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Effects of sub-lethal doses of glyphosate on honeybee navigation

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Abstract. Glyphosate (GLY) is a herbicide that is widely used in agriculture for weed control. Although reports about the impact of GLY in snails, crustaceans and amphibians exist, few studies have investigated its sub-lethal effects in non-target organisms such as the honeybee *Apis mellifera*, the main pollen vector in commercial crops. Here, we tested whether exposure to three sub-lethal concentrations of GLY (2.5, 5 and 10 mg/L corresponding to 0.125, 0.250 and 0.500 µg/animal) affects the homeward flight path of honeybees in an open field. We performed an experiment in which forager honeybees were trained to an artificial feeder, and then captured, fed with sugar solution containing GLY traces and released from a novel site (the release site, RS) either once or twice. Their homeward trajectories were tracked using harmonic radar technology. We found that honeybees that had been fed with solution containing 10 mg/L GLY spent more time performing homeward flights than control bees or bees treated with lower GLY concentrations. They also performed more indirect homing flights. Moreover, the proportion of direct homeward flights performed after a second release at the RS increased in control bees but not in treated bees. These results suggest that, in honeybees, exposure to GLY doses commonly found in agricultural settings impairs the cognitive capacities needed to retrieve and integrate spatial information for a successful return to the hive. Therefore, honeybee navigation is affected by ingesting traces of the most widely used herbicide worldwide, with potential long-term negative consequences for colony foraging success.

Keywords: *Apis mellifera*, glyphosate, sub-lethal effects, navigation, harmonic radar tracking.

Introduction

Honeybees (*Apis mellifera*) are the main pollinators in agricultural settings (Aizen *et al*., 2009) and as such are highly exposed to any perturbation occurring in the surroundings of crop fields. Consequently, this eusocial insect can serve as a biosensor to accurately determine environmental pollutants (Devillers and Pham-Delègue, 2002). Any foreign substance present in gathered resources (i.e. pollen and nectar) may also be stored and accumulated inside the nest for long periods, potentially affecting nest mates of all stages (Devillers and Pham-Delègue, 2002). This applies in particular to highly water-soluble agrochemicals such as the herbicide glyphosate *N*-(phosphonomethyl) glycine, which may remain on crops after application for long periods (Zhang e*t al*., 2011). Any subsequent accumulation of agrochemicals inside the hive could have negative effects which are often inconspicuous in the short-term (Giesy *et al*., 2000), but which could impair individual behaviors and social organization in the long-term (Kirchner, 1999).

The use of glyphosate (GLY) as a broad-spectrum post-emergent herbicide for weed control has spread rapidly in the last few decades (Goldsborough and Brown, 1988) to become one of the most commonly used agrochemicals worldwide (Zhang *et al*., 2011). The typical methods of administration involve spraying it directly onto foliage and aerial application (Giesy *et al*., 2000). As a consequence, traces of the herbicide can also be found in the surroundings of fields cultivated with the target crop. GLY deters plant growth by inhibiting the aromatic amino acid pathway apparently present only in plants, microorganisms and fungi, but not in animals (Amrhein *et al*., 1980; Carlisle and Trevors, 1988; Duke *et al*., 1989; Franz *et al*., 1997).

Several studies have reported negative effects of this herbicide on vertebrates and invertebrates. GLY doses of between 0.1 and 10 mg acid equivalent / L (a.e./L) have been found to reduce growth in the earthworm *Aporrectoden caliginom* (Springett and Gray, 1992) and affect reproduction and development in the freshwater snail *Pseudosuccinea columella* (Tate *et al*., 1997). A negative effect has also been reported in amphibians after chronic exposure to different concentrations of glyphosate (3.8-18 mg/L; Howe *et al*., 2004; Relyea, 2005a, b). Despite these findings and others which report negative and lethal effects on invertebrates such as amphipods (Dutra *et al*., 2011), the sub-lethal impacts of GLY on non-target organisms such as insect pollinators have so far been poorly evaluated (Herbert *et al*. 2014; Thompson *et al*. 2014). In this study, we used sub-lethal GLY concentrations ranging from 2.5 to 10 mg/L.

Honeybees show a behavioral repertoire that allows the evaluation of perturbations in well-known stereotypical responses. The behavior in which bees protrude their probosces after being stimulated by applying sucrose solution to their antennae is one of these responses, and it can be used to test the effects of environmental pollutants on appetitive behavior (Devillers and Pham-Delègue, 2002). A recent study found that a concentration of glyphosate (2.5 mg/L), within the recommended range for aquatic and terrestrial weed control (Giesy *et al*. 2000), affects gustatory responsiveness and learning performance in harnessed bees (tested with Proboscis Extension Response, PER, assays). However, no effect was observed on locomotive activity when foragers collected sucrose solution contaminated with the herbicide at an artificial feeder, suggesting that GLY may accumulate inside the hive (Herbert *et al*., 2014). Also, Herbert and co-workers (2014) found that an acute exposure to sub-lethal GLY concentrations offered during olfactory PER conditioning decreased short-term memory and impaired more complex forms of associative learning in foragers.

Studies have already shown that other agrochemical compounds used for pest control, such as neonicotinoids, negatively affect honeybee gustatory sensitivity and even their dance manoeuvres (Eire and Nieh, 2012). Non-lethal doses of imidacloprid (75-1000 ppb), a neonicotinoid insecticide which acts on cholinergic pathways of insect synaptic transmission (Gauthier, 2010), affect homing abilities (Bortolotti *et al*., 2003) and impair the retrieval of memory acquired during exploratory orientation flights (Fischer *et al*., 2014).

Honeybees are well established as a model for studies on animal navigation (von Frisch, 1967; Menzel *et al*., 2012; Menzel, 2012). In a typical experiment (catch-and-release experiment) bees are displaced within a previously explored area to evaluate their homing behavior using different tracking technologies (Decourtye *et al*., 2011; Schneider *et al*., 2012; Fischer *et al*., 2014). Exploration during orientation flights familiarizes bees with the sun compass, their distance measure (odometer) and the landmarks in the environment (Menzel *et al*., 2005; Menzel *et al*., 2012). Further information about the landscape is added during flights between the hive and the feeding sites. Integration of the multiple sources of spatial information leads to a reference memory that allows bees to perform shortcuts between important locations (hive, feeding sites and release sites). As a result, honeybees are able to refer to a

common frame of spatial reference that allows them to return to the hive even from an unfamiliar location by taking novel shortcuts (Menzel *et al*., 1998).

Although the GLY concentrations recommended for weed control as well as those previously detected in aquatic and agricultural systems are within the range of 1.4 mg/L to 3.7 mg/L (Couture *et al*., 1995; Giesy *et al*., 2000; Solomon and Thompson, 2003; Perkins *et al*., 2000), intensive use of the herbicide during the last two decades has led to an exponential increase in the realistic-field doses of GLY present in genetically modified (GM) crops (USDA data source, NASS). This situation implies that GLY concentrations found in close proximity to GM crops today should be much higher than the range previously reported. In the present study we propose that honeybees foraging on 'nectar' containing traces of GLY may have difficulty integrating complex information from their environment which they need for navigation. To evaluate whether sub-lethal doses of glyphosate affect *Apis mellifera* orientation and navigation, we performed a catch-and-release experiment in which honeybees flying to the hive were displaced during foraging trips.

Results

In a catch-and-release experiment as performed here, we expect that bees captured at the feeder, and then released from the release site (RS), are motivated to return to the hive (H). After ingesting food contaminated with glyphosate, we expected that these treated bees would perform irregular homeward flights or at least take more time than untreated control bees to return to the hive. Our results show that animals either start immediately with a straight flight from the release site (Fig. 1A, B) or they perform less regular flights (Fig. 1C). Some of the straight flights follow the vector the bees would have taken if they had not been relocated to the release site. These flights were either directed towards the hive and finished at the hive or they were be directed towards the feeder and then followed the trained route from the feeder to the hive (Fig. 1A). Some of these initially straight flights at the beginning of their homing behavior were followed by a single loop before the bees return to the hive (Fig. 1B). Therefore, we distinguish between two major flight categories: direct flights (straight flight with or without one loop, Fig. 1A, B) and indirect flights (flights with loops Fig. 1C).

First release

Figure 2 shows the proportion of bees performing different homeward flights after being relocated from the feeder to the RS and released from the RS for the first time. As already mentioned, these homeward paths involve: i) straight and rapid flights directly to the hive, with or without a single loop before returning to H ("direct flights"); or ii) irregular flights, in which bees changed direction frequently ("indirect flights"). Both control and treated bees showed similar proportions of direct flights to the hive (Test of heterogeneity: X^2= 2.604; p= 0.457; N= 79). However, we found statistical differences in the time spent performing direct flights between treatments (Fig. 3A; Kruskal-Wallis test: H= 10.008, p= 0.019, df= 3, N= 50). Specifically, bees that had ingested sucrose solution containing 10 mg/L of GLY spent more time flying from RS to H than control bees or bees that had ingested 2.5 or 5 mg/L of GLY (Mann-Whitney test: 0 mg/L vs. 10 mg/L: U= 28.5, p= 0.004; 2.5 mg/L vs. 10 mg/L: U= 13.5, p= 0.016; 5mg/l vs. 10 mg/L: U= 8.0, p= 0.003). No statistical difference in the flight time was found between control and treated bees performing indirect flights (Fig. 3B; Kruskal-Wallis test: H= 5.197, p= 0.158, df= 3, N= 29).

We observed that during some homeward flights a small number of bees passed through the feeder area. The proportion of bees that flew via the feeder was higher among control bees and bees that ingested sucrose solution with 2.5 mg/L GLY than among bees treated with 5 or 10 mg/L (see Table 1). After flying close to the feeder, those bees followed the trained flight route to the hive.

Second release

It is known that bees learn to improve their homing flights during sequential releases from the same site (Menzel *et al*., 2005). Therefore, we next asked whether this form of learning is compromised in bees that were exposed to the herbicide. To test this, bees were captured at the feeder, relocated to the RS, and released for a second time. Then, these bees were exposed twice to the same amount of GLY.

Control bees and bees that were exposed to 2.5 or 5 mg/L of GLY showed a tendency to perform direct flights more frequently than indirect flights (Fig. 4). Conversely, bees that had ingested sucrose solution with 10 mg/L of GLY showed the inverse tendency, with more bees performing indirect flights. Nevertheless, no statistical differences for the time spent in direct flights were found between control and treated

bees (Fig. 5A; Kruskal-Wallis test: H= 3.332, p= 0.343, df= 3, N= 27). It was not possible to perform a statistical analysis of data for indirect flights (Fig. 5B) because the sample size was too small (0 mg/L: N= 4, 2.5 mg/L: N= 1, 5 mg/L: N= 2, 10 mg/L: N= 3). When we compared the proportion of control and treated bees that performed direct and indirect flights after the first and second release, we found statistical differences between control bees released once or twice, but not between treated bees (Fig. 6A; Fisher´s exact test: Control bees: X^2= 10.80; p= 0.001; Treated bees: X^2= 1.07; p= 0.245, N= 32). Control bees modified their tendency to perform more indirect flights after the first release than after the second one, while the proportion of treated bees performing direct or indirect flights after one or two releases was similar. Furthermore, when studying the transitions (or lack thereof) from direct or indirect flights (or vice versa) performed after the first release to direct or indirect flights performed after the second release (direct-direct: D-D, direct-indirect: D-I, indirect-direct: I-D, and indirect-indirect: I-I), we observed a tendency to perform more I-D transitions in control bees than in treated bees. Interestingly, bees that had ingested the higher GLY concentration showed a tendency to perform more transitions to indirect flights (D-I, I-I) after the second release (Fig. 6B).

Discussion

We evaluated the effect of recommended concentrations of glyphosate (GLY) used in agricultural settings on honeybee navigation (up to 3.7 mg/L of GLY; Giesy *et al*. 2000) and two additional concentrations that resulting to be sub-lethal (5 and 10mg/L). Our results show that a single exposure to a concentration of GLY within this range delays the return of the foraging honeybee to the hive. In some cases the flight trajectories were also affected after successive exposures to the herbicide, suggesting that the spatial learning process is impaired by ingestion of the herbicide during the feeding. This impairment of navigation in the explored area increased when the concentration of GLY ingested was higher. Indeed, bees fed with 10 mg/L of GLY took more time to perform direct homeward flights and performed more indirect flights after the second release than bees treated with lower GLY concentrations. Bees that had ingested low concentrations of GLY (2.5 or 5 mg/L) and showed indirect flight trajectories after the first release performed direct flights after the second release. Accordingly, more

experimental honeybees found the hive regardless of the herbicide concentration ingested. However, subtle effects on the homing behavior within this concentration range were seen, indicating that the GLY concentrations used in this study caused only sub-lethal effects on honeybees.

Regarding the kind of flight trajectories performed, we found that honeybees treated with GLY exhibited more indirect homing flights after the second release than the control bees. As reported by Menzel *et al*. (2005), we expected that the bees released more than once from the same location improve their homeward flights. This means we expected a lower proportion of bees to execute indirect flights from RS to H after the second release. Our results show that indeed a higher proportion of control bees performed indirect flights during the first release and changed to direct flights during the second one, whereas animals treated with the highest dose of GLY were impaired in terms of improving their navigation performance. Bees released twice from the RS have fed on the contaminated food twice, a fact that might promote physiological stress and/or learning impairment. We propose that both a single exposure and repeated exposures to GLY have an effect on the retrieval and formation of memory. The effect of GLY on memory retrieval is indicated by a reduced probability to take a shortcut to the hive or the feeder, longer search flights and a lack of improvement by the experience of the homing behavior.

A recent study using the PER paradigm showed that acute exposures to GLY (2.5 mg/L) affect the retention of olfactory memory in honeybees evaluated in both simple and complex associative learning tasks. The learning process for both kinds of paradigm is faster for untreated bees and, specifically for a kind of negative pattern learning, in the presence of GLY in the reward (Herbert *et al*., 2014). Navigation requires several rather complex cognitive capacities during memory formation and retrieval that allow them to integrate current and previously acquired environmental information. These processes would compromise by the uptake of higher concentrations of GLY used as it showed for 5 (Fig. 6) and 10mg/L (Fig. 3, 4 and 6). A plausible explanation for this response is that the herbicide impairs appetitive behaviors, disturbing not only those processes involved in acquiring and associating chemosensory information, as has been proposed in a previous study (Herbert *et al*., 2014), but also the use of stored information about the environment acquired during the exploratory orientation flights of foragers and the experience from homing flights in the course of the experiment. Thus feeding on nectar containing traces of GLY might affect the

learning and retrieval of memory relevant for the recognition of food sources and for navigating between those food sources and the hive.

The ingestion of specific insecticides in sub-lethal concentrations increases sugar response thresholds (Eiri and Nieh, 2012) and affects homing in honeybees (Henry *et al*., 2012; Fischer *et al*., 2014). Herbert and co-workers (2014) have shown that chronic exposure to GLY traces reduce responsiveness to sucrose and learning performance during olfactory PER conditioning. Furthermore, when honeybees were exposed to this herbicide acutely they showed impaired associative learning, but no clear effects on their dancing behavior were observed (Herbert *et al*., 2014). These data support the view that exposure to GLY, even in low concentrations, negatively affects gustatory responsiveness and thus the motivation to forage for food also in free-flying honeybees during the experiments reported here. This motivational effect, however, was not strong enough to eliminate homing behavior but appeared to reduce the acquisition of new navigational memory.

The experiment performed here focused on the action of GLY over a short period of time (hours) but a chronic exposure to the herbicide could have additional effects and may affect the general performance of the entire colony.

Usually, genetically modified herbicide-tolerant (GMHT) crop fields are surrounded by native flora (Bohan *et al*., 2005). As we mentioned above, honeybees are the main pollinator in agricultural ecosystems but also play a key role in native flora pollination (Aizen *et al*., 2009). As a consequence of GLY application in those agricultural crops and its drift (Chang *et al*., 2011) to neighboring areas, the native species in the surrounding areas could be affected (Matthews, 2006), as well as their pollinators. Moreover, in countries that have introduced glyphosate resistant GM crops, traces of GLY were detected in honey (Chile: CIAP, 2012; Rubio *et al*., 2014), air particles and rain samples (USA: Chang *et al*., 2011; Argentina: Alonso *et al*., 2014) and in the surface of bodies of water located close to treated fields that could be visited by honeybees (Canada: CCME, 1989). In addition, we focus on agricultural settings and their surrounding, a system that includes the wild flora. The presence of GM crops in some countries where monoculture is common often is linked to the use of aerial spraying to inoculate pesticides, a situation that promotes drift of agrochemicals to non-target areas (Matthews, 2006; Chang *et al*., 2011). Moreover, herbicides are used beyond the surrounding of commercial crops; nowadays its scope has reached domestic

use in homes and gardens (Matthews, 2006), where honeybees can potentially collect food resources.

As organism resistance to agrochemicals increases, higher concentrations are used to treat agricultural crops (ARMS, 2014), and pollinators like the honeybee would expose to higher concentrations. As a consequence, higher proportions of 'disoriented' foragers could decrease foraging efficiency, leading to a reduction in the honeybee population. Such effects have been seen in neonicotinoid treatments (Henry *et al*., 2012; Schneider *et al*., 2012; Fischer *et al*., 2014). Schneider and co-workers (2012) recorded a significant reduction in the number of honeybees visiting the food source and returning to the hive after the exposure to imidacloprid and clothianidin. Moreover, bees spent longer periods inside the hive before restarting the foraging process to the food source. As a consequence of this impairment, the foraging efficiency of the colony as a whole might be affected.

The concentrations of herbicide used in our study were based on concentrations recommended for spraying fields and measured in natural environments (0 to 3.7 mg/L range (Couture *et al*., 1995; Mann and Bidwell, 1999; Giesy *et al*., 2000; Perkins *et al*., 2000; Solomon and Thompson, 2003), even though higher concentrations have not been previously measured in the environment, they were selected to represent a potential worst-case exposure scenario that a pollinator could encounter while foraging in flowers located in- or outside the GM crops (Chang *et al*., 2011). Interestingly, the locomotive activity of bees tested in our study was not impaired after the incubation phase and they did not reject the sucrose solution offered, whether with or without GLY. As a result, honeybees continued foraging at our feeding station and thus also on plants that expose bees to similar GLY concentrations, and the contaminated nectar or pollen could be brought back by honeybees to the hive and accumulated there. Rubio and co-workers (2014) found traces of glyphosate in both organic and non-organic honey samples (26-93 ppb, mean 50 ppb; 17-163 ppb, mean 66ppb; respectively) made in several countries, purchased in a metropolitan area of the USA. Moreover, they found the presence of GLY traces in honey samples by wild and melliferous flora. Although the amounts they reported are lower than the GLY concentration that we used in this study, it does not mean that this was representative of those concentrations the forager bees are exposed in the field. Since we used recommended concentration of GLY in food, it is expected that some bees could find this concentration in the field and brought it back to the hive. With this in mind, further studies in GLY exposed commercial crops and its

surrounding are necessary to evaluate the actual exposure of forager bees to the herbicide, and the relationship between the concentration of GLY collected by the honeybees in exposed environments and the traces found in the stored honey or pollen.

Despite the lack of data of the actual level of GLY exposed by forager honeybees in the field, present results show that exposure to non-lethal concentrations of glyphosate causes sub-lethal effects modifying their foraging behavior. However, further studies are necessary to evaluate to what extent this chemical influences foraging behavior of honeybees in a natural environment and whether prolonged exposures to this herbicide might contribute to worsen the health status of beehives. Since that GMHT crop fields are usually surrounded by native flora which is visited by honeybees, it would also be necessary to analyze traces of glyphosate present in collected and stored honey and pollen, as well as in larvae and adult bees from hives located in the surroundings of agricultural crops treated with GLY, before and after the herbicide application.

Material and Methods

Animals and study site

We used bees from a colony of approximately 30,000 bees (*Apis mellifera*). The experiment was conducted from August to September of 2013 in an open field (coordinates: N 50°48′53.01”, E 8°52′21.36”) located close to the village of Großseelheim (Hessen), Germany.

Experimental procedure

A group of forager bees was trained to collect unscented 0.5M sucrose solution from an artificial feeder located 400 m north of the hive and fitted with colored number tags on the thorax for individual identification. At 15-minute intervals, numbered bees were captured individually at the feeder before they began to ingest the sucrose solution offered and were immediately confined in plastic tubes, and transported to the release site (henceforth: RS) located 460 m east of the feeder location. The RS was located within the area explored during orientation flights, but otherwise it was novel for the trained bees. Each plastic tube contained a small feeder providing 50 µl of unscented 2M sucrose solution, either with or without glyphosate. The tube was kept in a dark box

for one hour (incubation), allowing bees to ingest all the solution offered. Three different concentrations of GLY were used (diluted in 2M sucrose solution, see next section for more details): 2.5 mg/L, 5 mg/L and 10 mg/L. Control bees were handled in the same way but were fed the solution without herbicide (0 mg/L).

After incubation a radar transponder was glued to the number tag fixed on the thorax of each bee and the homeward flight trajectory (from the RS to the hive) was tracked with harmonic radar. Bees were released at 15-minute intervals to ensure the same incubation time for all the individuals. One experimenter at the radar station passed on information about the flight trajectories of the released bees by walkie-talkie. Once the bee arrived at the hive it was caught, the transponder was removed, and then the honeybee was allowed to enter the hive. Whenever possible, these bees were captured at the feeder and released from the RS once more ("second release") in order to test whether learning during homing flights was compromised. The total number of bees tested was 108 for the first release, and 44 for the second. The number of flight trajectories obtained was 79 for first release and 37 for the second (see Tables 1 and 2).

We measured the following variables: capture time, release time, arrival time at the hive and the flight trajectory recorded with the harmonic radar. If a bee was observed on the radar but then disappeared from the radar range and was not seen arriving at the hive, it was classified as a non-arriving bee.

Herbicide

A stock solution of glyphosate (Glyphosate PESTANAL, Sigma-Aldrich, Steinheim, Germany) at a concentration of 100 mg acid equivalent /L was prepared with distilled water and kept refrigerated. New stock solution was prepared every 7 days. The stock solution was diluted in sucrose solution 2M to obtain the different GLY concentrations used in the experimental procedure. The concentrations of herbicide used were: 0 mg (control), 2.5 mg, 5 mg and 10 mg of glyphosate per liter of sucrose solution. Each bee ingested 50 µl of sucrose solution 2M with or without GLY, so the concentrations used were equivalent to the following doses: 0 ng, 125 ng, 250 ng and 500 ng of glyphosate per bee.

Harmonic radar tracking

Tracking bees with a harmonic radar system is described in Riley *et al*. 1996, 2005; Menzel *et al*. 2011 and Scheiner *et al*. 2013. We used a system with a sending unit which consisted of a 9.4 GHz radar transceiver (Raytheon Marine GmbH, Kiel, NSC 2525/7 XU) combined with a parabolic antenna of approx. 44 dBi that provided a signal from the transponder on the bee thorax every 3s. The transponder consisted of a dipole antenna with a Low Barrier Schottky Diode HSCH-5340 of centered inductivity. The second harmonic component of the signal (18.8 GHz) was the target for the radar. The receiving unit consisted of an 18.8 GHz parabolic antenna with a low-noise preamplifier directly coupled to a mixer (18.8 GHz oscillator) and a downstream amplifier with a 90 MHz ZF-Filter. The transponder was made of a silver wire with a diameter of 0.3 mm, a length of 11 mm, a weight of 10.5 mg and a loop inductance of 1.3 nH. The range of the harmonic radar had a radius of 900 m.

Statistical analysis

A heterogeneity Chi-square analysis was used to compare the proportion of bees performing direct or indirect flights from the release site back to the hive. A Kruskal-Wallis test was performed to compare the time bees spent between the RS and the hive, according to the treatments (control bees and bees exposed to GLY: 2.5 mg/L, 5 mg/L and 10 mg/L).

To compare the proportion of bees performing direct or indirect flights according to whether bees were released once or twice, we applied Fisher´s exact test (Sokal and Rohlf, 1995).

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.S.B, R.M. and W.M.F. conceived and designed the experiments. M.S.B., U.W. M-L.H., L.T. and R.M. performed the experiments. M.S.B., R.M. and W.M.F. performed data analysis. M.S.B, R.M. and W.M.F. drafted the manuscript. All authors revised and commented on the manuscript.

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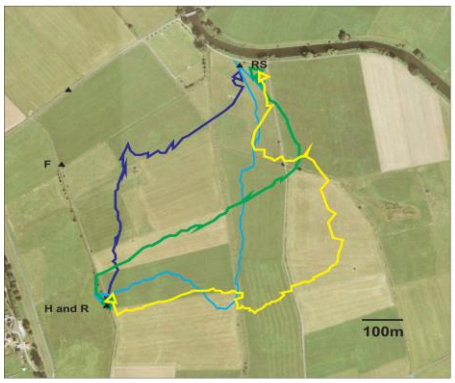
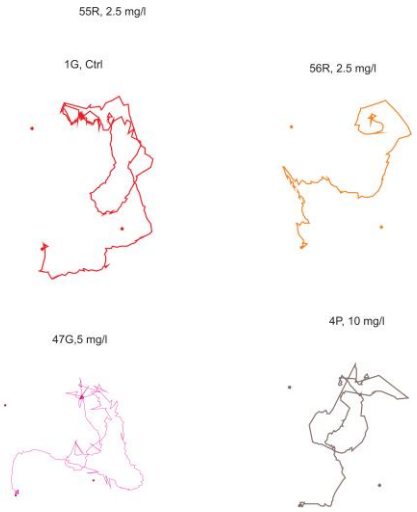
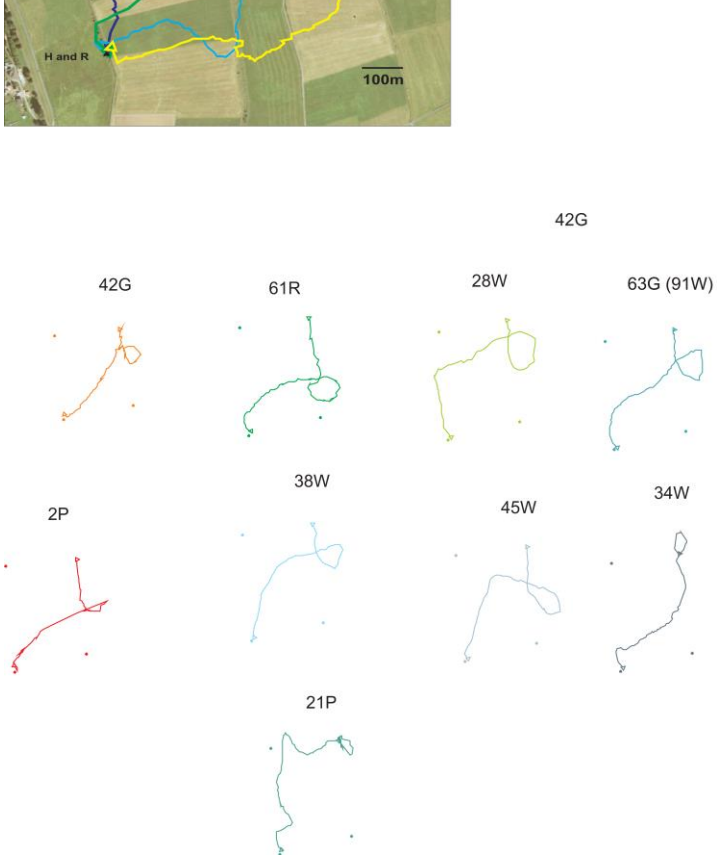
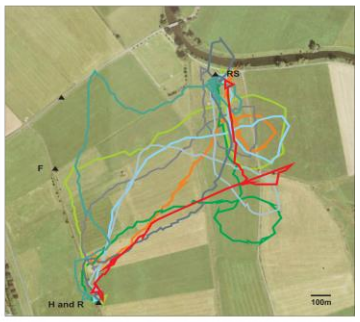
Figures









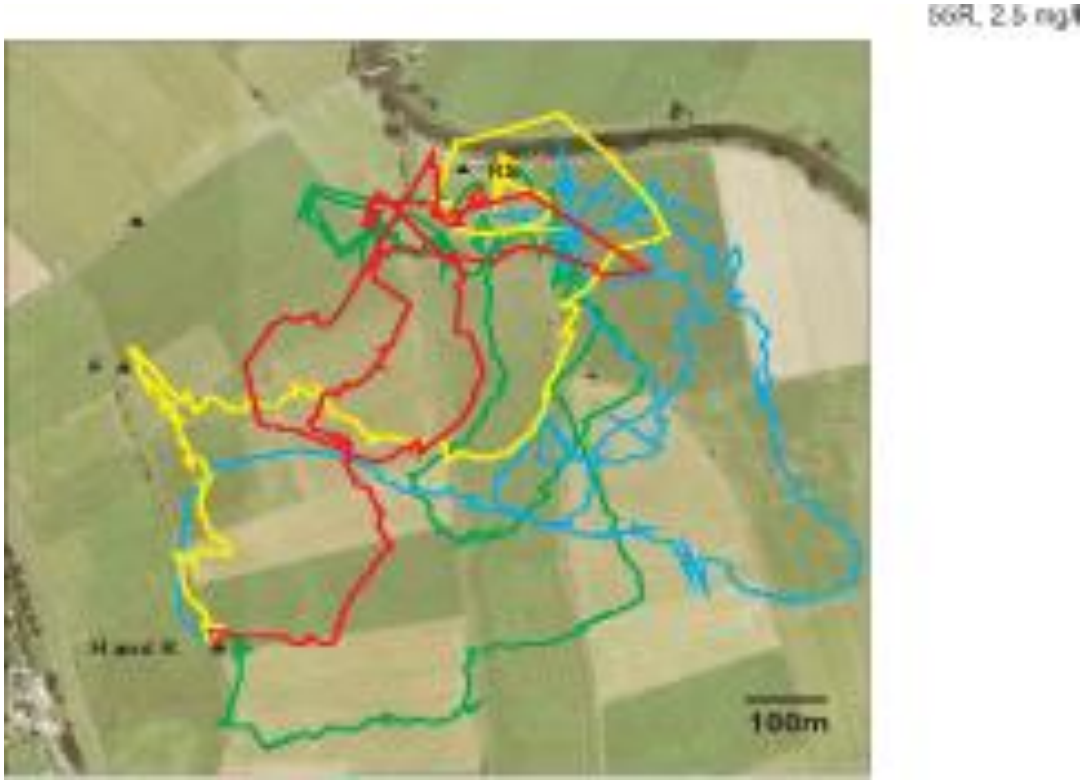


Figure 1. Examples of homeward flights during the first release after treatment. Flight paths were categorized as *direct* (**a**), *single-loop* (**b**) or *indirect* (**c**). Colors indicate: light blue and red for control bees, blue and orange for bees treated with 2.5 mg/L of glyphosate (GLY), yellow and pink for bees treated with 5 mg/L of GLY, and green and gray for bees treated with 10 mg/L of GLY.. H, R, F, RS indicate the location of hive, radar, feeder, and release site, respectively.

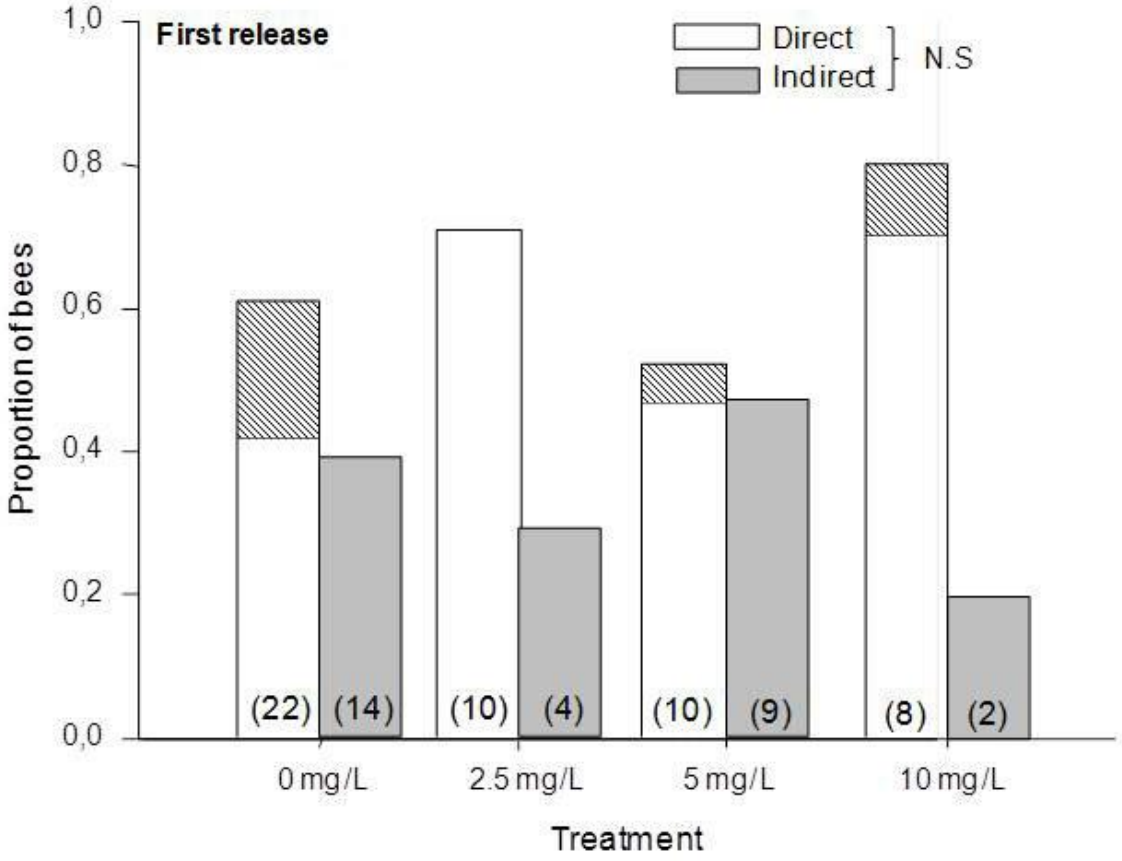


Figure 2. Proportion of bees performing *direct* and *indirect* homeward flights after the first release. Proportion of bees performing direct (white bars) and indirect (gray bars) homeward flights were pooled according to the treatment; looped flights are shown in hatched bars. 0 mg/L: control bees; 2.5 mg/L, 5 mg/L and 10 mg/L: bees exposed to different concentrations of GLY (corresponding to 0.125, 0.250 and 0.500 µg/animal). N.S, no significant differences ($p > 0.05$). Numbers inside the bars indicate the number of bees for each treatment.

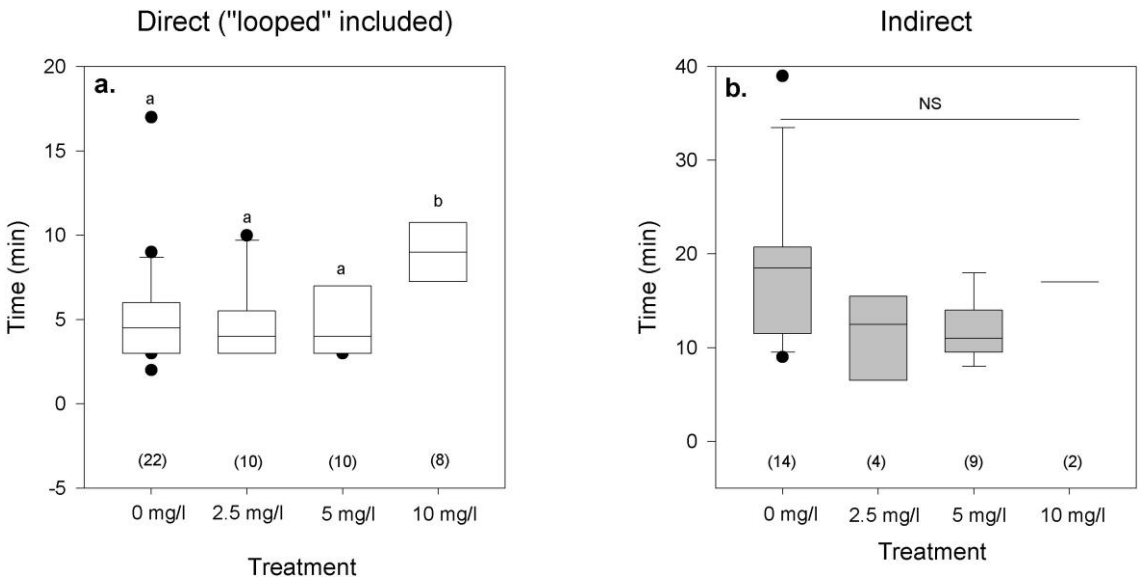


Figure 3. Times of homeward flights after the first release. Flying times from the release site to the hive according to different treatments (0 mg/L: control bees; 2.5 mg/L, 5 mg/L and 10 mg/L: bees exposed to 2.5 mg/L, 5 mg/L and 10 mg/L GLY, respectively). **a**, *direct* and "*single-loop*" flights; **b**, *indirect* flights. Boxes with different letters are significantly different at $p<0.05$. N.S, no significant differences ($p > 0.05$). Numbers in brackets indicate the number of bees for each treatment.

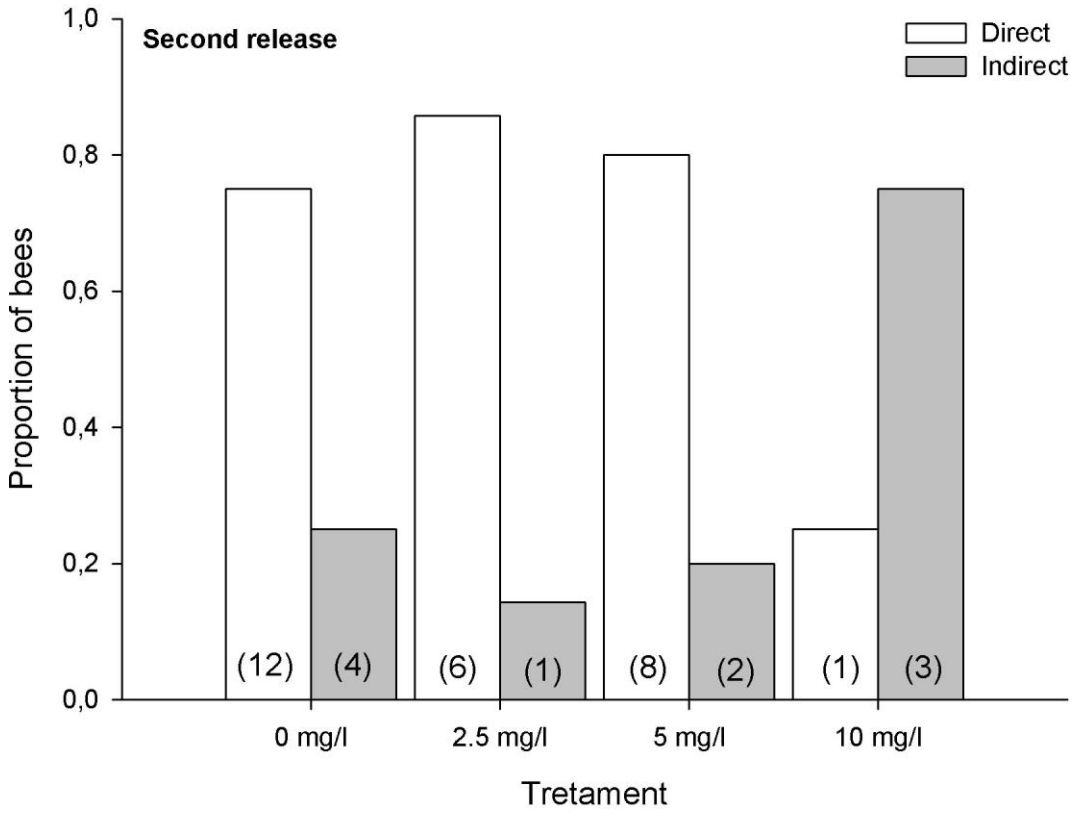


Figure 4. Proportion of bees performing *direct* and *indirect* homeward flights after the second release. Bees performing *direct* (white bars) and i*ndirect* (gray bars) homewards flights for the different treatments. Numbers in brackets indicate the number of bees for each treatment.

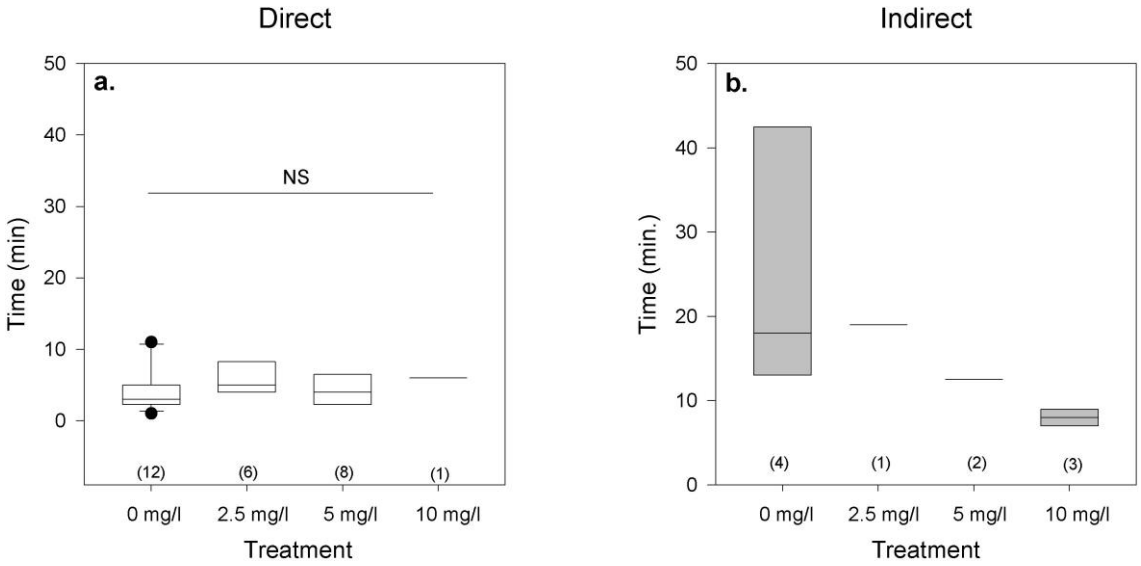


Figure 5. Times of the homeward flights after the second release. Flying times from the release site to the hive represented for the different treatments (0 mg/L: control bees; 2.5 mg/L, 5 mg/L and 10 mg/L: bees exposed to 2.5 mg/L, 5 mg/L and 10 mg/L GLY, respectively). a, *direct* flights; b, *indirect* flights. N.S., no significant differences ($p > 0.05$). Numbers in brackets indicate the number of bees for each treatment.

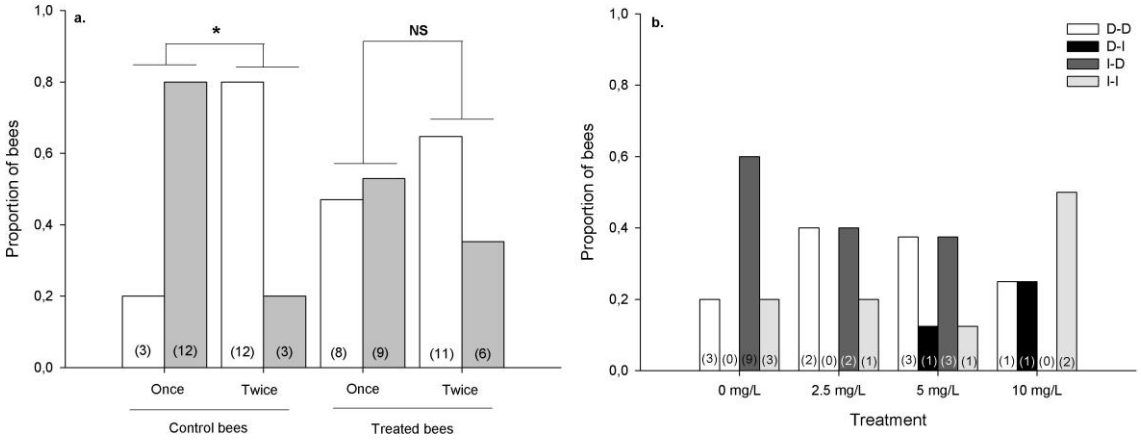


Figure 6. Proportion of transitions in the performance after the first and the second release according to treatment. a, Control and treated bees were categorized according to *direct* (white bars) or *indirect* flights (gray bars) after the first release (they fed GLY *once*) or the second release (they fed GLY *twice*). **b**, Flight transitions between the first and second release was considered per experimental bee (D: *direct* flight, I: *indirect* flight). The categories of both flights are the following: D-D (both flights were direct), D-D (both flights were indirect), D-I (the first flight was direct and the second indirect), I-D (the first flight was indirect and the second direct). Asterisk indicates $p<0.05$; N.S, no significant differences ($p > 0.05$). Numbers in brackets indicate the number of bees for each treatment.

Tables

Table 1. Total number of bees released for the *first time* and those that returned to the hive.

Treatment	Total number of bees	Arrived at the hive	Arrived at the hive via feeder	Not arrived
0 mg/L	46	36 (0.78)	6 (0.17)	10 (0.22)
2.5 mg/L	25	14 (0.56)	6 (0.42)	11 (0.44)
5 mg/L	22	19 (0.86)	2 (0.11)	3 (0.14)
10 mg/L	15	10 (0.67)	2 (0.2)	5 (0.33)

Numbers in parenthesis indicate the proportion of bees for each treatment.

Table 2. Total number of bees released for the *second time* and those that returned to the hive.

Treatment	Total number of bees	Arrived at the hive	Not arrived
0 mg/L	19	16 (0.84)	3 (0.16)
2.5 mg/L	11	7 (0.64)	4 (0.36)
5 mg/L	10	10 (1)	0 (0)
10 mg/L	4	4 (1)	0 (0)

Numbers in parenthesis indicate the proportion of bees for each treatment.