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Roundup causes high levels of mortality following contact exposure in bumble bees

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Abstract

1. Pollinators underpin global food production, but they are suffering significant declines across the world. Pesticides are thought to be important drivers of these declines. Herbicides are the most widely applied type of pesticides and are broadly considered 'bee safe' by regulatory bodies who explicitly allow their application directly onto foraging bees. We aimed to test the mortality effects of spraying the world's most popular herbicide brand (Roundup®) directly onto bumble bees *Bombus terrestris audax*.
2. We used three Roundup® products, the consumer products Roundup® Ready-To-Use and Roundup® No Glyphosate, the agricultural product Roundup® ProActive, as well as another herbicide with the same active ingredient (glyphosate), Weedol®. Label recommended pesticide concentrations were applied to the bees using a Roundup® Ready-To-Use spray bottle.
3. Bees exhibited 94% mortality with Roundup® Ready-To-Use® and 30% mortality with Roundup® ProActive®, over 24 hr. Weedol® did not cause significant mortality, demonstrating that the active ingredient, glyphosate, is not the cause of the mortality. The 96% mortality caused by Roundup® No Glyphosate supports this conclusion. Dose-dependent mortality caused by Roundup® Ready-To-Use, further confirms its acute toxicity. Roundup® products caused comprehensive matting of bee body hair, suggesting that surfactants, or other co-formulants in the Roundup® products, may cause death by incapacitating the gas exchange system.
4. These mortality results demonstrate that Roundup® products pose a significant hazard to bees, in both agricultural and urban systems, and that exposure of bees to them should be limited.
5. *Synthesis and applications*. Surfactants, or other co-formulants, in herbicides and other pesticides may contribute to global bee declines. We recommend that, as a precautionary measure until co-formulant identities are made public, label guidelines for all pesticides be altered to explicitly prohibit application to plants when bees are likely to be foraging on them. As current regulatory topical exposure toxicity testing inadequately assesses toxicity of herbicide products, we call for pesticide companies to release the full list of ingredients for each pesticide formulation,

as lack of access to this information hampers research to determine safe exposure levels for beneficial insects in agro-ecosystems.

KEYWORDS
bees, contact toxicity, herbicide, inert ingredient, pesticide, roundup, surfactants, topical toxicity

1 | INTRODUCTION

Bees provide the crucial ecosystem service of pollination (Potts et al., 2016), but are under threat, with 37% of EU bee species with known trends exhibiting population declines (Nieto et al., 2014). One apparent cause of these declines is pesticides (McArt et al., 2017; Rundlöf et al., 2015; Woodcock et al., 2016). Pesticide usage is pervasive, with 4.1 billion kilograms of active ingredient applied globally in 2017, nearly double the amount used in 1990 (FAOSTAT, 2019). Pesticides have received significant attention from the public and policymakers due to their apparent detriment to non-target organisms, such as pollinators, but this attention has largely focused on insecticides. A recent systematic review found that only 29 studies had tested the effects of herbicides on bees (Cullen et al., 2019). Additionally, research into herbicides relative to insecticides is disproportionate to their usage, with, for example, 24 times more herbicide applied in the United Kingdom than insecticide in 2018 (FERA, 2019).

For most classes of pest, pesticide usage varies by crop and region, with a range of active ingredients being employed (Garthwaite et al., 2016a,b). However, herbicides are unique in that one substance, glyphosate, is applied at a far greater rate than any alternative (FERA, 2019). In 2014, 826 million kilograms of glyphosate were applied globally (Benbrook, 2016), accounting for around 20% of all pesticide application (Benbrook, 2016; FAOSTAT, 2019). Glyphosate (applied in products called glyphosate-based herbicides—GBHs) has a favourable toxicity profile as a broad-spectrum herbicide, being the only herbicide to target the shikimate pathway (Duke, 2018). Its low toxicity to the majority of non-target organisms (EFSA, 2015a), has led to most regulatory regimes placing minimal restrictions on its application (Beckie et al., 2020). Bee exposure to glyphosate is poorly characterised, although it is known to be extensive, with surveys finding that 59% of honey samples had glyphosate present above the limit of detection, with a mean of 64 ppb (Rubio et al., 2014).

High acute doses (oral and contact) of glyphosate, applied as the active ingredient (glyphosate) alone, or in a single representative formulation (MON 52276 commercially called Roundup® Bioflow in Italian markets (EFSA, 2015b; Mesnage et al., 2021)), do not cause mortality in honeybee workers (EFSA, 2015b). Consequently, it has passed lower tier testing in the United States and Europe, facilitating its approval in both territories. However, GBHs contain additional components, called co-formulants, that can have serious, but systematically underestimated risks (Cox & Surgan, 2006; Mesnage & Antoniou, 2018; Mullin et al., 2016).

Co-formulants are chemical additives that increase the efficiency of the active ingredient (Hazen, 2000). Without co-formulants, pesticide formulations would be much less effective (Hazen, 2000), and more active ingredient would need to be applied, potentially leading to more environmental damage. Most co-formulants are considered 'inert' by regulatory bodies, and thus are not subject to equivalent testing to active ingredients. Consequently, there are no requirements to test their toxicity to bees (EC, 2009), meaning that potentially toxic substances are used abundantly (Cox & Surgan, 2006; Mullin, 2015; Mullin et al., 2015). As they are not tested for in food or environmental residue monitoring programmes (Mesnage et al., 2019), our understanding of their prevalence and environmental fate is highly limited. Bee exposure to these co-formulants is likely commensurate to that of active ingredients but is poorly studied.

While our understanding of co-formulant exposure is limited, studies of hazard (i.e. the damage they cause) are more informative. Nagy et al. (2019) reported that 24 of 36 studies showed formulations to be more toxic in non-target organisms than active ingredients alone. In human cell lines and rats, Roundup® products specifically were more toxic than the active ingredient alone in five of six studies, with just one study finding equivalent toxicity (Nagy et al., 2019). While only one formulation per active ingredient is typically submitted to the full range of toxicity tests in the EU (EFSA, 2015a), dozens of formulations per active ingredient are produced, each with a unique composition posing unique hazards to non-target organisms (Mesnage et al., 2019). For glyphosate in the United Kingdom there are 284 distinct consumer or agricultural formulations (Health & Safety Executive UK, 2020), making it the most formulation diverse AI in the United Kingdom. Co-formulants present in Roundup® have been found to have sub-lethal effects in human cell lines (Defarge et al., 2016; Mesnage et al., 2013), demonstrating that they present a relevant hazard to health, although almost nothing is known of their effects on bees (Mullin, 2015; Mullin et al., 2015). One class of co-formulants, surfactants (surface acting agent), were found in 100% of American honey, pollen and beeswax samples (n = 27; Chen & Mullin, 2014), demonstrating their pervasiveness.

Surfactants in herbicides like Roundup® spread the sprayed droplets out over target leaves, increasing glyphosate absorption and toxicity. Surfactants are major co-formulants in Roundup® products, typically accounting for 15% of the concentrated weight (Mesnage et al., 2019). Surfactants are environmental pollutants that have been shown to have a range of negative impacts on honey bees (Ciarlo et al., 2012; Fine et al., 2017; Goodwin & McBrydie, 2000; Moffett & Morton, 1973, 1975) and solitary bees (Artz & Pitts-Singer, 2015).

In agriculture, direct spraying of insecticides onto bees, or bee attractive flowers, is banned as part of their mitigation strategy (EFSA, 2013) in order to prevent bees contacting the pesticide as it is being sprayed, or the residues on flowers after it is sprayed. No such restrictions apply for herbicides, with the Environmental Information Sheet for Roundup® ProActive stating "Roundup ProActive is of low toxicity to honeybees; there is no requirement to avoid application of the product when bees are foraging on flowering weeds in treated crops" (Roundup® ProActive Environmental Information Sheet, 2020). Consequently, with both glyphosate and the co-formulants/surfactants in GBHs being considered safe by regulators (EFSA, 2015a), there should not be lethal effects from GBHs when used following label guidelines. Abraham et al. (2018) however, found significant mortality through indirect exposure to a GBH, Sunphosate 360 SL (Zhejiang Xinan Chemical Industrial Group, Zhe-jiang, China), which is a generic GBH available in Ghana. The study found that honeybees *Apis mellifera* and stingless bees *Hypotrigona ruspolii* exposed to the formulation via a branch of a flowering tree *Senna siamea* that had previously been sprayed with Sunphosate 360 SL suffered 28% and 23% mortality respectively, which was significantly higher than the 4% and 6% mortality for the water control. As glyphosate does not cause such mortality via contact or oral exposure (EFSA, 2015b), the mortality seen in this experiment is likely to be driven by co-formulants.

Risk assessment of the threat a pesticide poses to bees relies on the Risk = Hazard × Exposure model, where Hazard is a measure of toxicity, and Exposure is a measure of environmental contact. GBHs are currently believed to combine low to no hazard and high exposure, because they can be directly applied to bees, making them low to intermediate risk. Here we test how hazardous a range of GBHs, including Roundup® products are to bumble bees. We use a study design that can distinguish between the effects of co-formulants and the active ingredient, to allow us to test how these factors affect mortality. We predict that the GBHs will cause moderate mortality with direct exposure, in line with Abraham et al. (2018).

2 | MATERIALS AND METHODS

Ten commercial bumble bee, *Bombus terrestris audax*, colonies were used in the experiments (Agralan). On arrival 10 workers per colony were removed and their faeces screened for micro-parasites. No infections were detected, and all colonies were thus retained in the experiment.

In all experiments over 50 bees were exposed per treatment (excluding the control treatment in Experiment 4) in groups of five or six, as detailed in Table S2. Bees were sprayed in groups for efficiency and because an even coating could still be achieved with this number of bees in a box. For each experiment multiple source colonies were used to account for inter-colony variation, allocating them evenly across treatments. Workers were moved from source colonies into clear acrylic boxes (6.7 × 12.7 × 4.9 cm), with a plastic mesh grate bottom (6.7 × 7.3 cm). Within each box, bees were only taken from one source colony and were left to acclimatise for 10 min prior to exposure.

A mortality check was carried out prior to exposure. Mortality was defined as any moribund bee being entirely unresponsive to physical agitation with a pair of forceps. Following this, the acrylic box was sprayed in a X shape from corner to corner with two squeezes of the trigger of a Fast Action Roundup® Ready-To-Use bottle (Roundup® Ready-To-Use; total exposure = 1.327 ± 0.005 ml *SE*); the spray came out as a cone of droplets which ensured consistent and even coverage across the whole box. This amount was chosen to ensure the bees were evenly coated while keeping control mortality <10%, pilot work found this methodology to deliver the treatment evenly to all bees sprayed when visually assessed. Roundup® Ready-To-Use and Roundup® No Glyphosate are sold in these spray bottles, and Weedol® in a similar bottle. Bees were sprayed under red light to prevent flying, we did not attempt to influence their behaviour beyond this, and they were exhibiting normal resting behaviour when sprayed. This methodology is not designed to replicate field realistic exposure (spraying conditions or label recommended application rates), it is instead designed to assess the lethality (hazard) the herbicide products pose to bumble bees. One investigator performed the spraying and mortality checks. A series of practice sprays were performed to ensure consistency. Mortality was recorded immediately after spraying, and at 10, 20 and 30 min. After 30 min a source of sucrose (50% w/w) and small portion of pollen (1-2 g) was added. At 24 hr post-exposure mortality was recorded for a final time. Boxes that flooded due to sugar water spillage between 30 min and 24-hr observations were excluded ($n = 2$, both in Experiment 2, Control), as were individual bees who drowned themselves in the sucrose gravity feeder ($n = 1$, Experiment 5, Control).

We used a total of four herbicide products across our experiments. Fast Action Roundup® Ready-To-Use (MAPP 14481; henceforth referred to as Roundup® Ready-To-Use), Roundup® Speed Ultra (MAPP 18692; henceforth referred to as Roundup® No Glyphosate; both Scotts Miracle-Gro Company, Surrey, UK under licence from Monsanto, Cambridge, UK), and Weedol® Gun! Rootkill Plus (MAPP 14554; henceforth referred to as Weedol®, Scotts Miracle-Gro Company, Surrey, UK) are all consumer products that can be bought in supermarkets. Consumer products require no licence or training in the United Kingdom and are intended for garden use. Roundup® ProActive (MAPP 17380, Monsanto, Cambridge, UK) can be bought online without a licence in the United Kingdom, but a licence is required to spray the substance in agriculture or horticulture (Roundup® ProActive Label, 2019). All products were purchased in 2019 online or in person in the United Kingdom (full details of all products used are provided in Table S1). Table 1 shows the glyphosate and other active ingredient concentrations, as reported on the product labels, and the dilutions for the test solutions used across experiments. For pre-mixed consumer products, we used the concentration as sold, or diluted it further as in Experiments 2 and 3. For the agricultural product Roundup ProActive we used field realistic concentrations of the treatment solutions, with the product diluted as directed on the label to produce a concentration equivalent to that used in agricultural spraying. This is distinct from the rate of application, which is the amount of substance applied per area, typically expressed as AI g/ha or L/ha of a pesticide mixture. We did not attempt to replicate field realistic application rates for the agricultural product Roundup

Experiment	Treatment	Product concentration used (%)	Glyphosate concentration g/L
All	Control	0	0.0
1	Roundup® Ready-To-Use	100	7.2
1	Roundup® ProActive	6.25	22.5
2	Roundup® Ready-To-Use 50%	50	3.6
3	Roundup® Ready-To-Use 25%	25	1.8
4	Weedol®	100	7.2 (0.02 g/L pyraflufen-ethyl)
5	Roundup® No Glyphosate	100	0.0 (60 g/L acetic acid)

TABLE 1 The concentrations of the products used, based on the amount of water added to dilute them to, or below, label concentrations, and respective glyphosate concentrations. Concentrations of other active ingredients present in formulations given in parentheses

ProActive for the following reasons. While we know the application rates for this product based on ground surface area (from 1 to 6 L/ha of formulation, 0.6%–33% product concentration and 10-400 L/ha of mixed solution), the exposure, or application rate on bees will be a function of the height from which the product is sprayed, the height of either crop or weed flowers and the height at which bees are present when the product is applied (which may be either the same as the flowers, or above or below this if bees are flying between flowers). As each of these factors will vary both within crops, and from crop to crop, and as the only one for which good data exist are crop height, it is currently impossible to extrapolate from surface area application rate to bee exposure. Similarly, in the absence of label guidance on application rates for consumer products, we cannot compare our exposure to usage in gardens. Fundamentally, our experiment was designed to enable the detection of hazardous effects from substances previously reported to be non-hazardous. More complex designs using field realistic apparatus and application rates could determine the risk these substances pose.

Controls throughout were pure distilled water and were sprayed from an identical Roundup® Ready-To-Use bottle at room temperature. Both the Weedol® and Roundup® products tested (Experiments 1 and 2) contain glyphosate at equivalent concentrations. Because Weedol® is likely to have a different co-formulant composition to the Roundup® products it served as a glyphosate control. A series of five independent experiments were conducted to answer the following questions:

> Experiment 1: Are the impacts of consumer and agricultural Roundup® products comparable?
> Bumble bees in three treatment groups were sprayed with either the consumer product Roundup® Ready-To-Use (at its pre-mixed concentration), the agricultural product Roundup® ProActive at the highest label recommended concentration of 6.25%, which covers a range of applications, or the water control.
>
> Experiment 2: Does mortality still occur with a 1:1 dilution of consumer Roundup®?
> Bumble bees in two treatment groups were sprayed with either the consumer product (Roundup® Ready-To-Use) diluted 1:1 with pure distilled water, or the water control.
>
> Experiment 3: Does mortality still occur with a 1:3 dilution of consumer Roundup®?
> Bumble bees in two treatment groups were sprayed with either the consumer product (Roundup® Ready-To-Use) diluted 1:3 with pure distilled water, or the water control.
>
> Experiment 4: Does an alternative GBH (Weedol®) cause mortality?
> Bumble bees in two treatment groups were sprayed with either the generic consumer product GBH Weedol® at its pre-mixed concentration, or the water control.
>
> Experiment 5: Does the Roundup® formulation without glyphosate cause mortality?
> Bumble bees in two treatment groups were sprayed with either the consumer product (and GBH alternative) Roundup® No Glyphosate at its pre-mixed concentration, or the water control.

All statistical analyses were carried out in 'R' programming software version 3.6.2 (R Core Team, 2019). Plots were produced using the package 'GGPLOT$_2$' version 3.2.1 (Wickham, 2016) and 'SURVMINER' version 0.4.6 (Kassambara et al., 2019). Mixed effects Cox proportional hazards models were used to analyse mortality, utilising 'SURVIVAL' version 3.1-8 (Therneau, 2020a), 'COXME' version 2.2-16 (Therneau, 2020b) and 'MuMIn' version 1.43.17 for model averaging (Bartoń, 2020). AIC model simplification was used, with model averaging where no single model had ≥95% AIC support. The candidate set of models was chosen by adding the next best supported model until a cumulative ≥95% support was reached. Parameter estimates and 95% confidence intervals are reported. The full model used was (Survival ~ Treatment + Colony of Origin + (1|Box ID)). There was no correlation between variables. For comparisons between Roundup® Ready-To-Use concentrations in Experiments 2 and 3 Colony of Origin was not included as a variable, as it correlated with Treatment owing to different colonies being used for each experiment. Consequently, the final model was (Survival ~ Treatment + (1|Box ID)). Model parameters, AIC weights and final models are presented in Tables S3. Proportionality of hazards was checked for each experiment to validate the Cox proportional hazards assumption, where this was violated (Experiments 4 and 5) a Chi-squared Test of Independence was used with the model (Survival ~ Treatment).

3 | RESULTS

3.1 | Experiment 1: Comparing the impacts of consumer and agricultural Roundup® products

There was a significant difference in mortality between both Roundup® products (Ready-To-Use and ProActive) and the control (Cox proportional hazards model: parameter estimate (PE) = 5.17, 95% CI [3.52-6.82], and PE = 2.18, 95% CI [0.52-3.84] respectively), with 94% and 30% mortality respectively compared to 4% mortality in the control treatment (Figure 1). There was also a significant difference between Roundup® Ready-To-Use and Roundup® ProActive (Cox proportional hazards model: (PE) = 2.95, 95% CI [1.93-3.96]), with the Roundup® Ready-To-Use causing faster and higher mortality. Of the Roundup® Ready-To-Use treated bees, 38% died immediately after exposure compared to just 7% of Roundup® ProActive and 0% of control bees. Ad hoc behavioural observations also noted bees in all Roundup® treatments spent considerable time self-grooming after exposure. This may have been in response to, and potentially exacerbated, the matting of bee body hair that can be seen in Figure 4.

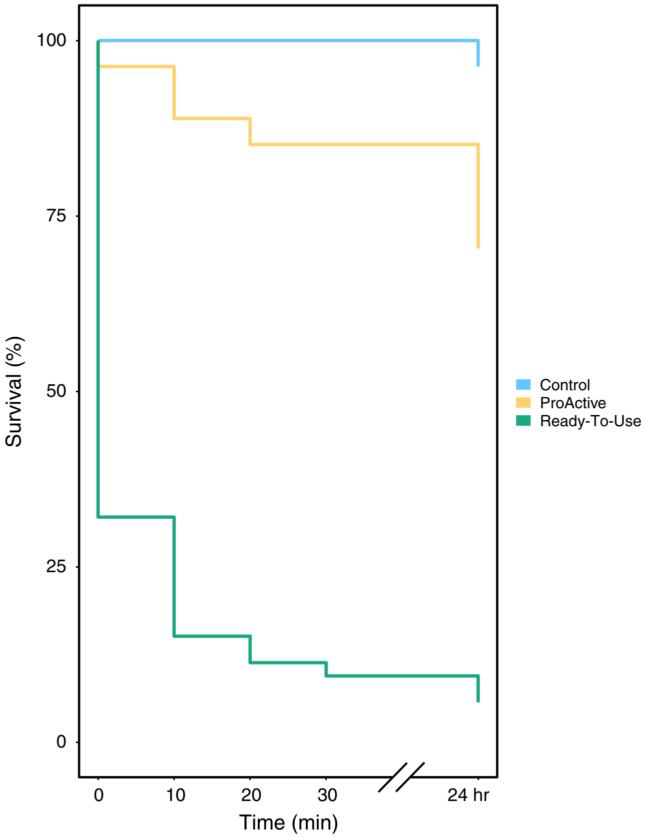

FIGURE 1 Experiment 1: Comparing the impacts of consumer and agricultural Roundup® products against the control, demonstrating high mortality with the Ready-To-Use treatment and intermediate mortality with the ProActive treatment

3.2 | Experiment 2: Does mortality still occur with a 1:1 dilution of consumer Roundup®?

The half strength Roundup® Ready-To-Use solution significantly increased mortality (Chi-squared test of Independence: $\chi^2 = 78.26$, $p < 0.0001$), with 98% mortality respectively compared to 3% mortality in the control treatment (Figure S1).

3.3 | Experiment 3: Does mortality still occur with a 1:3 dilution of consumer Roundup®?

The quarter strength Roundup® Ready-To-Use solution also produced significantly higher mortality than the control (Chi-squared test of Independence: $\chi^2 = 47.16$, $p < 0.0001$), with 78% mortality as opposed to 8% mortality in the control treatment (Figure S2). However, the mortality was less than either half or full strength (98% and 94% respectively; Figure 1; Figures S1 and S2). Furthermore, the mortality was delayed with only 10% of bumble bees dying within 30 min.

There was a significant difference between full-strength and both half and quarter-strength Roundup® Ready-To-Use solutions in their effects on mortality (Cox proportional hazards model: (PE) = 1.23, 95% CI [0.766-1.70], and 2.33, 95% CI [1.54-3.20] respectively), with the highest and fastest mortality in the whole strength treatment, followed by the half strength.

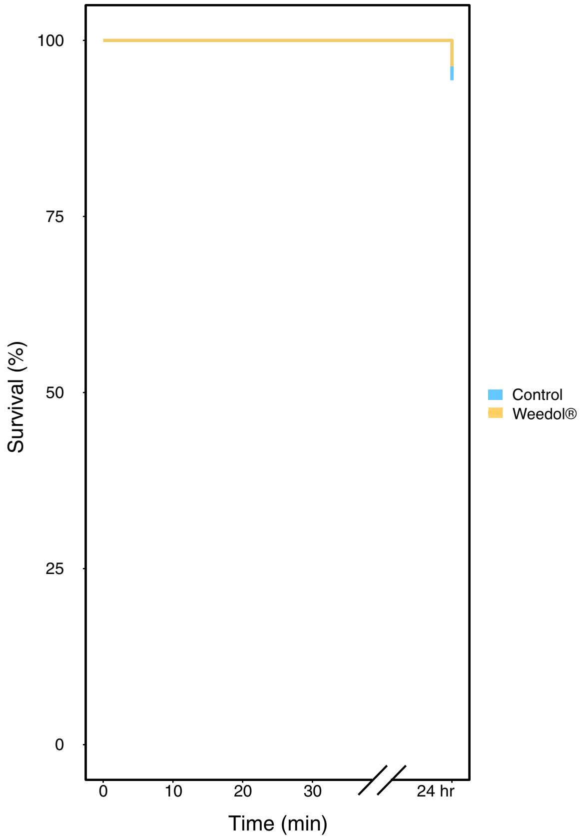

FIGURE 2 Experiment 4: Consumer product, and GBH alternative, Weedol® does not cause mortality relative to the control

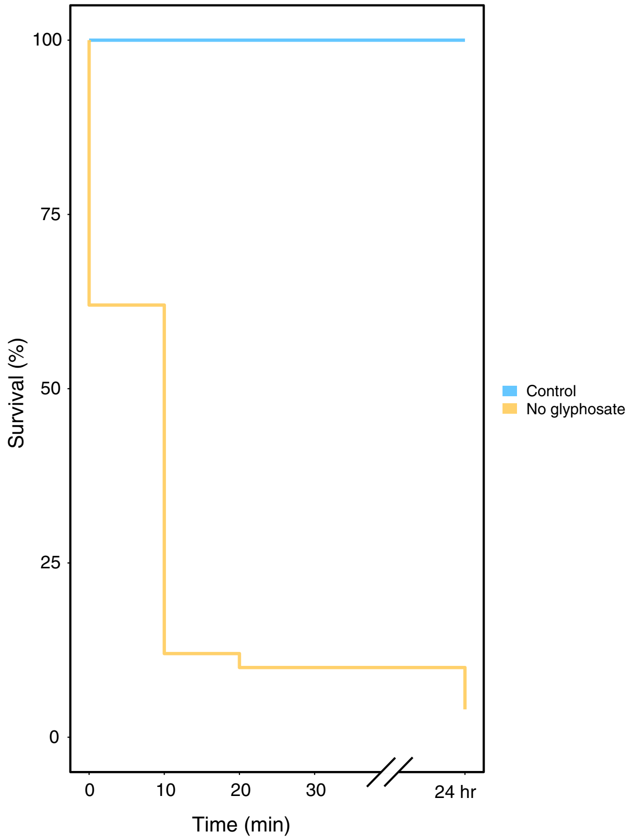

FIGURE 3 Experiment 5: The consumer product, and alternative to GBHs, Roundup® No Glyphosate causes high mortality

3.4 | Experiment 4: Does an alternative GBH (Weedol®) cause mortality?

Weedol® did not cause a significant difference in mortality relative to the control.

(Chi-squared test of Independence: $\chi^2 = 0.00$, $p = 0.983$), with 4% and 6% mortality respectively (Figure 2).

3.5 | Experiment 5: Does the roundup® formulation without glyphosate cause mortality?

Roundup® No Glyphosate produced significantly higher mortality than the control (Chi-squared test of Independence: $\chi^2 = 87.51$, $p < 0.0001$), with 96% mortality respectively compared to 0% mortality in the control treatment (Figure 3).

4 | DISCUSSION

Our results are the first to show that contact exposure to either consumer or agricultural Roundup® products at label recommended concentrations can cause high levels of mortality in bumble bees. The consumer product Roundup® Ready-To-Use caused 94% mortality at the pre-mixed concentration, and still caused significant mortality at a quarter strength. The agricultural product Roundup® ProActive also caused significant mortality, although over a longer time period. Interestingly, Roundup® No Glyphosate caused 96% mortality while the generic GBH Weedol® did not significantly increase mortality. Together, this demonstrates that the co-formulants in these Roundup® products, not the active ingredient glyphosate, are driving mortality. We suggest that the mechanism driving this mortality may be surfactants in the formulations blocking the tracheal system of the bees, which is essential for gas exchange. Given the hazard demonstrated here with all tested Roundup® products, and the extensive exposure of bees to such GBHs world-wide, GBHs may pose a high risk to bees, and thus may be an as yet unidentified driver of the bee declines that are occurring around the globe.

At a quarter strength, the consumer product Roundup® Ready-To-Use still caused 78% mortality, demonstrating that the formulation is sufficiently toxic to cause mortality despite being 75% water. The dose dependency shown in our experiments confirms the products' toxicity and aids our understanding of how to use them safely. At a quarter strength the mortality seen is equivalent to the double strength Sunphosate 360 SL used in Abraham et al. (2018), suggesting that Roundup® Ready-To-Use would also cause indirect contact mortality as even exposure to a severely reduced concentration caused high mortality. While consumer herbicides are unlikely to be applied directly to bees, they are likely to be applied to bee-attractive weeds which could drive mortality, with the Roundup® Ready-To-Use label even advising 'Treat established perennial weeds at the start of flowering to give best results' (Roundup® Ready-To-Use Label, 2019). Consequently, label restrictions should explicitly caution against application to flowering plants. While the agricultural product Roundup® ProActive requires a licence to spray, and has clear label instructions, the product label of Roundup® Ready-To-Use has no guidance pertaining to bees. A first step should be to amend household product labels to reflect the hazard posed to bees. Finally, whether consumers need access to potent pesticides, especially when nearly half of consumers either do not follow or take no notice of label recommendations (Grey et al., 2005), requires revisiting by policymakers; consumer pesticide products should not be overlooked in policy initiatives to reduce pesticide use.

The consumer product Roundup® Ready-To-Use caused more and faster mortality than the agricultural product Roundup® ProActive, but the latter still caused 30% mortality over 24 hr. The Material Safety Data Sheet (MSDS) for Roundup® ProActive MSDS (2020) lists Nitroryl (CAS no. 226563-63-9) and Alkylpolyglycoside (CAS no. 68515-73-1) as ingredients, possibly acting as a surfactants (US Patent 20100113274A1, 2010; US Patent 5266690A, 1993), although we do not know what, or if, other surfactants are in the formulation. If these substances are driving the mortality in the Roundup® ProActive treatment, this would be concerning as they are common in recently introduced products (Mesnage et al., 2019). We would suggest that the topical toxicity of these substances be assessed by regulatory agencies, to allow judgement to be made on their safety for inclusion in products bees are exposed to. This Roundup® ProActive driven mortality is in contrast to the guidance in the product's UK

Environmental Information Sheet stating, "Roundup ProActive is of low toxicity to honeybees; there is no requirement to avoid application of the product when bees are foraging on flowering weeds in treated crops" (Roundup® ProActive Environmental Information Sheet, 2020). This means that on-label guidance explicitly allows application directly onto bees, along with spraying onto flowering weeds, which are frequently visited by bees (Wood et al., 2019). This means that the exposure bees will face is incredibly high, with no attempt being made to mitigate their exposure. Furthermore, in the United States, Roundup® products can be directly applied to genetically modified glyphosate resistant (Roundup® Ready) crops, in order to knockdown weeds growing among the crop (Roundup® Ready Plus Information Sheet, 2020). For Roundup® Ready Soybeans this includes allowing application to the crop during flowering (Roundup® Ready Plus Information Sheet, 2020). As soybean flowers are an attractive floral resource for bees (EFSA, 2013), this will lead to direct exposure of bees to Roundup® products, which we have shown can drive significant mortality. Exposure through such herbicide tolerant crops is likely to be significantly higher than through flowering weeds, with herbicide tolerant soybeans covering 84.5 million hectares globally in 2014 (James, 2014 cited in Benbrook's, 2016, Supporting Information). Agricultural labels should preclude application to flowering plants or bees to reduce exposure.

Previous studies have examined the contact toxicity of surfactant adjuvants and Roundup® products. Results vary for studies testing similar surfactant spray adjuvants, with Goodwin and McBrydie (2000) finding 100% mortality below label recommended concentrations, while Donovan and Elliott (2001) found no mortality even in their highest treatments. This is likely explained by the different methodologies, with the former using a Potter spray tower which is close to field realistic spray conditions and the latter using pipette application using OECD 214 (OECD, 1998). Following OECD 214 1–2 μl of a solution is pipetted onto the backs of anaesthetised bees and then mortality assessed for 48 hr (OECD, 1998). This protocol is appropriate to assess the toxicity of AI, particularly potent insecticides, but inappropriate for assessing the toxicity of more dilute surfactant solutions. Due to EU law protecting co-formulant composition (EC, 2009), we do not know if the components of the adjuvants used in either study are present in any of the formulations tested here.

Our study diverges from the previously described results of Abraham et al. (2018) by using direct application onto bees, rather than indirect exposure (spraying flowers for the bees to then visit). We also used bumble bees, not honeybees or stingless bees, and still found high mortality suggesting the effects of GBH formulations on bees is widespread. The results presented here expand our understanding of how GBH formulations can cause mortality through contact exposure by isolating the co-formulants as driving the mortality and suggesting a mechanism behind the mortality. Recent work suggests similar mortality impacts in honey bees using a different Roundup® formulation (Motta et al., 2020).

The only regulatory studies of contact mortality with GBHs have used honey bees and the protocol OECD 214 (see above, OECD, 1998). This protocol does not accurately assess contact toxicity for formulations like Roundup® products, which can be sprayed directly onto bees. Regulatory testing should assess the contact toxicity of all formulations prior to approval/renewal using more field realistic methodologies than OECD 214, incorporating label recommended spraying apparatus and concentrations.

Our results clearly show that Weedol® does not produce higher mortality than the water control, and together with results from regulatory assessments (EFSA, 2015b), this confirms that the mortality seen in our experiments is not driven by glyphosate. This is supported by the findings of Motta et al. (2020), who found spraying honeybees with glyphosate did not cause mortality. Furthermore, Roundup® No Glyphosate caused 96% mortality, which demonstrates that the co-formulants in Roundup® products are toxic, and that the mortality we see does not derive from an interaction between co-formulants and glyphosate. This is encouraging, as it indicates the mortality could be eliminated entirely with a change to the co-formulants, without affecting the active ingredient content. The contrast between Weedol® and Roundup® products, which both use glyphosate as their active ingredient, demonstrates that co-formulants and formulations as well as active ingredients should be tested and regulated individually. This is especially true as active ingredient registrations have been greatly outstripped by novel formulation production, as pesticide manufacturers improve the efficiency of their products through changes to their co-formulants (Green & Beestman, 2007). That two of the three GBH's tested here produced significant mortality is concerning given that there are 281 other GBH's currently licenced for use in the United Kingdom.

The three Roundup® substances tested produced significant mortality, which shows that the current regulatory testing for contact toxicity is inadequate to detect mortality effects. While the testing performed here was not agriculturally field realistic, it highlights that these products pose a legitimate hazard that requires risk assessment through field realistic testing. These results contradict the regulatory assessment that GBHs are entirely bee-safe and do not require mitigation measures. Finally, for each active ingredient only a single representative formulation is mandated for testing at an EU level (EFSA, 2013). The only contact toxicity testing on bees with whole formulations presented in the EFSA, 2015 renewal assessment report is on the original version of Roundup® (MON 2139) in 1972 and the representative formulation Roundup® Bioflow (MON 52276), which lacks the alkylamine ethoxylates common in other GBH's, instead using a quarternary ammonium compound (EFSA, 2015b).

While we have not explicitly tested the mechanism through which this mortality is generated, we suggest that the surfactants in the formulations are interfering with the action of the spiracles, or tracheal system more broadly. Insects conduct gas exchange through the tracheal system, with spiracles (surface holes on the thorax and abdomen) enabling airflow into the tracheal system, and the tracheae carrying air to tissues and cells where gas exchange occurs (Bailey, 1954). Our observations show that the Roundup® products are spreading the formulation over the surface of the bumble bees, possibly limiting gas exchange. This spread may have been exacerbated by the self-grooming behaviour observed in the

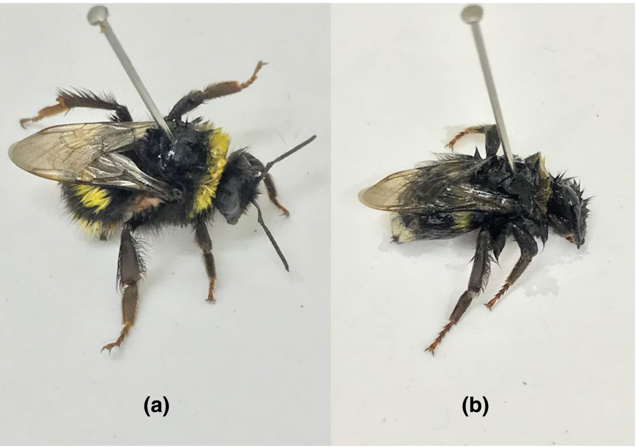

FIGURE 4 (a) Control and (b) Roundup® Ready-To-Use full concentration bumble bees sprayed and photographed within 5 min. Matting of the hairs over the bee's whole body can be seen in (b)

Roundup® treatments, and future research should formally assess this. This could be through a range of mechanisms, either by matting hairs down over the spiracles and physically smothering them, by blocking narrow sections in the respiratory system, or by coating the surface of the whole system in a non-permeable lining (see Figure 4; Figure S3). Stevens (1993) noted that insect spiracles are similar in size to plant stomata, which GBHs are designed to penetrate, and suggested therefore that the surfactants allow water penetration into the tracheal system, causing drowning. It is unlikely that the immediate mortality seen most prominently in the standard strength Roundup® Ready-To-Use treatment is caused by oral ingestion as even high doses of potent insecticides require several hours to produce mortality (Edward Straw, pers. obs.). We do not know if the mechanism driving the 38% immediate mortality in the Roundup® Ready-To-Use treatment is the same mechanism driving the further 56% mortality in the 30 min to 24-hr timeframe. Surfactant driven mortality in honeybees, which typically act as a sentinel for all beneficial insects, is unlikely to have been detected by beekeepers as the knockdown of bees is so fast they are unlikely to return to the hive before dying; this would mean the only symptom beekeepers would see is a reduced worker population (Goodwin & McBrydie, 2000).

Further work is required to elucidate the mechanism by which these products produce mortality. However, a significant difficulty in isolating this mechanism is that formulation composition is protected under EU law (EC, 2009), preventing researchers from knowing the identity and concentration of the surfactants involved, or what other co-formulant groups are present (Cox & Surgan, 2006). This severely impedes our ability to understand what mechanism(s) is/are at play and hinders academic testing of relevant ecological pollutants. If the MSDS that accompanies a product included a list of all the components, then each component could be tested individually to isolate the compounds (or interaction of compounds) causing the observed mortality. We suggest that the necessity to properly test pesticide effects on wildlife outweighs company rights to withhold proprietary information.

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AUTHORS' CONTRIBUTIONS

E.A.S. and E.N.C. carried out the experiment, and E.A.S. performed the statistical analyses; E.A.S., E.N.C. and M.J.F.B. designed the experiment and wrote the paper; E.A.S. and M.J.F.B. conceived the project. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.80gb5mkqn (Straw et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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