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ORIGINAL ARTICLE

Effects of glyphosate and glyphosate-based herbicide on learning and memory of the buff-tailed bumblebee (*Bombus terrestris*)

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Abstract

The decline of insect pollinators is a global concern, and the use of pesticides has been identified as a potential cause for it. Glyphosate-based herbicides (GBHs) are the world's most used pesticides, but until recent years they have been claimed to be safe for non-target organisms, such as pollinators. Using controlled arena experiments, we investigated the effects on the learning and long-term memory of buff-tailed bumblebees, *Bombus terrestris* (L.) (Hymenoptera: Apidae), of a single field-realistic dose of glyphosate, both in its pure form and as a commercial herbicide (Roundup Gold) containing the active ingredient (a.i.) glyphosate and other co-formulants. We found that glyphosate in its pure form caused deterioration in the learning ability of bumblebees in a 10-color discrimination experiment; the glyphosate-treated bees discriminated colors over 10% worse than the untreated control bees. However, the commercially used GBH (Roundup Gold) had no observable effect on the learning ability of the bumblebees, despite the fact that this herbicide contained the same amount of glyphosate as its pure form. These findings shed light on the potential risks associated with agrochemicals previously considered safe for pollinators and emphasize the need for comprehensive risk assessments of pesticides, including evaluations of cognitive functions in pollinators. Therefore, we propose that future pesticide testing should incorporate broader assessments to ensure the safety of pollinators in agricultural landscapes.

KEYWORDS
active ingredient, Apidae, bee, color discrimination, ecosystem service, herbicide, Hymenoptera, learning ability, non-target organism, pesticides, pollinator, pollinator decline

INTRODUCTION

Insect pollination is an important ecosystem service that is crucial for both agriculture and terrestrial biodiversity. Approximately 35% of crops and 78–94% of flowering plants worldwide are pollinated by animals (Klein et al., 2006; Ollerton et al., 2012), mostly by insects. However, populations of insect pollinators have been declining globally during the past decades (Ghazoul, 2005; Zattara & Aizen, 2021). The most important drivers of the global pollinator decline are land-use changes, such as fragmentation and loss of habitats (Hendrickx et al., 2007; Brown & Paxton, 2009; Potts et al., 2010), environmental pollution, alien animal and plant species (Goulson, 2003a; Stout & Morales, 2009), pathogens (Cox-Foster et al., 2007), climate change (Williams et al., 2007; Dormann et al., 2008; Soroye et al., 2020), and the increasing use of pesticides (Dicks et al., 2021). Pesticides include for example insecticides, fungicides, rodenticides, and herbicides, with herbicides being the most commonly used. Herbicides are agrochemicals intended to prevent the normal growth and development of unwanted plants, and they are used for example by farmers, gardeners, landscapers, and sports field managers. Herbicides and other pesticides are essential in modern agriculture because pests, and primarily weeds, cause over 40% of yield loss worldwide (Oerke, 2007).

Glyphosate is globally the most used active ingredient (a.i.) in herbicides (Benbrook, 2016) with more than 750 glyphosate-based herbicide (GBH) commercial products (Guyton et al., 2015). As glyphosate inhibits 5-enol pyruvylshikimate-3-phosphate (EPSP), synthesizing enzymes in the shikimate pathway, and the pathway is only found in all green plants, fungi, and some bacteria (Duke & Powles, 2008; Leino et al., 2021), it has had a reputation as being non-toxic to other non-target organisms, such as insects, humans, and other animals. However, recent studies have raised concerns about the harmful non-lethal effects of glyphosate on non-target organisms, including pollinators. Glyphosate has been discovered to affect pollinators' feeding and appetitive behavior, such as sensitivity to sucrose and associative olfactory conditioning (Herbert et al., 2014; Mengoni Goñalons & Farina, 2018; Faita et al., 2020). Moreover, it may disrupt pollinators' navigation (Balbuena et al., 2015), sensory and cognitive abilities (Farina et al., 2019; Vázquez et al., 2020), delay larval development (Vázquez et al., 2018; Odemer et al., 2020; Weidenmüller et al., 2022), and modify gut microbiota (Motta et al., 2020; Helander et al., 2023; Motta & Moran, 2023).

In addition to glyphosate, commercial GBHs include many co-formulants, that for example, help glyphosate to penetrate the plant's epidermal surface, reduce drifting or foam forming, and allow adherence for more prolonged exposure. Some of these co-formulants are suspected to be even more harmful to pollinators than the a.i. glyphosate (Mesnage et al., 2019; Straw et al., 2021; Straw & Brown, 2021). Unfortunately, detailed information about the co-formulants in commercial GBHs is sparse as the contents of the final products are usually considered confidential business information (Weinhold, 2010). Even though the a.i. of GBHs and other commercial herbicides are tested, only a few tests are performed on the final products before registration by regulatory authorities (Mesnage et al., 2019). Such tests would be necessary to assess the harmful effects of co-formulants (if any) on pollinators.

To date, most studies on glyphosate and pollinators are conducted with western honey bee (*Apis mellifera* L.), whereas other important pollinator groups have been largely ignored (Cullen et al., 2019). Bumblebees (*Bombus* spp.) are important natural pollinators in temperate areas (Plowright & Laverty, 1984) and similar to many other insect pollinators, the abundance of wild bumblebees has also declined (Cameron et al., 2011; Rollin et al., 2020; Martinet et al., 2021). Their high foraging rate, foraging preference for pollen instead of nectar, and large, hairy body that is capable of accommodating large pollen loads make bumblebees effective pollinators. Due to their long proboscis and ability of buzz pollination, bumblebees can pollinate flowers that other pollinators (e.g., honey bees) are not able to pollinate (Goulson, 2003b). Because they have a smaller colony size and less aggressive behavior compared to honey bees, bumblebees are suitable pollinators in greenhouses (Wahengbam et al., 2019) where they efficiently pollinate a large number of crop plants (Macfarlane et al., 1994; Willmer et al., 1994; Chen & Hsieh, 1996; Stanghellini et al., 1997; Dimou et al., 2008). Bumblebees are at a risk of being exposed to herbicides and other pesticides because of their generally large body size and dense hair. In particular, foraging bumblebee workers that visit more flowers per trip and make more daily trips than honey bees have a significant risk for herbicide exposure via direct spraying, air particles, plant surface residues, pollen, and nectar (Gradish et al., 2019). Unlike honey bees, which typically nest in hives above ground, many bumblebee species build their nests and hibernate below ground. This increases the risk of exposure to herbicides through soil residues for bumblebee queens and larvae, as they come into direct contact with the contaminated soil (Gradish et al., 2019; Farina et al., 2019; Vázquez et al., 2020; Helander et al., 2023). Unlike other pollinators, including honey bees, bumblebees have a higher likelihood of herbicide exposure due to their nesting habits and behavioral characteristics. They are active in unfavorable weather conditions, have an extended foraging period, and are active earlier and later in the day, all of which increase their exposure window to herbicides and highlight the importance of evaluating the impact on their populations and behaviors (Gradish et al., 2019). Lastly, bumblebees typically have a smaller foraging range (<1.5 km) (Knight et al., 2005) than honey bees (3–15 km) (Beekman & Ratnieks, 2000), making them more vulnerable to herbicide residues in agricultural landscapes where herbicides are used (Raine & Gill, 2015; Gradish et al., 2019).

The ability of bumblebees to learn is strongly related to their foraging performance under natural conditions, and is thus a significant factor in their overall fitness (Raine & Chittka, 2008). Indeed, Helander et al. (2023) stated that an acute dose of GBH has both immediate and lasting effects on the fine-color discrimination of bumblebees, which may harm the foraging of individual bees and, consequently, reduce the fitness of their colony. However, they also found that the GBH exposure did not affect olfaction or the general vision in bumblebees (Helander et al., 2023), suggesting that apart from color learning, GBH may not affect their cognition and learning in general.

In this study, we investigated the effects of field-realistic doses of both pure glyphosate and a GBH (Roundup Gold, Monsanto Europe, Antwerp, Belgium) on the cognition of adult buff-tailed bumblebees, *Bombus terrestris* (L.) (Hymenoptera: Apidae), by conducting controlled arena experiments to evaluate their capacity to learn color–reward associations and their long-term memory retention (i.e., their ability to remember what they learned 48 h ago). Studying both pure glyphosate and a GBH is crucial for a comprehensive understanding of the potential effects on bumblebees' cognition. By investigating both forms, we can differentiate between the impacts of glyphosate itself and the potential contributions of co-formulants present in the GBH. This knowledge is essential for accurately assessing the risks posed by these substances to bumblebee populations and informing appropriate risk management strategies. More specifically, we addressed the following two questions: (1) do glyphosate or GBH affect bumblebees' cognition in terms of color learning and long-term memory?; and (2) if so, is the effect primarily due to glyphosate or the co-formulants of the commercial GBH? We hypothesized that bees treated with either glyphosate or GBH would perform worse in learning and memory experiments than untreated bees. We also hypothesized that bees treated with GBH would perform worse in the experiments than those treated with the same amount of pure glyphosate; this is because the co-formulants present in GBH might have additional effects or interactions with glyphosate that could further impact the bees' cognition.

MATERIALS AND METHODS

Bumblebees and nest boxes

To explore the cognition of bumblebees, we conducted a 10-color discrimination experiment (Li et al., 2017; Helander et al., 2023; Kaila et al., 2023) that lasted 11 days and consisted of learning and memory tests (described in detail in the sections 'Learning phase' and 'Memory test' below; Figure 1). For the experiment, we used 10 colonies of commercial buff-tailed bumblebees (*B. terrestris*) from Koppert (Berkel en Rodenrijs, The Netherlands) which were placed in nest boxes (14.5 × 15 × 9 cm) with entrance halls of the same size. The nest boxes and entrance halls were connected to a pollination arena (60 × 45 × 25 cm) with a transparent corridor. The corridor had doors which could be opened to control which bees could enter the arena. The nest boxes were covered with a piece of cardboard to prevent light from entering the nests because bumblebees prefer dark nesting sites (Wahengbam et al., 2019). All the experiments were conducted indoors in the spring of 2022 and the winter of 2023 at Turku University Bumblebee Laboratory (Turku, Finland) at 20–23 °C and L10:D14 (light from 07:00 h to 17:00 h).

After placing the colonies in the nest boxes (day 0), the bumblebees were allowed to freely access a gravity feeder filled with 40% sucrose water in the arena and familiarize themselves with their new environment. The following day (day 1), we marked the most active large (length >1.2 cm) foragers (i.e., individuals that were trying to enter the arena) with individual number tags (Bienen-Voigt & Warnholz, Ellerau, Germany). The number tags were glued to the top of the thorax of the bees with super glue. We excluded the smallest bees (length <1.2 cm) because the number tags easily smeared their wings. The size of individual *B. terrestris* workers varies within a single colony (Goulson et al., 2002) and the benefit of choosing larger active individuals ensured that the bees used later in the experiment were probably foragers rather than in-nest workers (Free, 1970; Goulson et al., 2002).

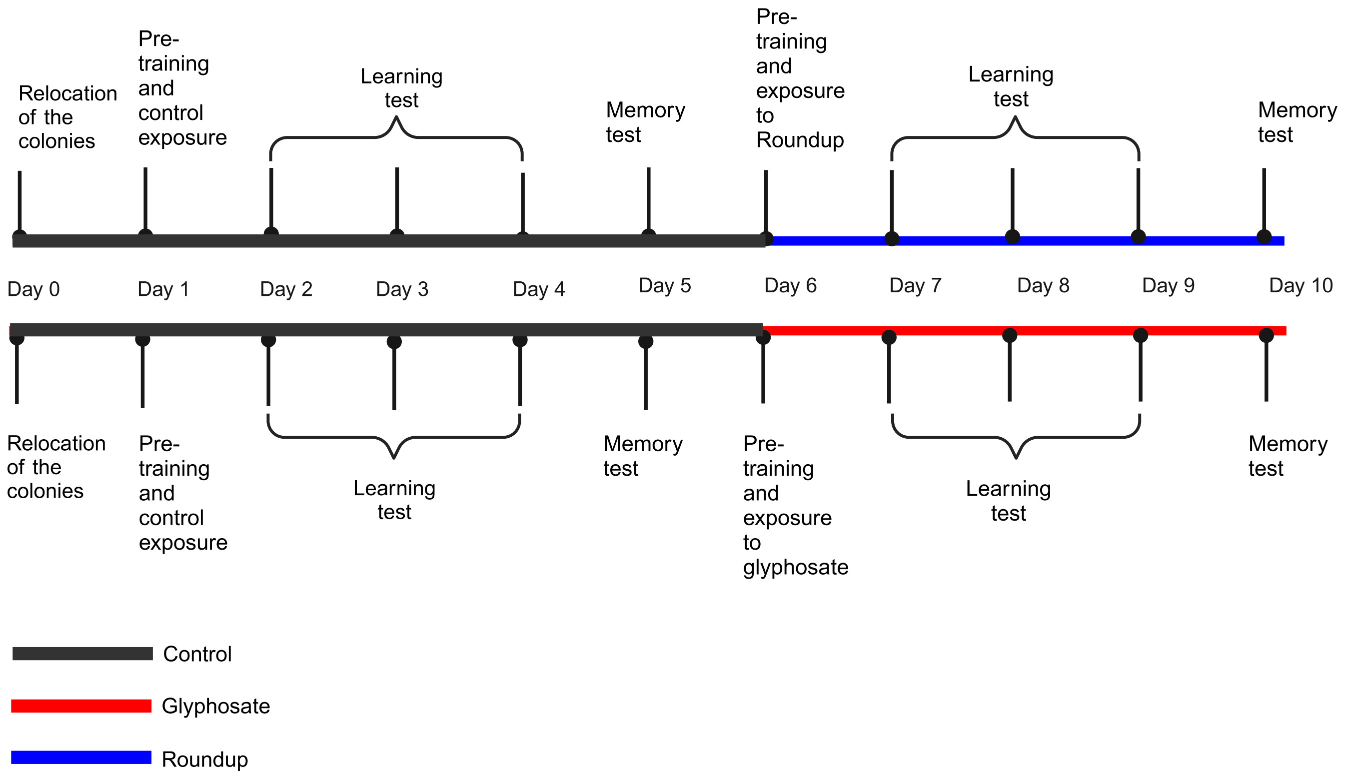

FIGURE 1 The timeline for the 10-color discrimination experiment for a single colony of buff-tailed bumblebees (*Bombus terrestris*). In order to prevent cross-contamination, bees were exposed to either pure glyphosate or the glyphosate-based herbicide (GBH) Roundup Gold after the control group bees from the same colonies had undergone both learning and memory test. Created with BioRender.com.

Even though bumblebee workers do not have a strong age-based division of labor (Free, 1955; Cameron, 1989), some workers are more concentrated on in-nest working than on foraging. Furthermore, specialization in the collection of either nectar or pollen has been observed among some foraging bumblebees in multiple colonies (O'Donnell et al., 2000). To account for these variations, we marked as many foragers as possible and included only the most active ones in our experiment. This approach ensured that the results represented the foraging behavior of active bee foragers.

Pre-training

On day 1, after marking the bumblebees with the identification tags, we pre-trained them to feed on artificial flowers in the arena. Ten colorless artificial flowers (i.e., transparent rectangular prisms; 5 cm high, 2.5 cm diameter) with 7 μL of 40% sucrose water on the top were placed in the arena; nine of them were placed randomly inside the arena and one was placed immediately in front of the entrance. The marked bees were released into the arena one at a time and allowed to forage for 5 min. This pre-training was repeated 5× for each bee selected for the experiment.

Exposure to glyphosate

After the pre-training, we selected 5–10 bees from each colony for the learning experiment. All of these bees had completed five foraging bouts and had successfully learned to forage for sugar water on colorless artificial flowers. On day 1, we fed the chosen bees with 7 μL of 60% sugar water per bee. Groups of control, glyphosate, and GBH exposed bees were established as follows. Following the initial feeding, control group (n = 28, 10 colonies) bees underwent the full 10-color discrimination experiment (described in the sections 'Learning phase' and 'Memory test' below). After the experiment was completed with control bees, we removed them from the colony and started pre-training with new naïve bees. After the second pre-training, 5–10 bees were allocated to either a glyphosate group (n = 23, 5 colonies) that received a 7 μL mixture comprising of 60% sugar water and pure glyphosate per bee (>98% purity, CAS: 1071-83-6, 5 g glyphosate L^{-1} sugar water; Sigma-Aldrich, St. Louis, MO, USA), or a Roundup group (n = 15, 4 colonies) that was provided with a 7 μL mixture consisting of 60% sugar water and a GBH per bee (commercial product Roundup Gold, registration no. 1934, a.i. glyphosate concentration 450 g L^{-1}, as glyphosate isopropylamine salt, CAS: 3864194–0, 11 mL Roundup Gold L^{-1} sugar water; Monsanto Europe). Unfortunately, all bees died in one colony before the second learning trials for Roundup bees, resulting in four colonies in the Roundup group and five colonies in the glyphosate group, whereas there were 10 colonies in the control group. Given the limited genetic variation within *B. terrestris* colonies, which are founded by single-mating queens (Schmid-Hempel & Schmid-Hempel, 2000), we conducted the experiment first with the control group bees on day 1 and subsequently with either the glyphosate or Roundup group bees on day 6. To ensure that the control group bees were not exposed to pure glyphosate or Roundup, we completed the entire experiment with the control group before commencing the treatments with the other groups (Figure 1). It was not feasible to choose all three treatment groups from a single colony, primarily because the colonies did not survive long enough under laboratory conditions without adequate nest cleaning. Additionally, bumblebees empty their crop loads into a common honeypot within the nest, potentially exposing all colony members to glyphosate.

We offered the prescribed exposure to the bees as a single droplet. Following the administration, the bees were individually isolated under transparent glass containers until they consumed the entire droplet. Thus, all treated bees in the glyphosate and Roundup groups received 35 μg of a.i. glyphosate the day before the learning experiment. This amount of glyphosate given to bees is based on the following factors. Recently GBH-treated flowers might contain up to 30 mg kg^{-1} of glyphosate residues in nectar and up to 629 mg kg^{-1} of glyphosate residues in pollen (Thompson et al., 2014). Importantly, bumblebees do not seem to discriminate between flowers that have been recently treated with either GBHs (K Kaakinen et al., unpubl.) or pure glyphosate (Thompson et al., 2022), confirming that they can be exposed to glyphosate residues while foraging. We determined that a field-realistic amount of glyphosate that a foraging bumblebee can potentially obtain orally while visiting recently treated flowers is 35 μg. This estimation considers the significant variation in loads of nectar and pollen carried by foraging bumblebees, with large *B. terrestris* foragers reported to collect over 200 mg of nectar in 1 h and complete up to 12 foraging bouts per day (Spaethe & Weidenmüller, 2002).

Learning phase

On day 2 (for controls) and day 7 (for glyphosate or GBH bees), we started the learning phase that lasted up to 2 days per colony. Throughout the five learning bouts, we observed the bumblebee's capacity to associate particular colors with a reward and other colors with a bitter quinine (CAS: 6119-47-7, 1 g L^{-1}; Sigma-Aldrich, St. Louis, MO, USA). We released the marked and pre-trained bees into the arena one at a time to forage from 20 artificial flowers. The artificial flowers were otherwise similar to the ones in pre-training, but they were painted in 10 colors (i.e., two flowers of each color) (Li et al., 2017). Five colors contained 7 μL of 40% sugar water solution, and the other five contained 7 μL bitter-tasting quinine water solution, resulting in 10 flowers in each group. In each bout, a bee was allowed to forage for 10 min or until they left the arena voluntarily. To prevent an immediate return to the nest without foraging, the bees were allowed to leave the arena only after 2 min from their release. All landings on the artificial flowers when the bee drank sugar or quinine water (i.e., touched either one with its proboscis) were recorded both in writing and by video. Events where the bee only landed on an artificial flower without touching the sugar/quinine solution were ignored. Choosing an artificial flower with sugar water was marked as a 'success' whereas choosing an artificial flower with bitter-tasting quinine water was marked as a 'failure'. Instances in which the bumblebee did not visit or consume any artificial flower were excluded from the count, and the bee was given an additional learning bout. If the bee failed again to complete any landings, it was removed from the experiment. After each bout, the flying arena and all the artificial flowers were cleaned with 70% ethanol to remove any scent marks left for the next bumblebee (Goulson et al., 1998). To ensure that all foraging bees had the same amount of foraging time in the experiment, we prevented them from entering the arena after the pre-training when they were not performing learning bouts.

Memory test

To examine whether the bees could remember which colors represented the rewarding sugar droplets, we performed a single-bout memory retention test 48 h after the last learning bout (day 5 for controls, day 10 for exposed bees) for all the bees that had completed all five learning bouts. In the memory test, bees were presented with artificial flowers of various colors, similar to the learning phase. However, unlike the learning phase where the flower colors were associated with either rewarding sugar water or aversive quinine water, in the memory test, all the artificial flowers contained plain, unrewarding water. As bumblebees do not collect water (Gradish et al., 2019), they only quickly visited the artificial flowers during the memory test. All flower visits were recorded both in writing and by video.

To prevent bees from succumbing to starvation and to mitigate torpor in the workers (Heinrich, 1979) during the 48 h break between the last learning bout and the memory experiment, we placed a small gravity feeder (50 × 33 mm) in the nest box's corridor filled with 40% sugar water solution. Because the tasks of bumblebee workers may change over time based on the needs of a given colony (O'Donnell et al., 2000), we did not give any sugar solution to bees 12 h before any of the experiments to ensure that the colony needed sugar during the experimental bouts. We had to take a break a few times during the learning and memory experiments because the bumblebees were not motivated to continue foraging. In addition to the sugar water solution, we also gave the bees one tablespoon (15 mL) of honey bee pollen (Foodin, Jyväskylä, Finland) every other day to enable the normal function of the colony.

Statistical analysis

Statistical analyses were performed using R v.1.4.1717 (R Core Team, 2021). We used two generalized linear mixed models (GLMM) with a binomial distribution and a logit-link function (lme4::glmer) (Bates, 2005). We first tested whether exposure to pure glyphosate or GBH Roundup Gold affected bumblebees' performance during the five learning bouts. Performance (proportion of correct landings) was used as a binomial response variable. The learning bout (five levels), treatment (control, glyphosate, and Roundup), and their interaction were used as fixed categorical explanatory variables, whereas the colony was used as a random effect. Moreover, bee identity nested within the colony was included as a random factor to consider the five repeated observations from the same bees.

We then tested whether the exposure to pure glyphosate or GBH Roundup Gold affected the memory retention of the bumblebees from the last learning bout to the memory test. Treatment (control, glyphosate, and Roundup), learning bout (bout five and memory test) and their interaction were used as a fixed explanatory variable, and bee identity nested within the colony was used as a random factor.

For both models, we determined the significance of the fixed variables with type II Wald's χ^2 test (car::Anova) (Fox & Weisberg, 2019) and assessed pairwise differences in mean values with a Tukey's test to compare the differences between treatments and (learning) bouts (emmeans::emmeans) (Lenth et al., 2019). We validated all models visually from residual plots and by checking the model dispersion; the first model (the effect of the exposure during the five learning bouts) was slightly underdispersed (0.68) (DHARMa::testDispersion) (Hartig & Hartig, 2017), which leads to a slight loss of statistical power.

RESULTS

Learning phase

Both the treatment (GLMM: $\chi^2 = 16.67$, d.f. = 2, $P < 0.001$) and learning bout (GLMM: $\chi^2 = 112.83$, d.f. = 4, $P < 0.001$) affected the bumblebees' performance (i.e., the percentage of correct landings on a rewarding flower), but there was no interaction between the two (GLMM: $\chi^2 = 10.87$, d.f. = 8, $P = 0.21$). Pairwise comparisons revealed that the glyphosate-treated bees had, on average, 14.4% lower performance than the control bees in all learning bouts; no difference in performance was detected between the other treatment groups (Figure 2A). The mean performance of the bumblebees increased over the five learning

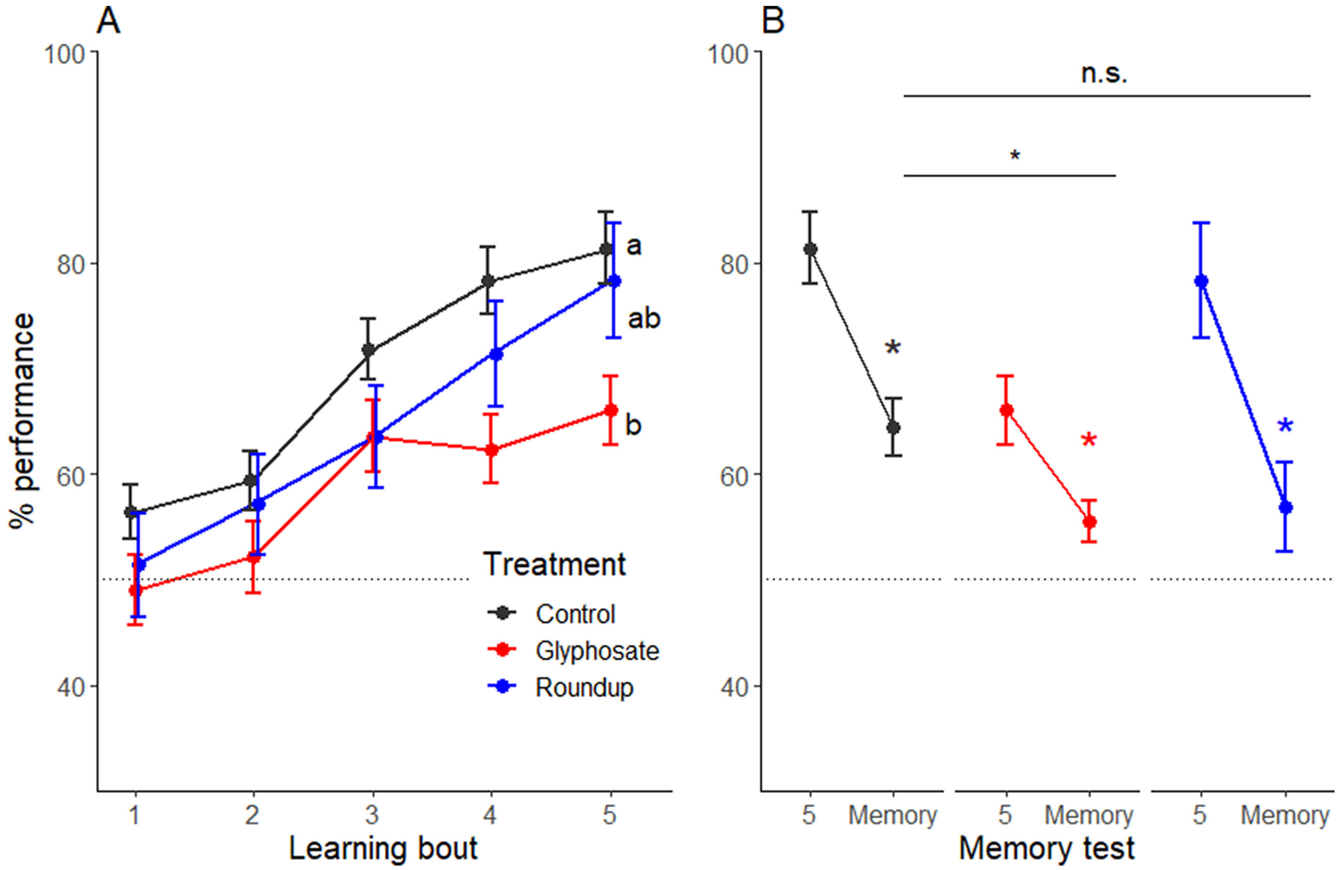

FIGURE 2 (A) Back-transformed least square mean (± SE) percent performance (i.e., percentage of correct landings on a rewarding flower) of buff-tailed bumblebees (*Bombus terrestris*) exposed to glyphosate, the glyphosate-based herbicide (GBH) Roundup Gold, or non-exposed (control) bees in (A) a five-bout learning test and (B) a single-bout memory test conducted 2 days after the last learning experiment bout. In the five-bout learning phase, foraging bumblebees were allowed to choose between artificial flowers of 10 colors representing either a rewarding sugar water solution or an unpalatable quinine water solution. The horizontal dotted line represents the chance level, set at 50%. (A) Means are jittered to prevent the overlap of error bars. Time courses followed by different letters indicate significant differences between the treatments (Tukey's test: $P < 0.05$). (B) Asterisks indicate significant differences in performance means between the fifth learning bout and the memory test within each treatment (Tukey's test: $P < 0.05$). The asterisk above the bracket indicates a significant difference in performance in the memory test between the control and glyphosate treatments; n.s. indicates no significant difference between the control and Roundup treatments.

bouts in all three treatments, particularly during the first three bouts, but then slowed down, especially in the pure glyphosate group. By the final (fifth) bout, performance for the controls reached 80.5%, 66.1% for the pure glyphosate group, and 79.0% for the Roundup group (Figure 2A).

During the first learning bout, the bumblebees chose artificial flowers at random because they did not have any foraging experience with different colors and could not discriminate colors with a bitter quinine taste (Figure 2A).

Memory test

In the memory test, conducted 48 h after the last (fifth) learning bout, again, treatment (GLMM: $\chi^2 = 20.59$, d.f. $= 2$, $P < 0.001$) and bout (GLMM: $\chi^2 = 30.47$, d.f. $= 1$, $P < 0.001$) affected the bumblebees' memory (i.e., the percentage of correct landings on a rewarding flower). In addition, the treatment and bout had a significant interaction (GLMM: $\chi^2 = 7.71$, d.f. $= 2$, $P = 0.021$). In the memory test, pairwise comparisons were conducted to assess the differences between the control group and the Roundup group, as well as between the control group and the glyphosate group. The results indicated that there was no statistically significant difference between the control and Roundup groups (Tukey test: $SE = 0.16$, $z = 1.16$, $P = 0.48$). However, performance was lower in the glyphosate than in the control group. (Tukey test: $SE = 0.13$, $z = 4.67$, $P < 0.001$). Further comparisons revealed that the bees' memory deteriorated in all three treatment groups compared to the previous (fifth) bout, but this deterioration was greatest in the Roundup group (18.9%), followed by the control (13.8%) and glyphosate (10.5%) groups (Figure 2B).

DISCUSSION

We found no difference between the performance of the control and GBH-treated bumblebees as regards learning in the 10-color discrimination task, but the bees treated with pure glyphosate had lower performance than the control bees. The ability of glyphosate-treated bees to discriminate colors was 14.4% worse during the last learning bout. Earlier studies have suggested that co-formulants in commercial GBH products are harmful for honey bees or bumblebees, or even more harmful than the a.i. glyphosate (Mesnage et al., 2019; Straw et al., 2021; Straw & Brown, 2021). A previous study (Helander et al., 2023), using a similar color discrimination task and the same commercial GBH (Roundup Gold) as used in this experiment, demonstrated that GBH reduced bumblebees' color discrimination by over 10%. However, Helander et al. (2023) used a 22.3% higher amount of GBH and thus of the a.i. (45 μg) to treat the individual bumblebees than we used in the present study (35 μg), which might explain the different results between the two studies. Regardless of the discrepancy, our study, together with the previous one (Helander et al., 2023), suggests that the negative effects of GBH Roundup Gold on bumblebees' cognition are due to the glyphosate itself, and not the other ingredients present in this commercial product. Unfortunately, neither this study nor the literature provides information on why the same amount of glyphosate in a pure form would be more harmful to bumblebees' performance than the glyphosate in the commercial product, although similar results have been obtained before (Thompson et al., 2023). A possible explanation for the more severe effect of pure glyphosate might be that the commercial product used here had some co-formulants that prevented the absorption of glyphosate from the bumblebee's honey stomach to their body before the bee emptied its load to the nest's honey pot.

The Roundup group exhibited the most significant decline in memory performance, with an average decrease of 18.9% compared to the last learning bout. In comparison, the control group showed a relatively lower decrease of 13.8%. The observed statistically significant difference in the average decrease of memory performance between the Roundup and control groups (18.9 vs. 13.8%) highlights the varying impact of these treatments on bumblebees' memory. The greater decline in memory performance in the Roundup group suggests a potentially stronger disruptive effect of Roundup on the cognitive abilities of bumblebees compared to the control group. These findings raise concerns about the potential negative impacts of Roundup on pollinator health and underscore the importance of considering the effects of pesticide exposure on memory and learning abilities in these essential insects. Further investigations are warranted to elucidate the underlying mechanisms contributing to these observed differences and to inform conservation strategies aimed at protecting pollinators in pesticide-exposed environments.

To ensure consistency in exposure, we gave the bumblebees a single dose of glyphosate in this study. We consider the dose field-realistic based on estimates of glyphosate in nectar and the amount of nectar that foraging bumblebees can collect in a day. However, it is important to note that bumblebees do not typically receive a single large dose (except during the application), but rather many small doses over the course of several days while foraging recently treated flowers (Thompson et al., 2022). A repeated exposure to small doses of glyphosate may thus have different effects on bees than a single large dose. Further research is needed to fully understand the impact of glyphosate exposure on bees, considering both the dose and frequency of exposure.

The impairment of cognitive skills may have a serious impact on bumblebees. Bumblebees' learning ability and foraging performance are closely intertwined as foraging success plays a vital role for both individual bees and their colonies (Raine & Chittka, 2008). Foraging and flight are exceptionally costly for bumblebees (Wolf et al., 1999) and they use a significant amount of energy for flying (Heinrich, 1975). The foraging activity of bumblebees is inversely related to their longevity. In-nest workers can live for several months (Brian, 1952) and therefore, they have a significantly longer lifespan compared to foraging bumblebees, which typically only survive for a few weeks (Rodd et al., 1980). Foraging workers are more vulnerable to predation and parasitism than in-nest workers, and foraging causes faster wing wear (Rodd et al., 1980) and immune system depression (König & Schmid-Hempel, 1997) that shorten the lifespan of foragers. Importantly, time and energy spent on foraging have consequences for the entire colony, even though eusocial bees do have a buffering capacity against environmental stressors (Hölldobler & Wilson, 2009). Even a short-time food and energy shortage may affect the colony by increasing its vulnerability to predators and parasites (Cartar & Dill, 1991), lowering the brood temperature (Heinrich, 1979), and lengthening the development time of larvae (Cartar & Dill, 1991). With their memory and cognition skills, bumblebees can minimize the time and energy spent on foraging. However, if the bumblebees' long-term memory is impaired by pesticides, they spend their resources looking for rewards and visiting unrewarding flowers.

There are at least two possible explanations why glyphosate impairs color discrimination in bumblebees. First, changes in color discrimination could be due to alterations in gut microbiota. Recent studies have shown that glyphosate alters the gut microbiota of bumblebees (Blot et al., 2019; Motta et al., 2020; Helander et al., 2023; Motta & Moran, 2023) which, in turn, is linked with their long-term memory (Li et al., 2021). However, all of the aforementioned studies involved prolonged glyphosate exposure in bees, rather than a single exposure event. Thus, it is unlikely that the single field-realistic dose of glyphosate used in the present study could have altered the gut microbiota to cause the observed decline in performance after 1–2 days from the glyphosate treatment. Second, changes in color discrimination could be related to altered brain functions after glyphosate exposure. Many pesticides are known to affect the brain functions of bees and even mild brain damage can have a negative effect on foraging performance

(Klein et al., 2017). So far, most studies have focused on neonicotinoids (Godfray et al., 2015) and studies on the effects of glyphosate on bees' brain functions are sparse. As optimal development and function of the brain are necessary for bees' learning and memory (Menzel, 2012), and glyphosate does impair the color discrimination of bees, further research is needed to better understand the mechanisms behind the effects that glyphosate has on bees. Interestingly, earlier studies have shown that glyphosate does not seem to have an effect on bumblebees' olfactory learning ability (Hernández et al., 2021; Helander et al., 2023), suggesting that the impact of glyphosate may be limited to visual learning. Therefore, it is still necessary to explore whether these mechanisms in bumblebees are associated with the brain, gut microbiota, or both, potentially through the brain–gut microbiota axis (Leger & McFrederick, 2020), or other factors that have yet to be discovered. Further investigation is required to unravel the underlying mechanisms involved in these relationships.

The results of our study support the idea that a single dose of glyphosate is harmful to bumblebees. Most studies on the effects of GBHs and other pesticides have focused on a few widespread and economically important pollinators such as honey bees and certain species of bumblebees, primarily *B. terrestris* also used in this study. Even though all bee species seem to have similar cognitive capacities and brain organization (Klein et al., 2017), it would be important to widen pollinator studies to other important insect pollinator species in order to understand the potential reasons for the global decline in insect pollinators.

AUTHOR CONTRIBUTIONS

Kimmo Iisakki Kaakinen: Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); project administration (supporting); software (equal); validation (equal); visualization (equal); writing – original draft (lead); writing – review and editing (equal). **Satu Ramula:** Conceptualization (supporting); data curation (supporting); formal analysis (equal); investigation (supporting); methodology (supporting); project administration (supporting); software (supporting); supervision (equal); validation (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal). **Olli Loukola:** Conceptualization (supporting); data curation (equal); formal analysis (supporting); investigation (supporting); methodology (lead); project administration (supporting); software (supporting); supervision (equal); validation (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal). **Marjo Helander:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (lead); investigation (supporting); methodology (equal); project administration (equal); resources (lead); software (supporting); supervision (equal); validation (supporting); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

According to the Ministry of Agriculture and Forestry in Finland, bumblebees are not classified as experimental animals and, consequently, project authorization is not required (Act of the Use of Animals for experimental purposes 2006/62 §4*)*. However, we considered ethical issues when designing the experiment. We pursued using bumblebees as effectively as possible to reduce the number of colonies in our experiment. After the experiment, we euthanized the bumblebees causing as little pain as possible by stunning them with CO_2 gas and then placing them in the freezer.

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