

## Honey bees' behavior is impaired by chronic exposure to the neonicotinoid thiacloprid in the field

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1 **Honey bees' behavior is impaired by chronic exposure to the neonicotinoid thiacloprid**  
2 **in the field**

3

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16

**17 Abstract**

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

## 29 Introduction

30 Bees, including honey bees, bumble bees and solitary bees represent the most prominent  
31 group of pollinators worldwide and contribute largely to agriculture as 35 % of the food crop  
32 production depends on them<sup>1</sup>. The recent loss of pollinator populations can be attributed to multiple  
33 factors such as habitat loss and fragmentation, colony management, bee pests and parasites, and  
34 additional environmental and anthropogenic elements. Doubtlessly the use of pesticides for crop  
35 protection contributes to the loss of pollinator abundance both at the species level and the quantity of  
36 a particular species<sup>2,3,4</sup>. It has also become evident that neonicotinoids (and other insecticides like  
37 fipronil) play a crucial role as the promoters of pathogen and parasite infections that effectively drive  
38 colony losses<sup>5,6,7</sup>. Thanks to their systemic properties, neonicotinoids are present in the pollen and  
39 nectar and will thus be continuously collected by pollinators for as long as flowering persists. They  
40 are agonists of nicotinic acetylcholine receptors (nAChR) which are normally activated by the  
41 neurotransmitter acetylcholine<sup>8</sup>. Nicotinic synaptic transmission is a major component of neural  
42 integration in the circuits related to sensory integration and functions related to the mushroom bodies,  
43 mediating multisensory integration, learning, and memory formation<sup>9,10</sup>. Neonicotinoids negatively  
44 affect the mushroom bodies' physiology<sup>11</sup> and function<sup>12</sup> in honey bees. It was already proven that  
45 neonicotinoids compromise olfactory learning<sup>13</sup> as well as the ability of worker bees to forage and to  
46 communicate<sup>14,15,16,17</sup>. The toxicity of sublethal doses is also expected to be reinforced over time<sup>18,19</sup>.  
47 However, a detailed analysis of the chronic exposure to thiacloprid on foraging, navigation, and social  
48 communication is lacking.  
49 The cyano-substituted neonicotinoid thiacloprid is declared less toxic to bees than nitro-substituted  
50 compounds like imidacloprid and thiamethoxam<sup>20,21,22,23</sup>. The formulations based on thiacloprid are  
51 registered and sold in more than 70 countries worldwide<sup>24</sup> and act against sucking and chewing pest  
52 insects of more than 50 crops<sup>25,26</sup>. The formulations based on thiacloprid are used in the field for  
53 spraying treatment at application rates much higher than for the 3 neonicotinoids suspended in Europe  
54 <sup>21,27</sup>. These formulations are allowed to be sprayed during flowering because less damage to  
55 pollinators is expected. Thiacloprid is also used in a maize seed treatment since the withdrawal of  
56 clothianidin and thiamethoxam on maize across Europe in 2013.

57 Toxicity tests performed by the company at the time before releasing thiacloprid on the market  
58 evaluated only the short term and lethal effects on worker honeybees. In contrast to acute effects, no  
59 standardized protocol exists for measuring chronic effects on individual bees under semi natural  
60 conditions<sup>23</sup>. The value of tests on single animals has been questioned because a whole colony may be  
61 more robust to pesticide exposure<sup>29</sup>. However, honey bees are acting as single animals during  
62 foraging; they need to adjust their behavior to the changing availability of food sources, return to the  
63 colony for survival, deliver the collected food and communicate with other foragers. Therefore,  
64 testing single foraging honeybees represents best conditions faced by honey bee foragers and other  
65 insect pollinators in nature. A few lab studies have shown that chronic exposure to sublethal doses of  
66 thiacloprid affects honey bees' sensitivity to the gut pathogen *Nosema cerenae*<sup>30,31,32</sup> and a field study  
67 has shown that navigation is compromised when thiacloprid was given as a single acute dose<sup>33</sup>.  
68 Chronic and sub-lethal exposure to the substance is the most likely exposure scenario in the field<sup>26,34</sup>  
69 but no field study to our knowledge has yet uncovered any specific behavioral effect of such condition  
70 of exposure. In our experiments honey bee foragers were exposed chronically for several weeks in the  
71 field to a concentration similar or lower to those used in previous chronic exposure studies with  
72 thiacloprid<sup>30,31,32</sup>. The concentration of thiacloprid in the contaminated sucrose solutions was 5.4 ng/μl  
73 whereas the concentration of thiacloprid in the formulation Calypso® directly sprayed on plants and  
74 flowers at a distance of 30 to 40 cm is 150 ng/μl.  
75 Since most of the collected sucrose solution will be deposited by the forager inside the hive, and only  
76 a small proportion will be taken up and metabolized by the bee during its return flight from the feeder  
77 to the hive, only a small amount of thiacloprid will reach the brain and interfere with nicotinic  
78 synaptic transmission.  
79 We found that a chronic exposure to thiacloprid significantly impaired honeybees' foraging  
80 behaviour, communication, and navigation. The substance increased in the foragers over time  
81 affecting also the animals indirectly exposed in the colony. We found no avoidance of or preference to  
82 the substance, supporting the idea that a neural impairment was responsible for affecting the honey  
83 bees' abilities to forage, communicate, and navigate rather than a repelling effect.

84

**85 Material and methods****86 Preparation of the solutions**

87 Stock solution: 10 mg thiacloprid ([3-[(6-chloro-3-pyridinyl) methyl]-2-thiazolidinylidene]  
88 cyanamide, Sigma-Aldrich Pestanal) diluted in 1 mL acetone ( $\geq 99.9\%$ , Sigma-Aldrich) plus 39 mL  
89 distilled water leading to a concentration of 0.25 g/L. Acetone was chosen as the solvent following the  
90 EPPO guidelines<sup>35</sup>. The final concentration of acetone (0.05 %) in the contaminated sucrose solutions  
91 was shown to not have an effect on honeybee navigation<sup>33</sup>. The thiacloprid sucrose solutions used in  
92 the field (0.02 mM, 4.5 ppm) as well as for the taste and choice experiments (0.025 mM, 5 ppm) were  
93 freshly made every morning from the stock solution. The concentration of thiacloprid at the treated  
94 feeder was always the same regardless of the sucrose solution concentration. The concentration of the  
95 solutions used were confirmed by LC-MS/MS (Methods S1).

96

**97 Field experimental design**

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

**107 Training to the feeders**

108 Two feeders (F1 and F2) were established 350 meters northeast and 340 southeast respectively and  
109 were separated by an angle of 90° as seen from the cabin. The release site (RS) was located 780  
110 meters east of the cabin. A group of foragers from each of the two colonies was trained to its  
111 respective feeder and marked individually with number tags. The origin of each newly marked bee  
112 from the two colonies was controlled at the respective hive entrance. In Experiment 1, one group of

113 bees (treated group) foraged during 19 days on a sucrose solution containing thiacloprid (4.5 ppm),  
114 and the other group (control group) foraged over the same time at a feeder containing only sucrose  
115 solution. In Experiment 2, the control hive became the treated hive and the treated hive was removed  
116 and replaced by a new control hive. The feeders' locations were exchanged between Experiment 1  
117 and 2 in order to exclude any possible landscape effect related to the feeders' position. In Experiment  
118 2, the two groups of foragers were feeding at their respective feeder during 29 days. Each feeder was  
119 placed in a little wooden box to allow counting the entrances and exits of foragers with a retro-  
120 reflective sensor (Baumer GmbH). The total number and the identity of bees visiting their feeder  
121 throughout each day was known as well as the amount of sucrose solution consumed at both feeders.  
122 The concentration of the sucrose solution at each feeder was adjusted during the day in order to  
123 regulate the traffic at the feeder (25 - 40 bees) following evaluation by the experimenter of the number  
124 of trained foragers visiting the feeder. Dance recruitment was induced 19 times on 19 different days  
125 (time: 1500 - 1700 hours) by first halving the sucrose concentration at both feeders for one hour and  
126 then increasing it twofold for another hour.

#### 127 Homing experiment

128 Colonies were settled in the field for at least a week before the homing experiments started. After a  
129 certain number of days foraging at the feeders, single bees were caught on their departure at their  
130 respective feeder and transferred into a glass vial after they had freely drunk either a 1 M sucrose  
131 solution (control bees) or a 1 M sucrose solution containing 4.5 ppm thiacloprid (treated bees). They  
132 were kept in the dark for 45 min while they were transported to the release site. Then a transponder  
133 was fixed to thorax and the bee was released (time: 1100 - 1700 hours, temperature: 17-30°C, wind <  
134 15 km/h). No release was made when the sky was evaluated too cloudy or totally overcast, nor when  
135 it was raining. Care was taken that the number of control and treated bees released every day were  
136 evenly distributed and it was ensured that each bee was released only once. The radar was shut down  
137 not before 120 min after the last bee was released if the bee was not yet back to its hive. Since none of  
138 the bees that did not return to the hive after being released was seen at the feeder or at the hive  
139 entrance on the same or the following days, we assume that they died in the field.

140 The method used for tracking bees with a harmonic radar system has been described before<sup>36,37,38</sup>. The  
141 transponders were built by ourselves following the procedure from Riley et al. (1996), their  
142 attachment and carrying by the bees do not alter honeybees' flight behavior<sup>39,40</sup>. The flights of the  
143 released bees carrying a transponder were monitored using the radar system over a distance of up to  
144 900 meters radius and at a temporal resolution of 1/3 Hertz<sup>37</sup>.

#### 145 Electric field recordings

146 The electric fields emitted by dancing bees<sup>41</sup> consist of low-frequency (movements of the abdomen,  
147 16 Hz on average) and high-frequency (buzzing of the wings, 230 Hz) components synchronization,  
148 leading to an average of three to seven electric pulses per waggle. The distance from the hive to a  
149 feeding site is encoded in the number of waggle runs and 1 sec is known to represent a distance of  
150 about 1 km<sup>42</sup>. The feeders were located 350 meters northeast (F1) and 340 southeast (F2) of the hives  
151 and since very few natural food sources existed in the experimental area and none of them were  
152 present at the same distance as the feeders, the distinction between dances from trained and untrained  
153 foraging bees was possible. Electric field measurements were performed at the same time on both  
154 sides of the lower comb in the control and treated hives using 4 copper wires with a silver coating  
155 positioned in the dance area (12 cm<sup>2</sup> covered), connected on each side to a stereo amplifier (USB -  
156 Soundbox 7.1, Conrad electronics SE) with a sample rate of 44.1 KHz. Each amplifier was connected  
157 to a laptop and the software Presonus Studio One (version 2.4) was used for saving the data as wave  
158 files. We recorded in total 340 hours of electric fields on 32 different days (average of 2.67 hours per  
159 day).

160

#### 161 **Thiacloprid residues analysis**

162 Bees were caught at their feeder after foraging for a certain number of days and after they had filled  
163 their crop with a 1 M sucrose solution contaminated or not. They were then kept in the dark for 45  
164 minutes before being killed by chilling and put into a -20° C deep-freezer. We also collected  
165 unmarked forager bees at the entrance of the treated and control hives when flying out on a foraging  
166 trip in order to assess the in-hive contamination of foragers not visiting the feeders but exposed



167 indirectly to thiacloprid inside the hive via the stored food. See Methods S1 for details about the  
168 residue analysis by LC-MS/MS.

169

## 170 **Repellent effect**

### 171 PER experiment

172 The Proboscis Extension Response (PER) was used to sample hungry bees' sensitivity to varying  
173 concentrations of sucrose<sup>43,44</sup> containing or not thiacloprid (5 ppm). Honeybees were captured at 1400  
174 hours when leaving the hive, immobilized by chilling, and mounted in small brass tubes which  
175 restrained body movements but allowed the antennae and the mouthparts to move freely<sup>43</sup>. One hour  
176 later they were tested in the laboratory by touching both antennae with a droplet of ascending  
177 concentrations of sugar concentrations (dry sugar diluted in tap water + 0.05 % acetone, 0.1 %, 0.3 %,  
178 1 %, 3 %, 10 %, 30 % and 50 %, w/v). Only the bees which showed a PER for the 50 % sugar  
179 concentration were considered as the non-responders (control: 1/74, treated: 3/74) were considered  
180 physically unable to extend their proboscis.

### 181 Choice experiment

182 In May, a group of bees was trained to a training/feeding platform located about 30 meters from the  
183 hive. The platform was composed of a yellow background and 10 blue squares randomly distributed  
184 and containing a mini-feeder from which the bees could freely drink a 1 M sucrose solution. The test  
185 platform contained only 6 mini-feeders. During testing of single bees three feeders contained 8  $\mu$ l of a  
186 1 M control sucrose solution each and the other three 8  $\mu$ l of a 1 M sucrose solution with thiacloprid  
187 (5 ppm) each. The positions of the control and treated mini-feeders were randomly allocated on the  
188 platform. The number of feeders drunk and the time a bee took to drink at each of the 6 feeders was  
189 recorded. At the end of the test the bee was killed and the same test was repeated with a new naive  
190 bee.

191

## 192 **Flight tracks and statistical analysis**

193 From the x/y coordinates collected by the radar, the length and duration of the flight from the first to  
194 the last signal was calculated. The x/y-coordinates were fitted into a google map scaled in meters

195 using CorelDraw.X5. The criteria used to categorize the different flight parameters were arbitrarily  
196 defined. A “vector flight” was considered as such when fitting into an angle of  $45^\circ$  as seen from the  
197 release site ( $\pm 22.5^\circ$  each side of the feeder-hive vector direction, F1:  $313^\circ$ , F2:  $222^\circ$ ) and had a  
198 minimal length of 200 m. The angle of a vector component is the angle between the crossing point of  
199 the vector track with the 200 m circle around the release and the direction towards north. The criterion  
200 “pass close to F” and “Return to RS” was attributed respectively to bees getting closer than 100 m  
201 from their feeder or from the release site during their flight.

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

207 For the statistical analysis of the data, we used R and Prism 5 and 6. The normality of the data was  
208 tested using the D'Agostino-Pearson omnibus test. If the data were normally distributed, we used a  
209 paired/unpaired t.test or an analysis of variances with Tukey's post-hoc tests. Otherwise non-  
210 parametric tests were performed (Mann-Whitney test, Wilcoxon signed rank test). The Fischer's  
211 Exact Test was used to compare proportions. . For the PER data we performed a mixed effects logistic  
212 regression in R (lme4 package) with “Bee” and “Date” as random effects to account for the difference  
213 between individuals and the date. This was followed by Overall Likelihood Ratio Tests and Tukey's  
214 post-hoc tests (multcomb package). The Wheeler-Watson test was used to calculate the angular  
215 distribution of the vector components. The survival analysis was conducted using censored Kaplan  
216 Meier Log-Rank in R and the influence of multiple variables was investigated using a Cox-regression  
217 model. The numbers of bees tested for each experiment and test groups are indicated in the legends of  
218 the figures and in the text.

219

## 220 **Results**

221 **Honey bees' foraging behavior and dance communication are compromised by chronic**  
222 **exposure to thiacloprid.**

223 The total foraging span of honey bees foraging at the control feeder was significantly longer than the  
224 foraging span of honey bees foraging at the treated feeder (Table 1, Kruskal-Wallis,  $P < 0.0001$ ).  
225 Control bees foraged at their feeder on average 0.78 days longer than treated bees (“Total”, Table 1).  
226 The significance was different between the groups according to the Experiment (see Table 1).  
227 Sucrose consumption at the control and treated feeder was significantly different in both experiments  
228 (Paired t-test,  $P < 0.0001$ ). Control bees consumed 1.7 times more sugar solution per day than treated  
229 bees (Table S1). The average amount of thiacloprid collected per bee and per day at the treated feeder  
230 was estimated at  $12118 \pm 900$  ng in Experiment 1 and  $10990 \pm 833$  ng in Experiment 2 (Table S1).  
231 Treated bees performed on average 1.8 times and 1.4 times less foraging trips per day than control  
232 bees in Experiment 1 and 2 respectively. On one trip, we estimate that a bee collected on average 216  
233 ng of thiacloprid (40  $\mu$ l of solution). The total amount of thiacloprid metabolized by a bee per day  
234 during the return flights to the hive ranges between 141 and 212 ng (Table S1). This calculation is  
235 based on the data related by Rortais et al.<sup>45</sup> that a bee needs 8 - 12 mg of sugar per hour to fly<sup>45,46</sup> and  
236 on our measurements (treated bees collected on average 1 M sucrose solution and flew on average 2  
237 minutes from the feeder to the hive).  
238 The reduced sugar consumption is linked to a reduced visitation rates of foragers at the contaminated  
239 feeder. Indeed, treated bees visited their feeder significantly less frequently than the control bees and  
240 higher sucrose concentrations were needed at the contaminated feeder in order to keep the bees  
241 visiting the feeder (Fig. 1 a). The median sucrose concentration used for regular foraging was 0.5 M at  
242 the control feeder and 1 M at the treated feeder. Recruitment of foragers via the waggle dance was  
243 induced by raising the sucrose concentration at the feeder<sup>42</sup>. First the sucrose concentration at both  
244 feeders was reduced to half of the current concentration for one hour, then it was increased twofold  
245 for another hour. Sucrose concentrations as high as 2 M during dance induction did not significantly  
246 increase the traffic at the treated feeder (ANOVA,  $F_{3,72} = 14.01$ ,  $P < 0.0001$ ), whereas a median  
247 concentration of 1 M increased significantly the number of visits at the control feeder ( $p < 0.05$ , Fig.  
248 1b).

249 Reduced recruitment at the feeder could indicate less waggle dances or compromised dance  
250 performance. Therefore, we monitored and estimated the number of waggle runs performed by the  
251 dancing bees in both colonies, taking advantage of the fact that waggle dances can be measured by the  
252 temporal modulation of the electrostatic field emanating from the dancing bee<sup>41</sup>. The number of  
253 waggles performed by the bees trained to the control feeder was significantly higher than those of the  
254 bees trained to the contaminated feeder (Fig. 2, Wilcoxon signed rank test,  $p < 0.0001$ ) although the  
255 sucrose concentration during dance induction was higher at the contaminated feeder (Fig. 1.a). Indeed,  
256 honey bees foraging at the control feeder performed on average 3.2 times more waggles per hour than  
257 honey bees foraging at the treated feeder. The reduced dance activity of treated bees explains the  
258 lower foraging activity at the contaminated feeder.

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

264

#### 265 **No repellent effect of thiacloprid.**

266 One explanation for lower foraging activity found in treated bees could be an aversive taste of the  
267 substance in contaminated sucrose solution. In the laboratory experiment, we tested the proboscis  
268 extension response (PER) of hungry foragers to water and 7 different sucrose concentrations (0.1 %,  
269 0.3 %, 1 %, 3 %, 10 %, 30 % and 50 % w/v) containing thiacloprid (5 ppm) or not (Fig. 3). No  
270 difference was found in the PER of bees stimulated either with the control sucrose solutions or the  
271 contaminated sucrose solutions (logistic regression with random effects “Bee” and “Date”, Sugar  
272 concentration x Treatment:  $\chi_6^2 = 2.5224$ ,  $P = 0.866$ ). The results of the Tukey’s post-hoc tests between  
273 the control and treated groups for each of the different sucrose concentrations tested can be found in  
274 Table S2.

275 In the free flight experiment, 45 bees had to choose between feeders containing a 1 M sucrose  
276 solution with or without thiacloprid (5 ppm). No significant difference was found in the visitation rate

277 of the bees to the control (64 %) and contaminated (65 %) feeders (n=135 feeders, Fischer Exact test,  
278  $P = 0.8989$ ). The average ( $\pm$  s.e.m.) drinking time per bee and feeder was  $6.88 \pm 0.27$  sec at the  
279 control feeders, and  $7.37 \pm 0.36$  sec at the contaminated feeders (no significant difference, Mann  
280 Whitney,  $P = 0.5578$ ). These results rule out the possibility that thiacloprid has a repellent taste for  
281 honeybees.

282

### 283 **Thiacloprid residue levels increase in foragers.**

284 The amount of thiacloprid in bees foraging at the contaminated feeders in Experiment 1 and 2 was  
285 analyzed by LC-MS/MS (Methods S1). Fig. 4 shows how it accumulated in different body parts over  
286 time. The amount of thiacloprid residues found in bees can be seen as the status of intoxication at the  
287 moment a bee is released with a transponder after foraging chronically during 2, 3 or 4 days at the  
288 contaminated feeder.

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

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

304

305 **Honey bees' homing success and navigation performance are impaired.**

306 Navigation requires the integration of multisensory cues and the retrieval of appropriate memory  
307 about the landscape structure. We tested navigation abilities of the bees trained to feeder 1 and 2  
308 during the Experiments 1 and 2. We found that treated bees returned to their hive at a significantly  
309 lower proportion than control bees (Fig. 5, homing success: control 91.76 %, treated 76 %, Fischer  
310 Exact Test,  $P < 0.01$ ). Based on the crop-emptying measurements by Fournier et al.<sup>47</sup> we calculated  
311 that the foragers released with a transponder could have assimilated in 45 min up to 7  $\mu\text{l}$  and thus 38  
312 ng thiacloprid in addition of the residues already assimilated over  $n$  days foraging at the feeder. This  
313 value is a higher estimate because the amount of assimilated sucrose during the 45 minute waiting  
314 time may well be much lower depending on the activity of the waiting bee<sup>48</sup>. In any case the partial  
315 acute treatment component involved in the navigation experiments adds to the chronic effect.  
316 A survival analysis was conducted on the data and a significant influence of thiacloprid on honey bee  
317 homing success was found (Kaplan Meier Log Rank test,  $\chi_1^2 = 12.9$ ,  $P < 0.001$ ). For the survival  
318 analysis, a flight duration of 120 min was settled for bees that flew out of the radar range and did not  
319 come back within the radar range or to the hive during this time. The flight duration of all other bees  
320 was the flight time in minutes from the release site to the hive or from the release site to a point inside  
321 of the radar range where the signal was lost. The influence of multiple variables was tested in a cox-  
322 regression model (Table 2). The variable "Treatment" shows a significant negative effect on honey  
323 bee survival. The hazard rate of the treated bees, representing the likelihood of returning to the hive, is  
324 almost half the hazard rate of the control bees. The period during which the experiment was  
325 performed ("Experiment"), the number of days a bee foraged at its feeder before being released  
326 ("Time foraging"), as well as the number of days from the first day of the experiment until a bee was  
327 released ("Time exposure") had no significant effect on honey bee homing abilities. The duration of  
328 the exposure had no effect possibly because 45 % of the treated bees individually released foraged at  
329 the contaminated feeder for less than 3 days. The temperature at the release time did not seem to play  
330 a role in the ability of honey bees to come back to their hive. At their release, 76.5 % of the control  
331 honey bees and 61 % of the treated honeybees waited for a short time at the release site before starting  
332 to fly. This waiting time ("Time before flying") was not different between the control and the treated

333 bees (mean  $\pm$  s.e.m control =  $3.17 \pm 0.33$  min, treated =  $4.53 \pm 0.69$  min, Mann Whitney,  $P = 0.5067$ )  
334 and had no influence on the homing success (Table 2).  
335 During the flight, 9 pauses were recorded in the control group and 24 in the treated group with a  
336 maximum of 3 pauses per bee (Table S5). The probability of making a pause during the return flight  
337 to the hive was not found significantly different between the control (13 %) and treated groups (24 %, Fischer Exact test,  $P = 0.0617$ ). However, the mean ( $\pm$  s.e.m.) pause duration was higher for the  
338 treated bees ( $20.13 \pm 5.28$  min) than for the control bees ( $5.29 \pm 2.12$ ) but not significantly different  
339 between the two groups (Mann Whitney,  $P = 0.0974$ ) possibly because of the limited number of cases  
340 and the large variance. The duration of the pause was deleted from the total flight duration in order to  
341 calculate an accurate flight speed (Tables S4 and S5). The total flight duration including pauses was  
342 however considered for every other analysis. If we take out the duration of the pauses from the total  
343 flight duration of the concerned bees and run the survival analysis again, the variable "Treatment"  
344 remains significant (Kaplan Meier Log Rank test,  $\chi_1^2 = 8.8$ ,  $P < 0.01$ ; cox regression Model 1:  $P =$   
345  $0.00435$ ) and none of the other variables tested before become significant.  
346  
347 Among the bees returning to their respective hives, no significant difference was found between the  
348 flight duration of control and treated bees (Table S4, median control = 7.8 min, treated = 7.4 min,  
349 Mann Whitney,  $P = 0.5741$ ), and no significant difference was found in the distance flown (Median  
350 control = 2032 m, treated = 1908 m, Mann Whitney,  $P = 0.4778$ ). However, the treated bees flew  
351 significantly slower than the control bees (Table S4, mean  $\pm$  s.e.m., speed treated =  $4.32 \pm 0.13$  m/s,  
352 control =  $4.78 \pm 0.15$  m/s, Unpaired t-test,  $P < 0.05$ ). In a catch and release situation like in the test  
353 performed here, bees usually fly first along a vector they would have taken if they were departing  
354 from the feeder in direction to the hive (vector flight)<sup>49</sup>. Then they usually search for some time before  
355 flying back to the hive rather straightly. The proportion of vector flights performed did not differ  
356 between the control ( $n = 55$ , 71 %) and treated ( $n = 57$ , 76 %) bees which returned to their hive  
357 (Fischer Exact test = 0.4703). There was a difference in the duration of the vector component between  
358 the control bees in Experiment 1 and 2 ( $P < 0.05$ ). Also, control bees from Experiment 2 flew the  
359 vector component faster than control bees from Experiment 1 and treated bees from Experiment 2 ( $P$   
360  $< 0.01$  and  $P < 0.05$  respectively). Since these bees foraged at different feeding locations the effect

361 indicates a site specific component. Therefore, we compared the parameters of the flights of control  
362 and treated bees separately for the two training sites, and found no differences with respect to the  
363 duration, length and the spatial distribution of the vector component (Table S5). The homing flight  
364 was considered as the flight component from the end of the vector to the hive. No difference was  
365 found in the length, duration, or speed of the homing flight between control and treated bees (Table  
366 S5). However, we found that more control bees returned less than 100 m from their release site at  
367 least once during their search flight (Fisher Exact test,  $P < 0.05$ ) indicating their ability to remember  
368 where they were released and use this location to start over the homing flight. Also, significantly more  
369 control bees flew less than 100 meters close to their feeder (Fisher Exact test,  $P < 0.01$ ) before  
370 heading to the hive indicating the use of known landmarks for a successful homing. Indeed, all the  
371 bees which passed close to their feeder flew directly back to the hive from the feeder.  
372 The bees which did not return to the hive performed different kinds of flight trajectories before getting  
373 lost (Fig. 6). None of the control bees got lost out of the radar range whereas 9 treated bees out of 20  
374 were lost bees in experiment 2 and flew in the opposite direction of the hive, left the radar range and  
375 did not return within the range or to the hive. Interestingly, some treated bees (Fig. 6 c) terminated  
376 their flights at the end of the vector component. These bees did not initiate search flights or homing  
377 flights and did not arrive at the hive.

378

## 379 Discussion

380 Our study documents important sublethal effects of a low concentration (4.5 ppm) of  
381 thiacloprid taken up chronically by foraging bees. We found that higher-order functions like  
382 navigation according to a learned landscape memory, motivation to forage and to communicate in a  
383 social context were compromised.  
384 Honey bees visiting a feeder containing thiacloprid foraged over shorter periods of time probably  
385 because they died earlier than the control bees. This result is not surprising, since a 10-day exposure  
386 to a sublethal concentration of another neonicotinoid, thiamethoxam, reduced honey bees' life span by  
387 41 %<sup>50</sup>. Exposure to pesticide residues in brood comb was also shown to shorten adult longevity<sup>51</sup>.  
388 Overexpression of the vitellogenin transcript in the honey bee brains could be one of the molecular



389 indicators for the alteration in foraging activity and accelerated aging upon neonicotinoid exposure<sup>6</sup>.  
390 Previous studies also demonstrated a reduced foraging activity of honey bees on sucrose solutions  
391 contaminated with thiacloprid<sup>52</sup>, imidacloprid<sup>15,53,54</sup>, or clothianidin<sup>14</sup>. These effects could be  
392 explained by a prolonged stay inside the hive before returning to the feeder<sup>14</sup>. We found that if  
393 occurring, a prolonged stay inside the hive was not used for dance communication, as dance activity  
394 was highly affected by a chronic uptake of thiacloprid, as already shown with imidacloprid<sup>15</sup>.  
395 We tried to compensate for the reduced foraging activity by increasing the sucrose concentration at  
396 the contaminated feeder, but the reduced dance activity could not be totally compensated for even  
397 though very high sucrose concentrations were applied during the dance induction periods. Thiacloprid  
398 increased the minimum sucrose concentration that honey bee foragers are willing to gather at the  
399 feeder as was found for imidacloprid<sup>15</sup>. Since increasing sucrose concentration could partially  
400 compensate for the reduced foraging activity observed at the contaminated feeder, it is most likely  
401 that thiacloprid did not alter the sensory or motor components of foraging but rather the motivation to  
402 forage. The results on dance performance point in the same direction. Pollination would be disturbed  
403 because of a reduced visitation of the flower by bees<sup>28</sup> leading to less flowers pollinated and thus  
404 reduced yields for farmers. In addition, honey bee colonies may suffer from a reduced food inflow,  
405 making them more susceptible to other disturbances (weather conditions, additional pesticides  
406 intoxication, parasites and pathogens).  
407 Several studies reported low toxicity of thiacloprid<sup>20,55</sup>. Laurino et al.<sup>55</sup> reported that acute uptake of  
408 thiacloprid (144 ppm) appeared to be not dangerous unless the honey bees were starved. It was thus  
409 suggested that thiacloprid acts as a repellent leading to reduced uptake and thus to lower toxicity.  
410 Here we disprove this hypothesis, documenting that thiacloprid does not have a repellent effect on  
411 honey bees. Furthermore, we show drastic effects on honey bee behavior for a concentration 32 times  
412 lower than the one used by Laurino et al. The results of our field study, especially the impairment of  
413 the foraging behavior and social communication, cannot be related to an avoidance of the substance,  
414 corroborating recent findings with other neonicotinoids<sup>56</sup>.  
415 The chronic exposure to thiacloprid lead to an accumulation over time in both the honey bee foraging  
416 at the contaminated feeder as well as in bees of the same colony via a contamination of the stored

417 food. The estimated amount of thiacloprid metabolized by a foraging honey bee can be estimated by  
418 the energy supply necessary to perform the return trips from the feeder to the hive assuming that all  
419 energy for the return flight is taken up from the collected sucrose solution. Applying a concentration  
420 of 5.4 ng/ $\mu$ l at the feeder, we calculated that a foraging bee collected on average 216 ng of thiacloprid  
421 (40  $\mu$ l of solution) on one trip (80 times less than the acute oral LD50<sup>(48h)</sup> of 17320 ng a.s per  
422 bee). Based on the data about metabolic rates in flying bees<sup>45,46</sup> the bee will metabolize only 0.53 - 0.8  
423  $\mu$ l of the sucrose solution and thus incorporates 2.86 - 4.32 ng thiacloprid while flying back to the  
424 hive from the feeder (2 min return flight, 1 M sucrose solution). In natural conditions, foraging bees  
425 can be exposed to different concentrations of the substance in nectar. Pohorecka et al.<sup>57</sup> report data on  
426 thiacloprid residues in nectar from flowers, combs and in honey up to 208.8 ng/g. The amount of the  
427 substance a bee will metabolize when foraging on nectar sources contaminated with 208.8 ng/g (0.25  
428 ng/ $\mu$ l) thiacloprid depends on the distance from the food source to the hive, the flight time during  
429 foraging, the motivational state<sup>46</sup> and the reward rate<sup>46,47</sup>. If a bee performs a 20 minutes foraging  
430 flight and forages on a 50 % nectar concentration, we can estimate that it will metabolize rather  
431 similar amounts of thiacloprid (2.6 - 4 ng) as in our study.”

432 Furthermore, we estimated an amount of metabolized thiacloprid between 141 and 212 ng per day and  
433 per bee foraging at the contaminated feeder. The lower range of this estimation, which is the most  
434 probable, is not far from the daily consumption and thus exposure of  $112.1 \pm 4.4$  ng per bee and per  
435 day measured by Vidau et al.<sup>32</sup> in his experiment.

436 Homing flight performance has been considered by the EFSA as a relevant criterion for measuring  
437 sublethal effects in free-ranging pollinators<sup>21</sup>. Indeed, in order to perform a successful homing flight, a  
438 bee has to use its sensory, motor and cognitive functions for successful foraging trips. We showed  
439 here that the sensory and motor functions are not compromised but rather specifically their cognitive  
440 abilities, such as retrieval of spatial memory about the landscape and motivation to forage and  
441 communicate. The homing success of the foragers exposed to thiacloprid was impaired, supporting  
442 previous findings on the effects of thiacloprid, imidacloprid, clothianidin<sup>33</sup> and  
443 thiamethoxam<sup>16,29</sup>. Honeybee colonies are behaving like a ‘superorganism’<sup>58</sup> and a sufficient number  
444 of honey bees in each class is needed to perform the various and different tasks in order to keep the

445 information flow going and to adapt efficiently to changing environmental conditions<sup>59</sup>. High forager  
446 death rates can induce a shift in the age that honey bees are starting to forage<sup>60</sup> and a change in the  
447 relative proportions of worker brood versus drone brood production<sup>29</sup> which might affect the fitness of  
448 the colony<sup>59</sup>.

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

468 Moreover, thiacloprid is often used together with other pesticides in mixtures<sup>61</sup> and some synergism  
469 effect between thiacloprid and ergosterol biosynthesis inhibiting fungicides has already been observed  
470 in honey bees, increasing the toxicity by up to 560-fold<sup>22,48</sup>. For Mullin et al.<sup>62</sup> “the formulation and  
471 not just the dose makes the poison”. Future studies should concentrate their efforts on investigating  
472 the effects of neonicotinoids not only as active substances but also as formulations. It should also be

473 noted that the risk of neonicotinoids to bumble bees or solitary bees is about two to three times as high  
474 as for honey bees, due to the different sensitivity among the species<sup>63</sup>. Dramatic consequences on  
475 honey bees and more generally pollinators chronically exposed to very low concentrations of  
476 thiacloprid are thus to be expected. Therefore, thiacloprid cannot be considered a less harmful  
477 neonicotinoid. Our results also demonstrate how important it is to include field test procedures  
478 directed towards chronic exposure to sublethal doses of these pesticides and how essential it is to test  
479 a large range of possible behavioral effects of a substance before commercializing it.

480

**481 Supporting Information Available:**

482 Information about residues analysis by LC-MS/MS can be found in Methods S1. Number of waggle  
483 runs performed by bees foraging at food sources other than the feeders (Fig. S1), sucrose consumption  
484 at the feeders and estimated amounts of thiacloprid collected and metabolized (Table S1), Tuckey's  
485 post-hoc tests of the Proboscis Extension Response experiment (Table S2), pesticide residues analysis  
486 of honey bees directly and indirectly exposed to thiacloprid (Table S3), flight data of honey bees  
487 returned to the hive (Table S4), detailed flight parameters of honey bees returned to the hive (Table  
488 S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

489

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501

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675 (4), e94482.

676 **Table 1: Foraging span in days of the trained honey bees at the control or treated feeder.**

	<b>Experiment 1</b>	<b>Experiment 2</b>	<b>Total <sup>§</sup></b>
<b>Control</b>	5.21 ± 0.32 ( <i>n</i> = 67) * <b>a</b>	4.19 ± 0.24 ( <i>n</i> = 72) <b>a</b>	4.68 ± 0.20 ( <i>n</i> = 139)
<b>Treated</b>	4.7 ± 0.22 ( <i>n</i> = 79) <b>a</b>	3.34 ± 0.14 ( <i>n</i> = 111) * <b>b</b>	3.91 ± 0.13 ( <i>n</i> = 190)

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Numbers shown are means (days foraging) ± s.e.m.

<sup>§</sup> Mann-Whitney, *P* < 0.01

\* The control group in Exp. 1 and the treated group in Exp. 2 correspond to the same colony, as the control colony in Exp. 1 became the treated colony in Exp. 2 and continued to forage at the same feeder (F1).

Different letters indicate significant differences (post-hoc tests with Bonferroni correction): a-b (Exp.2), *P* < 0.05, a-b (Treated), *P* < 0.001, a-b (F1), *P* < 0.001.

687 **Table 2: Summary of the Cox regression model.**

Variables	Model 1				Model 2			
	regression coefficient	exp (coef) *	Z	P	regression coefficient	exp (coef) *	Z	P
<b>Treatment</b>	-0.577213	0.561461	-3.408	<b>0.000656</b>	-0.5866	0.5562	-3.505	<b>0.000456</b>
<b>Experiment</b>	-0.372878	0.688749	-1.563	0.117983	-0.2864	0.7510	-1.745	0.080899
<b>Time foraging ‡</b>	-0.035163	0.965448	-0.674	0.500248				
<b>Time exposure §</b>	-0.013654	0.986439	-0.838	0.402182				
<b>Temperature</b>	-0.007925	0.992106	-0.238	0.811991				
<b>Time before flying</b>	0.017345	1.017496	1.133	0.257266				
	<i>Rsquare: 0.091 (max possible= 0.999), Likelihood Ratio Test: 17.71 on 6 df, P=0.007007</i>				<i>Rsquare: 0.08 (max possible= 0.999), Likelihood Ratio Test: 15.52 on 2 df, P=0.0004268</i>			

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689 A backward selection on the AIC was performed on Model 1 in order to obtain Model 2

690

690 Values in bold indicate significant differences

691

691 \*exp (coef) = Hazard ratio

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692 ‡ **Time foraging** is the time in days during which a bee is foraging at its feeder before being released

693

693 § **Time exposure** is the time in days from the first day of the experiment until the day the bee is released

694 **Figure 1**695 **Required sucrose concentrations and foraging activity at the control and treated feeders.**

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

703

704 **Figure 2**705 **Number of waggles runs performed by the trained bees from the control and treated feeders.**

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

710

711 **Figure 3**712 **Proboscis Extension Response (PER) to different sucrose concentrations containing 5 ppm**

713 **thiacloprid (treated) or not (control).**  $N$  control = 73.  $N$  treated = 71. No difference was found

714 between the two groups (logistic regression with random effects, Sugar conc x Treatment:  $\chi_6^2 =$   
715 2.5224,  $P = 0.866$ ).

716

717 **Figure 4**718 **Accumulation of thiacloprid residue in heads, thoraces, abdomens and in the whole body**

719 **(representing the sum of the measurements) of honey bees foraging at the contaminated feeder**

720 **over time.** Honey bee foragers were collected at the end of 2, 3 or 4 days of foraging after they had  
721 filled their crop at the feeder containing thiacloprid (4.5 ppm). 10 bees per foraging group.

722

723 **Figure 5**

724 **Probability of homing success as a function of time until return.** Treated honey bees returned to  
725 their hive at a significantly lower proportion than control bees ( $n_{\text{treated}} = 100$ , 76 % return;  $n_{\text{control}} = 85$ ,  
726 91.76 % return, Fischer Exact Test,  $P < 0.01$ ). The origin of the temporal axis represents the moment  
727 of release.

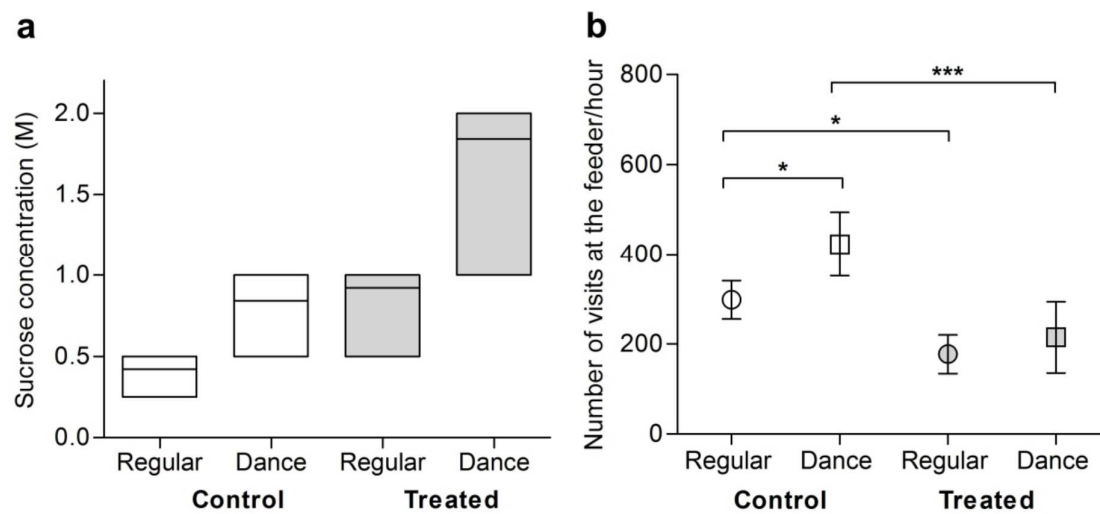
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729 **Figure 6**

730 **Flight trajectories of the non-returning bees.** Map data provided by: Google Earth and GeoBasis -  
731 DE BKG. The figures show the flight trajectories of individual bees, each in a different color within a  
732 group (**a, b, c** and **d**). The trained route of the bees released at the release site (RS) is represented with  
733 a red line between the hive (H) and the feeders (F1 and F2). In Experiment 1, F1 was the feeder of the  
734 control bees and F2 the feeder of the treated bees. In Experiment 2 the situation was reversed (F1:  
735 treated bees, F2: control bees). The circle (black dashed line) represents the edge of the radar range  
736 (900 m from the radar). Bees leaving the radar range and then returning into it are marked with a  
737 black arrow directed to the East (leaving the range) or to the West (returning into the radar range)  
738 respectively. A square at the beginning of each flight track marks the first radar signal, and the  
739 triangle at the end of the flight marks the last radar signal. See Table S4 for the number of bees lost  
740 within each group.

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742 Fig. 1:



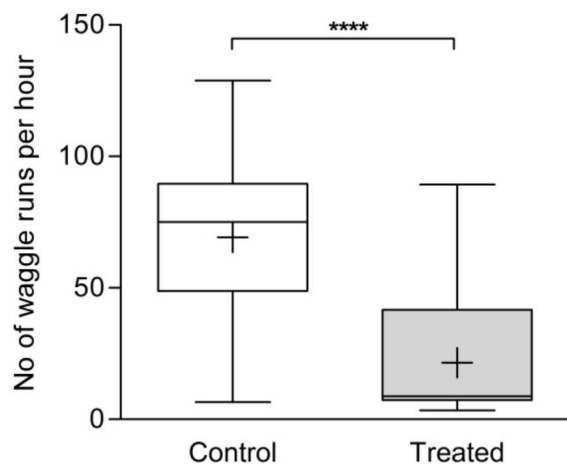
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745 Fig. 2:

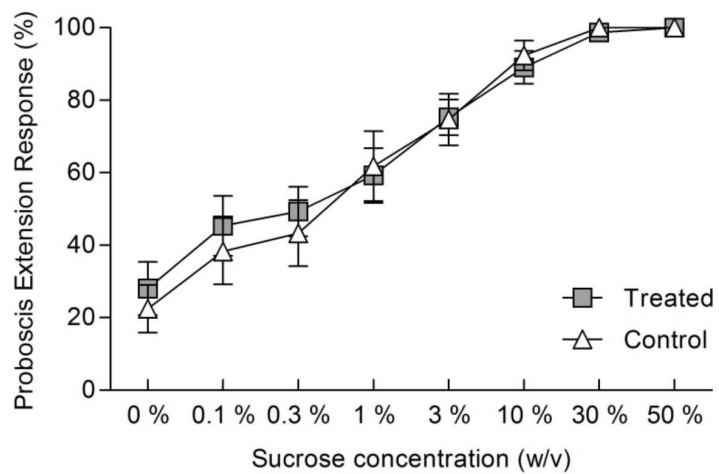
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748 Fig. 3:

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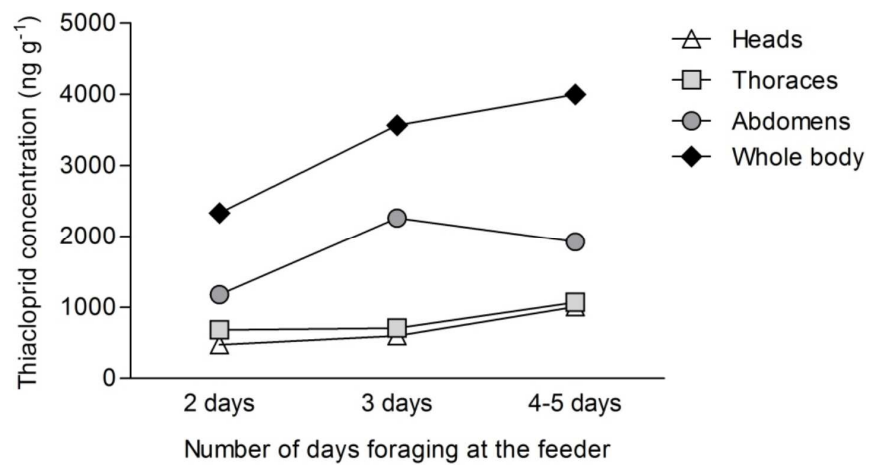


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752 Fig. 4:

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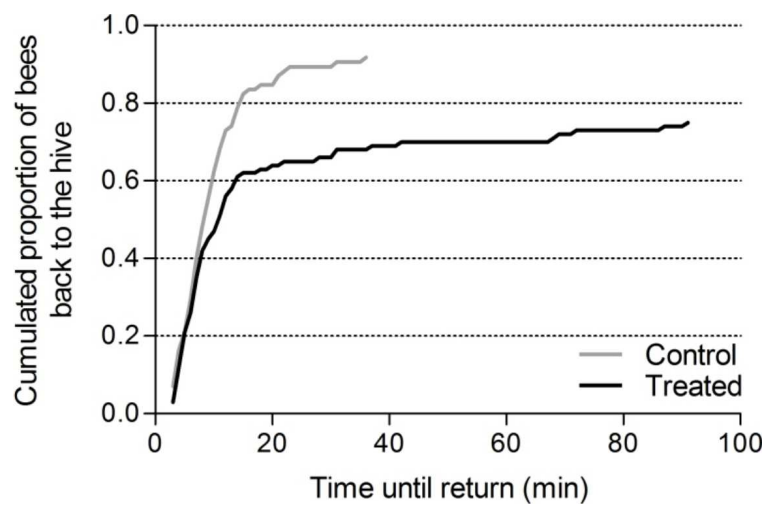


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756 Fig. 5:

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759 Fig. 6:

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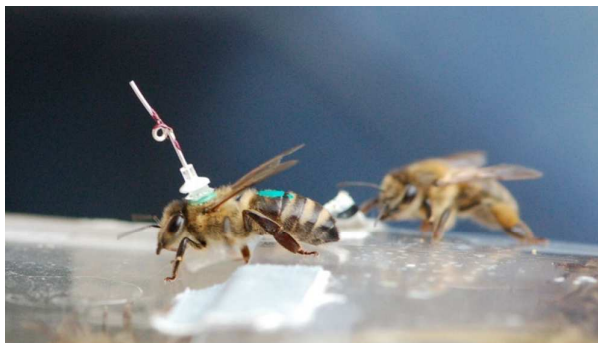


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763 TOC/Abstract Art:

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