Manuscript on the influence of agrochemicals on nutritional intake in bees

Deliverable 5.3

August 2022

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PoshBee

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

Prepared under contract from the European Commission
Grant agreement No. 773921
EU Horizon 2020 Research and Innovation action

Project acronym:	**PoshBee**
Project full title:	**Pan-european assessment, monitoring, and mitigation of stressors on the health of bees**
Start of the project:	June 2018
Duration:	60 months
Project coordinator:	Professor Mark Brown Royal Holloway, University of London www.poshbee.eu

Deliverable title:	Manuscript on the influence of agrochemicals on nutritional intake in bees
Deliverable n°:	D5.3
Nature of the deliverable:	Report
Dissemination level:	Public
WP responsible:	WP5
Lead beneficiary:	UMONS
Citation:	Michez D., Barraud A., Barascou L., Schwarz J.M., Andreu B., Depris J., Arnet N. L., Knauer A.C., Sene D., Le Conte Y., Albrecht M., Alaux C. (2022). *Manuscript on the influence of agrochemicals on nutritional intake in bees*. Deliverable D5.3 EU Horizon 2020 PoshBee Project, Grant agreement No. 773921.
Due date of deliverable:	Month n° 51
Actual submission date:	Month n° 51

Deliverable status:

Version	Status	Date	Author(s)
1.0	Final	25 August 2022	Michez D., Barraud A., Barascou L., Schwarz J.M., Andreu B., Depris J., Arnet N. L., Knauer A.C., Sene D., Le Conte Y., Albrecht M., Alaux C. UMONS, INRAE, Agroscope

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Summary

Bee exposure to single or multiple pesticide compounds (i.e. herbicide, fungicide, insecticide) in agro-ecosystems may be a driver of the negative population trends observed in many species. The sublethal effects of these compounds on bee behaviour, including nutritional intake, are still poorly understood. We explored the impact of agrochemicals on the collection of nectar by three important managed bee species: *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis.* We developed original protocols for each three species, adapted to their specific ecology, behaviour and sociality.

To inform pesticide risk assessment for honey bees, we studied the risk posed by pesticides to two behavioural castes, nurses and forager bees, which represent the majority of a colony's workers and which exhibit large differences in their physiological backgrounds. We determined the sensitivity of nurses and foragers to azoxystrobin (fungicide) and sulfoxaflor (insecticide) upon chronic exposure. Azoxystrobin was found to be weakly toxic to both types of bees. However, foragers were more sensitive to sulfoxaflor than nurses upon chronic exposure. This phenomenon was not explained by better sulfoxaflor metabolization in nurses, but rather by differences in body weight (nurses being 1.6 times heavier than foragers). Foragers consistently consumed more sugar syrup than nurses, and this increased consumption was even more pronounced with pesticide-contaminated syrup (at specific concentrations). Altogether, the stronger susceptibility and exposure of foragers to sulfoxaflor contributed to 2- and 10-fold increases, respectively, in the acute and chronic risk quotients, compared to nurses. In conclusion, to increase the safety margin and avoid an under-estimation of the risk posed by insecticides to honey bees, we recommend that regulatory tests for honey bees should systematically include forager bees.

In bumble bees, we tested the impact of 3 compounds (herbicide, fungicide, insecticide) in single exposure or combined exposure on proboscis extension. Our results show that the nutritional intake of bumble bees can be altered during chronic pesticide exposure. The reduction in the number of proboscis extensions, associated with a decrease in proboscis extension length of bumble bees exposed to the highest concentration of insecticide (cyantraniliprole), and the low number of feeding individuals, suggests significant physiological effects even on single exposure. Herbicide (glyphosate) alone showed little effect on the food intake of *B. terrestris* workers, only impacting the amount consumed per extension at the highest concentration (50,000 ppb). When *B. terrestris* workers were exposed to a fungicide (boscalid), they performed more extensions to consume the same amount of nectar. This implies an impact of boscalid on foraging ability. Multiple simultaneous exposure led mainly to additive effects.

In mason bees, we tested the single and combined effects of sulfoxaflor and azoxystrobin exposure on female nectar intake. We found a drastic reduction of the volume of ingested sugar solution after exposure to sulfoxaflor. Interestingly, however, we found an antagonistic interaction of the two pesticides, showing that the reduction in nectar intake was only statistically significant in the absence, but not in the presence of azoxystrobin.

Overall, our results show that the foraging behaviour of bees can be altered after pesticide exposure. We additionally show that honey bee workers are not all equal regarding the risk posed by pesticides and that, depending on the honey bee behavioural caste, this risk might be under or over-estimated. The growing agreement across studies that foragers or old bees are more sensitive to insecticides than nurse or young bees therefore suggests consistent inclusion of forager bees in regulatory tests should allow for an increase in the safety margin of pesticide risk assessment.

1. Introduction

In temperate and tropical zones, animal-pollinated plants represent respectively 78 and 94% of the plant species (Ollerton et al., 2011). While the majority of animal pollinators are insects, a large part of the

crop pollination is carried out by bees, which are needed for the pollination of more than 70% of the main agricultural crops (Klein et al., 2007; Geslin et al., 2016). Despite their importance, bees are affected by global changes (Nieto et al., 2014), as are several other insect pollinator groups, e.g. butterflies (Parmesan et al., 1999) and hoverflies (Miller-Struttmann et al., 2015). Since the last century, shifts in wild bee species distribution, species ecology and community composition have been recorded from around the world (Rasmont et al., 2005; Biesmeijer et al., 2006; Cameron et al., 2011; Duchenne et al., 2020; Zattara and Aizen, 2021). Very high national negative trends have been recorded in the United Kingdom and Belgium (Drossart et al., 2019; Powney et al., 2019). For example, 61% of the Belgian wild bee species have been shown to be in decline over the past 70 years (Duchenne et al., 2020). Nowadays, the causes of bee decline seem to be mainly anthropogenic, including factors such as climate change, habitat losses and agricultural intensification (Goulson et al., 2015).

The increasing demand for food has led to global productivity enhancement in the agricultural sector (Deguine et al., 2014). This agricultural intensification led to the emergence of large monocultural crops to maximize food production. For over a century, agrochemicals, including synthetic fertilizers and pesticides, have been applied on fields to maximize crop yields (Boardman, 1986; Deguine et al., 2014). The aggregation of thousands of plant individuals from the same species in a limited area, as well as limited genetic diversity, provide optimal conditions for pest outbreaks. Pesticides are therefore needed to control their populations (Hillocks, 2012; Godfray et al., 2014; Botías et al., 2015). Pesticides comprise a broad variety of molecules targeting different organisms, and largely break down into three groups, (i) herbicides, which target unwanted plants (e.g., glyphosate), (ii) fungicides, which target parasitic fungi, (e.g., strobilurins), and (iii) insecticides, which target insect pests (e.g. neonicotinoids and sulfoxamines).

Concerns about pesticide effects on human health emerged soon after pesticide use became widespread (Hayes et al., 1956). Carson (1962) originally started to raise public awareness about the consequences of agrochemical use on health and environment. Through their reliance on floral resources (Michener, 2007), and their high presence on flowering crops and surrounding areas, bees are frequently exposed to pesticides through different routes of exposure (Godfray et al., 2014). Systemic pesticides are transported through the plant and can be found in pollen and nectar (Krupke et al., 2012). Adult bees can thus ingest these molecules while they are foraging and bring them back to the nest (Krupke et al., 2012). While being sprayed, pesticides can either directly be in contact with the foraging bees, or diffuse in the soil and surrounding waters. When seeds are coated with pesticides, seed coating treatment dust from the seed drilling process can also contain large agrochemical concentrations that can be deposited on crop soil, or diffuse in the surrounding areas (Krupke et al., 2012; Goulson et al., 2015; Sgolastra et al., 2019). Ground-nesting or stem-nesting bees that use mud for nest construction can therefore be exposed to pesticides directly through contaminated soil. Indeed, soils of crops and their surrounding areas can accumulate high quantities of pesticides, such as neonicotinoids, which were applied as seed treatments (Willis Chan et al., 2019). Furthermore, exposure to mixtures of pesticides that could present a synergistic effect can occur directly while the bee is foraging on various crops treated at the same time with diverse pesticides (Pettis et al., 2013). According to pollen samples found on honey bees, they forage not only on crops, but also on weeds which can be subject to pesticide drift from other treated crops (Baron et al., 2014). Moreover, tank mixtures are also used on crops to either increase the spectrum of a product's activity, delay the appearance of resistant strains by minimizing the selection strength, or benefit from synergistic effects (BLISS, 1939; Koziol and Witkowski, 1982). Therefore, there is a high risk for bees to be exposed to multiple agrochemicals at the same time, through various routes of exposure, increasing the risk of synergisms (Siviter et al., 2021).

Insecticides partly explain bee decline through direct lethal effects or sublethal effects, as has been demonstrated many times, for example, for neonicotinoids (Cresswell et al., 2012; Laycock et al., 2012; Feltham et al., 2014; Gill and Raine, 2014; Phelps et al., 2018; Barraud et al., 2020). Despite the substitution potential of sulfoxaflor over neonicotinoids on the market, the fact that they share a similar mode of action raises concerns about the potential similar sub-lethal effects of the molecule on pollinators (Centner et al., 2018). For example, several field or laboratory studies highlighted negative

impacts on bumble bee colony fitness through lowered reproductive performances (Siviter et al., 2018a). However, in follow-up studies results have been mixed, with no impacts of sulfoxaflor on bee cognition, but reduced egg-laying in microcolony experiments (Siviter et al., 2018a, 2019, 2020). Some studies suggested that this can be linked to nutritional intake (Pettis et al., 2013; Siviter et al., 2020). Therefore, more studies are needed to understand how sulfoxaflor exposure can impact bee behaviour, food intake, cognition, and reproductive capacity.

While many studies have focused their efforts on the adverse effects of insecticides on bees, fungicides and herbicides have received far less attention. Systemic fungicides can be found in high quantities in soil, nectar and pollen from treated crops (Pettis et al., 2013). As an example, fungicidal molecules were found to increase gut cell mortality in honey bees, and as a consequence, their susceptibility to gut parasites, e.g. *Nosema* spp. (Pettis et al., 2013). Several colony disorder symptoms, brood abnormalities, queen failure, and adverse effects on bee gut microbiota due to fungicide exposure have also been highlighted (Bartlett et al., 2002; Bartlewicz et al., 2016; Steffan et al., 2017). Therefore, there is a need to explore more broadly the sub-lethal effects of fungicide exposure on food intake and nutrition. Several studies already highlighted adverse effects of strobilurin on the digestive system of bees. Indeed, as some of these molecules have been found to affect yeast and microbes present in nectar, they could act on bee digestive flora, and, therefore, impact their nutritional intake and feeding behaviour (Campbell et al., 2016). In addition, it has been highlighted that picoxystrobin inhibits ATP production by mitochondria of bee thorax cells in vitro (Domingues et al., 2017), induces changes in morpho-physiology of the hepato-nephrotic system, reduces survival time (Batista et al., 2020), and causes cytotoxic effects on bee midgut epithelial cells which may lead to malnutrition and poor nutrient absorption (Degrandi-Hoffman et al., 2015).

The most common herbicide in the world, glyphosate, was first considered as non-dangerous for bees (Edwards et al., 1980; Spurrier 1973). However, across the admittedly still scarce studies, there are conflicting results about glyphosate impact on bee health. While some studies concluded an absence of significant impact (Giesy et al., 2000; Dill et al., 2010; Rolando et al., 2017; Blake and Pallett, 2018), others found significant effect on bees with either lethal or sublethal effect on adults and larvae (Motta et al., 2018; Vázquez et al., 2018; Blot et al., 2019; Nocelli et al., 2019; Tomé et al., 2020), impacting, e.g., navigation (Balbuena et al., 2015) or sleep (Vázquez et al., 2020). More information on the potential effect of this molecule is needed in order to properly assess its risk.

In addition to the effects of individual molecules, synergies between them can play an important role in the overall side effects of pesticides. Synergisms have been known since before the late 1930s, and have been studied for their capacity to modify the LD50 of the individual components. However, their sub-lethal effects on bees were not investigated until 1992, with the synergy between deltamethrin, a pyrethroid insecticide, and prochloraz, an imidazole fungicide (Colin and Belzunces, 1992). Since then, decreases of bee LD50 of at least one of the mixture components due to synergisms between several widely used insecticides and fungicides have been observed (Schmuck et al., 2003; Thompson and Wilkins, 2003; Thompson et al., 2014; Robinson et al., 2017; Sgolastra et al., 2018; Wade et al., 2019). The appearance of sub-lethal effects has also been highlighted (Brittain et al., 2013). For example, larval exposure of Africanized honey bees to both a neonicotinoid insecticide and a strobilurin fungicide were found to impact the behaviour and the survival of emerged bees (Tadei et al., 2019). Recently, it has been shown that, while sulfoxaflor had a higher bee LD50 than the remaining authorized neonicotinoids, this value decreases when co-exposed to the fungicide fluxapyroxad, a succinate dehydrogenase inhibitor (Azpiazu et al., 2021).

Moreover, single pesticide dose or concentration does not necessarily induce a single response, as the level of the measured toxicity endpoint may vary depending on the physiological state of bees (Poquet et al. 2016). Investigating this intraspecific variability or modulation of response is therefore important to better screen the risks posed by pesticides to bees. In this regard, some studies have shown that heavier honey bees are less sensitive to pesticides than lighter honey bees (Tahori et al. 1969; Gerig 1991; Nogueira-Couto et al. 1996). In addition, sensitivity may depend on age, with younger bees being

more sensitive to certain pesticides, but less to others, than older bees (Mayland and Burkhardt 1970; Ladas 1972; Bendahou et al. 1997; Rinkevich et al. 2015; Zhu et al. 2020). This is likely related to the changes in endocrine and metabolic activity that occur during age-related behavioural maturation (transition from nurse to forager tasks) (Robinson 2002). For instance, foragers can weigh two times less than nurse bees (Vance et al. 2009), and the activity of glutathione S-transferase, an enzyme involved in detoxification pathways (Claudianos et al. 2006; Berenbaum and Johnson 2015), is significantly higher in forager bees than in nurses (Smirle and Robinson 1989). As a result, pesticide sensitivity might vary strongly depending on the behavioural state of honey bee individuals. Confirmation of this hypothesis was given by Tosi and Nieh (2019) who found that foragers were consistently more susceptible to flupyradifurone (fourfold greater effect) than in-hive bees. Nevertheless, this intraspecific difference in pesticide sensitivity between behavioural castes, as well as the underlying mechanisms, has rarely been studied, although it could better inform pesticide risk assessment for honey bees.

Current knowledge about bee sensitivity to pesticides is mainly based on mortality tests performed on a few species. There is a need to gather more data about sub-lethal effect of pesticides on multiple bee species. Therefore, the present work aims to evaluate the effects of a chronic exposure to different pesticides on the food intake ability of three different bee species: *Apis mellifera*, *Bombus terrestris* and *Osmia bicornis*. Experiments were designed to account for variation across the three species in their ecology, behaviour and sociality.

2. Methodology

2.1 Overview of the study

According to the grant agreement, we were planning to develop protocols for three model bee species (*A. mellifera*, *B. terrestris* and *O. bicornis*) to assess the impact of pesticide exposure on nutritional intake. We were planning to test effects of chronic exposure (at the level of the individual bee) of the same agrochemical classes used in Task 5.2 on nutritional intake. After preliminary tests with field realistic concentrations, the herbicide was tested only on *B. terrestris*. For *A. mellifera* and *B. terrestris*, nectar consumption was supposed to be tested by the proboscis extension reflex. Nectar consumption of *O. bicornis* was planned to be quantified in individually caged males and females (under controlled conditions) using a novel protocol with acute exposure. We were also planning to evaluate pollen consumption in adults using the same pollen diets and endpoint measurements described in Task 5.1 (published in Barraud et al. 2022). However, analysis of pollen collection after exposure to pesticide was not feasible under laboratory conditions as it requires a semi-field or field experiment. However, as analysis of pollen collection after exposure to sulfoxaflor and azoxystrobin was undertaken in WP7, the results of this WP7 analysis can feed directly into Task 5.3.

The study on honey bees was published in 2022 under the following reference:

Barascou L., Sene D., le Conte Y., Alaux C. (2022). Pesticide risk assessment: honeybee workers are not all equal regarding the risk posed by exposure to pesticides. Environmental Science and Pollution Research, 3 (1). https://doi.org/10.1007/s11356-022-21969-2

The publications of the results on *Bombus terrestris* and *Osmia bicornis* are under preparation:

Schwarz J.M., Arnet N.L., Knauer A.C., Albrecht M. Pesticide effects on solitary bee (*Osmia bicornis*) nectar intake, foraging performance and learning ability.

Barraud A., Dewaele J., Andreu B., Depris J., Vanderplanck M., Michez D. Pesticides impact on nutritional intake in *Bombus terrestris*.

2.2 Bee model species

This study was conducted on three common European pollen generalist bee species that forage in the same habitat for part of the year (Michez et al., 2019). We selected the Western honey bee *Apis mellifera* (Hymenoptera, Apidae, Apini), a domesticated eusocial species; the buff-tailed bumble bee *Bombus terrestris* (Hymenoptera, Apidae, Bombini), a wild social species (Rasmont et al., 2008); and one mason bees (*Osmia bicornis*; Hymenoptera, Megachilidae, Osmiini), a wild solitary species. They are commonly used as model species because of their easy management in laboratory conditions.

2.3 Experimental protocols

As the three genera (i.e. *Apis*, *Bombus* and *Osmia*) show very different life cycles and behaviour, they could not be tested following the same protocol in laboratory conditions. Thus, we developed a different experimental setup for each of the three bee genera.

2.3.1 Honey bee (*Apis mellifera*)

Experiments were conducted with honey bees obtained from a local apiary at the "Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement" (INRAE) in Avignon (France). To determine whether pesticide sensitivity differs between bees of different behavioural castes, nurses and foragers were collected the same day from four different colonies. Nurses were identified by removing brood combs from colonies and detecting bees that dipped their heads into multiple cells containing larvae. Foragers were identified as bees returning to the colony with pollen loads, thereby discarding bees performing orientation or cleansing flights.

To validate the nurse sampling method, we checked whether bees collected as nurses had more developed hypopharyngeal glands (HPG) than forager bees, as HPGs, where jelly is produced to feed larvae, the queen and drones (Crailsheim 1992), are consistently more developed in nurse than forager bees (Knecht and Kaatz 1990; Robinson et al. 1992). For that purpose, 10 bees of each behavioural caste were sampled from all colonies and stored at − 20 °C. Glands from 10 bees per caste and colony were dissected in distilled water under a binocular magnifier (LEICA MZ 12). Pictures of each gland were taken with a digital camera (Toupcam™) and ToupView image capturing software (v3.7.5660). Then, the gland development was assessed by measuring the maximum diameter of 10 to 15 randomly chosen ovoid acini per gland using ImageJ v1.53e (http:// rsb. info. nih. gov/ ij/ index. html). The diameters of acini were significantly larger in nurse (80.34 ± 9.66 µm) than in forager bees (53.54 ± 9.45 µm; Kruskal–Wallis test, $\chi 2 = 1274.4$, $p < 0.001$), validating our sampling method.

After collecting bees from the four different colonies, nurses and foragers were immediately placed in different cages (10.5 cm × 7.5 cm × 11.5 cm) (Pain 1966; Williams et al. 2013) containing a feeding tube with a solution of 50% (w/v) sucrose and brought back to the lab. They were then placed in an incubator under controlled conditions (28 °C and 50–70% relative humidity).

Nurse and forager bees were chronically exposed to a low and a high concentration of pesticides. Groups of 20 nurse or forager bees from the same four colonies were placed in different cages (n = 1 or 2 cages per colony giving n = 6 cages per pesticide concentration and behavioural caste). Bees were provided with a solution of 50% (w/v) sucrose, 0.1% acetone and azoxystrobin (0.2 or 2 µg/ml) or sulfoxaflor (0.02 or 0.2 µg/ml). Control groups were fed with pesticide-free sugar solution (50% w/v sucrose, 0.1% acetone).

The concentrations were chosen based on pesticide residue data found in nectar. Depending on different application rates of sulfoxaflor and the crops, field studies reported levels of the neurotoxin ranging from 0.04 to 2.37 mg/kg in nectar (Niesen 2019). Residues of azoxystrobin have been found at high concentrations (up to 1.45 mg/kg) in nectar collected by honey bees, shortly after the application

day (Schatz and Wallner 2009). The chronic pesticide treatments were performed over 5 days and the syrup feeders were replaced every day. Since forager lifespan is on average 8 days (Prado et al. 2020), chronic toxicity tests were performed over 5 days to minimize the risk of natural forager mortality. For each cage, individual syrup consumption was assessed daily, by weighing feeders and dividing the consumed food by the number of remaining live bees. Dead bees were counted daily and removed over the 5-day period. The exact concentrations of sulfoxaflor and azoxystrobin were determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS, see Barascou et al. 2021), giving low and high concentrations of 0.021 and 0.223 µg/ml for sulfoxaflor and 0.16 µg/ml and 1.46 µg/ml for azoxystrobin.

2.3.2 Bumble bee *(Bombus terrestris*)

We used three queen-right colonies of *Bombus terrestris*. All colonies were maintained in the same room in constant darkness at 26°C with a relative humidity of 60–65%. They were manipulated under red light to minimize disturbance (Sadd, 2011).

In this experiment, three different pesticides were used: cyantraniliprole (insecticide), boscalid (fungicide) and glyphosate (herbicide). Experiments on Sulfoxaflor and Amistar were also developed but are not presented in the present manuscript (data processing is still in progress). For each pesticide, three concentrations were tested, corresponding to: i) the residual concentration found in the field in pollen and/or nectar, ii) twice the residual concentration found in the field and iii) a high concentration corresponding to 10 times the residual concentration, plus iv) a control concentration without pesticide (Table 1). Concentration iii) is deliberately high in order to test whether an effect of the pesticides on food intake can be observed (i.e. positive control). For cyantraniliprole, concentration iii) was replaced by a concentration representing 50% of the residual concentration, after initial tests showed a low proportion of individuals feeding after exposure to the pesticide at the residual concentration. Data from field and laboratory experiments reported in the literature were taken into account. Thus, concentrations of 1550 (C0.5), 3100 (C1) and 6200 (C2) ppb were determined for cyantraniliprole (Dinter and Samel, 2015; Kyriakopoulou et al., 2017); 5000 (G1), 10,000 (G2) and 50,000 (G10) ppb for glyphosate (Herbert et al., 2014; Balbuena et al., 2015); and 3000 (B1), 6000 (B2) and 30,000 (B10) ppb for boscalid (David et al., 2016; Simon-Delso et al., 2017).

Table 1. Single exposure treatments tested in the *Bombus terrestris* experiment.

	Fungicide			Insecticide			Herbicide			Controls
Condition	Boscalid			Cyantraniliprole			Glyphosate			Control
Abbreviation	B			C			G			T
Condition	B1	B2	B10	C0.5	C1	C2	G1	G2	G10	T
Concentration (ppb)	3000	6000	30000	1550	3100	6200	5000	10000	50000	0
Individuals	25	25	25	25	25	25	25	25	25	50

Bumble bees were also exposed to mixtures of these pesticides. The previously stated residual concentrations were used, except again for cyantraniliprole where the concentration corresponding to 50% of the field concentration was used (Table 2). The exposure period lasted 7 days under red light and at an optimum temperature of 26 ± 1 °C and a humidity of 60 ± 5%.

Table 2. Multiple exposure treatments tested in the *Bombus terrestris* experiment.

Condition	Cyantraniliprole & Boscalid		Cyantraniliprole & Glyphosate		Glyphosate & Boscalid		Controls
Abbreviation	C-B		C-G		G-B		T
Pesticide	C	B	C	G	G	B	T
Concentration (ppb)	1550	3000	1550	5000	5000	3000	0
Individuals	25		25		25		50

Feeding tests were carried out following pesticide exposure. These tests mimic the plant-bee interaction. When the bee lands on the flower, its taste receptors are stimulated by nectar and in response, the bee extends its proboscis, collects nectar and memorises floral odours, which are then recognised during subsequent foraging (Nocelli et al., 2018). It has been shown that foraging test data correlate with the olfactory responses of bees under free-flying conditions, suggesting that the effects found in foraging test responses under laboratory conditions reflect the effects produced in nature (Pham-Delègue et al., 2002).

Feeding test protocols are generally not adapted to non-*Apis* bees and therefore require adjustments (Nocelli et al., 2018). An adaptation of this protocol, based on tests conducted by Ma et al. (2016), was performed. After the 7 day exposure period, the bumble bees from the micro-colonies were placed into a 2-hour starvation period. Beforehand, the selected bumble bees were numbered so that they could be followed individually throughout the experiments. The starvation period was carried out at 26°C and in complete darkness. Then, each worker was placed in a 15 mL Falcon tube with a hole at the end facing a 100µl capillary (Fig 1). After 5 minutes of acclimatisation, bumble bees were tested with syrup containing 50% sugar to avoid viscosity bias (Depris, 2018). The tested syrup was placed in the scanned capillary once filled and presented to the bumble bee. The measurement phase started as soon as the proboscis came into contact with the tested sugar solution inside the micro-capillary and lasted 5 minutes. This phase was carried out under artificial light in order to maximise food intake by the bumble bee (Depris, 2018). The solution was maintained in contact with the bumble bee with the help of a syringe connected upstream of the micro-capillary. A camera was placed 5 cm above the end of the tube and the micro-capillary in order to film the intake of the sugar solution by the bumble bee (Fig 3). Each pesticide treatment (single or in cocktail) was tested on 25 different workers. A bumble bee was considered as a non-feeder if it did not feed within 5 minutes. A capillary scan was used to determine the volume of solution consumed. Slow motion video analysis (x0.25) and photographic measurements using Image J software were used to determine the number of proboscis extensions taken in 5 minutes and the maximum proboscis extension length. Finally, we monitored mortality during the exposure period by checking the micro-colonies every day.

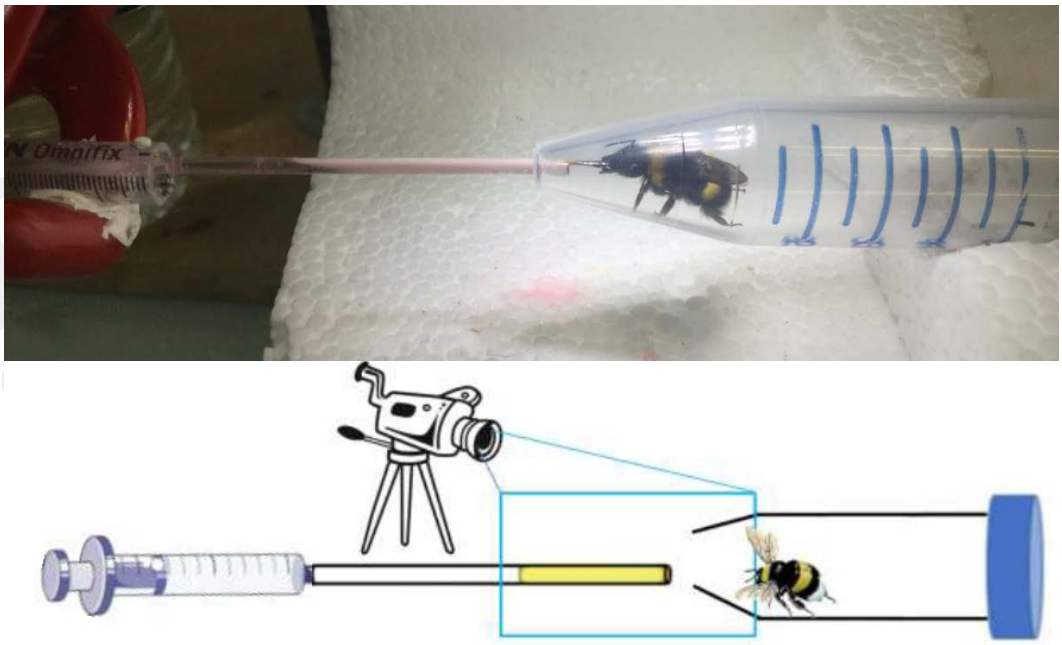

Figure 1. Experimental set-up used to measure food intake after pesticide exposure for *Bombus terrestris.*

2.3.3 Mason bee (*Osmia bicornis*)

Nectar Intake Experiment

We tested the nectar intake of adult female solitary bees, *Osmia bicornis*, at Agroscope Zürich, Switzerland between April and June 2021. Approximately 200 freshly hatched females (Wildbiene + Partner AG, Switzerland) were introduced into a flight cage (1.4 m × 1.4 m × 1.4 m, model "Grünhaus M", Aerarium Nets GmbH, Switzerland) located in the greenhouse. We offered the bees ad libitum sugar solution (33% w/w) through artificial paper flowers equipped with an Eppendorf tube. These were made of wooden cylinders with a small hole in the middle on which round paper disks were attached. The cage also contained a bee home for roosting, a dish offering organic apple pollen (honey bee-collected, purchased from Abeille heureuse, France) and a small water dish. The bees were kept in the cages for one and a half days, before the sugar solution and pollen were removed for a 1-day starvation period before the nectar intake experiment. On the day of the experiment, bees were exposed to a single acute oral dose of sulfoxaflor, azoxystrobin, their combination or a water-acetone control in NICOT systems (small cages used for queen rearing in honey bees). After consuming the pesticide or control solutions, the bees were offered a specific amount of sugar solution and left to feed on it for three hours before measuring the ingested amount by weighing. For each treatment condition, 45 female *O. bicornis* were used. Nine additional NICOT systems containing sugar solution but with no bee inside were used to measure the amount of solution that evaporated during the three hour feeding period. The bees were afterwards released into the wild next to nesting aids close to the Institute.

Pesticide treatments

The bees were exposed to one acute oral dose of one of four pesticide treatments in a full-factorial design (1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor+azoxystrobin (mix) and 4) water-acetone control). These doses corresponded to approximate residue levels detected in nectar of treated plants shortly after pesticide application (worst-case scenario). For sulfoxaflor, we tested a scenario where females would be exposed to nectar containing 0.1 ppm sulfoxaflor (EFSA 2019). For azoxystrobin, we tested an

exposure to nectar containing 2 ppm azoxystrobin based roughly on residue levels found in nectar of sprayed oilseed rape (*Brassica napus*) (Schatz and Wallner 2009) as well as levels found in bee-collected pollen in France (Observatory of Pesticide Residue, ITSAP – Institute de l'Abeille 2014, personal communication). We orally exposed bees to only 5 µL of pesticide or control solution (treatment solution), in order to keep them hungry during the subsequent nectar intake phase. To simulate a worst-case scenario, we exposed bees to a concentration of pesticides expected to be ingested across one day of foraging. Under laboratory conditions, *O. bicornis* consume ca. 30 µL sugar solution per day (Azpiazu et al. 2021). Therefore, to simulate a 0.1 ppm and 2 ppm scenario for sulfoxaflor and azoxystrobin, respectively, we exposed bees to a 6-times higher concentration dissolved in only 5 µL of solution. This resulted in an acute oral exposure of bees to 5 µL of 0.6 ppm (µg/g) sulfoxaflor, 12 ppm azoxystrobin, their mix, or a water-acetone control.

The pesticide powders were dissolved in acetone and subsequently diluted to the desired concentrations using 33% (w/w) sugar solution. The proportion of acetone in the solutions was adjusted, so that each treatment solution contained identical proportions of acetone (< 1%).

2.4 Statistical analyses

2.4.1 Honey bee (*Apis mellifera*)

Data were analyzed using the statistical software R v3.3.3 (R Core Team 2020). Variation in body weights between nurses and foragers was analyzed using a Kruskal–Wallis test, followed by Dunn's multiple comparison tests with the Benjamini–Hochberg correction. Syrup consumption between nurses and foragers, and among experimental groups in the chronic toxicity experiment, was analyzed using a Kruskal–Wallis test, followed by Dunn's multiple comparison test with the Benjamini–Hochberg correction. Survival data from the chronic toxicity tests were analyzed with a Cox proportional hazards regression model (coxph function of the survival package in R (Cox 1970)).

In order to assess the potential risk posed to nurse and forager bees by chronic exposure to pesticides we used the NOED from the chronic toxicity test:

$$\mathrm{RQ} = \frac{\text{Exposure concentration (µg/kg)} \times \text{Consumption (kg/day)}}{\text{chronic 5 day oral NOED (µg/bee/day)}}$$

The acute and chronic RQ threshold levels of concern (LOC) are 0.4 and 1, respectively. If the RQ is less than 0.4 or 1, the risk posed by the pesticide is acceptable, but if the RQ is equal or greater than 0.4 or 1, the risk is not acceptable (Thompson 2021).

2.4.2 Bumble bee *(Bombus terrestris*)

The data analyses of the food intake experiment were performed using glmmTMB linear models on R software with different random factors (mass, mother colony, micro-colony from which the individuals originated, consumption over 7 days). These models (glmmTMB) were used to deal with the large number of 0's due to the non-feeders. A quasi-poisson distribution was used for the number of proboscis extensions and a Gaussian distribution for the other variables. Sqrt transformations were used when the residuals did not give a satisfactory result with a visual analysis and a simulated residuals normality p-value (DHARMa). The final models were chosen on the basis of their AIC. Multiple comparisons were performed with a variant of the glht function for glmmTMB. Glm's were used to analyse survival and feeders/non-feeders data. All the graphs were produced using ggplot2 package v3.3.5 (Wickham, 2016). The standard error is shown. Letters are shown from the multcompletter package. The significance level was set at $\alpha = 0.05$ for all tests.

2.4.3 Mason bee *(Osmia bicornis*)

The statistical analysis of the data was performed in R version 4.2.1 (R Development Core Team 2022) using the package nlme (Pinheiro et al. 2020). Type II ANOVA was used for calculating statistical inferences. The volume of consumed sugar solution was analysed using a generalized least-squares fitted linear model (GLS) to account for non-homogeneous variances in the different treatment groups. The model included sulfoxaflor (present or absent), azoxystrobin (present or absent) and their interaction as explanatory variable. The average volume of sugar solution that had evaporated during the time of the experiment was subtracted from the calculated volume of solution consumed by the bees. Of the 180 bees included in the experiment, 134 (74.4%) were feeders (i.e., they consumed the treatment solution; feeders per treatment: sulfoxaflor: 33, azoxystrobin: 34, sulfoxaflor + azoxystrobin (mix): 37, control: 30). During the subsequent handling, five bees escaped, resulting in the following sample sizes in the experimental treatment groups: sulfoxaflor: 31, azoxystrobin: 32, mix: 37, control: 30.

3. Results

3.1 Honey bee (*Apis mellifera*)

Regardless of the pesticide and its concentration, forager bees consistently consumed more sugar solution than nurse bees (sulfoxaflor: Kruskal–Wallis test, $\chi2 = 31.49$, $p < 0.001$ and azoxystrobin: $\chi2 = 37.67$, $p < 0.001$; Fig. 2). Overall, forager bees ingested 1.4 times more syrup than nurse bees. Although we did not find any effect of pesticide concentration on sugar consumption by nurse bees (sulfoxaflor: $p = 0.38$ and azoxystrobin: $p = 0.528$), a significant effect was observed in forager bees (sulfoxaflor: $p = 0.024$ and azoxystrobin: $p < 0.01$; Fig. 2). Foragers exposed to 0.021 µg/ml of sulfoxaflor consumed more syrup (68.79 ± 19.76 mg/day) than control foragers (53.90 ± 14.90 mg/day, Dunn's test, $p = 0.022$) but this was not true for foragers exposed to 0.223 µg/ml of sulfoxaflor ($p = 0.07$). Similarly, foragers exposed to 1.46 µg/ml of azoxystrobin consumed more syrup (72.16 ± 26.71 mg/day) than bees exposed to 0.16 µg/ml of azoxystrobin (54.16 ± 14.11 mg/day, $p < 0.01$) and control bees (53.90 ± 14.90 mg/day, $p < 0.01$).

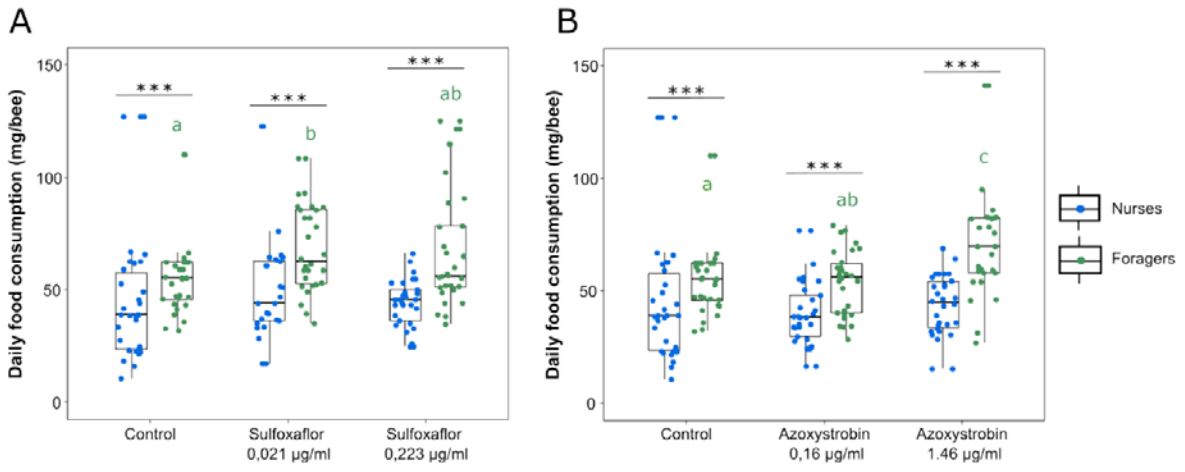

Figure 2. Individual syrup consumption according to pesticide treatments in nurse and forager honey bees. Daily individual consumption (mg/bee) is shown for foragers and nurses exposed to A sulfoxaflor and B azoxystrobin (n = 20 bees per cage and 6 cages per pesticide concentration and behavioural caste). Boxes indicate the first and third interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters and number of asterisks

indicate significant differences between pesticide concentrations and between nurse and forager bees, respectively (Kruskal–Wallis tests followed by Dunn's multiple comparison test, *** denotes $p < 0.001$)

Chronic exposure to azoxystrobin (0.16–1.46 µg/ml) and sulfoxaflor (0.02–0.22 µg/ml) did not affect the survival of nurse bees (Cox model, p = 0.99; Fig. 3A). While we did not find any effect of either azoxystrobin concentration or the lowest concentration of sulfoxaflor (0.021 µg/ml) on forager mortality, the highest concentration of sulfoxaflor (0.223 µg/ml) reduced their survival probability by around 50% within 5 days (Cox model, p < 0.001, Fig. 3B).

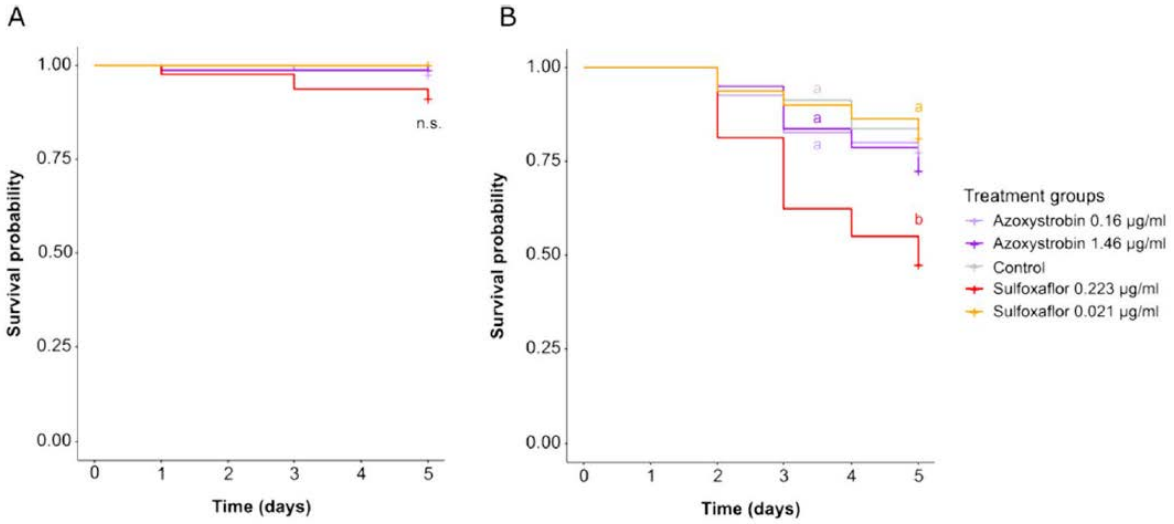

Figure 3. Chronic toxicity of azoxystrobin and sulfoxaflor on **A** nurse and **B** foragers honey bees. Data represent the survival probabilities of bees (n = 20 bees per cage and 6 cages per pesticide concentration and behavioural caste). Different letters indicate significant differences (Cox model).

3.2 Bumble bee (*Bombus terrestris*)

Bumble bees exposed to cyantraniliprole extended their proboscis less often, with a significantly lower number of extensions than the control for condition C2 (p= 0.0360) (Fig 4.a). For the maximum length of proboscis extension, a marginal difference with the control emerged for treatment C1 (p=0.0567) and a significant difference appeared for C2 (p=0.0074). The quantities consumed per extension were not significantly different from the control (Fig 4.d). Finally, the proportion of individuals exposed to conditions C1 and C2 that did not feed (Fig 4.f) was higher than the control, at 56 and 64%, respectively (p= 0.0429 and 0.0196).

The number of extensions for B1 was lower than the control and the other two conditions (p=0.0451). No impact of boscalid was observed on the number of extensions for the two highest concentrations (Fig 4.a) nor on the length of proboscis extension (Fig 4.c). The amount consumed per extension (Fig 4.d) of bumble bees exposed to B10 was significantly lower compared to the control (p=0.0136). A significant difference between individuals in the B1 and B10 condition (p=0.0347) was also observed, with individuals in the B10 condition consuming less per extension. Although a significant number of individuals did not feed, no significant difference with the control was observed (Fig 4.f).

No significant differences were observed between control and glyphosate-exposed individuals regarding the number of extensions (Fig 4.a) and the length of proboscis extension (Fig 4.c). The amount consumed per extension (Fig 4.d), on the other hand, was significantly impacted in G10 condition (p=0.034). Overall, when exposed to a single pesticide, no significant differences were observed in syrup consumption compared to controls (Fig 4.b), nor regarding mortality (Fig 4.e).

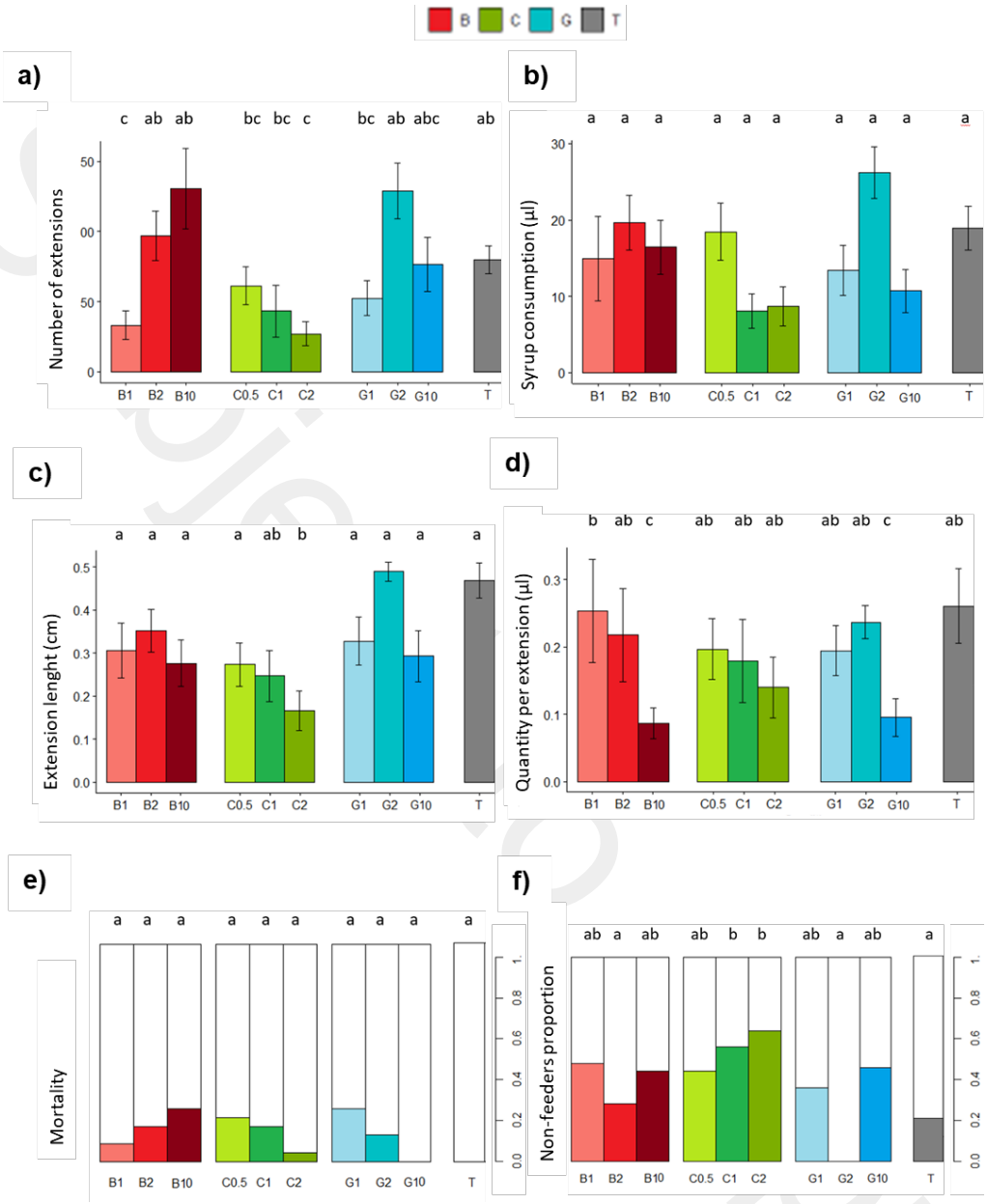

Figure 4. Effects of single pesticide exposure on food intake of *Bombus terrestris*. The letters above each of the different conditions are the result of pairwise comparison tests. C = cyantraniliprole, G = glyphosate and B = boscalid. The different variables presented are a) the number of extensions, b) the total syrup consumption in five minutes, c) the maximum extension length of the proboscis, d) the amount consumed per extension, e) the mortality after the week of chronic exposure and f) the number of individuals that responded positively to the food intake test.

The mixtures of the different pesticides had an impact on feeding behaviour regardless of the treatment. The maximum length of proboscis extension (Fig 5.c) was lower for individuals that consumed pesticides (pCB-T=0.003), (pCG-T=0.0004), (pGB-T=0.0002). For individuals consuming cyantraniliprole in combination with boscalid, there were significantly fewer extensions than in the control individuals (pCB-T=0.0039) (Fig 5.a) and significantly lower consumption (Fig 5.b). Individuals in the CG treatment also performed fewer extensions than controls (p=0.0229) (Fig 5.a). This treatment also had a negative effect on other measured variables, including syrup consumption (p=0.0024) (Fig 5.b) and the amount

consumed per extension (p=0.038) (Fig 5.d). The GB treatment induced a lower consumption of microcapillary syrup (p-T=0.0009) as well as a lower amount consumed per extension (p=0.0009). Finally, neither mortality nor food intake were affected by any of the treatments (p=0.3636) (Fig 5.e-5.f).

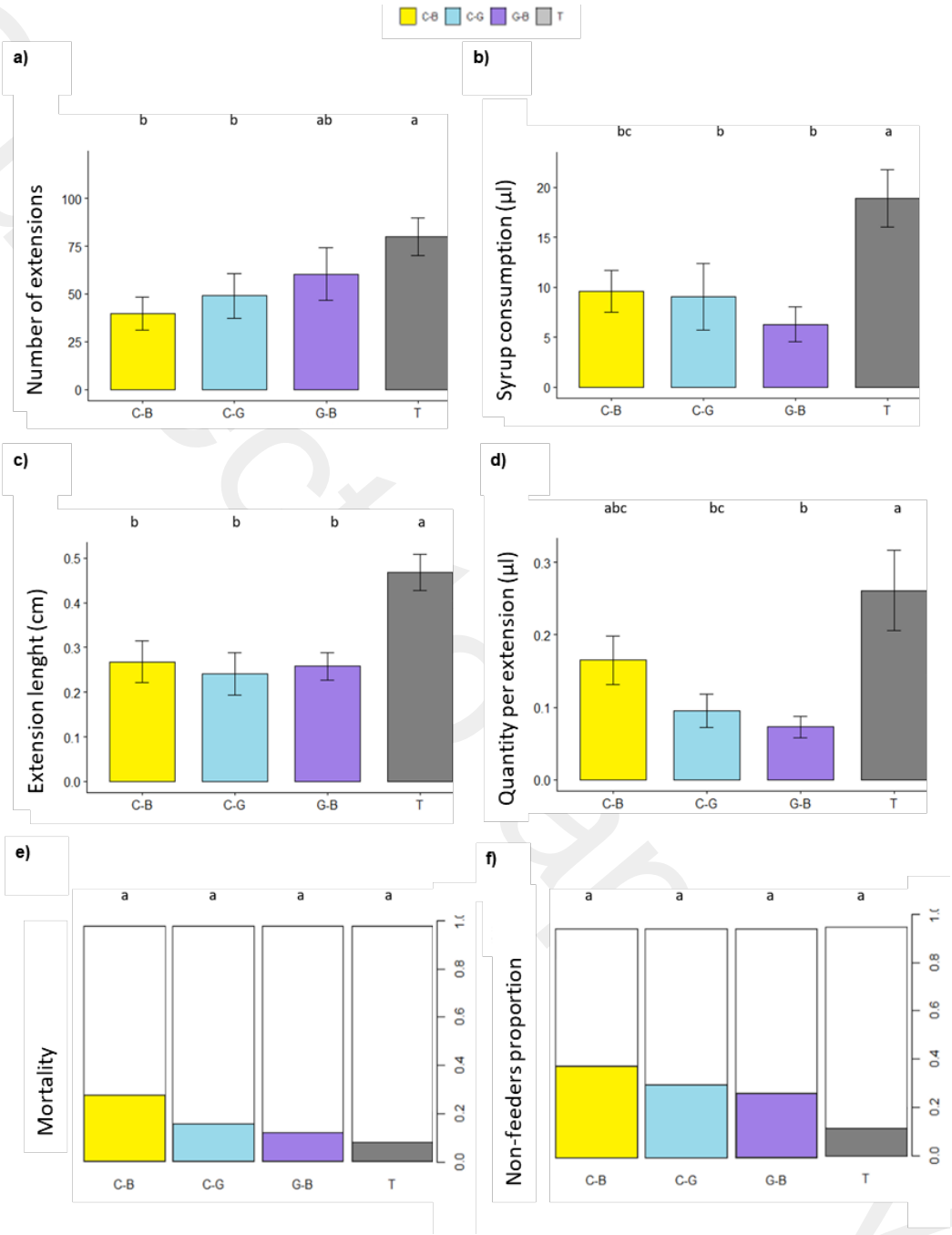

Figure 5. Effects of mixture of pesticides exposure on food intake of *Bombus terrestris*. The letters above each of the different conditions are the result of pairwise comparison tests. CB = cyantraniliprole and boscalid, CG = cyantraniliprole and glyphosate, GB = glyphosate and boscalid, and T = control. The different variables presented are a) the number of extensions, b) the total syrup consumption in five minutes, c) the maximum extension length of the proboscis, d) the amount consumed per extension, e) the mortality after the week of chronic exposure and f) the number of individuals that responded positively to the food intake test.

3.3 Mason bee (*Osmia bicornis*)

On average, 26.4 ± 4.2 µL (mean ± SE) of sugar solution evaporated during the three hours of the experiment. Sulfoxaflor exposure negatively affected the volume of ingested solution by *O. bicornis*. On average, consumption was reduced by 23.8% compared to bees not exposed to sulfoxaflor (Fig. 6). Additionally, we found an antagonistic interaction of sulfoxaflor and azoxystrobin on the amount of sugar solution consumed (Fig. 6). In the absence of azoxystrobin, sulfoxaflor lowered the amount of consumed solution by 34.2%, whereas no significant reduction was detected in presence of azoxystrobin.

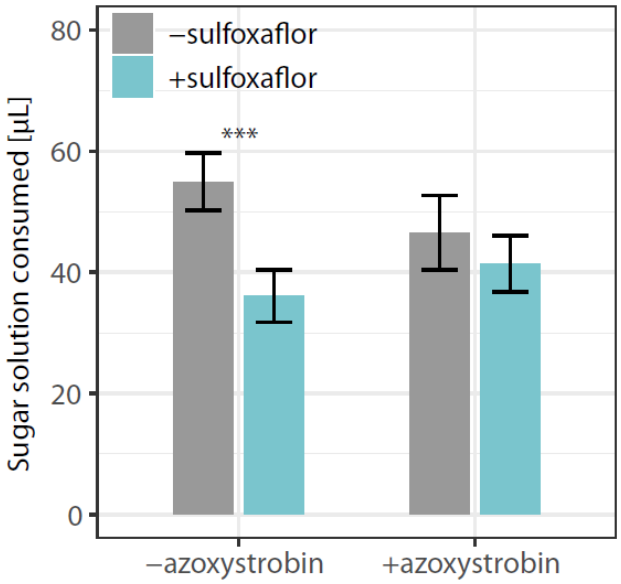

Figure 6. Effect of sulfoxaflor and azoxystrobin on the volume of 33% sugar solution consumed by female *O. bicornis* over three hours inside a NICOT system after a single acute oral dose of the pesticides. The average volume of evaporated sugar solution was subtracted from the measurements. Bars depict model predictions (emmeans) and 95% confidence intervals. Grey bars: no sulfoxaflor exposure, blue bars: sulfoxaflor exposure.

4 Discussion

Honey bees (Apis mellifera)

Responses to pesticides can be highly variable between species (Uhl et al. 2016; Sgolastra et al. 2017; Spurgeon et al. 2020; Adams et al. 2021; Azpiazu et al. 2021), but also within species (Graves and Mackensen 1965; Calow 1996; Dahlgren et al. 2012; Szabó et al. 2021). This intraspecific variance might not only add another level of complexity to ecotoxicological studies but also provide highly relevant information on the risk posed to populations by pesticides (Calow 1996). Such levels of information are especially important for better understanding the risk associated with pesticide exposure in honey bees, which exhibit high interindividual variability in their physiological backgrounds.

We found a significantly higher consumption of sugar syrup by forager bees compared to nurses, and this propensity to consume more syrup was even more pronounced when it was laced with pesticide (at a specific concentration). This phenomenon confirms the often observed preferences of forager bees for sugar solutions containing pesticides (Kessler et al. 2015; Liao et al. 2017), but also has the consequence of intensifying the risk posed by pesticides to foragers. Indeed, the higher consumption of pesticide-contaminated syrup (sulfoxaflor and azoxystrobin) combined with the stronger susceptibility

to pesticide (sulfoxaflor) in foragers contributed to an increase by 2 and 10-fold of the acute and chronic RQ, respectively (see Barascou et al 2022). The magnitude of RQ differences might however be lower in the case of pollen contamination by pesticides given that nurse bees can additionally consume on average 5-10 mg pollen/day.

How to explain the differences in sulfoxaflor sensitivity between nurse and forager bees? To survive toxic compounds, insects have developed detoxification mechanisms, which prevent their accumulation in organs and tissues (Smith 1955; Panini et al. 2016; Lu et al. 2021). Accordingly, we expected a more efficient elimination of sulfoxaflor by nurse bees when compared to foragers. However, the analysis of sulfoxaflor residues showed that its concentration did not differ between nurse and forager bees at 8 h post-exposure (see Barascou et al 2022). On the contrary, sulfoxaflor metabolization was stronger in foragers within 2 h post-exposure, suggesting faster sulfoxaflor elimination by forager bees in the very short term. These results agree with a study that showed that the expression level of three genes encoding cytochrome P450 monooxygenases (involved in the detoxification pathway) was higher in forager bees than in nurses (Vannette et al. 2015). This was later confirmed at the enzymatic level, in which cytochrome P450 monooxygenase activity gradually increased with bee age (Zhu et al. 2020). However, we cannot exclude that in the long term (> 8 h post-exposure), nurse bees are more efficient at eliminating sulfoxaflor than foragers. This is suggested by the improved ability of bees to metabolize pesticides after the consumption of pollen (Ardalani 2021; Ardalani et al. 2021; Barascou et al. 2021), which in honey bees is only consumed by nurses. Lastly, given that the impact of pesticides depends not only on the fate of the molecule in the body, but also on its interaction with the biological target and consecutive effects on the organism, we could also expect that sulfoxaflor affected nurses and foragers in different ways. A recent study demonstrated that nurse and forager bees were differentially affected by neonicotinoids at the gene expression level in the brain: while the expression of genes involved in cognition and development was predominantly affected in foragers, the expression of genes involved in metabolism was modified in nurses (Tsvetkov and Zayed 2021). Although it is not known how such effects might affect honey bee survival or performance, it could help to explain differences in pesticide sensitivity. However, we believe that the most likely explanation is the difference in body weight between nurses and foragers, because for any given bee species, the heavier the individual bees are, the less sensitive they are to a given dose of pesticide (Tahori et al. 1969; Gerig 1975; Nogueira-Couto et al. 1996; Thompson and Hunt 1999). When converted to ng/g of bee, the sulfoxaflor LD50 did not differ between nurses and foragers. However, the fold change in body weight was much stronger in favour of nurses (1.6 times heavier than foragers), which likely explains the higher sensitivity of foragers compared to nurses at the individual level. For a given dose, the concentration of pesticide in the bee body will be higher in foragers than in nurses.

Bumble bees (Bombus terrestris)

Our results show that the nutritional intake of bumble bees can be altered during chronic pesticide exposure. The reduction in the number of extensions, associated with a decrease in proboscis extension length of bumble bees exposed to the highest concentration of cyantraniliprole, and the low number of feeding individuals, suggests significant physiological effects even on single exposure. Toxicity of this molecule has previously been demonstrated for honey bees via the oral and contact routes, but at a moderate magnitude with little variation between different formulated products (Dinter and Samel, 2015). This toxicity, observed in both *A. mellifera* and *B. terrestris*, can be explained by various factors. It has been shown that activation of detoxification in response to insecticide intake can impair the nervous system in bumble bees, reducing their ability to locate floral resources and derive rewards, thus exacerbating nutritional stress and potentially explaining the results observed here (Goulson et al., 2015; Stanley et al., 2015). Furthermore, metabolic detoxification, particularly that induced by P450s, contributes significantly to insecticide tolerance in bees (Gong and Diao, 2017; Manjon et al., 2018). Yet, pesticide exposure can alter detoxification gene expression pathways in addition to immune responses, thus adversely affecting bee health (Boncristiani et al., 2012).

Glyphosate alone showed little effect on the food intake of *B. terrestris* workers. We found a significant negative impact only for the amount consumed per extension at the highest concentration (50,000 ppb). We also observed a tendency of the workers to collect more nectar at the medium concentration (10,000ppb) but this was not significant. Our results are in line with the study of Herbert et al. (2014) who showed that prolonged exposure to high concentration of glyphosate can result in sublethal effects during the first 15 days of adult life. The negative impact was associated with a loss in sensory sensitivity and cognitive deficits. Regarding the potential impact at medium concentration (i.e. increase of consumption), it has also been shown that honey bees can consume more nectar in the presence of glyphosate (Liao et al., 2017). This behaviour could be explained by the excitement of finding new different resources (Köhler et al., 2012).

When *B. terrestris* workers were exposed to boscalid alone, they performed more extensions to consume the same amount of nectar. This implies an impact of boscalid on feeding ability. Such an effect of boscalid has not yet been demonstrated in the literature. This decrease in the amount taken per extension seems to be counterbalanced by an increase in the number of extensions, leading to stable levels of syrup consumption between treatments.

We did not observe synergistic effects when bumble bee workers were exposed to multiple pesticides. Regarding the interaction between glyphosate and boscalid, we found an additive effect of these pesticides. The amount consumed, the extension length and the amount consumed per extension were significantly impacted. When mixed with cyantraniliprole, boscalid negatively affected different feeding parameters including the number of extensions and the extension length of the proboscis. However, this effect does not appear to be additive, being similar to the effects of cyantraniliprole or boscalid when used alone. The cyantraniliprole/glyphosate mixture had a negative impact on the different parameters measured. The effect of cyantraniliprole in the experiments with the pesticides alone suggests that the effect found in this interaction is mainly due to cyantraniliprole. These results are in line with previous studies showing many lethal and sublethal effects of pesticide mixtures including delayed ovarian maturation (Raimets et al., 2018; Sgolastra et al., 2018). Our results are also in line with studies showing additive or antagonistic effects of pesticides (e.g. Thompson et al. 2014; Tosi & Nieh 219).

Mason bees (Osmia bicornis)

We tested the single and combined effects of sulfoxaflor and azoxystrobin exposure on *O. bicornis* nectar intake. We found a drastic reduction in the volume of ingested sugar solution after exposure to sulfoxaflor. Interestingly, however, we found an antagonistic interaction of the two pesticides, showing that the reduction in nectar intake was only statistically significant in the absence, but not in the presence of azoxystrobin. This finding can suggest that the two substances might interact with each other directly at the molecular level, but other physiological mechanisms inside the bee could also explain this antagonistic interaction. In order to explore the nature of this potential interaction, further studies are needed.

The reduction of nectar intake is in agreement with previous studies testing feeding rates after exposure to sulfoxaflor (Linguadoca et al. 2021), neonicotinoid insecticides (Kessler et al. 2015, Thompson et al. 2015), or flupyradifurone (Tosi et al. 2021, Wu et al. 2021). Bees are apparently not able to detect and avoid neonicotinoid insecticides in their food, and evidence suggests that they even prefer contaminated nectar (Kessler et al. 2015). Since we exposed bees to sulfoxaflor before letting them feed on sugar solution, the negative impact observed is likely a result of impaired locomotion (Tosi and Nieh 2017, Williamson et al. 2014), reduced sucrose responsiveness (Démares et al. 2018, Hesselbach and Scheiner 2018) or reduced feeding motivation (Siviter and Muth 2022). A reduced intake of nectar could result in nutritional stress, with negative implications for fitness-related measures such as foraging behaviour, immune function or reproduction. In combination, nutritional stress and pesticide exposure

could lead to additive or to synergistic negative impacts on bees with harmful consequences for population dynamics (Tosi et al. 2017, Linguadoca et al. 2021, Siviter et al. 2021, Knauer et al. in review).

Conclusion

Our results show that the foraging behaviour of bees can be altered after pesticide exposure. We additionally show that honey bee workers are not all equal regarding the risk posed by pesticides and that, depending on the honey bee behavioural caste, it might be under or over-estimated. The growing agreement across studies that foragers or old bees are more sensitive to insecticides than nurse or young bees therefore suggests consistent inclusion of forager bees in regulatory tests should allow for an increase in the safety margin of pesticide risk assessment.

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