration of 5.4 ng/μL at the feeder, we calculated that a foraging bee collected, on average, 216 ng of thiacloprid (40 μL of solution) on one trip (80 times less than the acute oral $LD50^{(48h)}$ of 17320 ng active substance per bee). Based on the data about metabolic rates in flying bees,[45,46] we calculated that the bee will metabolize only 0.53−0.8 μL of the sucrose solution and thus incorporates 2.86−4.32 ng thiacloprid while flying back to the hive from the feeder (2 min return flight; 1 M sucrose solution). In natural conditions, foraging bees can be exposed to different concentrations of the substance in nectar. Pohorecka et al.[57] report data on thiacloprid residues in nectar from flowers and combs and in honey up to 208.8 ng/g. The amount of the substance a bee will metabolize when foraging on nectar sources contaminated with 208.8 ng/g (0.25 ng/μL) thiacloprid depends on the distance from the food source to the hive, the flight time during foraging, the motivational state,[46] and the reward rate.[46,47] If a bee performs a 20 min foraging flight and forages on a 50% nectar concentration, we can estimate that it will metabolize rather similar amounts of thiacloprid (2.6−4 ng) as in our study."

Furthermore, we estimated an amount of metabolized thiacloprid between 141 and 212 ng per day and per bee foraging at the contaminated feeder. The lower range of this estimation, which is the most probable, is not far from the daily consumption and thus the exposure of 112.1 ± 4.4 ng per bee and per day measured by Vidau et al.[32] in his experiment.

Homing-flight performance has been considered by the EFSA as a relevant criterion for measuring sublethal effects in free-ranging pollinators.[21] Indeed, to perform a successful homing flight, a bee has to use its sensory, motor, and cognitive functions for successful foraging trips. We showed here that the sensory and motor functions are not compromised but rather specifically their cognitive abilities, such as the retrieval of spatial memory about the landscape and motivation to forage and communicate. The homing success of the foragers exposed to thiacloprid was impaired, supporting previous findings on the effects of thiacloprid, imidacloprid, clothianidin,[33] and thiamethoxam.[16,29] Honey-bee colonies are behaving like a "superorganism",[58] and a sufficient number of honey bees in each class is needed to perform the various and different tasks to keep the information flow going and to adapt efficiently to changing environmental conditions.[59] High forager death rates can induce a shift in the age that honey bees are starting to forage[60] and a change in the relative proportions of worker-brood versus drone-brood production,[29] which might affect the fitness of the colony.[59]

The radar-tracking method applied here allows the identification of which components of navigational tasks necessary for a successful return to the hive are compromised. The catch-and-release test exposes the bee to the condition of localizing itself after being released at an unexpected place within the area around the hive, which it had explored during its orientation flights.[39] Treated bees were more frequently lost than control bees, particularly during the initial part of their homing flight. Treated bees also had a higher probability to start their flight by taking a wrong direction, and they had a tendency to interrupt their flights toward the hive, indicating their inability to recall their memory and locate themselves. Our results also corroborates previous findings[33] that the vector flight of bees acutely treated with thiacloprid was not altered, indicating an uncompromised application of the recently learned vector memory if it is retrieved. Homing, however, requires the activation of a remote memory acquired during exploratory orientation flights and the recognition of landmarks as indicators for the route toward the hive from an unexpected location. The flight trajectories recorded in the Fischer et al. study[33] and here strongly indicate a loss of memory retrieval that differs from the recently learned route flight. Neonicotinoids affect predominantly higher-order cognitive functions of the bee brain that are related to the integrative properties of the mushroom bodies. These structures are known to be essential for across sensory integration, learning, and memory formation,[9,10] and they require functional nicotinic acetylcholine synaptic transmission both at their input site and their output site. It is thus likely that neonicotinoids at low-level doses interfere predominantly with mushroom-body functions.[11,12]

Moreover, thiacloprid is often used together with other pesticides in mixtures,[61] and some synergism effect between thiacloprid and ergosterol biosynthesis inhibiting fungicides has already been observed in honey bees, increasing the toxicity by up to 560-fold.[22,48] For Mullin et al.,[62] "the formulation and not just the dose makes the poison". Future studies should concentrate their efforts on investigating the effects of neonicotinoids not only as active substances but also as formulations. It should also be noted that the risk of neonicotinoids to bumble bees or solitary bees is about 2 to 3 times as high as for honey bees due to the different sensitivity among the species.[63] Dramatic consequences on honey bees and, more generally, pollinators chronically exposed to very low concentrations of thiacloprid are thus to be expected. Therefore, thiacloprid cannot be considered a less-harmful neonicotinoid. Our results also demonstrate how important it is to include field-test procedures directed toward the chronic exposure to sublethal doses of these pesticides and how essential it is to test a large range of possible behavioral effects of a substance before commercializing it.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b02658.

Information about residues analysis by LC−MS/MS (Methods S1). Number of waggle runs performed by bees foraging at food sources other than the feeders (Figure S1), sucrose consumption at the feeders and

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estimated amounts of thiacloprid collected and metabolized (Table S1), Tuckey's post-hoc tests of the proboscis extension response experiment (Table S2), pesticide residues analysis of honey bees directly and indirectly exposed to thiacloprid (Table S3), flight data of honey bees returned to the hive (Table S4), and detailed flight parameters of honey bees returned to the hive (Table S5). (PDF)

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Notes

The authors declare no competing financial interest.

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