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ABSTRACT

Pollination is an essential process for the sexual reproduction of seed plants, and the majority of flowering plants worldwide benefit from insect pollinators. However, insect pollinators have been declining globally, and their decreasing numbers and species diversity could have serious environmental, food production, and economic consequences. Several factors have been proposed to explain pollinator decline, including land-use changes, biodiversity loss, climate change, and pesticide use. In this dissertation, I investigated the impacts of glyphosate, the world’s most widely used herbicide, and its commercial formulations (glyphosate-based herbicides) on the buff-tailed bumblebee (*Bombus terrestris*). Although concerns have arisen in recent decades about glyphosate being more harmful than previously thought, there is no consensus on the extent of its effects, specific mechanisms, or exposure routes. First, I investigated whether bumblebees could be exposed to glyphosate while visiting flowers. The results showed that bumblebees did not avoid glyphosate-sprayed plants, even when untreated plants were available. Second, I studied the effects of glyphosate on cognitive abilities considered crucial for bumblebee survival. Glyphosate-based commercial formulations did not affect the duration of daily foraging trips in freely flying bumblebees, but the number of trips nearly doubled, while the number of flower visits per trip decreased. Exposure to the commercial formulation did not impair the bees' ability to return to the colony. While the formulation had no impact on learning or memory, the same amount of pure glyphosate weakened learning performance. Third, I examined the effects of glyphosate and its commercial formulation on the gut microbiota of bumblebees, as some gut bacteria are potentially sensitive to glyphosate. The results indicated that pure glyphosate increased microbiota diversity, whereas the commercial formulation decreased it. However, both treatments reduced the abundance of *Snodgrassella alvi*, a bacterium beneficial to bumblebee immunity. Overall, my findings suggest that both glyphosate and its commercial formulations have harmful effects on bumblebees. My research provides new insights into exposure routes and individual-level effects, but further studies are needed, particularly on glyphosate’s mechanisms and its impact on entire colonies.

KEYWORDS: pollinator, pesticides, pollinator behavior, foraging activity

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TIIVISTELMÄ

Pölytys on siemenkasvien suvullisen lisääntymisen kannalta välttämätöntä ja valtaosa kukkakasveista hyötyy hyönteisten suorittamasta pölytyksestä. Pölyttäjähyönteiset ovat kuitenkin vähentyneet maailmanlaajuisesti ja niiden vähenemiseen on esitetty monia syitä, kuten maankäytön muutokset, luonnon monimuotoisuuden vähentyminen, ilmastonmuutos ja torjunta-aineet. Väitöskirjassani tutkin maailman käytetyimmän rikkakasvien kemiallisen torjunta-aineen, glyfosaatin, ja sen kauppavalmisteiden vaikutuksia kontukimalaisiin (*Bombus terrestris*). Vaikka glyfosaatin epäilläänkin olevan luultua haitallisempaa ei-kohde-eliöille, sen vaikutusten laajuudesta, tarkoista vaikutusmekanismeista ja altistumisreiteistä ei olla yksimielisiä. Tutkin ensin, voivatko kimalaiset altistua glyfosaatille vieraillessaan kukilla. Tulosten perusteella kimalaiset eivät välttelleet glyfosaatilla ruiskutettuja kasveja, vaikka käsittelemättömiä kasveja olisi ollut tarjolla. Toiseksi tutkin, miten glyfosaatti vaikuttaa kimalaisten selviytymisen kannalta tärkeinä pidettyihin kognitiivisiin ominaisuuksiin. Glyfosaatin kauppavalmiste ei vaikuttanut vapaana lentävien kimalaisten päivittäisten ravinnonhakumatkojen kestoon, mutta päivässä tehtyjen matkojen määrä lähes kaksinkertaistui ja matkan aikana tehtyjen kukkavierailujen määrä laski. Kauppavalmisteelle altistuminen ei vaikuttanut kimalaisten kykyyn löytää takaisin yhdyskuntaan. Kauppavalmiste ei vaikuttanut kimalaisten oppimiskykyyn tai muistiin, mutta sama määrä puhdasta glyfosaattia heikensi oppimistuloksia. Kolmanneksi selvitin glyfosaatin ja sen kauppavalmisteen vaikutuksia kimalaisten suolistomikrobistoon, sillä jotkut suoliston bakteereista ovat potentiaalisesti herkkiä glyfosaatille. Tulosten perusteella puhtaalla glyfosaatilla ja kauppavalmisteella oli erilainen vaikutus, sillä puhdas glyfosaatti lisäsi, kun taas kauppavalmiste vähensi suolistomikrobiston monimuotoisuutta. Molemmat kuitenkin vähensivät kimalaisten vastustuskyvylle hyödyllisen *Snodgrassella alvi*-bakteerilajin runsautta. Tulosteni perusteella sekä glyfosaatilla, että glyfosaattipohjaisilla kauppavalmisteilla on haitallisia vaikutuksia kimalaisiin. Väitöskirjani tuo uutta tietoa glyfosaatin altistumisreiteistä ja vaikutuksista yksittäisille kimalaisille, mutta jatkotutkimukset glyfosaatin vaikutusmekanismeista ja vaikutuksista kokonaisiin kimalaisyhdyskuntiin ovat tarpeen.

ASIASANAT: pölyttäjä, torjunta-aineet, pölytyskäyttäytyminen, ravinnonhaku

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Abbreviations

E4P	Erythrose 4-phosphate
ECHA	European Chemicals agency
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPSP	Enzyme 5-enolpyruvylshikimate-3-phosphate
EU	European Union
FAO	Food and Agriculture Organization
GBH	Glyphosate-based herbicide
GLMM	Generalized linear mixed model
GM	Genetically modified
LMM	Linear mixed model
NMDS	Nonmetric multidimensional scaling
PEP	Phosphoenolpyruvate/2-phosphoenolpyruvate
PERMANOVA	Permutational analysis of variance
RFID	Radio frequency identification
VOC	Volatile organic compound
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I	Kaakinen, K., Ramula, S., Fuchs, B., Blande, J. D., Vaajamo, E-M., & Helander, M. Bumblebees (*Bombus terrestris*) forage on plants treated with glyphosate-based herbicides despite potential behavioral consequences. *Manuscript*.
II	Kaakinen, K., Ramula, S., & Helander, M. Glyphosate-based herbicide increases the number of foraging trips but does not affect the homing of *Bombus terrestris*. *Apidologie*, 2025; 56(54).
III	Kaakinen, K., Ramula, S., Loukola, O. J., & Helander, M. Effects of glyphosate and glyphosate-based herbicide on learning and memory of the buff-tailed bumblebee (*Bombus terrestris*). *Entomologia Experimentalis et Applicata*, 2024; 172(4), 324–333.
IV	Helander, M., Jeevannavar, A., Kaakinen, K., Mathew, S. A., Saikkonen, K., Fuchs, B., Puigbò, P., Loukola, O. J., & Tamminen, M. Glyphosate and a glyphosate-based herbicide affect bumblebee gut microbiota. *FEMS Microbiology Ecology*, 2023; 99(7).

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Author contributions to the original publications

	Chapter I	**Chapter II**	**Chapter III**	**Chapter IV**
Conceptualization	**KK**, SR	**KK**	**KK**, MH	MH, KS, PP, OJL
Methodology	**KK**, SR, BF, EMV	**KK**, MH, SR	**KK**, MH	MH, **KK**, SAM, PP, MT
Investigation	**KK**, BF, JDB, EMV	**KK**	**KK**	**KK**, SAM
Formal analysis	**KK**, SR, BF	**KK**, SR	**KK**, SR	AJ, PP
Supervision	SR, MH	MH, SR	SR, MH, OJL	MT
Writing – original draft	**KK**	**KK**	**KK**	MH
Writing – review and editing	**KK**, SR, MH, BF, JDB	**KK**, MH, SR	**KK**, SR, OJL, MH	MH, AJ, **KK**, SAM, KS, BF, PP, OJL, MT

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1 Introduction

1.1 Pollinator decline

Insect pollination is important for biodiversity and agriculture and is the most economically valuable ecosystem service provided by insects (Breeze et al., 2016). Of terrestrial flowering plants, 78–94% need or benefit from insect pollinators (Ollerton et al., 2011), and over 75% of globally cultivated plant species rely, at least in part, on insect pollination (Klein et al., 2007). These crops contribute an estimated $235–577 billion annually to the global economy (IPBES, 2016), including key food crops, such as fruits (23% decline without pollinators), vegetables (16%), nuts and seeds (22%), and cash crops, such as coffee and cocoa (Smith et al., 2015). Although 60% of global crop production is derived from self- and wind-pollinated plants that do not rely on insect pollination (Klein et al., 2007) and only 3–8% of total crop production would be lost without insect pollinators (Aizen et al., 2009), pollinators are directly responsible for up to 40% of the world's supply of some micronutrients (Eilers et al., 2011). For instance, Smith et al. (2015) estimated that a complete loss of pollinators would result in 71 million people experiencing vitamin A deficiency and 173 million people experiencing folate deficiency, both vitamin A and folate being important, especially for children and pregnant women.

Insect pollinators include flies, butterflies, beetles, thrips, wasps, moths, and more than 20,000 species of bees, including a few widely managed honeybees (e.g., *Apis mellifera* L. and *Apis cerana* F.). Natural populations of insect pollinators have declined globally in recent decades (Burkle et al., 2013; Ghazoul, 2005; Zattara & Aizen, 2021). Recent studies have suggested an overall annual decline of 1–2% in insect abundance (Dirzo et al., 2014; Forister et al., 2019; Hallmann et al., 2017; Wagner et al., 2021, 2021; Wepprich et al., 2019). Declines in the abundance, occurrence, and diversity of wild bee and butterfly populations have been recorded, particularly in Europe and North America, during the last century (Biesmeijer et al., 2006; Goulson et al., 2008; IPBES, 2016; Potts et al., 2010; Settele et al., 2008; Williams & Osborne, 2009). According to Zattara and Aizen (2021), the number of bee species has declined by approximately 25% since the 1990s. In Europe, 9% of bees and butterfly species are threatened, while in Finland, 17% of bees and 30% of butterflies were classified as threatened in 2019 (Hyvärinen et al., 2019). However, the proportion of crops that depend on pollinators has increased by over 300% during the last 60 years (Aizen et al., 2009), and managed pollinators alone cannot suffice the need for pollination.

Pollinators are subjected to various stressors, often experiencing multiple stressors simultaneously. The stressors assumed to cause the global pollinator decline are land-use changes, including agricultural intensification (Goulson et al., 2008), urbanization (Fisogni et al., 2020; Wenzel et al., 2020), habitat fragmentation and degradation (Brown & Paxton, 2009; Hendrickx et al., 2007; Potts et al., 2010), the spread of invasive animal and plant species (Goulson, 2003; Stout & Morales, 2009), pathogens and parasites (Cox-Foster et al., 2007; Meeus et al., 2011), climate change (Dormann et al., 2008; Soroye et al., 2020; Williams et al., 2007), and the escalating use of pesticides (Dicks et al., 2021).

In this dissertation, I study the effects of glyphosate, one of the most frequently used pesticides, on pollinators. I focus on the direct effects of glyphosate and glyphosate-based herbicides (GBHs) on individual *Bombus terrestris* L. pollinators. The dissertation consists of four related chapters that combine field and laboratory experiments that employ both novel and well-established methodologies.

1.2 Pesticides

Pesticides are agrochemicals intended to reduce the damage pests cause to crops, livestock, and the environment. The global use of pesticides in agriculture has almost doubled in the last three decades (FAOSTAT, 2024). Pesticides have enabled an enormous increase in food production (Alexandratos & Bruinsma, 2012) and are vital in modern agriculture, since pests, primarily weeds, account for over 40% of global yield losses (Oerke, 2007).

Pesticides include insecticides, fungicides, and herbicides. Insecticides designed to target insect pest populations are generally considered to cause the most significant risk to pollinators. In recent years, research on the effects of pesticides on pollinators has focused on neonicotinoids (Lundin et al., 2015), one of the most frequently used classes of insecticides (Klingelhöfer et al., 2022). Because of low application doses and selective binding, neonicotinoids are presumed to cause a lower risk for mammals, birds, fish, and the environment in general than other insecticides (Casida & Durkin, 2013; Klingelhöfer et al., 2022; Tomizawa & Casida, 2005). However, they have been strongly associated with a decline in pollinator populations and negative impacts on individual pollinator health (Laycock et al., 2014; Singla et al., 2021; Stanley et al., 2016; Tsvetkov et al., 2017; Whitehorn et al., 2012; Woodcock et al., 2017).

Herbicides are intended to prevent or interrupt the normal growth of unwanted plants (i.e., weeds). Herbicide use saves energy and the labor of mechanical weeding in agriculture. In addition to agriculture, herbicides are used in silviculture, private gardens, landscaping, roadside and railway management, etc. (Giesy et al., 2000). Although these nonagricultural uses of herbicides may cause serious environmental issues (Kristoffersen et al., 2008; Spliid et al., 2004), the use of herbicides outside agriculture is relatively small (Benbrook, 2016). Several countries have banned the noncommercial use of certain herbicides and other pesticides in public spaces (Arcuri & Hendlin, 2019).

Herbicides can be classified by the time of application as pre- or postemergent. Pre-emergent herbicides are applied to the soil before crop planting to prevent the germination of weeds. They are nonselective (or very broad spectrum) and prevent the normal growth of all plants, while postemergent herbicides target either monocotyledon or dicotyledon weeds. Postemergent herbicides can be applied to emergent crops and soil (Duke & Dayan, 2018). Another practice of classifying herbicides is by their absorbance characteristics. Contact herbicides are absorbed by plant surfaces and cause damage to the plant tissue they encounter. Systemic herbicides, which penetrate plant tissue and are transported throughout the plant, are effective against perennial weeds. Systemic herbicides can be applied on soil or sprayed on weeds (Duke & Dayan, 2018).

Commercial herbicides consist of active ingredients and many coformulants, inerts, adjuvants, and other ingredients. The active ingredients of commercial herbicides—the substances that have specific effects on plant metabolism—have multiple different modes of action, such as inhibition of enolpyruvyl shikimate phosphate synthase (glyphosate) (Amrhein et al., 1980), inhibition of photosynthesis (e.g., atrazine) (Shimabukuro & Swanson, 1969), inhibition of lipid synthesis (quizalofop) (Stoltenberg et al., 1989), disturbance in the cell membrane (e.g., paraquat and diquat) (Dodge & Harris, 1970), and inhibition of plant growth (e.g., dicamba and 2,4-D) (Gleason et al., 2011).

The costs of registration, research, and development of herbicides have increased in recent decades because of stricter requirements and regulations (McDougall, 2016). Furthermore, the slowed discovery of new herbicides, the reduced cost of existing herbicides due to expired patents, and the widespread reliance on glyphosate have made registering new active ingredients virtually nonexistent (Duke, 2012). In addition to lobbying for existing products (Heisey & Schimmelpfennig, 2006), the major pesticide companies have focused on developing other ingredients that improve the efficiency of existing active ingredients. In herbicides, these ingredients are called coformulants, inerts, or adjuvants. Strictly speaking, coformulants and adjuvants are not synonymous, since coformulants refer to ingredients added to an herbicide product when it is formulated and adjuvants are added by the applicator just before the product is used (Mesnage & Zaller, 2021). In this work, I use the term coformulant to refer to any nonactive ingredient added to commercial herbicide products. Although coformulants are not active substances themselves, they play a crucial role in enhancing the performance of active ingredients. They can help active ingredients penetrate a plant's epidermal surface, reduce drifting or foam forming, and allow adherence for longer exposure to the target plant (Duke & Dayan, 2018).

The global use of herbicides has significantly increased over time. In 1990, approximately 700,000 tons of active ingredients were applied, but by 2022, this amount had risen to nearly 1,950,000 tons of herbicide active ingredients used worldwide in agriculture (FAOSTAT 2024). Although the upward trend in herbicide use has stabilized and even declined in some regions, such as Europe, in recent years (FAOSTAT 2024), global herbicide consumption is projected to increase due to the growing need to feed an expanding global population. This growth is particularly evident in developing countries where agricultural intensification is ongoing (Gianessi, 2013; Haggblade et al., 2017). Additionally, the widespread use of herbicides, particularly in conjunction with herbicide-tolerant genetically modified (GM) crops, has contributed to the proliferation of herbicide-resistant weed species. Climate change is also projected to influence herbicide and pesticide usage. The demand for pesticides is likely to increase as wintertime pest control may become less effective and pest reproduction rates may increase due to milder winters (Ehrlich & Harte, 2015). Consequently, herbicide resistance and changing environmental conditions are expected to drive greater reliance on herbicides and other pesticides in agricultural practices.

1.3 Glyphosate and glyphosate-based herbicides

The most commonly used active ingredient worldwide in commercial herbicides is glyphosate [N-(phosphonomethyl)glycine] (Myers et al., 2016). Glyphosate has more than 750 commercial formulations (Guyton et al., 2015) called glyphosate-based herbicides (GBHs). It was first tested in 1970 by John E. Franz of Monsanto Co. (Franz et al., 1997) and was patented as an herbicide in 1971. In 1974, the first commercial GBH product, Roundup®, was released to market (Duke & Powles, 2008). After the introduction of glyphosate-resistant genetically modified crop plants in 1996 (Guyton et al., 2015) and the expiration of Monsanto's patent in 2000, the use of glyphosate dramatically increased (Duke & Powles, 2008). In Finland, GBHs have been used since the mid-1970s; some GBH products are now only allowed for professional use, and buying and applying them require plant protection certification approved by the Finnish Safety and Chemicals Agency (Tukes, 2024). In addition to its use as a weed killer, glyphosate is employed for forced ripening. This practice is particularly beneficial in mitigating the impact of unfavorable weather conditions, such as untimely rainfall, which could otherwise ruin crops before they can be harvested (Malalgoda et al., 2020). However, in the European Union (EU), using glyphosate for forced grain ripening is not considered a good agricultural practice, based on the Commission Implementing Regulation (EC) 2016/1313.

Glyphosate is a nonselective, postemergent herbicide and a systemic herbicide (Duke & Dayan, 2018) that is absorbed through plant surfaces and transported to all actively growing tissues of the plant (Servaites et al., 1987). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an essential enzyme in the shikimate pathway found in all green plants and in many microbes (Leino et al., 2021). The shikimate pathway produces chorismate, which is needed to synthesize the essential aromatic amino acids tryptophan, phenylalanine, and tyrosine for the plant's growth and function (Duke & Powles, 2008; Herrmann & Weaver, 1999) (Figure 1).

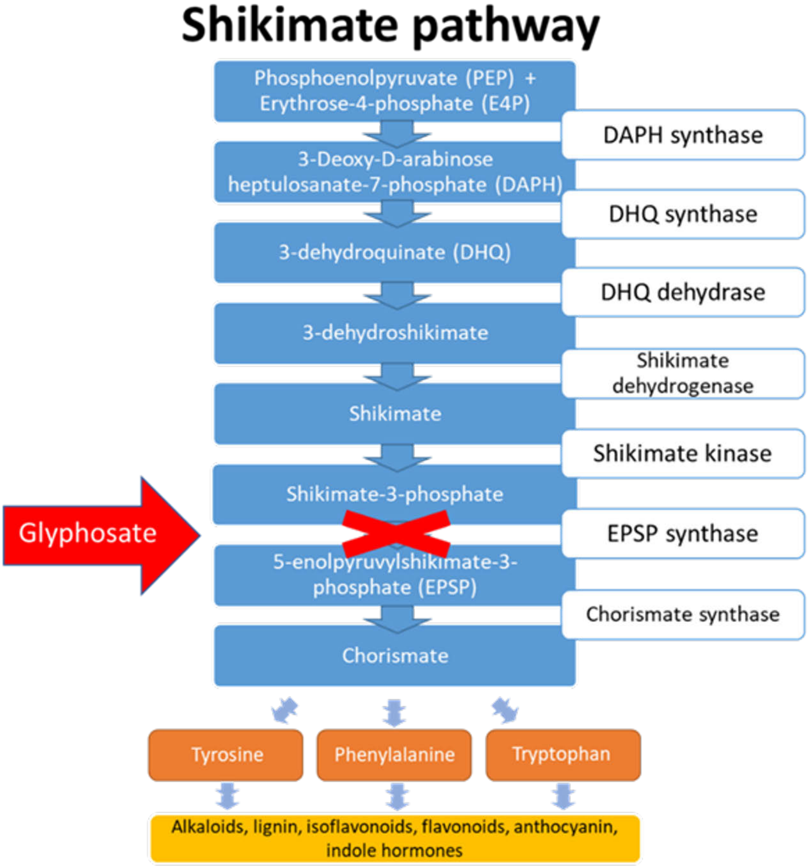

Figure 1. Shikimate pathway. In the shikimate pathway, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate in several metabolic steps (on the right). Chorismate is a precursor for many aromatic metabolites and the essential aromatic amino acids tryptophan, phenylalanine, and tyrosine, which are important for a plant's growth and function. Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase.

Because the shikimate pathway is only found in plants and the majority of microbes, such as fungi and some bacteria (Duke & Powles, 2008; Leino et al., 2021), glyphosate has had a reputation as being relatively safe and nontoxic to other nontarget organisms, such as insects, humans, and other animals (Duke & Powles, 2008). Glyphosate is considered nonpersistent because its half-life in favorable conditions is less than 30 days (Lewis et al., 2006). It is generally expected to be adsorbed rapidly through sorption onto variable-charge soil minerals and to degrade rapidly through microbial degradation via two metabolic pathways: the aminomethylphosphonic acid (AMPA) and sarcosine pathways, facilitated by glyphosate-degrading bacteria (Borggaard & Gimsing, 2008; Franz et al., 1997; Vereecken, 2005). Consequently, the risk of glyphosate leaching into groundwater is considered limited (Borggaard & Gimsing, 2008).

However, recent studies have shown that glyphosate may have adverse effects on soil microbes and the rhizosphere (Caggìa et al., 2023; Fuchs et al., 2021; Helander et al., 2018; Newman et al., 2016; Ruuskanen et al., 2023), water ecosystems (Brovini et al., 2021; Carles et al., 2019; Folmar et al., 1979; Milan et al., 2018; Vera et al., 2010), and fauna below (Gaupp-Berghausen et al., 2015; Maderthaner et al., 2020; Stellin et al., 2018; Zaller et al., 2014) and above ground (Berger et al., 2013; Carpenter et al., 2016; Evans et al., 2010; Jarrell et al., 2020; Takahashi, 2007). Recent studies have indicated that glyphosate may persist in soil longer than previously understood, influenced by soil composition (Székács & Darvas, 2012), reduced microbial activity (Borggaard & Gimsing, 2008; Franz et al. 1997), and climatic factors. For example, glyphosate and its metabolite AMPA, a known phytotoxin (Duke, 2011; Hoagland, 1980; Reddy et al., 2004), may exhibit extended half-lives in cold northern ecosystems, where growing seasons are short and microbes are inactive during cold and long winters (Hagner et al., 2024; Helander et al., 2012; Laitinen et al., 2006).

Glyphosate residues in soil may affect plants' physiological processes and, consequently, species interactions, including pollination (Fuchs et al., 2021). Glyphosate inhibits the production of essential amino acids (Amrhein et al., 1980), which are building materials for plant secondary chemicals, including volatile organic compounds (VOCs) (Vogt, 2010). VOCs are secondary defense metabolites that deter herbivores, recruit natural enemies of herbivores, and induce defensive responses in nearby plants (Arimura et al., 2005; Heil & Ton, 2008). Some VOCs attract pollinators, and pollinators with specialized olfactory systems can use VOCs to find, recognize, and distinguish between flowers (Kunze & Gumbert, 2001). Glyphosate, even at sublethal residue levels, can alter the emission of VOCs and thus disrupt plant–pollinator interactions (Fuchs et al., 2021). Phenylalanine, an amino acid synthesized via chorismate produced in the shikimate pathway, serves as a vital precursor for multiple VOCs (Vogt, 2010).

Several regulatory bodies have recently linked herbicides, including glyphosate, to the ongoing insect decline (EFSA, 2023a; IPBES, 2016). The most evident risk glyphosate poses to pollinators is its intended function of eliminating vegetation. By reducing the diversity of plant species, glyphosate indirectly diminishes the availability of foraging resources for pollinators, thereby contributing to declines in their populations (Albrecht, 2005; Goulson et al., 2015). In addition, several studies have shown that glyphosate and its commercial products have harmful, nonlethal effects on pollinators. Glyphosate has been discovered to affect pollinators' **feeding and appetitive behavior**, such as reducing sensitivity to sucrose and impairing associative olfactory learning (Herbert et al., 2014; Mengoni Goñalons & Farina, 2018), **navigation** (Balbuena et al., 2015), and **sensory and cognitive abilities** (Helander et al., 2023; Herbert et al., 2014; Vázquez et al., 2020); **delay larval development** (Odemer et al., 2020; Vázquez et al., 2018; Weidenmüller et al., 2022); and **modify gut microbiota** (Motta et al., 2018, 2020; Motta & Moran, 2023) (Table 1).

Table 1. Example studies investigating the effects of pure glyphosate and glyphosate-based herbicides (GBHs) on bees. This table includes observed effects on various behavioral and cognitive traits in bees, specifying the type of glyphosate exposure, study species, and experimental setting (laboratory or field). References correspond to studies reporting the effects.

Effect	Specific effect	Study species/ Glyphosate type	Laboratory/ Field	Example study
Feeding and appetitive behavior	Reduced sensitivity to sucrose	Honeybees/ Pure glyphosate	Laboratory	Herbert et al., (2014)
	Reduced food uptake	Honeybees/ Pure glyphosate	Field	Mengoni Goñalons & Farina (2018)
Navigation	Increased inward homing flight time	Honeybees/ Pure glyphosate	Field	Balbuena et al., (2015)
Sensory and cognitive abilities	Reduced learning performance	Honeybees/ Pure glyphosate	Laboratory	Herbert et al., (2014)
	Reduced learning performance	Bumblebees/GBH	Laboratory	Helander et al., (2023)
	Decreased antennal activity and sleep bout frequency	Honeybees/ Pure glyphosate	Laboratory	Vázquez et al., (2020)

Effect	Specific effect	Study species/ Glyphosate type	Laboratory/ Field	Example study
Delay in larval development	Delayed molting and reduced brood weight	Honeybees/ Pure glyphosate	Laboratory	Vázquez et al., (2018)
	Delayed brood development and reduced brood weight	Honeybees/ GBH	Field	Odemer et al., (2020)
	Impaired thermo-regulation (possibly affecting brood development and colony growth)	Bumblebees/ Pure glyphosate	Laboratory	Weidenmüller et al., (2022)
Changes in gut microbiota	Altered gut microbiota composition, increased mortality, and a decrease in the bacteria *Snodgrassella alvi* and *Commensalibacter*	Honeybees/ GBH	Both	Motta et al., (2020)
	Altered gut microbiota diversity, decrease in *S. alvi*	Honeybees/ Pure glyphosate	Laboratory	Motta et al. (2018)
	Decrease in *S. alvi*	Bumblebees/Both	Laboratory	Motta and Moran (2023)

Coformulants in GBHs are assumed to be even more toxic than the active ingredient glyphosate itself (Mesnage et al., 2019; Straw, 2024; Straw et al., 2022; Straw & Brown, 2021). A few studies have shown that the mortality of bees increases after oral or topical exposure to GBH (Motta et al., 2020; Straw et al., 2021), but these effects were unlikely caused by glyphosate alone, as elevated mortality has been observed only with commercial formulations, suggesting a role for coformulants. However, toxicity testing for nontarget organisms is often limited to the active ingredient alone (Mesnage et al., 2019), and detailed information about the coformulants in commercial GBHs is limited, as the composition of final products is typically classified as confidential business information (Weinhold, 2010). Among these coformulants, surfactants that help glyphosate penetrate plant cuticles and reduce runoff by rain are of particular concern to insects. Surfactants have been shown to be highly harmful to bees, potentially blocking their tracheal system (Straw et al., 2021), which is critical for gas exchange.

1.4 Bumblebees

Bumblebees (*Bombus*) are part of the Anthophila clade, which includes approximately 250 known bumblebee species. They are primarily found in temperate, alpine, and arctic regions in the northern hemisphere (Williams, 1994) and are vital pollinators in colder climates because they can forage under less favorable weather conditions than other pollinators (Heinrich, 1972).

Bumblebees are important generalist pollinators in natural ecosystems and agriculture for several reasons. They have a high foraging rate, even making 4000 flower visitations in 1 day (Peltotalo, 2010). Because bumblebee hives are annual and only the queen hibernates, bumblebees do not produce a large amount of spare food (i.e., honey); thus, they prefer pollen instead of nectar. The morphology of bumblebees is excellent for pollination, with their large and hairy bodies capable of accommodating large pollen loads. Because bumblebees, especially species such as *B. consobrinus* and *B. hortorum*, have a very long proboscis, they can pollinate flowers with deep corollas that other insects cannot. Bumblebees are also capable of buzz pollination, a process in which pollen from the poricidal anthers of flowers is extracted using vibrations (De Luca & Vallejo-Marín, 2013; King & Buchmann, 2003). Buzz pollination is essential for approximately 15,000–20,000 flowering plant species (Buchmann, 1983). Bumblebees are used for pollination services in open fields (Javorek et al., 2002; MacKenzie, 1994) and especially greenhouses (Dimou et al., 2008; Kwon & Saeed, 2003; Pritts et al., 1999). Their ability to perform buzz pollination and their suitable colony size make them more effective greenhouse pollinators than honeybees. Currently, bumblebees are used commercially to pollinate over 240 crop plants, especially tomatoes (Velthuis & Doorn, 2006). Whereas *B. impatiens* is the primary species used in North America, *B. terrestris* is the primary species used for crop pollination in Europe (Velthuis & Doorn, 2006).

Like many other insect pollinators, bumblebee populations have declined in recent decades (Goulson et al., 2008; Potts et al., 2010; Williams & Osborne, 2009). The drivers behind the decline in bumblebee populations are similar to those in all other insect pollinators (Goulson et al., 2008; Potts et al., 2010). Among the key drivers, pesticide use has been identified as a significant contributor to bumblebee population decline (Goulson et al., 2015; Williams & Hemberger, 2023).

Glyphosate is typically applied directly to foliage (Giesy et al., 2000), and in recently treated flowers, glyphosate residues may reach up to 629 mg/kg in pollen and up to 30 mg/kg in nectar (Thompson et al., 2014). Pollinator insects can be exposed to pesticides orally while foraging nectar or pollen through body contact by touching sprayed matrices or being sprayed directly when pesticide is applied (Böhme et al., 2018; Gradish et al., 2019). However, the susceptibility and routes of pesticide exposure may differ between pollinators, even between eusocial central-place foragers. Bumblebees, in particular, face heightened pesticide exposure both orally and through body contact (Gradish et al., 2019). They visit significantly more flowers per foraging trip—two to three times more—than honeybees and remain active under harsher weather conditions, as well as earlier and later in the season and throughout the day, extending their exposure window. As a result, bumblebees are especially likely to encounter pesticides, particularly during herbicide applications (Gradish et al., 2019). Their large body size and dense hair further increase the risk of pesticide contact exposure. Moreover, bumblebee queens forage in early summer and late autumn, increasing their chances of direct pesticide exposure during critical colony establishment and reproductive periods. Bumblebees and other eusocial bees may bring herbicides into their colonies via contaminated floral rewards, which can expose larvae, queens, and in-hive workers to both herbicide residues and their degradation products (Bokšová et al., 2023; El Agrebi et al., 2020; Wilmart et al., 2021). In addition, bumblebees typically build their nests underground. This nesting behavior creates an additional route of pesticide exposure for queens, workers, and larvae through soil residues. Lastly, bumblebees generally have a slightly smaller foraging range than honeybees (Beekman & Ratnieks, 2000; Greenleaf et al., 2007; Knight et al., 2005), which, in agricultural landscapes where herbicides are used, places them at a higher risk of encountering herbicide residues (Gradish et al., 2019; Raine & Gill, 2015).

1.5 The mind of the bee

The ability to learn is critical for pollinators. To be effective foragers, bumblebees must learn when and which flowers they should visit, how to handle flowers effectively, and how to find a way back to the colony with flower rewards (Klein et al., 2017). The ability to learn demands the optimal development, function, and plasticity of the brain (Giurfa, 2013; Menzel, 2012). Pesticides (Farina et al., 2019; Palmer et al., 2013; Peng & Yang, 2016), pathogens (Iqbal & Mueller, 2007), and a lack of certain nutrients (Arien et al., 2015) are known to affect the function of the brain and, thus, cognition.

Bumblebees' foraging behavior is shaped by innate preferences (instinct) and learned experiences. Bumblebees have an innate color preference toward the blue (and violet) range of the spectrum (Chittka et al., 2004; Lubbock, 1881; Willmer, 2011) and an innate shape preference for radiating and symmetric shape patterns (Lehrer et al., 1997; Møller, 1995). However, the strength and persistence of innate color preferences differ among species and between colonies (Ings et al., 2009). Moreover, the concept of pollination syndrome, whereby a plant's common pollinators can be predicted by the flower's color and/or shape, is oversimplified (Ollerton et al., 2009; Waser et al., 1996).

Flowers employ an array of floral traits, encompassing attributes such as flower color, flower accessibility, and specific geometries to attract pollinators with their reproductive structures. Indirect (e.g., floral display) and direct (reward) flower signals can be honest signals that correlate with reward quality and/or quantity (Raguso, 2008; Stanton & Preston, 1988; Wright & Schiestl, 2009). Pollinators use floral signals to find the most rewarding plants and are known to discriminate between flowers offering different and different amounts of rewards using visible morphological characteristics, such as floral displays (Russo et al., 2020) and flower colors (Gumbert, 2000; Kunze & Gumbert, 2001; Peitsch et al., 1992). Although bumblebees show some innate preference toward specific flower colors and shapes, which is already present in naïve bumblebees during their first flower visits (Lunau, 1990; Lunau & Maier, 1995), bumblebees also learn to associate rewards with colors (Heinrich et al., 1977).

Beyond their visible morphological traits, flowers utilize olfactory stimuli to attract pollinators (Kunze & Gumbert, 2001). They emit a diverse array of VOCs, also known as biogenic volatile organic compounds, which are produced through the anabolic and catabolic pathways of secondary metabolism in plants (Knudsen et al., 2006). These VOCs create unique scents that pollinators can use to find, recognize, and discriminate between flowers (Kunze & Gumbert, 2001). In addition to attracting pollinators, VOCs serve as secondary defense metabolites, deterring herbivores, recruiting natural enemies of herbivores (Arimura et al., 2005), and inducing defensive responses in nearby plants (Heil & Ton, 2008).

Bumblebees learn efficient handling techniques, quickly mastering flowers with exposed nectar, but they require more practice with complex floral structures in which rewards are hidden (Laverty, 1994). Social learning also plays a role since bumblebees can observe and imitate foraging strategies, including robbing from conspecifics (Goulson et al., 2013; Leadbeater & Chittka, 2007). Once a rewarding flower species has been identified, bumblebees exhibit floral constancy (Von Frisch, 1966), although they will adapt if rewards decline or the needs of the colony change (Raine & Chittka, 2012).

In addition to flower recognition and handling abilities, efficient foraging relies heavily on navigation, which is essential for eusocial bees to locate high-quality food sources and return to their colonies (Klein et al., 2017). Bees use a variety of environmental cues for navigation, including the sun's position and polarized light patterns (Dovey et al., 2013; El Jundi et al., 2014; Wehner et al., 1994), fine magnetic-field variations (Wajnberg et al., 2010), and visual landmarks (Collett et al., 2013; Goulson & Stout, 2001). *B. terrestris*, a central-place forager, generally operates within 2 km of the colony (Walther-Hellwig & Frankl, 2000) but can return from distances of up to 9.8 km (Goulson & Stout, 2001). This impressive homing capability relies mainly on familiar landmarks and landscape features for guidance (Brebner et al., 2021; Goulson & Stout, 2001).

Eusocial bees are particularly vulnerable to environmental stressors. Effective foraging requires complex cognitive abilities, including navigating large distances and learning how to recognize and handle diverse flowers. Any decline in foraging efficiency or homing accuracy poses a significant threat to colony survival (Bryden et al., 2013). Bees possess advanced learning and memory capacities to tackle tasks (Giurfa, 2013), which depend on optimal brain development and plasticity. However, pathogens (Iqbal & Mueller, 2007), nutritional deficiencies (Arien et al., 2015), and many pesticides, especially neonicotinoids (Cabirol & Haase, 2019; Palmer et al., 2013; Paoli & Giurfa, 2024; Peng & Yang, 2016; Rigosi et al., 2022), are known to impair brain functions, weakening cognitive abilities even with minor neurological damage.

Glyphosate and GBHs have been shown to interfere with bees' sensory processing, cognitive performance (Farina et al., 2019; Helander et al., 2023; Herbert et al., 2014; Mengoni Goñalons & Farina, 2018), and navigation ability (Balbuena et al., 2015). However, research on the underlying mechanisms of glyphosate's effects on bumblebees is limited.

1.6 The gut microbiota of bumblebees

Bumblebees host specialized gut microbial communities called gut microbiota. Gut microbiota are important for the well-being of bees (Dosch et al., 2021; Kwong, Mancenido, et al., 2017; Steele et al., 2021; Zheng et al., 2017) and have been shown to protect bumblebees against intestinal parasites such as *Crithidia bombi* (Koch & Schmid-Hempel, 2011, 2012) and pollutants (Rothman et al., 2019, 2020) and to affect the memory of bumblebees (Cryan & Dinan, 2012; Li et al., 2021). Bumblebees' gut microbiota are dominated by only a few bacterial taxa, including *Snodgrassella*, *Gilliamella*, Lactobacillaceae (*Bombilactobacillus* and *Lactobacillus*), Bifidobacteriaceae (especially *Bifidobacterium* and *Bombiscardovia*), *Schmidhempelia*, and *Bombiscardovia* (Hammer et al., 2021). In addition to these core symbionts, the bumblebee gut has low levels of other noncore bacteria (Hammer et al., 2021; Praet et al., 2018).

Most knowledge about social bee gut microbiota comes from honeybee research (Zheng et al., 2018), but since transmission routes and compositions of gut microbiota differ widely between honeybees and bumblebees, knowledge about the former cannot be directly applied to the latter (Hammer et al., 2021). For example, *Schmidhempelia* and *Bombiscardovia* are often present in bumblebee gut microbiota but are not found in honeybee gut microbiota (Hammer et al., 2021; Kwong, Medina, et al., 2017). Furthermore, honeybees and bumblebees have different but closely related strains of some bacteria, and the strain-level diversity within individuals is lower in bumblebees (Powell et al., 2016). In addition to bacterial composition, the transmission routes of gut microbiota differ. Newly emerged workers (NEWs) of social bees are nearly free of gut bacteria (Powell et al., 2014). In annual bumblebee colonies, the initial cohort of workers acquire gut microbiota from the foundress queen. Subsequently, the following broods of NEWs acquire microbial communities from older workers and the queen. Finally, new queens inherit the colony's microbiota, which they preserve over the winter, acting as vectors for its transmission when founding new colonies the following summer (Hammer et al., 2021). Sometimes, bumblebee queens lose their core microbial symbionts when entering and exiting diapause for winter (Bosmans et al., 2018; Koch et al., 2013). Although the transmission of bumblebee gut microbiota can be considered vertical (Hammer et al., 2021), the transmission also happens between adult bumblebees (gut microbiota is obtained after emerging), and horizontal transmission among adults within colonies occurs (Kwong & Moran, 2016). Because bumblebees acquire their gut microbiota from the foundress queen and older workers (Hammer et al., 2021), and since all bumblebees in the colony are offspring of the same single-mating queen (Schmid-Hempel & Schmid-Hempel, 2000), the genetic variation of bumblebees is limited, and the microbiota composition is similar within a colony. In contrast, in perennial honeybee colonies that reproduce through swarming, the establishment of microbiota in a new colony is facilitated by thousands of worker bees, and the honeybee queen does not contribute to this microbiota-seeding process (Powell et al., 2018).

As stated earlier, the efficacy of glyphosate is based on its inhibition of the EPSP synthesizing enzyme in the shikimate pathway. In addition to green plants, the shikimate pathway is also found in some bacteria (Leino et al., 2021). EPSP synthesizing enzymes can be classified into two types based on their tolerance to glyphosate: type I EPSP synthesizing enzymes, which are sensitive to glyphosate, have been identified in plants and bacteria, and type II EPSP synthesizing enzymes, which are tolerant to glyphosate, have been identified in only a few bacteria (Cao et al., 2012; Priestman et al., 2005; Stalker et al., 1985). Bees have both type I and type II bacteria in their gut microbiota (Motta et al., 2018).

The bacterial symbiont *Snodgrassella alvi* is a core member of the gut microbiota in bumblebees, (Powell et al., 2016). *S. alvi* colonizes the ileum, establishing close contact with the gut epithelium and providing resistance against pathogens. Another core symbiont, *Gilliamella apicola*, layers on top of *S. alvi*, forming a dense biofilm (Martinson et al., 2012). This biofilm is believed to protect bees from opportunistic pathogens (Horak et al., 2020; Raymann et al., 2017), although the specific protective mechanisms remain unknown. Potential mechanisms include physical blockage by the biofilm (Martinson et al., 2012), pathogen antagonism through *S.*

alvi’s type VI secretion system (Steele et al., 2017), and immune priming, whereby *S. alvi* activates the bee’s innate immune system to enhance pathogen resistance (Engel & Moran, 2013). Research has suggested that *S. alvi* may contain a type I EPSP synthase enzyme (Blot et al., 2019; Motta et al., 2018, 2020), making it susceptible to glyphosate. Notably, glyphosate treatment has been linked to a reduction in *S. alvi* abundance in the bumblebee gut microbiota, potentially affecting microbial community stability (Blot et al., 2019; Castelli et al., 2021; Motta et al., 2018, 2020; Motta & Moran, 2023).

1.7 Aims of the dissertation

In this dissertation, I study the effects of glyphosate and its commercial formulations, GBHs, on bumblebees. Specifically, my research addresses the following questions:

1. Can bumblebees be exposed to GBHs while foraging (Chapter I)?

I examine whether individual bumblebees discriminate between GBH-treated and control plants. I hypothesize that bumblebees visit GBH-treated plants less frequently than control plants. Additionally, I study the longevity of GBH-treated flowering plants and the exposure window for pollinators.

2. What are the effects of glyphosate and GBHs on certain important abilities of bumblebees (Chapters I, II, and III)?

I examine the effects of glyphosate and GBHs on bumblebees’ foraging behavior, homing ability, learning, and memory retention.

First, I investigate whether oral exposure to GBHs and the presence of an invasive plant species with showy inflorescences alongside crop plants influence the foraging behavior of bumblebees. I predict that GBH-exposed bumblebees will visit fewer plants than control bumblebees and that the presence of invasive plants will reduce visits to crop plants.

Second, I study whether oral exposure to GBHs alters bumblebee foraging activity and whether exposed bees can navigate back to their colony as efficiently as control bees. Additionally, I examine whether GBH exposure at the colony level leads to measurable effects on colony weight. I hypothesize that bumblebees from GBH-treated colonies exhibit fewer foraging bouts and prolonged foraging times compared to control colonies. Furthermore, I predict that GBH-exposed bees will experience higher rates of homing failure and longer homing times than control bees. I anticipate that the GBH-exposed colonies will be lighter at the end of the experiment compared to the control colonies due to longer foraging bouts, fewer gathered resources, and disoriented bumblebees that have not returned to the colony.

Third, I investigate how oral exposure to GBHs and pure glyphosate affects the learning of color–reward associations and long-term memory retention of bumblebees. I hypothesize that bees treated with glyphosate or GBH perform worse in learning and memory experiments than control bees.

3. How do glyphosate and GBHs affect the gut microbiota of bumblebees (Chapter IV)?

Finally, I study the potential reasons behind the effects of glyphosate on bumblebees. I examine how pure glyphosate and GBH affect the gut microbiota of bumblebees. I hypothesize that exposure to glyphosate and GBH alters microbiota by lowering the abundance of glyphosate-sensitive bacteria.

2 Materials and Methods

2.1 Study organisms

The bumblebees used in all experiments (I–IV) were commercial buff-tailed bumblebees (*Bombus terrestris*) from Koppert (Berkel en Rodenrijs, the Netherlands).

B. terrestris is a widespread and commercially widely used bumblebee species. Originally from the Palearctic region, *B. terrestris* has spread widely, such as to New Zealand (Hopkins, 1914), Argentina (Torretta et al., 2006), and Japan (Matsumura et al., 2004), as a greenhouse escapee. In Finland, it was first discovered at the beginning of the 1990s but is today one of the most common bumblebee species in southern Finland (Pekkarinen & Kaarnama, 1994).

Most studies on the effects of herbicides on bees have focused on honeybees (Cullen et al., 2019; Klein et al., 2022; Straw et al., 2022) because they are the most abundant and among the most important generalist pollinators in agricultural ecosystems. However, their role as pollinators for wild plants has often been exaggerated (Breeze et al., 2016; Ollerton et al., 2012; Westerkamp & Gottsberger, 2000; Winfree & Kremen, 2008), with 20,000 other bee species receiving less attention. Therefore, I chose the less-studied bee species *B. terrestris* as the study organism.

Bumblebees have been used as model animals (Lihoreau et al., 2025) to study cognition (Gibbons et al., 2022; Leadbeater & Chittka, 2005; Loukola et al., 2017), navigation (Lihoreau et al., 2012, 2012b; Woodgate et al., 2016), nutrition (Kraus et al., 2019; Ruedenauer et al., 2015; Stabler et al., 2015; Zhou et al., 2024), host–parasite interactions (Durrer & Schmid-Hempel, 1997; Gómez-Moracho et al., 2022; Koch & Schmid-Hempel, 2012; Tobin et al., 2024), and the effects of environmental stressors, such as pesticides (Gill et al., 2012; Helander et al., 2023; Kessler et al., 2015; Nicholson et al., 2024). *B. terrestris* and bumblebees in general have suitable colony sizes (from tens of workers to a few hundred workers) (Lopez-Vaamonde et al., 2003) for controlled indoor and flight cage experiments, and they are not very aggressive (Duchateau & Velthuis, 1988; Free, 1955; Jandt & Dornhaus, 2009; Spaethe & Weidenmüller, 2002). Small bumblebee colonies are easy to handle, and their manageable size allows for experiments that assess the effects of environmental stressors at both the colony and individual levels. Colonies of *B. terrestris* are founded by a single mating queen (Estoup et al., 1995; Schmid-Hempel & Schmid-Hempel, 2000); thus, all workers in colonies are full-sisters, and genetic diversity is low. Additionally, findings from bumblebee studies can be more reliably extrapolated to wild pollinator populations than results obtained from domesticated honeybee colonies (Lihoreau et al., 2025).

In Chapters I, II, and III, I chose the largest worker bumblebees as the study subjects. Bumblebees do not exhibit a strong age-based division of labor but instead display a size-based division (alloethism), whereby smaller workers typically perform tasks within the hive and larger workers act primarily as foragers (Cameron, 1989; Free, 1955; Goulson et al., 2002; Spaethe & Weidenmüller, 2002). Larger workers can carry more resources and have better optical quality of the eye (Spaethe & Chittka, 2003) and better antennal olfactory sensitivity (Spaethe et al., 2007).

In Chapter I, I used two plant species as study organisms: oilseed rape (*Brassica napus* ssp. *oleifera*, Brassicaceae) and garden lupine (*Lupinus polyphyllus* Lindl., Fabaceae). Oilseed rape was chosen due to its attractiveness to pollinators (Ion et al., 2012; Rašić et al., 2018) and its significance as one of the world's most productive oleaginous crops (FAOSTAT, 2025). It is widely cultivated in northern climatic regions (Vuorinen et al., 2014) and is among the most commonly grown glyphosate-resistant GM crops globally (Bansal & Kour, 2022). Oilseed rape is known to produce both nectar and pollen (Ion et al., 2012) and it benefits, at least partially, from insect pollination (Ouvrard & Jacquemart, 2019).

Garden lupine is an invasive ornamental herb chosen because of its floral attractiveness to bumblebees (Ramula & Sorvari, 2017), even though it does not produce nectar (Haynes & Mesler, 1984). Lupine has a large blue, pink, or white inflorescence. Bumblebees have an innate color preference for the blue range of the spectrum (Chittka et al., 2004; Müller, 1881; Willmer, 2011). Invasive plant species may reduce pollinator visits to native plants through competition and cause heterospecific pollen transfer (Brown et al., 2002; Thijs et al., 2012). However, because some invasive plant species can act as magnet species for pollinators (Lopezaraiza–Mikel et al., 2007), the native plants near plant such invaders may have increased flower visitation rates and richness of pollinators, especially generalist pollinators, such as bumblebees (Jakobsson et al., 2015; Jakobsson & Padrón, 2014; Lopezaraiza-Mikel et al., 2007).

2.2 Experimental setups

In all experiments, colonies of commercial bumblebees (queen, 50–100 workers, larvae, and combs) were transferred into wooden nest boxes (14.5 cm × 15 cm × 9 cm) with entrance halls of the same size. The nest boxes had transparent tops covered with pieces of cardboard to prevent light from entering the nests, since bumblebees prefer dark nesting sites (Wahengbam et al., 2019). When colonies were not actively used in the experiments, the bees were fed a 40% sugar water solution and bee pollen (Foodin, Jyväskylä, Finland) to prevent starvation.

In Chapter I, nest boxes were connected either to an outdoor flight cage or to a Y-maze. The outdoor flight cage (110 cm × 195 cm × 190 cm) was connected to the nest boxes by a transparent tunnel (Figure 2A) with doors that could be opened to control which bees were allowed to enter the cage. The same tunnel was used when the nest boxes were connected to the Y-maze (Figure 2B), a hollow, transparent glass device consisting of a main stem and two separate arms used to monitor insect responses or preferences to different odors and volatile compounds.

In Chapter II, a transparent tunnel was attached to the nest boxes, and the bumblebees were able to exit and enter the colony freely (Figure 2C, D). In Chapters III and IV, nest boxes were connected to a foraging arena (60 cm × 45 cm × 25 cm) with a transparent tunnel. The tunnel had doors that could be opened to control which bees could enter the arena (Figure 2E).

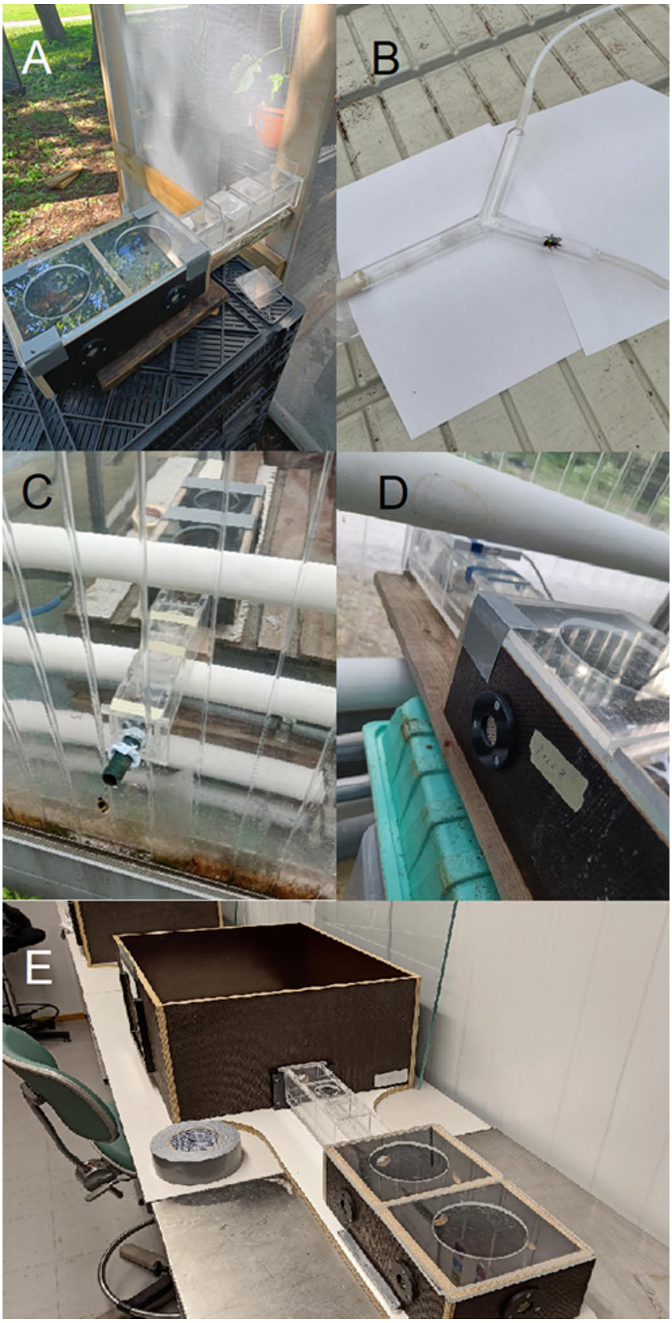

Figure 2. (**A**) Nest box attached to an outdoor flight cage with oilseed rape plants. (**B**) A bumblebee in the Y-maze. (**C**) Nest box with the bumblebee colony in the greenhouse. (**D**) Bumblebees were able to enter and exit the colony via a transparent tunnel that went through the greenhouse wall. (**E**) An empty nest box connected to a foraging arena. The transparent top is laid sideways on the entrance hall.

2.3 Field realistic exposure and exposure methods

Most studies of herbicides' effects on bees have been conducted using only active ingredients instead of commercial products (Cullen et al., 2019). In my experiments, I used both pure glyphosate (>98% purity, CAS: 1071-83-6, Sigma-Aldrich, St. Louis, MO, USA) and two different GBHs: Roundup Gold® (registration no. 1934; glyphosate concentration 450 g/L, as glyphosate isopropylamine salt, CAS: 3864-194–0, Monsanto Europe) and Roundup Flex® (registration no. 3072; glyphosate concentration 480 g/L, as glyphosate potassium salt CAS: 6229-014-3, Bayer Agriculture BVBA, Belgium). I used pure glyphosate and GBHs in laboratory experiments (in Chapters III and IV) to examine the difference between the effects of commercial products and glyphosate itself. In field experiments (in Chapters I and II), I used only commercial products (either Roundup Flex® or Roundup Gold®) to limit the fixed effects to a manageable scope and to ensure our investigation reflected the real-world agricultural use of these widely applied formulations.

Early herbicide toxicity experiments consisted of spraying various herbicides (e.g., MSMA, paraquat, 2,4-D, and 2,4,5-T) directly onto caged honeybees to determine whether the selected herbicides increased bee mortality (Moffett et al., 1972). Although pollinators may be topically exposed to GBH when it is applied in the field or when they touch contaminated matrices, here, I focused only on oral exposure. I used two methods to expose the bumblebees: a single acute dose given with a pipette and colony-level exposure offered with a gravity feeder. When studying the effects of glyphosate or GBHs on individual bumblebees (Chapters I, II, and III), it was essential to ensure that each bumblebee was exposed to an identical dose of the herbicide. To achieve this, selected bumblebees were fed a single acute dose of glyphosate mixed with sugar water. Each bumblebee was offered a droplet of the prepared solution, which the bumblebee consumed in an isolated cage. The administered dose ranged from 35–37 μg of glyphosate as the active ingredient. While bumblebees are unlikely to encounter such a dose at once, except in cases of direct herbicide spray during application (Gradish et al., 2019), they may visit thousands of flowers daily, making this exposure field realistic. Additionally, the administered dose aligns with previous studies on *Bombus terrestris* (Helander et al., 2023), ensuring comparability across research.

When investigating the effects of GBHs on foraging activity (Chapter II) and gut microbiota (Chapter IV), I employed a more field-realistic exposure method. Bumblebees are typically exposed to pesticides through continuous, low-level exposure rather than a single large dose, except in cases of direct spraying. By using a gravity feeder, I was able to expose the entire colony at once and observe colony-level differences in behavior.

In Chapter II, a gravity feeder containing 30 mL of glyphosate mixed with sugar water (10 mg/L of glyphosate) was placed on the nest boxes. The concentration of glyphosate was based on earlier studies with both honeybees and bumblebees, in which the concentration of glyphosate typically ranged between 2.5 mg/L and 10 mg/L (Balbuena et al., 2015; Herbert et al., 2014; Weidenmüller et al., 2022). Since bumblebees face additional exposure routes and may be exposed to larger amounts of pesticides (Gradish et al., 2019), I decided to use 10 mg/L of glyphosate. According to Thompson et al. (2014), nectar in recently treated flowers may have at most up to 30 mg/kg of glyphosate residues; hence, 10 mg/L is considered a field-realistic dose. A limitation of the gravity feeder is that the exact amount of herbicide consumed by individual bees is not known.

In Chapter IV, I used different concentrations of glyphosate (both pure glyphosate and GBH). The low concentration (10 mg/L) represented a field-realistic concentration that bumblebees may face in the agricultural landscape for several days (Thompson et al., 2022) after the application of herbicide. The higher concentration (5g/L) represented the worst-case scenario, whereby bumblebees are exposed to glyphosate directly after its application.

In Chapter I, I used a 6% GBH solution to treat the plants. This solution was made by blending 0.6 L of Roundup Flex® with 10 L of water. I used Bürkle handheld pressure sprayer to apply approximately 6.5 mL of solution to each plant. This volume and concentration align with the standard glyphosate treatment dosage and represent the maximum concentration recommended for weed control management in Finland, as specified on the product label.

2.4 Summary of methods in Chapters I-IV

This dissertation consists of four individual chapters that study the effects of glyphosate and GBHs on pollinators. I used novel and well-established approaches and performed experiments in the field and in the laboratory. Field experiments (Chapters I and III) were conducted in southwestern Finland, in Ruissalo Botanic Garden's (60° 26′ 00″ N, 22°10′ 23″ E) fields and greenhouses during the summers of 2022 and 2023. Laboratory experiments (Chapters II, IV) were conducted in the Bumblebee Laboratory at the Department of Biology at the University of Turku in 2022 and 2023.

2.4.1 Chapter I

The first study can be divided into two parts: the first one examines **whether individual bumblebees discriminate between GBH-treated and control oilseed rape** plants, and the second examines **how oral exposure to GBH and the presence of the invasive plant lupine influences the foraging behavior of bumblebees**.

To answer the first question, I performed a choice test in the field, a flight cage experiment, and a Y-maze olfactory test. I also observed the longevity of oilseed rape plants after GBH exposure and performed a volatile analysis of GBH-exposed oilseed rape.

In the field study, 16 observation squares (1 m² each) were established in a flowering field, half of which were sprayed with GBH (GBH treatment) and the other half with water (control). I began pollinator observations 1 h after spraying and recorded all the insect pollinators that visited flowers within the squares. Each square was observed six times over the following 3 days (twice a day), with each observation period lasting 10 min. A pollinator landing on any flower in the same or a different plant within a square was recorded as a visitation.

In the flight cage setup, bumblebee hives were connected to an outdoor flight cage via a tunnel, allowing individual naïve bumblebees to forage freely among four oilseed rape plants (Figure 2A). Two plants (oilseed rape) were sprayed with Roundup Flex® (GBH treatment), while the other two received only water (control). Treatments were applied 16–24 h or 40–48 h before the experiment. Each bumblebee's foraging behavior was observed for 10 min, starting from its first interaction—defined as the moment it landed on a flower. All plant and flower visits were recorded throughout the observation period.

In the Y-maze olfactometer experiment, bumblebees were given a choice between a control flower and a flower sprayed with GBH, relying solely on the flowers' odors/volatiles to make their selection. Two oilseed rape plants, one treated with GBH and one with water, were enclosed in separate bags. A high-pressure pump was used to transfer volatile compounds from each bag into separate arms of the Y-maze. Individual bees entered the Y-maze, where they had 2 min to choose between the two arms.

In the volatile analysis, we studied **whether GBH treatment affected the VOCs emitted by oilseed rape plants**. We collected VOCs from control and GBH-exposed oilseed rape plants 6 and 24 h after treatment. The plants were enclosed in oven bags connected to a flow of clean air via a Teflon tube attached to an air pump into the bag (225 ml/min) and a suction of 200 ml/min through a Tenax TA adsorbent filter. The VOC samples were analyzed using gas chromatography–mass spectrometry, and ChemStation software was used for peak identification and quantification. Most of the detected compounds belonged to one of three compound classes: green leaf volatiles, monoterpenes, or sesquiterpenes.

To answer the second question, I performed a flight cage experiment and a Y-maze olfactory test. In the flight cage setup, individual naïve bumblebees entered an outdoor cage containing two test plants (two oilseed rape plants or oilseed rape and lupine). First, I observed the bees foraging freely for 10 min, recording all visits to flowers. The experiment was then repeated after feeding the bees with an acute GBH dose. I then conducted a Y-maze olfactory experiment for the control and exposed bumblebees. A lupine and an oilseed rape plant were enclosed in separate bags, and a high-pressure pump was used to transfer volatile compounds from each bag into the separate arms of the Y-maze. Individual bees entered the Y-maze, where they had 2 min to choose between the two arms. After that, I repeated the setup with bees treated orally with GBH.

2.4.2 Chapter II

In Chapter II, I studied **the effects of GBH on the foraging activity and homing ability of bumblebees.** A two-phase field experiment was conducted with 10 colonies of commercial buff-tailed bumblebees. In both experiments, I used a radio frequency identification (RFID) device provided by Microsensys GmbH. This RFID device contained a controller (ID® BEEcontroller), two tandemly arranged antennae (iID® science reader device AEB-03.C2D) (Figure 3A), and hundreds of passive RFID tags (BEE-TAG mic3® Q1.6) (Figure 3B) that contained unique identification numbers.

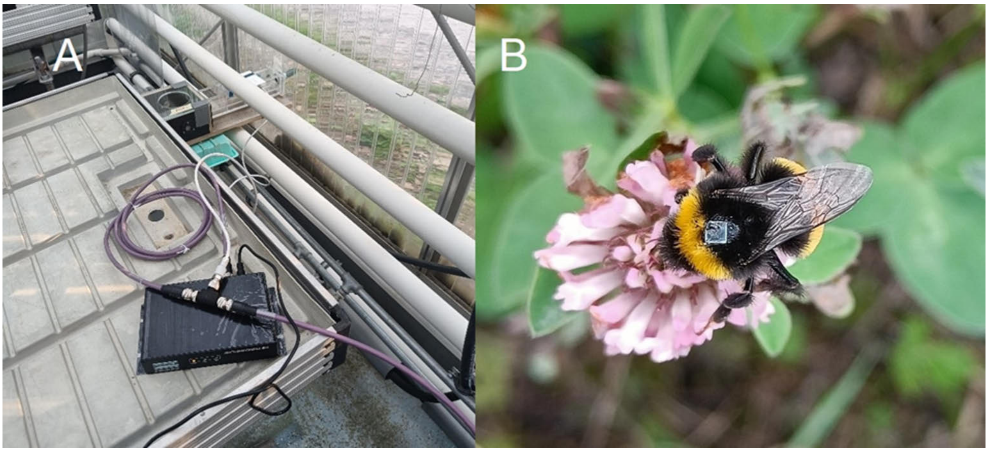

Figure 3. (**A**) Radio frequency identification device: Controller (ID® BEEcontroller) attached to a reader placed on top of the entrance tunnel. (**B**) A bumblebee tagged with an RFID tag with a unique identification number.

First, I transferred colonies to nest boxes and marked 10–15 foraging bumblebees per colony (140 bees in total) with tiny (1.5 mm^3) RFID tags. Then, I placed the colonies (two colonies simultaneously) with the marked bumblebees in the nest boxes inside the greenhouse. The bumblebees were able to move freely between the colony and the external environment via a tunnel that went through the greenhouse wall (Figure 2C, D).

Because the two colonies in each replicate were close to each other, I painted their entrances with different colors to ease their identification. After the bees familiarized themselves with the environment for 24 h, I placed a gravity feeder in both nest boxes. One colony (GBH treatment) received a gravity feeder containing 30 mL of 60% sugar water with 10 mg/L of Roundup Flex®, while the other colony received a similar gravity feeder with only sugar water. In all 10 colonies, the bumblebees had emptied the feeder by the next day.

Next, I began the foraging activity experiment by activating the RFID tracking devices positioned at the entrances of each colony. When a tagged bee came within the reach of a reader, the tag received an electromagnetic signal from the reader. Then, the energy of the signal was stored in the tag's capacitor, and when the capacitor built up enough energy, the tag transmitted a modulated signal containing the tag's information (e.g., its identification number). The reader received the signal, demodulated it, and stored the tag's information in its external memory, together with the time when the tag was detected (Nayak, 2019).

Tagged bumblebees were then observed during a 5-day free-flying period, along with all arrivals and departures. The information was stored in the device's memory with a time stamp, which enabled us to count the number of bouts of a marked bee during the observation period and estimate the duration of its foraging trips.

During the foraging activity experiment, I began the homing ability experiment. I selected 8–12 nontagged foragers from each colony, marked them with RFID tags, and recorded their original colony. These bees had already undergone colony-level exposure to either a plain sugar solution or a GBH solution at 10 mg/L for 24 h. I applied an additional acute exposure treatment by feeding them either 7 µL of 60% sugar water or 7 µL of 60% sugar water containing 5.28 g/L glyphosate in Roundup Flex®. For each paired replicate (GBH treatment and control), I randomly selected two release sites located at distances of either 500 m or 1000 m from the greenhouse (Figure 4). I transported the marked bees to the release sites in transfer boxes and released half of the bees at the 500 m site and the other half at the 1000 m site. The bees were released individually from the transfer boxes. Each bee had 2.5 days to return to its colony, after which the colonies in the greenhouse were removed and replaced with new colony pairs. Any bee that did not return to its colony within this timeframe was considered lost.

Figure 4. Release sites (yellow dots) for the homing ability experiment. The greenhouse and bumblebee colonies are located at the center of the circles. The radii of the inner and outer circles are 500 m and 1000 m, respectively.

2.4.3 Chapter III

In Chapter III, I studied **the effects of glyphosate and GBH on the learning and memory of bumblebees.** I conducted a two-phase 10-color discrimination experiment in the laboratory with 10 colonies of commercial buff-tailed bumblebees. These bumblebees were placed in nest boxes connected to a pollination arena with a corridor (Figure 2E). The corridor had doors that could be opened to control which bees were allowed to enter the arena.

First, bumblebees were marked with identification tags and pretrained to feed on sucrose water on colorless artificial flowers placed in the arena. The marked bees were released into the arena one at a time and allowed to forage for 5 min. This pretraining was repeated five times for each bumblebee selected for the experiment.

I then selected 5–10 pretrained bees for the learning experiment. One by one, selected bees were let into the arena, which contained artificial flowers of 10 different colors (Li et al., 2017). Five colors had 7 µL of 40% sugar water solution, and the other five had 7 µL of bitter-tasting quinine water solution (Figure 5A). The bees were allowed to forage in the arena for 10 min or until they left the arena voluntarily. I recorded all visits to the artificial flowers when the bee drank sugar or quinine water (i.e., touched either one with its proboscis) in writing and by video.

Choosing an artificial flower with sugar water was marked as a success, while choosing an artificial flower with bitter-tasting quinine water was marked as a failure. The learning experiment contained five 10-min learning bouts in the arena and was conducted over a 2-day period (Figure 5B).

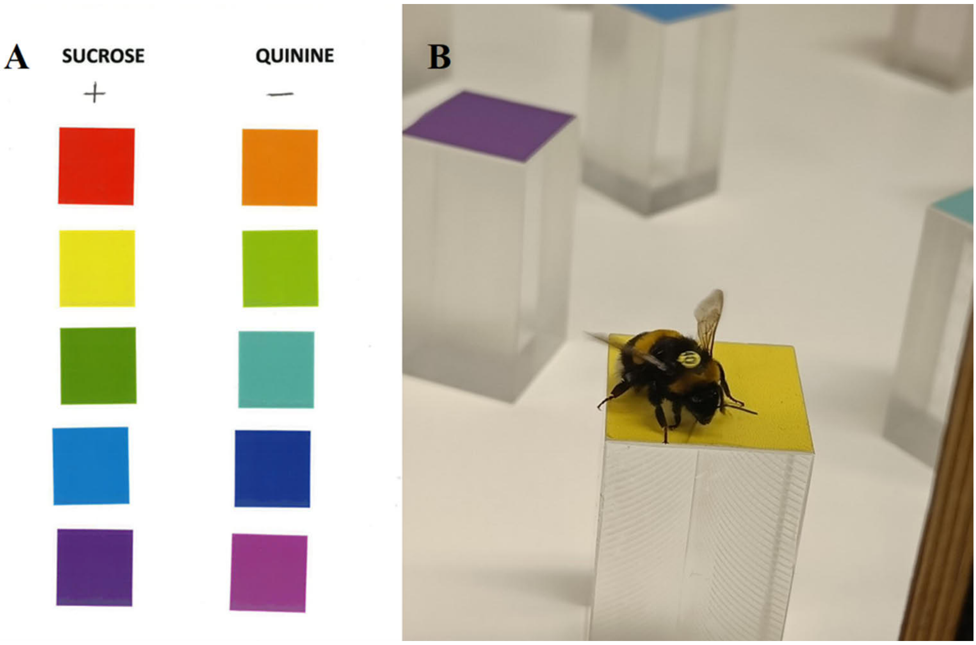

Figure 5. (**A**) Color codes for sugar and quinine water. (**B**) Bumblebee marked with an identification tag lands on a yellow artificial flower containing sugar water.

Forty-eight hours later, I performed a single-bout memory retention experiment to examine whether the bees could remember which color represented the rewarding sugar droplets. The experimental bees underwent a memory test similar to the learning phase. However, all the artificial flowers contained plain, unrewarding water instead of rewarding sugar or aversive quinine water. As in the learning test, bees had 10 min to forage in the arena, and all visits were recorded in writing and by video.

After the memory test, I repeated the whole experiment, from the pretraining to the memory test, with new bees from the same colonies. However, this time I exposed the chosen bees after the pretraining either to a 7 µL mixture consisting of 60% sugar water and pure glyphosate or to a 7 µL mixture consisting of 60% sugar water and Roundup Gold®.

2.4.4 Chapter IV

In Chapter IV, I studied **the effects of pure glyphosate and GBH on the gut microbiota of bumblebees** in the laboratory. First, the bumblebee colonies were transferred to nest boxes connected to a foraging arena by a tunnel. Bumblebees were allowed to move freely between the hive and the flight arena, where they could feed on a 60% sucrose solution for 2 days. From each of the six colonies, five actively foraging bumblebees (a total of 30 bees) were removed, placed into Eppendorf tubes, immediately snap-frozen in liquid nitrogen, and stored in a deep freezer at −80°C. Then, the rest of the bumblebees were exposed to either pure glyphosate or GBH Roundup® using a gravity feeder.

Including a control treatment for each colony, the study comprised five treatment groups: two colonies exposed to a low concentration (10 mg/L) of pure glyphosate, one colony exposed to a high concentration (5 g/L) of pure glyphosate, two colonies exposed to a low concentration (10 mg/L) of glyphosate as the active ingredient in Roundup Gold®, and one colony exposed to a high concentration (5 g/L) of glyphosate in Roundup Gold®.

After the bumblebees from each colony had fed on either pure glyphosate or Roundup Gold® mixed with sugar water for 3 or 5 days, five randomly selected actively foraging bumblebee workers in the foraging arena were removed from each colony, placed in an Eppendorf tube, flash-frozen in liquid nitrogen, and stored at −80°C.

Individual bees were rinsed in 70% ethanol, and their intestines (crop, midgut, and hindgut) were aseptically removed and placed on ice. Genomic DNA was extracted using ZymoBiomics DNA Microprep kits (Zymo Research), and DNA libraries were prepared following a modified Earth Microbiome Project protocol for 16S Illumina amplicons. The V4 region of the 16S rRNA gene was amplified using 515FB and 806RB primers with Illumina adapters and barcodes. Negative extraction and PCR controls were included, as well as a mock community sample (ZymoBIOMICS Microbial Community DNA Standard). The PCR products were quantified with a Qubit DNA HS assay, pooled equimolarly, and purified with SPRI beads. Sequencing was performed on an Illumina MiSeq platform (2 × 250 bp) at the Finnish Functional Genomics Center (Turku, Finland).

To examine the effects of colony-level exposure to glyphosate and GBH on bee mortality, I marked 30 individual forager bees from each experimental hive as well as from four additional hives not previously used in the experiment. Each bee was marked with a small plastic number tag (Bienen-Voigt & Warnholz, Germany) glued to its thorax with super glue. For 7 days following the start of the treatments, all deceased marked bumblebees were collected and counted daily from each hive.

2.5 Statistical analysis

All statistical analyses were performed in R mostly using generalized linear mixed models (GLMMs) and linear mixed-effects models (LMMs; lme4::lmer) (Bates, 2010). For the GLMMs, I evaluated the fixed effects using type II Wald's χ^2 test (car::Anova) (Fox & Weisberg, 2019), and for the LMMs, I used F-tests with type III Kenward–Roger's method (lmerTest::anova) (Kuznetsova et al., 2017). Model assumptions were verified from residual plots for all models and from dispersion analysis for the GLMMs (DHARMa::testDispersion) (Hartig, 2018).

In Chapter I, I analyzed bumblebee foraging behavior in flight cages on GBH-exposed oilseed rape using GLMMs with a binomial distribution and a logit-link function (glmmTMB::glmmTMB) (Magnusson et al., 2017). I tested whether treatment (GBH-exposed vs. unexposed plants), exposure duration (>24 h or >40 h), their interaction, temperature, and the number of open flowers per plant influenced foraging probability. Plant tetrad and colony were included as random factors. I further examined whether treatment, time from treatment, their interaction, temperature, and flower number influenced plant and flower (log-transformed) visit frequency using LMMs, with colony included as a random factor.

In the field study, I examined whether GBH treatment of squares with flowering plants, the time of the treatment, their interaction, and temperature affected flower visitation (sqrt-transformed) in a field setting using an LMM. Flowering squares were used as a random factor. This analysis was conducted separately for all insect pollinators and for the observed bumblebees.

In the choice test on GBH-exposed bumblebees in the flight cage, I used a GLMM with a binomial distribution and logit-link function to assess whether oral exposure and the presence of invasive lupine influenced foraging probability. Fixed effects included oral exposure, lupine presence, their interaction, temperature, and flower number, with colony as a random factor. To evaluate plant and flower (log-transformed) visit frequency, I used LMMs with oral exposure, the presence of an invasive plant, temperature, and flower number as fixed effects.

Differences in the choices made by bumblebees in both Y-maze experiments (GBH-exposed flowers and GBH-exposed bees) were tested for significance using a chi-square test. I examined only the first decisions that the bumblebees made.

To assess the impact of GBH treatment 6 h and 24 h after application on leaf VOC composition, I conducted nonmetric multidimensional scaling (NMDS) analyses of all 31 compounds (vegan::metaMDS) (Oksanen et al., 2025) using a Bray–Curtis dissimilarity matrix (vegan::vegdist) with Wisconsin double standardization and two dimensions (stress = 0.167) for ordination. I tested whether the time after GBH application affected VOC emissions via permutational analysis of variance (PERMANOVA; 9999 permutations; vegan::adonis), followed by post hoc tests for individual compounds.

In Chapter II, I used LMMs to assess whether foraging bout number and duration (both log-transformed) differed between the two treatments (BGH-treated and control colonies) in the foraging activity experiment. I also included date as a continuous explanatory variable and the paired replicate and bee's identification number as random factors. To investigate the effects of GBH exposure and release distance on the homing rate, I used a GLMM with a binomial distribution and a logit-link function (glmmTMB::glmmTMB) (Magnusson et al., 2017), and for the homing time, I used an LMM. The homing rate was defined as the binary outcome of whether an individual bumblebee returned to the colony, while the homing time was measured as the duration in minutes required for the bumblebee to return (log-transformed). The study included acute exposure treatment (GBH-treated and control bees), colony-level exposure treatment (GBH-treated and control colonies), and release distances (500 m and 1,000 m) as fixed categorical explanatory variables. I also analyzed all possible two- and three-way interactions among these variables. Date was included as a continuous explanatory variable, and paired replication was included as a random effect.

In Chapter III, I used two GLMMs with a binomial distribution and a logit-link function (lme4::glmer) (Bates, 2010). In the learning test, the learning bout (five levels), treatment (control, glyphosate, and Roundup®), and their interaction were used as fixed categorical explanatory variables, while the colony was used as a random effect. Bee identity nested within the colony was included as a random factor. In the memory test treatment (control, glyphosate, and Roundup®), learning bout (bout five and memory test) and their interaction were used as a fixed explanatory variable, and bee identity nested within the colony was used as a random factor. For both models, I assessed pairwise differences in mean values with a Tukey's test to compare the differences between treatments and (learning) bouts (emmeans::emmeans) (Lenth, 2012).

In Chapter IV, sequences were grouped into amplicon sequence variants (ASVs) and classified with DADA2 (Callahan et al., 2016) using the Ampliseq pipeline (version 2.4.0). Diversity metrics (alpha and beta) were computed using the mia (Ernst et al. 2022) and vegan (Oksanen et al., 2025) packages, and community shifts were analyzed using PERMANOVA. Genus-level phylogeny was inferred in mia, and significantly responding ASVs were identified using ALDEx2 (Fernandes et al., 2013), ANCOM-BC (Lin et al., 2022), MaAsLin2 (Mallick et al., 2021), LinDA (Zhou et al., 2022), and DESeq2 (Love et al., 2014).

3 Results

Bumblebees do not discriminate against flowers exposed to GBH over uncontaminated flowers (Chapter I). GBH treatment, time from treatment, and their interaction did not affect the visitation rate of all pollinators or bumblebees in the field study. In the flight cage experiment, neither treatment (exposed or control), time from treatment (exposed <24 h or <48 h earlier), nor their interaction affected bumblebees' visits to oilseed rape plants or flowers (Figure 6A, B). Neither did the treatment of plants affect the bumblebees' choices in the y-maze experiment.

In the second choice test with oral GBH exposure and two different plant species, **oral exposure to GBH lowered bees' foraging activity** (GLMM: df = 1, $\chi^2 = 5.56$, $p = 0.018$) by 49% compared to the control bumblebees (Figure 6C). The interaction between oral exposure and the presence of an invasive plant did not significantly affect the number of plants or flowers visited, but it was significantly correlated with the number of plants visited. In the presence of an invasive plant, control bumblebees tended to visit fewer plants than GBH-exposed bumblebees, while there was no difference in the number of plant visits when only oilseed rape plants were available (Figure 6D).

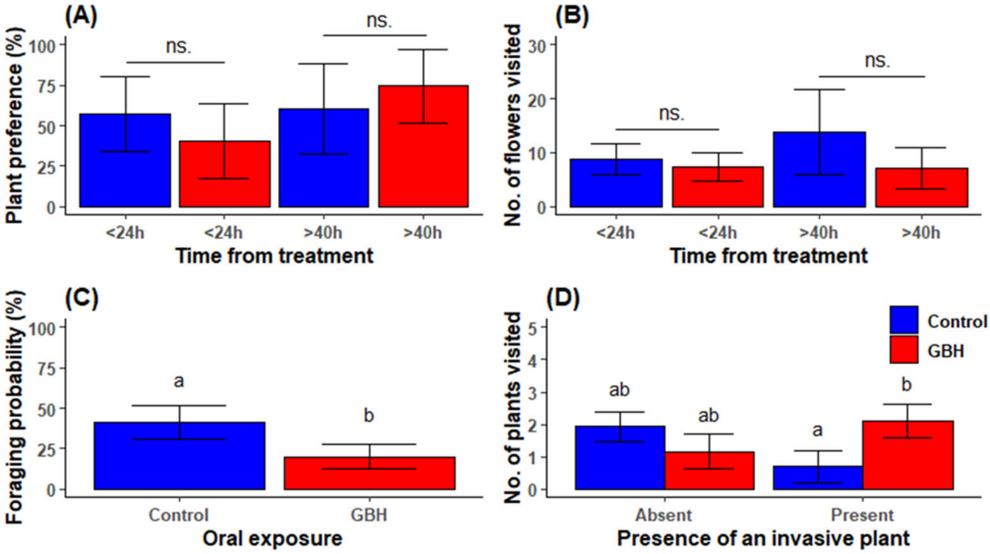

Figure 6. Back-transformed least square mean (± SE) for the effects of glyphosate-based herbicides (GBHs) and plant combination on bumblebee foraging behavior. (**A**) Probability of bumblebees landing on oilseed rape plants exposed to GBH. (**B**) Number of oilseed rape flowers exposed to GBH visited by bumblebees. (**C**) Effect of oral GBH exposure on overall foraging probability. (**D**) Effects of oral GBH exposure and presence of an invasive plant on the number of plants visited. Differences between treatments are indicated by different letters ($p < 0.05$ for foraging probability and $p = 0.07$ for the number of plants visited).

The bumblebees did not frequently visit lupines. When the two plant species (oilseed rape and lupine) were available, they visited 16 (out of 58) different oilseed rape individuals at least once but only four individual lupines.

In the longevity observation, the oilseed rape plants remained alive at least 3 days after the application of GBH. After this period, the GBH-treated plants began to lose flowers, and their green parts started to turn yellow (Figure 7).

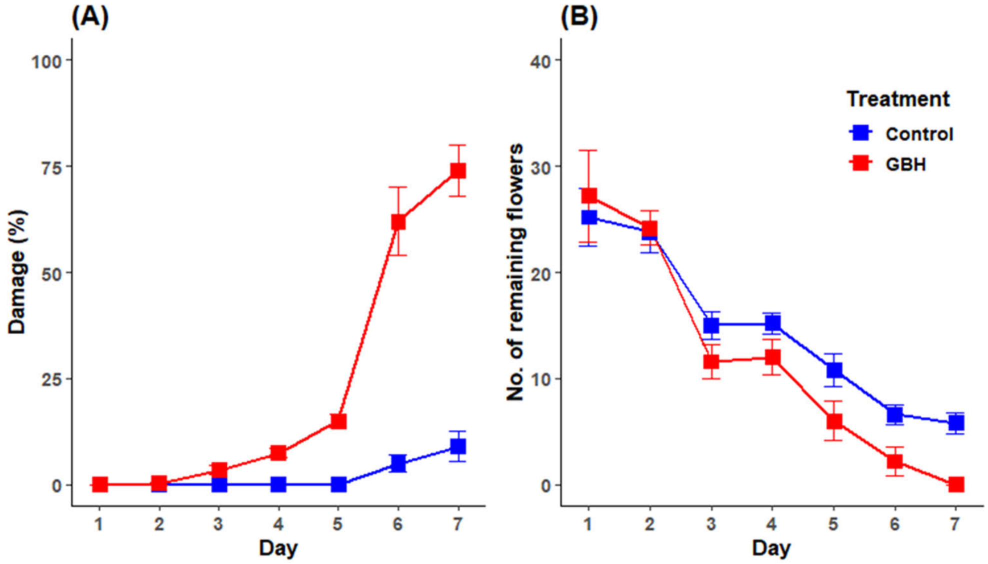

Figure 7. Summary of longevity observations for oilseed rape plants sprayed with water (control) and glyphosate-based herbicide (GBH). Damage (%) represents the mean percentage of yellow parts, and the number of remaining flowers indicates the mean number of flowers remaining on oilseed rape plants (mean ± SE).

When examining the effects of GBH on leaf VOC emissions, we identified 31 compounds. NMDS analysis revealed a shift in volatile composition between the control and GBH-treated plants (df = 2, F = 3.89, $R^2 = 0.28$, p = 0.024; Figure 8), most prominent 24 h after GBH treatment. At the individual compound level, the concentrations of α-farnesene, β-ocimene, and myrcene were significantly lower 24 h after GBH treatment compared to control and those sampled after 6 h.

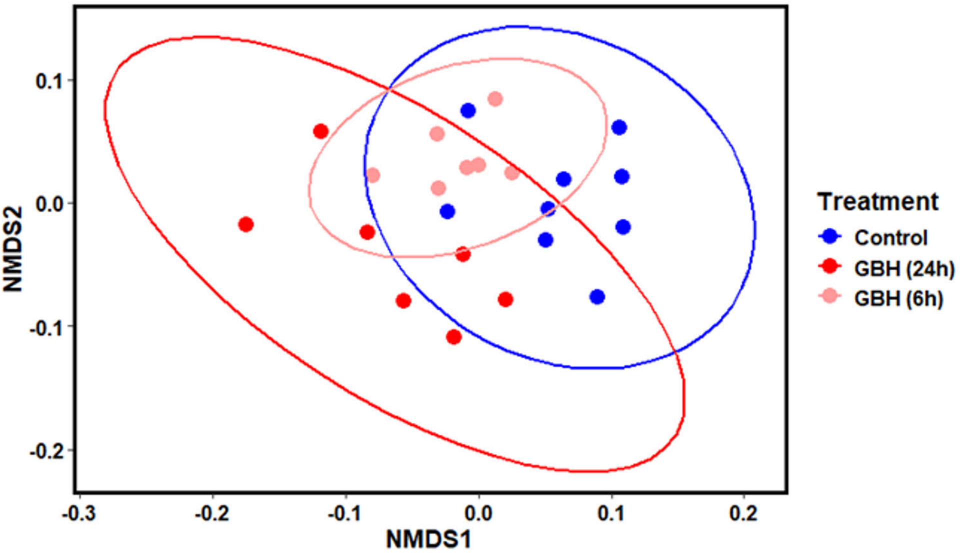

Figure 8. Nonmetric multidimensional scaling ordination based on 31 leaf volatile organic compound (VOC) emissions rates (ng g−1 min−1) collected from the flowerheads of individua oilseed rape plants. Ellipses with 95% confidence intervals are drawn by treatment. GBH (24 h) = 24 hours after glyphosate-based herbicide (GBH) treatment, GBH (6 h) = 6 hours after GBH treatment.

The results from the foraging activity and homing experiment (Chapter II) indicate that colony-level **exposure to GBH led to a 94% increase in the number of foraging bouts** (LMM: df = 1, ddf = 77.5, F = 6.39, p = 0.01) among bumblebees compared to the control colonies (Figure 9A). However, **GBH exposure did not affect the duration of these bouts.** In the homing ability experiment, **colony-level exposure did not impact the overall homing rate** (Figure 9B). Nevertheless**, a marginally significant interaction was observed between acute individual-level exposure and release distance** (GLMM: df = 1, $\chi^2 = 3.38$, p = 0.066). Specifically, GBH-treated bees released at a shorter distance (500 m) showed a tendency toward a higher homing rate than control bees. Acute exposure, colony-level exposure, release distance, or date did not affect the homing time of the bees.

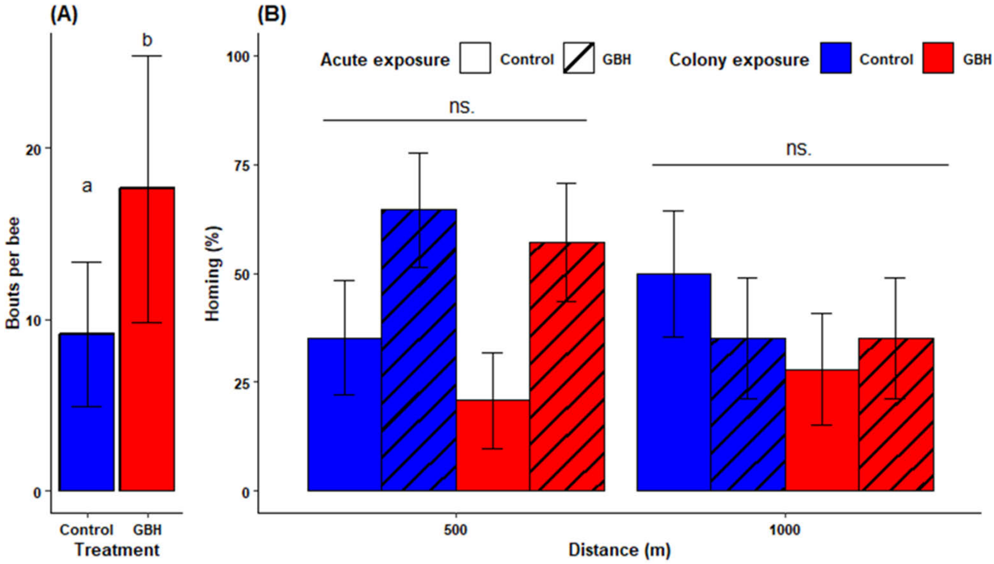

Figure 9. (**A**) Number of foraging bouts per bumblebee from the control colonies and colonies exposed to a glyphosate-based herbicide (GBH) during the foraging activity experiment. (**B**) Homing rate (%) of displaced bumblebees (500 m and 1000 m) with 24-hour and acute GBH exposure in the homing ability experiment. The figures show back-transformed least square means (±SE). The bars with different letters indicate significant differences ($p < 0.05$).

The results of the 10-color discrimination experiment (Chapter III) indicate that **both treatment** (control, pure glyphosate, and Roundup®) (GLMM: $\chi^2 = 16.67$, df = 2, $p < 0.001$) **and the number of learning bouts** (GLMM: $\chi^2 = 112.83$, df = 4, $p < 0.001$) **significantly impacted bumblebees' performance in landing on rewarding flowers,** although no interaction was found between the two. Pure glyphosate impaired the bumblebees' learning ability in a 10-color discrimination experiment, but the GBH (Roundup Gold®) had no observable effect. Glyphosate-treated bees had a 14% lower performance, on average, than control bees in all learning bouts. The bumblebees' performance improved in all three treatments over the five learning bouts, with the most progress seen in the first three bouts, after which improvement slowed. By the fifth and final bout, the control group reached a performance level of 80%, the pure glyphosate group reached 66%, and the GBH group reached 79% (Figure 10A). In the memory test conducted 48 h after the final learning bout, **both treatment** (GLMM: $\chi^2 = 20.59$, df = 2, $p < 0.001$) **and bout** (GLMM: $\chi^2 = 30.47$, df = 1, $p < 0.001$) **significantly impacted the bumblebees' memory performance,** with a notable interaction between the two factors (GLMM: $\chi^2 = 7.71$, df = 2, $p = 0.021$). Pairwise comparisons revealed no significant difference between the control and Roundup® groups, but the glyphosate-treated bees performed significantly worse than the controls (Tukey's test: SE = 0.13, z = 4.67, p < 0.001). Memory declined in all three groups compared to the fifth bout, with the largest reduction in the Roundup® group (19%), followed by the control (14%) and glyphosate (11%) groups (Figure 10B).

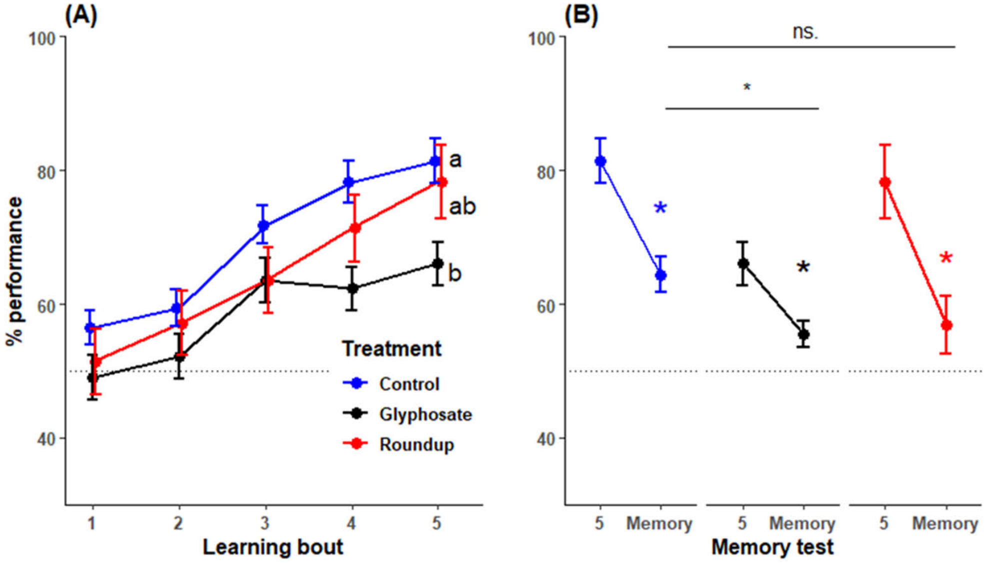

Figure 10. (**A**) Back-transformed least square mean (± SE) of correct landings (%) on rewarding flowers by buff-tailed bumblebees (*Bombus terrestris*) exposed to glyphosate, the glyphosate-based herbicide (GBH) Roundup Gold®, or no exposure (control) in (**A**) a five-bout learning test and (**B**) a single-bout memory test conducted 2 days after the previous learning bout. During the learning phase, bees chose between artificial flowers of 10 colors that contained rewarding sugar water or unpalatable quinine water. The dotted line indicates the chance level (50%). The different letters show significant treatment differences (Tukey's test, p < 0.05). (**B**) The asterisks denote significant performance changes between the fifth learning bout and the memory test within each treatment, with an asterisk above the bracket indicating significant memory test differences between control and glyphosate, while ns. indicates no significant difference between control and Roundup®.

Glyphosate increased gut microbiota diversity, while GBH reduced it (Chapter IV), suggesting that the negative effects were likely due to coformulants. Both glyphosate and GBH treatments significantly reduced the relative abundance of the potentially glyphosate-sensitive bacterial species *Snodgrasella alvi*. In contrast, the relative abundance of the potentially glyphosate-sensitive species *Candidatus schmidhempelia* increased in the bumblebees exposed to glyphosate.

After day 7 of the experiment, cumulative mortality rates were observed as follows: control bees at 16%, low Roundup® treatment (10 mg/L) at 29%, high Roundup® treatment (5 g/L) at 66%, low glyphosate treatment (10 mg/L) at 15%, and high glyphosate treatment (5 g/L) at 34% (Figure 11). The low Roundup®

treatment (10 mg/L) can be classified as sublethal exposure, while **the high Roundup® treatment significantly increased bumblebee mortality.**

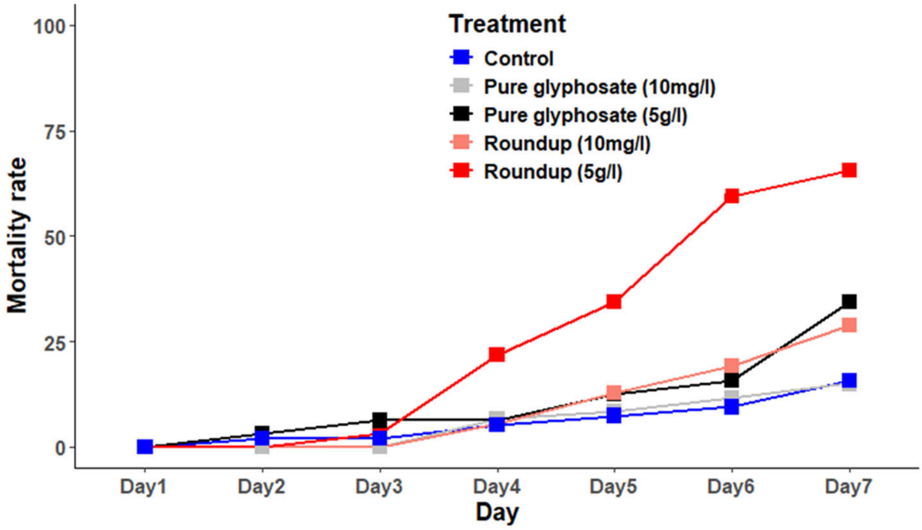

Figure 11. Mortality rates of bumblebees exposed to different treatment groups.

Table 2. Summary of the main results from each chapter. The table also specifies whether the study was conducted in a laboratory or field setting, the method of exposure (acute: a single dose administered via pipette; colony-level: continuous exposure via gravity feeder for a designated period), and the amount of active ingredient glyphosate administered to bumblebees (*Bombus terrestris*).

Chapter	Main results	Field or laboratory experiment	Exposure
I. Bumblebees (*Bombus terrestris*) forage on plants treated with glyphosate-based herbicides (GBHs) despite potential behavioral consequences.	1. Bumblebees visit GBH-exposed plants even if there are uncontaminated plants available. 2. Oral GBH exposure decreases the foraging activity of bumblebees. 3. GBH alters the composition of volatile compounds emitted by flowers.	Field and laboratory	For bees: Acute: 35 µg of glyphosate/bee (Roundup Gold®) For plants: ~180 mg of glyphosate/plant ~290 mg of glyphosate/square (according to recommendations for Roundup Flex®)
II. Glyphosate-based herbicide (GBH) increases the number of foraging trips but does not affect the homing of *B. terrestris.*	1. GBH increases the number of bouts made by bumblebees. 2. GBH does not affect the time spent on bouts. 3. GBH does not affect homing (time or percentage of homed bumblebees).	Field	Colony-level exposure (24 h): 10 mg/L of glyphosate (Roundup Flex®) Acute: 37 µg of glyphosate/bee (Roundup Flex®)
III. Effects of glyphosate and glyphosate-based herbicide on the learning and memory of the buff-tailed bumblebee (*B. terrestris*).	1. Pure glyphosate impairs the learning and memory of bumblebees. 2. GBH does not affect learning and memory.	Laboratory	Acute: 35 µg of glyphosate/bee (Roundup Gold® and pure glyphosate)
IV. Glyphosate and a glyphosate-based herbicide affect bumblebee gut microbiota.	1. A high treatment of Roundup is lethal for bumblebees, while a lower treatment is sublethal. 2. Glyphosate increases and GBH decreases gut microbiota diversity. 3. Both decrease the abundance of *Snodgrasella alvi.*	Laboratory	Colony-level exposure (3 days and 5 days): 10 mg/L and 5 g/L of glyphosate (Roundup Gold® and pure glyphosate)

4 Discussion

Pollinator insects can be exposed to glyphosate and other pesticides through body contact by touching sprayed matrices, being sprayed directly when pesticide is applied, or orally while foraging nectar or pollen from recently exposed flowers (Böhme et al., 2018; Gradish et al., 2019). Bumblebees are suspectable to obtain large amounts of pesticide residues because of their morphology and foraging behavior (Thompson et al., 2014). The unsolved question is whether bumblebees or other pollinators can recognize plants contaminated with pesticides. Although sublethal levels of glyphosate (Russo et al., 2020) and dicamba (Bohnenblust et al., 2016) have been shown to reduce the visits of pollinators, it has been stated that bumblebees are poor at detecting contamination in flowers (Kessler et al., 2015; Tiedeken et al., 2014). The results in Chapter I show that bumblebees do not discriminate against plants or flowers exposed to GBH over uncontaminated flowers. Although we found a minor shift in the emitted volatile composition between control plants and plants sprayed with GBH 24 hours previously, bumblebees did not discriminate against plants exposed to GBH, even when their choice was based solely on emitted VOCs.

I found that exposure to GBH altered the foraging behavior of bumblebees in contrasting ways across experimental setups. In the free-flying experiment (Chapter II), GBH exposure significantly increased the number of foraging bouts. The bumblebees exposed to GBH made 94% more foraging bouts than the control bumblebees over the 5 days following exposure. Because the duration of foraging bouts did not differ between the control and GBH-exposed bumblebees, GBH exposure seemed to increase the foraging activity. Increased foraging activity is typically considered a positive response because more active bees can forage more resources and support colony growth and survival, especially during less favorable seasons. Unfortunately, with the RFID system, I could only monitor the number and duration of foraging bouts, and I was unable to observe whether foraging bumblebees visited any flowers or how many resources they brought to the colony. Since earlier studies have shown the impairment of learning after exposure to glyphosate (Helander et al., 2023) and difficulty in gathering resources after exposure to other pesticides (Stanley et al., 2016), the increasing foraging activity may have been due to the decreased efficiency while foraging, and thus exposed bumblebees made additional foraging trips to gather resources for the colony. The results from the flight-cage experiment in Chapter I strengthen this hypothesis. GBH exposure decreased the foraging activity of bumblebees in the flight cage experiment in Chapter I; that is, a greater proportion of GBH-exposed bumblebees compared to control bees did not visit any plants at a given time.

GBH-treated and control bees were not significantly different in their homing rates or homing times. I observed only a weak interaction between acute GBH treatment and release distance. A higher proportion of GBH-exposed bumblebees released closer to the colony (500 m) successfully returned home compared to the control bees. A similar trend was reported by Stanley et al. (2016), who studied the effects of neonicotinoids on bumblebees using similar methods. They proposed that the reason for the greater homing rate of pesticide-exposed bumblebees might be caused by the pesticide's brain-accelerating effect or hormesis. My findings, based on a 10-color discrimination experiment (Chapter III), revealed that pure glyphosate impaired bumblebee learning ability, while GBH had no observable effect despite containing the same amount of glyphosate as the pure form.

Because bumblebees and animals generally do not have the shikimate pathway, glyphosate has been considered relatively safe for pollinators. However, some bumblebee gut microbiota bacteria are known to be sensitive to glyphosate. Gut microbiota may protect bumblebees from parasites and environmental pollutants (Koch & Schmid-Hempel, 2011; Rothman et al., 2019) and influence cognitive functions, such as memory (Li et al., 2021). Our findings revealed that glyphosate increased but GBH decreased gut microbiota diversity (Chapter IV). Both treatments significantly reduced the abundance of *Snodgrasella alvi*, a glyphosate-sensitive bacterial species critical to bumblebees' defense against harmful pathogens (Horak et al., 2020; Raymann et al., 2017). In addition, 50% of the bacterial genera in the gut microbiota were classified as resistant to glyphosate, but 36% were sensitive. At higher concentrations, GBH also contributed to increased bumblebee mortality.

The strengths of this dissertation include the diverse range of environments in which the experiments were conducted, the focus on bumblebees rather than honeybees, the use of multiple methodologies to assess the effects of glyphosate and GBHs on specific bumblebee abilities, and the application of field-realistic doses of both pure glyphosate and GBH. However, the dissertation is limited by the absence of a long-term, colony-level examination of the effects of glyphosate.

I was able to investigate the specific effects of glyphosate on bumblebees under laboratory conditions in Chapters III and IV, whereby variables such as temperature, nutrition, and light were controlled and second stressors (parasites, resource limitations, etc.) were minimized. In Chapter III, where GBH- and glyphosate-exposed and control bumblebees learned to associate the reward with colors, I used artificial flowers. This approach allowed the elimination of all other variables that could influence choice, ensuring that color was the only determining factor. In Chapter IV, by conducting experiments under controlled laboratory conditions, I was able to eliminate all external factors that could influence the gut microbiota of the bumblebees. This ensured that GBH or glyphosate was the only variable affecting the results. After the laboratory experiments, I extended the research to outdoor flight cages in which bumblebees could choose between real plants instead of artificial flowers, and detailed observation of individual bumblebees was possible. In the flight cages, most variables and additional stressors were controlled, allowing for the precise manipulation of bumblebee and plant treatments according to experimental requirements. Finally, I studied the effects of GBH on bumblebees and other pollinators in their natural environment. I repeated the study by examining the bumblebees' preferences between GBH-treated and control plants in the field, whereby natural pollinators could choose freely between differently treated plants. I confirmed that pollinators are poor at detecting the contamination of plants while foraging.

Most previous studies investigating the effects of herbicides on bees have focused on honeybees (Cullen et al., 2019; Klein et al., 2022); therefore I used *B. terrestris* as a study organism in my dissertation. The results from studies on *B. terrestris* can be better generalized to other eusocial bees and other pollinators than the findings from domesticated honeybees (Lihoreau et al., 2025).

I often compared the results with earlier studies in which pollinator insects were exposed to different neonicotinoid insecticides. This was because research on the effects of pesticides on insects has concentrated on neonicotinoids and other insecticides (Lundin et al., 2015), which are associated with the decline of pollinator populations (EFSA, 2023a; Laycock et al., 2014; Singla et al., 2021; Stanley et al., 2016; Tsvetkov et al., 2017; Whitehorn et al., 2012; Woodcock et al., 2017) and the colony collapse disorder of honeybees (Lu et al., 2014; Pereira et al., 2020; Wahengbam et al., 2019). However, the modes of action of neonicotinoids and glyphosate are very different. While neonicotinoids are neurotoxins that act as agonists of the nicotinic acetylcholine receptors of insects (Manjon et al., 2018), glyphosate inhibits the essential EPSPS enzyme in the shikimate pathway found in plants, fungi, and bacteria. Since there is no clear consensus on the role of this enzyme, which is absent in animal cells, and its impact on pollinators, I have minimized speculation about the underlying mechanisms in my dissertation. An exception is Chapter IV, in which I examined the effects of GBHs and pure glyphosate on the gut microbiota of bumblebees. Although our findings are interesting and show that glyphosate has implications for bumblebees' gut microbiota, they do not explain why glyphosate has an effect on bees. Some bacteria species belonging to the Lactobacillus genus are known to enhance bees' memory

(Li et al., 2021) and promote hive productivity and immune functionality (Yu et al., 2024), but here, the sensitivity of Lactobacillus to glyphosate was left undetermined. In our experiments, the exposure times were very short, and the effects were immediate. For instance, in the 10-color discrimination experiment (Chapter III), in which pure glyphosate caused a significant decline in learning ability, bees were exposed to glyphosate 1 day prior to the experiment. The rapid impact of glyphosate on bumblebee learning and memory is unlikely to be attributed to microbiota changes. This highlights the need for more studies on the underlying mechanisms of glyphosate's impact on pollinators.

Most studies of herbicides' effects on bees have been conducted using only active ingredients instead of commercial products (Cullen et al., 2019). Therefore, I aimed to use both pure glyphosate and GBH in our experiments. Some coformulants present in GBHs are suspected to be more harmful for nontarget organisms than the active ingredient glyphosate itself (Mesnage et al., 2019; Straw, 2024; Straw et al., 2022). When forced to choose between the use of pure glyphosate and GBH, I considered it essential to investigate effects of glyphosate using the commercial formulation applied in agriculture. However, since details about the coformulants in GBHs are usually considered confidential business information (Weinhold, 2010), I do not know the exact composition of the coformulants and the differences between the two commercial GBH products (Roundup Gold® and Roundup Flex®) I used in my experiments.

The term "field realistic exposure" used in our studies has been criticized (Carreck & Ratnieks, 2014). Bumblebees and other pollinators are exposed to different doses of glyphosate and other pesticides, depending on the application method, pesticide concentration, duration of exposure, and pollinator's choice of plants applied with pesticides (Carreck & Ratnieks, 2014). Our "field-realistic" doses were determined based on the amounts used in previous studies and the estimated residual levels of glyphosate found in pollen and nectar (Thompson et al., 2014). The selected doses were intended to represent a worst-case scenario for oral exposure that foraging bees might encounter without overestimating the potential risk. In addition, when discussing the field-realistic levels of specific pesticides, an important consideration is often overlooked. In natural environments, particularly agricultural landscapes, bumblebees and other pollinators are frequently exposed to mixtures of multiple pesticides in addition to other stressors, such as parasites, fluctuating weather conditions, competition, and limited resource availability (Cartar & Dill, 1991; Crone & Williams, 2016; Goulson et al., 2015; Spiesman et al., 2017; Weidenmüller et al., 2022). Most studies of the effects of pesticides, especially herbicides, have been conducted in the laboratory instead of the field (Cullen et al., 2019), and even in well-organized field experiments, only one pesticide is often studied at a time, and second stressors (parasites, resource limitations, etc.) are controlled. A limitation of even the most robust studies is that they often fail to account for the combined effects of these multiple stressors.

Our experiments and exposure methods focused on the short-term effects of glyphosate on individual bumblebees. The European Food and Safety Authority (EFSA) recommends 10 days as a standard chronic assessment for pesticide safety for bees (EFSA, 2023b), while in our experiment employed either single acute exposures or colony-level exposures that lasted either 24 hours (in Chapter II) or a few days (in Chapter IV). Exposure methods, short exposure periods, and especially short observation periods (all experiments took 10 days or less per colony to complete) were suitable for studying the short-term effects of glyphosate on individual bumblebees, but in such a short time, possible colony-level effects were left without much attention. Although a slightly longer exposure and observation period might have brought more insight into the colony-level effects of glyphosate, the decision to focus on short-term effects was made because of the unique nature and use of glyphosate. Glyphosate-resistant GM crops that allow several applications of herbicides during the growing season are not cultivated in the European Union (ECHA, 2024), where GBHs are applied only before planting or after harvest. Thus, the time window when pollinators can be exposed to glyphosate in arable land is relatively short, and I aimed to keep our methods as “field-realistic” as possible. Future research is needed to evaluate the impact of glyphosate on entire pollinator colonies, explore the effects on different pollinator species beyond eusocial bees, and investigate the underlying mechanisms driving glyphosate’s impact on pollinator insects.

5 Conclusion and future perspectives

Since the introduction of glyphosate in the 1970s, it has been an important herbicide in agriculture. After the expiration of Monsanto's patent in 2000 and the introduction of resistant GM-crops, glyphosate became the most used herbicide worldwide. Glyphosate has been considered an environmentally safe herbicide because of its unique mode of action and low toxicity to animals. Less than two decades ago, glyphosate was praised as "a once-in-a-century herbicide," and the only concerns around it were related to potential glyphosate-resistant weeds evolving because of its extensive use (Duke & Powles, 2008).

Today, glyphosate has been shown to have negative effects on terrestrial (Caggìa et al., 2023; Helander et al., 2018; Newman et al., 2016) and water ecosystems (Brovini et al., 2021; Carles et al., 2019; Folmar et al., 1979; Milan et al., 2018; Vera et al., 2010). The possible effects of glyphosate on humans have also been discussed because it has been connected with non-Hodgkin lymphoma (Eriksson et al., 2008; Guyton et al., 2015; Roos et al., 2003) and perinatal health, including reduced birthweight and gestational length (Reynier & Rubin, 2025).

Glyphosate has been linked directly and indirectly to ongoing pollinator decline. My dissertation provides new information on the effects of glyphosate and GBH on pollinators. In my research, glyphosate and/or GBHs were shown to alter gut microbiota diversity, impact foraging behavior, and impair the learning and memory of bumblebees. Furthermore, bumblebees were unable to discriminate against glyphosate-exposed flowers, which increases their risk of encountering herbicide residues post-application.

After a long debate, the European Commission approved the renewal of glyphosate in Europe for 10 years. This decision was validated by a risk assessment by EFSA and a hazard assessment by the European Chemicals Agency (ECHA). EFSA concluded that it "did not identify any critical areas of concern in its peer review of the risk assessment of the active substance glyphosate in relation to the risk it poses to humans and animals or the environment" (EFSA, 2023b), and ECHA stated that glyphosate "did not meet the scientific criteria to be classified as a carcinogenic, mutagenic, or reprotoxic substance" (ECHA, 2023).

Glyphosate use in Europe is set to continue until at least 2033, and the global use of pesticides is likely to rise due to the increasing demand for food to feed the world's growing population, the widespread adoption of herbicide-tolerant GM-crops, weed resistance, and milder winters resulting from climate change to boost weed growth (Ehrlich & Harte, 2015; Mesnage & Zaller, 2021). In conclusion, this dissertation demonstrates that glyphosate, the most widely used herbicide globally, and its commercial formulations can pose a risk to pollinators. The findings propose that toxicity testing for nontarget organisms should be expanded to include coformulants and not just active ingredients.

Ethical statement

According to the Ministry of Agriculture and Forestry in Finland, bumblebees are not classified as experimental animals and, consequently, project authorization is not required (Act of the Use of Animals for experimental purposes 2006/62 §4). However, I considered ethical issues when designing the experiment. I pursued using bumblebees as effectively as possible to reduce the number of colonies in our experiment. After the experiment, I euthanized the bumblebees by stunning them with CO2 gas and then placing them in a freezer, thereby causing as little pain as possible.

In the preparation of this dissertation, I utilized AI-based tools in a limited but transparent manner. Specifically, ChatGPT was used to rephrase individual sentences for improved clarity, while ensuring that the integrity of the original meaning was maintained. Also, minor figure refinements were made based on AI-assisted suggestions. Grammarly was used for basic grammar and spelling checks, ensuring linguistic accuracy without altering scientific content. All AI-assisted modifications were reviewed and validated by me to ensure ethical and responsible use.

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