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RESEARCH ARTICLE

Sublethal glyphosate exposure reduces honey bee foraging and alters the balance of biogenic amines in the brain

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ABSTRACT

Glyphosate is a broad-spectrum herbicide that inhibits the shikimate pathway, which honey bees (*Apis mellifera*), a non-target beneficial pollinator, do not endogenously express. Nonetheless, sublethal glyphosate exposure in honey bees has been correlated to impairments in gustation, learning, memory and navigation. While these impacted physiologies underpin honey bee foraging and recruitment, the effects of sublethal glyphosate exposure on these important behaviors remain unclear, and any proximate mechanism of action in the honey bee is poorly understood. We trained cohorts of honey bees from the same hives to forage at one of two artificial feeders offering 1 mol l^{-1} sucrose solution, either unaltered (*N*=40) or containing glyphosate at 5 mg acid equivalent (a.e.) l^{-1} (*N*=46). We then compared key foraging behaviors and, on a smaller subset of bees, recruitment behaviors. Next, we quantified protein levels of octopamine, tyramine and dopamine, and levels of the amino acid precursor tyrosine in the brains of experimental bees collected 3 days after the exposure. We found that glyphosate treatment bees reduced their foraging by 13.4% (*P*=0.022), and the brain content of tyramine was modulated by a crossover interaction between glyphosate treatment and the number of feeder visits (*P*=0.004). Levels of octopamine were significantly correlated with its precursors tyramine (*P*=0.011) and tyrosine (*P*=0.018) in glyphosate treatment bees, but not in control bees. Our findings emphasize the critical need to investigate impacts of the world's most-applied herbicide and to elucidate its non-target mechanism of action in insects to create better-informed pollinator protection strategies.

KEY WORDS: Pollinators, Foraging, Herbicide, Insects, Waggle dances

INTRODUCTION

Pesticides, including insecticides, herbicides and fungicides, are globally ubiquitous, and their use is projected to increase alongside agricultural intensification (Zhang et al., 2011; Benbrook, 2016; Brovini et al., 2021). Therefore, it is critical that we balance the benefits of pesticide use with the cost of impacts to non-target organisms, and particularly beneficial insects such as pollinating bees (Pimentel, 1997, 2005). The presence and degree of such impacts depend on the pesticide encountered (Decourtye et al., 2004; Charreton et al., 2015), the susceptibility of the organism to that pesticide (Wu et al., 2017; Goñalons and Farina, 2018), the route of exposure (Krupke et al., 2012), the dosage experienced (Almasri et al., 2021), and the duration (chronic versus acute) of that exposure (Tosi et al., 2017). While such non-target effects of insecticides on bee health have received increased attention in recent years (Abati et al., 2021; Bernardes et al., 2022; Gonçalves et al., 2022), those of fungicides and herbicides are less studied (Desneux et al., 2007; Schmidt-Jeffris, 2023), partly because regulatory requirements for toxicity testing on insects are generally less stringent for non-insecticidal chemistries.

The herbicide glyphosate is a weedkiller in the *N*-(phosphonomethyl) glycine chemical group that targets the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) within the shikimate pathway (Steinrücken and Amrhein, 1980; Sikorski and Gruys, 1997; Duke and Powles, 2008). Although glyphosate was initially approved in 1974 for the limited removal of all vegetation, the introduction of crops genetically modified to resist glyphosate, such as RoundUp Ready® (RR) maize, soybean and cotton varieties in 1996, followed by alfalfa in 2005 and sugar beet in 2008, led to a dramatic increase in its use as a broadcast herbicide: between 1974 and 2014, glyphosate use increased more than 15-fold in the USA (Duke and Powles, 2008; Duke, 2018), with a corresponding global increase as well (China: Zhang et al., 2011; Brazil: Sharma et al., 2019; Brovini et al., 2021). Although glyphosate is no longer sold directly to consumers in the USA, its large-scale use in agriculture continues, and today, glyphosate is the most widely used pesticide in the world (Benbrook, 2016).

This widespread use of glyphosate is due in part to its relatively low acute oral and contact toxicity to honey bees. With an LD50 value over 100 µg per bee (Frasier and Jenkins, 1972; Chen et al., 2023), the herbicide has been touted as being pollinator friendly (Frasier and Jenkins, 1972; Duke and Powles, 2008). However, a growing body of literature suggests that glyphosate may nevertheless cause sublethal, non-target effects on both the behavior and physiology in a variety of bee taxa, including the Western honey bee (*Apis mellifera*). Recent work has documented reduced honey bee survival after exposure to glyphosate in formulation (Abraham et al., 2018; Motta et al., 2020), as well as sublethal effects of non-formulated glyphosate, including altered physiological homeostasis (Almasri et al., 2022), immune dysregulation (Motta et al., 2022), altered sleep patterns (Vázquez et al., 2020), reduced navigational capabilities (Balbuena et al., 2015), decreased sucrose responsiveness (Herbert et al., 2014; Farina et al., 2019), impaired associative learning (Herbert et al., 2014; Goñalons and Farina, 2018) and reduced olfactory memory retention (Hernández et al., 2021). As the honey bee is the most economically impactful pollinator in agricultural systems, contributing to the pollination of 44% of commodity and food crops (Klein et al., 2007) and providing billions of dollars annually in pollination services (Southwick and Southwick, 1992), understanding these non-target impacts to honey

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bees is critically important. In particular, more field studies are needed in order to better understand whether and to what extent the sublethal effects of glyphosate documented in laboratory settings are borne out in freely flying and foraging bees.

Disruptions to navigation, sucrose responsiveness, associative learning and memory hold particular importance for forager honey bees, which rely on these functions to find food successfully in a dynamically changing landscape. Foragers evaluate the profitability of a floral resource as its net energetic efficiency, which balances its sugar reward versus energetic cost (primarily flight distance) associated with working that resource (Schmid-Hempel, 1987; Garbuzov et al., 2015; Seeley, 1995). These foragers may also recruit nestmates to a profitable resource by performing a waggle dance, which communicates a distance and direction to that resource (von Frisch, 1967), thereby increasing colony-level foraging success and ultimately fitness (Seeley, 1995; Sherman and Visscher, 2002; Grüter and Farina, 2009; Grüter and Ratnieks, 2011; Schürch and Grüter, 2014; Nürnberger et al., 2019; although see Price et al., 2019). Successful foragers must therefore not only learn the location of a resource in the landscape but also assess its profitability and adjust visitation frequency and waggle dance recruitment accordingly (Grüter and Farina, 2009; Couvillon, 2012). Despite the evidence that glyphosate may disrupt the physiological processes that underpin and modulate foraging and recruitment, the effects of sublethal exposure on these behaviors themselves remain incompletely understood (but see Herbert et al., 2014). Furthermore, the mechanism of non-target toxicity in bee physiology is poorly understood (Battisti et al., 2021, 2023; Schmidt-Jeffris, 2023).

Here, we conducted a feeder training experiment to study the impact of glyphosate on behavior and brain neurochemistry in the honey bee. In particular, we investigated the effects of sublethal, field-realistic glyphosate exposure on key honey bee foraging and recruitment behaviors via quantification of foraging frequency (our primary behavioral outcome), foraging persistency, waggle dance propensity, waggle dance frequency and waggle run repetitions per dance (our secondary behavioral outcomes, studied with a subset of bees). As these behavioral metrics tend to scale positively alongside a bee's evaluation of a resource's profitability (von Frisch, 1967; Seeley et al., 1991, 2000; Seeley, 1995), and as glyphosate can disrupt the underlying functions of gustation, associative learning and memory, we predicted that sublethal glyphosate exposure would reduce the incidence of these behaviors. Next, we conducted a follow-up experiment with a subset of the same experimental bees to examine the effect of sublethal glyphosate exposure on total and relative brain content (Lim et al., 2016) of certain key biogenic amines involved in foraging, recruitment and the neurological signaling associated with these functions, including octopamine, tyramine and dopamine (reviewed in Bicker, 1999; Scheiner et al., 2002; Farooqui, 2012; Blenau and Baumann, 2016), as well as their biosynthetic precursor, the amino acid tyrosine (Karlson and Herrlich, 1965; Roeder, 2005; Matsuyama et al., 2015).

MATERIALS AND METHODS

Study organism

We studied three small queenright honey bee, *Apis mellifera* L., colonies, each containing brood and approximately 5000 workers. Each experimental colony was housed indoors in observation hives consisting of three American Standard Deep frames and transparent Plexiglas walls to enable direct observation of activity on the comb. Colonies were connected to the outdoors via a plastic tube measuring approximately 5 cm×30 cm. We conducted three experimental trials in all, each with a separate experimental colony.

We created observation hives that would possess enough empty cells for incoming nectar storage. This aspect of management is important in feeder training experiments where insufficient availability of empty cells may result in suppression of recruitment related to nectar unloading delays (Seeley, 1989, 1995; Seeley and Tovey, 1994).

We collected data from 14 July 2021 to 1 August 2021, on days with favorable foraging conditions. We chose to work in high summer because we have found it easier to train bees to feeders when there is a relative dearth of natural forage in the surrounding landscape (Couvillon et al., 2014; Ohlinger et al., 2022a). It was sometimes necessary to provide colonies with supplemental sugar solution, which we removed at least 24 h before the beginning of data collection.

Training forager bees to feeders and glyphosate treatment

We trained an initial cohort of worker honey bees from our study colonies to visit artificial feeder stations containing 2 mol l^{-1} sucrose solution, scented with lavender (10 µl l^{-1}) to encourage strong recruitment (Tautz, 2022), using our step-wise training method (Ohlinger et al., 2022b; Couvillon et al., 2023, 2024). Briefly, on the day prior to the experiment (Day −1), we loaded a Petri dish with a few drops of scented solution, placed that Petri dish inside a small box, placed the box on a platform mounted on a tripod (approximately 1 m in height), then used the box to cover the exit tube from the hive such that exiting worker bees would be able to explore the box and encounter the sugar solution. Importantly, this method prevented bees from the nearby apiary from discovering the Petri dish. As soon as there were at least three bees drinking the solution from the Petri dish, we removed the box and gently transferred the dish to the tripod platform just outside the tube entrance. Stepwise training began as soon as any bee made a new visit to the feeder (i.e. landed on the Petri dish and began to collect the sucrose solution).

Two researchers participated in Day −1 training, one marking visiting foragers with numbered plastic discs (BetterBee, Greenwich, NY, USA) and the other confirming the membership of marked foragers to the experimental colony. To exclude bees from the nearby apiary, we removed any marked bee that returned to the feeder but that we could not confirm inside the observation hive. As soon as 3–5 marked bees from the experimental colony had made at least three visits to the Petri dish, we swapped out the dish for the full feeder setup, which consisted of a 4 oz glass jar (approximately 29.6 ml; Ball, Westminster, CO, USA) on a plastic base with collection wells that contained the same scented training solution.

In this stepwise training phase, we progressively moved the feeder in a straight line away from the colony entrance and across an open field in sequential steps (10–12 steps, with 10–15 m between steps) until reaching the penultimate location 5 m in front of and centered between what would ultimately be the final two experimental feeder locations, referred to as 'left' and 'right', as seen from the direction of the observation hive. At each step along the way, new, unmarked bees arriving at the feeder were marked, confirmed or removed as before. We allowed marked bees to visit at least three times before making the next step to ensure they had learned the feeder's current location. We kept the feeder at this penultimate location until 5–15 confirmed foragers had made at least three visits each, typically by mid- to late afternoon on Day −1. The accumulation of multiple visits at the final Day −1 feeder location provided a highly rewarding experience for these foragers, thus increasing the likelihood that they would return to the feeder the next day (Al Toufailia et al., 2013).

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The next morning (Day 0), we positioned two feeders containing the Day −1 solution side by side on a tripod at the end location from Day −1. We allowed trained foragers from Day −1 to self-assemble across the two feeders. A forager was considered 'committed' once she had visited one of the two feeders at least five times. When multiple (approximately 3–5) committed foragers were observed simultaneously collecting sucrose solution, we delicately relocated the feeders, along with the drinking bees, to the designated experimental feeder locations 5 m apart and equidistant from the colony: we placed one feeder on the 'left' tripod and the other on the 'right' tripod, each with a randomly assigned yellow or blue background color cue, which we alternated between right and left in each trial. The bees trained on Day −1 were then free to forage and to recruit to the feeders. These Day −1 training bees were not ultimately destined to be part of our experiment but rather served to recruit bees that would.

As newly recruited (Day 0) foragers arrived at either feeder, we marked them and confirmed their colony membership as before. The color of the plastic discs assigned to each Day 0 forager corresponded to the feeder they visited first, while also distinguishing them from the Day −1 training foragers. We recorded each visit made by the Day 0 foragers as a training visit. Once a consistent pattern of feeder visits and recruitment emerged among the Day 0 foragers, we steadily removed the Day −1 foragers from the experiment to prevent overcrowding at the feeder. As soon as 5–15 Day 0 foragers had made at least three training visits to only one feeder (either 'left' or 'right'), we began the experimental phase.

At the start of the experimental phase, we simultaneously removed both training phase feeders and replaced them with experimental feeders, each containing unscented 1 mol l^{-1} sucrose solution, either unaltered (control) or with 5 mg acid equivalent (a.e.) l^{-1} glyphosate [treatment; *N*-(phosphonomethyl) glycine, product no. 89432-100MG, TraceCERT®, Sigma-Aldrich Production GmbH, Buchs, Switzerland]. We selected this concentration of glyphosate for two reasons. First, it falls within the range of glyphosate residues found in bee-collected nectar between 3 and 7 days after the application of glyphosate according to maximum label specifications (Thompson et al., 2014, Fig. 1A); and second, it falls within the concentration range commonly used by other studies investigating the sublethal effects of glyphosate (e.g. Balbuena et al., 2015; Farina et al., 2019; Almasri et al., 2022), thus facilitating easier cross-comparison of results. We assigned glyphosate treatment and control solutions to the 'left' and 'right' feeders randomly with a coin toss, and thereafter alternated between trials. Researchers collecting feeder visitation data were unaware of treatment assignment between feeders.

For the next 3 h experimental phase, we allowed the marked bees to forage freely at their designated feeder. We recorded the bee identity and time of every feeder visit ('foraging frequency', see below). Any unmarked bee arriving at a feeder during the experimental phase was captured and removed. During these 3 h, we also filmed the observation hive (see below). At the end of the experimental phase, both feeders were simultaneously and promptly removed. Each experimental phase took place between 12:00 h and 17:00 h, as it generally took until at least midday for enough bees to be trained to allow us to begin the experiment.

To study the effect of glyphosate exposure on bees' persistency to a previously rewarding, now-unrewarding food source, on the following 2 days (Day 1 and Day 2), we re-erected feeder stations identical to those for the Day 0 experimental phase, but this time empty and unrewarding. We then recorded all visits in which a marked bee made physical contact with any part of the feeder itself ('persistency', see below). We did not consider it a visit if the bee contacted the platform but not the feeder. We monitored the feeders for the 8 h period between 08:00 h and 16:00 h on Day 1 and Day 2.

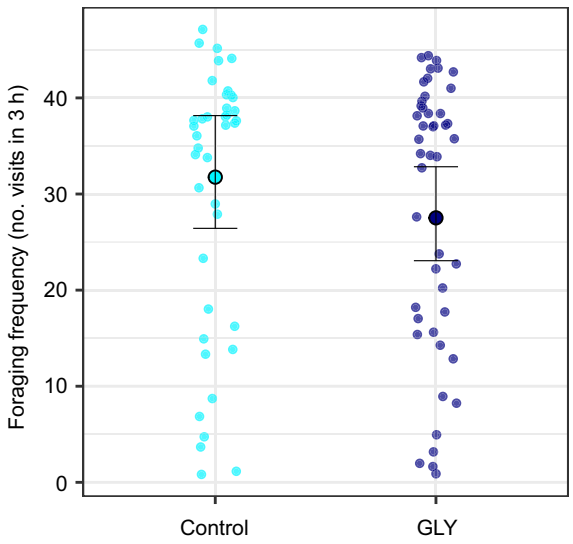

Fig. 1. Sublethal glyphosate exposure reduces honey bee foraging. Honey bee foragers collecting 1 mol l^{-1} sucrose solution containing glyphosate (GLY) at 5 mg a.e. l^{-1} (n=46) foraged 13.34% less compared with foragers collecting unaltered equimolar solution (n=40) from artificial feeders over the course of a 3 h experimental phase [Poisson GLMM; estimated marginal mean (95%CI): control: 31.76 (26.43 to 38.16), treatment: 27.52 (23.06 to 32.84); ratio: 1.15 (1.02 to 1.30); d.f.=81; Z=−2.29; N=40 control, N=46 glyphosate; P=0.022]. Estimated marginal means and 95% confidence intervals are shown over raw data points, where each point represents data for an individual bee.

In order to confirm that our experimental bees experienced the dosage of glyphosate as sublethal, we performed mortality censuses on the mornings of Day 1 and Day 2 to determine which marked bees were still alive in the colony. Any bee that could not be visually confirmed during a 1 h search from 06:45 h to 07:45 h, and that did not thereafter make a persistency feeder visit, was presumed dead. Any bee presumed dead via mortality census data was excluded from persistency analysis to avoid zero-inflation in our data.

On the afternoon of Day 3, we collected all the bees that participated in our behavioral experiment on dry ice and froze them for quantification of brain analytes (see below).

Video recording and monitoring of observation hives

We filmed each observation hive continuously during the experimental phase to record Day 0 bees' recruitment behaviors ('dance propensity', 'dance frequency' and 'waggle run repetitions' – see below). To do this, we set up two Canon Vixia HF R82 camcorders mounted on tripods and equipped with SanDisk Extreme SD cards recording at 30 frames s^{-1}, one on either side of the observation hive, with the lens of each positioned 1 m from the Plexiglas surface of the observation hive. We focused the camera on the 'dance floor', the portion of the bottom-most frame of comb where most waggle dance activity occurs (approximately 25×20 cm). After the completion of each experimental phase, we backed up all SD cards to a Google Team Drive (GTD) for analysis.

Behavioral response variables

Honey bees are sensitive to the profitability of a resource, which depends on the net benefit of its sucrose reward versus the energetic cost required to reach it on the wing, and they will tune their foraging

and waggle dance behavior accordingly (von Frisch, 1967; Seeley, 1995; Seeley, et al., 2000; Couvillon, 2012; Couvillon et al., 2015). In this experiment, the two feeders presented equal profitability in that they offered identical sucrose reward (1 mol l^{-1}) and identical energetic cost (equal flight distance from the colony). Therefore, any difference in measured behaviors between glyphosate treatment and control would indicate an effect of glyphosate.

'Foraging frequency', or the number of visits to a food source per unit time, is one such behavioral metric that correlates positively with a resource's profitability (von Frisch, 1967; Seeley, 1995; Seeley et al., 2000). Once treatment (with glyphosate) and control (without glyphosate) feeders were in place, we measured foraging frequency as the number of foraging trips that each experimental bee made to her 1 mol l^{-1} sucrose feeder for the 3 h duration of the experimental phase on Day 0. We counted visits for analysis according to a specific and consistent set of criteria. First, the marked bee must alight on the experimental feeder, extend her proboscis and collect the sucrose solution. Second, we only counted a visit for each bee if at least 3 min had elapsed following that bee's previous visit. We implemented this condition because a foraging bee might occasionally visit the feeder a second time after a short period of flight but before returning to the colony, and previous work has shown that even highly motivated foragers take at least 3 min to make the return trip to the colony, unload their collected sucrose solution and then return to the feeder (Couvillon et al., 2015) at a foraging distance similar to ours (145 m).

A bee may not advertise all food sources, however: there is a range of middling profitability in which a bee will continue to forage upon a resource but will not dance to recruit nestmates to it. The higher the profitability, the higher the likelihood a bee will dance at all for that resource, or the higher her 'dance propensity' (von Frisch, 1967; Seeley, 1995; Couvillon et al., 2015). We measured each bee's dance propensity as a binomial outcome reflecting whether she performed at least one waggle dance ('1', dancers) or foraged but did not dance ('0', non-dancers) during the experimental phase.

When a bee does dance to advertise a resource, she tunes her dance behavior according to her perception of its profitability in several measurable ways. For instance, bees will dance more frequently when foraging upon a highly profitable resource, and those dances tend to contain greater numbers of waggle runs, the repeating subunit of the waggle dance behavior that encodes the vector information (von Frisch, 1967; Seeley et al., 2000). We therefore monitored video data to quantify each dancer's 'dance frequency', defined here as the number of waggle dances performed during the 3 h experimental phase, as well as 'waggle run repetitions', defined as the mean number of waggle runs per dance for each experimental bee.

Finally, another behavior that a forager bee tunes according to her perception of a resource's profitability is the frequency with which she will reinvestigate a formerly rewarding food source after it has become unrewarding, called 'persistency'. Persistency is adaptive in natural settings because floral resources that have gone dry one afternoon may yet be re-rewarding within hours to days, as plants replenish their floral nectaries or open fresh blooms. The more profitable the resource, the more persistently a forager bee will reinvestigate it (Seeley et al., 1991; Seeley, 1995; Al Toufailia et al., 2013). Furthermore, it is known that the presence of pharmacologically active substances in nectar can modulate persistency (caffeine, persistency increased: Couvillon et al., 2015; a neonicotinoid, persistency decreased: Ohlinger et al., 2022b). We therefore measured bees' persistency to the experimental feeders for 2 days (Day 1 and 2) following the experimental phase.

Analyte selection rationale

We selected octopamine, tyramine and dopamine as our primary biogenic amines of interest in this study because of their roles in behaviors and physiologies relevant to honey bee foraging and recruitment. Octopamine is an important neurotransmitter, neuromodulator and neurohormone that is intricately involved in a diverse suite of insect physiological processes (reviewed in Bicker,1999; Farooqui, 2012) including those important for foraging and recruitment, our interest. For instance, octopamine can decrease olfactory response thresholds (Mercer and Menzel, 1982; Barron et al., 2002), improve olfactory memory formation and retrieval (Menzel et al., 1999), and increase propensity to reinvestigate previously rewarding food sources (persistency) (Linn et al., 2020). Tyramine is octopamine's biosynthetic precursor and a neurotransmitter in its own right (Blenau et al., 2000; Lange, 2009; Blenau and Baumann, 2016), and often (but not always) exerts opposing effects: honey bees treated with octopamine initiate flight more often, while bees treated with tyramine fly less (Fussnecker et al., 2006); octopamine stimulates precocious foraging, while tyramine suppresses it (Barron et al., 2002); and both octopamine and tyramine can increase sucrose sensitivity (Scheiner et al., 2002, 2017; Pankiw and Page, 2003). Dopamine also plays a role in the honey bee forager brain: foragers have higher dopamine levels than bees at work inside the colony (Wagener-Hulme et al., 1999; Taylor et al., 1992), and it can both decrease sucrose responsiveness (Scheiner et al., 2002) and increase dance-following behavior (Linn et al., 2020). We also opted to include it because, like octopamine, it is biosynthesized ultimately from tyramine as a precursor. Finally, we chose to include the amino acid tyrosine in our brain analyses, as it is the biosynthetic precursor for all three biogenic amines of interest. We did not expect to correlate tyrosine levels with any behavior, as it is not a neurotransmitter. Rather, we included it because we wanted to be able to examine relative levels of precursors and products along the biosynthetic pathway.

Brain dissection and quantification of analytes via ultra high performance liquid chromatography with tandem mass spectrometry

To investigate the effect of glyphosate on the brain content and relative levels of tyrosine, tyramine, dopamine and octopamine, we recaptured all available experimental bees on Day 3 after the behavioral portion of the experiment (Day 0–Day 2) was complete. Collecting the bees on Day 3 was a reasonable compromise that we thought, based on previous work from our lab (Ohlinger et al., 2022b; Couvillon et al., 2015), would allow enough time for us to complete the behavioral tests but not so long that any potential physiological effect of exposure from Day 0 would disappear. We captured the bees, both glyphosate treatment and control, by temporarily capping the colony entrance for approximately 10 min between 14:00 h and 17:00 h, then capturing all marked bees that accumulated into individual sterile Falcon tubes and immediately stored them over dry ice. Then we flash-froze the bees in liquid nitrogen and stored them at −80°C.

Brain dissection and analyte quantification occurred April–May 2023. We dissected each frozen brain, one at a time, using sterile dissection tools under a dissecting microscope and on chilled dissecting plates set on a bed of dry ice to prevent brains from thawing during dissection, which degrades analytes. Additionally, we rotated our use of dissecting tools, with one set chilling over dry ice while the other was in use, and swapping back in the re-chilled tools every 15–30 s. We dissected entire brains, including optic and antennal lobes, but excluding the subesophageal ganglia. After

dissection, we immediately transferred each brain to a sterile 150 µl microcentrifuge tube and returned it to storage at −80°C.

We performed individual chemical extractions in batches of six. For each brain, we added 100 µl of extraction solution containing all four isotopically labeled internal standards in methanol and 1% formic acid (v/v), and immediately sonicated the sample at 35,000 Hz for two 10-s bursts with a QSonica Q55A-110 Sonicator using a 2 mm probe (Genesee Scientific Corporation). After sonication, we re-sealed each microcentrifuge tube and stored it over dry ice until the batch of six was complete. We then centrifuged that batch of sonicates in a refrigerated centrifuge at 10,000 ***g*** for 20 min. If any particulates were still visible in suspension via visual inspection, we repeated the centrifugation step for those tubes, balancing the centrifuge with blanks. We transferred each supernatant into a brown glass microvial, which we stored at −80°C until all extractions were complete.

We analyzed the samples at the Virginia-Maryland College of Veterinary Medicine Analytical Chemistry Research Laboratory. Analyte concentrations in bee brain samples were determined by ultra high performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS). We obtained reference standards of dopamine hydrochloric (DA), racemic *p*-octopamine hydrochloride (OA), tyramine (TA), L-tyrosine sodium salt hydrate (TS), isotopically labeled dopamine-d4 hydrochloride (Dd4) and L-tyrosine-$^{13}C_9$,^{15}N (TSIS) from Cayman Chemical. We obtained isotopically labeled octopamine-$^{13}C_2$,^{15}N acetic acid (OIS) and tyramine-d4 hydrochloride (TAIS) from Toronto Research Chemicals (Vaughan, ON, Canada). Stock solutions of the dopamines, octopamines and tyramines were made up in methanol with 1% v/v formic acid (FA) to acidify and solubilize and 0.1% w/v ascorbic acid (AA) as an antioxidant to stabilize the analytes in solution. The tyrosines, with the most acidic pK_a, required 1% v/v hydrochloric acid (HCl)+0.1% AA in methanol for dissolution. We made up all stock solutions individually at a concentration of 1 mg ml^{-1} and then diluted each in 50/50/0.5% methanol/H_2O/FA to their final standard concentrations.

Sample extracts were subjected to chromatographic separation performed on a Waters H-Class UPLC system with an HSS T3 reverse phase column (Waters Acquity UPLC HSS T3, 100 mm length×2.1 mm i.d.×1.8 µm) and matching guard column (Waters Acquity UPLC HSS T3 VanGuard Pre-Column, 5 mm length×2.1 mm i.d.×1.8 µm) maintained at 40°C. We injected 5 µl of sample onto the column using a refrigerated autosampler maintained at 5°C. Mobile phase A consisted of 0.02% heptafluorobutyric acid (HFBA)+0.02% trifluoroacetic acid (TFA) in water, mobile phase B consisted of 0.025% HFBA+0.05% TFA in methanol. The mobile phase was delivered to the UPLC column at a flow rate of 0.4 ml min^{-1}. The gradient elution program is shown in Table 1.

To reduce mass spectrometer contamination, we used the divert valve to transfer the column effluent to the mass spectrometer from 1.0 to 2.00 min. From 0 to 0.99 min and 2.01 to 4.25 min, all the column effluent was transferred to waste. Simultaneous and efficient separation of all analytes of interest was achieved using this ion pairing methodology with octopamine, dopamine, tyrosine and tyramine eluting at approximately 1.13, 1.32, 1.50 and 1.54 min, respectively. The UPLC column effluent was pumped directly without any split into a triple-quadrupole mass spectrometer (Waters Xevo TQD) equipped with a Zspray ionization source which was operated in positive-ion electrospray mode (ESI+) using multiple reaction monitoring (MRM). The parent and product ion transitions for the compounds of interest are shown in Table 2.

Table 1. UPLC gradient method used for the ion-pairing analysis

Time (min)	% A	% B
0.00	80	20
1.00	0	100
2.50	0	100
2.51	80	20
4.25	80	20

UPLC (ultra high-performance liquid chromatography) solution composition: A – 0.02% HFBA+0.02% TFA in H_2O; B – 0.025% HFBA+0.05% TFA in methanol. HFBA, heptafluorobutyric acid; TFA, trifluoroacetic acid.

We used commercial software (MassLynx) to analyze the data. Tuning was performed on each analyte by direct infusion of standard solution (5 ng µl^{-1}) at a rate of 20 µl min^{-1}. Mass spectrometer parameters used for the detection of the analytes of interest are shown in Table 3.

We then made up a nine-point calibration curve in solution containing the same isotopically labeled internal standard as the extracts with a range of approximately 1–1000 ng analytes ml^{-1} solution for all analytes. Using these standards, calibration curves were constructed for each of the individual analytes using the MassLynx software to determine analyte concentration in samples based on the sample/IS ratio.

Amino acid and biogenic amine response variables

We quantified brain levels of the biogenic amines octopamine (OA), dopamine (DA), tyramine (TA) and their amino acid precursor tyrosine (Tyr) in terms of total brain content (ng analyte/brain homogenate). We analyzed individual brains for the experimental bees that could be recaptured on Day 3 and which were also eligible for behavioral analysis (i.e. only visited either the glyphosate treatment or control feeder during the experimental phase, see below, n=25).

Statistical analysis

Foraging and waggle dance response analysis

We performed all statistical analyses in R 4.2.3 (http://www.R-project.org/). We present in graphical form both the original data as well as data summarized by treatment and trial phase (experimental phase and persistency phase). In these summaries, we present both means (and 95% confidence intervals, CIs) and medians (and upper and lower quartiles). We used generalized linear mixed-effect models (GLMMs) to infer any differences between treatment groups, and report point estimates for any treatment differences, including two-sided 95% CIs and associated P-values. We selected for analysis only those bees that foraged exclusively at one or the other feeder (i.e. collected only glyphosate treatment or control sucrose solution) for the entire duration of the 3 h experimental phase (N=86). In other words, we excluded all bees that visited both feeders at any point during the experimental phase from this analysis (N=18), as those bees experienced both glyphosate treatment and control conditions.

Unfortunately, when we began our video analysis of recruitment behavior, we found that the videos for hives 2 and 3 were of surprisingly poor quality, and this precluded us from reading bee tag numbers. We were only able to analyze the video data from the first experimental colony, and we therefore decided to present dance data as secondary outcomes given the lower statistical power available for the analysis of these waggle dance response variables.

We analyzed count data for foraging frequency during the experimental phase and for persistency visits during the persistency phase using Poisson GLMMs. We modeled these data using the

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Table 2. Multi-reaction monitoring transitions and specific mass spectrometry tuning parameters for the quantification of the analytes of interest

Analyte	Parent ion (amu)	Product ion (amu)	Cone energy (V)	Collision energy (eV)	Quant/qual transition
Dopamine	137.0 $[M+H]^+$	91.0	40	18	Quantifier
	137.0 $[M+H]^+$	118.9	40	12	Qualifier
Dopamine-d4 (IS)	141.1 $[M+H]^+$	94.8	46	18	Quantifier
Octopamine	136.0 $[M+H]^+$	91.0	32	16	Quantifier
	136.0 $[M+H]^+$	118.9	32	12	Qualifier
Octopamine-$^{13}C_2$,^{15}N (IS)	139.1 $[M+H]^+$	92.0	30	16	Quantifier
Tyramine	121.0 $[M+H]^+$	77	40	20	Quantifier
	121.0 $[M+H]^+$	103	40	15	Qualifier
Tyramine-d4 (IS)	125.1 $[M+H]^+$	106.3	40	16	Quantifier
Tyrosine	182.1 $[M+H]^+$	136.0	22	16	Quantifier
	182.1 $[M+H]^+$	165.1	22	10	Qualifier
Tyrosine-$^{13}C_9$,^{15}N (IS)	192.1 $[M+H]^+$	145.1	24	12	Quantifier

glmer() function from the lme4 package (Bates et al., 2015). For dance frequency, we used instead the Quasi-Poisson model family, as we detected overdispersion in those data, without an offset as each experimental phase was standardized at 3 h for each trial. We modeled waggle run repetitions using the Gamma model family, as we detected both overdispersion and a right-tailed skew in those data. Finally, we modeled dance propensity as a binomial response variable, with a binomial GLMM [function glmer(), package lme4; Bates et al., 2015]. For all models, we used the emmeans R package to calculate treatment group contrasts and extract 95% CIs (https://CRAN.R-project.org/package=emmeans). For each, we modeled the response variable with respect to categorical treatment group, feeder color cue (blue or yellow) and feeder position (left or right, from the outbound forager bee's view) as fixed effects, and included hive as a random effect to control for possible inter-colony differences (Harris and Woodring, 1992), and generated a set of models for each outcome variable using backwards elimination of non-significant terms. We then selected the final model for each outcome according to the second-order Akaike information criterion (AICc; Portet, 2020), the use of which is indicated when the number of modeled terms (k) exceeds the number of observations divided by 40 (n/40), as is the case with our dataset. To do this, we first used the AICc() function in the MuMIn package (https://CRAN.R-project.org/package=MuMIn) to compare identical models with and without hive as a random effect, selecting the model with the lowest AICc value as the most parsimonious model, and proceeding from there with backwards elimination of non-significant terms.

Amino acid and biogenic amine analysis

We analyzed log-transformed brain content of each of our four analytes of interest as continuous numeric data using Gaussian GLMMs [function glmer(), package lme4], specifying the following as fixed effects: treatment group, feeder color cue (blue or yellow), feeder position (left or right from the outbound forager bee's view), total number of feeder visits made during the experimental phase and finally an interaction term combining treatment group with number of feeder visits. We included this interaction term to account for the incremental accrual of exposure to glyphosate on a per-feeder visit basis; that is, bees in the glyphosate treatment group accrued an additional unit of exposure each time they collected sucrose solution containing glyphosate. We therefore included this interaction term to account for this variability in exposure arising from unequal numbers of feeder visits between bees within each treatment group. We obtained regions of significance for interactions by supplying the model object to the sig_regions() function from the package reghelper (https://CRAN.R-project.org/package=reghelper).

We also conducted exploratory analysis of the log-transformed brain analyte data using the pairs() and pairs.panels() functions in ggplot2, which allowed us to visualize and identify correlative relationships between analyte levels between glyphosate treatment and control bees. Finally, when these exploratory analyses indicated a significant correlation between analytes, we then performed *post hoc* analysis of correlations between log-transformed analyte levels by treatment group using the cor.test() function in R to quantify Pearson correlations, 95% CIs and P-values. In a scenario where there may be large variability in baseline values of analytes or where sample size is limited, examining relationships between levels of precursors and products rather than raw amounts can be more informative of biosynthetic pathway activation (Lim et al., 2016) and physiological outcomes (Latshaw et al., 2023).

Table 3. Mass spectrometer tuning parameters for the detection of the analytes of interest

Parameter	Value
Capillary (kV)	0.50
Cone (V)	40
RF (V)	2.50
Extractor (V)	3.00
Source temperature (°C)	150
Desolvation temperature (°C)	600
Cone gas flow (l h^{-1})	10
Desolvation gas flow (l h^{-1})	750

RF, radio frequency.

Confirming glyphosate exposure as sublethal

To test whether our glyphosate exposure regimen was indeed sublethal, we implemented a binomial GLMM to test for an effect of treatment group on a bee's survival to Day 2, including hive as a random effect.

RESULTS

Glyphosate exposure confirmed as sublethal

Across the three trials, a total of 86 bees foraged at an experimental feeder and remained site-specific to that feeder for the duration of the 3 h experimental phase and were therefore eligible for analysis (control: 40 bees; treatment: 46 bees). The probability of survival to the morning of Day 2 predicted by our mixed model approach did not differ significantly between control bees and glyphosate treatment bees [binomial GLMM; estimated marginal mean (95% CI): control: 0.84 (0.65 to 0.94), treatment: 0.73 (0.53 to 0.86); odds ratio: 1.94 (0.63 to 5.98); d.f.=83; Z=−1.15; P=0.251]. In other words, our glyphosate exposure was sublethal.

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Glyphosate decreased foraging frequency

Forager honey bees collecting glyphosate-containing sucrose solution (5 mg a.e. l^{-1} glyphosate) made 13.34% fewer feeder visits than bees collecting unaltered sucrose solution (Fig. 1). This was our primary outcome.

Feeder color also had a significant effect on foraging: bees collecting from a feeder with a yellow color cue foraged 20.64% more than bees collecting from a feeder with a blue color cue [Poisson GLMM; estimated marginal mean (95% CI): blue feeder: 26.91 (22.61–32.03), yellow feeder: 32.47 (26.99–39.06); ratio: 0.82 (0.74 to 0.93); Z=3.10, d.f.=81; Z=3.10; N=59 blue, N=27 yellow; P=0.002]. The model selected as most parsimonious, with the lowest relative AICc via the methods described above, also retained hive as a random effect, as well as feeder position as a non-significant fixed effect.

Glyphosate did not affect recruitment behaviors or persistency

Dance propensity

Of the 26 bees belonging to the first experimental replicate that was suitable for video analysis, 16 performed at least one waggle dance during the experimental phase (control: 6 dancers, 6 non-dancers; treatment: 10 dancers, 4 non-dancers).

The predicted probability of a bee performing a waggle dance, the dance propensity, did not differ between control and glyphosate treatment bees [binomial GLMM; estimated marginal probability (95% CI); control: 0.50 (0.19 to 0.80); treatment: 0.65 (0.30 to 0.88); odds ratio: 0.54 (0.08 to 3.54); Z=0.65; d.f.=23; N=26; P=0.519; data not shown].

Dance frequency

For the bees that did perform at least one dance, we first investigated their dance frequency qualitatively [geometric mean (95% CI)]: control bees danced 5.32 (1.75 to 16.20) times during the experimental phase and glyphosate treatment bees danced 8.66 (5.37 to 14.00) times during the same period. The median number of dances was 4 for control bees and 7.5 for glyphosate treatment bees.

However, when compared via our mixed modeling approach, the number of dances performed did not differ between control and glyphosate treatment bees (Fig. 2A).

Waggle run repetitions

We then examined the mean number of waggle run repetitions performed by each dancer [geometric mean (95% CI)] by treatment group: control bees' dances contained 14.90 (7.49 to 29.70) waggle runs and glyphosate treatment bees' dances contained 16.20 (10.90 to 24.20) waggle runs. The median number of was 14.4 for control bees and 15.9 for glyphosate treatment bees.

Among bees that danced during the experimental phase, the predicted mean number of waggle run repetitions per dance did not differ between control and glyphosate treatment bees (Fig. 2B).

Persistency

We performed an initial qualitative examination of persistency visits using arithmetic means and 95% CIs for persistency data, as some experimental bees were confirmed to be alive in the observation colony but made no persistency visits, and geometric means are inappropriate for data including values of zero.

On Day 1, 72 of our 86 analysis-eligible Day 0 bees were still alive in the colony, and 66 of these (92%) made at least one persistency visit. Control bees made 4.26 (3.03 to 5.50) persistency visits and glyphosate treatment bees made 5.55 (4.39 to 6.71) persistency visits. The median number of Day 1 persistency visits was 3.5 for control bees and 5 for glyphosate treatment bees. Treatment did not significantly affect Day 1 persistency (Fig. 2C). However, the color cue that bees experienced on Day 0 did affect their Day 1 persistency: bees that had visited a yellow feeder on Day 0 made 30.9% more persistency visits on Day 1 than their counterparts who had visited a blue feeder [Poisson GLMM; estimated marginal mean (95% CI): blue: 4.47 (3.91 to 5.12); yellow: 5.85 (4.80 to 7.13); ratio: 0.76 (0.59 to 0.98); Z=2.10; d.f.=69; N=50 blue, N=22 yellow; P=0.035].

On Day 2, 66 of our 86 analysis-eligible Day 0 bees were still alive in the colony, and 31 of these (47%) made at least one

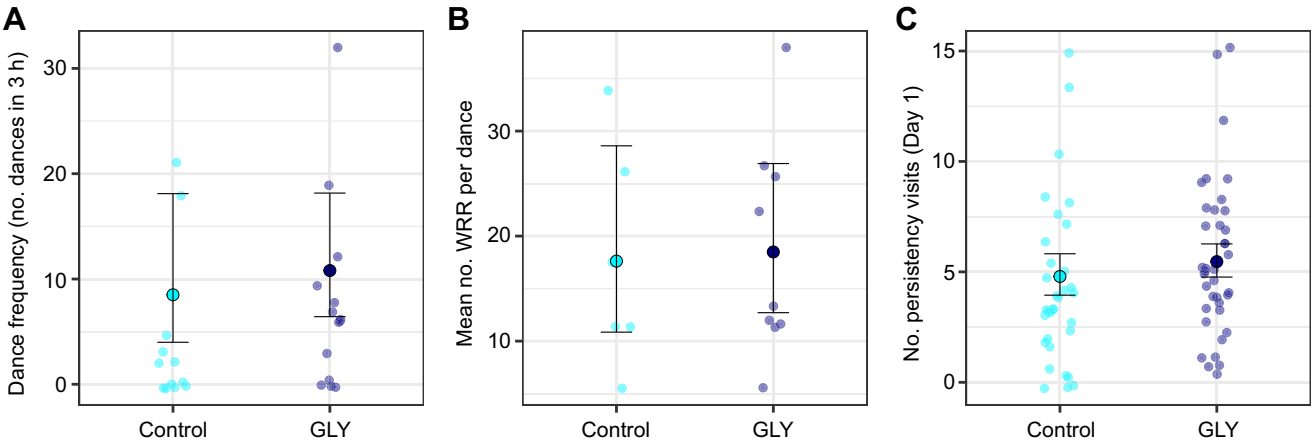

Fig. 2. Sublethal glyphosate exposure did not affect certain honey bee foraging and recruitment behaviors. Exposure to glyphosate did not affect (A) dance frequency, (B) waggle run repetitions (WRR) or (C) persistency (all not significant) in honey bees trained to collect sucrose solution containing either glyphosate at 5 mg a.e. l^{-1} or unaltered sucrose solution (control) from artificial feeders over a 3 h experimental phase. Estimated marginal means and 95% CIs over raw data points for (A) dance frequency [Quasi-Poisson GLM; estimated marginal mean (95% CI); control: 8.50 (3.99 to 18.11); treatment: 10.80 (6.42 to 18.16); ratio: 0.79 (0.31 to 1.98); T=0.51; d.f.=14; N=6 control, N=10 glyphosate; P=0.617] and (B) mean waggle run repetitions per dance [Gamma GLM; estimated marginal mean (95% CI); control: 17.62 (10.85 to 28.59); treatment: 18.49 (12.71 to 26.91); ratio: 0.95 (0.52 to 1.76); T=0.17; d.f.=14; N=6 control, N=10 glyphosate; P=0.919] and (C) number of Day 1 persistency visits [Poisson GLMM; estimated marginal mean (95% CI); control: 4.79 (3.95 to 5.82); treatment: 5.46 (4.77 to 6.27); ratio: 0.88 (0.68 to 1.12); Z=01.04; d.f.=69; N=34 control, N=38 glyphosate, P=0.30].

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Table 4. Summary of outputs of selected models for biogenic amine data

Analyte	Group	Median (range) (ng per brain)	emmean (95% CI)	Predictor, *P*-value
Tyrosine	Glyphosate	103.00 (91.30–132.00)	4.67 (4.52–4.83)	Treatment, 0.694
	Control	114.00 (98.60–129.00)	4.71 (4.55–4.88)	
Tyramine	Glyphosate	0.28 (0.24–0.33)	NA	Treatment×foraging frequency, **0.004**
	Control	0.27 (0.24–0.33)	NA	
Octopamine	Glyphosate	2.45 (2.22–2.89)	2.50 (2.23–2.81)	Treatment, 0.635
	Control	2.52 (2.28–2.96)	2.57 (2.28–2.91)	
Dopamine	Glyphosate	2.43 (2.10–2.81)	2.54 (2.16–2.98)	Treatment, 0.758
	Control	2.55 (2.13–2.70)	2.42 (1.94–3.03)	

There was no effect of glyphosate exposure on brain content of tyrosine, octopamine or dopamine (ng per brain homogenate), but tyramine level was modulated by the interaction between treatment group and foraging frequency. Estimated marginal means (emmeans) are excluded for tyramine, as they may be misleading in the presence of a significant interaction. CI, confidence interval. Bold indicates significance.

persistency visit. Control bees made 0.85 (0.39 to 1.31) persistency visits, and glyphosate treatment bees made 1.09 (0.54 to 1.65) persistency visits. The median number of Day 2 persistency visits was 0 for both glyphosate treatment and control bees. For Day 2 persistency, treatment was the least significant predictor in the full model, and we therefore removed it from the model first according to our backwards-elimination method. However, the most parsimonious model indicated a significant effect of the Day 0 feeder color cue on Day 2 persistency.

Bees that had foraged at a feeder with a yellow color cue on Day 0 made 92.4% more persistency visits on Day 2 than did bees that had foraged at a feeder with a blue color cue [Poisson GLMM; estimated marginal mean (95% CI); blue: 0.77 (0.55 to 1.06); yellow: 1.47 (1.02 to 2.13); ratio: 0.52 (0.32 to 0.85); Z=2.60; d.f.=64; N=47 blue, N=19 yellow; P=0.009; data not shown].

Interaction of glyphosate exposure and foraging frequency modulated brain content of tyramine, but tyrosine, octopamine and dopamine were unaffected

In total, we quantified brain content of tyrosine, tyramine, octopamine and dopamine in the brains of 25 recaptured experimental bees with 12 bee brains for control and 13 for treatment. We present descriptive, untransformed results [medians and minimum–maximum range (ng analyte per brain homogenate)], estimated marginal means and P-value outputs of mixed effect models using log-transformed data for each analyte in Table 4.

Our mixed model approach indicated a significant effect of the interaction between the number of feeder visits and treatment group (glyphosate versus control) on brain content of tyramine (Fig. 3), with the significance of this effect occurring between 25.13 and 45.42 feeder visits [given by the sig_regions() function in the reghelper R package, as described above]. The visualization of the data indicates that it is likely a crossover interaction. In these circumstances, because the two-way interaction is driving the significance of the effect, interpreting the fixed effects (glyphosate treatment or foraging frequency) alone is not appropriate (Schielzeth, 2010).

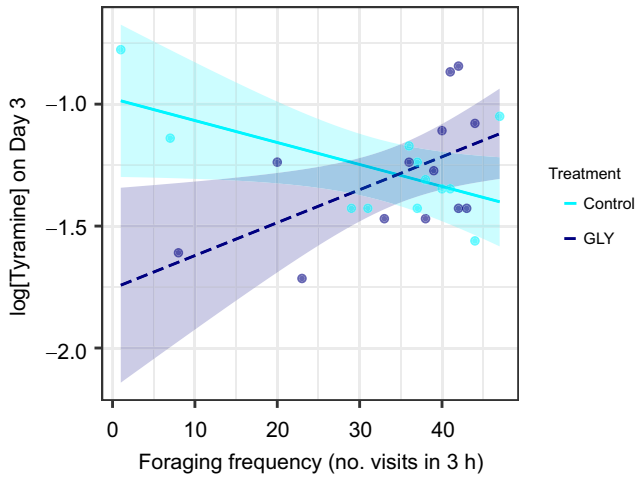

Fig. 3. Sublethal exposure to glyphosate modulated brain content of tyramine after a threshold exposure. There was a significant interaction between treatment (glyphosate or control) and foraging frequency on the brain content of tyramine (ng per brain), with the region of significance ranging from 25.13 to 45.42 foraging visits [Gaussian GLMM; T=3.21; d.f.=21; N=12 control, N=13 glyphosate; P=0.004]. Tyramine levels were measured for bees that were recaptured and flash frozen approximately 72 h after the experimental phase concluded (afternoon of Day 3, following a 3 h period of exposure on the afternoon of Day 0).

Octopamine significantly correlated with tyrosine and tyramine in glyphosate treatment bees

We found a significant correlation between log-transformed brain content of octopamine (OA) and its two precursors, tyrosine (Tyr) and tyramine (TA), in bees exposed to glyphosate (N=13) but not in control bees (N=12) (Fig. 4).

DISCUSSION

Here, we report that honey bee foragers experiencing a field-realistic, sublethal exposure to glyphosate foraged significantly less (−13.34%) compared with control bees (Fig. 1). Glyphosate did not affect recruitment (dance propensity, dance frequency and waggle run repetitions) in our trial 1 bees, nor was bees' persistency altered by the treatment (Fig. 2). However, our data also indicate that exposure to glyphosate can affect the neurochemical state of the forager bee brain. We found that the brain content of tyramine was modulated by an interaction between glyphosate treatment and foraging frequency, such that tyramine was higher in bees that foraged on glyphosate sucrose solution at least 25 times during the experimental phase (Fig. 3). Lastly, we found that octopamine levels correlate with the levels of its precursors tyrosine and tyramine (Fig. 4), but only in the bees exposed to glyphosate. Overall, these data indicate that the most widely used herbicide worldwide generates subtle but significant effects on the behavior and physiology of exposed foraging honey bees.

Foraging frequency reduction

Bees foraging on sucrose solution containing glyphosate at 5 mg a.e. l^{-1} decreased their foraging by 13.34% compared with control bees. Interestingly, this result is in contrast with previous work that showed no effect on foraging frequency under glyphosate exposure conditions (Herbert et al., 2014). However, the foraging experiment

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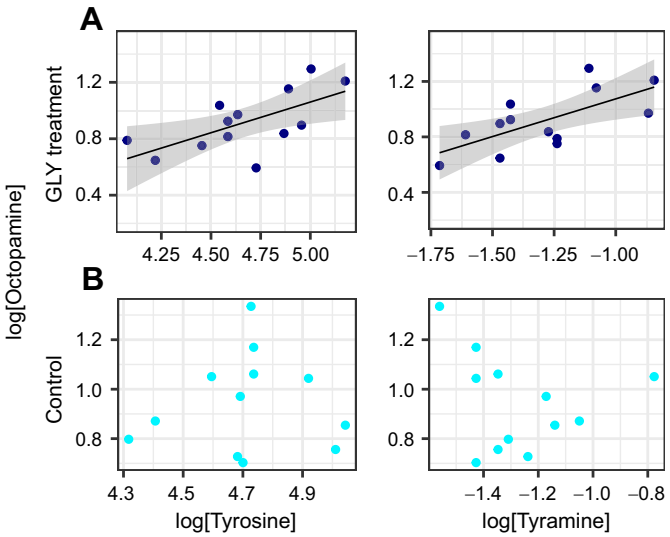

Fig. 4. Sublethal glyphosate exposure induced positive correlations between octopamine and both tyramine and tyrosine. Forager honey bees trained to artificial feeders collected 1 mol l^{-1} sucrose solution either containing 5 mg a.e. l^{-1} glyphosate (N=13) or unaltered sucrose solution (control) (N=12). Each point represents the brain of a bee that experienced the 3 h experimental phase (Day 0) and was collected at the end of the persistency phase (Day 3). In glyphosate-exposed bees (A), brain content of octopamine (ng per brain) significantly correlated with that of tyrosine (ng per brain) (left; Pearson's product-moment correlation; T=2.77; R=0.68; d.f.=12; P=0.018) and with that of tyramine (ng per brain) (right; Pearson's product-moment correlation; T=0.18, R=0.06, d.f.=12; P=0.864). In control bees (B), no correlation was found with either tyrosine (Pearson's product-moment correlation; T=0.18; R=0.06; d.f.=11; P=0.864) or tyramine (Pearson's product-moment correlation; T=−0.68; R=−0.21; d.f.=11; P=0.512).

in that study used a lower glyphosate concentration than we did (2.5 mg a.e. l^{-1}), with double the molarity of sucrose solution (2 mol l^{-1}). Sucrose solution at 2 mol l^{-1} offers a highly rewarding foraging experience, which is why we used this concentration for training bees when we wanted to maximize their commitment to that particular feeder. However, such a highly concentrated sucrose reward may mask any effect of treatment if the effect is not sufficient to overcome such a strong reward. This is why we used 1 mol l^{-1} during the experimental phase. Additionally, the study by Herbert et al. (2014) examined a bee's foraging cycle time for three foraging trips before and after the addition of glyphosate, rather than studying separate side-by-side treatment groups, as we did. As foraging and recruitment scale positively with a bee's experience at a rewarding food source (Seeley, 1995; Couvillon et al., 2015), any effect of treatment may have additionally been impacted by the order effect (with all glyphosate visits happening after all control visits, concurrent with the foragers having more experience at the feeder).

Why did we not find differences in the other behavioral metrics?

In our study, glyphosate did not affect recruitment (waggle dance propensity, waggle dance frequency, waggle run frequency) or persistency. This was surprising because the foraging and recruitment behaviors in these studies typically scale positively together for a given food source, according to the bee's perception of its profitability. In other words, the higher her assessment of a resource's profitability, (1) the more frequently she will visit it to collect food, (2) the more likely she is to dance to advertise it to nestmates, (3) the more frequently she will perform independent bouts of dances, (4) the greater the number of waggle runs she will perform per dance (von Frisch, 1967; Seeley, 1995; Couvillon et al., 2015) and, finally, (5) the more persistently she will revisit that food source after it has become unrewarding (Al Toufailia et al., 2013). In our study, however, glyphosate elicited a reduction only in foraging, while the other behaviors remained unaffected.

This apparent uncoupling of behaviors that are expected to increase or decrease in tandem may be due to any effect of treatment being subtle enough that our statistical power was insufficient to capture any difference. In the case of our analyses of waggle dance behaviors, this may well be the case, as only a subset of dance data was suitable for analysis because of insufficient video quality. It is also worth noting that any effects on persistency, data that were fortunately not affected by the quality issues impacting our dance analysis, might be more difficult to detect compared with effects on foraging frequency, as we would expect the range distribution of persistency visit counts to be smaller. A forager bee working a highly profitable resource may visit as frequently as she can, limited only by her physiological constraints, the distance of the resource and any nectar unloading delays. So long as her previous trip was sufficiently rewarding, she has incentive to return. In contrast, a forager bee making a visit to a previously rewarding but now unrewarding food source would be ill-served by continuing to visit at such a high frequency, as the likelihood of resource replenishment would not outweigh the cost of such frequent repeated visitation. In other words, the maximum visit frequency that is adaptive is higher for rewarding food sources and lower for spent food sources that may or may not become rewarding again. Thus, the range distribution of persistency visits would be expected to be smaller than that of rewarded visits, and any change in persistency may therefore be more difficult to detect. Nevertheless, our results are consistent with a scenario in which a honey bee foraging workforce collecting glyphosate-laced nectar in the field might reduce its foraging frequency while maintaining recruitment. Such a scenario could reduce the total nectar inflow while still maintaining a steady inflow of glyphosate residue to the colony, which could ultimately produce deleterious health outcomes at the colony level (Farina et al., 2019). Future studies should further investigate recruitment impacts under glyphosate exposure.

Effect of glyphosate on octopamine, tyramine and tyrosine in the brain

We found that bees exposed to sublethal glyphosate experienced alterations in both total brain content and relative levels of tyrosine, tyramine and octopamine. First, we found that tyramine levels in bee brains were related to an interaction effect between treatment group (glyphosate or control) and foraging frequency, and that the region of significance for this effect was from 25.13 to 45.42 feeder visits. (As feeder visits cannot be fractional, we therefore interpret the biologically relevant region of significance of this effect as 26–45 visits, or all the integer values contained in that range.) In other words, once glyphosate treatment bees accrued at least 26 increments of exposure to glyphosate during the experimental phase, the amount of tyramine in the brain increased more per additional feeder visit than did that of control bees.

The tyramine receptor AmTYR1 is expressed in multiple regions of the honey bee brain (Mustard et al., 2005; Sinakevitch et al., 2017; Thamm et al., 2017), and the gene that encodes this receptor exerts a pleiotropic suite of effects on behaviors and physiologies related to foraging and recruitment (sucrose sensitivity: Pankiw and Page, 2003; Thamm et al., 2017; Scheiner et al., 2017; proclivity to forage nectar or pollen: Page et al., 2000; Arenas et al., 2021; division of labor in foraging: Scheiner et al., 2014; Cook et al., 2019; latent inhibition: Cook et al., 2019; Latshaw et al., 2023).

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Importantly, activation of the AmTYR1 receptor by tyramine results in a reduction in levels of 3′-5′-cyclic adenosine monophosphate (cAMP) in a dose-dependent manner (Blenau et al., 2000). Treatment with tyramine can also produce behavioral changes that align with a reduction in foraging: worker bees injected with tyramine spend less time flying (Fussnecker et al., 2006) and are less apt to initiate precocious foraging (Schulz and Robinson, 2001). Altogether, our observed increase in tyramine in glyphosate treatment bees at higher increments of exposure therefore seems consistent with our observed reduction in foraging frequency.

It is important to note that this interaction between treatment group and foraging frequency could be interpreted in the other direction: a positive relationship between brain content of tyramine and higher foraging frequency values could have also emerged in a scenario where individual bees that naturally have higher tyramine levels (e.g. scout bees have higher tyramine than recruit bees; see Cook et al., 2019) might tend to forage more frequently. However, this interpretation is inconsistent with the documented functions and effects of tyramine in bees as an overall suppressor of foraging behaviors (Scheiner et al., 2002; Pankiw and Page, 2003; Schützler et al., 2019; Latshaw et al., 2023). It is also unlikely that greater foraging frequency alone contributed to higher tyramine levels, as tyramine has been shown to be unrelated to cumulative foraging experience (Peng et al., 2021; Latshaw et al., 2023).

Our experimental bees that were exposed to sublethal glyphosate also experienced alterations in the relative brain content of octopamine with respect to both tyramine and tyrosine. Specifically, glyphosate exposure induced a positive correlative relationship between brain levels of octopamine and tyramine, as well as between octopamine and tyrosine, 3 days following the 3 h sublethal exposure. We found no such correlations between any of our studied analytes in control bees (Fig. 3). The observed correlations between octopamine levels with those of both tyramine and tyrosine in glyphosate treatment bees should be interpreted in the context of their biosynthetic relationship. Tyrosine, the precursor to all three biogenic amines, is converted to tyramine by tyrosine decarboxylase (Karlson and Herrlich, 1965); tyramine is then acted upon by tyramine-beta-hydroxylase and converted to octopamine (Roeder, 2005). Ratios of precursor to product are often used as metabolomic biomarkers that may indicate relative levels of biosynthetic pathway activation (Schröcksnadel et al., 2006; Torres et al., 2007; Gupta et al., 2012; Lim et al., 2016). In addition, relative levels between constituents of this biosynthetic pathway may offer more insight than total brain levels, especially when precursor concentrations vary widely between individuals, as was the case for tyrosine brain content in our bees (Table 4). Precursor to product ratios can also be related to the experience of an individual (e.g. tyramine to octopamine; Kononenko et al., 2009). The positive correlations we found between octopamine and its precursors tyramine and tyrosine in glyphosate-exposed bees therefore suggest that sublethal glyphosate exposure may affect the neurochemical balance of these amines in the context of their synthesis. Although we found no significant differences in tyrosine levels (Table 4), the observed correlations between levels of co-metabolites tyramine and octopamine in glyphosate treatment bees (Fig. 4) would be consistent with a scenario where their precursor tyrosine becomes more limiting with glyphosate exposure. The idea that available tyrosine may limit tyramine and octopamine is also supported by evidence that dietary supplementation with tyrosine can increase brain levels of tyramine and octopamine (Matsuyama et al., 2015). In our study, it is possible that total bioavailable tyrosine might have been limited, even if such limitation was not detectable in brain tissue (Table 4).

A potential explanatory mechanism

Although our study did not directly investigate the mechanism that might be behind the effects of glyphosate, we nonetheless wondered, based on our results, whether the sublethal effects of glyphosate on the neurochemical state of the bee brain might arise indirectly via impacts on the gut microbiome. Although honey bees themselves do not endogenously possess the molecular target of glyphosate, namely EPSPS within the shikimate pathway, some members of the gut microbiota do. Honey bees possess a relatively simple and conserved microbiome (Martinson et al., 2011; Moran et al., 2012) organized into five core lineages clustering within the genera *Bifidobacterium*, *Bombilactobacillus* (previously *Lactobacillus* Firm-4; Zheng et al., 2020), *Lactobacillus* (previously *Lactobacillus* Firm-5; Zheng et al., 2020), *Snodgrassella* and *Gilliamella*, with additional non-core bacteria of the genera *Bartonella*, *Commensalibacter* and *Frischella* commonly present (Kwong et al., 2017). Previous work has demonstrated that the bee gut microbial community is susceptible to perturbations by glyphosate. For example, bees fed glyphosate at concentrations ranging from 0.01 to 0.1 mmol l^{-1} not only show a reduced total abundance of beneficial bacteria (Motta et al., 2018; Motta and Moran, 2020) but also demonstrate impacts to microbial biodiversity via changes in the relative abundance of some bacterial groups (Motta et al., 2018; Motta and Moran, 2020; Blot et al., 2019; Castelli et al., 2021). Notably, most *Snodgrassella alvi* strains express a susceptible class I EPSPS (Motta et al., 2018), and usually drop in relative abundance in the presence of glyphosate, while other groups increase, though there is some between-strain variation (Motta et al., 2018).

Under normal conditions, *S. alvi* forms a biofilm on the chitin-layered wall of the bee ileum and rectum (Callegari et al., 2021; Motta and Moran, 2024), and enriches the lumen with amino acids, notably tyrosine and other products of the shikimate pathway (Zheng et al., 2017). While it is not confirmed whether microbe-produced tyrosine specifically is bioavailable to the bee host, microbial provisioning of an insect host with tyrosine is documented in other insect taxa (e.g. beetles; Anbutsu et al., 2017), and the gut microbiome is known to donate other amino acids to bee nutrition and physiology (including tryptophan, another product of the shikimate pathway) (Motta and Moran, 2024). In a glyphosate exposure scenario in which the shikimate pathway is inhibited in the gut microbiome, microbial production of these amino acids, and any contribution thereof to the host physiology, would likewise be expected to decrease. A decrease in available tyrosine would be consistent with our results, where a more limited precursor might result in significant correlations with levels of its downstream metabolites, as we found here.

Possible evidence for a downstream limitation of tyrosine resulting from glyphosate exposure also arises from studies of glyphosate's impact on insect melanization, where tyrosine is also a biosynthetic precursor of melanin (Rzepka et al., 2016), which serves a key role in immune function (Nappi and Vass, 1993; Zhao et al., 1995; Carton and Nappi, 1997; Nappi and Ottaviani, 2000; Dubovskiy et al., 2016). Glyphosate exposure inhibits melanization and increases susceptibility to infection in two non-Hymenopteran taxa (Smith et al., 2021). It would be informative to investigate the effect of glyphosate on melanization in honey bees as well to further explore whether inhibition of the shikimate pathway in the bee gut microbiome could reduce tyrosine availability in such a way that it becomes limiting in its various functions within the bee host physiology.

Conclusions and future directions

We have shown here that sublethal glyphosate exposure can reduce honey bee foraging frequency concurrent with exposure, and that it

affects the neurochemical balance of octopamine, tyramine and tyrosine in the bee brain 3 days after exposure. The behavioral and molecular portions of the current study are closely linked in that we studied the same bees in both; however, our experimental design did not allow for a Day 0 biogenic amine analysis group, or for a Day 3 behavioral analysis group. This is a key limitation of our study. Future work is therefore needed to clarify any effect on total levels and correlations between brain content of these neurotransmitters in the time window between the end of exposure and Day 3, as well as to discover how long post-exposure the effects we found may persist, for both the biogenic amines and foraging frequency.

Intriguingly, we now see an emerging picture of an overlap between the lists of behaviors and physiologies that (1) underpin bee foraging, (2) are disrupted by glyphosate exposure and (3) are modulated by neurotransmitters biosynthesized from Tyr, an amino acid produced in the gut microbiome and plausibly donated to the bee host by the very pathway that glyphosate inhibits. We therefore suggest that the gut microbiota–brain axis in insects (Cryan and Dinan, 2012; Liberti and Engel, 2020; Liberti et al., 2022; Motta and Moran, 2024) may be an important possible route of pesticide exposure in insect pollinators and warrants further study. Clarifying the mechanism of action of glyphosate in insect systems will ultimately better equip us to make informed pesticide regulatory decisions that protect non-target beneficial insects such as pollinators.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.C.M., A.D.G., M.J.C.; Data curation: L.C.M., R.S., M.J.C.; Formal analysis: L.C.M., R.S., M.J.C.; Funding acquisition: L.C.M., M.J.C.; Investigation: L.C.M., R.S., M.C.-T., L.E.J., B.D.O., M.J.C.; Methodology: L.C.M., R.S., M.C.-T., A.D.G., B.D.O., M.J.C.; Project administration: L.C.M., M.J.C.; Resources: M.C.-T., A.D.G., M.J.C.; Software: M.J.C.; Supervision: R.S., M.J.C.; Validation: L.C.M., R.S., M.J.C.; Visualization: L.C.M., R.S., M.J.C.; Writing – original draft: L.C.M., R.S., M.C.-T., M.J.C.; Writing – review & editing: L.C.M., R.S., M.C.-T., A.D.G., L.E.J., B.D.O., M.J.C.

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Data and resource availability
All data and analysis code are available from Newman Library at Virginia Tech: https://doi.org/10.7294/28778912.

ECR Spotlight
This article has an associated ECR Spotlight interview with Laura McHenry.

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