

1 **Running title:** Effects of glyphosate traces on honeybee appetitive behaviour

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6 **Effects of field-realistic doses of glyphosate on**
7 **honeybee appetitive behaviour**

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

25

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

50

51 **Introduction**

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

63 Although GLY inhibits aromatic amino acid pathways present only in
64 plants, microorganisms and fungi, but not in animals (Amrhein et al., 1980;
65 Carlisle and Trevors, 1988; Duke et al., 1989), there are studies that have
66 found different negative effects in invertebrate and vertebrate species. For
67 instance, common application concentrations have been found to cause
68 growth deficit in the earthworm *Aporrectodea caliginosa* (Springett and
69 Gray, 1992) and concentrations higher than 10 mg/L have been proven to
70 have an effect on body growth in the freshwater snail *Pseudosuccinea*
71 *columella* (Tate et al., 1997). In vertebrates, studies indicate that chronic
72 exposure to different formulates with GLY concentrations ranging between
73 3.8 and 18 mg acid equivalent/L (a.e./L) may negatively affect amphibians
74 (Howe et al., 2004; Relyea, 2005a, b).

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

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

100 olfactory information has been shown to be well retained even when it occurs
101 at young ages of the adult stage (Arenas and Farina, 2008; Arenas et al.,
102 2009a; Arenas et al., 2012). Young workers that remain inside the hive can
103 learn rewarded odours when fed with resources recently collected (Nixon and
104 Ribbands, 1952; Grüter et al., 2006) or with food stored in the hive (Winston,
105 1987). Moreover, experiences acquired inside the colony can increase the
106 efficiency of a colony's foraging related tasks (Arenas et al., 2009b;
107 Balbuena et al., 2012a). These learning abilities can be evaluated under
108 laboratory experimental conditions through the proboscis extension response
109 (PER). Bees extend their proboscis after their antennae have been stimulated
110 with sucrose solution and this response can be conditioned if a neutral
111 stimulus (e.g., an odour or another sensory stimulus) is paired with the
112 reward (Kuwabara, 1957; Takeda, 1961; Bitterman et al., 1983; Matsumoto et
113 al., 2012).

114 The proboscis extension response can also be used to measure reward
115 sensitivity. Reward sensitivity is intimately bound to associative learning
116 (Scheiner et al., 1999; Page and Erber, 2002) and therefore, inseparable from
117 foraging behaviours (Page et al., 1998). Changes in food source profitability
118 found by foragers affect their threshold for appetitive responses to the extent
119 that they modify a series of stereotyped movements used to convey
120 information, known as the waggle dance (von Frisch, 1967). The dancers'
121 manoeuvres encode information about the location and profitability of the
122 discovered food source which is transmitted to the rest of the colony during
123 the dance (von Frisch and Lindauer, 1955; Riley et al., 2005; Thom et al.,
124 2007; Grüter and Farina, 2009a, b). This complex behavioural repertoire and

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

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

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

150

151 **Results**

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

153 ***Survival, food ingestion and locomotive activity.*** We first investigated the
154 effect of a prolonged exposure to GLY on the behaviour of laboratory-reared
155 bees. Table 1 shows the results obtained for survival, ingestion and
156 locomotive activity measured at 15 days of age on bees exposed to different
157 GLY concentrations during the first 15 days of adult life. Although bees
158 exposed to GLY showed a higher level of mortality than untreated bees, we
159 found no significant differences between both groups (one-way ANOVA:
160 $F_{2,12}=3.67$, $p=0.057$, Table 1). This result, together with the fact that the
161 highest accumulated mortality recorded during 15 days only reached 24%,
162 led us to regard the GLY doses used as sub-lethal.

163 Before evaluating the effect of a prolonged exposure to GLY on
164 sensitivity to sucrose and learning abilities, we studied whether it had an
165 effect on the overall behaviour of 15 day old bees. Food intake, mortality,
166 mortality due to harnessing and locomotive and orientation activity did not
167 vary between bees exposed to different GLY concentrations (food intake:
168 $F_{2,12}=1.32$, $p=0.305$, one-way ANOVA; survival between harnessing and
169 PER conditioning: $G_H=0.76$, $p=0.683$, $N=579$, $df=2$, G -test; locomotive
170 activity: main effect GLY concentration: $F_{2,9}=0.07$, $p=0.936$, GLY
171 concentration x LED colour interaction: $F_{2,4}=0.85$, $p=0.493$, three-way RM-
172 ANOVA; for details see Table 1). These results show that all bees,
173 independently of the GLY concentration to which they were exposed,
174 presented similar behavioural responses and survival rates at 15 days of age.

175

176 **Sensitivity to sucrose.** With the general behavioural results in mind, we
177 investigated whether sensitivity to sucrose and learning performance were
178 also intact. We first tested the sensitivity to sucrose of bees through a
179 proboscis extension response and gustatory response score protocol (PER-
180 GRS protocol). GRS scores of bees exposed to GLY were lower than those of
181 non-exposed bees (Kruskal-Wallis test: $H=9.54$, $p=0.007$, $N=203$, $df=2$; Fig.
182 1A). This indicates that 15-day-old bees that were reared with sub-lethal
183 concentrations of GLY present an increased response threshold for sucrose.

184

185 **Olfactory PER conditioning.** Next, we assayed bees' performance in an
186 absolute olfactory classical conditioning protocol of the proboscis extension
187 response (PER). Figure 1B shows the %PER towards the conditioned
188 stimulus (CS: linalool, henceforth: LIO) for bees of 15 days of age for the
189 course of 3 acquisition trials in which the reward did not contain GLY. Bees
190 that were exposed to sub-lethal concentrations of GLY during the first 15
191 days of adult life showed a lower performance than non-exposed bees. We
192 performed a two-way repeated measures analysis of variance and found a
193 significant interaction between factors (two-way RM-ANOVA; main effect
194 GLY concentration: $F_{2,282}=7.76$, $p<0.001$; interaction GLY concentration x
195 acquisition trial: $F_{2,4}=5.14$, $p<0.001$; Fig. 1B). We therefore computed simple
196 effects for GLY concentration and found statistical differences for GLY
197 concentration effects for the second acquisition trial (One-way ANOVA:
198 $F_{2,282}=9.19$, $p<0.001$). Tukey post hoc comparison tests revealed that the
199 effects of the three GLY concentrations on the second acquisition trial differ

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

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

213

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

215 ***Elemental olfactory learning.*** After studying the effects of a prolonged
216 exposure to GLY at pre-foraging ages we wondered whether an acute
217 exposure to GLY at foraging ages could also have an effect on honeybees.
218 We started by performing an elemental PER conditioning assay with 0 or
219 2.5 mg GLY per litre of 1.8 M sucrose solution as reward. Figure 2 shows the
220 overall performance of both groups of bees for the duration of 8 acquisition
221 trials and 5 extinction trials. Right away, from trial 2 of the acquisition phase,
222 bees that received GLY in the reward showed a lower PER towards the CS
223 (LIO). The difference between both groups remained throughout the rest of
224 the protocol: bees that were acutely exposed to GLY responded consistently

225 less than bees that were not exposed (Mann-Whitney test: $U=338.50$,
226 $N_1=N_2=32$, $Z=2.33$, $p=0.019$; Fig. 2).

227

228 ***Non-elemental olfactory learning.*** To further investigate acute exposure
229 effects of GLY on hive-reared bees, we carried out a non-elemental PER
230 conditioning assay using a negative patterning discrimination assay. Figure
231 3A shows %PER averaged across all trials of A+ (LIO or 2-Octanol), B+ (1-
232 Hexanol or limonene), and AB- (LIO and 1-Hexanol or 2-Octanol and
233 limonene), respectively, for each group of bees exposed to a different GLY
234 concentration. A GLY concentration \times Element (2×2) ANOVA yielded no
235 differences for the elements A+ versus B+ (two-way ANOVA: $F_{1,134}=0.82$,
236 $p=0.367$; Fig. 3A). We therefore pooled the reinforced elements (A+ and B+)
237 within each GLY group for the next analysis. Figure 3B shows the course of
238 conditioned responses to the compound AB- and the average responding to
239 the elements A+ and B+ across blocks of trials for each group. Bees in both
240 groups could correctly discriminate the reinforced elements (A+, B+) from
241 the non-reinforced element (AB-), as shown by the increase in response
242 towards the reinforced elements throughout the trials whilst the response to
243 the non-reinforced element remains constant. We then evaluated total
244 acquisition (and therefore overall amount of differentiation) by computing the
245 average level of responding to the pooled CSs+ and to the CS- for each GLY
246 group. Bees rewarded with GLY during the negative patterning
247 discrimination assay had an overall lower acquisition than non-exposed bees
248 (two-way ANOVA: $F_{1,134}=5.92$, $p=0.016$; Fig. 3B). These results indicate that

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

251

252 ***Foraging related behaviour.*** We investigated the effects of an acute GLY
253 exposure in a more realistic and natural context by training bees to an
254 artificial feeder and measuring different foraging variables for each bee,
255 before and after the artificial feeder contained sucrose solution with GLY.
256 We started by analysing the cycle time (min) and visit frequency
257 (cycles/hour) of each bee, before and after the exposure. Bees continued
258 visiting and collecting at the artificial feeder at a constant rate regardless of
259 whether the artificial feeder contained GLY or not (Wilcoxon matched pairs
260 test; cycle time: $Z=1.15$, $N=6$, $p=0.249$, Fig. 4A; visit frequency: $Z=1.57$,
261 $N=6$, $p=0.116$, Fig. 4B).

262 Having established that foragers return to the hive and complete
263 foraging cycles in the same manner even when GLY is present at the food
264 source, we then focused on the transfer of information that occurs inside the
265 hive. Dance probability did not differ before or after GLY exposure
266 (Wilcoxon matched pairs test; dance probability: $Z=0.944$, $N=9$, $p=0.345$,
267 Fig. 4C). Thus, we assayed the dance event in itself. We found no change in
268 the mean number of waggle-runs per hive when GLY was added to the food
269 source (Wilcoxon matched pairs test: $Z=0.024$, $N=17$, $p=0.981$, Fig. 4D). The
270 mean percentage of dance errors per hive stay was not affected either by the
271 presence of GLY in the sucrose solution (Wilcoxon matched pairs test:
272 $Z=0.639$, $N=17$, $p=0.523$, Fig. 4E).

273

274

275 **Discussion**

276 We set out to evaluate the effects of chronic and acute exposures to field-
277 realistic doses of glyphosate (GLY), the main herbicide currently used for
278 weed control in agriculture, on the behaviour of the honeybee *Apis mellifera*.
279 Our results show that both chronic and acute exposure to GLY traces produce
280 sensory sensitivity and cognitive deficits on adult honeybees of the worker
281 caste. The concentrations used (within a 0 to 3.7 mg e.a./L range) were based
282 on concentrations recommended for spraying and on those measured in
283 natural environments, from 1.4 to 7.6 mg e.a./L (Goldsborough and Brown,
284 1988; Feng et al., 1990; Giesy et al., 2000), and were shown to be sub-lethal
285 for honeybees. Young adult bees chronically exposed to concentrations of
286 2.5 and 5.0 mg/L of GLY showed reduced sensitivity to sucrose (reward) and
287 impaired acquisition dynamics during elemental associative olfactory
288 learning. This impairment cannot be explained by deterioration of the general
289 state or motor skills of the subjects, since measurements such as survival,
290 food uptake and locomotive activity did not differ between experimental
291 groups. Furthermore, acute exposure to GLY significantly decreased short-
292 term memory retention and negatively affected non-elemental associative
293 learning at foraging ages. Nevertheless, an acute exposure to GLY in a
294 foraging context did not have a detrimental effect on foraging activity and
295 dancing behaviour. Altogether, these results imply that GLY at
296 concentrations that can be found in nature as a result of standard spraying
297 reduce sensitivity to nectar reward and also impair associative learning in
298 honeybees. Since no effect on foraging activity was found, successful forager

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

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

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

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

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

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

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

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

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

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

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

402

403 **Materials and Methods**

404 *Study site and animals*

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

410 To study the effect of prolonged exposures to GLY we worked with
411 adult bees reared under laboratory conditions (laboratory reared bees). Bees
412 were obtained from sealed brood frames placed in an incubator (36°C, 55%
413 relative humidity, RH, and darkness). Recently emerged adults (0–1 days old)
414 were collected in groups of about 100 individuals in wooden cages (10 x 10 x
415 10 cm) that had a wire mesh door on one side. Bees were fed with a 1.8M
416 sucrose solution with different GLY (Sigma-Aldrich, Steinheim, Germany)
417 concentrations, in addition to water and pollen *ad libitum*. Three GLY
418 concentrations were used: 0 mg (control group), 2.5 mg and 5 mg per litre of
419 sucrose solution. Caged bees were kept in an incubator (31°C, 55% RH and
420 darkness) until 15 days of age. Feeding tubes were refilled every 48 hours in
421 order to reduce any effects that high incubator temperatures might have on
422 GLY and to avoid bacterial proliferation, which is known to shift the pH in
423 sucrose solutions.

424 Experiments to study the effect of acute exposures to GLY were
425 performed using worker bees caught at the entrance of outdoor hives at the
426 beginning of each experimental procedure (hive-reared bees). In order to
427 study foraging-related behaviour a colony of 3000 to 4000 worker bees,
428 queen and brood was placed in a two-frame observation hive (von Frisch,
429 1967) located inside the laboratory. The experimental hive consisted of two
430 see-through acrylic walls and had a lateral opening so that bees could forage
431 freely. Individually labelled colony bees (with plastic tags on thorax,
432 *Opalithplättchen* (von Frisch, 1967), or with acrylic paint marks) were
433 trained to forage on a feeder further than 100 m away from the hive. To
434 ensure that marked individuals belonged to the experimental colony, those
435 bees with marks that were not seen inside the observation hive were captured
436 at the artificial feeder and removed from the experiment.

437

438 *Experimental Series*

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

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

443

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

449 was recorded daily (and dead bees were removed). In order to quantify food
450 intake, the volume of solution remaining in the feeding tubes was recorded
451 daily for each cage and made relative to the number of bees alive each day.
452 Additionally, other variables were measured to evaluate the general state of
453 sensory sensitivity and locomotive activity in bees after a prolonged exposure
454 to GLY. First, spontaneous response to an unconditioned stimulus was
455 measured as follows: the antennae of test bees were touched with a drop of
456 1.8M sucrose solution and the number of responses was recorded. Mortality
457 between harnessing and conditioning protocol was also measured.

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

473

474 ***Sensitivity to sucrose.*** Individuals exposed to GLY during the first 15 days of
475 adult stage were taken from their cages, anaesthetised at 4°C and harnessed
476 on plastic holders that restrained body movement but allowed free movement
477 of antennae and mouthparts (Page et al., 1998). After awakening, bees were
478 offered water to drink and housed in an incubator (30°C, 55% RH and
479 darkness) for at least 1 hour before the protocol was carried out. In order to
480 measure sensitivity to reward, the antennae of test bees were stimulated with
481 droplets of sucrose solution of increasing concentration. Prior to performing a
482 PER-GRS assay (Page et al., 1998; Scheiner et al., 1999), water was offered
483 again in order to avoid confounding thirst effects. PER was quantified as bees
484 were presented with sucrose solutions of increasing concentration (0.1, 0.3, 1,
485 3, 10, 30 and 50% w/w). The lowest sucrose concentration at which an
486 individual responded by extending its proboscis was interpreted as its sugar
487 response threshold (SRT). Bees were lined up in groups of 20–35 individuals
488 and tested for each concentration sequentially: i.e. all bees were tested first at
489 0.1%, then at 0.3%, and so on. All bees were tested for their response to
490 water between each concentration of sucrose solution. This serves to control
491 for potential effects of repeated sucrose stimulation that could lead to
492 increased sensitization or habituation. The inter-stimulus interval between
493 water and sucrose solution depended on the number of individuals tested at a
494 given time, but averaged 3 min. At the end of the procedure, a GRS was
495 obtained for each bee. This score was based on the number of sucrose
496 concentrations to which the bees responded (which correlates with the SRT
497 since bees normally respond to all concentrations above their threshold). The
498 response was arbitrarily quantified with scores from one to seven, where one

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

510

511 ***Olfactory PER conditioning.*** After an exposure to GLY during the first 15
512 days of adult stage, individuals were taken from their cages, anaesthetised
513 and harnessed as described above and kept in an incubator (30°C, 55% RH
514 and darkness) for about 2 to 3 h before the protocol of olfactory PER
515 conditioning (Takeda, 1961; Matsumoto et al., 2012) was carried out. During
516 classical conditioning, a constant airflow of 50 ml/s was delivered to the head
517 of bees through a tube (1 cm diameter) placed 2 cm in front of the bee, using
518 an electronic device. A piece of filter paper was impregnated with the odour
519 (4 µl a pure odorant, linalool, on 30 x 9 x 3 mm) and placed inside a syringe
520 located in the electronic device to add the odour to the airflow when required.
521 The volatile was delivered through a secondary air-stream (6.25 ml/s)
522 injected in the main airflow during the delivery of the odour. During the
523 experiment in the PER setup, a fan extracted the released odours to avoid

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

537

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

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

542

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

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

553

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

574 presentation without reward. After experiments were finished, all animals
575 were again tested for PER. If an animal did not respond, it was discarded
576 (<10%). All bees received a total of 16 training trials, four A+ trials, four B+
577 trials, and eight AB- trials. The sequence of CSs+ and CS- trials was
578 randomized.

579

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

- 588 (1) Cycle Time (min) taken by a forager to arrive to the feeder, collect,
589 fly back to the hive and leave the hive for the next cycle. It was
590 calculated as time between first and final visits, over the total number
591 of cycles completed.
- 592 (2) Visit Frequency (feeding cycles/hour) calculated as the inverse of the
593 cycle time.
- 594 (3) Dance Probability (%) calculated as the number of hive visits in
595 which a dancing event was recorded, over the total number of
596 complete hive visits.

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

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

607

608 *Statistical analysis*

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

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

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

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

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

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

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

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

654 The alpha level was set to 0.05 for all analyses.

655

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662

663 **Competing interests**

664 The authors declare no competing financial interests.

665

666 **Author contributions**

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

671

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679

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801
802

803 **Figure legends**

804

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

821

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

828 1.8 M sucrose solution or a compound of 1.8 M sucrose solution and 2.5 mg
829 GLY per litre of sucrose solution. The switch from acquisition to extinction
830 occurred on Trial 8. The number of bees tested is shown in brackets beside
831 each curve (Mann-Whitney: $*p < 0.05$).

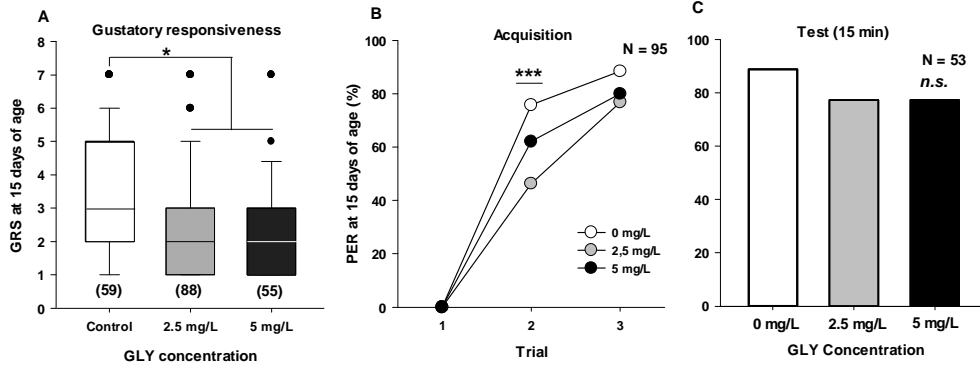
832

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

845

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

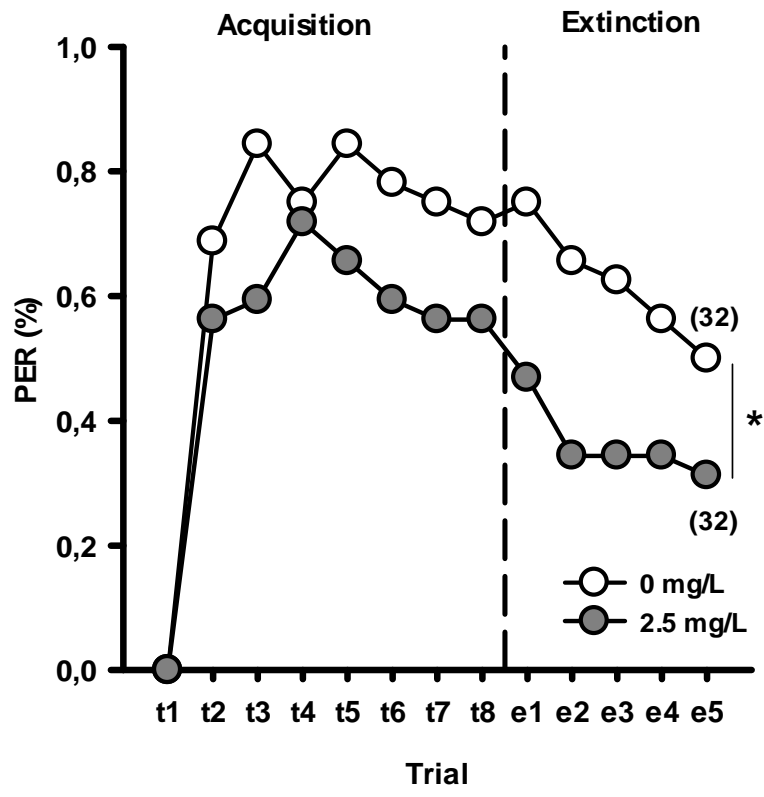
853 GLY (control). On the fourth visit and for the next three bouts the sucrose
854 solution contained 2.5 mg of GLY per litre of sucrose solution. Bars indicate
855 means \pm s.e.m. The number of bees evaluated for each variable is shown in
856 the top right corner of each graph. *n.s.*, no significant differences.
857



858

859 **Figure 1**

860

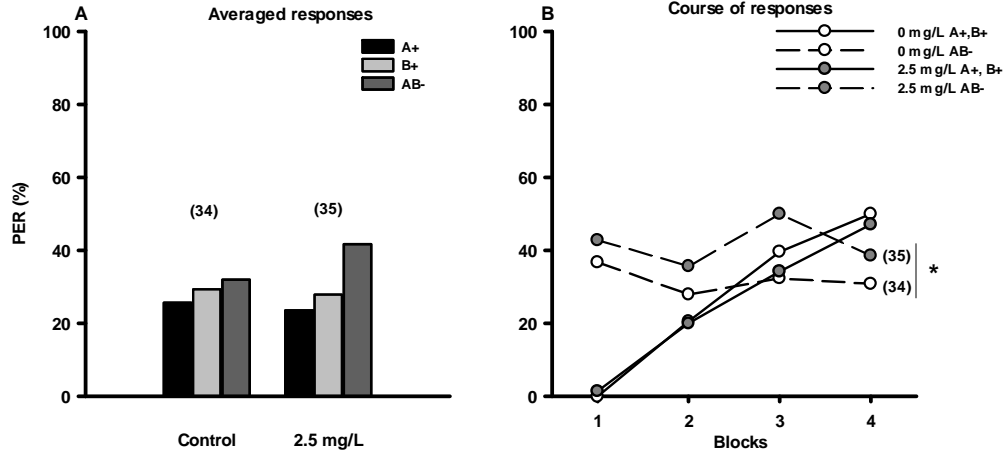


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863 **Figure 2**

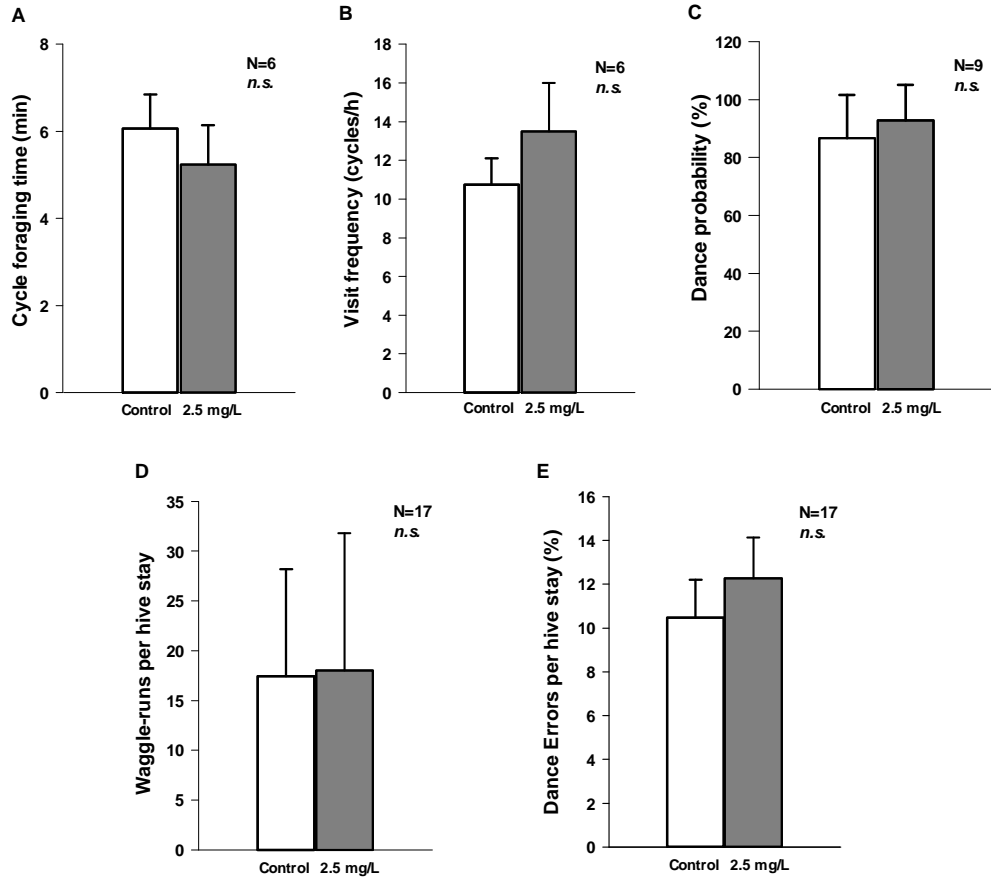
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866 **Figure 3**

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870 **Figure 4**

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874 **Tables**

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

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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 \pm 3.7	24.1 \pm 3.7	20.1 \pm 3.7	F _{2,12} =3.67	5	0.057
Accumulated intake upto day 14 per cage (ml/bee) ^a	0.28 \pm 0.04	0.33 \pm 0.04	0.36 \pm 0.04	F _{2,12} =1.32	5	0.305
Survival between harnessing and conditioning protocol (%) ^b	86.0	92.8	93.8	G ₂ =0.76 (df=2)	193	0.685
Locomotive activity: log10 time between same color lights (s) ^c	8.5 \pm 0.8	11.0 \pm 2.4	14.8 \pm 3.5	F _{2,9} =0.07	28	0.936
	14.4 \pm 3.2	10.7 \pm 1.3	12.8 \pm 2.8			

^aOne-way ANOVA

^bHomogeneity Test (G-test)

^cThree-way RM-ANOVA

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