## Article

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

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### Just Accepted

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

2    **in the field**

3

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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[1]. 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[2,3,4]. 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[5,6,7]. 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[8]. 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[9,10]. Neonicotinoids negatively

44    affect the mushroom bodies' physiology[11] and function[12] in honey bees. It was already proven that

45    neonicotinoids compromise olfactory learning[13] as well as the ability of worker bees to forage and to

46    communicate[14,15,16,17]. The toxicity of sublethal doses is also expected to be reinforced over time[18,19].

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[20,21,22,23]. The formulations based on thiacloprid are

51    registered and sold in more than 70 countries worldwide[24] and act against sucking and chewing pest

52    insects of more than 50 crops[25,26]. 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    [21,27]. 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[23]. The value of tests on single animals has been questioned because a whole colony may be

61    more robust to pesticide exposure[29]. 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*[30,31,32] and a field study

67    has shown that navigation is compromised when thiacloprid was given as a single acute dose[33].

68    Chronic and sub-lethal exposure to the substance is the most likely exposure scenario in the field[26,34]

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[30,31,32]. 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 (≥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[35]. The final concentration of acetone (0.05 %) in the contaminated sucrose solutions

91    was shown to not have an effect on honeybee navigation[33]. 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   <u>Training to the feeders</u>

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    <u>Homing experiment</u>

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.

6

140    The method used for tracking bees with a harmonic radar system has been described before[36,37,38]. 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[39,40]. 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[37].

145    Electric field recordings

146    The electric fields emitted by dancing bees[41] 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[42]. 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 $cm^2$ 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

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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[43,44] 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[43]. 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 µl of a

186     1 M control sucrose solution each and the other three 8 µl 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

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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° as seen from the

197    release site (± 22.5° each side of the feeder-hive vector direction, F1: 313°, F2: 222°) 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.**

9

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 ± 900 ng in Experiment 1 and 10990 ± 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 µl 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.[45] that a bee needs 8 - 12 mg of sugar per hour to fly[45,46] 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[42]. First the sucrose concentration at both

244 feeders was reduced to halve 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).

10

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[41]. 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[41,42]. 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

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277    of the bees to the control (64 %) and contaminated (65 %) feeders (n=135 feeders, Fischer Exact test,

278    P = 0.8989). The average (± s.e.m.) drinking time per bee and feeder was 6.88 ± 0.27 sec at the

279    control feeders,  and 7.37 ± 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

12

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.[47] we calculated

311   that the foragers released with a transponder could have assimilated in 45 min up to 7 µl 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[48]. 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 ± s.e.m control = 3.17 ± 0.33 min, treated = 4.53 ± 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 %,

338    Fischer Exact test, P = 0.0617). However, the mean (± s.e.m.) pause duration was higher for the

339    treated bees (20.13 ± 5.28 min) than for the control bees (5.29 ± 2.12) but not significantly different

340    between the two groups (Mann Whitney, P = 0.0974) possibly because of the limited number of cases

341    and the large variance. The duration of the pause was deleted from the total flight duration in order to

342    calculate an accurate flight speed (Tables S4 and S5). The total flight duration including pauses was

343    however considered for every other analysis. If we take out the duration of the pauses from the total

344    flight duration of the concerned bees and run the survival analysis again, the variable "Treatment"

345    remains significant (Kaplan Meier Log Rank test, $\chi_1^2$ = 8.8, P < 0.01; cox regression Model 1: P =

346    0.00435) and none of the other variables tested before become significant.

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 ± s.e.m., speed treated = 4.32 ± 0.13 m/s,

352    control = 4.78 ± 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)[49]. 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

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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 %[50]. Exposure to pesticide residues in brood comb was also shown to shorten adult longevity[51].

388     Overexpression of the vitellogenin transcript in the honey bee brains could be one of the molecular

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389    indicators for the alteration in foraging activity and accelerated aging upon neonicotinoid exposure[6].

390    Previous studies also demonstrated a reduced foraging activity of honey bees on sucrose solutions

391    contaminated with thiacloprid[52], imidacloprid[15,53,54], or clothianidin[14]. These effects could be

392    explained by a prolonged stay inside the hive before returning to the feeder[14]. 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[15].

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[15]. 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[28] 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[20,55]. Laurino et al.[55] 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[56].

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

16

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/µl at the feeder, we calculated that a foraging bee collected on average 216 ng of thiacloprid

421     (40 µl of solution) on one trip (80 times less than the acute oral LD50[(48h)] of 17320 ng a.s per

422     bee). Based on the data about metabolic rates in flying bees[45,46] the bee will metabolize only 0.53 - 0.8

423     µl 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.[57] 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/µl) thiacloprid depends on the distance from the food source to the hive, the flight time during

429     foraging, the motivational state[46] and the reward rate[46,47]. 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 ± 4.4 ng per bee and per

435     day measured by Vidau et al.[32] 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[21]. 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[33] and

443     thiamethoxam[16,29]. Honeybee colonies are behaving like a 'superorganism'[58] 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[59]. High forager

446  death rates can induce a shift in the age that honey bees are starting to forage[60] and a change in the

447  relative proportions of worker brood versus drone brood production[29] which might affect the fitness of

448  the colony[59].

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[39]. 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[33] 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[33] 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[9,10], 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[11,12].

468  Moreover, thiacloprid is often used together with other pesticides in mixtures[61] 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[22,48]. For Mullin et al.[62] "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

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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[63]. 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.

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

|         | **Experiment 1** | **Experiment 2** | **Total [§]** |
|---------|------------------|------------------|---------------|
| **Control** | $5.21 \pm 0.32$ (n = 67) [*][a] | $4.19 \pm 0.24$ (n = 72) [a] | $4.68 \pm 0.20$ (n = 139) |
| **Treated** | $4.7 \pm 0.22$ (n = 79) [a] | $3.34 \pm 0.14$ (n = 111) [*][b] | $3.91 \pm 0.13$ (n = 190) |

677
678
679    Numbers shown are means (days foraging) $\pm$ s.e.m.
680
681    [§] Mann-Whitney, $P < 0.01$
682    [*] 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
683    became the treated colony in Exp. 2 and continued to forage at the same feeder (F1).
684
685    Different letters indicate significant differences (post-hoc tests with Bonferroni correction): a-b (Exp.2), $P < 0.05$, a-b
686    (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 |
| **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 ǂ** | -0.035163 | 0.965448 | -0.674 | 0.500248 | | | | |
| **Time exposure §** | -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 | | | | |
| | *Rsquare: 0.091 (max possible= 0.999), Likelihood Ratio Test: 17.71 on 6 df, P=0.007007* | | | | *Rsquare: 0.08 (max possible= 0.999), Likelihood Ratio Test: 15.52 on 2 df, P=0.0004268* | | | |

688
689    A backward selection on the AIC was performed on Model 1 in order to obtain Model 2
690    Values in bold indicate significant differences
691    *exp (coef) = Hazard ratio
692    ǂ **Time foraging** is the time in days during which a bee is foraging at its feeder before being released
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 (± 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**

29

720     **over time.** Honey bee foragers were collected at the end of 2, 3 or 4 days of foraging after they had

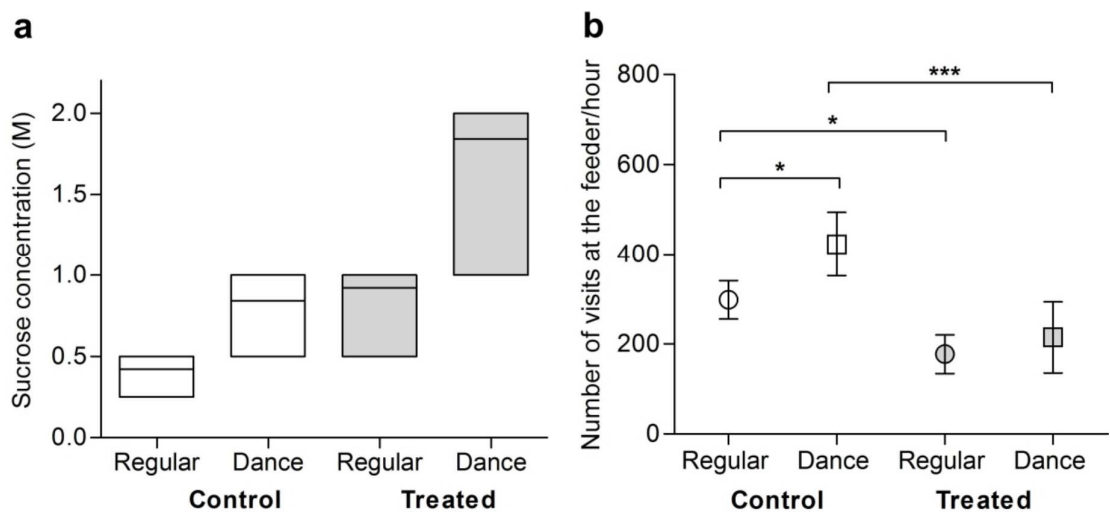721     filled their crop at the feeder containing thiacloprid (4.5 ppm). 10 bees per foraging group.

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 ($n_{treated}$ = 100, 76 % return; $n_{control}$ = 85,

726     91.76 % return, Fischer Exact Test, $P < 0.01$). The origin of the temporal axis represents the moment

727     of release.

728

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
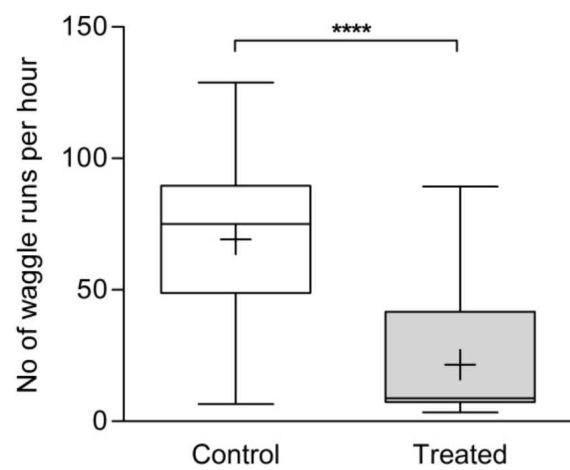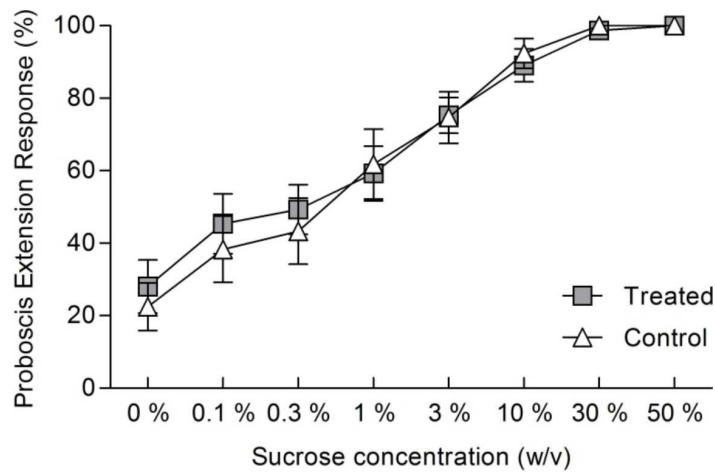
740     within each group.

741

30

742    Fig. 1:



743

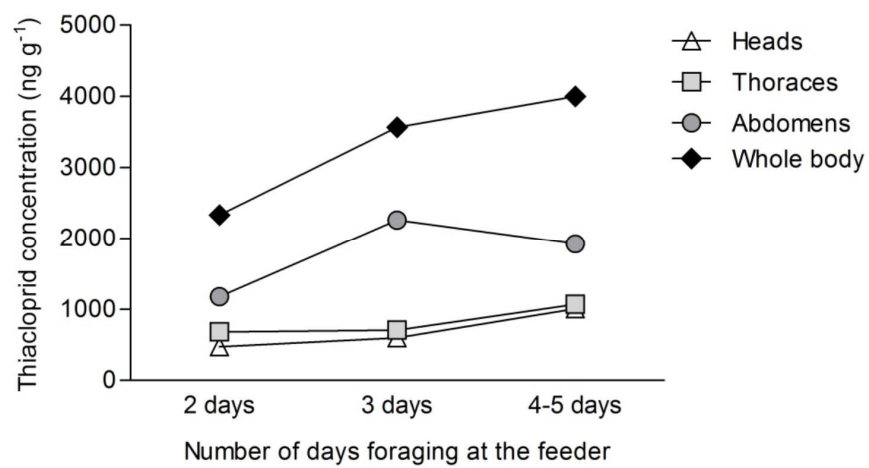744

745     Fig. 2:

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748     Fig. 3:

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751

752    Fig. 4:

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754    Number of days foraging at the feeder
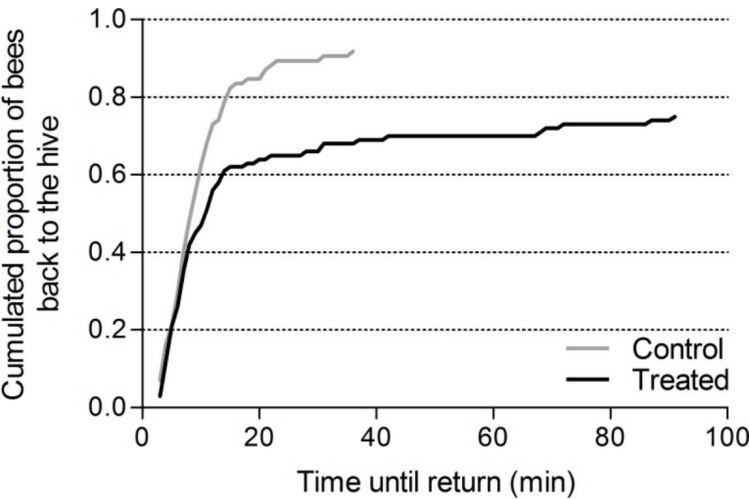
755

756    Fig. 5:

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758

759     Fig. 6:

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