Running title: Effects of glyphosate traces on honeybee appetitive behaviour

Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour

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Keywords: *Apis mellifera*, glyphosate, sub-lethal effects, associative learning, sensitivity to reward.

Abstract. Glyphosate (GLY) is a broad spectrum herbicide used for weed control. Presently, sub-lethal impact of GLY on non-target organisms such as insect pollinators has not been evaluated yet. *Apis mellifera* is the main pollinator in agricultural environments and a well-known model for behavioural research. Moreover, honeybees are accurate biosensors of environmental pollutants and their appetitive behavioural response is a suitable tool to test sub-lethal effects of agrochemicals. We studied the effects of field-realistic doses of GLY on honeybees exposed chronically or acutely to it. We focused on sucrose sensitivity, elemental and non-elemental associative olfactory conditioning of the proboscis extension response (PER) and on foraging related behaviour. We found a reduced sensitivity to sucrose and learning performance for the groups chronically exposed to GLY concentrations within the range of recommended doses. When olfactory PER conditioning was performed with sucrose reward with the same GLY concentrations (acute exposure), elemental learning and short-term memory retention decreased significantly compared to controls. Non-elemental associative learning was also impaired by an acute exposure to GLY traces. Altogether, these results imply that GLY at concentrations found in agro-ecosystems due to standard spraying can reduce sensitivity to nectar reward and impair associative learning in honeybees. However, no effect on foraging related behaviour was found. Therefore, we speculate that successful forager bees could become a source of constant inflow of nectar with GLY traces that could then be distributed among nest mates, stored in the hive and have long-term negative consequences on colony performance.

Introduction

Glyphosate (GLY), N-(phosphonomethyl) glycine, is a broad spectrum herbicide applied for weed control (Goldsborough and Brown, 1988). In the last decades its consumption has increased sharply and it has become one of the most used agrochemicals worldwide (Zhang et al., 2011). Due to the upscale in monocultures and genetically modified crops, aerial applications of GLY have become the most common application method and have widened its spread area (Giesy et al., 2000). This and other methods of application generate spray drift which carries the herbicide away from the limits of the field cultivated with the target crop. Therefore, its widespread presence in agricultural ecosystems and their surroundings has inevitably made us wonder what effects, if any, it has on non-target organisms.

Although GLY inhibits aromatic amino acid pathways present only in plants, microorganisms and fungi, but not in animals (Amrhein et al., 1980; Carlisle and Trevors, 1988;Duke et al., 1989), there are studies that have found different negative effects in invertebrate and vertebrate species. For instance, common application concentrations have been found to cause growth deficit in the earthworm *Aporrectodea caliginosa* (Springett and Gray, 1992) and concentrations higher than 10 mg/L have been proven to have an effect on body growth in the freshwater snail *Pseudosuccinea columella* (Tate et al., 1997). In vertebrates, studies indicate that chronic exposure to different formulates with GLY concentrations ranging between 3.8 and 18 mg acid equivalent/L (a.e./L) may negatively affect amphibians (Howe et al., 2004; Relyea, 2005a, b).

Honeybees *Apis mellifera* are the main pollinators in agricultural ecosystems (Aizen et al., 2009). Each foraging honeybee makes trips several times a day to gather resources from several kilometres away and, in doing so, takes any foreign substances present in those resources back to the hive. Since honeybee foragers take back to the hive substances present in the resources they gather (von Frisch, 1967), agrochemicals with a high solubility in water such as GLY, which might be present in the flowers visited after a spray application (Bohan et al., 2009), may also be present in the stored honey. Substances that are taken into the hive can remain stored for long periods of time and accumulate until they are used as supplies for the colony (Devillers and Pham-Delègue, 2002). Hence, agrochemicals accumulated inside the hive could have subtle negative effects, often inconspicuous within the short term (Giesy et al., 2000), that could impair behavioural processes in the long-term (Kirchner, 1999). As a result, honeybees are very sensitive biosensors of changes in the environment and respond even to subtle variations caused by pollutants (Devillers and Pham-Delègue, 2002). Sub-lethal effects of agrochemicals can be evaluated on honeybees through standardized laboratory assays based on appetitive behavioural responses, learning abilities and foraging and communication skills.

Honeybee foragers can obtain information and retain a variety of cues from the environment by perceiving different sensory stimuli and establishing associations between them (Menzel, 1999). In this way, bees can learn to associate a specific odour with a reward (elemental learning) or even that an odour predicts reward only when it is part of a complex blend (e.g., non-elemental learning; (Deisig et al., 2001; Giurfa, 2003, 2007). Acquisition of

olfactory information has been shown to be well retained even when it occurs at young ages of the adult stage (Arenas and Farina, 2008; Arenas et al., 2009a; Arenas et al., 2012). Young workers that remain inside the hive can learn rewarded odours when fed with resources recently collected (Nixon and Ribbands, 1952; Grüter et al., 2006) or with food stored in the hive (Winston, 1987). Moreover, experiences acquired inside the colony can increase the efficiency of a colony's foraging related tasks (Arenas et al., 2009b; Balbuena et al., 2012a). These learning abilities can be evaluated under laboratory experimental conditions through the proboscis extension response (PER). Bees extend their proboscis after their antennae have been stimulated with sucrose solution and this response can be conditioned if a neutral stimulus (e.g., an odour or another sensory stimulus) is paired with the reward (Kuwabara, 1957; Takeda, 1961; Bitterman et al., 1983; Matsumoto et al., 2012).

The proboscis extension response can also be used to measure reward sensitivity. Reward sensitivity is intimately bound to associative learning (Scheiner et al., 1999; Page and Erber, 2002)and therefore, inseparable from foraging behaviours (Page et al., 1998). Changes in food source profitability found by foragers affect their threshold for appetitive responses to the extent that they modify a series of stereotyped movements used to convey information, known as the waggle dance (von Frisch, 1967). The dancers' manoeuvres encode information about the location and profitability of the discovered food source which is transmitted to the rest of the colony during the dance (von Frisch and Lindauer, 1955; Riley et al., 2005; Thom et al., 2007; Grüter and Farina, 2009a, b). This complex behavioural repertoire and

the specialized skill set of workers are highly relevant and fine-tuned for colony survival and susceptible to sub-lethal effects of noxious substances.

Glyphosate toxicity tests on *Apis mellifera* for product approval did not consider sub-lethal nor prolonged exposure effects. Studies were only focused on obtaining LD50 (lethal dose, 50%) as a measure of the effect of an acute exposure, but nevertheless, they were carried out on the basis that honeybees might in fact be exposed to GLY in their natural environment, either through the consumption of contaminated resources or through a direct exposure as a result of inadvertent spraying (Giesy et al., 2000). Even though LD50 results seem to indicate that GLY is not harmful for honeybees, the fact that honeybees are potentially exposed to GLY motivated us to pursue further analysis and to address the lack of chronic studies.

We were specifically interested in the possible sub-lethal effects of GLY on *Apis mellifera*. To evaluate these effects we used GLY concentrations within a range of 0 to 3.7 mg a.e./L which do not exceed those recommended for aquatic and terrestrial weed control nor those measured in natural environments that arefound within a 1.4 to 7.6 mg a.e./L. range (Goldsborough and Brown, 1988; Feng et al., 1990; Giesy et al., 2000). We focused on reward sensitivity (sensitivity to sucrose) and learning abilities of honeybees, processes that involve appetitive behaviours. First we evaluated the effect of prolonged exposures to GLY at pre-foraging ages (henceforth: laboratory-reared bees) on sensitivity to sucrose and on associative learning. We then studied the effect of acute exposures to GLY at foraging ages (henceforth: hive-reared bees) on elemental and non-elemental associative learning and on foraging behaviour.

Results

I. Effect of prolonged exposures to glyphosate on laboratory-reared bees

Survival, food ingestion and locomotive activity. We first investigated the effect of a prolonged exposure to GLY on the behaviour of laboratory-reared bees. Table 1 shows the results obtained for survival, ingestion and locomotive activity measured at 15 days of age on bees exposed to different GLY concentrations during the first 15 days of adult life. Although bees exposed to GLY showed a higher level of mortality than untreated bees, we found no significant differences between both groups (one-way ANOVA: $F_{2,12}$=3.67, p=0.057, Table 1). This result, together with the fact that the highest accumulated mortality recorded during 15 days only reached 24%, led us to regard the GLY doses used as sub-lethal.

Before evaluating the effect of a prolonged exposure to GLY on sensitivity to sucrose and learning abilities, we studied whether it had an effect on the overall behaviour of 15 day old bees. Food intake, mortality, mortality due to harnessing and locomotive and orientation activity did not vary between bees exposed to different GLY concentrations (food intake: $F_{2,12}$=1.32, p=0.305, one-way ANOVA; survival between harnessing and PER conditioning: G_H=0.76, p=0.683, N=579, df=2, G-test; locomotive activity: main effect GLY concentration: $F_{2,9}$=0.07, p=0.936, GLY concentration x LED colour interaction: $F_{2,4}$=0.85, p=0.493, three-way RM-ANOVA; for details see Table 1).These results show that all bees, independently of the GLY concentration to which they were exposed, presented similar behavioural responses and survival rates at 15 days of age.

Sensitivity to sucrose. With the general behavioural results in mind, we investigated whether sensitivity to sucrose and learning performance were also intact. We first tested the sensitivity to sucrose of bees through a proboscis extension response and gustatory response score protocol (PER-GRS protocol). GRS scores of bees exposed to GLY were lower than those of non-exposed bees (Kruskal-Wallis test: H=9.54, p=0.007, N=203, df=2; Fig. 1A). This indicates that 15-day-old bees that were reared with sub-lethal concentrations of GLY present an increased response threshold for sucrose.

Olfactory PER conditioning. Next, we assayed bees' performance in an absolute olfactory classical conditioning protocol of the proboscis extension response (PER). Figure 1B shows the %PER towards the conditioned stimulus (CS: linalool, henceforth: LIO) for bees of 15 days of age for the course of 3 acquisition trials in which the reward did not contain GLY. Bees that were exposed to sub-lethal concentrations of GLY during the first 15 days of adult life showed a lower performance than non-exposed bees. We performed a two-way repeated measures analysis of variance and found a significant interaction between factors (two-way RM-ANOVA; main effect GLY concentration: $F_{2,282}$=7.76, p<0.001; interaction GLY concentration x acquisition trial: $F_{2,4}$=5.14, p<0.001; Fig. 1B). We therefore computed simple effects for GLY concentration and found statistical differences for GLY concentration effects for the second acquisition trial (One-way ANOVA: $F_{2,282}$=9.19, p<0.001). Tukey post hoc comparison tests revealed that the effects of the three GLY concentrations on the second acquisition trial differ

(p<0.05). These results show that a prolonged exposure to sub-lethal concentrations of GLY during the first 15 days of adult life hinders the acquisition dynamics of the ability to establish an association between an odour and a reward.

However, this effect was not carried through to the evaluation stage (Fig. 1C). The conditioned response towards the trained odour alone measured 15 minutes after acquisition did not differ between GLY concentrations (G-test: G_H=0.550, p=0.760, N=159, df=2; Fig. 1C). Overall, these results show that a prolonged exposure to sub-lethal concentrations of GLY does not have an effect on the establishing of short-term memories but it does have an impairing effect on the ability to establish odour-reward associations, which could be related to the detrimental effect found on gustatory responsiveness.

II. Effect of acute exposure to glyphosate on hive-reared bees

Elemental olfactory learning. After studying the effects of a prolonged exposure to GLY at pre-foraging ages we wondered whether an acute exposure to GLY at foraging ages could also have an effect on honeybees. We started by performing an elemental PER conditioning assay with 0 or 2.5 mg GLY per litre of 1.8 M sucrose solution as reward. Figure 2 shows the overall performance of both groups of bees for the duration of 8 acquisition trials and 5 extinction trials. Right away, from trial 2 of the acquisition phase, bees that received GLY in the reward showed a lower PER towards the CS (LIO). The difference between both groups remained throughout the rest of the protocol: bees that were acutely exposed to GLY responded consistently

less than bees that were not exposed (Mann-Whitney test: U=338.50, N_1=N_2=32, Z=2.33, p=0.019; Fig. 2).

Non-elemental olfactory learning. To further investigate acute exposure effects of GLY on hive-reared bees, we carried out a non-elemental PER conditioning assay using a negative patterning discrimination assay. Figure 3A shows %PER averaged across all trials of A+ (LIO or 2-Octanol), B+ (1-Hexanol or limonene), and AB− (LIO and 1-Hexanol or 2-Octanol and limonene), respectively, for each group of bees exposed to a different GLY concentration. A GLY concentration × Element (2 × 2) ANOVA yielded no differences for the elements A+ versus B+ (two-way ANOVA: $F_{1,134}$=0.82, p=0.367; Fig. 3A). We therefore pooled the reinforced elements (A+ and B+) within each GLY group for the next analysis. Figure 3B shows the course of conditioned responses to the compound AB− and the average responding to the elements A+ and B+ across blocks of trials for each group. Bees in both groups could correctly discriminate the reinforced elements (A+, B+) from the non-reinforced element (AB-), as shown by the increase in response towards the reinforced elements throughout the trials whilst the response to the non-reinforced element remains constant. We then evaluated total acquisition (and therefore overall amount of differentiation) by computing the average level of responding to the pooled CSs+ and to the CS− for each GLY group. Bees rewarded with GLY during the negative patterning discrimination assay had an overall lower acquisition than non-exposed bees (two-way ANOVA: $F_{1,134}$=5.92, p=0.016; Fig. 3B).These results indicate that

an acute exposure to sub-lethal GLY concentrations impairs non-elemental learning abilities of hive-reared bees.

Foraging related behaviour. We investigated the effects of an acute GLY exposure in a more realistic and natural context by training bees to an artificial feeder and measuring different foraging variables for each bee, before and after the artificial feeder contained sucrose solution with GLY. We started by analysing the cycle time (min) and visit frequency (cycles/hour) of each bee, before and after the exposure. Bees continued visiting and collecting at the artificial feeder at a constant rate regardless of whether the artificial feeder contained GLY or not (Wilcoxon matched pairs test; cycle time: Z=1.15, N=6, p=0.249, Fig. 4A; visit frequency: Z=1.57, N=6, p=0.116, Fig. 4B).

Having established that foragers return to the hive and complete foraging cycles in the same manner even when GLY is present at the food source, we then focused on the transfer of information that occurs inside the hive. Dance probability did not differ before or after GLY exposure (Wilcoxon matched pairs test; dance probability: Z=0.944, N=9, p=0.345, Fig. 4C).Thus, we assayed the dance event in itself. We found no change in the mean number of waggle-runs per hive when GLY was added to the food source (Wilcoxon matched pairs test: Z=0.024, N=17, p=0.981, Fig. 4D). The mean percentage of dance errors per hive stay was not affected either by the presence of GLY in the sucrose solution (Wilcoxon matched pairs test: Z=0.639, N=17, p=0.523, Fig. 4E).

Discussion

We set out to evaluate the effects of chronic and acute exposures to field-realistic doses of glyphosate (GLY), the main herbicide currently used for weed control in agriculture, on the behaviour of the honeybee *Apis mellifera*. Our results show that both chronic and acute exposure to GLY traces produce sensory sensitivity and cognitive deficits on adult honeybees of the worker caste. The concentrations used (within a 0 to 3.7 mg e.a./L range) were based on concentrations recommended for spraying and on those measured in natural environments, from 1.4 to 7.6 mg e.a./L (Goldsborough and Brown, 1988; Feng et al., 1990; Giesy et al., 2000), and were shown to be sub-lethal for honeybees. Young adult bees chronically exposed to concentrations of 2.5 and 5.0 mg/L of GLY showed reduced sensitivity to sucrose (reward) and impaired acquisition dynamics during elemental associative olfactory learning. This impairment cannot be explained by deterioration of the general state or motor skills of the subjects, since measurements such as survival, food uptake and locomotive activity did not differ between experimental groups. Furthermore, acute exposure to GLY significantly decreased short-term memory retention and negatively affected non-elemental associative learning at foraging ages. Nevertheless, an acute exposure to GLY in a foraging context did not have a detrimental effect on foraging activity and dancing behaviour. Altogether, these results imply that GLY at concentrations that can be found in nature as a result of standard spraying reduce sensitivity to nectar reward and also impair associative learning in honeybees. Since no effect on foraging activity was found, successful forager

bees can become a source of inflow of nectar with GLY traces into the hive, which in turn could have long-term negative consequences on colony survival.

Our first results shed light on the effects of a prolonged exposure to sub-lethal concentrations of GLY during the first 15 days of adult honeybee life. An exposure to GLY during this period caused both a lower sensitivity to reward and a reduction in the dynamics of acquisition without an effect on memory retention, compared with non-exposed bees. One plausible explanation for these results is that a prolonged exposure to GLY promotes an increase in sugar response thresholds and that this is expressed by a lower PER percentage to the rewarded odour during training. There is evidence that sub-lethal concentrations of insecticides, such as neonicotinoids can in fact affect behaviours involved in honeybee foraging, as for example the sugar response thresholds that increase with traces of these insecticides (Eiri and Nieh, 2012) and impair learning and memory processes (Williamson and Wright, 2013; Fischer et al. 2014). However, we have not found any record of similar effects due to the use of herbicides. It is important to note that survival and behavioural variables after a prolonged exposure to GLY show that all bees, independently of whether they had been exposed to GLY and of the GLY concentration to which they were exposed, had a similar general state at 15 days of age.

In what respects to the acute exposure of adult bees to the herbicide, we also showed that honeybees present a diminished capacity to associate an odour to a reward through elemental associative learning, as was observed through an exposure to a low GLY concentration (2.5 mg/L). Furthermore,

acute exposures to GLY not only show effects on the acquisition of an odour-reward association, but also on retention of olfactory memory. This can be deduced by the faster extinction process found in bees trained with reward that contained sub-lethal concentrations of GLY. Moreover, we found a similar deficit when we exposed bees to GLY during a non-elemental associative learning protocol that requires a more complex cognitive process. Even though the response towards the unrewarded mix of odours (AB-) did not decay along conditioning as was expected (Giurfa, 2003), the differences between PER values towards rewarded and unrewarded stimuli along the learning process were increasingly higher for untreated bees. Consequently, a negative patterning learning paradigm can be better resolved without the presence of the herbicide in the reward. Overall, these results suggest that an acute exposure to GLY affects the nervous system of bees either by acting on chemo-sensory stimuli perception (gustatory and/or olfactory) or by directly hindering the association between the unconditioned and the conditioned stimulus. In both cases, individuals exposed to this herbicide would need more learning events in order to reach response levels similar to those not exposed.

Honeybees roam the countryside when foraging. During their trips, they interact both with plants that are targeted by agrochemical spraying and with non-target plants that have become contaminated by drift or accidental spraying they do not always identify foreign substances in nectar as noxious and so continue gathering it. Subtle negative effects promoted by handling nectar with GLY traces may impair important processes that play a fundamental role in the framework of foraging activities, such as response

thresholds for reward and odour-reward learning. When we then evaluated the behaviour of free flying bees, focusing specifically on foraging and recruitment behaviour (measured through the waggle dance) we found no effect when we added traces of GLY to an artificial food source. In fact, honeybees did not interrupt foraging activity nor were they impeded from intensely displaying a complex motor pattern such as the waggle dance once back in the hive. This result is consistent with the lack of effect on locomotive activity after a prolonged exposure to GLY.

The constant inflow of GLY into the hive means that the agrochemical would accumulate in the hive's stores which would then be fed to larvae and young bees and used as sustenance for the whole colony during the winter. In this sense, a recent study found no effects of GLY on brood survival, development, and mean pupal weight in a realistic exposure scenario (Thompson et al. 2014). In this study, honeybee colonies were exposed to the herbicide when the glasshouse where the colonies were settle was sprayed with GLY (i.e., higher glyphosate doses than in the present study would income into the hive). Despite of these results, bees chronically exposed to GLY or any other agrochemical found in the food sources of the hive may perform tasks with diminished cognitive capacities, as we showed in this study. Therefore, it is likely that activities that require a decision making process based on information previously acquired through learning and memory, such as which nectar to process (Goyret and Farina, 2005), which dances to follow (Balbuena et al., 2012a) or which source to visit (Balbuena et al., 2012b), will be affected. This in turn might have negative consequences in the search and collection of resources as well as in the

coordination of collective activities. In the long term, this could affect the survival of these colonies.

Our results have shown that the presence of sub-lethal concentrations of GLY in this context has the following consequences: i) a lower sensitivity to reward, ii) the formation of weak associative memories that can be extinguished rapidly and iii) a difficulty to establish non-elemental associations. These difficulties to establish associative memories would in turn make the gathering of resources inefficient. However, our results have also shown that foraging behaviour is not immediately affected by the presence of GLY in the food source. Therefore, these same forager bees become vectors of the herbicide that is taken back to the hive, disseminated between the individuals of the hive and stored in their reserves for long periods of time (Kirchner et al., 1988).

Bearing in mind the results we found regarding the effects of GLY on sensory sensitivity and associative learning, it is hard not to wonder what effect GLY has on survival and sanitary state of honeybee hives exposed to this agrochemical. This is the first study on the sub-lethal effects of an herbicide on honeybee behaviour and we hope it contributes to understanding how honeybee hives situated in agricultural environments are affected by agrochemicals. Many questions fan out from our results. For instance, how would honeybees exposed to sub-lethal doses of GLY be affected by experiencing stress from infestation with parasites or pathogens? Could an exposure to a combination of a pesticide and GLY have a synergistic effect on honeybees? What are the mechanisms underlying the effects found in the present study? It is therefore essentialto examine the real exposure of

honeybees to GLY in agricultural environments in order to determine how feasible chronic exposure is and what risks it actually implies for honeybee colony survival.

Materials and Methods

Study site and animals

Experiments were performed during the austral spring, summer and fall seasons between 2010 and 2013. European honeybees *Apis mellifera* L. of the worker caste were reared either in the laboratory or in hives from our apiary located at the experimental field of the University of Buenos Aires, Buenos Aires, Argentina (34° 32’ S, 58° 26’ W).

To study the effect of prolonged exposures to GLY we worked with adult bees reared under laboratory conditions (laboratory reared bees). Bees were obtained from sealed brood frames placed in an incubator (36°C, 55% relative humidity, RH, and darkness). Recently emerged adults (0–1 days old) were collected in groups of about 100 individuals in wooden cages (10 x 10 x 10 cm) that had a wire mesh door on one side. Bees were fed with a 1.8M sucrose solution with different GLY (Sigma-Aldrich, Steinheim, Germany) concentrations, in addition to water and pollen *ad libitum*. Three GLY concentrations were used: 0 mg (control group), 2.5 mg and 5 mg per litre of sucrose solution. Caged bees were kept in an incubator (31ºC, 55% RH and darkness) until 15 days of age. Feeding tubes were refilled every 48 hours in order to reduce any effects that high incubator temperatures might have on GLY and to avoid bacterial proliferation, which is known to shift the pH in sucrose solutions.

Experiments to study the effect of acute exposures to GLY were performed using worker bees caught at the entrance of outdoor hives at the beginning of each experimental procedure (hive-reared bees). In order to study foraging-related behaviour a colony of 3000 to 4000 worker bees, queen and brood was placed in a two-frame observation hive (von Frisch, 1967) located inside the laboratory. The experimental hive consisted of two see-through acrylic walls and had a lateral opening so that bees could forage freely. Individually labelled colony bees (with plastic tags on thorax, *Opalithplättchen* (von Frisch, 1967), or with acrylic paint marks) were trained to forage on a feeder further than 100 m away from the hive. To ensure that marked individuals belonged to the experimental colony, those bees with marks that were not seen inside the observation hive were captured at the artificial feeder and removed from the experiment.

Experimental Series

I. Effect of prolonged exposures to glyphosate on laboratory-reared bees

To study the effect of prolonged exposures to GLY we evaluated survival and food ingestion during the two-week experimental period as well as a set of distinct in 15-day-old bees.

Survival, food ingestion and locomotive activity. Mortality and food intake were quantified for all the laboratory-reared groups exposed to different GLY concentrations during the complete laboratory rearing period (15 days). These recordings were carried out to corroborate whether GLY concentrations were sub-lethal. In order to quantify mortality, the number of dead bees per cage

was recorded daily (and dead bees were removed). In order to quantify food intake, the volume of solution remaining in the feeding tubes was recorded daily for each cage and made relative to the number of bees alive each day. Additionally, other variables were measured to evaluate the general state of sensory sensitivity and locomotive activity in bees after a prolonged exposure to GLY. First, spontaneous response to an unconditioned stimulus was measured as follows: the antennae of test bees were touched with a drop of 1.8M sucrose solution and the number of responses was recorded. Mortality between harnessing and conditioning protocol was also measured.

Then, we used an adapted protocol to record the locomotive and orientation activity of 17 day old bees (Rueppell et al., 2007). Each bee was taken from the cage and introduced into a darkened circular arena that had a video camera (Sony HandycamHDR-SR11) on infrared mode located on the top section and four LED lights at equal distances around the perimeter. Four lights of two different colours were placed equidistantly around the arena, alternating colours so lights of the same colour pair faced each other. After an initial acclimatization of 2 minutes, the first light was turned on until the bee oriented and moved towards it. Once the bee was in the vicinity of the first light, it was turned off and the one opposing it was turned on. This was repeated sequentially (first a green light, then the opposing green light, then a yellow light and finally the opposing yellow light) until the bee had visited all lights twice. The time taken by each bee to complete the circuit was recorded using a self-written event-recording program and then discriminated by LED colour.

Sensitivity to sucrose. Individuals exposed to GLY during the first 15 days of adult stage were taken from their cages, anaesthetised at 4°C and harnessed on plastic holders that restrained body movement but allowed free movement of antennae and mouthparts (Page et al., 1998). After awakening, bees were offered water to drink and housed in an incubator (30ºC, 55% RH and darkness) for at least 1 hour before the protocol was carried out. In order to measure sensitivity to reward, the antennae of test bees were stimulated with droplets of sucrose solution of increasing concentration. Prior to performing a PER-GRS assay (Page et al., 1998; Scheiner et al., 1999), water was offered again in order to avoid confounding thirst effects. PER was quantified as bees were presented with sucrose solutions of increasing concentration (0.1, 0.3, 1, 3, 10, 30 and 50% w/w). The lowest sucrose concentration at which an individual responded by extending its proboscis was interpreted as its sugar response threshold (SRT). Bees were lined up in groups of 20–35 individuals and tested for each concentration sequentially: i.e. all bees were tested first at 0.1%, then at 0.3%, and so on. All bees were tested for their response to water between each concentration of sucrose solution. This serves to control for potential effects of repeated sucrose stimulation that could lead to increased sensitization or habituation. The inter-stimulus interval between water and sucrose solution depended on the number of individuals tested at a given time, but averaged 3 min. At the end of the procedure, a GRS was obtained for each bee. This score was based on the number of sucrose concentrations to which the bees responded (which correlates with the SRT since bees normally respond to all concentrations above their threshold). The response was arbitrarily quantified with scores from one to seven, where one

represented a bee that only responded to one concentration of sucrose (usually 50% w/w), while a score of seven represented an individual that responded to all concentrations tested. If a bee failed to respond to sucrose concentration in the middle of a response series (e.g. responded to 0.1, 0.3, 3 and 10% w/w, but did not respond to 1%), this 'failed' response was considered to be an error and the bee was deemed to have responded to that concentration as well. A bee that did not respond to any of the sucrose concentrations (score of 0) was excluded from further analyses. In addition, those bees that responded to all sucrose concentrations and all presentations of water were excluded from analyses as they appeared not to be able to discriminate between sucrose solution and water.

Olfactory PER conditioning. After an exposure to GLY during the first 15 days of adult stage, individuals were taken from their cages, anaesthetised and harnessed as described above and kept in an incubator (30°C, 55% RH and darkness) for about 2 to 3 h before the protocol of olfactory PER conditioning (Takeda, 1961; Matsumoto et al., 2012) was carried out. During classical conditioning, a constant airflow of 50 ml/s was delivered to the head of bees through a tube (1 cm diameter) placed 2 cm in front of the bee, using an electronic device. A piece of filter paper was impregnated with the odour (4 µl a pure odorant, linalool, on 30 x 9 x 3 mm) and placed inside a syringe located in the electronic device to add the odour to the airflow when required. The volatile was delivered through a secondary air-stream (6.25 ml/s) injected in the main airflow during the delivery of the odour. During the experiment in the PER setup, a fan extracted the released odours to avoid

contamination. Before odour presentation, bees were left to rest for 15s in the airflow for familiarization as well as for testing their response towards the mechanical stimulus. Only bees that showed the unconditioned response (UR) after applying 50 % w/w (1.8 M) sucrose solution sucrose solution onto the antennae and that did not respond to the mechanical stimulus (airflow) were used. For the training procedure the proboscis extension response towards the trained odour (%PER) was quantified over the course of three acquisition trials. We presented the conditioned stimulus LIO for 6s and each learning trial lasted 40s. Reinforcement (1.8 M sucrose solution without GLY) was presented on the proboscis and occurred for 3 s, 3 s after the onset of the CS. The conditioned response towards the trained odour on its own (Test) was measured 15 minutes after acquisition by quantifying PER during the first 3 s of a single presentation of the test odour (LIO).

II. Effect of acute exposure to glyphosate on hive-reared bees

To study the effect of acute exposure to GLY we evaluated learning abilities in worker bees caught at the entrance of outdoor hives. The foraging related behaviours were tested in free-flying bees that collected at an artificial feeder.

Elemental olfactory learning. Individuals were anaesthetised and harnessed as described previously. For this experimental procedure PER towards the trained odour was quantified over the course of eight acquisition trials (%PER). Reinforcements consisted of 0 mg/L GLY or 2.5 mg/L GLY per litre of 1.8 M sucrose solution and were presented on the proboscis. Extinction of the conditioned response was evaluated by quantifying PER to

LIO over the course of five trials in which the CS was presented without any reward. Extinction followed 15 minutes after acquisition. Experimental setup, CS, reward times and criteria for discarding individuals were defined as described previously.

Non-elemental olfactory learning. This experimental procedure was based on a negative patterning (A+, B+, AB−) non-elemental conditioning protocol (Deisig et al., 2001). In this procedure, elements A and B were rewarded with either 0 or 2.5 mg GLY per litre of 1.8M sucrose solution (reinforced elements A+ and B+) whilst the compound AB was not rewarded (non-reinforced element AB-). This assay incorporates an additional complexity for the bee because the discrimination between elements cannot be achieved through an elemental solution, it can only be solved by recognising a certain rule. Individuals were anaesthetised and harnessed as described previously. The CSs were the odorants linalool and 1-Hexanol for one group of bees and limonene and 2-Octanol for another (Sigma-Aldrich, Steinheim, Germany).We only report analyses of the pooled data. The experimental setup and reward times were as described previously. In this case, during periods of odorant delivery, the airflow was shunted through a syringe containing the odorant. In that way, a single odorant or a compound of two odorants could be delivered to the bee. In the latter case, the valves corresponding to two different syringes were opened simultaneously so the airflow arriving at the antennae of the bee contained the two odours as a compound. PER was quantified over the course of the protocol, both for reinforced and non-reinforced trials. Non-reinforced trials consisted of 6-s CS

presentation without reward. After experiments were finished, all animals were again tested for PER. If an animal did not respond, it was discarded (<10%). All bees received a total of 16 training trials, four A+ trials, four B+ trials, and eight AB− trials. The sequence of CSs+ and CS− trials was randomized.

Foraging related behaviour. The experiment consisted of six successive visits to the artificial feeder for each bee. During the first three visits, the feeder offered 2M sucrose solution without GLY. During the last three visits, solution was changed to 2.5 mg/L GLY per litre of 2 M sucrose solution. At the observation hive, we video recorded (Sony Handycam HDR-SR11) the behaviour of the returning foragers, during all the visits. Data were obtained from videotapes and quantified using a self-written event-recording program. Five variables were evaluated for each bee:

(1) Cycle Time (min) taken by a forager to arrive to the feeder, collect, fly back to the hive and leave the hive for the next cycle. It was calculated as time between first and final visits, over the total number of cycles completed.

(2) Visit Frequency (feeding cycles/hour) calculated as the inverse of the cycle time.

(3) Dance Probability (%) calculated as the number of hive visits in which a dancing event was recorded, over the total number of complete hive visits.

(4) Mean Number of Waggle-runs per Hive Stay calculated as the number of waggle phases completed for each complete hive stay, over the total number of complete hive visits.

(5) Dance Errors per Hive Stay (%). When a forager performs a waggle dance, she normally turns alternately to the left or to the right to begin the return phase at the end of the waggle phase (von Frisch 1967). Deviations from the alternate left and right turns (e.g. two consecutive right turns) appear to be a measure of how disordered the dance is. We therefore counted the correct and incorrect turns for all the dances of each bee, over the total number of complete hive visits.

Statistical analysis

Mortality is expressed as percentage accumulated mortality for the complete exposure period per cage. Cumulative food intake is expressed as cumulative ml per bee. The means of mortality (percentage accumulated mortality for the complete exposure period per cage) and of food intake (cumulative ml of food ingested per bee) were analysed using a one-way analysis of variance, one-way ANOVA (Sokal and Rohlf, 1995). Normality and homoscedasticity assumptions were met for all data. Mortality between harnessing and conditioning protocol for the different GLY concentrations was analysed through a *G*-test of homogeneity. Time taken by bees exposed to different GLY concentrations between each pair of LED lights in the locomotive and orientation procedure was analysed using a three-way repeated measures analysis of variance (RM-ANOVA) with GLY concentration and LED colour

as fixed factors and cage and bees as random factors. Data met normality, homogeneity and sphericity assumptions after log10 transformation.

GRS data was treated as nonparametric because the assumption of normality was not met. Median GRSs were compared between GLY concentrations using Kruskal-Wallis (K-W) ANOVA tests.

PER proportions for each GLY concentration during each acquisition trial were assayed using analyses of variance for repeated measurements (RM-ANOVA). Monte Carlo studies have shown that it is possible to use ANOVA on dichotomous data (Lunney, 1970). Where necessary, simple effects were computed and Tukey tests were used to perform post hoc comparisons. PER proportion for each GLY concentration towards the trained odour on its own (Test) were assayed using a *G*-test of homogeneity.

PER for the different GLY concentrations throughout acquisition and extinction (elemental learning procedure) were analysed by assigning a value to each bee corresponding to the total number of trials during which they exhibited PER across the thirteen trials of the procedure. This value, which ranged from zero to thirteen, was assayed using a Mann-Whitney *U*-test for independent samples to compare overall performance levels between groups (Zar, 1999).

The percentage of conditioned responses (%PER) in successive CS+ trials (omitting the randomly interspersed CS− trials) and in successive CS− trials (omitting the randomly interspersed CS+ trials) were measured for the non-elemental learning procedure. Bees received four A+, four B+, and eight AB− trials. Data were grouped to obtain four blocks of two CS+ trials and four blocks of two CS− trials. A two-way analysis of variance (two-way

ANOVA) was used for comparisons between elements and a further two-way ANOVA was used for comparisons between GLY concentrations. Monte Carlo studies have shown that it is possible to use ANOVA on dichotomous data (Lunney, 1970).

Finally, all foraging variables were analysed in the same manner. A mean for the first three visits and a mean for the last three visits were obtained for each bee. Means for each variable were compared using a Wilcoxon matched pairs test (Zar, 1999).

The alpha level was set to 0.05 for all analyses.

Acknowledgements

We thank the CONICET, ANPCYT and the University of Buenos Aires for financial support. We are grateful to H. Pizarro for her valuable contributions and to V. M. Fernandez for support with statistical analysis. We also thank H. Verna for technical assistance and the two anonymous referees for their valuable comments and suggestions on an early version of this manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions

L.T.H., A.A. and W.M.F conceived and designed the experiments. L.T.H., D.E.V. and A.A. performed the experiments. L.T.H. and D.E.V. performed data analysis. L.T.H., A.A. and W.M.F. drafted the manuscript. All authors revised and commented on the manuscript.

Funding

Funding was provided by the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET; Agencia Nacional de Promoción Científica y Tecnológica, ANPCYT and the University of Buenos Aires.

The experiments comply with the ‘‘Principles of animal care’’, publication No. 86-23, revised 1985 of the National Institute of Health, and also with the current laws of the country in which the experiments were performed.

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Figure legends

Figure 1.Effect on sensitivity to sucrose and learning performance after a prolonged exposure to glyphosate (GLY). Caged bees were exposed to different GLY concentrations (0, 2.5 and 5 mg GLY per litre of 1.8 M sucrose solution) during the first 15 days of their adult life. Behavioural parameters of bees at 15 days of age were tested through: A, sensitivity to reward that was evaluated with a gustatory response score (GRS) test; B, an absolute classical conditioning protocol in which the proboscis extension response towards the trained odour (%PER) was quantified over the course of three acquisition trials; and C, the conditioned response (%PER) towards the trained odour alone measured 15 minutes after acquisition. The number of bees tested is shown in brackets below each box (A) or in the top right corner (B, C). Boxes indicate the inter-quartile range, horizontal lines within boxes indicate the medians, whiskers include all points within 1.5 times the inter-quartiles, solid circles indicate outliers (Dunn comparisons: * $p<0.05$, in A; Tukey post hoc comparisons: $p<0.05$, in B; ***stands for significant differences between treatments in the second trial).

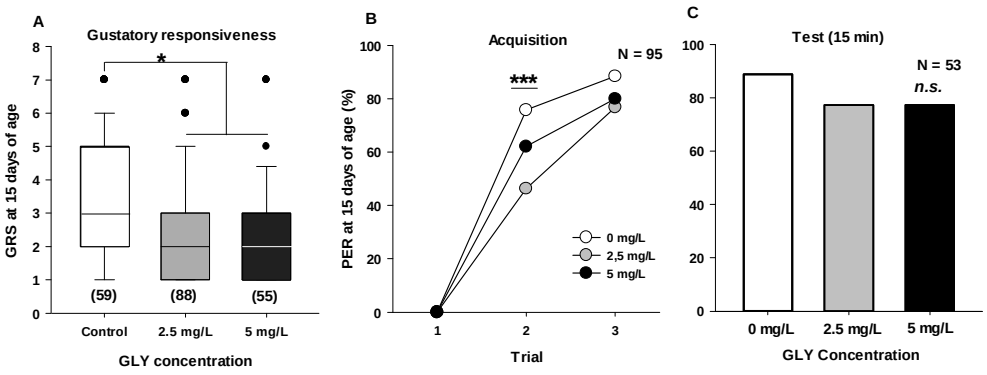
Figure 2.Effect on elemental olfactory learning during an acute exposure to glyphosate (GLY). Learning abilities of bees captured at the hive entrance and exposed acutely to GLY were tested through an absolute classical conditioning procedure. The proboscis extension response towards the trained odour (%PER) was quantified over the course of 8 acquisition and 5 extinction trials in which the unconditioned stimulus (US) consisted of either

1.8 M sucrose solution or a compound of 1.8 M sucrose solution and 2.5 mg GLY per litre of sucrose solution. The switch from acquisition to extinction occurred on Trial 8. The number of bees tested is shown in brackets beside each curve (Mann-Whitney: *p< 0.05).

Figure 3.Effect on non-elemental olfactory learning during an acute exposure to glyphosate (GLY). Non-elemental learning abilities of bees captured at the hive entrance and exposed acutely to GLY were tested through a negative patterning olfactory conditioning procedure in which the US consisted of either 1.8 M sucrose solution or a compound of 1.8M sucrose solution and 2.5 mg GLY per litre of sucrose solution. A, averaged %PER across all trials of A+, B+, and AB− for both groups. B, course of %PER to the reinforced elements (A+, B+; solid line) and to the non-reinforced compound (AB−; dashed line) for both groups. The number of bees in each group is shown in brackets above each bar (A) and beside each curve (B), while asterisk indicates *p < 0.05(two-way ANOVA).*n.s.*, no significant differences.

Figure 4.Effect on foraging and dancing behaviour during an acute exposure to glyphosate (GLY).A, cycle foraging time, in min; B, visit frequency to the feeder, expressed in foraging cycles per hour; C, dance probability, percentage; D, number of waggle-runs displayed per hive stay; and E, dance errors per hive stay, percentage. The reward program consisted first of three foraging bouts in which single foragers collected at a feeder located 150 m from the hive which offered a 2 M sucrose solution without

GLY (control). On the fourth visit and for the next three bouts the sucrose solution contained 2.5 mg of GLY per litre of sucrose solution. Bars indicate means ±s.e.m. The number of bees evaluated for each variable is shown in the top right corner of each graph. *n.s.*, no significant differences.



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859 **Figure 1**

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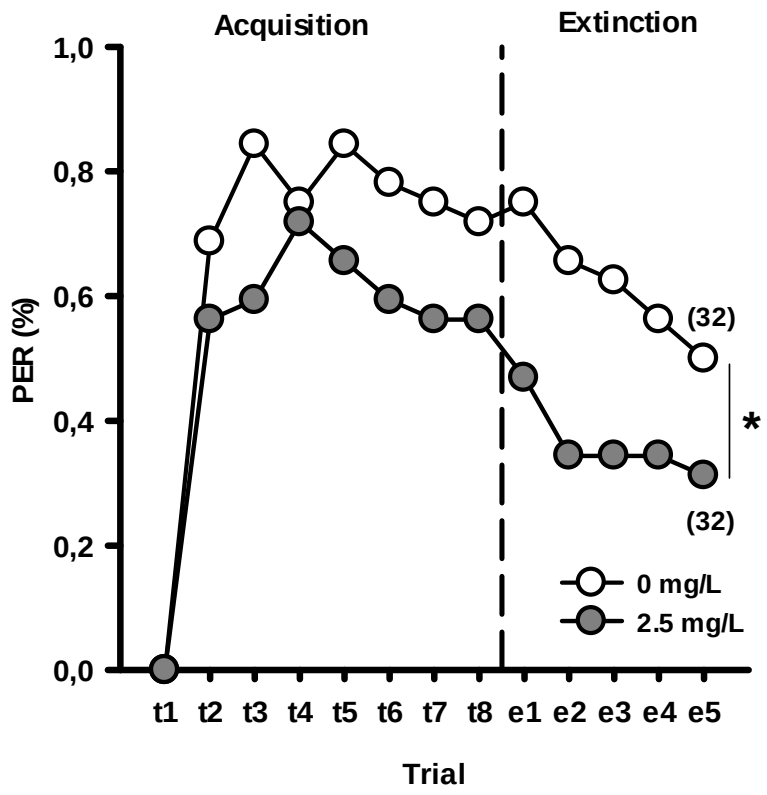

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863 **Figure 2**

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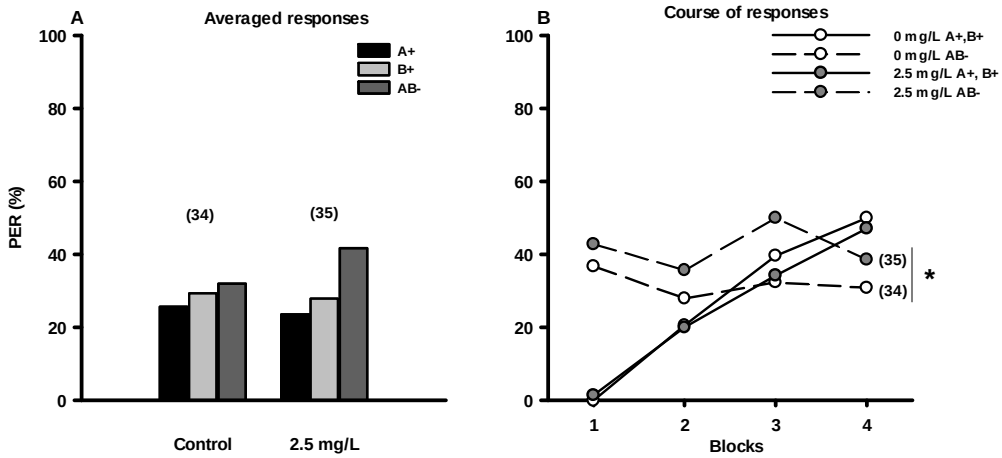

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866 **Figure 3**

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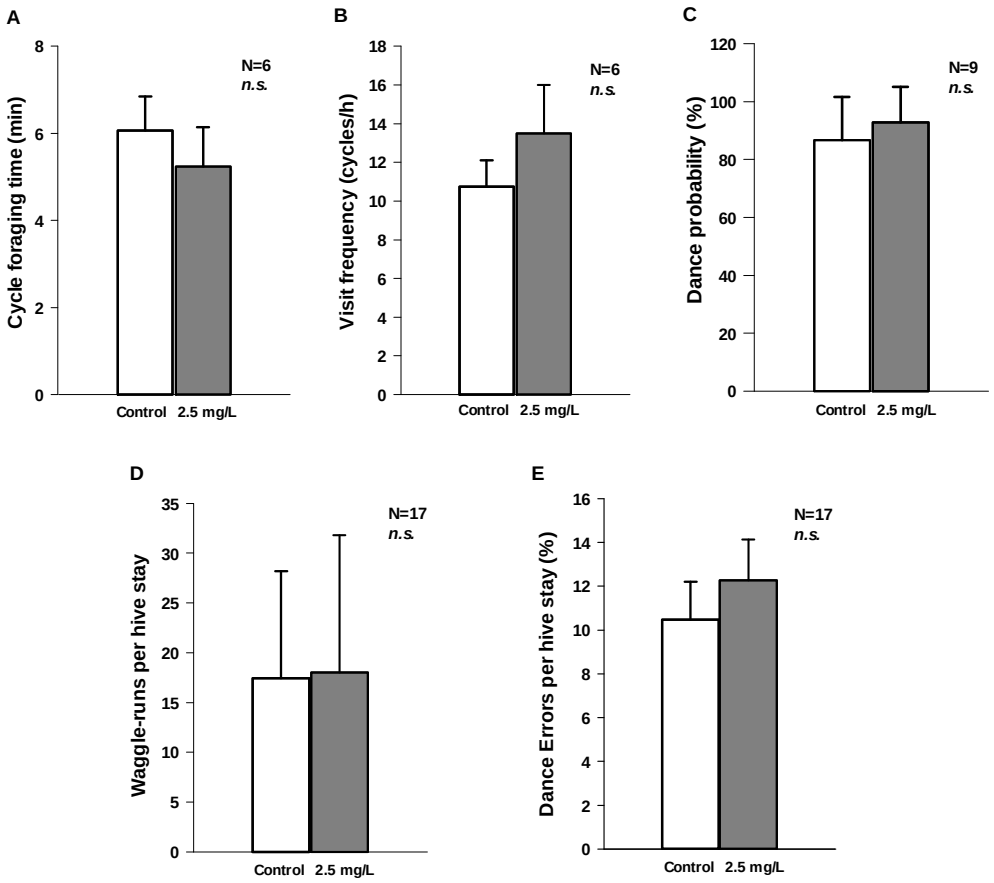

Figure 4

Tables

Table 1.Survival and behavioural variables after a prolonged exposure to glyphosate (GLY). Caged bees were exposed to different GLY concentrations (0, 2.5 and 5 mg GLY per litre of sucrose solution) during the first 15 days of adult life. Locomotive activity was measured for two pairs of LED lights: yellow-yellow (top row) and green-green (bottom row). All values expressed as mean ± s.e.m., with the exception of those corresponding to survival between harnessing and conditioning protocol.

Survival and behavioural variables	*GLY concentration*			Test statistic	N	P
	0 mg/L	2.5 mg/L	5 mg/L			
Accumulated mortality upto day 14 per cage (%)[a]	10.3±3.7	24.1±3.7	20.1±3.7	$F_{2,12}$=3.67	5	0.057
Accumulated intake upto day 14 per cage (ml/bee)[a]	0.28±0.04	0.33±0.04	0.36±0.04	$F_{2,12}$=1.32	5	0.305
Survival between harnessing and conditioning protocol (%)[b]	86.0	92.8	93.8	G_H=0.76 (*df=2*)	193	0.685
Locomotive activity: log10 time between same color lights (s)[c]	8.5±0.8 14.4±3.2	11.0±2.4 10.7±1.3	14.8±3.5 12.8±2.8	$F_{2,9}$=0.07	28	0.936

[a]One-way ANOVA
[b]Homogeneity Test (*G*-test)
[c]Three-way RM-ANOVA