ORIGINAL PAPER

Sublethal doses of glyphosate impair olfactory memory retention, but not learning in the honey bee (*Apis mellifera scutellata*)

Juan Hernández[1] · Andre J. Riveros[2] · Marisol Amaya-Márquez[1]

Abstract

Learning and memory are important biological processes that allow for optimized honey bee behavior. Factors negatively affecting bee cognition are important contributors to declines in pollination and food security. Agrochemical use, including herbicides, is one of the primary stressors linked to bee decline. Predicted agricultural expansion and associated increased use of glyphosate combined with scarcity of honey bees further highlights the need to understand the relationship between glyphosate and honey bee cognition and health. Here we investigated the effect of field-realistic doses of glyphosate on honey bee olfactory learning and memory. We used the conditioning of the Proboscis Extension Reflex (PER) to evaluate olfactory absolute conditioning. We found no differences in olfactory PER performance between glyphosate-exposed and control bees. We also did not find differences in olfactory memory retrieval at 15 min or 24 h after conditioning between exposed and non-exposed bees. However, we found that sublethal doses of glyphosate impaired memory retention; bees exposed to sublethal doses of glyphosate showed a decay trajectory of learned information, while in non-exposed bees the trajectory had a positive increment with time. This trend in memory retention was significantly different between bees exposed to 1500 ng and controls. *Implications for insect conservation*: Field-realistic doses of glyphosate had negative effect on memory dynamics in the honey bee. These results suggest glyphosate affects time-dependent neural mechanisms underlying information processing. This negative effect contributes to declines in pollination function and food security. We highlight the need to critically evaluate the cost-benefit analysis of indiscriminate glyphosate use.

Keywords Bee health · Cognition · Pollinator-Conservation · Time-dependent effect · Food security · Pesticides

Introduction

Bees are key organisms for ecosystem functioning by pollinating the world's flowering plants (Kearns et al. 1998; Ollerton et al. 2011; Potts et al. 2016). Specialization on floral resources as the only source of nutrients makes these insects reliable pollinators (O'Toole and Raw 1991; Michener 2007) and important nodes of ecological networks underlying biodiversity (Kearns et al. 1998; Memmott 1999; Thompson 2006; Bascompte and Jordano 2007; Potts et al. 2016; Gill et al. 2016). Moreover, bees frequently improve crop yield and quality (e.g., Ángel-Coca et al. 2011; Klatt

et al. 2013). The honey bee (*Apis mellifera*) is the main managed pollinator species in agroecosystems (Aizen et al. 2009; Breeze et al. 2011) because of its positive impact on crop production and, therefore, on local and global economies (Gill 1991; Southwick and Southwick 1992; Mwebaze et al. 2010; Maggi et al. 2016; Ponisio et al. 2015; Garibaldi et al. 2013, 2016; Blaauw and Isaacs 2014; Pywell et al. 2015). Honey bees, besides wild insects, play an important role in food security (Potts et al. 2016, Rader et al. 2016, Garibaldi et al. 2013).

However, the use of agrochemicals aimed at increasing food yield may, paradoxically, negatively impact food production if pollinator health is impaired. The use of pesticides, lack of floral resources, and pathogens are some of the main known stressors to pollinator bees (e.g., Kearns et al. 1998; Potts et al. 2010; Vanbergen 2013; Godfray et al. 2014, 2015; Pisa et al. 2015; van der Sluijs et al. 2015; Goulson et al. 2015; Collison et al. 2016; Klein et al. 2017). The increased demand for food imposes pressure on agricultural

✉ Marisol Amaya-Márquez
  mamayam@unal.edu.co

[1] Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia

[2] Departamento de Biología, Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia

expansion, which in turns drives larger inputs of agrochemicals into the system (Gill et al. 2017).

While there is evidence demonstrating the negative effect of insecticides on pollinator bees (Siviter et al. 2018), it is less known how herbicides affect bee health. From a general perspective, herbicides should not pose a threat to pollinators given that these agrochemicals are created for plant control, mainly weeds or undesired crops, and not for animals. Consequently, herbicide use is not often a factor considered in models predicting honey bee decline (e.g., Smith et al. 2013; Becher et al. 2013). However, glyphosate, a wide spectrum herbicide (Goldsborough and Brown 1988) may remain in agroecosystems for long periods of time and in areas beyond local application due to high solubility in water (Kolpin et al. 2006; Zhang et al. 2011) and low abiotic degradation (Rueppel et al. 1977; Kujawa 1996). Several studies have shown that glyphosate has adverse effects on different groups of animals (vertebrates and invertebrates, including the honey bee) and on diverse biological processes (e.g., genotoxicity, cytotoxicity, DNA damage) affecting organism health (see Gill et al. 2017 for a review). The IPBES assessment discussed the risks and knowledge gaps related to the use of herbicides (IPBES 2016).

Furthermore, the frequent development of glyphosate resistance by many weeds can promote the use of larger amounts of the herbicide to control them (Takano et al. 2019). The weeds absorb, assimilate, and translocate the glyphosate, with concentrations between 5 and 15 mg/L in a time interval of 8 days after its application (Sammons and Gaines 2014). Moreover, glyphosate residues are present in nectar ranging in concentration from 2.78 mg/L to 31.3 mg/L (Thompson et al. 2014). Given that social bees have foraging ranges that may reach up to 4 km from the hive (Osborne et al. 1999; Hagler et al. 2011; Redhead et al. 2016) and that foraging occurs in sequential visitation of flower sets, forager bees are repeatedly exposed to glyphosate. Substances present in the nectar or pollen of visited flowers are collected, stored and accumulated along with the floral resource itself in the hive (Kirchner 1999; Grüter and Farina 2007). The accumulation of toxic substances can occur rapidly as just one individual forager bee can make on average three round trips between the floral patch and the hive, collecting on average 20 µL of nectar per trip (Fewell and Winston 1996). Based on the amounts of glyphosate collected from the nectar by the honey bees (Thompson et al. 2014), forager bees likely ingest concentrations of glyphosate ranging from 139 ng/bee to 1565 ng/bee per 50 µL nectar harvested.

Current recommendations for glyphosate use are based on ecotoxicity tests of sublethal doses (LD50) on honey bees (Kujawa 1996). Recent studies have evaluated the impact of sublethal doses on bee survival (Thompson et al. 2014), metabolism (Boily et al. 2013; Helmer et al. 2015), internal microbiota balance (Motta et al. 2018; Blot et al. 2019), navigation (Balbuena et al. 2015), and gustatory perception and olfactory learning (Herbert et al. 2014; Mengoni Goñalons and Farina 2018). These studies indicate that sublethal doses of glyphosate can impact bee health. As such, understanding the consequences of glyphosate use beyond targeted organisms is highly warranted.

Trends in agriculture intensification predicts an increment in the use of glyphosate for the near future, so the commercial honey bee will frequently be exposed to this agrochemical. Given that sublethal doses of glyphosate impair learning and long-term memory (LTM) in honey bees (Herbert et al. 2014; Balbuena et al. 2015; Farina et al. 2019), both relevant cognitive skills for survival, foraging efficiency, and navigation of bees (Gould 1984; Menzel 2001a, it becomes urgent to investigate on the effects of glyphosate on bee pollinator health. This is especially relevant given the honey bee population declines reported in North America, Europe (Stokstad 2007; Currie et al. 2010; Pettis and Delaplane 2010; Potts et al. 2010; Neumann and Carreck 2010; vanEngelsdorp et al. 2010), and South America (Maggi et al. 2016) and the increased need of this pollinator with agriculture expansion (Aizen et al. 2009, Aizen and Harder 2009).

Long-term memory (LTM) is a relevant biological process for foraging and survival of honey bee colonies (Menzel 2001a) and is a time-dependent process (Hammer and Menzel 1995). The time-frame window between short-term memory (STM) and LTM consolidation is an active period of cellular and molecular functioning affecting neural and behavioral responses (Menzel 1990; Hammer and Menzel 1995; Menzel and Müller 1996; Müller 2012) where the presence of pesticides may interfere. Previous studies with the honey bee have found that sublethal doses of glyphosate negatively affect the navigational skills of adult bees suggesting that LTM can be affected (Balbuena et al. 2015). Additionally, Pavlovian olfactory learning, STM (Herbert et al. 2014), and differential olfactory learning (Mengoni Goñalons and Farina 2018) have also been shown to be affected by glyphosate. Despite previous advances in the understanding of the effect of this pesticide on honey bee cognition, the effect of sublethal doses of glyphosate on the consolidation of the STM to LTM remains poorly understood. Furthermore, previous studies on olfactory learning have been conducted with young bees (≤ 14 days old), though one of these studies found that bees ≥ 9 days old are not affected by sublethal doses of glyphosate (Mengoni Goñalons and Farina 2018). Given that adult foraging bees (≥ 15 days old) are the ones that need to face cognitive challenges outside of the hive, learn trajectory routes between the hive and the floral patches, associate floral cues with reward quality, and optimize food exploitation by making correct floral choices, here we asked whether sub-lethal doses of glyphosate affect cognition in adult bees. To answer this

question, we evaluate whether sub-lethal doses of glyphosate affect olfactory learning and memory dynamics (MTM and LTM) in the honey bee (*Apis mellifera scutellata*).

We conducted an experiment in which we evaluated the effect of (1) total sublethal dose ingestion (field realistic doses: 350 and 1500 ng) and (2) exposure type (acute and repeated) on bee cognitive performance, using the olfactory conditioning of the Proboscis Extension Reflex (PER) following an absolute learning protocol. PER is a robust protocol used to test Pavlovian learning in honey bees (Takeda 1961; Bitterman et al. 1983; Abramson et al. 1997; Giurfa and Sandoz 2012), the technique has been used to evaluate specific olfactory learning and memory in the honey bee (Matsumoto et al. 2012; Menzel 2012), and has been implemented as a tool to evaluate the effect of exogenous substances on learning in bees (e.g., Decourtye and Pham-Delègue 2002; Abramson et al. 2004, 2006; Smith and Burden 2014).
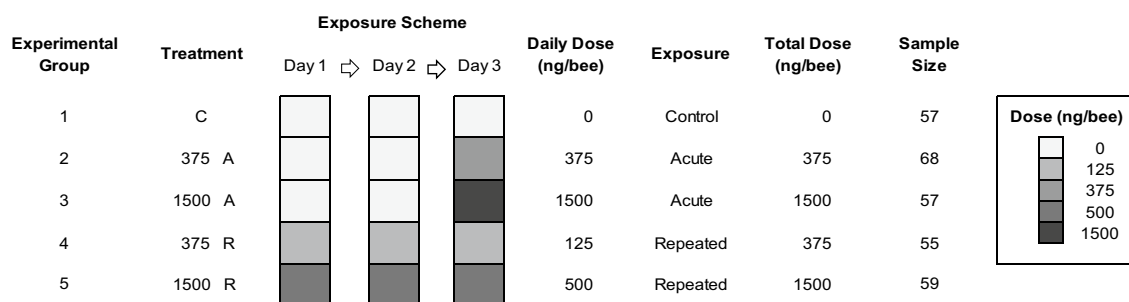
Materials and methods

Collection and maintenance of specimens

The study was conducted from March to August 2019 at the Universidad Nacional de Colombia, Bogotá, Colombia (4°38′30.98″N, 74°4′52.66″W), average temperature and relative humidity 20.3 °C ± 1.9 °C and 55.8% ± 11.5% respectively for the period of the study. Forager bees used in the experiments (Fig. 1) were captured when departing from the hive using an ultraviolet translucent plexiglass pyramid (Matsumoto et al. 2012). Based on the preliminary observations conducted for one of us (JH) on the experimental-hive, and considering that bees learn the time of the day floral resources are available (Moore 1989), we only used individuals captured within the time-framework window between 08:00 and 10:00 h, when the bees were more active visiting floral resources located nearby the hive. The restriction of

time gathering aimed to increase the probability of obtaining adult individuals that were in the foraging phase of their life cycle. Bees were then carefully transported to the laboratory and transferred one by one to individual scintillation vials (4 cm long × 2 cm wide) using the pyramid-apical aperture. The bees were cold-anesthetized by putting the vials inside an open plastic container with water and ice (Matsumoto et al. 2012; Smith and Burden 2014). Once the bees were motionless (Matsumoto et al. 2012), we harnessed them in plastic-resin tubes (Fig. 2a) and left them to warm and accustomed to the system 1 h before exposure to the treatment. Bees from each set used in a 5-day experiment were randomly assigned to one of the five experimental groups (Fig. 2a). Bees were maintained at temperature of 20 °C and relative humidity of 85% in conditions of natural day and night light cycle, receiving a daily food quota of 15 µL of sucrose solution 30% w/v with or without glyphosate corresponding to bee's exposure scheme (Fig. 1).

Experimental procedures

We controlled the *total amount* of glyphosate ingested per bee, being either 375 or 1500 ng, and the *exposure* to the glyphosate, being either acute (total amount distributed in a single intake: A) or repeated (total amount distributed across three days: R). Therefore, we had four treatments (field realistic doses: 375 A, 1500 A, 375R, 1500R) and a control group (C) of bees fed only with the vehicle (sucrose solution 30 %w/v). Treatment was administered on Days 1–3 according to the experimental group: (1) Treatment **C**: bees were fed every day with 15 µL of pure sucrose solution 30% w/v. (2) Treatment **375 A**: an acute dose of 375 ng was administered on day 3 with the daily food regime of 15 µL of sucrose solution 30% w/v, the previous day 1 and day 2 bees received 15 µL of sucrose solution 30% w/v. (3) Treatment **1500 A**: an acute dose of 1500 ng was administered on day 3 with the daily food regime of 15 µL of sucrose solution 30% w/v, the previous day 1 and day 2 bees received

Experimental Group	Treatment	Exposure Scheme Day 1 → Day 2 → Day 3	Daily Dose (ng/bee)	Exposure	Total Dose (ng/bee)	Sample Size
1	C		0	Control	0	57
2	375 A		375	Acute	375	68
3	1500 A		1500	Acute	1500	57
4	375 R		125	Repeated	375	55
5	1500 R		500	Repeated	1500	59

Dose (ng/bee): 0, 125, 375, 500, 1500

Fig. 1 Experimental groups employed to evaluate *total amount* of glyphosate ingested per bee (375 or 1500 ng), and *exposure* to glyphosate: Acute (total amount distributed in a single intake: A) or Repeated pulses (total amount distributed across three days: R). Five experimental independent groups of bees were used to applied treatments: exposed bees (treatments: 375 A, 1500 A, 375R, 1500R) and non-exposed control (C)

Fig. 2 Experimental procedure to evaluate the effect of sublethal doses of glyphosate on bee performance in olfactory learning and memory. **a** Bees captured on day-1 were harnessed and randomly assigned to one of five experimental groups. **b** On days 1–3 bees received the treatment with the daily food quota of 15 μL of a sucrose solution (30% w/v). **c** on Day-4 bees were olfactory PER conditioned. **d** on Day-4 and Day-5 bees were tested for consolidation of olfactory memory for trained odor CS (1-Hexanol)
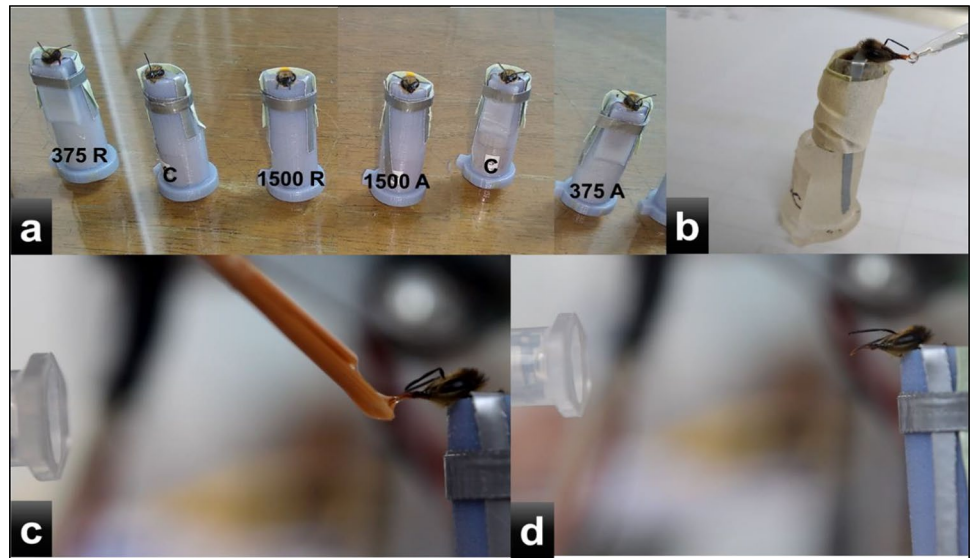
15 μL of sucrose solution 30% w/v. (4) Treatment **375R**: a total dose ingestion of 375 ng was administered in repeated chronic doses of 125 ng each day (day 1, day 2, and day 3) with the daily food regime of 15 μL of sucrose solution 30% w/v. (5) Treatment **1500R**: a total dose ingestion of 1500 ng was administered in repeated pulse doses of 500 ng each day (day 1, day 2, and day 3) with the daily food regime of 15 μL of sucrose solution 30% w/v (Fig. 2b). On day 4, bees of all experimental groups were first conditioned (Fig. 2c), and then tested for memory retention 15 min after the last training trial (medium-term memory: MTM) (Fig. 2d). After completing training and testing, the bees were provided with the daily food quota. On day 5, bees received a memory test 24 h after the last training trial (long-term memory: LTM) (Fig. 2d). By following the previous process, we assured all five experimental groups of bees were under the same environmental conditions and received the same amount of food and manipulation during the 5-day experiment. The process was repeated each week until a sample size ≥ 55 bees per treatment was reached (Fig. 1). Experimental individual bees were not reused. We used Glyphosate PESTANAL® Germany, ref. 45,521 (purity > 98.55%) to prepare the solutions.

Olfactory conditioning using PER

We trained bees using absolute conditioning (Giurfa 2007) in an appetitive context in which the conditioned scent stimulus (CS) was always paired to an unconditioned stimulus (US). We used 1-Hexanol (SIGMA, Aldrich) as the CS, and a sucrose solution (50% w/v) as the US reward. As in Amaya-Márquez et al. (2019) we included in the experiment only individuals that (1) did not show spontaneous response (SR) to the CS scent (i.e., those that did not extend the proboscis in response to the CS before training), and (2)

did show the unconditioned reflex (UR) of extending the proboscis when the antenna was contacted with a sucrose solution 50% (w/v). The use of these tests guarantee: that the CS was a neutral stimulus before conditioning (Pavlov 1927) and the individuals were motivated and able to respond to the US (Matsumoto et al. 2012). We utilized bees that do not presented visible wing wear to exclude adult bees that were too old.

Olfactory conditioned PER response was evaluated over six CS-US contingency trials (Fig. 2c). We used an overlapped stimuli presentation protocol: the CS was presented 6 s with the last 3 s overlapped with the US. The inter-stimuli interval (ISI) was 3 s and the stimuli overlapping time was 3 s. The inter-trial-interval (ITI) was 10 min. We used syringes of 10 mL to dispense the CS (Giurfa and Sandoz 2012; Matsumoto et al. 2012); we attached to the syringe's plunger a strip of Whatman 1 filter paper 3 cm × 1 cm impregnated with 10 μL of 1-Hexanol. Syringes were replaced every three trials, working with sets of five to seven bees, with the aim to maintain the same level of odor intensity along training trials (Smith and Burden 2014). The syringe was adjusted to a computer-handled machine controlling air flow time and duration of the parameters: CS exposition, ISI, and ITI. The scent emission syringe was located 3 cm apart from the bee's head and the scented flow air was dispensed at 0.02 L/s. The system was programmed to extract air from the chamber after each CS-US contingency presentation. The system signaled the experimenter when to provide the US and when to record latency.

Latency is the time that it takes the bee to respond with a conditioned PER to the CS stimulus presentation before receiving the US reward (Smith and Burden 2014). We measured latency in the first three seconds of the CS presentation; the response was recorded with 1 s precision.

This parameter was evaluated for a subset of individuals in each experimental group: (1) Treatment **C**: n = 35 bees. (2) Treatment **375 A**: n = 33 bees. (3) Treatment **1500 A**: n = 37 bees. (4) Treatment **375R**: n = 32 bees. (5) Treatment **1500R**: n = 33 bees. According to Domjan (2003), latency quantifies the behavioral response of the individual when the reinforced CS is presented to the animal; it measures the strength of the reinforcement, and in classical and operant conditioning latency may decrease along training trials.

We recorded the PER conditioned response as binary: (1) for a positive response of extending the proboscis in response to the conditioned odor CS, and (0) for a negative response. We evaluated bees' response to the US reward sucrose solution at each training trial to verify individuals were in good physiological condition (*Suppl 1* Fig S1).

Test of memory retention

We conducted a double purpose test 15 min after the last training trial in absolute olfactory conditioning to evaluate: (1) whether *olfactory PER response was specific to the trained odor* CS (1-Hexanol) indicating associative learning, and (2) whether olfactory information acquired in absolute olfactory conditioning had been *consolidated MTM*. We evaluated if the conditioned PER response was associative learning, to do that we used a novel odor as an internal control (e.g., Frost et al. 2012; Matsumoto et al. 2012). We performed a single trial unpaired to the US reward, using as the novel odor stimuli CS (1-Nonanol) and then a single trial unpaired with the US reward using the conditioned odor CS (1-Hexanol). Olfactory memory response was recorded as binary: (1) when the bee exhibited the conditioned PER response to the trained CS (1-Hexanol) and (0) when there was not conditioned PER response to the presentation of the trained CS. Only bees that showed at least one conditioned PER response in olfactory training trials, and did not showed spontaneous response to the CS (1-Hexanol), were included in the final analyses (*Suppl 1* Fig S2). Bees giving a negative response in the specific memory test were evaluated to rule out an impaired physiological condition of the bee (*Suppl 1* Fig S3), bees that did not respond to the US reward in this test were discarded from the memory analysis. Here we used *MTM* and *LTM* as in Menzel's model (e.g., Erber et al. 1980; Menzel 1999, 2000b, 2012; Menzel et al. 1993 Hammer and Menzel 1995; Menzel and Müller 1996).

24 h after olfactory conditioning, we conducted a test to evaluate retention, presumably supported by a *consolidated LTM*. We performed a single trial unpaired with the US reward using the conditioned odor CS (1-Hexanol). Olfactory memory response was recorded as binary: (1) when the bee exhibited the conditioned PER response to the trained CS (1-Hexanol) and (0) when there was not conditioned PER response to the presentation of the CS (1-Hexanol).

Statistical analysis

To analyze the effect of sublethal doses of glyphosate on *olfactory conditioning* we fitted a generalized linear mixed model (GLMM) with the conditioned PER as the response variable, dose amount, exposure, and training trials as fixed factors, and individual bee as a random factor. We used a binomial distribution and the logit link function. Non-exposed group (C) was used as control for both type of exposed groups (acute and repeated). We started out with the most complex model including all factors and interactions, and then iteratively went through the models eliminating those in which factors or interactions were not significant at the probability level of 0.05 (Faraway 2016). Among obtained models we used parsimony to choose the final model using Akaike information criteria (AIC) and Bayes information criteria (BIC) (Faraway 2016). Model overdispersion was calculated using Pearson residuals, the reference value for acceptable overdispersion was ≤ 1.5 (Zuur et al. 2009). The significance of the factors contributing to the model, and the significance among levels of each factor was evaluated with the Wald Chi-Squared (Fox and Weisberg 2019).

To analyze the effect of sublethal doses of glyphosate on *latency* we fitted a GLMM with latency as the response variable, dose amount, exposure type, and training trials as fixed factors, and individual bee as a random factor. We used a Poisson distribution and the logit link function. The rest of the procedure was the same as in the first model.

We evaluated the *specificity of the CR to the trained odor* using the McNemar's test, we tested the null hypothesis that the probability of bees giving a PER response to the conditioned CS (1-Hexanol) was equal to the probability of bees giving a PER response to the novel odor CS -Nonanol). This hypothesis was tested for the five experimental groups.

To analyze the effect of sublethal doses of glyphosate on *memory retention after 15 min* we fitted a GLMM with the CR as the response variable, dose amount and exposure as fixed factors, and individual bee as a random factor. We used a binomial distribution and the logit link function. The rest of the procedure was the same as in the first model.

To analyze the effect of sublethal doses of glyphosate on *memory retention after 24 h* we fitted a GLMM with the CR as the response variable, dose amount and exposure as fixed factors, and individual bee as a random factor. We used a binomial distribution and the logit link function. The rest of the procedure was the same as in the first model.

To evaluate the effect of sublethal doses of glyphosate on *memory dynamics* (i.e., change in olfactory performance between MTM and LTM) we fitted a GLMM with conditioned PER as the response variable, dose amount, exposure, and type of memory as fixed factors; and individual bee as a random factor. We used a binomial distribution and the

logit link function. The rest of the procedure was the same as in the first model.

GLMM analyses were run with R version 3.6.1 (R Core Team 2019); we used the function "glmer" from the lme4 package (Bates et al. 2015), "drop1" from the stats package, "summary" from the base R package (R Core Team 2019), "Anova" from the car package (Fox and Weisberg 2019), and "effect" from the effects package (Fox 2003). Sample size used in the analyses is shown in a flow chart (*Suppl 1* Fig S2).

Results

Effect of sublethal doses of glyphosate on olfactory learning

Olfactory conditioned PER response of bees exposed to sublethal doses of glyphosate was affected by dose *amount* (Wald Chisq = 5.7842, p = 0.05), training *trials* (Wald Chisq = 67.4342, p < 0.001), and by the *interaction* among these two factors (Wald Chisq = 8.2153, p < 0.05), while *exposure* (acute or repeated) was not significant. Furthermore, variability in olfactory learning performance in individual *bees* was a significant factor in the model (*Suppl 2* Table S1). The negative effect of sublethal doses of glyphosate ingestion was significant for 375 ng per bee (p < 0.05) and marginally significant for 1500 ng per bee (p = 0.059). Training had a positive effect on bee olfactory PER performance along trials (p < 0.001), with significant positive interactions among dose and trials on the CR, both for the dose amount of 375 ng per bee (p < 0.05) and 1500 ng per bee (p < 0.05) (*Suppl 2* Table S2). Thus, both exposed and control bees were able to reach similar conditioned PER performance to odor CS (1-Hexanol) along six training trials (Fig. 3). Bees exposed to glyphosate showed a conditioned PER response that was lower than the control bees until the 3rd trial, but from trial 4th and ahead the conditioned PER response of exposed bees was higher than the PER response of control bees (Fig. 3). The conditioned PER response to the CS + was significantly distinct from the PER response elicited by the control odor CS (1-Nonanol) in all experimental groups, indicating that specific olfactory learning occurred (MacNemar's test: 1.) Treatment **C**: ch-sq = 22.042, df = 1; p < 0. 001 (n = 57 bees). 2.) Treatment **375 A**: ch-sq = 18.375, df = 1; p < 0.001 (n = 68 bees). 3.) Treatment **1500 A**: ch-sq = 30.031, df = 1; p < 0.001 (n = 57 bees). 4.) Treatment **375R**: ch-sq = 28.03, df = 1; p < 0.001 (n = 55 bees). 5.) Treatment **1500R**: ch-sq = 24.038, df = 1; p < 0.001 (n = 55 bees), (Fig. 4). The bee's response to the US along the six training trials was high for all treatments (*Suppl 1* Fig S1) indicating that bees were in good physiological condition to respond in the test. Thus, PER
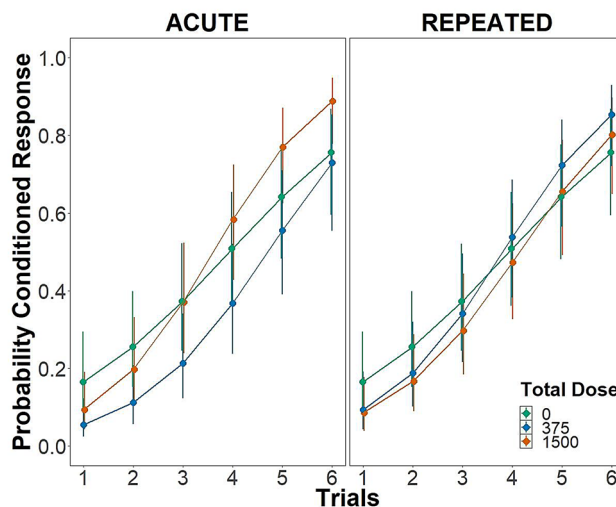
Fig. 3 Probability of the conditioned Proboscis Extension Reflex (PER) response along six training trials in olfactory absolute PER conditioning in honey bees exposed to glyphosate ingestion doses of 375 ng and 1500 ng per bee under acute and repeated pulses exposure. (95% confidence intervals are shown)
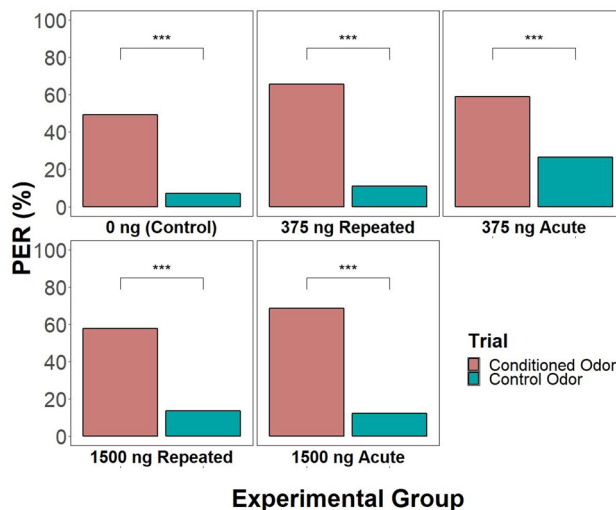

Fig. 4 Percentage of bees responding with the Proboscis Extension Reflex (PER) to the conditioned odor CS (1-Hexanol) unpaired to the US sucrose reward and to the novel odor (control) CS (1-Nonanol) unpaired to the US sucrose reward 15 min after olfactory conditioning

performance in absolute olfactory conditioning cannot be attributed to the physiological condition of bees.

Effect of sublethal doses of glyphosate on latency

Latency was affected by training *trials* (Wald Chisq = 8.059, p < 0.01), but not by sublethal doses of glyphosate, *amount*
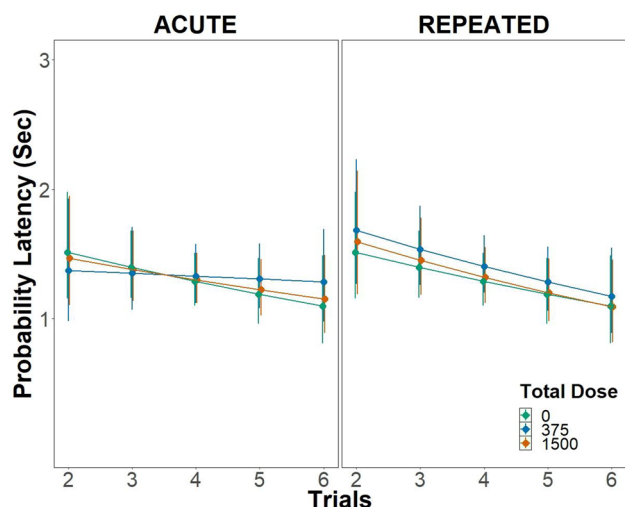
Fig. 5 Probability of latency (i.e., time it takes the bee to respond with the conditioned PER) along six training trials in olfactory absolute PER conditioning, in bees exposed to ingestion of glyphosate doses of 375 ng and 1500 ng per bee under acute and repeated pulses exposure. Control bees did not ingested glyphosate (0 ng). (95% confidence intervals are shown)

or *exposure* (acute vs. repeated). The only fixed factor in the model affecting latency was trial with a decreasing response along training trials ($p < 0.01$) in all experimental groups (Fig. 5), and bee individual differences as a random factor (*Suppl 2* Table S3); no interaction among factors was found significant in the model (*Suppl 2* Table S4).

Effect of sublethal doses of glyphosate on olfactory memory

Medium-term memory (MTM)

Sublethal doses of glyphosate ingestion did not affect olfactory MTM, neither dose amount ($p = 0.9641$) nor exposure ($p = 0.9801$) were significant factors in the model; only the random factor of bee individual differences explained the observed differences in memory (*Suppl 2* Table S5).

Long-term memory (LTM)

Sublethal doses of glyphosate ingestion did not affect olfactory LTM, neither dose amount ($p = 0.752$) nor exposure ($p = 0.9087$) were significant factors in the model; only the random factor of bee individual differences explained the LTM memory differences (*Suppl 2* Table S6).

Memory dynamics

Olfactory memory performance was affected by *memory type* (Wald Chisq = 3.7518, df = 1, p-value = 0.05), dose

amount (Wald Chisq = 8.8694, df = 2, p-value < 0.05), and by the *interaction* among these two factors (Wald Chisq = 9.8111, df = 2, p-value < 0.01); *exposure* type was not significant in the model. Furthermore, the random factor *bee* individual differences affected changes in memory along time (*Suppl 2* Table S7). Bees exposed to sublethal doses of glyphosate ingestion showed a decreased probability in olfactory memory performance being LTM lower than MTM, this tendency in memory dynamics was contrary in non-exposed (control) bees, that showed a LTM performance higher than the MTM (Figs. 6 and 7); the effect of dose amount was significant for 1500 ng ($p < 0.01$); but not for 375 ng; the interaction among types of memory and doses was significant for dose amount both 375 ng per bee ($p < 0.05$) and 1500 ng per bee ($p < 0.01$) (*Suppl 2* Table S8).

Discussion

We investigated the effect of field-realistic doses of glyphosate on olfactory learning and memory in the honey bee. We experimentally evaluated the effect of dose *amount* and *exposure* on olfactory absolute conditioning and memory using the conditioned PER protocol. We found that the probability of a conditioned PER to the CS + was negatively affected by dose *amount*, but positively affected by *trial* and by the interaction between dose *amount* and *trial*, while dose *exposure* was not an important factor in predicting the conditioned PER performance. Interestingly, despite of the negative effect of sublethal dose *amount*, exposed bees
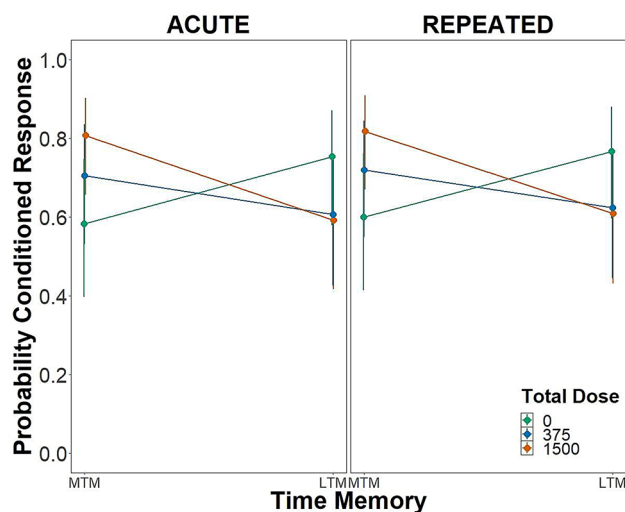
Fig. 6 Probability of conditioned PER response in memory tests conducted 15 min and 24 h after absolute olfactory conditioning in bees exposed to glyphosate ingestion doses of 375 ng and 1500 ng per bee under acute and repeated pulses exposure. Control bees did not ingested glyphosate (0 ng). (95% confidence intervals are shown)
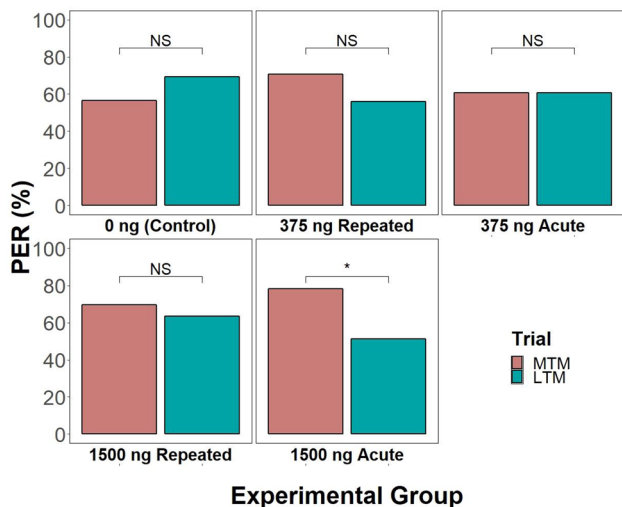
Fig. 7 Percentage of bees responding with the conditioned Proboscis Extension Reflex (PER) in medium-term memory (MTM) and long-term memory (LTM) in tests conducted 15 min and 24 h after absolute olfactory conditioning, respectively. Paired memory responses to conditioned stimuli CS (1-Hexanol) were compared in all experimental groups with the McNemar's test, 1 df, $p < 0.05$ (*)

were able to learn the CS odor to the same level than control bees. Nevertheless, the acquisition pattern differed from the typical olfactory learning curve in the honey bee, which usually reaches an asymptotic value between 80 and 90% by trial 3 (Menzel 1990), while in this study, bees reached the conditioned PER performance around 80% (78–88%) by trial 6. As this pattern occurred both in exposed and control groups, we attribute that difference to the experimental procedure followed in this study, where olfactory conditioning was evaluated at day 4 after the bees had spent three days harnessed in the lab to receive the treatment.

The potential stress caused in bees by the procedure may explain the observed underperformance of bees along trials 2–5 in both exposed and control groups. Consequently, we distinguish conditioned PER performance related to the experimental procedure from the one related to glyphosate ingestion. Apart from the procedural management of bees related to the glyphosate-exposure-scheme, the olfactory conditioning PER protocol followed in our study was the standardized PER protocol (Bitterman et al. 1983; Matsumoto et al. 2012; Frost et al. 2012; Smith and Burden 2014), with optimal interstimulus time of 3 s and intertrial time of 10 min (Bitterman and Menzel 1983). The conditioned PER response characterized and modelled in this study was specific to the trained odor indicating that associative olfactory learning occurred, and non-associative learning was ruled out as a potential factor eliciting PER. Latency response diminished along training trials indicating that the stimuli association CS-US was getting stronger along training trials as predicted by Domjan (2003). Also, bees exhibited MTM

and LTM for the CS at 15 min and 24 h after the last training trial.

We detected olfactory cognition impairment in exposed bees at two moments in the continuous process of acquisition and formation of memory: trials ≤ 3 and then on memory decay, separated between them by a phase in which the negative effect of glyphosate dose amount was unnoticed due to both increased PER performance in trials ≥ 4, and odor specific memories (*MTM* and *LTM*) formation. However, individual exposed bees showed lower performance in the *LTM* than in *MTM*, while these negative memory trajectories did not occur in control bees, indicating that impairment in memory retention was caused by the ingestion of sublethal doses of glyphosate. According to the honey bee memory model (e.g., Menzel 1999, 2001a, b, 2012) the LTM evaluated 24 h after the last training trial in this study, was expected to be stable, specific, and a protein-synthesis-dependent form of memory derived from multiple trials (Menzel 1999, 2001a, or single trial (Villar et al. 2020), therefore decreasing in this memory, in exposed but not in control bees, indicates affectation on LTM typology. The consolidation of a stable LTM requires of long-lasting changes in the intracellular levels of enzymes such as protein kinase A (PKA) and protein kinase C (PKC) (Hammer and Menzel 1995; Eisenhardt 2006; Müller 2012), PKA activity is essential for LTM consolidation, and is activated since the first trials by the activity of the cyclic Adenosine Mono Phosphate (cAMP); interestingly, when the PKA is experimentally reduced there is a selective impairment in LTM, but not in acquisition, STM or MTM (Menzel 1999). Here in this study, our model detected the negative effect of glyphosate dose *amount* on acquisition, observed only in the first three trials, and exposed bees were able of olfactory acquisition by reinforcement along training trials. Similarly, Herbert et al. (2014) in their study on the effect of sublethal doses of glyphosate on olfactory learning, also detected impairment in acquisition at trial 2 in young honey bees. Thus, the negative impact of glyphosate ingestion by honey bees on olfactory cognition behaviorally described in this study, coincides with the temporal activity of cAMP/PKA signaling pathway which is critical to the formation of stable form of *LTM* in the honey bee (Menzel 1999; Müller 2000, 2012), and highlights that memory consolidation begins since the very first conditioning trials. Future studies with a neuroethological approach focused on glyphosate affectation on the cAMP/PKA pathway and correlated behavioral performance may lead to the discovery of the mechanism by which glyphosate impairs cognition in the honey bee. An additional hypothesis to be evaluated in future studies is on the interaction between glyphosate and AMPA receptors. AMPA and NMDA receptors are key players in the formation of LTM. AMPA and NMDA and Glutamate receptors and Glyphosate is predicted to bind strongly to AMPA. Glutamatergic

neurons are certainly present within the mushroom bodies and are involved in learning, yet most attention is paid to cholinergic nicotinic neurons, as they comprised most of the population. As glutamate is not only present in the brain but also in the neuromuscular junction of insects, glyphosate is therefore predicted to impair also motor control (Zachepilo et al. 2018; Zgurzynski and Lushington 2019). Our study was conducted with adult bees of foraging age (≥ 15 days), though we did not specify the age further. Explicitly evaluating the role of age on the susceptibility of bees to herbicides is an important future direction (e.g., Mengoni Golaños and Farina 2018). While our experiments were conducted with individuals from one colony, individual differences on cognitive performance were evident in all models analyzed in the study. Evaluating patterns across different honey bee colonies with distinct genetic backgrounds is a necessary next step to further support the findings of this study.

Type of *exposure* (acute o repeated pulses) was not a significant factor in the model predicting the bee probability to elicit the conditioned PER performance to the CS (1-Hexanol). This result indicates that the negative effects of field-realistic sublethal doses of glyphosate on honey bees occur even after just one exposure. The negative effect of sublethal doses of glyphosate on honey bees is magnified at the colony level in this social insect, as forager honey bees remain collecting and carrying nectar to the hive, despite of the presence of glyphosate in it (Farina et al. 2019). Thus, the accumulation of toxic substances in the hive, leads to chronical exposure of adult bees and larvae to agrochemicals within the hive (Giesy et al. 2000; Kirchner 1999); Herbert et al. (2014) showed that accumulated glyphosate in the hive affected larva development and adult abilities of navigation and optimal foraging activity.

Our results add to the increasing evidence that glyphosate has negative effects on cognitive functioning of forager honey bees (see Farina et al. 2019), even when exposure was limited to sublethal doses. We found that field-realist doses of glyphosate ingested by honey bees negatively affect olfactory acquisition and impair olfactory memory retention. Another study with the honey bee has also shown a negative affectation of glyphosate on navigational memory (Balbuena et al. 2015). Foraging and navigation of a central place forager relies on the processes of learning and memory occurring in the brains of individual bees. Learning is one of the most important and expensive biological process, it allows organisms to modulate and optimize behavior according to changing environmental conditions (Dukas 1998). Acquisition implies changes in neural and cellular substrates underlying behavioral performance which are based on elemental and non-elemental types of learning affecting diverse biological processes of the social honey bee (e.g., flower decision-making, learned floral constancy, homing to the correct hive, communication with hive mates). Our findings on memory

retention impairment caused by the ingestion of sublethal doses of glyphosate has an effect on duplicated cognitive costs paid by bees for knowing: (1) investment in learning new information (CS-US association), keeping it along different phases of memory, and finally storing it in a form of protein-dependent-LTM that by the effect of the glyphosate is not stable as expected from the honey bee memory models (e.g. Menzel 2001b, 2012), and (2) losing capacity to retain and use the acquired information. The negative affectation on LTM has consequences on the capacity of bees to forage efficiently, navigate among productive floral patches, and between floral patches and hive. Additionally, damage in LTM retention will affect the capacity of individual bees to choose the correct flower type when economical choice is the challenge. Further, the honey bee is a very important pollinator as its evolutionary specialization on flowers for food is performed via generalist behavior, allowing this species to adapt to worldwide floras and to pollinate a diverse number of angiosperm species. Efficiency of this pollinator is based on concerted activity of hundreds of individual bees from the same hive communicating information with other members in the colony. Honey bees are important pollinators for crop pollination. Thus, the negative impact of glyphosate ingestion by honey bees on olfactory cognition will also contribute to declines in pollination function and food security, as these three processes are interdependent (Kevan and Menzel 2012).

Lastly, losses to pollinator health not only affect bee fitness but also can result in cascading impacts of relating biological and ecological processes: pollinator limited pollination in flowering plants, crop yield reduction, and functional biodiversity based on networks of ecological interactions via specialized mutualism (Potts et al. 2016; IPBES 2016). Given the growing literature on the negative effect of glyphosate on honey bee cognition (Farina et al. 2019) and other animals (Gill et al. 2017), as we further report in this study, we highlight the need to critically evaluate the cost-benefit analysis of indiscriminate glyphosate use aimed to increase food production and support food security, since this could actually result in the opposite effect by reducing pollinator health.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Abramson CI, Aquino IS, Silva MC, Price JM (1997) Learning in the Africanized honey bee: *Apis mellifera* L. Physiol Behav 62:657–674

Abramson CI, Squire J, Sheridan A, Mulder PG (2004) The effect of insecticides considered harmless to honey bees (*Apis mellifera*): proboscis conditioning studies by using the insect growth regulators tebufenozide and diflubenzuron. Environ Entomol 33:378–388. https://doi.org/10.1603/0046-225X-33.2.378

Abramson CI, Singleton JB, Wilson MK, Wanderley PA, Ramalho FS, Michaluk LM (2006) The effect of an organic pesticide on mortality and learning in Africanized honey bees (*Apis mellifera* L.) in Brasil. Am J Environ Sci 2:37–44. https://doi.org/10.1007/s002449900548

Aizen MA, Harder LD (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr Biol 19:915–918. https://doi.org/10.1016/j.cub.2009.03.071

Aizen MA, Garibaldi LA, Cunningham SA, Klein AM (2009) How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. Ann Bot 103:1579–1588. https://doi.org/10.1093/aob/mcp076

Amaya-Márquez M (2019) Olfactory learning in the stingless bee Melipona eburnean Friese (Apidae:Meliponini). Insects 10:412. https://doi.org/10.3390/insects10110412

Ángel-Coca C, Nates-Parra G, Ospina-Torres R, Melo Ortiz CD, Amaya-Márquez M (2011) Biología floral y reproductiva de la gulupa (pasiflora edulis Sims f. edulis). Caldasia 33:413–431

Balbuena MS, Tison L, Hahn ML, Greggers U, Menzel R, Farina WM (2015) Effects of sublethal doses of glyphosate on honey bee navigation. J Exp Biol 218:2799–2805. https://doi.org/10.1242/jeb.117291

Bascompte J, Jordano P (2007) Plant-Animal mutualistic networks: the architecture of biodiversity. Ann Rev Ecol Evol Syst 38:567–593. https://doi.org/10.1146/annurev.ecolsys.38.091206.095818

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48. https://doi.org/10.18637/jss.v067.i01

Becher MA, Osborne JL, Thorbek P, Kenedy PJ, Grimm V (2013) Towards a system approach for understanding honey bee decline: a stocktaking and synthesis of existing models. J Appl Ecol 50:868–886. doi:https://doi.org/10.1111/1365-2664.12112

BIOLOGÍA FLORAL Y REPRODUCTIVA DE LA GULUPA PASSIFLORA EDULIS SIMS F. EDULIS (scielo.org.co)

Bitterman ME, Menzel R, Fietz A, Schäfer S (1983) Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). J Comp Psych 97:107–119. https://doi.org/10.1037/0735-7036.97.2.107

Blaauw BR, Isaacs R (2014) Flower plantings increase wild bee abundance and the pollination services provided to a pollination-dependent crop. J Appl Ecol 51:890–898. https://doi.org/10.1111/1365-2664.12257

Blot N, Veillat L, Rouzé R, Delatte H (2019) Glyphosate, but not its metabolite AMPA, alters the honeybee gut microbiota. PLOS ONE 14(4):e0215466. https://doi.org/10.1371/journal.pone.0215466

Boily M, Sarrasin B, DeBlois C, Aras P, Chagnon M (2013) Acetylcholinesterase in honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. Environ Sci Pollut Res 20:5603–5614. https://doi.org/10.1007/s11356-013-1568-2

Breeze TD, Bailey AP, Balcombe KG, Potts SG (2011) Pollination services in the UK: how important are honeybees? Agric Ecosyst Environ 142:137–143. https://doi.org/10.1016/j.agee.2011.03.020

Collison E, Hird H, Cresswell J, Tyler C (2016) Interactive effects of pesticide exposure and pathogen infection on bee health—a critical analysis. Biol Rev Camb Philos Soc 91:1006–1019

Currie RW, Pernal SF, Guzmán-Novoa E (2010) Honey bee colony losses in Canada. J Apic Res 49:104–106. https://doi.org/10.3896/IBRA.1.49.1.18

Decourtye A, Pham-Delègue MH (2020) The proboscis extension response: assessing the sublethal effects of pesticides on the honey bee. In: Devillers J, Pham-Delegue MH (eds) Honey Bees: estimating the environmental impact of chemicals. Taylor &Francis Inc, New York, pp 72–88

Domjan M (2003) Principios de aprendizaje y conducta, 5th edn. Thomson-Paraninfo, Madrid

Dukas R (1998) Evolutionary ecology of learning. In: Dukas R (ed) Cognitive ecology: the evolutionary ecology of information processing and decision making. The University of Chicago Press, Chicago, pp 129–174

Eisenhardt D (2006) Learning and memory formation in the honeybee (Apis mellifera) and its dependency on the cAMP-protein kinase A pathway. Anim Biol 56:259–278. https://doi.org/10.1163/157075606777304249

Erber J, Masuhr T, Menzel R (1980) Localization of short-term memory in the brain of the bee, *Apis mellifera*. Physiol Entomol 5:343–358. https://doi.org/10.1111/j.1365-3032.1980.tb00244.x

Faraway JJ (2016) Extending the linear model with R: generalized linear, mixed effects and nonparametric regression models, 2nd edn. CRC Press, Boca Raton

Farina WM, Balbuena MS, Herbert LT, Mengoni Goñalons C (2019) Effects of the herbicide glyphosate on honey bee sensory and cognitive abilities: individual impairments with implications for the hive. Insects 10:354. https://doi.org/10.3390/insects10100354

Fewell JH, Winston ML (1996) Regulation of nectar collection in relation to honey storage levels by honey bees, *Apis mellifera*. Behav Ecol 7:286–291. https://doi.org/10.1093/beheco/7.3.286

Fox J (2003) Effect displays in R for generalised linear models. J Stat Softw 8:1–27

Fox J, Weisberg S (2019) An {R} companion to applied regression, 3rd edn. Sage, Thousand Oaks

Frost EH, Shutler D, Hillier NK (2012) The proboscis extension reflex to evaluate learning and memory in honey bees (*Apis mellifera*): some caveats. Naturwissenschaften 99:677–686. https://doi.org/10.1007/s00114012-0955-8

Garibaldi et al (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. Science 339:1608–1611

Garibaldi LA et al (2016) Mutually beneficial pollinator diversity and crop yieldoutcomes in small and large farms. Science 351:388–391

Giesy JP, Dobson S, Solomon KR (2000) Ecotoxicological risk assessment for Roundup® Herbicide. In: Ware GW (ed) Reviews of environmental contamination and toxicology. Springer-Verlag, New York, pp 35–120. https://doi.org/10.1007/978-1-4612-1156-3_2

Gill RA (1991) The value of honey bee pollination to society. Apiacta 26:97–105

Gill RJ, Baldock KCR, Brown MJF, Creswell JE, Dicks LV, Fountain MT, Garratt MPD, Gough LA, Heard MS, Holland JM, Ollerton J, Stone GN, Tang CQ, Vanbergen AJ, Vogler AP, Woodward G, Arce AN, Boatman ND, Brand-Hardy R, Breeze TD, Green M, Hartfield CM, O'Connor RS, Osborne JL, Phillips J,

Sutton PB, Potts SG (2016) Protecting an ecosystem service: approaches to understanding and mitigating threats to wild insect pollinators. Adv Ecol Res. https://doi.org/10.1016/bs.aecr.2015.10.007

Gill JPK, Sethi N, Mohan A, Datta S, Girdhar M (2017) Glyphosate toxicity for animals. Environ Chem Lett 16:401–426. https://doi.org/10.1007/s10311-017-0689-0

Giurfa M (2007) Behavioral and neural analysis of associative learning in the honey bee: a taste from the magic well. J Comp Physiol A 193:801–824. https://doi.org/10.1007/s00359-007-0235-9

Giurfa M, Sandoz JC (2012) Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honey bees. Learn Mem 19:54–66. https://doi.org/10.1101/lm.024711.111

Godfray HCJ et al (2015) A restatement of recent advances in the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc R Soc Lond B 282:20151821

Godfray HCJ, Blacquière T, Field LM, Hails RS, Petrokofsky G, Potts SG, Raine NE, Vanbergen AJ, McLean AR (2014) A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators. Proc R Soc B 281:20140558. https://doi.org/10.1098/rspb.2014.0558

Goldsborough LG, Brown DJ (1988) Effect of glyphosate (Roundup®) formulation on periphytic algal photosynthesis. Bull Environ Contam Toxicol 41:253–260. https://doi.org/10.1007/BF01705439

Gould JL (1984) Natural history of honey bee learning. In: Marler P, Terrace HS (eds) The biology of learning. Springer, New York, pp 149–180

Goulson D, Nicholls E, Botias C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347:1255957. Doi:https://doi.org/10.1126/science.1255957

Grüter C, Farina WM (2007) Nectar distribution and its relation to food quality in honey bee (Apis mellifera) colonies. Insectes Soc 54:87–94. https://doi.org/10.1007/s00040-007-0915-z

Hagler JR, Mueller S, Teuber LR, Machtley SA, Deynze A (2011) Foraging range of honey bees, Apis mellifera, in Alfalfa seed production fields. J Insect Sci 11:1–12. https://doi.org/10.1673/031.011.14401

Hammer M, Menzel R (1995) Learning and memory in the honey bee. J Neurosci 15:1617–1630. https://doi.org/10.1523/jneurosci.15-03-01617.1995

Helmer SH, Kerbaol A, Aras P, Jumarie C, Boily M (2015) Effects of realistic doses of atrazine, metolachlor, and glyphosate on lipid peroxidation and diet-derived antioxidants in caged honey bees (Apis mellifera). Environ Sci Pollut Res 22:8010–8021. https://doi.org/10.1007/s11356-014-2879-7

Herbert LH, Vazquez DE, Arenas A, Farina WM (2014) Effects of field-realistic doses of glyphosate on honey bee appetitive behaviour. J Exp Biol 217:3457–3464. https://doi.org/10.1242/jeb.109520

IPBES, Imperatriz-Fonseca VL, Ngo HT (eds) (2016) The assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. Potts SG. Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, Bonn

Kearns C, Inouye D, Waser NM (1998) Endangered mutualisms: the conservation of plant-pollinator interactions. Ann Rev Ecol Syst 29:83–112. https://doi.org/10.1146/annurev.ecolsys.29.1.83

Kevan PG, Menzel R (2012) The plight of pollination and the interface of neurobiology, ecology and food security. Environmentalist 32:300–310. https://doi.org/10.1007/s10669-012-9394-5

Kirchner WH (1999) 17. Mad-Bee-Disease? Subletale Effekte von Imidacloprid (Gaucho®) auf das Verhalten von Honigbienen. Apidologie 30:421–422

Klatt BK, Holzschuh A, Westphal C, Clough Y, Smit I, Pawelzik E, Tscharntke T (2013) Bee pollination improves crop quality, shelf life and commercial value. Proc R Soc Lond B Biol Sci 281:20132440. https://doi.org/10.1098/rspb.2013.2440

Klein S, Cabirol A, Daevaud JM, Barron AB, Lihoreau M (2017) Why bees are so vulnerable to environmental stressors. Trends Ecol Evol 33:268–278. https://doi.org/10.1016/j.tree.2016.12.009

Kolpin DW, Thurman EM, Lee EA, Meyer MT, Furlong ET, Glassmeyer ST (2006) Urban contributions of glyphosate and its degradate AMPA to streams in the United States. Sci Total Environ 354:191–197. https://doi.org/10.1016/j.scitotenv.2005.01.028

Kujawa M (1996) Glyphosate. Environmental Health Criteria 159, 177 Seiten, 3 Abbildungen und 25 Tabellen. World Health Organization, Geneva 1994. Preis: 27,—Sw.fr. Food / Nahrung 40(3):166–166. https://doi.org/10.1002/food.19960400341

Maggi M, Antúnez K, Invernizzi C, Aldea P, Vargas M, Negri P, Brasesco C, De Jong D, Message D, Teixeira EW, Principal J, Barrios C, Rufinengo S, Rodriguez Da Silva R, Eguaras M (2016) Honey bee health in South America. Apidologie 47:835–854

Matsumoto Y, Menzel R, Sandoz J-C, Giurfa M (2012) Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: a step toward standardized procedures. J Neurosci Meth 211:159–167. https://doi.org/10.1016/j.neumeth.2012.08.018

Memmott J (1999) The structure of plant-pollinator food web. Ecol Lett 2:276–280. https://doi.org/10.1046/j.1461-0248.1999.00087.x

Mengoni Goñalons C, Farina WM (2018) Impaired associative learning after chronic exposure to pesticides in young adult honey bees. J Exp Biol 221:jeb176644. https://doi.org/10.1242/jeb.176644

Menzel R (1990) Learning, memory, and "cognition" in honey bees. In: Kesner RP, Olten DS (eds) Neurobiology of comparative cognition. Erlbaum Inc, New Jersey

Menzel R (1999) Memory dynamics in the honey bee. J Comp Physiol A 185:323–340. https://doi.org/10.1007/s003590050392

Menzel R (2001a) Behavioral and neural mechanisms of learning and memory as determinants of flower constancy. In: Chittka L, Thomson JD (eds) Cognitive ecology of pollination: animal behavior and floral evolution. Cambridge University Press, Cambridge, pp 21–40

Menzel R (2001b) Searching for the memory trace in a mini-brain, the honey bee. Learn Mem 8:53–62

Menzel R (2012) The honey bee as a model for understanding the basis of cognition. Nat Rev Neurosci 13:758–768. https://doi.org/10.1038/nrn3357

Menzel R, Mülller U (1996) Learning and memory in honey bees: from behavior to neural substrate. Ann Rev Neurosci 19:379–404. https://doi.org/10.1146/annurev.ne.19.030196.002115

Menzel R, Greggers U, Hammer M (1993) Functional organization of appetitive learning and memory in a generalist pollinator, the honey bee. In: Papaj DR, Lewis AC (eds) Insect learning. Chapman & Hall, Inc, London, pp 79–125. https://doi.org/10.1007/978-1-4615-2814-2_4

Michener CD (2007) The bees of the world, 2nd edn. Johns Hopkins University Press, Baltimore

Moore D, Siegfried D, Wilson R, Rankin MA (1989) The influence of time of day on the foraging behavior of the honeybee Apis mellifera. J Biol Rhythms 4:305–325

Motta EVS, Raymann K, Moran NA (2018) Glyphosate perturbs the gut microbiota of honey bees. PNAS 115:10305. https://doi.org/10.1073/pnas.1803880115

Müller U (2000) Prolonged activation of cAMP dependent protein kinase during conditioning induces long-term memory in honey bees. Neuron 27:1–20. https://doi.org/10.1016/s0896-6273(00)00017-9

Müller U (2012) The molecular signalling processes underlying olfactory learning and memory formation in honey bees. Apidologie 43:322–333. https://doi.org/10.1007/s13592-011-0115-8

Mwebaze P, Marris GC, Budge GE, Brown M, Potts SG, Breeze TD, Macleod A (2010) Quantifying the value of ecosystem services: a case study of honey bee pollination in UK. In: 12th Annual BIOECON conference from the Wealth of Nations to the Wealth of Nature: rethinking economic growth 27–28 September, Venice, Italy

Neumann P, Carreck NL (2010) Honey bee colony losses. J Apic Res 49:1–6. https://doi.org/10.3896/IBRA.1.49.1.01

Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals? Oikos 120:321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Osborne JL, Clark SJ, Morris RJ, Williams IH, Riley JR, Smith AD, Reynolds DR, Edwards AS (1999) A landscape-scale study of bumble bee foraging range and constancy, using harmonic radar. J Appl Ecol 36:519–533. https://doi.org/10.1046/j.1365-2664.1999.00428.x

O'Toole C, Raw a (1991) Bees of the world. Blandford Publishing, an imprint of Cassell plc, London

Pavlov IP (1927) Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex (Anrep GV translation). Oxford University Press, London

Pettis JS, Delaplane KS (2010) Coordinated responses to honey bee decline in the USA. Apidologie 41:256–263. https://doi.org/10.1051/apido/2010013

Pisa LW et al (2015) Effects of neonicotinoids and fipronil on non-target invertebrates. Environ Sci Pollut Res 2:68–102. DOI:https://doi.org/10.1007/s11356-014-3471-x

Ponisio LC et al (2015) Diversification practices reduce organic to conventional yield gap. Proc R Soc B 282:20141396

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol 25:345–353. https://doi.org/10.1016/j.tree.2010.01.007

Potts SG, Imperatriz-Fonseca V, Ngo HT, Aizen MA, Biesmeijer JC, Breeze TD, Dicks LV, Garibaldi LA, Hill R, Settele J, Vanbergen AJ (2016) Safeguarding pollinators and their values to human well-being. Nature 540:220–229. https://doi.org/10.1038/nature20588

Pywell RF et al (2015) Wildlife-friendly farming increases crop yield. evidence forecological intensification. Proc R Soc B 282:20151740

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rader et al (2016) Non-bee insects are important contributors to global crop pollination. Proc Natl Acad Sci USA 113:146–151

Redhead JW, Dreier S, Bourke AFG, Heard MS, Jordan WC, Sumner S, Wang J, Carvell C (2016) Effects of habitat composition and landscape structure on worker foraging distances of five bumble bee species. Ecol Appl 26:726–739

Rueppel ML, Brightwell BB, Schaefer J, Marvel JT (1977) Metabolism and degradation of glyphosate in soil and water. J Agric Food Chem 25:517–528. https://doi.org/10.1021/jf60211a018

Sammons RD, Gaines TA (2014) Glyphosate resistance: state of knowledge. Pest Manag Sci 70:1367–1377. https://doi.org/10.1002/ps.3743

Siviter H, Koricheva J, Brown MJF, Leadbeater E (2018) Quantifying the impact of pesticides on learning and memory in bees. J Appl Ecol 55:2812–2821. https://doi.org/10.1111/1365-2664.13193

Smith BH, Burden CM (2014) A proboscis extension response protocol for investigating behavioral plasticity in insects: application to basic, biomedical, and agricultural research. J Vis Exp JoVE 91:e51057. https://doi.org/10.3791/51057

Smith KM, Loh EH, Rostal MK, Zambrana-Torrelio CM, Mendiola L, Daszak P (2013) Pathogens, pests, and economics: drivers of honey bee colony declines and loose. Ecohealth 10:434–445

Southwick EE, Southwick L (1992) Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States. J Econ Entom 85:621–633. https://doi.org/10.1093/jee/85.3.621

Stokstad E (2007) The case of the empty hives. Science 316:970–972. https://doi.org/10.1126/science.316.5827.9

Takano HK, Mendes RR, Scoz LB, Lopez Ovejero RF, Constantin J, Gaines TA, Westra P, Dayan FE, Oliveira RS Jr (2019) Proline-106 EPSPS mutation imparting glyphosate resistance in goosegrass (*Eleusine indica*) emerges in South America. Weed Sci 67:48–56. https://doi.org/10.1017/wsc.2018.71

Takeda K (1961) Classical conditioned response in the honey bee. J Insect Physiol 3:168–179. https://doi.org/10.1016/0022-1910(61)90060-9

Thompson JN (2006) Mutualistic webs of species. Science 312:372–373. https://doi.org/10.1126/science.1126904

Thompson HM, Levine SL, Doering J, Norman S, Manson P, Sutton P, von Mérey G (2014) Evaluating exposure and potential effects on honey bee brood (*Apis mellifera*) development using glyphosate as an example. Integr Environ Assess Manag 10:463–470. https://doi.org/10.1002/ieam.1529

van der Sluijs JP, Amaral-Rogers V, Belzunces LP, van Lexmond MFIJB, Bonmatin JM, Chagnon M, Downs CA, Furlan L, Gibbons DW, Giorio C, Girolami V, Goulson D, Kreutzweiser DP, Krupke C, Liess M, Long E, McField M, Mineau P, Mitchell EAD, Morrissey CA, Noome DA, Pisa L, Settele J, Simon-Delso N, Stark JD, Tapparo A, Van Dyck H, Van Praagh J, Whitehorn PR, Wiemers M (2015) Conclusions of the Worldwide Integrated Assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. Environ Sci Pollut Res 22:148–154

Vanbergen AJ, the Insect Pollinators Initiative (2013) Threats to an ecosystem service: pressures on pollinators. Front Ecol Environ 11:251–259. https://doi.org/10.1890/120126

vanEngelsdorp D, Hayes J, Underwood RM, Pettis JS (2010) A survey of honey bee colony losses in the United States, fall 2008 to spring 2009. J Apic Res 49:7–14. https://doi.org/10.3896/IBRA.1.49.1.03

Villar ME, Marchal P, Viola H, Giurfa M (2020) Redefining single-trial memories in the honeybee. Cell Rep 30:2603–2613. https://doi.org/10.1016/j.celrep.2020.01086

von Frisch K (1967) The dance language and orientation of bees. Harvard University Press, Cambridge

Zachepilo TG, Davydova AA, Vaido AI, Lopatina NG (2018) Role of the GluR2 subunit of AMPA receptors in associative learning in the honey bee *Apis mellifera* L. J Evol Biochem Phys 54:449–456. https://doi.org/10.1134/S0022093018060042

Zgurzynski MI, Lushington GH (2019) Glyphosate impact on *Apis mellifera* navigation: a combined behavioral and chemioinformatics study. EC Pharmacol Toxicol 7:806–824

Zhang WJ, Jiang FB, Ou JF (2011) Global pesticide consumption and pollution: with China as a focus. Proc Int Acad Ecol Environ Sci 1:125–144

Zuur A, Ieno EN, Walker N, Saveliev AA, Smith GM (2009) Mixed effects models and extensions in ecology with R. Springer, Berlin