



Manuscript on single and combined effects of key chemicals and other stressors on bees under field conditions across Europe

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PoshBee

**Pan-european assessment, monitoring, and mitigation
of stressors on the health of bees**



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Table of contents

1. Summary	4
2. Introduction	6
3. Material and Methods	7
3.1. Study sites	7
3.2. Treatment	8
3.3. Floral resources.....	8
3.4. Glyphosate residue analysis.....	10
3.4.2. Residue analysis	11
3.5. Pollen analysis.....	11
3.5.1. Parameters.....	11
3.6. Experiments on honey bees: <i>Apis mellifera</i>	11
3.6.1. Honey bee colonies.....	11
3.6.2. Honey bee assessments.....	12
3.6.3. Parameters.....	13
3.7. Experiments on bumble bees: <i>Bombus terrestris</i>	14
3.7.1. Bumble bee colonies.....	14
3.7.2. Bumble bee assessments.....	14
3.7.3. Parameters.....	16
3.8. Experiments on mason bees: <i>Osmia bicornis</i> and <i>Osmia cornuta</i>	17
3.8.1. Trap nests.....	17
3.8.2. Mason bee assessments	17
3.8.3. Parameters.....	19
3.9. Molecular genetic screening of bees	19
3.9.1. Parameters.....	20
3.10. Data analysis	20
4. Results.....	22
4.1. Glyphosate residues.....	22
4.2. Pollen protein-lipid ratio	23
4.3. Honey bees	25
4.4. Bumble bees.....	27
4.5. Mason bees.....	28
4.6. Bee pathogens	30
5. Discussion.....	31

1. Summary

Bees, which are the most important pollinators of both crops and wild plants, have been experiencing declines worldwide. Pesticide use and the decline of floral resources, among other factors, have been identified as causes of bee decline. Glyphosate-based herbicides are the most widely used pesticides in the world, and although glyphosate has been considered safe for bees, several studies have reported harmful effects on bees. The risk of the herbicide is currently being re-evaluated in the European Union to decide whether its authorization shall be extended beyond 2023. Field studies on possible interactions with other stressors and with realistic herbicide exposure are scarce.

In this manuscript (D7.3), we report the findings of two field experiments conducted in Spain and Germany to measure interactive effects of the herbicide Roundup (active ingredient = glyphosate) and resource availability measured by the total nectar sugar amount provided by the flowering habitat on or bordering the study sites on four model bee species (conducted as part of the PoshBee Task 7.3: Europe-wide field experiments to assess the effects of a key chemical and another stressor on different model bee species). Glyphosate effects were investigated on the model species the western honey bee (*Apis mellifera*), the buff-tailed bumble bee (*Bombus terrestris*), the European orchard bee (*Osmia cornuta*) and the red mason bee (*Osmia bicornis*). The study consisted of 16 almond orchard sites in Spain (near Murcia) and 16 vineyard sites in Germany (near Freiburg). Half of the sites were mechanically weeded (control) and the other half were sprayed with Roundup Ultimate (in Spain) or Roundup PowerFlex (in Germany). These two products are identical in composition.

Placement at Roundup-treated sites resulted generally in higher glyphosate residues in honey bees and *Osmia* cell walls in Spain, as well as *Osmia* cell walls and soil in Germany. In bumble bees, no clear difference in glyphosate residues was found between treatments, with only a few bee samples containing detectable glyphosate, potentially because during sampling no distinction was made between bees leaving the hive and returning foragers. We did not find effects of placement at Roundup-treated sites on the protein-to-lipid ratio of bee-collected pollen, the intertegular distance of bumble bees, the number of bumble bee queen cocoons, the number of adult bumble bee workers and the number of adult bumble bee males. In honey bees, no significant treatment effects were found on the number of workers, the hive weight, or the Varroa mite load. Also, the number of nesting *Osmia* females, the number of *Osmia* brood cells, and pathogen loads of the investigated bees were not affected by Roundup treatment of the study sites. In contrast, bumble bee colony weight was positively influenced by Roundup treatment. Nectar sugar content of the surrounding flowering resources did not influence the intertegular distance, the number of worker/male cocoons, the number of adult workers and the number of adult males in bumble bee colonies. Further the pollen lipid ratio, the number of adult honey bees, the number of nesting *Osmia* females and the number of *Osmia* brood cells were not affected by nectar sugar amount. In Germany on sites with high nectar sugar availability, bumble bee colonies at Roundup-treated sites had more worker/male cocoons and at the start of the experiment honey bee hives in Spain were heavier at sites with high nectar sugar amounts, but this difference diminished until the end of the experiment. Varroa mite load of honey bee colonies increased more at sites with high nectar sugar contents. In Germany the number of adult bumble bee gynes in Roundup colonies was higher at sites with low nectar sugar amounts, but no difference in gyne numbers was observed at high sugar sites. Conversely, in Spain Roundup honey bee colonies at high sugar sites showed a higher flight activity shortly after the weed control and no impact of treatment was observed in honeybee colonies on low sugar content sites.

Our results do not indicate a risk of glyphosate for the tested bee species when applied in plant rows of almond or vineyard sites. However, more studies examining what factors govern glyphosate exposure and effects on bees are needed to conclude on the safety of the substance. It is particularly unclear whether bees are more exposed and/or affected when glyphosate is applied not only in plant

rows but on larger areas as is done in potato fields before planting. There is also a need to understand mechanisms behind potential glyphosate effects observed in artificial feeding experiments or the positive impact on colony weight that we observed in bumble bee colonies in both tested cropping systems/countries.

2. Introduction

Bees are the most important group of pollinators. They not only maintain wild plant diversity (Ollerton et al., 2011; Potts, 2016), but are also critical for the pollination of 75% of crop species (Klein et al., 2007; Potts et al., 2016b). Unfortunately, declines of wild bees have been reported in recent decades (Biesmeijer et al., 2006; Goulson et al., 2015; Potts et al., 2010; Cameron et al., 2011). Several stressors have been identified as contributing to pollinator decline, such as habitat loss and floral resource decline (Goulson et al., 2008; Winfree et al., 2009; Steffan-Dewenter et al., 2002; Hendricks et al., 2007), pathogens (Cox-Foster et al., 2007), invasive species (Stout and Morales, 2009; Thomson, 2006), climate change (Williams et al., 2007; Dormann et al., 2008) and the use of plant protection products in agriculture (Goulson et al., 2015; Potts, 2016; Potts et al., 2016b; Potts et al., 2010; Rortais et al., 2005; McArt et al., 2017; Potts et al., 2016a). Among all pesticides, glyphosate-based herbicides are the most used worldwide (Duke and Powles, 2008). Glyphosate was first approved in the European Union in 2002 and is currently evaluated for authorization beyond December 2023 (European Commission, 2022). The broad-spectrum, non-selective herbicide is the only pesticide that inhibits the enzyme 5-enolpyruvyl-3-shikimate phosphate synthase of the shikimate-pathway, which results in the death of meristematic tissue (Steinrücken and Amrhein, 1980; Duke, 2018). Since only bacteria, fungi and plants are known to carry that target enzyme, the toxicity of glyphosate to most non-target organisms was considered to be low (EFSA, 2015). However, evidence for detrimental effects of glyphosate, especially on bees, have been reported in recent years. For example, in *Apis mellifera*, glyphosate exposure negatively affected larval and adult mortality (Dai et al., 2018; Vázquez et al., 2018; Motta et al., 2020), larval food intake (Dai et al., 2018; Vázquez et al., 2018; Goñalons and Farina, 2018), larval development (Dai et al., 2018; Farina et al., 2019; Odemer et al., 2020), ecdysis (Vázquez et al., 2018), adult hatch weight (Farina et al., 2019) and immune response (Vázquez et al., 2018; Tomé et al., 2020). Glyphosate also increased mortality in *Tetragonisca angustula*, *Melipona quadrifasciata* (Ruiz-Toledo and Sánchez-Guillén, 2014) and *Hypotrigena rufipoli* (Motta et al., 2020). Additionally, glyphosate impaired thermoregulation abilities of *Bombus terrestris* workers (Weidenmüller et al., 2022). In *Apis mellifera*, glyphosate furthermore impaired bee navigation (Balbuena et al., 2015), associative learning and cognitive abilities (Luo et al., 2021), sleep frequency (Vázquez et al., 2020), sensory abilities, sucrose responsiveness and olfactory learning (Luo et al., 2021; Goñalons and Farina, 2018). Nevertheless, lethal or sublethal effects of glyphosate on bees and synergies between different stressors are not yet fully understood. Moreover, these questions are mostly investigated in semi-field or laboratory studies, so there is a lack of field studies with realistic exposure.

In addition, floral resources can alter the effects of pesticides on bees. For example, providing alternative floral resources reduced negative effects of an insecticide on mason bee offspring (Klaus et al., 2021). Environmental conditions and flowering habitats surrounding pesticide applications mitigated the harmful relationship between wild bees and pesticides (Park et al., 2015; Boff et al., 2020) and detrimental insecticide impacts on bumble bees were mitigated by the availability of non-crop flowers in the surrounding (Ingwell et al., 2021). Furthermore, the tolerance of bumble bees to a fungicide was dependent on the plant resource (Wintermantel et al., 2022), and on the other hand, nutritional stress exacerbated the effects of an insecticide on behaviour, reproduction and survival of *Osmia bicornis* (Knauer et al., 2022). These and other studies show the importance of the landscape context, especially floral resource availability and quality for bees.

To determine the interactive effects of the glyphosate-based herbicide Roundup and floral resource availability on honey bees, bumble bees and mason bees under typical European field conditions, we conducted a large-scale experiment in almond orchards in Spain and vineyards in Germany. In each country, 16 sites were selected and paired based on land use in the surroundings of which one site per pair was sprayed with Roundup Ultimate (in Spain) or Roundup PowerFlex (in Germany) and the other was mechanically weeded. Mechanical weeding was chosen as the control treatment, because

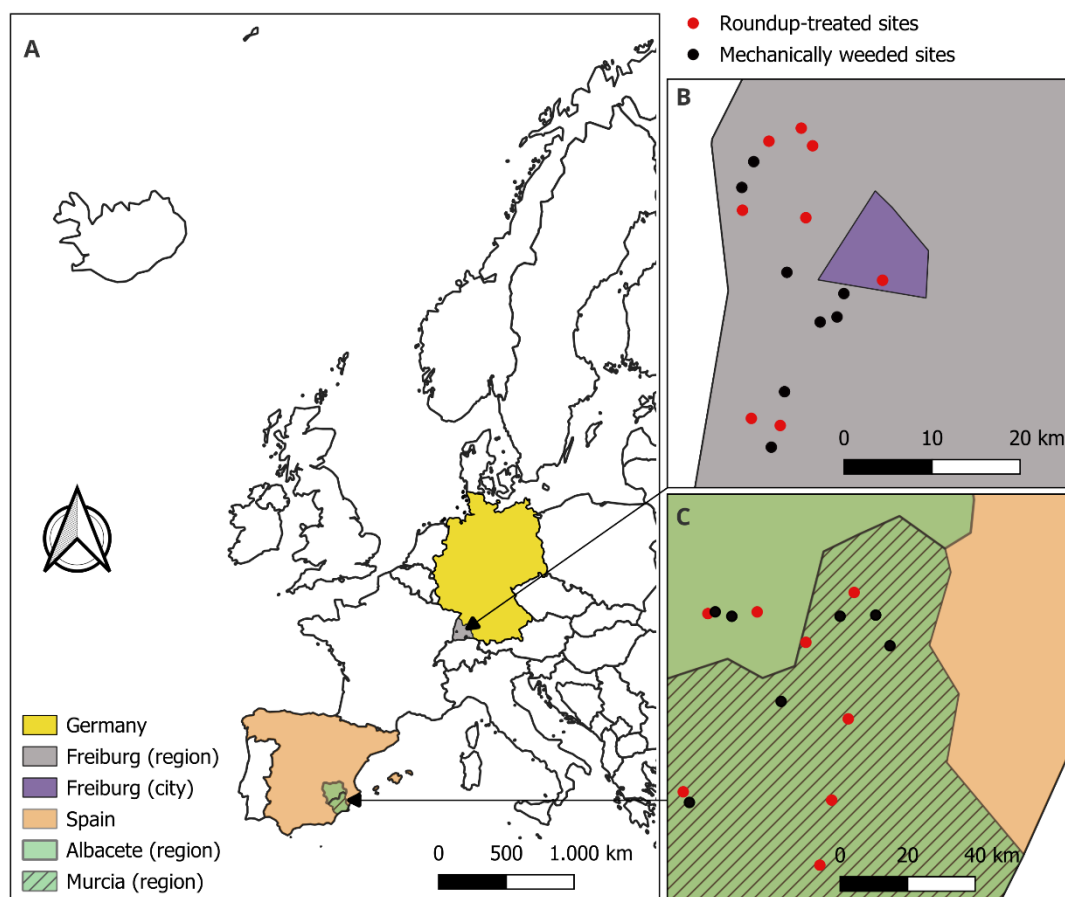
it is the most common alternative to glyphosate-based weed control. No weed control is often not a realistic alternative and the study does not aim at determining the indirect impact of reducing floral resources through Roundup but rather the direct toxic effects and their potential mitigation through increased floral resource availability.

3. Material and Methods

In 2022, two field studies were conducted in south-eastern Spain and south-western Germany. In both studies interactive effects of the glyphosate-based herbicide were investigated on the species *Bombus terrestris* and *Osmia cornuta*. In addition, the effects on *Apis mellifera* in Spain and *Osmia bicornis* in Germany were studied. Half of the sites were mechanically weeded (control) and the other half was sprayed with formulations of the herbicide Roundup (active ingredient = glyphosate) that are identical in composition (Roundup Ultimate in Spain and Roundup PowerFlex in Germany).

3.1. Study sites

The study in Spain was conducted on 16 almond orchards in the provinces of Murcia and Albacete (Figure 1). The study in Germany was conducted on 16 vineyards in the regions Kaiserstuhl and Markgräflerland (Figure 1). All sites were selected based on several criteria, such as non-organic management, distance from other field study sites (at least 3 km in Spain and at least 2 km in Germany), and expected almond flowering time between the beginning of February and the beginning of March (the latter only applies to Spain). **Figure 1: Overview map (A) with extracts**



showing 16 vineyard sites in the Freiburg region in Germany (B) and 16 almond sites in the regions of Albacete and Murcia in Spain (C).

In Spain, sites were paired based on land use composition within a 2 km radius, spatial proximity and expected onset of almond bloom and then of each pair one was randomly assigned to Roundup treatment and the other to mechanical weed control. In two field pairs, random assignment was, however, not possible as one of the fields was ploughed shortly before the experiment and was therefore assigned to the mechanical treatment. Furthermore, in Spain two fields were exchanged as neighbouring farmers objected to placement of honey bees near their fields. In Germany, sites to be treated with Roundup were chosen that were comparable in both their land use within 2 km and size to sites where mechanical weeding was planned.

Due to the assessment effort required and the long distances between sites, it was not possible to assess all sites on the same day. Therefore, in Spain sites were assessed in four groups separated by 1, 14 or 15 days and sites in Germany were assessed in two groups separated by 1 day. Thereby it was ensured that paired fields were assessed on the same day, which in turn ensured that on each day an equal number of Roundup-treated and mechanically weeded sites were assessed. In addition, assessment times relative to the treatment day were kept the same or at least comparable for all groups (as the treatment was delayed in groups that were assessed later).

3.2. Treatment

In both field studies half of the sites were weeded chemically with glyphosate-based herbicides produced by the company Bayer and half of the sites were weeded mechanically. In Spain chemical weeding was done with Roundup Ultimate (480 g/l glyphosate) with the maximum application rate of 4.5 l/ha. Mechanical weeding was done by ploughing. In Germany chemical weeding was done with Roundup Power Flex (480 g/l glyphosate), applied in the understock area of the vines at an application rate of 3.7 l/ha. These two herbicide products are identical in composition. Mechanical weeding was done by roller hake, brushing or mulching.

3.3. Floral resources

A field and a boundary area were defined for each of the 16 experimental sites. At every Spanish site, floral species richness and total floral abundance were recorded within two 50x2 m transect areas respectively in the field and the boundary. Information was gathered on non-crop flowering plants as well as the almond crop. In the case of the almond trees, only the flowers within the transects were estimated. The floral species richness describes the number of different species recorded at each experimental site respectively. In order to quantify floral abundance, the flowering species were identified and the number of floral units of those species found in the transect was counted. At the German sites, on four 1x1 m quadrats within the field and twelve within the boundary, floral species richness and abundance were surveyed accordingly. However, the crop vines did not flower here and were therefore not included in the German floral survey.

The amount of sugar, referring to the nectar sugar mass per 24 h per m², was derived from the floral abundance recorded on the experimental sites. For this purpose, reference values for the daily nectar sugar mass of each individual species were researched from existing scientific publications. When different nectar sugar mass values for a species were found in several studies, the mean of all found values was calculated and taken as the species' reference value. If only the genus was listed, the mean of different species' nectar sugar masses from this genus was provided.

For each species observation in each quadrat/transect, its number of floral units was multiplied by the respective number of single flowers per floral unit to obtain the total number of single flowers per species and quadrat/transect. Thereby, in Germany, three values for the number of single flowers per floral unit were sampled and their average was included in the calculation. Each total number of single flowers per species and quadrat/transect was then multiplied by the nectar sugar mass per 24 h of a single flower. Only in the case of *Asteraceae*, the number of floral units, here referring to a capitulum,

was directly multiplied by the nectar sugar mass per 24 h of a capitulum. Finally, the daily nectar sugar masses of all species of all field or boundary quadrats/transects of a site were summed up to the total daily nectar sugar mass of each site's field and boundary (respectively). Furthermore, a site average of sugar per site per 24 h per m² was calculated by scaling down the nectar sugar mass in field and boundary transects or quadrats.

Gaps in the databases were filled as follows: if no reference value for the daily nectar sugar mass could be found for a species, it was either replaced with the value for a species from the same genus, a mean value for the genus, or by the mean of the values of all other species from the same family recorded at the experimental sites (Table 1). If the number of single flowers per floral unit was missing for a species in a certain site quadrat or transect, it was either replaced by the mean of the number of single flowers per floral unit of the species in all other site quadrats or transects or it was estimated by reference to an image of the species. Further, values with less-than or greater-than indications have generally been included as just the reference number, e.g. if $x > 10$ it was calculated with $x = 10$.

Table 1: Original flowering species as recorded at the experimental sites (for which no nectar sugar mass was found) and their substitute species from which the nectar sugar mass was obtained as an approximation.

Original species	Substitute
<i>Amaryllis</i> sp.	mean of different <i>Amaryllidaceae</i> species
<i>Anacyclus clavatus</i>	mean of all other recorded <i>Asteraceae</i> species
<i>Biscutella auriculata</i>	<i>Biscutella laevigata</i>
" <i>Brassica</i> "	mean of all other recorded <i>Brassicaceae</i> species
<i>Campanula pratensis</i>	<i>Campanula patula</i>
<i>Carduus</i> sp.	<i>Carduus nutans</i>
<i>Carrichtera annua</i>	mean of all other recorded <i>Brassicaceae</i> species
<i>Carum carvi</i>	mean of different <i>Apiaceae</i> species
<i>Centaurea scabiosa</i>	<i>Centaurea</i> spp.
<i>Chrysanthemum coronarium</i>	mean of all other recorded <i>Asteraceae</i> species
<i>Cistus clusii</i>	<i>Cistus</i> spp.
<i>Crucifera "Amarilla"</i>	mean of all other recorded <i>Brassicaceae</i> species
<i>Dianthus carthusianorum</i>	<i>Dianthus</i> spp.
<i>Diplotaxis eruroides</i>	<i>Diplotaxis tenuifolia</i>
<i>Diplotaxis ilorcitana</i>	<i>Diplotaxis tenuifolia</i>
<i>Erodium malacoides</i>	<i>Erodium cicutarium</i>
<i>Fumaria parviflora</i>	<i>Fumaria</i> spp.
<i>Galium glaucum</i>	mean of all other recorded <i>Galium</i> species
<i>Geranium rotundifolium</i>	mean of all other recorded <i>Geranium</i> species
<i>Helianthemum almeriense</i>	<i>Helianthemum</i> spp. (ornamental)
<i>Helichrysum stoechas</i>	mean of all other recorded <i>Asteraceae</i> species
<i>Hippocrepis comosa</i>	mean of all other recorded <i>Fabaceae</i> species
<i>Hypocoum imberbe</i>	mean of all other recorded <i>Papaveraceae</i> species

Original species	Substitute
<i>Isatis tinctoria</i>	mean of all other recorded <i>Brassicaceae</i> species
<i>Knautia pratensis</i>	<i>Knautia arvensis</i>
<i>Leucanthemum ircutianum</i>	<i>Leucanthemum vulgare</i>
<i>Lobularia sp.</i>	<i>Lobularia maritima</i>
<i>Lysimachia arvensis</i>	<i>Lysimachia nemorum, nummularia</i>
<i>Moricandia arvensis</i>	mean of all other recorded <i>Brassicaceae</i> species
<i>Pyracantha coccinea</i>	<i>Pyracantha spp.</i>
<i>Reichardia tingitana</i>	mean of all other recorded <i>Asteraceae</i> species
<i>Reseda phyteuma</i>	<i>Reseda lutea</i>
<i>Reseda sp.</i>	<i>Reseda lutea</i>
<i>Rhinanthus alectorolophus</i>	<i>Rhinanthus minor</i>
<i>Rumex acetosella</i>	<i>Rumex spp.</i>
<i>Scabiosa pratensis</i>	<i>Scabiosa spp.</i> (ornamental)
<i>Sisymbrium irio</i>	<i>Sisymbrium officinale</i>
<i>Sisymbrium orientale</i>	<i>Sisymbrium officinale</i>
<i>Sonchus tenerrimus</i>	mean of all other recorded <i>Asteraceae</i> species
<i>Thymus hyemalis</i>	<i>Thymus vulgaris</i>
<i>Valerianella locusta</i>	<i>Valerianella spp.</i>

3.4. Glyphosate residue analysis

3.4.1. Samples

Residues in honey bees

The glyphosate residues in honey bee foragers were assessed in Spain only. 90 individuals were analysed per site, sampled 1 day after treatment. The residues are given in ppb.

Residues in bumble bees

In Spain, 18 individuals were sampled per site, sampled 1-2 days after treatment. In Germany, 9-19 individuals were analysed per site, sampled 1 and 7 days after treatment. The residues are given in ppb.

Residues in *Osmia*

To obtain samples, 1 *Osmia* trap nest per site was sacrificed 3-5 nights after treatment in Spain and 3 nights after the treatment in Germany. In Spain, males and females were pooled together to obtain sufficient bees for analysis (10 were required), which was the case on 12 sites. These samples contained 11-36 bees. In Germany, *Osmia bicornis* and *Osmia cornuta* nesting females were pooled together. On ten sites, sufficient nesting females were obtained for analysis. These samples contained 11-24 females. All residues are given in ppb.

Residues in soil

Per site, two soil samples of 50 ml were taken 1-2 days after treatment in Spain and 1 day after treatment in Germany. Each sample consisted of top soil collected in 5 different spots within the focal field. All glyphosate residues are given in ppb.

Residues in cell walls

From the *Osmia* trap nests that were sacrificed three days after treatment, mud from cell walls was extracted for residue analyses. In Spain, on 14 sites samples with 1.0-14.3 g of soil were obtained and analysed for glyphosate residues. In Germany, on 14 sites samples with 1.6-12.5 g of soil were obtained and analysed. All residues are given in ppb.

3.4.2. Residue analysis

For the analysis of glyphosate residues, 1 g of each sample was extracted with 10 ml acidified water (0.1 % HCOOH). Dichloromethane was then added to the extract to remove hydrophobic matrix components. The extract was then filtered and neutralised. Molecularly imprinted solid phase extraction (MIP-SPE) with AFFINIMIP glyphosate served as the first purification step. Subsequently, the extract was derivatized by addition of 9-fluorenyl methyl chloroformate (FMOC-Cl). After derivatization, solid phase extraction (SPE) was performed with Oasis HLB to remove FMOC-OH and residual borate buffer. Instrumental analysis was performed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

3.5. Pollen analysis

3.5.1. Parameters

Protein-lipid ratio

Pollen collected by honey bees (only in Spain), bumble bees, *Osmia cornuta*, and *Osmia bicornis* (only in Germany) was analysed for protein and lipid contents. Honey bee-collected pollen was sampled 2-11 days after treatment using pollen traps that had been activated for 24 hours. Pollen was only collected from one honey bee colony per site. Bumble bee-collected pollen was sampled directly from foraging bees 1-2 days after treatment in Spain and 14-15 days after treatment in Germany. *Osmia* pollen was collected 3-5 days after treatment in Spain and 3 days after treatment in Germany from the sacrificed trap nests. After the pollen composition was analysed, the protein-to-lipid ratio was calculated.

3.6. Experiments on honey bees: *Apis mellifera*

3.6.1. Honey bee colonies

In Spain, glyphosate effects were investigated on the honey bee species *Apis mellifera*. The honey bee colonies used in this study were selected on the basis of several criteria. The colonies had to have marked young queens (ideally 1 year old; 2 years max.) from similar genetic origin, be free from diseases (or have 'typical' acceptable low level of pathogens and pests) and have normal strength for the season and location. The colonies had to be similar in overall size (adult bees and brood), brood composition (relative amounts of eggs, open and capped brood) and in food resources (honey, pollen). They also had an adult bee population that covered at least 7 to 10 frames, containing at least: 5-6 frames of brood, 2-3 frames of food resources, and 1-2 empty frames in order to allow for colony growth (Figure 2)

Figure 2: Open honey bee hive with frames during assessment at a Spanish almond site.



3.6.2. Honey bee assessments

Placement on sites

Six colonies per site were placed 14 days before treatment at the edge of each field. The colonies were placed in two separate groups of three hives. A pollen trap and a dead bee trap were attached to two different hives per site (Figure 3).



Figure 3: Honey bee hives at the Spanish almond sites, left hive with pollen trap and right hive with dead bee trap.

Colony assessments

The honey bee colonies were assessed twice, once 12 to 10 days before the treatment and 21 (batch 1), and 23 (batch 2) days respectively after the treatment. In the colony assessments the parameters hive weight, number of adult bees, surface area of capped brood and stored pollen, number of adult female *Varroa* mites, presence/absence of signs of several diseases and the presence/absence of queen, queen cells and eggs were recorded. In addition, 15 adult bees were taken from each colony

for pathogen analysis, placed on dry ice and then stored at -20 °C. Flight activity was assessed 7 (batch 1) and 8 (batch 2) days before the treatment and one day after the treatment. One day after treatment, haemolymph was collected and bees and pollen were sampled for residue analysis. Starting one week after the pre-exposure assessment, weekly colony checks were done until the post-exposure assessment.

3.6.3. Parameters

Varroa mite infestation

The protocol for quantifying female *Varroa* mites per colony has been taken/adapted from [deliverable D1.1](#). To quantify levels of *Varroa* infestation, we used examination of debris in each hive based on a method given by the OIE Terrestrial Manual (2008). All hives were equipped with a bottom board that was protected by a mesh which allowed mites to fall through but prevented bees access, otherwise bees would have discarded the dead mites from the hive. After cleaning the bottom board, three days prior to each assessment a yellow sticky trap was inserted onto it. During the assessment, all adult female *Varroa* mites were counted directly on the sticky traps in the field. If easy detection of mites was prevented by a large amount of debris, the boards were covered in plastic (“cling”) film and examined later in the laboratory.

Number of adult bees

The protocol for colony strength assessment was adapted from [deliverable D1.1](#). Pollen was collected from only one colony per site.. Colony strength was assessed subjectively (using the COLEVAL method) following Delaplane et al. (2013) and Sandrock et al. (2014). The method is based on a human observer, who visually estimates the surface area of each comb covered with bees. To measure colony strength, colonies were opened and each comb was removed sequentially. All estimates were made to the nearest 5%. The total area refers to the inner border of the wooden frame. Both sides per comb were estimated separately. The observer looked at one side of a comb and visually estimated the percentage of the comb surface covered by bees, then turned the comb and estimated the percentage of bees on the second side. These estimates were done quickly as bees tend to move a lot and walk from one side of the frame to the other. Combs were labelled with continuous numbers; comb sides were referred to as A and B. So, 2A referred to the first side of the second comb.

Hive weight

Hives were weighed without the dead bee trap, the pollen trap or the sticky boards. The hives were placed on the scale and the weight was recorded. If it was not possible to place the entire hive on the scale at once, the boxes were placed separately and the recorded weights of the boxes added together.

Flight activity

Each hive was observed for two minutes and the total number of bees entering the hive and the number of bees carrying pollen were recorded using click counters. In addition, the weather conditions (temperature, sunniness, wind) during the assessment were documented.

3.7. Experiments on bumble bees: *Bombus terrestris*

3.7.1. Bumble bee colonies

The experimental bumble bee colonies were purchased in a standard-size hard plastic nest (23x30x20 cm) with two entrance options (a two-way opening hole and a one-way gate tube) and a sliding closure that doubled as a queen excluder. They were delivered with pollen and nectar supplements for transport.

3.7.2. Bumble bee assessments

Pre-placement assessment

After delivery, the bumble bee colonies were examined in an initial assessment in the laboratory (Spain: 7-5 days before treatment; Germany: 6-3 days before treatment). To reduce bee activity, the examinations were performed in a dark room under red light (as bees cannot perceive red light). The standard plastic cover was replaced by a transparent Plexiglas cover to be able to photograph the colonies without having to open the nest. Once the pollen supplement and nectar reservoir were removed from the interior of the nest, the entire nest box was weighed. Dead adults and dead pupae were counted and removed. The foundress queen was marked on its thorax. If a wax ceiling covered parts of the nest, a photograph of the nest was taken to estimate the covered area. The wax ceiling was subsequently removed and weighed. Under daylight conditions the number of cocoons was counted, and photographs of all nests were taken to count the number of living adults afterwards.

Allocation to sites

The previously examined parameters nest weight, number of dead adults, number of dead pupae, number of adults and number of cocoons were used with the R package “anticlust” (Papenberg and Klau 2020) to allocate the colonies semi-randomly to the study sites for each field study. The above parameters were used to determine the similarity of the colonies and were centred at mean = 0 and standardised at standard deviation = 1. In addition, the number of adults was weighted more heavily and therefore considered twice. Pairs of colonies, that were the most equal were formed with the function “matching”. For each study the 48 most similar pairs were selected and the remaining four colonies became surrogate colonies. The paired colonies were then divided into two groups with the function “anticlustering”. These groups were selected in a way, that within-group heterogeneity was high and between-group heterogeneity was low. These two groups were randomly assigned to the different treatments of mechanical weed control or Roundup application. Eight groups were formed within treatment groups, so that heterogeneity within groups was high and heterogeneity between groups was low. These groups were then randomly assigned to the different study sites.

Placement at sites

Six bumble bee colonies were placed at each study site 5-3 days (Spain), and 3 days (Germany) prior to the treatment. To inhibit access to a food source supplementary to the available floral resources, the large Biogluc reservoirs (containing a sugar solution for transport) underneath the colonies were closed. In Spain, two plastic nest boxes were packed into one polystyrene box, with the individual nests separated from each other (Figure 5A). These polystyrene boxes were placed at a minimum distance of 3 m from each other, facing the orchards to the south (Figure 4A). In Germany each plastic nest box was put into a bigger wooden box, with two holes in the front, which were aligned to the queen-excluder exit and the one-way entrance of the plastic nest boxes. Additionally, the wooden boxes had ventilation holes on the sides and an overhanging roof for weather protection. These wooden boxes were placed at a minimum distance of 1.5 m from each other, facing the vineyards to the south (Figure 4B). A bee counter was installed at the entrance of the third hive at each site to track bee activity (Figure 5B). In addition, two temperature measuring devices (iButtons) were also attached

to the third hive on every site (Figure 5B). One iButton was placed inside the colony and one was placed outside in the bee counter on the wooden box, which measured the temperature in 20-minute intervals inside and outside the colony.



Figure 4: Arrangement of bumble bee colonies in polystyrene boxes in Spanish almond orchards (A) and wooden boxes in German vineyards (B) facing the experimental sites to the south.



Figure 5: Two bumble bee colonies in a polystyrene box in a Spanish almond orchard with a pollen trap installed on the right-hand-side colony (A) and a bumble bee colony in a wooden box in a German vineyard with a bee counter installed (B).

In-field assessments timetable

The first colony assessments were done 3-5 days (Spain), and 3 days (Germany) after treatment application. The second assessments followed 9-12 days (Spain), and 9 days (Germany) after the treatment. In Spain the third assessments were done 18 days after treatment application only on sites from the first batch. In Germany the third and fourth assessments were done 16 and 23 days after the application. During the assessment the parameters adult and larval mortality, colony weight, wax weight and wax cover area, survival of the foundress queen and production and hatching of new queens were examined. Additionally, photographs of the nests were taken and in Spain, if the

assessment was done during the daytime, another photograph was taken at night to ensure the highest possible number of bumble bees would be present in the nest and therefore in the photograph.

Colony termination

Colonies were terminated either when their foundress queen was found dead or at the latest 28-29 days after treatment application. The terminated colonies were collected at night, to make sure most of the bees were inside the hives. After collection dead adults and dead juveniles were, if possible, removed and counted from the terminated colonies under red light. Subsequently these colonies were frozen and stored at -20 °C. After the experiment, all colonies were dissected and various parameters were recorded.

3.7.3. Parameters

Unless stated otherwise, each parameter was measured in the colony dissection after the field experiment.

Colony weight

The colony weight was continuously recorded for all assessments. Each colony including its nest plastic box was taken out of the polystyrene/wooden box and weighed on a digital scale.

Worker ITD

15 individual adult worker bees that were assumed to be alive during the termination, if possible completely developed and free of parasites or signs of DWV, were randomly picked. The intertegular distance of each individual was measured with a digital sliding calliper as a measure for individual bee size.

Number of worker/male cocoons

The final number of worker/male cocoons in the frozen nest was counted. Worker/male cocoons were identified by their width below 12 mm.

Number of queen cocoons

In the colony dissection after the field experiment, the final number of queen cocoons in the frozen nest was counted. Queen cocoons were identified by their width above 12 mm.

Number of live adult gynes

The final number of live adult gynes in the frozen nest was counted. Gynes were identified by the presence of a stinger, their larger size than a worker female and the lack of the foundress queen's marking and worn out appearance.

Number of live adult workers

The final number of live adult workers in the frozen nest was counted. Workers were identified by the presence of a stinger.

Number of live adult males

The final number of live adult males in the frozen nest was counted. Males were identified by the lack of a stinger and the two claspers in their genital region.

3.8. Experiments on mason bees: *Osmia bicornis* and *Osmia cornuta*

For both experiments mason bee cocoons were ordered from PolliNature. For the study in Spain 4000 male and 2000 female *Osmia cornuta* cocoons and for the study in Germany 5500 male and 3500 female cocoons of both *Osmia* species were purchased. The cocoons were stored at 2 °C prior to the experiments. However, as a large proportion of the mason bees in both trials had already begun to hatch in the laboratory, 1000 male *Osmia cornuta* cocoons (Spain), 3200 *Osmia cornuta* cocoons (Germany) and 1200 *Osmia bicornis* cocoons (Germany) had to be reordered. The cocoons were then put in trap nests which were installed at the study sites.

3.8.1. Trap nests

The trap nests consisted of 15 nesting boards each containing 10 brood channels with a length of about 15 cm and a diameter of 0.8 cm. Every nesting board was closed at the top with an acetate cover to make it possible to take photos of the dormant bees and the status of the brood cells at night. Additionally, every trap-nest and every nesting board was labelled with the specific nest ID and board ID (Figure 6A). On top of the nesting boards a release box containing the cocoons was placed. The release box was fashioned as a circular cavity containing opening(s) out of which the hatched adults could emerge. The cocoons were divided between sexes by a fitted cardboard strip with males on one and females on the other side of the release box. The trap-nest containing the nesting boards and the release box was fixed together with a lashing strap (Figure 6B).

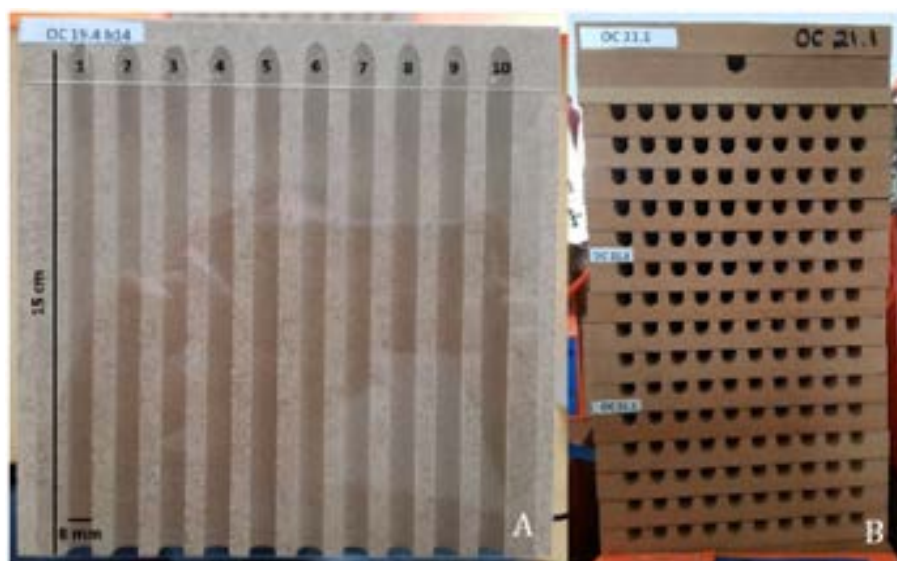


Figure 6: Nesting board with ten channels (15 cm x 8 mm x 8 mm), labelled with a board ID in the top left corner and topped with an acetate cover (A); Complete trap-nest containing 15 boards and one release box on top, held together with a lashing strap (B).

3.8.2. Mason bee assessments

Placement at sites

The trap nests were placed in wooden boxes, designed to shield them from varying weather conditions. These boxes were placed on wooden poles at a height of about 1.5 m facing south towards the experimental plots (Figure 7). A total of four trap nests with *Osmia cornuta* cocoons were placed at each trial site and additionally in Germany four trap nests with *Osmia bicornis* cocoons. To reduce the likelihood of bees nesting in trap nests of the other species (i.e. *Osmia cornuta* in trap nests

intended for *Osmia bicornis* and vice versa), in Germany the groups of nests of the same species were placed as far apart as the sites allowed.



Figure 7: *Osmia cornuta* trap-nest setup in Spanish almond orchards with attached camera and battery on the fourth nest.

For further research the fourth *Osmia cornuta* nest on each site was equipped with a camera, which filmed the trap-nest entrance and was connected to a battery box on the ground (Figure 7). All *Osmia cornuta* nests were equipped with 65 male and 45 female cocoons. Further, in Germany *Osmia bicornis* nests were equipped with 70 male and 50 female cocoons. Sex determination was done by cocoon size by PolliNature, as female cocoons tend to be notably larger than male cocoons in both species. The trap nests were equipped with cocoons 7-8 days (Spain), and 11 days (Germany) prior to the treatment application.

In-field assessments timetable

The first assessment took place the night before the treatment application. After that, the fields were visited regularly after sunset on a weekly schedule. In Spain, the assessments were done 3, 8, 16 and 35 days (first group of colonies), and 4 or 3, 11 or 10 and 34 days (second group of colonies) after treatment application. In Germany, assessments were done 3, 9, 16 and 23 days after treatment. In the assessments, photos of the nesting boards were taken and nesting behaviour and brood cell development in the trap nests was examined. Further to this the presence of mould, natural enemies, other insects, other wild bee species or wasps were recorded and if possible, identified to species level.

Further measurements were taken in addition to the parameters that were examined every week. Two individuals per trap nest were caught with an insect net for haemolymph sampling one day after the treatment application. In Germany, one female per trap nest was caught for pathogen analysis in the first and fifth assessment and 4 females/bees per nest in Spain after the almond blossom. During the second assessments the cocoons in the release box were sampled to determine the ratio of hatched and unhatched cocoons for both sexes. To obtain samples for further analyses of pollen and cell walls, one trap-nest per site was sacrificed after the second assessment. For this, in Spain, the *Osmia cornuta* trap-nest with the fewest roosting bees was selected, while in Germany generally the *Osmia bicornis* trap-nest with the fewest roosting sleeping females was selected, except in two sites where the *Osmia cornuta* trap-nest with the fewest roosting bees was sacrificed.

Termination

In Germany, after the last assessment, all adults were removed from the trap nests and collected to prevent further breeding cells from forming after the trial. To avoid interference by natural enemies or scavengers, all other insects in the trap nests were also removed. The nests were then covered with

fine mesh netting and sealed with strong outdoor tape to prevent further nesting and invasion by insects. The trap nests in Spain were also left on the experimental sites, but without further preparation.

3.8.3. Parameters

Number of nesting females

Nesting bees were counted individually in each nesting board. The resting *Osmia* were distinguished by sex and, in Germany, additionally by species.

Number of brood cells

The extent of new cell production was marked on the acetate cover with a permanent waterproof marker. This allowed for an easier determination of the temporal extent of brood development of the specimen after the treatment.

3.9. Molecular genetic screening of bees

RNA extraction, cDNA synthesis and PCR screening of bee parasites and members of the microbiome

Bulk samples of 8 (*Bombus terrestris*) or 15 (*Apis mellifera*) bees from each hive/colony and sampling period were transferred to separate plastic mesh bags (Bioreba, Reinach, Switzerland) with 15 ml of RLT-buffer and crushed using a semi-automated homogenizer (Bioreba, Reinach, Switzerland). Then 1 ml of homogenate was transferred to a new tube, centrifuged for 3 min, and 100 µl of supernatant was added to 500 µl of the 1% beta-mercaptoethanol-RLT buffer mix followed by RNA extraction using an RNeasy kit according to the manufacturer's protocol (RNeasy Mini Kit, Qiagen, Hilden, Germany) in a QiaCube robot (Qiagen, Hilden, Germany). For *Osmia bicornis*, individual bees were crushed in a 1.5 mL Eppendorf tube with 500 µL RLT-buffer, of which 100 µl of supernatant was added to 500 µl of the 1% beta-mercaptoethanol-RLT buffer mix followed by RNA extraction as described for *Bombus terrestris* and *Apis mellifera* above. cDNA synthesis using 800 ng RNA per sample was performed using oligo-dT primers (ThermoFisher Scientific, Hennigsdorf, Germany) and M-MLV Reverse Transcriptase, (Promega, Mannheim, Germany) according to the manufacturers' protocols.

All samples were screened by PCR for three widespread bee viral pathogens: DWV-A, DWV-B, and BQCV.

Viral screening was performed from cDNA by qPCR in a QuantStudio 3 thermal cycler (Applied Biosystems/ThermoFisher Scientific, Germany) using SYBRgreen Sensimix (Bioline, Luckenwalde, Germany). Samples were run in technical duplicates (primer sequences in Table 2 and qPCR protocols in Table 3). A cut-off quantification threshold of 35 cycles ($C_q=35$) of averaged duplicate C_q values was used to determine the presence of a viral pathogen. If the standard deviation of the two duplicates exceeded 1, the sample was rerun. The honey bee β -actin gene was used as a reference gene to ensure successful RNA extraction, cDNA synthesis and qPCR. Each qPCR plate contained two negative control wells (H₂O as template) and a positive control (PCR product of a viral-positive sample). To confirm the specificity of primer binding, a melting curve profile was generated (one cycle of 95 °C for 1 min and 50 °C for 1 min followed by 50°C to 95°C at 0.5°C per second increments) for each well at the end of each qPCR; all samples had a correct melt profile (a single peak at the correct dissociation temperature for the PCR product).

Table 2: PCR primers used in qPCR of viral targets and a bee reference gene.

Target	Name	Sequence 5' – 3'	Reference
BQCV	BQCV-F7893 BQCV-B8150	F: AGTGGCGGAGATGTATGC R: GGAGGTGAAGTGGCTATATC	Locke et al. 2012
DWV-A	DWVq-F2 DWVq-R2a	F: TGTCTTCATTAAAGCCACCT R: TTTCTTCATTAAGTGGCT	McMahon et al. 2015
DWV-B	VDVq-F2 VDVq-R2a	F: TATCTTCATTAAACCGCCAGGCT R: CTCCTCATTAAGTGGCTGTGTC	McMahon et al. 2015

Table 3: qPCR protocol for amplification of viral targets.

Targets	Protocol
DWV-A, DWV-B, BQCV	1. 95 °C – 5 min 2. 95 °C – 15 s 3. 57 °C – 30 s 4. 72 °C – 30 s 5. 95 °C – 1 min Melting curve: 6. 50 °C – 1 min 7. increase of 0.5 °C /1 s until 95 °C

3.9.1. Parameters

For the pathogen analysis in Germany, *Osmia bicornis* females were sampled from each site 23 days after treatment. For bumble bees, 8 individuals per colony were sampled 20-31 days after treatment. All samples were screened for the following pathogens.

BQCV loads

The loads of Black queen cell virus (BQCV) in *Osmia bicornis* and bumble bees were analysed.

DWV-A loads

The loads of Deformed wing virus type-A (DWV-A) in *Osmia bicornis* and bumble bees were analysed.

DWV-B loads

The loads of Deformed wing virus type-B (DWV-B) in *Osmia bicornis* and bumble bees were analysed.

3.10. Data analysis

All analyses were conducted in R (version 4.2.1). The data on bee parameters were modelled with (generalised) mixed effects models ((G)LMMs) that contained a two-way interaction between treatment (categories: Roundup and mechanical weeding) and the natural logarithm of sugar nectar amount. For variables with repeated measurements, a three-way interaction between experimental day (days relative to treatment day; continuous), treatment and the natural logarithm of sugar nectar amount was used. All models on bee parameters contained site identity as a random effect and those analysing intertegular distance, which was measured per bee, contained colony identity as an

additional random effect. LMMs were fitted using the lmer function of the lme4 package. GLMMs were fitted using the glmmTMB function and package. Model residuals were visually analysed using the Dharma and performance packages and the quasi-Poisson (family="nbinom1") or negative binomial distribution (family="nbinom2") was used for modelling overdispersed count data (Table 4).

Models were evaluated by calculating estimated marginal means (EMMs) using the emmeans (for simple/main effects) and emtrends (for slopes) functions of the emmeans package. Treatment effects were typically estimated at low and high sugar amount values, whereby the low and high values represent the extremes of the range that was encountered in both treatment groups. In Spain these were 7.8 and 1300.3 mg day⁻¹ m⁻² and in Germany 0.8 and 21.5 mg day⁻¹ m⁻².

Table 4: Error distributions of models on bee parameters in Spain and Germany.

Bee taxon	Parameter	Spain	Germany
Honey bee	Nest weight	Gaussian	n/a
	Number of bees per colony	Gaussian	n/a
	Number of Varroa mites per colony	negative binomial	n/a
Bumble bee	Nest weight	Gaussian	Gaussian
	Worker ITD	Gaussian	Gaussian
	Number of worker/male cocoons	quasi-Poisson	quasi-Poisson
	Number of queen cocoons	quasi-Poisson	quasi-Poisson
	Number of alive workers	quasi-Poisson	quasi-Poisson
	Number of alive males	quasi-Poisson	quasi-Poisson
	Number of alive gynes	quasi-Poisson	negative binomial
<i>Osmia</i>	Number of nesting <i>O. bicornis</i>	n/a	quasi-Poisson
	Number of nesting <i>O. cornuta</i> females	quasi-Poisson	quasi-Poisson
	Number of <i>O. bicornis</i> brood cells	n/a	negative binomial
	Number of <i>O. cornuta</i> brood cells	quasi-Poisson	negative binomial
Bumble bee / <i>Osmia</i>	Virus cycle number	n/a	Gaussian
Honey bee / Bumble bee / <i>Osmia</i>	Protein-lipid ratio of bee-collected pollen	Gaussian	Gaussian

4. Results

4.1. Glyphosate residues

In Spain, in bumble bees, no glyphosate was found in either treatment group. In contrast, in honey bees, *Osmia* bees, soil and *Osmia* cell walls, glyphosate was found in both treatment groups (Figure 8). Glyphosate tended to be higher at Roundup-treated sites with a few samples from control sites also containing high levels of glyphosate. Glyphosate levels in soil and the mud of cell walls were considerably higher than in bees.

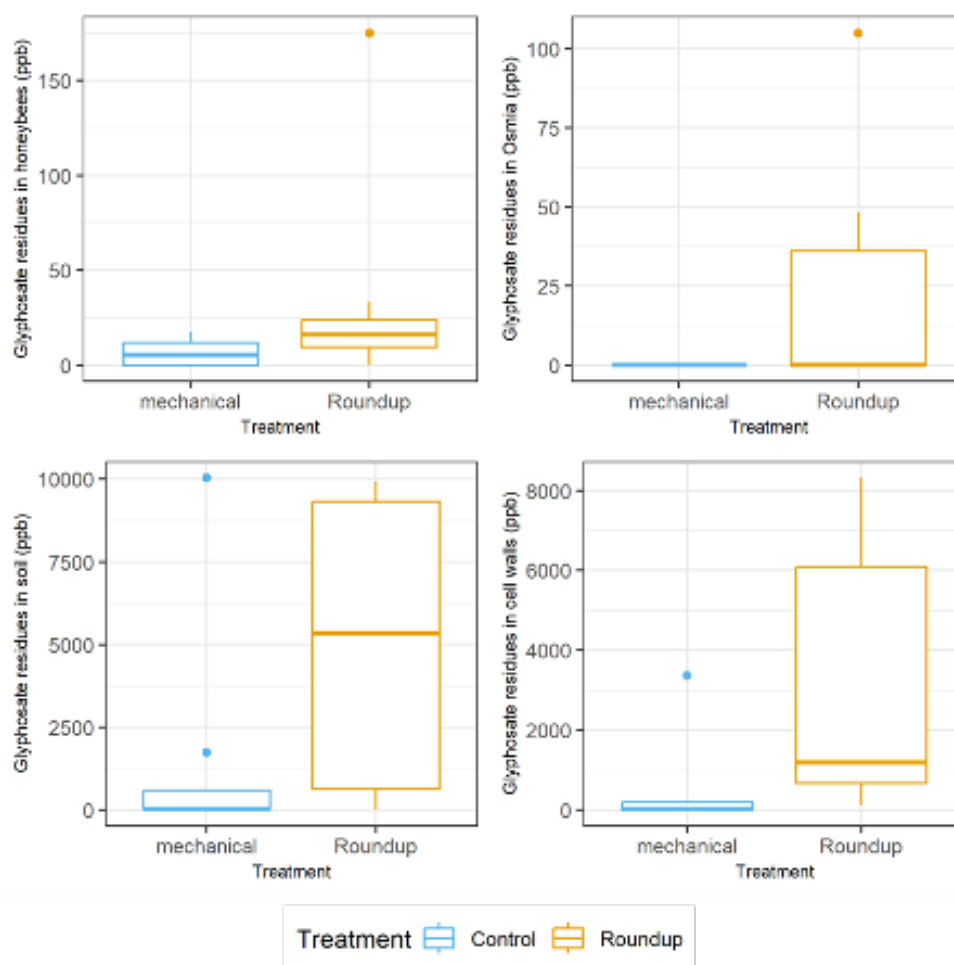


Figure 8: Glyphosate residues in honey bee foragers, *Osmia* foragers, soil and *Osmia* cell walls in relation to treatment in Spanish almond sites.

In bumble bees in Germany, glyphosate was found in two colonies of each of the two treatment groups. In *Osmia* bees, no glyphosate was found, but in their cell walls and soil, high levels of glyphosate were found (Figure 9). While Roundup sites had clearly higher soil residue levels than control sites, the difference was less pronounced in cell walls as a few control sites also exhibited high cell wall residue levels.

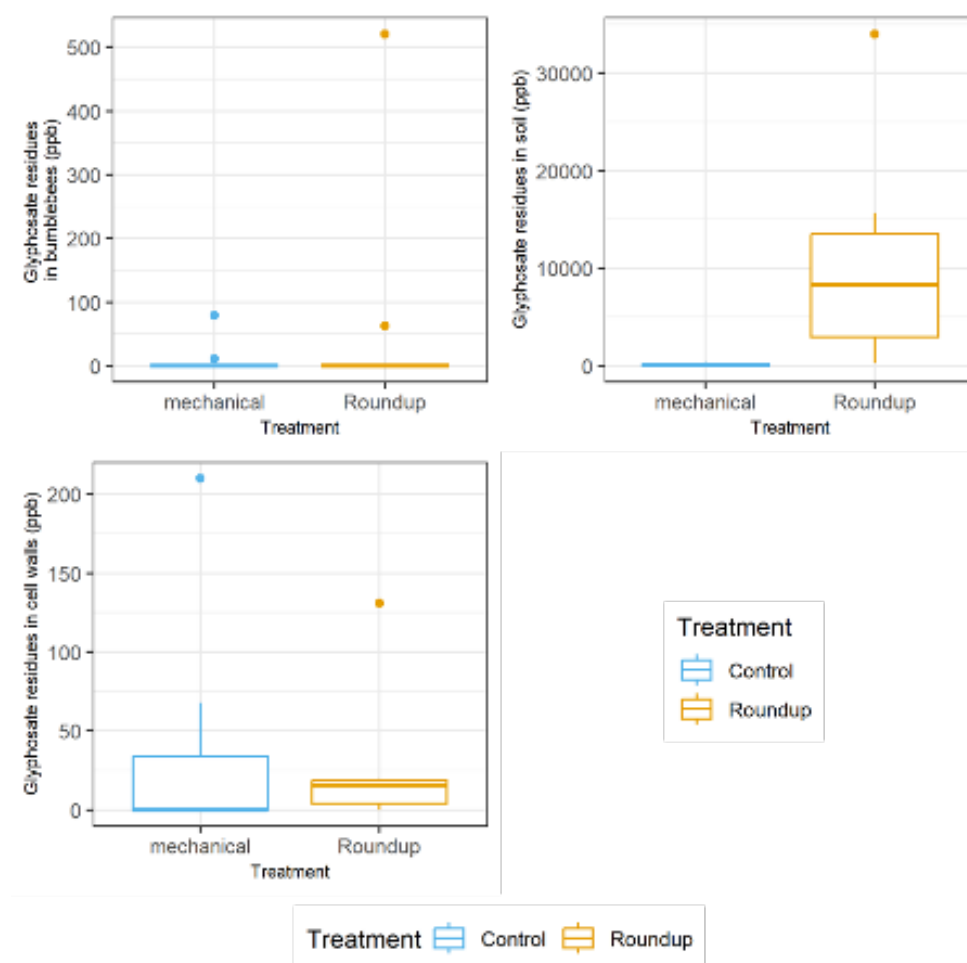


Figure 9: Glyphosate residues in bumble bee foragers, soil and *Osmia* cell walls in relation to treatment in German vineyard sites.

4.2. Pollen protein-lipid ratio

The protein-lipid ratio of pollen collected by the different experimental species was not affected by the available amount of sugar at the site, the treatment or bee species in Spain (Figure 10) or Germany (Figure 11).

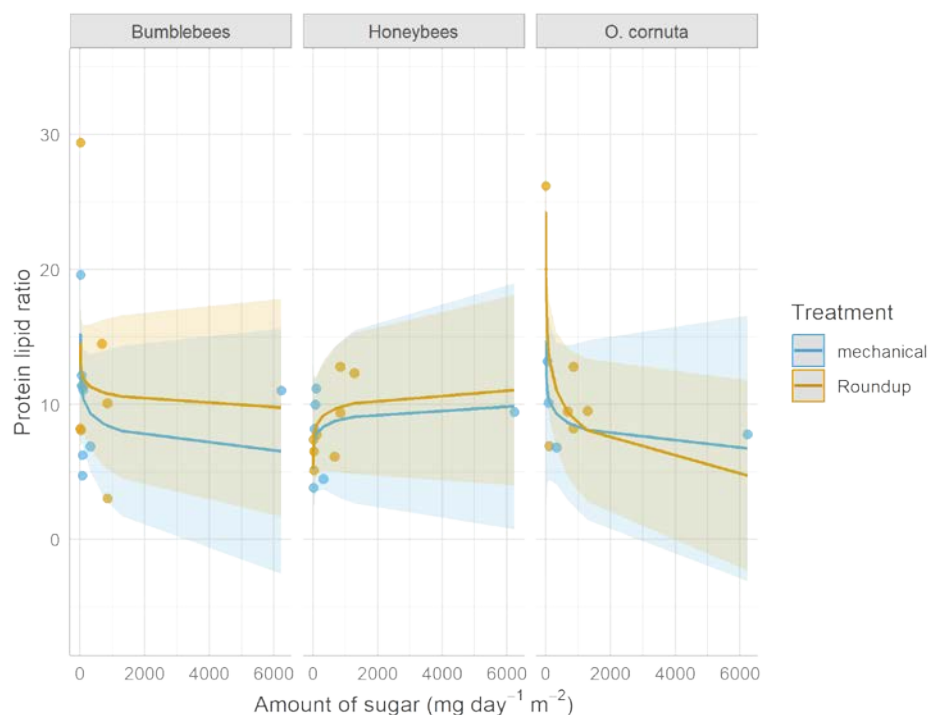


Figure 10: The pollen-lipid ratio of pollen per treatment at varying amounts of sugar at the Spanish sites, collected by bumble bees, honey bees and *Osmia cornuta*. The graphs are based on the fitted LM (with 95% confidence interval depicted transparently). The points display the observed values.

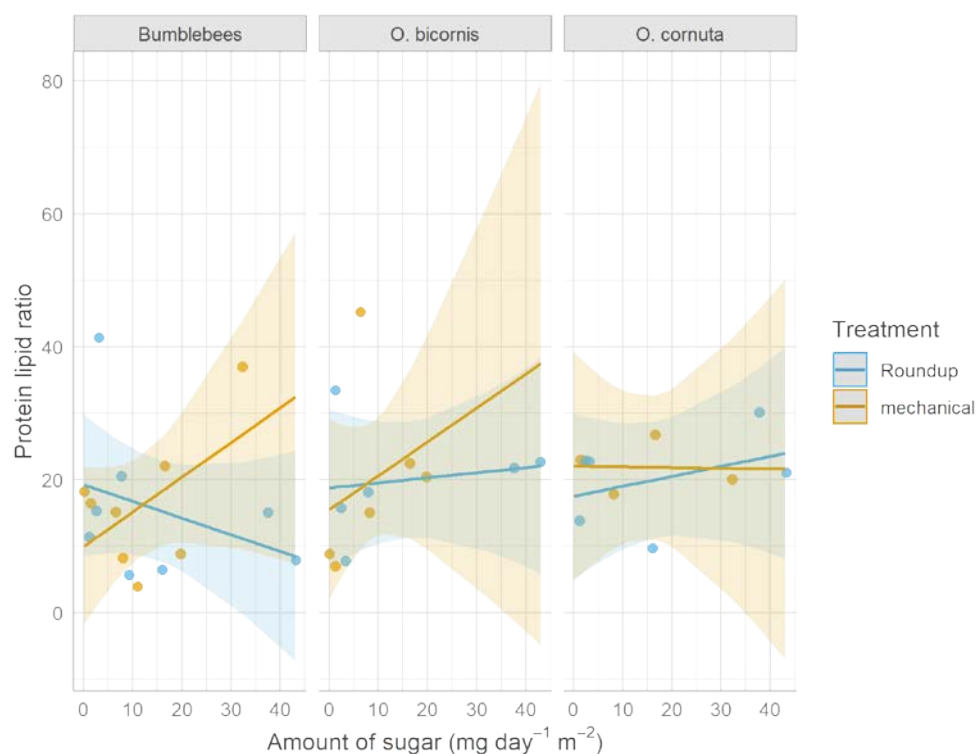


Figure 11: The pollen-lipid ratio of pollen per treatment at varying amounts of sugar at the German sites, collected by bumble bees, *Osmia bicornis* and *Osmia cornuta*. The graphs are based on the fitted LM (with 95% confidence interval depicted transparently). The points display the observed values.

4.3. Honey bees

Both the Roundup colonies and the colonies at mechanically weeded sites in Spain increased in weight over time (Figure 12). We found, however, no difference in weight gain between treatments ($p=0.24$). Colonies at sites with high sugar amount weighed 3.8 kg or 11% less at the start of the experiment than colonies at sites with low sugar amount ($p=0.047$). No difference in hive weight was found between low and high sugar amount sites at the end of the experiment ($p=0.179$). There were also no interactive impacts of sugar amount and treatment on weight gain ($p>0.2$).

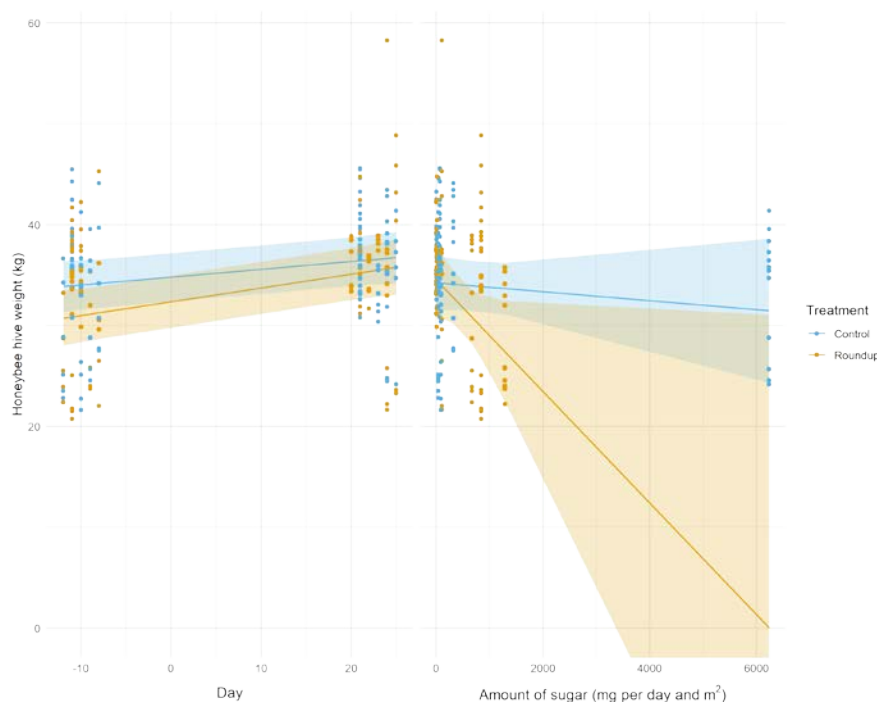


Figure 12: Honey bee hive weight in Spanish almond sites in relation to treatment, time, and amount of sugar. Shaded areas around the prediction lines obtained from a linear mixed-effect model depict 95% confidence intervals and dots represent observations.

The number of adult honey bees per hive increased over the study period in both treatment groups ($p<0.001$) with no apparent difference between treatments ($p=0.51$; Figure 13). We found no impact of sugar amount on the number of adult honey bees either before (day -10) or after the treatment (day 20).

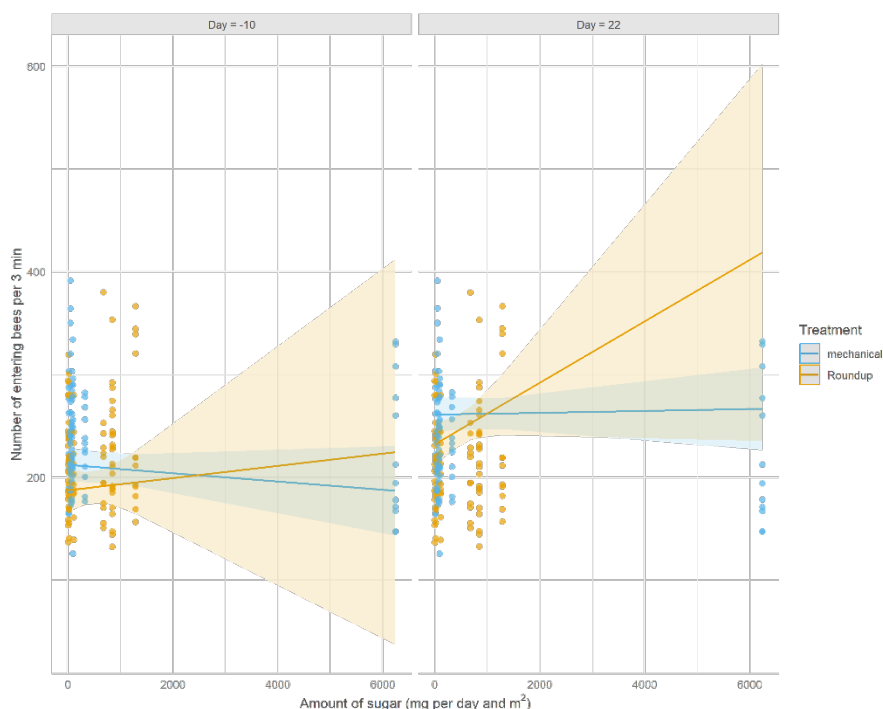


Figure 13: Number of honey bees per hive in Spanish almond sites in relation to treatment, time, and amount of sugar. Shaded areas around the prediction lines obtained from a linear mixed-effect model depict 95% confidence intervals and dots represent observations.

The flight activity shortly after the weed control was done depended interactively on treatment and sugar amount (Figure 14). While we found no impact of treatment in colonies at low sugar sites ($p=0.05$), colonies at Roundup-treated sites with high sugar had higher flight activity than colonies at mechanically weeded sites ($p=0.003$).

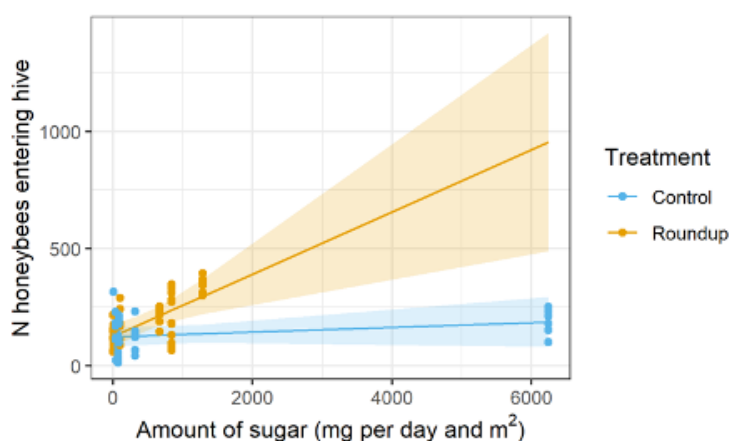


Figure 14: Number of honey bees entering their hive within 3 min in Spanish almond sites in relation to treatment and amount of sugar 1-3 days after treatment. Shaded areas around the prediction lines obtained from a linear mixed-effect model depict 95% confidence intervals and dots represent partial residuals.

The number of *Varroa* mites per colony increased over time ($p=0.002$) irrespective of treatment ($p=0.72$, Figure 15). However, this increase was not observed at sites with a low amount of nectar

sugar ($p=0.445$). In sites with high sugar amount, *Varroa* numbers in colonies at both mechanically weeded ($p=0.004$) and Roundup-treated sites ($p=0.032$) increased over time.

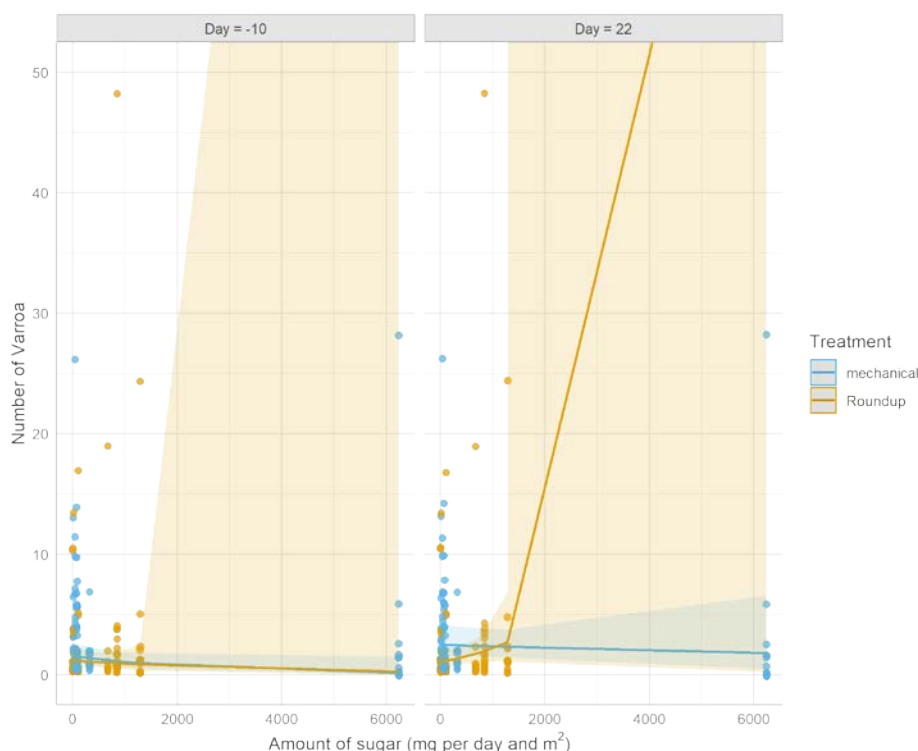


Figure 15: Number of *Varroa* per honey bee colony in Spanish almond sites in relation to treatment and amount of sugar (i.e. resource availability) before and after treatment. Shaded areas around the prediction lines obtained from a linear mixed-effect model depict 95% confidence intervals and dots represent partial residuals.

4.4. Bumble bees

The intertegular distance (ITD) of bumble bee workers did not differ significantly between Roundup and mechanical treatment at low or high nectar sugar sites in either Spain or Germany. While no difference between treatments was detected in the final number of worker/male cocoons at low and high nectar sugar sites in Spain as well as low nectar sugar sites in Germany, at German high nectar sugar vineyards, colonies at Roundup sites had 8.1% more final worker/male cocoons than those at mechanically weeded sites ($p=0.024$). The colony's weight gain was consistently positively influenced by the Roundup treatment. In Spain, the colonies at Roundup sites gained 6.6% more weight than those at mechanically weeded sites with low nectar sugar amounts ($p=0.048$) and 8.5% more at high nectar sugar levels ($p=0.028$). In Germany, the colonies at Roundup sites gained 11.3% more weight than those at mechanically weeded sites with low nectar sugar amounts ($p=0.002$) and 9.3% more at high nectar sugar levels ($p<0.001$) (Figure 16).

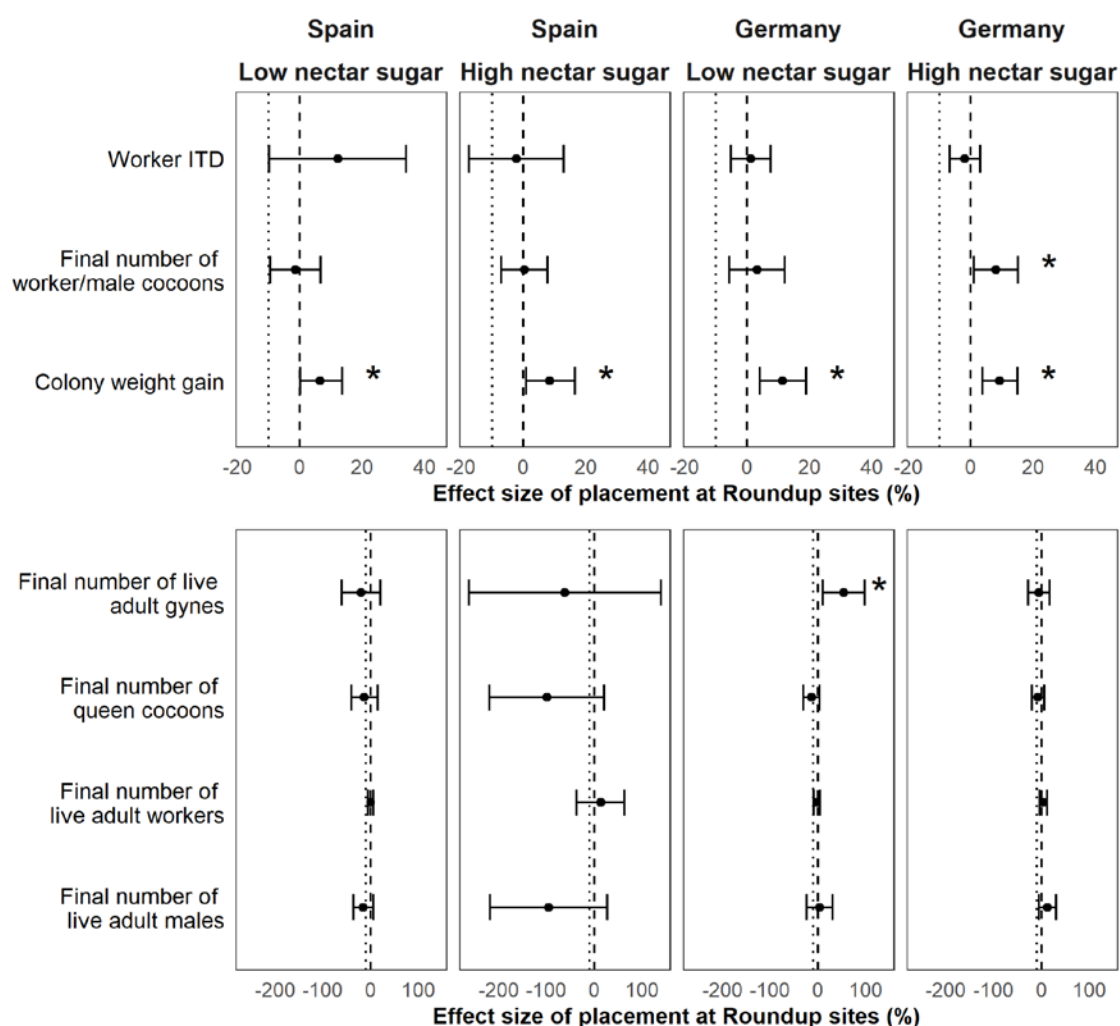


Figure 16: The effect sizes of placement at Roundup sites as opposed to placement at mechanically weeded sites for different parameters of individual and colony-level bumble bee development, divided according to country of the experimental sites and respectively low and high nectar sugar amounts.

The final number of live adult gynes was 54.1% higher at Roundup sites than at mechanically weeded sites with low nectar sugar amounts in Germany ($p=0.017$). No difference between treatments was detected at high nectar sugar sites in Germany and neither at any Spanish sites in the final number of live adult gynes. Moreover, the final number of queen cocoons, live adult workers and live adult males did not differ between Roundup and mechanically weeded sites with low or high nectar sugar amounts in Spain or Germany (Figure 16).

4.5. Mason bees

In Germany, no difference was found in the number of nesting *Osmia bicornis* females between treatments in relation to the available amount of sugar at the vineyard site. There was also no difference in the number of *Osmia bicornis* brood cells between treatments in relation to the amount of sugar (Figure 17).

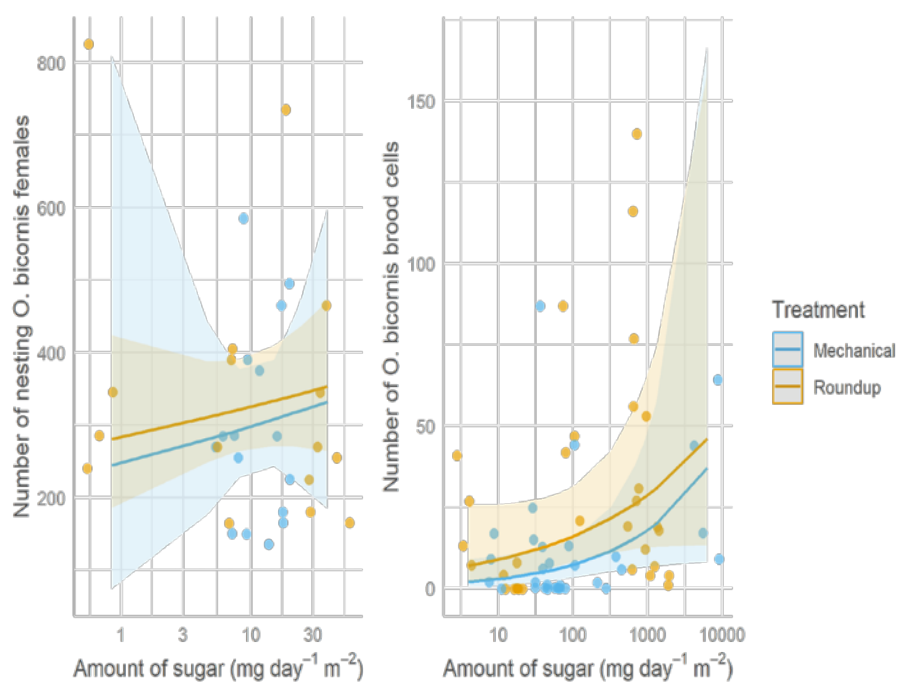


Figure 17: Number of nesting *Osmia bicornis* females and brood cells in German vineyard sites in relation to treatment and amount of sugar. Shaded areas around the prediction lines obtained from a linear mixed-effect model depict 95% confidence intervals and dots represent observations.

Similarly, for *Osmia cornuta* in Spain and Germany, there was no effect of the placement at Roundup sites on the number of nesting females or on the number of brood cells at low or high nectar sugar sites (Figure 18).

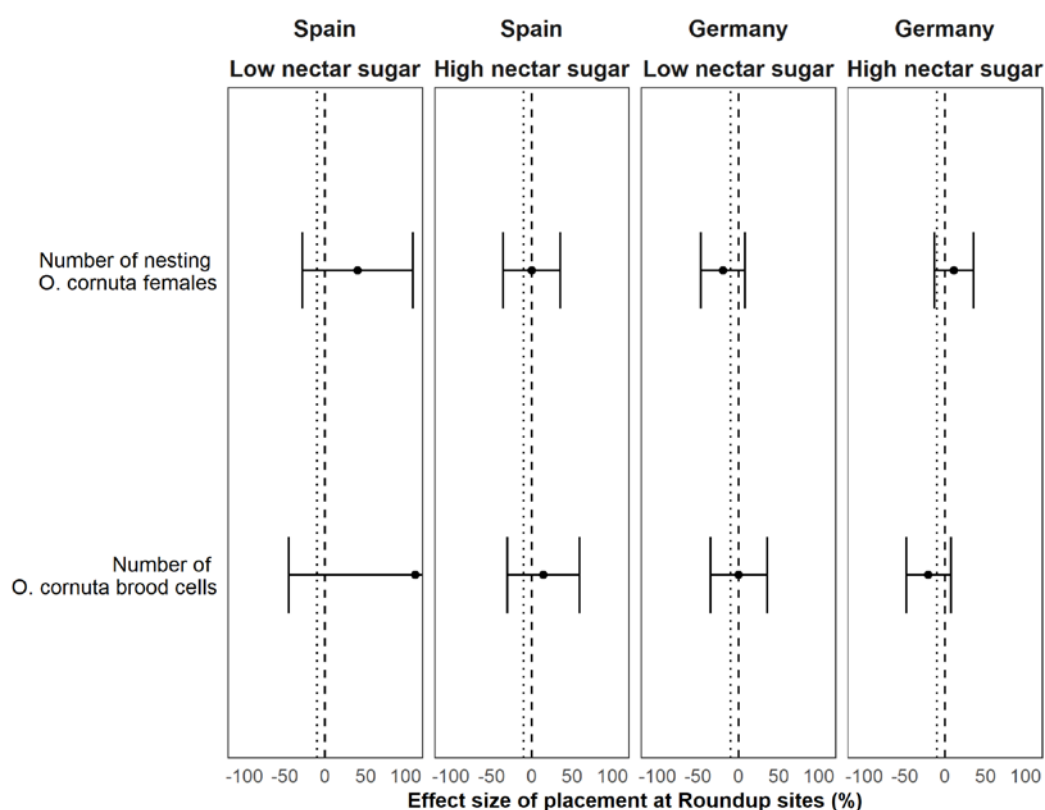


Figure 18: The effect sizes of placement at Roundup sites as opposed to placement at mechanically weeded sites for the number of nesting *Osmia cornuta* females and brood cells, divided according to country of the experimental sites and respectively low and high nectar sugar amounts.

4.6. Bee pathogens

While Black queen cell virus (BQCV) was found in both *Osmia* and bumble bees in Germany, Deformed wing virus type-B (DWV-B) was only found in *Osmia*. Deformed wing virus type-A (DWV-A) was not found in either bumble bees or *Osmia bicornis* (if cycle number < 35 was used as a detection threshold; Table 5). Generally, there were no marked differences between treatments. However, BQCV was found in a third of control *Osmia* samples but not in *Osmia* from Roundup-treated sites.

Table 5: Bee pathogens in *Osmia* and bumble bees sampled at German vineyard sites that were either mechanically weeded (control) or treated with Roundup.

	<i>Osmia</i>			Bumble bee		
	BQCV	DWV-A	DWV	BQCV	DWV-A	DWV
Control	33.3%	0%	5.6%	15.3%	0%	0%
Roundup	0%	0%	11.1%	27.1%	0%	0%

The pathogen loads of the viruses that were relatively frequently found in both treatments were analysed. However, no effect of treatment was found on DWV-B cycle numbers in *Osmia bicornis* ($p=0.613$) or on BQCV in bumble bees ($p=0.892$; Figure 19). We also found no interactive effects of the amount of nectar sugar ($p>0.4$), even though in Roundup bumble bee colonies, cycle number increased, i.e. loads decreased with amount of sugar ($p=0.049$).

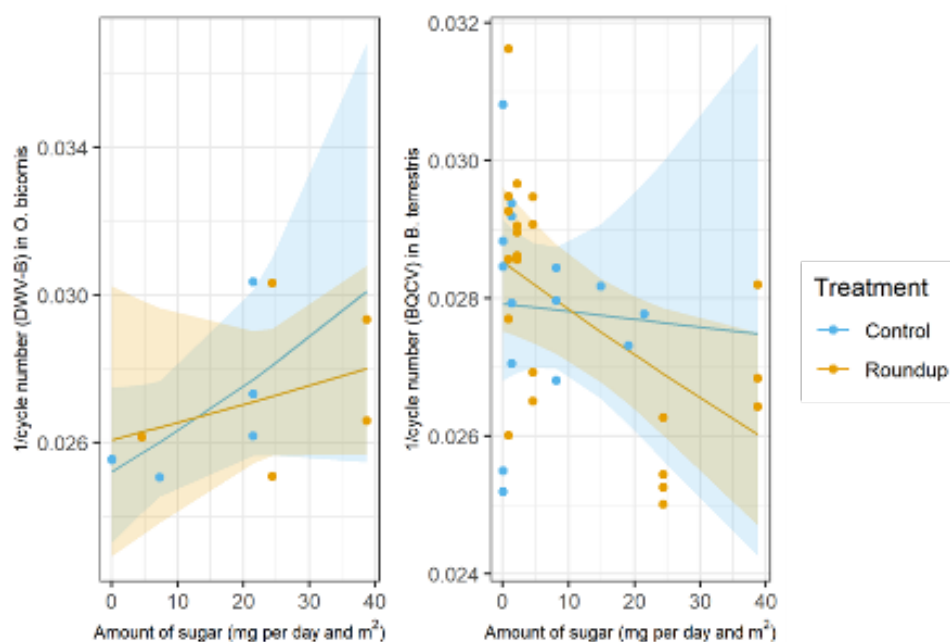


Figure 19: Loads (actually inverse cycle number) of DWV-B in *Osmia bicornis* and BQCV in *Bombus terrestris* in relation to treatment and amount of nectar sugar in German vineyards.

5. Discussion

The large-scale field experiments aimed to investigate how exposure to the glyphosate-based herbicide Roundup and floral resource availability, measured by the total nectar sugar amount provided by the flowering habitat in the direct surroundings of the study sites, affect *Apis mellifera* colonies, *Bombus terrestris* colonies, *Osmia cornuta* bees and *Osmia bicornis* bees individually, as well as interacting factors. Contrary to our expectations that Roundup treatment would negatively affect the investigated bee species, we found no effects of placement at Roundup treated sites on the pollen-lipid ratio of the floral resources in the close surrounding of the sites, the ITD of bumble bees, the number of bumble bee worker/male cocoons, the number of bumble bee queen cocoons, the number of adult bumble bee workers and the number of adult bumble bee males. Treatment also had no significant effect on the number of workers, the hive weight and the Varroa mite load of honey bee colonies. Similarly, the number of nesting *Osmia* females, the number of *Osmia* brood cells, and pathogen loads of the investigated bees were not affected by the treatment of the study sites. However, glyphosate residues in honey bees and *Osmia* cell walls in Spain, as well as *Osmia* cell walls and soil in Germany were higher at Roundup treated sites. Intriguingly, bumble bee colony weight was positively influenced by Roundup treatment. Nectar sugar content of the surrounding flowering resources did not influence the ITD, the number of worker/male cocoons, the number of adult workers and the number of adult males in bumble bee hives. Similarly, the pollen lipid ratio, the number of adult honey bees, the number of nesting *Osmia* females and the number of *Osmia* brood cells were not affected by the sugar amount in the nectar. In Germany on sites with high nectar sugar content, Roundup colonies had more worker/male cocoons and at the start of the experiment honey bee hives in Spain were heavier at sites with high nectar sugar amounts, but this difference diminished by the end of the experiment. Varroa mite load of honey bee colonies increased more at sites with high nectar sugar contents. In Germany the number of adult bumble bee gynes in Roundup colonies was higher at sites with low nectar sugar amounts, but no difference in gyne numbers was observed at

high sugar sites. Conversely, in Spain Roundup colonies at high sugar sites showed a higher flight activity shortly after the weed control while no impact of treatment was observed at colonies on low sugar content sites.

Increased colony weight gain in bumble bee colonies at Roundup-treated sites in both Spain and Germany at low- and high-sugar sites might be due to increased nectar and pollen storage triggered by glyphosate exposure. Glyphosate exposure was previously associated with increased bumble bee colony weight in bumble bees (Odemer et al., 2020) and increased food consumption in honey bees (Almasri et al. 2020; Faita et al. 2020), although one study found reduced food consumption after exposure to glyphosate and other pesticide mixtures (Zhu et al. 2017). *Bombus terrestris* colonies lose weight after switching the production of workers to queens and males. However, it is unlikely that the stronger weight increase at Roundup-treated sites was induced by a delayed switch point as colonies at Roundup-treated sites in Germany had slightly more adult gynes at high-sugar sites and worker/male cocoons at low-sugar sites compared to colonies at mechanically weeded sites. The effects were however, relatively small and inconsistent across countries and sugar amounts and no effects of placement at Roundup-treated sites was found on worker body size (ITD), the number of live adult workers and live adult males. As the weight of bumble bee colonies is mainly influenced by the number and weight of individuals, the extent of brood cell structures and food stores, it is likely that increased food storage caused the increased weight gain in colonies at Roundup-treated sites.

No significant effects of the Roundup treatment on the study sites on most bee parameters across the test species may be the result of lower glyphosate exposures than expected. Our hypotheses were based on previous studies which mostly investigated glyphosate effects on *Apis mellifera* in artificial feeding experiments (Cullen et al., 2019; Dai et al., 2018; Vázquez et al., 2018; Ruiz-Toledo and Sánchez-Guillén, 2014; Ruiz-Toledo and Sánchez-Guillén, 2014; Balbuena et al., 2015). Glyphosate exposure rates used in these artificial feeding experiments may be higher than the actual glyphosate load reaching the bees in an agricultural landscape, leading to less detectable or weaker effects on bees than in these studies. Pesticide exposure on the fields is influenced by various environmental factors such as meteorological conditions or geological properties of the soil. The sorption of the glyphosate compound to soil is strong compared to other pesticides, and consequently its soil activity is low. Due to its degradation by microorganisms to the primary metabolite aminomethylphosphonic acid (AMPA), the compound itself has a fairly short half-life of around 30 days (in temperate climates) in soil and water. AMPA is slightly more persistent (Duke, 2020; Gill et al., 2018). Glyphosate can enter ground- and surface water through leaching, surface runoff and spray drift (Duke, 2020b).

The absence of significant effects on the investigated mason bee species is in line with the findings of other studies. No effects of glyphosate and clothianidin on adult mortality and food consumption were found for *Osmia bicornis* (Strobl et al., 2020). Correspondingly, a study assessing the acute contact toxicity (48 h LD50) of 16 insecticides toward *Osmia bicornis* found the species to be less sensitive than *Apis mellifera* to a majority of the tested formulations (Uhl et al., 2019). Further, another study investigating the acute toxicity of the insecticide dimethoate found *Osmia bicornis* to be among the least sensitive out of five wild bee species considered in every scenario tested, with females being more resistant than males (Uhl et al., 2016). It has been suggested that the not yet fully developed cuticle of young worker honey bees possibly leads to their increased sensitivity compared to other bee species (Uhl et al., 2019).

In conjunction with the different risk indications, it is necessary to distinguish between the isolated active ingredient glyphosate and the full product formulation containing several co-formulants in addition to glyphosate. While glyphosate alone was often found to be non-hazardous for bumble bees, single herbicide co-formulants or full formulations were shown to have toxic effects (Bradberry et al., 2004; Nagy et al., 2020) – with several commercial formulations up to 1000 times more toxic than

glyphosate alone (Mesnage & Antoniou, 2018). In particular, the co-formulants in different Roundup products exhibited lethal toxicity (Straw et al., 2021). An interference of surfactants with the tracheal system is suggested as one mechanism of action: The formulation is spread over the surface of the bee where it might limit gas exchange (presumably through 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); future research should investigate this further (Straw et al., 2021). However, gaining a better understanding is complicated since formulation composition is protected under EU law (Straw et al., 2021). In total, there are more than 750 different glyphosate-based herbicide products available on the market, used in agricultural, forest, urban and domestic environments (Guyton et al., 2015), all with different compositions of co-formulants. Without knowing the identity and concentration of those co-formulants, compound-specific research and consequent realistic risk assessments for non-target species are severely hampered. Individually testing and regulating co-formulants and active ingredients, as well as full product formulations (to include potential interactive compound effects), would improve currently inadequate legal requirements. As of yet, for each active ingredient, only one representative formulation is required for testing at EU level (Straw et al., 2021). The experimental design of our study does not allow for a distinction between the formulated products Roundup Ultimate, Roundup Power Flex, and their active ingredient glyphosate. Thus, in this experiment observed effects cannot be ascribed to glyphosate alone or a specific co-formulant but only the full product formulation. Hence the herbicide is always referenced here in its entirety. Further research should focus on the impact of individual product ingredients or of co-formulants frequently used in different formulations to gain a better understanding of the risk they pose to non-target organisms.

In realistic environmental settings, stressors rarely act in isolation. In the context of this experiment, the impact of the two stressors nutrition and Roundup exposure was investigated. In addition, bees are exposed to numerous other factors with detrimental effects on bees. Studies have shown that, amongst others, synergies between multiple classes of pesticides, environmental parameters such as temperature or other known threats such as parasites and pathogens have the potential to compromise bee populations (Archer et al., 2014; Brunner et al., 2014; Cameron & Sadd, 2020; Sgolastra et al., 2017; Zaller & Brühl, 2019). These co-stressors and especially additive or synergistic effects of multiple stressors in combination must be subject of further ecotoxicological studies in order not to underestimate actual risks.

Our results do not indicate a risk for *Apis mellifera*, *Bombus terrestris*, *Osmia cornuta* and *Osmia bicornis* of Roundup PowerFlex/Ultimate or glyphosate when applied in plant rows of almond or vineyard sites. However, to conclude on the safety of the herbicide for bees, more studies examining potential drivers of glyphosate exposure and effects on bees are required. It is particularly unclear whether bees are more exposed and/or affected when glyphosate is applied not only in plant rows but on larger areas, as is done in potato fields before planting. Our study revealed a surprising positive association between placement at Roundup-treated sites and bumble bee colony weight gain that was consistent across the two cropping systems and occurred irrespective of the amount of floral resources available in the landscape. The mechanisms of this impact must be studied to better understand the direct and indirect effects Roundup treatment can have on bees.

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