Manuscript on the response of novel wild bees to diverse agrochemicals

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PoshBee

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

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Summary

Morphology, physiology and ecology differ greatly among wild bee species. Since the last century, first tier pesticide risk assessments have mainly focussed on LD50 determination for the honey bee *A. mellifera*. Recently *B. terrestris* and *O. bicornis* have also been included in these tests. However, in order to better understand the risks associated with pesticides, more data are needed on the maintenance of wild bees in the laboratory and on the effects of pesticides on them. In this study, several wild bee species were caught, kept under laboratory conditions and exposed to oral and topical acute doses of the insecticidal molecule sulfoxaflor. We then measured and compared their sensitivity towards the bumble bee model species *B. terrestris*. Our results revealed significant variability in survival in the laboratory among bee species. They also confirmed that, as suggested by recent comparative studies, some species such as *Anthophora plumipes, Osmia cornuta* and *Bombus pascuorum* were more sensitive than the model species. We also investigated the largely unexplored exposure route of soil to ground-nesting bees. We attempted unique assays in order to determine the risks of imidacloprid and sulfoxaflor soil exposure to adults and developing offspring of the novel wild bee species *A. plumipes* and *Colletes hederae.* We confirm that assays are possible with *A. plumipes,* and that no adverse effects of field-realistic imidacloprid exposure could be determined on various brood production parameters. The assays conducted with *C. hederae* were terminally hampered by the great difficulty in transferring the species to a laboratory or semi-field based assay system. Besides their ecological characteristics, there might be specific morphological and physiological traits and routes of exposure influencing the sensitivity of wild bee species to pesticides that remain to be determined. We contribute a novel assay method of soil residues to ground-nesting bees, a knowledge gap that deservers further attention. However, even more protocols need to be developed to keep wild bee species alive under laboratory conditions so as to perform better pesticide risk assessments.

1. Introduction

Animals are pollen vectors of more than 85% of angiosperms (Ollerton et al., 2011). Many animal clades are described as pollinators (e.g. butterflies, flies, beetles, wasps, bats, birds, lizards, and mammals). However bees are the most important for a majority of plant species (Potts et al., 2016). To in-part explain this dominance, and unlike other groups, the whole life cycle of bees relies almost exclusively on floral resource consumption (i.e. pollen and nectar) by both adults and larvae (Michener, 2007). Moreover, their morphology (e.g. hairiness), ubiquity in terrestrial environments and species diversity make bees very efficient pollinators. With more than 20,000 species recorded worldwide (Michener, 2007), and more than 2,000 species recorded in Europe (Rasmont et al., 2017), wild bee species exhibit significant variability in terms of phenology (e.g. adults active in spring, summer or autumn; univoltine like *Colletes hederae* or bivoltine like *Andrena bicolor*), foraging strategies (e.g. pollen generalist like *Bombus terrestris* or pollen specialist like *Heriades truncorum*), nesting behaviour (e.g. cavity-nesting like *Osmia cornuta* or soil-nesting like *Anthophora plumipes*), social and nesting behaviour (e.g. cleptoparasitic like *Nomada fulvicornis*, solitary like *Megachile rotundata* or social like *Bombus lapidarius*), and body size (ranging from 0.2 mm in *Perdita minima* to 63.5mm in *Megachile pluto*) (Michener, 2007; Danforth et al., 2013; Michez et al., 2019). The great species diversity of wild bees is directly linked to the diversity of plants, as co-evolutionary mechanisms are thought to have led to the appearance of traits specific to certain bee species that are necessary to feed on specific plant species (Goulson, 1999; Stang et al., 2009; Willmer, 2011; Pyke et al., 2012; Boberg et al., 2014).

In addition to their crucial role in the sexual reproduction of wild plants, bees are the main pollinators of 75% of agricultural crops that benefit from animal pollination (Klein et al., 2007). Due to the increasing demand for food, managed bees, including honey bees, bumble bees, and some solitary

bees, have become crucial for these pollinator-dependent crops (Aizen and Harder, 2009; Breeze et al., 2014). However, various studies have shown that wild species are equally effective or better pollinators than honey bees for many crops (Heard, 1999; Javorek et al., 2002; Kremen et al., 2002; Winfree et al., 2007), and that they can increase crop yield by up to twice as much as honey bees (Garibaldi et al., 2013; Weekers et al., 2022).

Despite their importance, losses and declines in managed and wild bee populations have been reported worldwide (Cameron et al., 2011; Goulson et al., 2015; Duchenne et al., 2020). Large-scale studies such as the European red list (Nieto et al., 2014) highlighted this global decline but also showed that many species lacked data to conclude their population trend or even their spatial distribution. National level studies with comprehensive assessments, such as the recent red list of Belgium, indicated 29.6% of species were at threat and 11.8% considered regionally extinct (Drossart et al., 2019). The drivers for these declines have been well documented in Europe, which include habitat loss (e.g. Persson et al., 2015; Vray et al., 2019), invasive species (e.g. Stout and Morales, 2009), parasites (e.g. Williams et al., 2008), pathogens (e.g. Ravoet et al., 2014), climate change (e.g. Kerr et al., 2015) and agricultural intensification (e.g. Winfree, 2010). Considering the crucial role that wild bees play for both natural ecosystems and in crop production, it is important to better understand how wild bee species can deal with these different drivers, particularly pesticide use through agricultural intensification, which may be particularly challenging because of the wide diversity of wild bee species and poor knowledge of how to maintain them in the laboratory.

The role of pesticides in the current decline of wild bees remains unclear (Goulson et al., 2015; Johnson and Corn, 2015). However, due to their reliance on floral resources and their frequent presence on flowering crops and on flowers in surrounding areas, bees are frequently exposed to pesticides (Godfray et al., 2014), either through contact exposure during pesticide application when nesting, or oral exposure via consumption of pesticide-treated resources (pollen and nectar). While more and more studies are showing adverse effects of pesticides on wild bee species, the methods currently used to assess the toxicity of these molecules for bees mainly rely, at least for initial tests, on LD50 determination (i.e. the determination of the dose that kills 50 percent of the tested sample), which for a long time has only been performed on the western honey bee, *Apis mellifera* (Boller et al., 2006). This well-known species is domesticated and easy to breed and maintain under laboratory conditions (Franklin and Raine, 2019), in contrast to the majority of wild bee species. Yet, considering the great ecological, physiological and morphological variability found in this insect group, using *A. mellifera* to predict hazards of pesticides for wild bees could lead to a considerable underestimation of their adverse effects (Wood et al., 2020). In 2013, the European Food Safety Authority (EFSA) thus suggested to include two other surrogate species in the pesticide risk assessments: the buff-tailed bumble bee, *Bombus terrestris* and solitary bee species from the genus *Osmia* (Spurgeon et al., 2017).

The species-specific sensitivity of wild bee species has already been highlighted through meta-analysis (Arena & Sgolastra, 2014), which indicated that, besides the intrinsic sensitivity of individual bee species, the effects of pesticides were also dependent on a species' specific life cycle, nesting activity and foraging behaviour. Besides ecological factors, the wide variation in size and body weight among wild bee species could also impact their sensitivity to pesticides. Sensitivity has been shown to increase with the surface-to-volume ratio of a target organism (Johansen, 1972) and it has recently been highlighted that the specific bee body weight seems to be a good predictor of sensitivity towards acute contact pesticide exposure (Pamminger, 2021).

So far, only a few wild bee species have been tested in laboratory conditions (Helson et al., 1994; Thompson, 2001; Scott-Dupree et al., 2009; Arena and Sgolastra, 2014), mainly due to the difficulty obtaining enough individuals to perform relevant experiments and in keeping them alive. Therefore, there is a need to develop new methods to maintain live wild bee specimens in laboratory conditions,

as well as to experiment on their sensitivity to pesticides. The work presented in this report aims to (i) assess methods to maintain live bees collected from the wild in laboratory conditions, (ii) determine suitable exposure routes (topical and/or oral) and (iii) compare the effects of oral and topical acute pesticide exposure on the individual mortality of several wild bee species. Here, we test the sulfoximine insecticide sulfoxaflor, which is one of the target chemicals investigated in PoshBee. We expect to see species-specific sensitivity towards acute sulfoxaflor exposure (Arena and Sgolastra, 2014).

The majority of wild bee species, globally and in Europe, nest underground in soil (Antoine & Forrest, 2021). Ground-nesting bees spend their larval, pupal and often hibernating stages in brood chambers built in soil by their mother, and could therefore be chronically exposed to agrochemical residues in the soil. Additionally, females spend most of their adult life constructing nests and therefore handling soil (Harmon-Threatt, 2020). Despite this, almost nothing is known about the potential exposure risk to bees of agrochemical residues in soil, and health hazards of this exposure (Willis Chan et al., 2019). This knowledge gap is due to the lack of suitable model organisms which can be readily reared and manipulated in large numbers. So far, no European ground-nesting bee has been discussed in a pesticide exposure context, though one North American species, the Hoary squash bee *Eucera pruinosel,* has been shown to suffer reduced reproductive success when exposed to imidacloprid-contaminated soil (Willis Chan & Raine, 2021; see also Fortuin et al., 2021). In the Poshbee project, we have introduced the hairy footed flower bee *Anthophora plumipes* and the ivy bee *Colletes hederae* as plausible ground-nesting wild bee test species. We tested the effects of a high, but field-realistic, concentration of the neonicotinoid insecticide imidacloprid on brood cell production and larval survival in a free-living population of *A. plumipes,* using methods described in the Poshbee Milestone MS9 (Hellström et al. 2020). Soil exposure of sulfoxaflor and azoxystrobin was also tested on *C. hederae* by collecting brood cells of *C. hederae* in the field and mixing soil from nesting sites with sulfoxaflor or azoxystrobin to monitor development from larval to adult stage. However, due to several problems encountered, we had to reduce and adapt the measured parameters and could not yet include the data in this report.

2. Acute exposure of Sulfoxaflor to various wild bee species

2.1. Materials and methods

Here we cover the details of our experiments on a diversity of wild bee species to oral or contact exposure to pesticides, with a focus on sulfoxaflor.

2.2. Species selection and sampling

This study was conducted on thirteen different bee species, including the buff-tailed bumble bee, *Bombus terrestris*, considered here as a reference species. Since 2016, toxicity test protocols for *B. terrestris* have been part of the international ICPPR and OECD ring-test groups for pesticide risk assessments. The annual colonies of this species can have a large number of workers (>500), and are commercially available. This allows toxicity tests that require a high number of individuals, such as the determination of LD50. To perform experiments, commercial queen-right colonies of 100 *Bombus terrestris* workers were maintained in a controlled room at 25 ± 5°C and 60 ± 5% humidity (Taseï and

Aupinel, 2008). They were fed with *ad libitum* sugar syrup (65% sugar, 35% water), and once a week with 10 g of freeze dried *Salix* spp. pollen per colony.

Females of the 15 other bee species were transported from the wild to the lab to first assess their ability to survive under these conditions and then, if their ability to survive was sufficient, to assess their sensitivity to sulfoxaflor compared to that of *B. terrestris*. We collected three bumble bee species, the red-tail bumble bee, *Bombus lapidarius*, the tree bumble bee, *Bombus hypnorum* and the common carder bee, *Bombus pascuorum*; two Andrenidae species, the grey-backed mining bee, *Andrena vaga* and *Andrena rufa*; two Collitidae species, *Colletes daviesanus* and *Hylaeus signatus*; one Halictidae species, the sweat bee, *Halictus scabiosae*; one Melittidae species, the pantaloon bee *Dasypoda hirtipes*; one Apidae species, the hairy-footed flower bee *Anthophora plumipes;* five Megachilidae species, the European orchard bee, *Osmia cornuta*, the blue mason bee, *Osmia caerulescens*, the large scissor-bee, *Chelostoma florisomne*, the European wool carder bee *Anthidium manicatum* and finally, the large-headed resin bee, *Heriades truncorum*. The bumble bee species were kept in the same conditions as the *Bombus terrestris* colonies (i.e. 25 ± 5°C and 60 ± 5% humidity), while the other bees were kept at room temperature (~21°C).

All species except *A. plumipes* were collected as adults using hand netting in their natural habitats around the city of Mons (Belgium). The collection of *A. plumipes* in Halle (Germany) took place as follows: clay blocks in which active nesting had taken place during 2021 were brought into the laboratory and brood cells were extracted from them by excavation. The brood cells were subsequently overwintered at 4°C. At the start of the experiments, brood cells were transferred to a 21°C incubator and emergence was checked daily. Freshly emerged adults were transferred back to 4°C until enough individuals had emerged to populate the tests.

2.2.1. Housing and acclimation

Before the beginning of experiments, each bee was weighed individually, placed under an inverted see-through plastic beaker (Figure 1), and fed with *ad libitum* 50% w/w sugar solution through soaked cotton capillaries. Other feeding methods were tested, such as the use of syringes. But apart from bumble bees, other wild bee species were not successful in using them to feed. Then, specimens were left at least twelve hours in a dark controlled room at a temperature of 25 ± 5°C and 60 ± 5% humidity for the *Bombus* spp., and at room temperature for the other species. They remained in these rooms for the whole duration of the experiment. For each species, individuals of the same mean weight were randomly assigned to treatment groups in an attempt to maintain an equal mean body size across treatment groups.

2.2.2. Sulfoxaflor exposure

The experimental setups of topical and oral exposure were adapted from the OECD guidelines (OECD, 2017b, 2017a), and the improved protocols for testing agrochemicals in bees (Medrzyck *et al.*, 2021). The OECD guidelines recommend the use of at least 30 individuals for each treatment group. But since the majority of wild bee species are not commercially available, we had to use fewer individuals in some cases. All the species with enough specimens that survived the acclimation phase (see below) were exposed to one single concentration of sulfoxaflor because we did not have enough specimens to test multiple concentrations. We selected oral and topical LD50 concentrations for *Bombus terrestris* (oral LD50: 0.563µg/g b.w.; topical LD50: 10.4 µg/bee; unpublished data). Preliminary tests on the model species were performed following the same protocols we used with wild bees. In most

tests, a positive control was used consisting of a 10 µg/bee dimethoate treatment. After the exposure period, sub-lethal effects were observed and mortality recorded under red light at 24 hours and 48 hours after exposure. At the end, the final rate of mortality was recorded.

2.2.3. Oral exposure

After the 12 hour acclimation period (Figure 1a), the sugar-soaked capillaries were removed from the see-through plastic beakers (Figure 1b) before a four-hour starvation period. Then, for each treatment group, spectrophotometer cuvettes were filled with 20µL of either treatment or control solution and placed under the beakers (Figure 1c). The cuvettes were left in situ for the four-hour exposure period, during which consumption of the solution was observed every 30 minutes. The negative control solution consisted of a 50% w/w sugar-water solution with 0.05% of acetone. Once a bee consumed the 20µL droplet, it was included in the test, and a new capillary containing a 50% w/w sugar-water solution was placed back under the beaker for the remainder of the observation period (Figure 1d). To control for evaporation during the exposure period, five additional doses were placed into spectrophotometer cuvettes under empty beakers. These cuvettes were weighed before and after the four hour exposure period.

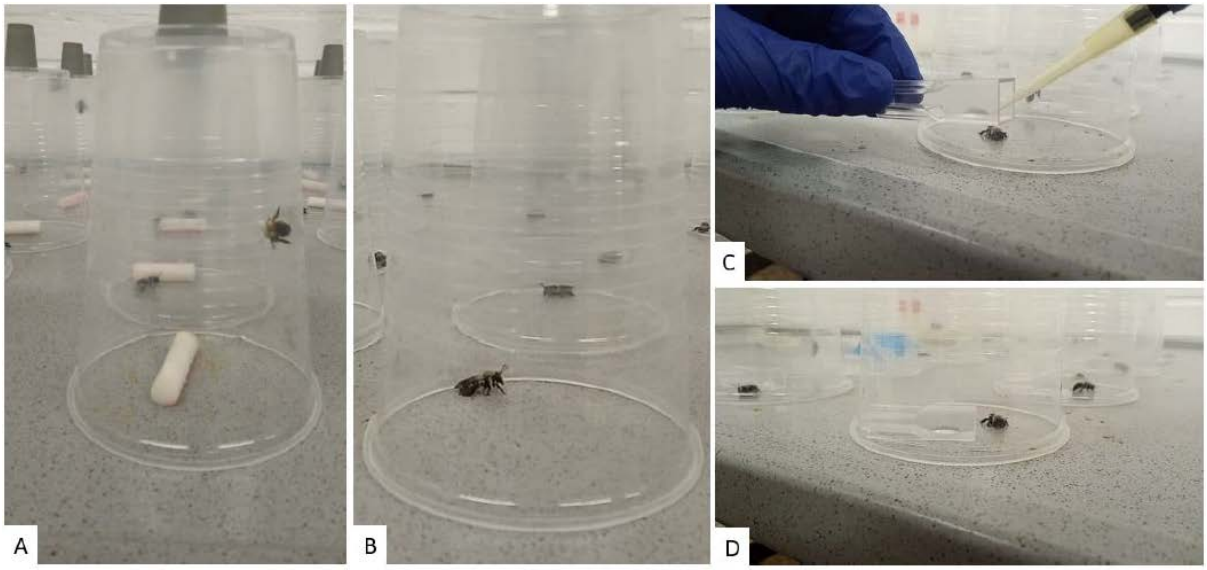

Figure 1: Example of acute oral exposure to sulfoxaflor with *Andrena vaga*. A) Acclimation period under an inverted see-through plastic beaker with a soaked capillary as food source, B) starvation period with the soak capillary removed from the beaker, C) positioning of the 20µL droplet inside the spectrophotometer cuvette, and D) exposure period with the cuvettes under the beaker (picture credits: Justine Dewaele).

2.2.4. Topical exposure

For each treatment group, bees were chilled until immobile (max. ten minutes in the freezer at -20°C for the largest species, or an hour in the fridge at +4°C) before handling (Figure 2b). They were then exposed by applying a 2µL droplet with a micro-pipette on the dorsal side of the thorax (Figure 2c). The negative control individuals were treated with 2µL of distilled water containing 0.05% acetone. To ensure even dispersal of the treatment and control solutions on the bee thorax, Triton X-100 (0.05%) was used as a surfactant. Once the 2µL droplet was applied, the individual was placed in a Petri dish until it recovered from chilling, and then placed back under an inverted see-through plastic beaker of the controlled room for the observation period (Figure 2d).

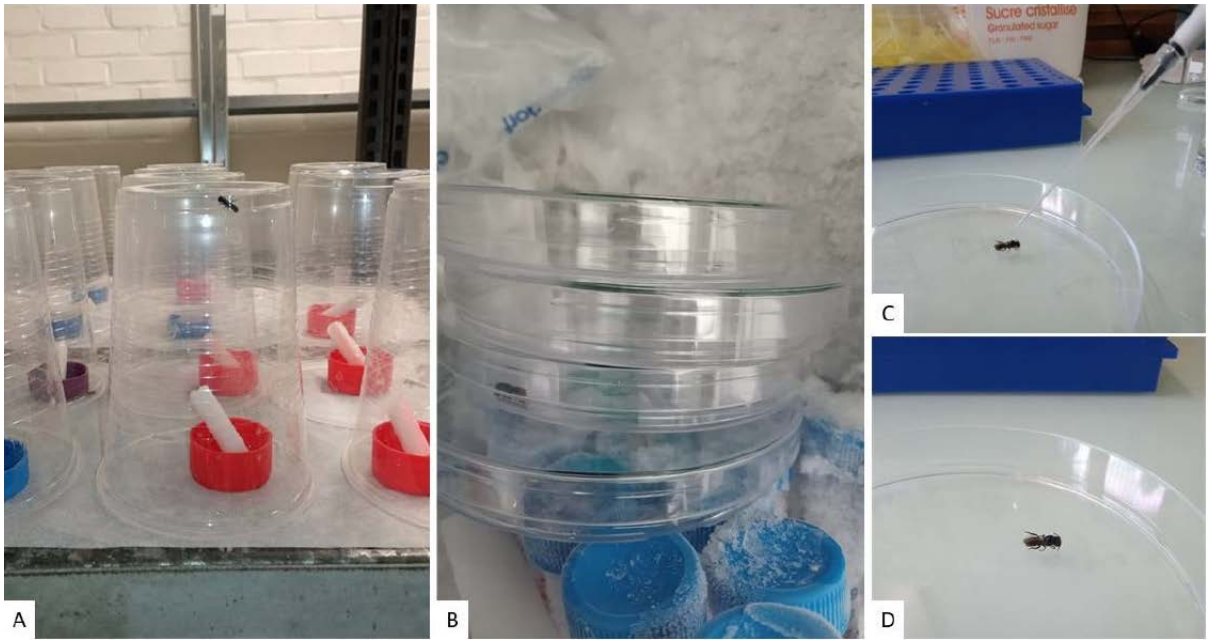

Figure 2: Example of acute topical exposure to sulfoxaflor with the *Heriades truncorum*. A) Acclimation period under an inverted see-through plastic beaker with a soaked capillary as food source, B) chilling process in Petri dishes, C) topical exposure by positioning a 2µL droplet on the bee thorax, and D) recovery period into the Petri dish after chilling and before going back under the plastic beaker (picture credits: Justine Dewaele).

2.2.5. Statistical analysis

All statistical analyses were carried out in the R environment v 4.1.0 (R Core Team, 2021). The Chi-squared and Fisher's tests were performed using the base stats package of R (R Core Team, 2021). All graphs were produced using the ggplot2 package v 3.3.5 (Wickham, 2016), associated either with ggmosaic v 0.3.3 (Jepson et al., 2021), or ggeffects v 1.1.0 (Lüdecke, 2018).

To evaluate the effect of acute sulfoxaflor exposure on the mortality of wild bees, Chi-squared tests were performed. When the expected frequencies were lower than five, we used the Fisher's exact test for count data. Before comparing the sensitivity of wild bees with *B. terrestris*, a correction of the control proportions was needed to take into account the mortality that was not linked to pesticide exposure (Puntener, 1992). Since the sample sizes and control mortality rates were different between the groups compared, we used the Sun-Shepard's formula (Puntener, 1992) expressed as

$$Corrected\ \% = \frac{mortality\ (\%)\ in\ treatment\ +\ change\ (\%)\ in\ control}{100\ +\ change\ (\%)\ in\ control} \times 100$$

Then, in order to compare the sensitivity of wild bees with *B. terrestris*, we used either a Chi-squared test followed by a pairwise test of the comparison of proportions, or, when at least one expected frequency was lower than five, a Fisher's exact count test followed by a pairwise Fisher's test. Both pairwise tests were performed using the Holm method to correct the p-value for multiple testing.

2.3. Results

In total, 11 species caught in the wild were placed under adapted conditions (cf. Material and Methods section 2.2): *Bombus hypnorum*, *Bombus pascuorum*, *Anthophora plumipes*, *Osmia cornuta*, *Osmia*

cearulescens, *Osmia leaiana*, *Heriades truncorum*, *Chelostoma florisomme*, *Andrena vaga*, *Andrena fulva*, and *Halictus scabiosae*. While a very small number of *Andrena fulva* and *Chelostoma florisomme* individuals were caught, they seemed to survive well in laboratory conditions. Moreover, the captured *Andrena fulva* individuals successfully fed from the cuvettes. However, they could not be considered in the oral and topical analyses because not enough individuals were caught.

This left nine species of wild bee to use in the experiments (Table 1). Their mortality after oral and topical exposure was compared to *B. terrestris*. It must be highlighted that the oral and topical LD50 under OECD conditions were not lethal for 50% of the tested *B. terrestris* samples under adapted conditions. Therefore, we decided to undertake the comparison based on mortality proportions obtained under the adapted conditions, i.e. the same as for the wild bees. Where insufficient individuals from a species were caught, namely for *O. leaiana* and *A. plumipes*, we did not perform both exposure experiments, but only topical exposure, except for *H. scabiosae* and *O. cornuta* that were only used in the oral exposure experiment (Table 1).

Table 1: Species morphological and ecological characteristics, as well as the experiments in which they were considered.

Species	Average mass ± SE (g)	Phenology	Lectism	Sociality	Experiment
Bombus terrestris (model species)	0.261 ± 0.003	Summer Spring	Polylectic	Primitively social	Oral+Topical
Bombus hypnorum	0.150 ± 0.004	Summer	Polylectic	Primitively social	Oral+Topical
Bombus pascuorum	0.147 ± 0.003	Summer	Polylectic	Primitively social	Oral+Topical
Anthophora plumipes	0.131 ± 0.002	Spring	Polylectic	Solitary	Topical
Heriades truncorum	0.014 ± 0.000	Summer	Oligolectic	Solitary	Topical
Osmia caerulescens	0.041 ± 0.001	Spring	Polylectic	Solitary	Topical
Osmia cornuta	0.122 ± 0.002	Spring	Polylectic	Solitary	Oral
Osmia leaiana	0.066 ± 0.005	Spring	Oligolectic	Solitary	Topical
Andrena vaga	0.140 ± 0.002	Spring	Oligolectic	Solitary	Oral+Topical
Halictus scabiosae	0.087 ± 0.004	Summer	Oligolectic	Primitively social	Oral

Among the species used in the experiments, the rate of survival of the control samples (i.e. untreated witrh pesticide) under lab conditions after 48 hours differed significantly among species (Fisher's exact test for count data with simulated p-value based on 2000 replicates, p-value = 0.0005, N = 610). The species that survived significantly better under laboratory conditions were *B. terrestris* (the model species), and *B. hypnorum* (Figure 3a).

The seven orally exposed species that were tested for their ability to feed on the control solution from the cuvettes differed significantly (Fisher's exact test for count data with simulated p-value based on 2000 replicates, p-value = 0.0005, N = 366). The species for which most individuals fed on the control solutions were *O. cornuta* and the three *Bombus* species, while no *O. caerulescens* individuals fed on the control solution (Figure 3b). *O. caerulescens* individuals were therefore only used for the topical exposure experiment.

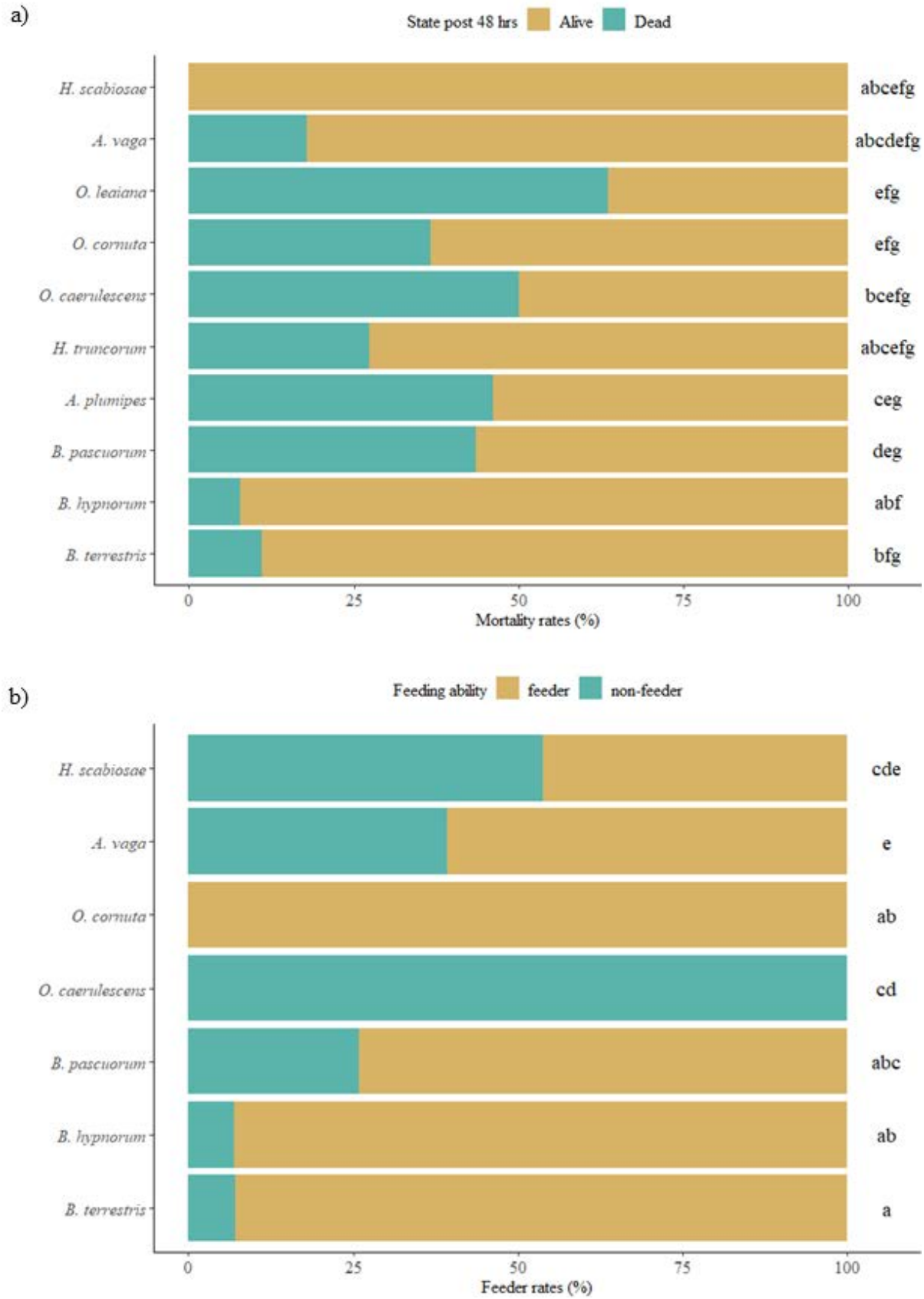

Figure 3: a) Rates of mortality (%) of the control samples towards laboratory conditions and b) proportion feeding (as a %) in each species used in the experiment. Species that do not share the same letter have significantly different proportions at p-value<0.05 (post-hoc, Fisher's pairwise comparison of proportions with Holm correction).

2.3.1. Oral exposure

In addition to the model species *B. terrestris*, we were successfully able to orally expose an additional five wild bee species to an acute sulfoxaflor dose of 0.563 µg/g body weight. These included two *Bombus* species, *B. hypnorum*, and *B. pascuorum*, as well as *O. cornuta*, *H. scabiosae*, and *A. vaga*.

Table 2: Effects of oral exposure to an acute sulfoxaflor dose (43.9 µg/g b.w.) on the rate of mortality of 6 bee species. Legend: Number of individuals (proportion of individuals).

Species	Treatments	Alive	Dead	p-value
B. terrestris (model species)	Control	91 (100%)	0 (0%)	1.4200e-25***
	Sulfoxaflor	48 (56.5%)	37 (43.5%)	
B. hypnorum	Control	56 (96.6%)	2 (3.4%)	0.6358
	Sulfoxaflor	50 (98.0%)	1 (2.0%)	
B. pascuorum	Control	16 (80.0%)	4 (20.0%)	0.0699
	Sulfoxaflor	10 (52.6%)	9 (47.4%)	
O. cornuta	Control	19 (63.3%)	11 (36.7%)	1.6971e-06***
	Sulfoxaflor	3 (8.1%)	34 (91.9%)	
A. vaga	Control	58 (81.7%)	13 (18.3%)	0.0023**
	Sulfoxaflor	42 (58.3%)	30 (41.7%)	
H. scabiosae	Control	6 (100%)	0 (0.0%)	0.1824
	Sulfoxaflor	3 (50.0%)	3 (50.0%)	
				Significance level: *p<0.05; **p<0.01; ***p<0.001

Of the five species selected for the analysis, only three exhibited significant sensitivity to acute sulfoxaflor exposure. The species were *B. terrestris* (the model species), *A. vaga* and *O. cornuta*, with respectively 37 (43.5%), 30 (41.7%) and 34 (91.9%) individuals that died 48 hours after feeding on the sulfoxaflor solution, compared to 0, 13 (18.3%) and 11 (36.7%) individuals in the control group (Pearson's chi-square test, *B. terrestris*: χ^2=50.16, df=1, p-value=1.4200e-12, n=176; *A. vaga*: χ^2=9.27, df = 1, p-value = 0.0023, n=143; *O. cornuta*: χ^2=22.91, df=1, p-value= 1.6971e-06, n=67; see Table 2). The Sun-Shepard's corrected proportions of *B. terrestris*, *A. vaga* and *O. cornuta* individuals that died 48 hours after exposure showed that *O. cornuta* was more sensitive than *B. terrestris* towards an acute oral sulfoxaflor exposure (post-hoc, Fisher's pairwise comparison of proportions with Holm correction, p=1.9e-09), while the sensitivity of *A. vaga* did not differ significantly from *B. terrestris*. Indeed, while 30 % of the *B. terrestris* individuals died after being exposed to a sulfoxaflor dose of 0.563 µg/g body weight, this proportion was higher in the *O. cornuta* treated group, at 93.5% mortality. However, only 29.2% of the *A. vaga* died after being exposed to a sulfoxaflor dose of 0.563 µg/g body weight (Chi-square test, χ^2=42.30, df=1, p-value=6.5117e-10, n=163; see Figure 4).

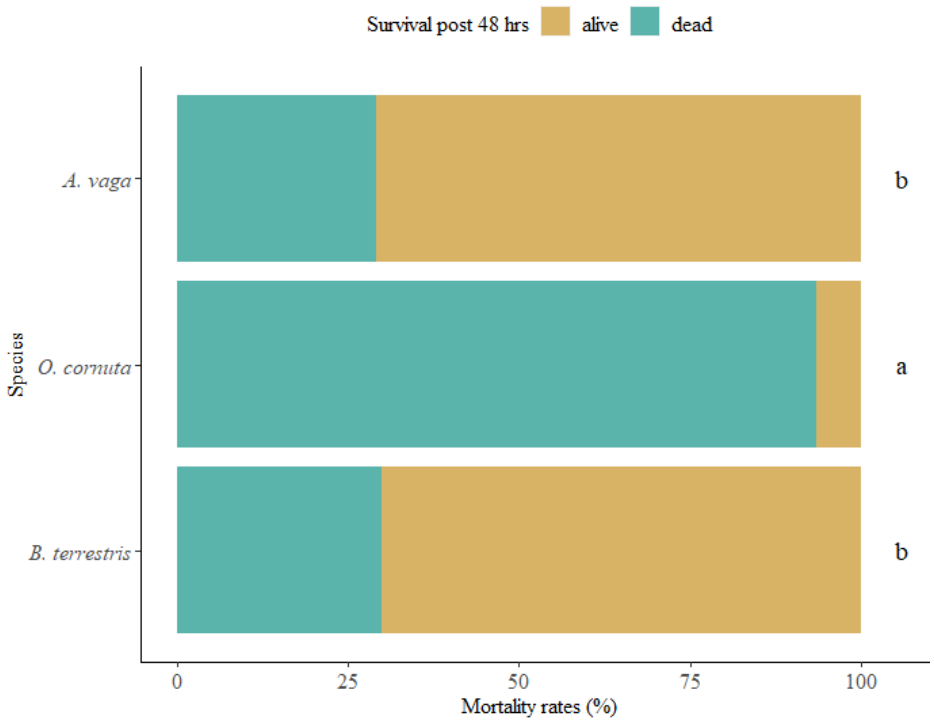

Figure 4: Rates of mortality (%) of treated individuals 48 h after ingestion of Sulfoxaflor (0.563 µg/g body weight) for each species, corrected with Sun-Shepard's formula. Species that do not share the same letter have significantly different proportions of dead and alive individuals 48 hours after exposure at a p-value<0.05 (Post-hoc, Chi-square pairwise comparisons with Holm correction).

2.3.2. Topical exposure

In addition to the model species *B. terrestris*, seven species were topically exposed to an acute sulfoxaflor dose of 10.4 µg/bee. Topical exposures to pesticides generally result from droplets that are deposited on the bee following application of the products. Therefore, unlike oral exposure, the amount of pesticide to which different bee species are exposed can be considered as similar. As we could not test more conditions due to the small number of individuals, it seemed more consistent to work with µg/bee rather than µg/g body weight. Two *Bombus* species, *B. hypnorum* and *B. pascuorum*, three Megachilidae species, *H. truncorum*, *O. leaiana* and *O. cearulescens*, as well as *A. vaga* and *H. scabiosae* were topically exposed.

Table 3: Effects of topical exposure to acute sulfoxaflor dose (43.9 µg/g b.w.) on the rate of mortality of 8 bee species.
Legend: Number of individuals (proportion of individuals).

Species	Treatments	Alive	Dead	p-value
B. terrestris (model species)	Control	61 (89.7%)	7 (10.3%)	1.1296e-11***
	Sulfoxaflor	22 (32.8%)	45 (67.2%)	
B. hypnorum	Control	8 (80%)	2 (20%)	0.0427*
	Sulfoxaflor	4 (33.3%)	8 (66.7%)	
B. pascuorum	Control	32 (60.4%)	21 (39.6%)	2.0272e-11***
	Sulfoxaflor	1 (1.8%)	56 (98.2%)	
A. plumipes	Control	21 (53.8%)	18 (46.2%)	5.6427e-06***
	Sulfoxaflor	3 (7.3%)	38 (92.7%)	
O. caerulescens	Control	6 (60%)	4 (40%)	0.0024**
	Sulfoxaflor	0 (0.0%)	11 (1.0%)	
O. leaiana	Control	4 (100%)	0 (0.0%)	0.0047**
	Sulfoxaflor	0 (0.0%)	4 (100%)	
H. truncorum	Control	16 (84.2%)	3 (15.8%)	2.3679e-07***
	Sulfoxaflor	0 (0.0%)	18 (100%)	
A. vaga	Control	38 (95%)	2 (50%)	9.9329e-13***
	Sulfoxaflor	8 (17.8%)	37 (82.2%)	
				Significance level: *p<0.05; **p<0.01; ***p<0.001

All of the species showed a significant mortality to acute sulfoxaflor exposure (Table 3). The Sun-Shepard's corrected proportion of dead individuals 48 hours after exposure for each species was then compared to *B. terrestris*. *Bombus pascuorum* and *A. plumipes* were significantly more sensitive than *B. terrestris* with respectively 98% and 90% mortality 48 hours after the exposure for *B. pascuorum* and *A. plumipes*, compared to 70% for *B. terrestris* (post-hoc, Fisher's pairwise comparison of proportions with Holm correction, $p_{B.\ terrestris\text{-}B.\ pascuorum}$=0.0005, $p_{B.\ terrestris\text{-}A.\ plumipes}$=0.0361; see Figure 5).

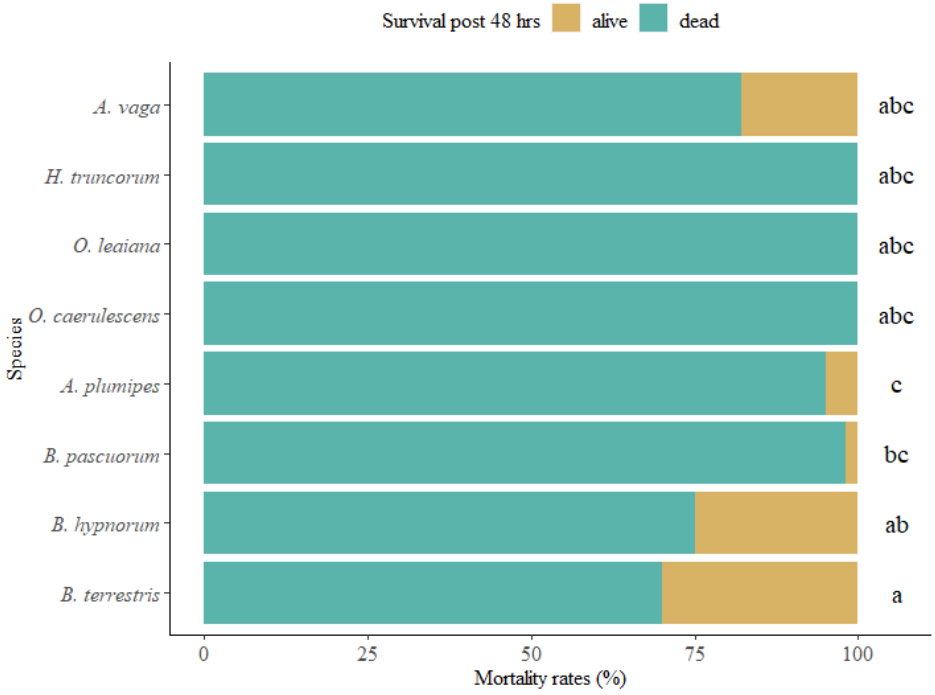

Figure 5: Rates of mortality (%) of treated individuals 48 h after exposure to sulfoxaflor (43.9 µg/g body weight) for each species, corrected with Sun-Shepard's formula. Species that do not share the same letter have significantly different proportions of dead and alive individuals 48 hours after the exposure at p-value<0.05 (post-hoc, Fisher's pairwise comparisons with Holm correction).

3. Soil exposure of *A. plumipes* to imidacloprid

3.1. Materials and methods

Using methods developed and described in MS9, we investigated the effects of soil contaminants on nest initiation and brood survival of *A. plumipes.* In brief, imidacloprid-contaminated and uncontaminated soil-filled nesting units were placed in a pesticide-free environment next to an existing aggregation of *A. plumipes*. Using this method, the propensity of free-living *A. plumipes* to nest in contaminated substrate was assessed, as well as the number of brood cells constructed per unit, the number of surviving brood, and the rate of brood cell parasitism.

The chosen contamination treatment was 100 µg/kg imidacloprid in soil. A stock solution of 333 mg/L was made by mixing 10 g pure imidacloprid (Chemservice) in 300 mL distilled water, mixed on a magnetic stirrer for 6 hours. The ensuing mix was diluted in water to 1.1 mg/L and mixed with dry soil substrate 1:10 w/w (100 mL liquid to 1 kg dry soil substrate). For the control treatment, soil was mixed using distilled water only. Separate tools and storage containers were used for control and treatment boxes, minimizing risk of cross-contamination.

For each nesting unit, 1.1 kg of wet soil substrate was added to a cardboard container measuring 5x9x14 cm. The soil was lightly compressed and 4 to 5 ‘entrance holes’ (1 cm diameter, 3 cm depth) were made in each unit (Figure 6). The nesting units were then allowed to dry for seven days at room temperature. Eight units were placed in Styrofoam boxes, always alternating control and treatment units within the same box in order to minimize nesting bias based on location or orientation. After drying, boxes were placed on a vertical wall in the Botanical Gardens of the University of Halle (Saale) on 30th March 2021, at a location directly adjacent to a nest aggregation of *A. plumipes*.

After nesting activity was completed and no more adult bees could be observed (July 2021), boxes were transferred to a shaded, secluded location for hibernation. In October the same year, the nest units were opened and the number of brood cells was counted per unit. The nest units were divided up into three parts, in order to determine at what depth in the block most brood cells could be found. Half of the brood cell contents were also assessed by poking a small hole in the brood cell, while the other half was left intact for hibernation until spring 2022 (Figure 6). The brood cell contents were sorted into the following categories: Adult female, adult male, dead larva, empty brood cell/pollen provision only, or parasitized. The brood parasites were determined to species level, when possible.

Figure 6. Nest units placed at the field location (left) and *Anthophora plumipes* adults in their brood cells after extraction in the laboratory (right).

Soil samples for residue analysis were taken when soil was first placed at the field location and at two additional time points (Table 3). Samples of white-eyed pupae were taken in August of 2021 by extracting brood cells from one control block and one nesting block, rinsing the pupae in distilled water and storing them at -20C until residue analysis.

Uptake of imidacloprid through soil by pupae was additionally tested in the following manner. A nesting unit containing freshly sealed brood cells was collected from the field site. The brood cells were excavated from it and placed into freshly mixed soil containing 800 µg/kg imidacloprid overwinter. Using a modified electrospray ionization tandem mass spectrometry (MS) protocol, the soil and pupal samples were screened for residues and the active ingredient Imidacloprid as well as the metabolites desnitro-imidacloprid, olefin-imidacloprid, 5-hydroxy-Imidacloprid, and 6-chloronicotinic acid (Limit of detection: 10 µg/kg).

3.2. Results

3.2.1. Residue analysis

Imidacloprid exposure in the soil nesting unit was determined to be maximally 84 µg/kg (Table 4). The maximum concentration in the 800 µg/kg treatment was determined to be 628 µg/kg. No evidence of uptake into pupae could be determined, as all pupal samples were below the limit of imidacloprid detection. None of the samples contained measurable levels of any metabolite of imidacloprid.

Table 4: The amount of imidacloprid residue in soil and pupal samples

Sample	Days post preparation	µg/kg
Soil 100 µg/kg	0	61±24
Soil 100 µg/kg	6	59±26
Soil 100 µg/kg	21	28±13
Soil 100 µg/kg	60	40±18
Soil 800 µg/kg	0	462±166
Soil 800 µg/kg	60	281±109
Pupae 100 µg/kg	-	<10
Pupae 800 µg/kg	-	<10
Pupae control	-	<10

3.2.2. Brood cell production and contents

A total of 1471 brood cells were detected and removed from the nest units, and the contents of 844 cells were determined. When considering these preliminary results, there was no difference in the number of brood cells constructed per nesting unit between the control and the imidacloprid treatment (t-test $p > 0.9$, Figure 7). Most brood cells were constructed at a depth of 5-10 cm, which did not differ between treatments (Figure 8). There was no effect of imidacloprid treatment on either the proportion adult bees, the proportion dead larvae or the rate of parasitism when considering the data per nesting unit (Figure 9) or as an overall rate across nesting units (Table 5).

Three prominent insect parasites were identified from the brood cells: the cleptoparasitic bee *Melecta albifrons,* the parasitic wasp *Monodontomerus obscurus,* and the cleptoparasitic meloid beetle *Sitaris muralis*.

The female to male sex ratio of *A. plumipes* that emerged from brood cells was 53% in the imidacloprid treatment and 47% in the control treatment, a minimal difference.

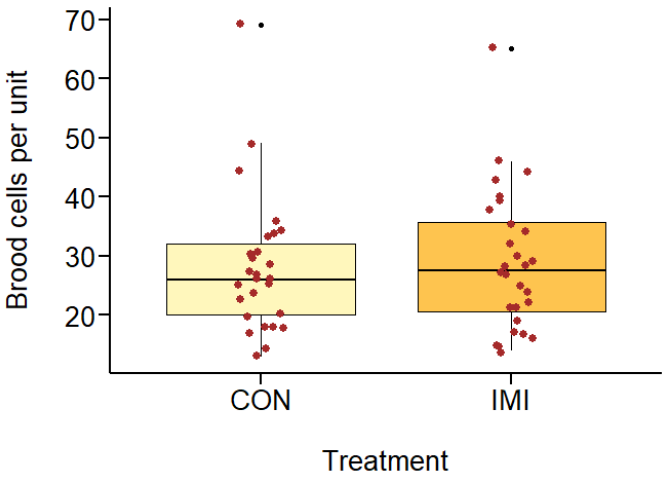

Figure 7. Boxplot showing number of brood cells constructed per nest unit in the control (left) and imidacloprid (right) treatments (t-test $p > 0.9$, n.s.).

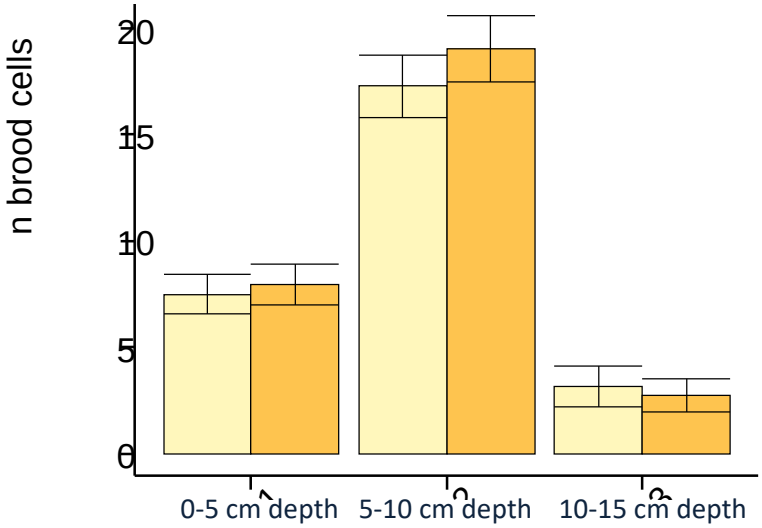

Figure 8.Frequency of brood cells at different depths of the nest block (mean, SD) per nesting unit and treatment. Most cells were at a depth of 5-10 cm.

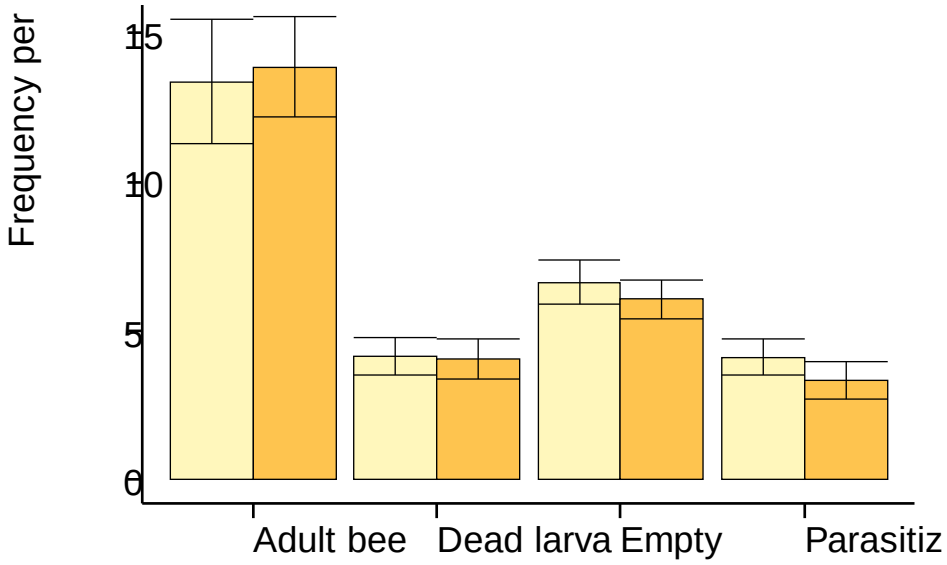

Figure 9. Frequency of brood cells per nest unit (mean, SD) containing adult (*A. plumipes*) bees, dead larvae, empty (e.g. pollen or only mould) or insect parasites (*Melecta albifrons, Monodontomerus obscurus, Sitaris muralis*). Light yellow represents the control treatment and orange represents the imidacloprid treatment. Frequencies did not differ between treatments (n.s.).

Table 5. Overall frequencies of parasitized, empty, dead larvae and eclosed adults from a subset of brood cells excavated from nesting units for the Imidacloprid and control treatments. Differences between treatments were minimal.

Treatment	Parasitized	Empty	Dead larvae	Adults
Imidacloprid	10.00%	22.50%	15.25%	37.75%
Control	14.86%	23.87%	13.06%	36.94%

4. Discussion

In our acute exposure assay on various wild bee species, we demonstrate how some novel species can be successfully studied in the laboratory. Of all the tested species, 9 survived, and 5 were able to feed on a given solution from a cuvette. Results from pesticide exposure experiments confirmed that wild bees can exhibit a higher sensitivity towards acute sulfoxaflor exposure than the model species currently used. Most of the orally treated species were not significantly more sensitive than *B. terrestris* to acute sulfoxaflor exposure, but these results may have been confounded by a lack of individuals or high mortality in the control treatment. *Osmia cornuta* exhibited a higher oral sensitivity compared to the model species *B. terrestris*. When exposed topically, all the species were impacted, with *A. plumipes* and *B. pascuorum* being more sensitive than *B. terrestris.*

The meta-analysis of Arena & Sgolastra (2014), incorporating data on 19 bee species, showed that the sensitivity of most bee species was lower than the sensitivity of the model species, *Apis mellifera*. Here, with a different model species (*B. terrestris*), we found both more sensitive species: *O. cornuta*, *B. pascuorum* (oral) and *A. plumipes* (topical); and less sensitive species: *B. hypnorum* (oral). Bees differ greatly in their ecology, and even if it could have been expected that closely related species would be similarly sensitive and, in comparison to *B. terrestris*, this was not the case in our study. The high variability in terms of pesticide sensitivity found amongst bee species is complicated to explain. Indeed, several intra- and inter-specific differences in physiological and morphological traits can influence the sensitivity of a bee to pesticide. First, at the intra-specific level, van der Steen (1994) showed a correlation between the body weight and the sensitivity of an individual towards pesticides. Across species, it has also been shown that the interspecific sensitivity is inversely proportional to the mean body weight of a species (Devillers et al., 2003). Indeed, the sensitivity of a bee has been shown to increase with the surface-to-volume ratio (Johansen, 1972). Moreover, regarding topical exposures, sensitivity may vary with cuticle composition. The first barrier protecting the insect body from external perturbation is the chitinous cuticle that covers its whole body (Reece & Campbell, 2011). The effect of an insecticide depends on the rate at which it enters the insect's body. Therefore, the surface-to-volume ratio, cuticular thickness as well as the cellular and molecular components of the cuticle can all play a role in determining the sensitivity of an insect towards pesticides (Lewis, 1980).

Another trait that can influence the sensitivity of an individual or of a species to pesticide is directly related to its mechanisms of detoxification. Some species have the physiological ability to detoxify toxins other than pesticides, such as alkaloids contained in the floral resources of some plant species. These abilities could help in the detoxification of pesticides, such as neonicotinoids (Cresswell et al., 2012). *Heriades truncorum*, and *O. leaiana* are oligolectic on the Asteraceae family, members of which have been shown to excrete toxic secondary compounds through their pollen and nectar (Vanderplanck et al., 2020). We did not find a significant difference in their sensitivity to pesticide compared *to B.* terrestris, possibly due to the lack of individuals available for us to test. It would therefore be interesting to continue experimenting on these species.

Finally, once a pesticide has entered an insect's body, its actual toxic effect on the individual depends on the organism's capacity to metabolize and subsequently excrete it. Such detoxification processes are controlled by enzyme activity (Uhl et al., 2016) which could differ among species because of, for example, differences in haemolymph pH. For example, while the pH of honey bee haemolymph was measured at 6.0, that of *Megachile rotundata* was measured at 6.8. It has been

hypothesized therefore that xenobiotics can be detoxified at different rates depending on the species (Ahmad and Johansen, 1973). Analysing the haemolymph of species with a different sensitivity to *B. terrestris* could provide more insight into the underlying mechanisms of detoxification efficiency and its variation in relation to sensitivity.

While most of the treated bee species were shown as being equally sensitive compared to *B. terrestris*, these results need to be interpreted cautiously. Indeed, for some species such as *H. scabiosae*, *H. truncorum*, *O. leaiana* or *O. cearulescens*, sample sizes were much lower than recommended by the OECD guidelines (OECD, 2017b, 2017a). Increasing the sample size in those cases could probably reveal some subtle effects of presticide sensitivity.

As shown by the laboratory survival tests, wild bees are difficult to maintain in captivity. Therefore, a high number of individuals needs to be taken from the wild in compensation, which is not always possible for small bee communities, and for bees that do not nest in aggregations. Moreover, the high percentage of non-feeders found during the oral exposure experiment, and the high rates of mortality found in the control groups, further diminished the sample size and showed that laboratory conditions must be improved to allow toxicological tests on a larger spectrum of wild bee species. For further experiments, and to increase the sample size, some methods that have been developed to adapt the feeding of bees to their natural feeding behaviour can be used. For example, a flower from which the reproductive column was removed and replaced by the test solution has already been used with *Megachile rotundata* and *Osmia lignaria* (Ladurner et al., 2003). While difficult to install, and while difficult to adapt to specialist bees, this technique has been improved by the “petal method” that seems to increase feeding success, and to be easily set up. This method consists of using a single petal as a visual clue to indicate the treatment solution and facilitate the feeding of solitary species (Hinarejos et al., 2014). To increase survival under laboratory conditions, the stress caused by having been caught in the wild could be avoided by rearing the species under laboratory conditions, from the first larval stages until emergence (Eeraerts et al., 2020). However, these methods are only known for a few species (Peterson & Artz, 2014). Finally, while our results show that many species do not survive well in the laboratory, they also show that these methods can work on some species with interesting ecological traits that are not shared with the main model species (i.e. *Anthophora plumipes* as a soil-nesting solitary bee, or *Andrena vaga* as an oligolectic bee species).

The results of our novel soil exposure assay with *A. plumipes* are promising in terms of nesting success in the provided substrates, the ease with which brood and brood parameters can be assessed, as well as the large sample sizes that can be achieved under the particular field conditions explored here. The limitation in this particular series of experiments is the lack of multiple populations and locations explored, as well as the obligate non-independence of the nest units, as one female could potentially nest in several units across her lifespan. There was no attempt to determine the per-female reproductive output, and due to the cluster-like structure of the *Anthophora* nests, per-nest brood assays were not possible. The use of soil units of a standardised size was an attempt to get around this issue.

We could not show any treatment effect on any of the measured parameters; however, the high rate of mortality of control brood (13%) might mask minor treatment effects. Thus, reducing control mortality by measures such as heat-sterilizing the soil should be further explored. In future assays,

the possibility of using semi-field setups with, for example, a known number of females per cage and nest unit, should be explored. In a semi-field setup, the external factor of brood parasitism, which affects around 10-15% of brood cells, would be excluded. Using the method at hand, brood and brood provisions can be extracted at various life stages and checked for pesticide residues, as demonstrated here.

It is noteworthy that no evidence of uptake of imidacloprid into the brood was evident. In further assays, additional samples of brood from soil of increasing concentrations will be analyzed. One can hypothesize that the waxy protective layer coating the inside of the brood cell, excreted from the Dufour's gland of Anthophorine bees (Norden et al. 1980), is also an effective barrier against chemical contaminants in the soil.

Trials with *Colletes hederae* have been carried out, but as we were not able to control the development from the larval to adult stage under laboratory conditions, we tried instead to understand the movement of pesticides from the soil to the brood cell in order to find out whether the larva and its pollen provision can be protected from agrochemicals. Unfortunately, as the analyses have not yet been completed, the results have not been included in this report. In addition, the bad weather during the flight season of *C. hederae* did not allow us to collect many brood cells. It may therefore be difficult to draw definitive conclusions from these analyses.

Overall, we demonstrate that experimental exposure of the ground-nesting species *A. plumipes* in a field setting is possible under the right conditions (the proximity of natural aggregations), that brood production and brood survival can be readily quantified, and that a field-realistic exposure of the insecticide Imidacloprid does not affect the parameters measured in our preliminary dataset.

5. Acknowledgements

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