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Palatability of glyphosate in ants: a field experiment reveals broad acceptance of highly polluted solutions in a Mediterranean ant

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Abstract

Glyphosate is a systemic herbicide still used in many countries, though there are several known detrimental effects on animals. Previous studies concerning its effects on social insects are available, but they are primarily focused on honeybees; little is known about the interactions of this compound with ants. Here, we assessed whether different concentrations of glyphosate can be perceived by ant workers and to what extent. As a model species, we used the Mediterranean ant *Crematogaster scutellaris*, commonly found in agroecosystems. We performed 3,000 individual tests of acceptance using ten different solutions of various concentrations of the herbicide. Half of the solutions contained added sucrose in order to test the possible masking effect of the sugar taste on glyphosate. We used comparable glyphosate concentrations to those previously used in other studies on social insects or suggested by the producer. We found that the acceptance of the solutions decreased as the concentration of the herbicide increased. However, a significant percentage of ants drank the solutions with concentrations up to dozens of times higher than those inducing toxic effects in bees. In light of these results, we urge further assessment of the effects of glyphosate on ants, particularly because the food ingested by workers is transferred to the brood and queens, posing a potential threat to the health of the entire colony. Surprisingly, we did not record any difference in acceptance between solutions with and without sugar; this point is discussed regarding drought stress.

Keywords glyphosate, herbicide, ants, palatability, sugar concentrations, acceptance, ecotoxicology

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Author contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by Filippo Frizzi, Valeria Palchetti Alberto Masoni, Paride Balzani, and Clara Frasconi Wendt. Analyses were performed by Filippo Frizzi and Giacomo Santini. The first draft of the manuscript was written by Filippo Frizzi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Introduction

Glyphosate, or [N-(phosphonomethyl)glycine], is an herbicide broadly used in agriculture since the early 1970s. It hampers the functionality of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is a crucial element of the shikimate pathway in plants, a primary metabolic pathway for producing essential aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. Since this process is not present in metazoans, the systemic functioning of glyphosate has led to the belief for many years that it is nearly innocuous to animals (Richmond 2018). Only in the last 20 years have studies begun to show that glyphosate can cause severe damage to some physiological functions in animals, such as hormone production, neuronal growth, and fertility (e.g., Soso et al. 2007; Schneider et al. 2009; Romano et al. 2012; Coullery et al. 2016). Moreover, this compound can be highly persistent in the environment. Indeed, almost one-fifth of the initial concentration can be found in the soil up to one year after its application (Feng and Thompson 1990; Bento et al. 2016), so effects on organisms can act in the long term (Bai and Ogborne 2016). These new insights into the effects of glyphosate on the fauna have led many countries to enact ad-hoc laws to regulate or even ban the use of glyphosate as a pesticide in agricultural and gardening activities (Arcuri 2018). Nonetheless, its use is still permitted and widespread in some areas, including in developed countries such as the US (US EPA, Docket Number EPA-HQ-OPP-2009-0361, January 2020) and, to a lesser extent, in the European Union, which renewed its license to use glyphosate until 15 December 2022 (Regulation [EU] 2017/2324, implemented on December 2017).

Several recent studies investigating the effects of glyphosate on insects have concentrated on social hymenopterans, particularly honeybees, likely because of their substantial environmental and economic value (Gallai et al. 2009; Breeze et al. 2011). Glyphosate negatively affects many key aspects of the biology of bees—such as navigation ability (Balbuena et al. 2015), short-term memory (Mengoni Goñalons and Farina 2018), larval development (Vàsquez et al. 2018), and royal jelly production—by triggering the degeneration of gland tissues (Faita et al. 2018). Even the gut microbiota, which is fundamental for protecting individuals from pathogens, can be altered by glyphosate ingestion (Blot et al. 2019). In some cases, intake can have lethal effects (Seide et al. 2018). Despite the evidence for these detrimental effects, very few studies have been conducted on other social insects such as wasps and ants. It is surprising that such little attention has been given to the interaction between ants and glyphosate given the well-known ecological relevance of these organisms (Holldöbler and Wilson 1990; Lach et al. 2010) and their widespread occurrence in agroecosystems, where interactions with glyphosate are highly likely (Hagner et al. 2019).

A large fraction of the studies dealing with honeybees have been conducted in the laboratory by supplying colonies with food supplemented with known concentrations of glyphosate. Researchers have used either the most common concentrations used in agriculture, following the instructions of the producer (Seide et al. 2018), or the concentrations directly measured in natural and agricultural ecosystems (Motta et al. 2018; Vàsquez et al. 2018). In most cases, specimens were "forced" to feed on polluted food because of the lack of uncontaminated alternatives. When selection was allowed, avoidance behaviors towards glyphosate-based pesticides was observed in other invertebrates, such as earthworms (Casabe et al. 2007, but see Niemeyer et al. 2018) and springtails (Santos et al. 2012). However, some questions about the behavioral response of social insects toward glyphosate-contaminated food in natural contexts remain unanswered. Specifically, are they able to detect glyphosate in the food? If so, at what concentration can they detect it? And finally, do they avoid feeding on that resource or do they consume it anyway?

To answer these questions, in this study we offered different concentrations of the commonly used glyphosate-based pesticide Roundup® to the Mediterranean acrobat ant *Crematogaster scutellaris*. We recorded the ability of this species to detect the pollutant in the solutions by individually testing their acceptance. This is a widespread and dominant species found in tree trunks and dead logs throughout the western Mediterranean basin (Casevitz-Weulersse 1972, 1991). We used this ant as a model species because many aspects of its biology and ecology are well-known (e.g., Marlier et al. 2004; Giannetti et al. 2019; Masoni et al. 2019). This species forms large polydomous colonies, is widespread in both natural and managed habitats (Gramigni et al. 2013; Frizzi et al. 2014), and has a generalist diet, being both an aphid tender and a top predator (Schatz et al. 2003; Ottonetti et al. 2008; Frizzi et al. 2016). Since glyphosate is usually applied by spraying, all resources, including water holes, can be affected by the compound and potentially used by workers of *C. scutellaris*. Moreover, their feeding preferences can be optimally tested by individual trials of acceptance (Frizzi et al. 2016). Hence, this represents a reliable model species for our purpose. This experiment aimed to improve our knowledge of whether this pollutant can be transferred from the abiotic to the biotic sphere via food ingestion, thus entering the trophic web, and to what extent.

Materials and methods

The study was carried out in June and July of 2019 on the Sesto Fiorentino University Campus and nearby sites (43°49’00’’N, 11°11’59’’E). The climate is typical of the Mediterranean region, with dry, hot summers and mild winters. During the experiments, mean temperatures ranged from 28°C to 30°C, and no rain events occurred during the ten days prior to the first trial (data from Servizio Idrologico della Regione Toscana [SIR], available at https://www.sir.toscana.it/, visited on 2 April 2020). The habitat is semi-urban, with tree-lined streets and managed parks partially surrounded by buildings. The area is included within an urban matrix, but it also borders fallow fields, meadows, and small shrublands. Most of the trees are ornamental, including oaks (*Quercus* spp.), cypresses (*Cupressus* spp.), and pines (*Pinus* spp.). The management of green areas is performed without using chemicals and mainly consists of periodic tree pruning and lawn mowing. For these experiments, we randomly selected 15 trees, irrespective of the species, that included a nest of *C. scutellaris*. One tree can be considered as a single nest (Frizzi et al. 2015). Since the species is polydomous (Santini et al. 2011), we selected trees that were at least 25 meters apart from each other in order to exclude nests belonging to the same colony.

We prepared four different water dilutions of Roundup® Power 2.0, a mixture providing 360 g/l of glyphosate acid (added as potassic salt), with exponentially decreasing glyphosate content: 1/10, 1/100, 1/1000, and 1/10000 (hereafter 1D, 2D, 3D, and 4D, respectively). Appropriate volumes of Roundup® were diluted in distilled water corresponding to concentrations of 36 g/l, 3.6 g/l, 0.36 g/l, and 0.036 g/l of glyphosate, respectively. We used pure distilled water as a control. Three of these concentrations are comparable with those suggested by the producer, which range from 1.2 g/l to 21.6 g/l depending on the pest being treated. The lowest concentration is comparable to the long-lasting values measured in crops treated with glyphosate (up to 0.02 g/l; Rubio et al. 2014). To evaluate the possible masking effect of food taste on the glyphosate content, we added sucrose to each solution, resulting in a final concentration of 4% (4 g sucrose per 100 ml solution). This concentration of sucrose is detectable by workers of *C. scutellaris* (Frizzi et al. 2016). In total, we tested ten solutions, including five with sucrose and five without.

Tests consisted of offering individual drops of one of the solutions to solitary ants. We took care not to use ants forming trails since the pheromone may distract them from the resource. For each drop, we recorded the acceptance. A solution was considered accepted if the ant touched the drop with its mouth for at least two seconds (Frizzi et al 2016). A solution was considered refused if the ant touched the drop with the mandibles and promptly left without drinking. For each of the 15 nests selected, we tested 200 ants—20 with each solution—for a total of 3,000 individual tests. Ants were removed and collected within a plastic container after the test in order to avoid using the same ants repeatedly or transferring the glyphosate into the nest. Furthermore, in order to ensure the independence of treatments, all tests were carried out in different randomly chosen locations around the tree trunk at least 30 cm apart.

To analyze the effects of both glyphosate concentration and sucrose on the acceptance rate, we used a two-step analysis. First, we ranked five different binomial Generalized Linear Mixed Models (GLMMs) by using the Aikaike's Information Criterion (AIC) index. Models included: the presence of sucrose only, the glyphosate concentration only, both factors, and both factors and their interaction. We also fitted a null model as a reference. In all models, we added the nest as a random factor. In the second step, we tested factors included in the best model using a Type II ANOVA with the Wald chi square test for assessing the significance. When necessary, we used multiple comparisons to test the differences between levels in pairs by computing and comparing Estimated Marginal Means (EMMs). All analyses were performed using the 3.6.3 version of the R software (R Core Team 2020) with the libraries "lme4" (Bates et al. 2015), "emmeans" and "car" (Fox and Weisberg 2019), and "AICcmodavg" (Mazerolle 2019).

Results

Table 1 shows the result of the model ranking. The complete model—which includes the type of solution, the glyphosate concentration, and their interaction—has the lowest AIC value. However, the model that includes only the concentration, despite being more parsimonious, has an AIC value that is 0.26 points higher. This means that the two models perform identically and that the presence of sugar in the solutions seems not to influence the level of acceptance by the ants. This is also confirmed by the fact that the model which included only the presence of sucrose performed very similarly to the null model (both ΔAICs were more than 1650 points higher than the best model). For this reason, we pooled the data from tests with and without sugar, then tested the effects of glyphosate concentration (Figure 1). Overall, the acceptance level was significantly different among concentrations (Type II ANOVA, Wald chi square test: $\chi^2 = 540.96$, df = 4, $P < .0001$), with the frequency of acceptance decreasing from water to the 1D solution. Multiple comparisons showed that all levels were significantly different from each other (Table 2).

Discussion

This study demonstrates that the frequency of acceptance of the test solutions decreased as the glyphosate concentration increased, although it remained surprisingly high even for highly concentrated glyphosate solutions. This suggests that workers of *C. scutellaris* can detect the presence of this pesticide in the solutions. Indeed, in all trials, the highest concentration (36 g/l) was almost completely disregarded by workers. Nonetheless, detection of the compound appears not to discourage the majority of foragers from drinking the solutions containing concentrations of glyphosate that, in other insects, have been demonstrated to have severe harmful effects. For example, the 3.6 g/l concentration was, on average, accepted by more than 60% of the tested workers. This concentration falls within the suggested range for the use of the product, and it can therefore easily be found in freshly treated crops. In *Apis mellifera* and *Hypotrigona ruspolii*, this concentration can be lethal within 24 hours, even after a simple contact with the body (Abraham et al. 2018). The lowest concentration tested in this study, 0.036 g/l, has been previously demonstrated to cause considerable perturbations in the gut microbiota of bees, increasing the risk of bacterial infections, particularly in larvae (Motta et al. 2018). Albeit low, this concentration is more than three times higher than the sublethal concentration tested by Balbuena et al. (2015) in homecoming experiments with honeybees (0.01 g/l), which showed significant impairments of their cognitive capabilities. Moreover, a similar concentration (2 µl of Roundup in 140 µl of food, ~0.02 g/l) can be dramatically toxic for larvae of the stingless bee *Melipona quadrifasciata* (Seide et al. 2018). In laboratory experiments, all larvae of this species that were fed with the contaminated diet died within a few days. In our trials, such a concentration was accepted by an average of more than 80% of the workers.

Although detrimental effects have also been documented in adults, the most affected categories appear to be the juvenile stages, such as larvae (Vàsquez et al. 2018 and references therein; also see Zhu et al. 2015). In social hymenopterans, food collected by foragers is partially shared with the rest of the colony via the mouth-to-mouth sharing behavior of trophallaxis. This process usually does not involve all workers equally and may vary based on hunger conditions or colony size (Buczkowski and Bennett 2009; Feigenbaum and Naug 2010). However, it is mandatory for providing nutrition to the nest-housed castes such as the queen and her brood, which are unable to forage outside of the nest by themselves. This food exchange can be very efficient and quick; within a few dozen minutes, most individuals can be fed (Sendova-Franks et al. 2010; Jung et al. 2018). If the effect of glyphosate is detrimental to ants, the continuous provision of this compound to queens and brood may lead to severe damage to the colony in a very short time. One of the most common methods of eradicating ant pests is based on this process; ant baits are filled with food that is polluted with specific insecticides which are then spread via trophallaxis to the rest of the colony (Hoffman et al. 2016). In this light, the use of solutions with glyphosate concentrations that are dozens of times higher than those causing toxic effects in other insects may have rapid and disastrous effects on ant communities. In turn, negative effects on this important group may result in top-down or intraguild effects on the trophic web (e.g., Mestre et al. 2016; Bisseleua et al. 2017; Goncalves et al. 2017). Furthermore, it should be recalled that glyphosate can persist in the environment for an extended period of time; thus, the risk of contamination may persist in the long term (Feng and Thompson 1990; Mercurio et al. 2014; Bento et al. 2016). Hence, the next step is to evaluate these effects in further ad-hoc experiments.

An additional and unexpected result is that the level of acceptance did not differ between solutions with and without sugar. This may suggest that the presence of sucrose did not mask the taste of the glyphosate or that sucrose is not an attractive resource for improving the acceptance rate of the solutions. Also, the taste of glyphosate may mask the sugar content; however, this does not seem to be the case because no significant difference was found between pure water and water with added sugar. Though surprising, this result could be partially explained by the fact that in the hottest months, *C. scutellaris* may suffer drought stress, thus preferring water over other food sources (Frizzi et al. 2016). The hot and dry climate may have led the ants to accept the solutions for their water content while ignoring their sucrose content. However, this result deserves further investigation, as does the aphid community dynamics in this habitat, since the availability of aphid honeydew can profoundly affect the feeding behavior of ants (Detrain et al. 2010).

In conclusion, to our knowledge, this is the first study assessing the palatability of a glyphosate-based herbicide in ants. While it appears that ants can detect the pollutant in their food, we found a significant level of acceptance of food containing high and potentially lethal glyphosate concentrations, irrespective of the sugar nutritive content. This result should encourage further analysis of the effects of this widespread pesticide on ants—a matter almost completely ignored thus far.

Model	AIC	ΔAIC
Null	3836.8	1656.06
Type	3838.80	1658.06
Conc	2181.00	0.26
Type + Conc	2183.00	2.26
Type * Conc	2180.74	0

Table 1 Model ranking according to the AIC index. Null = null model; Type = type of solution (sugary or watery); Conc = glyphosate concentration; ΔAIC = difference with the lowest AIC value.

Contrast	Estimate	z ratio	*P*
W - 4D	1.28	5.09	< 0.0001
W - 3D	1.91	7.99	< 0.0001
W - 2D	2.65	11.36	< 0.0001
W - 1D	6.81	20.72	< 0.0001
3D - 4D	0.64	3.95	0.0001
2D - 4D	1.38	9.04	< 0.0001
1D - 4D	5.54	19.99	< 0.0001
2D - 3D	0.74	5.56	< 0.0001
1D - 3D	4.90	18.37	< 0.0001
1D - 2D	4.16	15.94	< 0.0001

Table 2 Results of multiple comparisons between each concentration in pair. W = water; 1D = 36 g/l; 2D = 3.6 g/l; 3D = 0.36 g/l; 4D = 0.036 g/l.

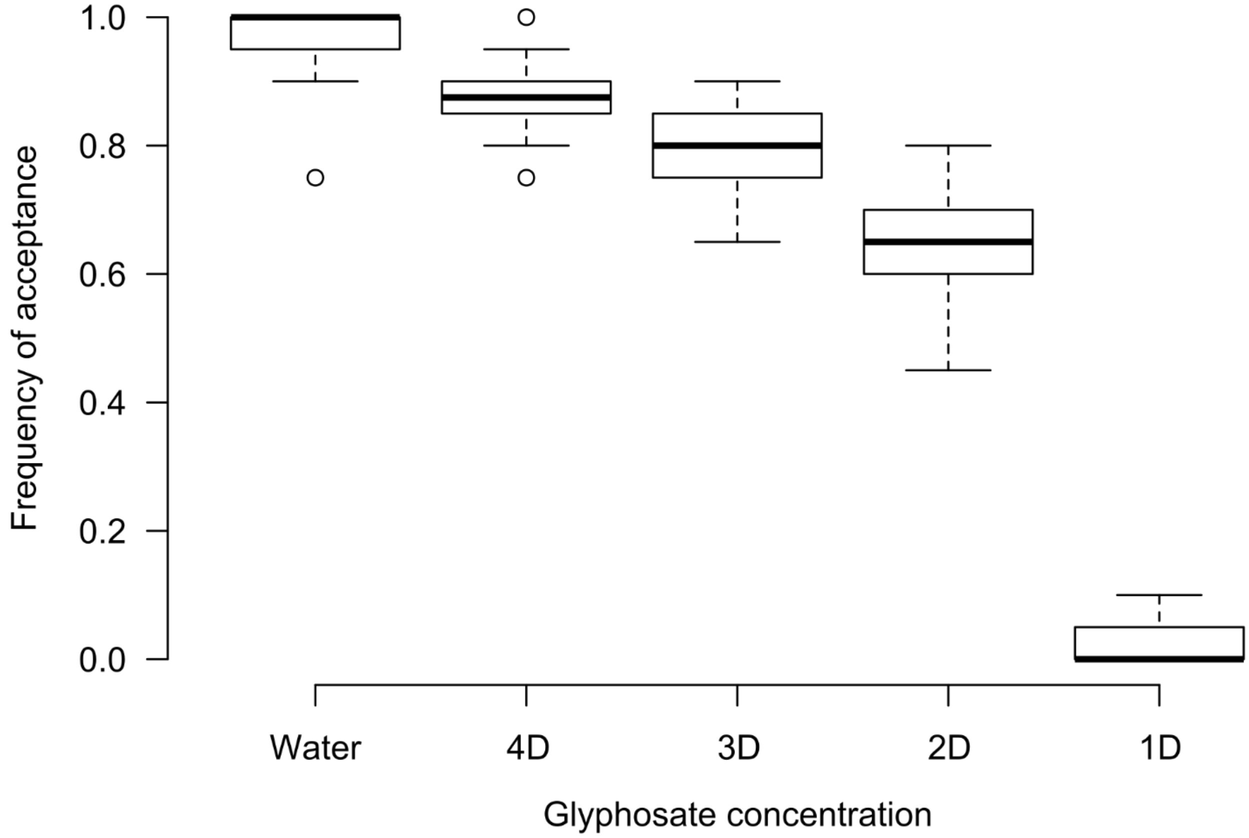

Fig. 1 Boxplot of the frequency of acceptance for all the four glyphosate solutions and for water in the 15 nests tested. Data from sugary and watery solutions are pooled. 1D = 36 g/l; 2D = 3.6 g/l; 3D = 0.36 g/l; 4D = 0.036 g/l.

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