PESTICIDE IMPACT ON WILD BEE MORTALITY AND FEEDING BEHAVIOUR

Master's thesis presented by

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ABSTRACT

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The agricultural intensification plays an important role in the current wild bee decline. The development of pest resistances has led the industry to develop an increasing diversity of molecules. Since the last century, the pesticide risk assessments mainly focus on the honeybee mortality, *Apis mellifera*. There is a need for gathering more data about the lethal and sub-lethal effects of pesticides on wild bees, as their morphology, physiology and ecology greatly differ from the honeybee.

In this study, wild bee species have first been exposed to oral and topical acute doses of the insecticidal molecule, sulfoxaflor. We measured and compared their sensitivity. *O. cornuta* and *B. pascuorum* were more sensitive than *B. terrestris*, the second most used model species in ecotoxicological studies. Besides the ecological characteristics, there might be morphological and physiological specific traits influencing their sensitivity.

In the second part of this work, workers of *B. terrestris* were chronically fed with syrup treated with two pesticides, sulfoxaflor and the fungicidal molecule, azoxystrobin, under its commercial formula, Amistar®, as well as a mixture between these two pesticides. Sulfoxaflor and Amistar® alone impacted the feeding behaviour at field-realistic doses. However, their mixture showed some additive effects. These results revealed that those molecules can impact the feeding behaviour of *B. terrestris*, probably by impairing their digestion and cognition.

Our results confirmed that, as suggested by recent comparative studies, wild bee sensitivity to pesticide varies, and some species are more sensitive than the model species. Moreover, we showed that field-realistic doses of sulfoxaflor and Amistar® can impair the feeding behaviour what could explain the decrease in bee reproductive performances observed in previous studies.

Keywords: Comparative ecotoxicology – Pesticides – Wild bees – Feeding behaviour – *Bombus terrestris* – Pollinators

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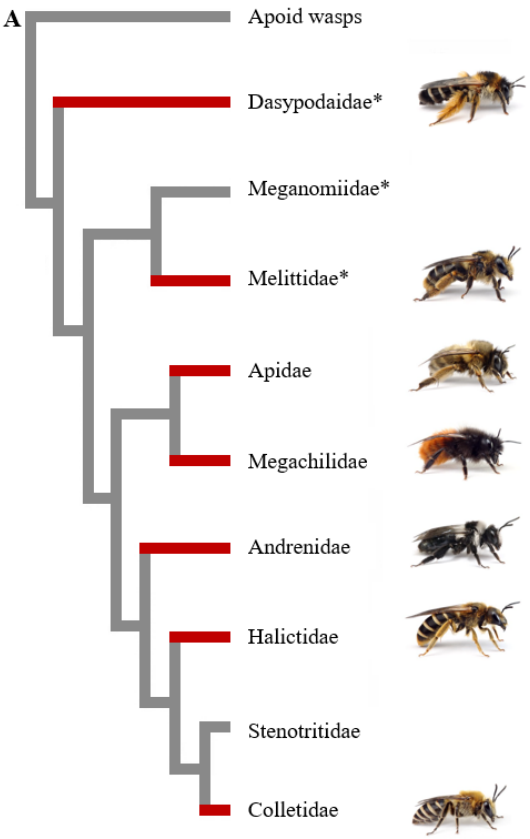
1. Introduction

1.1. An overview of the bees

1.1.1. Origin and evolution

Around 130 mya, the plants evolved towards a new group: the Angiosperms (Le Guyader & Lecointre, 2017). This group relied on pollen transport, commonly called pollination, for their reproduction (Reece & Campbell, 2011). This process can be performed either abiotically (e.g. wind), or biotically (e.g. through animals). The Anthophila group found within the Apoidea superfamily of the Hymenoptera order, specialized itself into biotic pollen transport. They evolved and differed from primary carnivorous wasps, to become specialized pollen and nectar feeders, better known as bees, that harbour branched hairs all over their body (Michez *et al.*, 2011, 2019). Nowadays, around 20,000 species of bees are described, of which 10% are found in Europe (Fig. 1; Nieto *et al.*, 2014).

A



B

Family	Number of species	Number of endemic species (% endemic)
Andrenidae	465	96 (20.6%)
Apidae	561	107 (19.1%)
Colletidae	146	40 (27.4%)
Halictidae	314	70 (22.3%)
Megachilidae	442	74 (16.7%)
Mellitidae	37	13 (35.1%)
Total	1965	400 (20.4%)

Fig. 1 - Families of bees. A) Phylogeny of the bees according to Danforth *et al.*, 2006. The families found in Europe are marked in red, and illustrated by an example species. *current taxonomy classifies these families under the family Melitidae *sensu lato*. (adapted from Michez *et al.*, 2010; picture credits: Mandy & Michael Fritzsche). B) Table summarizing the diversity and endemism in bee families found in Europe (adapted from Nieto *et al.*, 2014).

Although some other groups from the whole Hymenoptera order also specialized themselves in pollen transport, 15% of the hymenopteran pollinator species are found in the Anthophila group (Michez *et al.*, 2019).

1.1.2. The ecology of bees: a story of diversity

The buff-tailed bumblebee, *Bombus terrestris*, and the domesticated honeybee, *Apis mellifera*, are the most popular bee species. However, because of a big knowledge gap about their diversity and ecology, the other wild bee species are far less known (Wilson *et al.*, 2017). Yet, there is a high variability in the Anthophila group, and it can be described into three main elements: (i) the social behaviour, (ii) the nesting strategy, and finally, (iii) the lectism, i.e. the range of their floral choice.

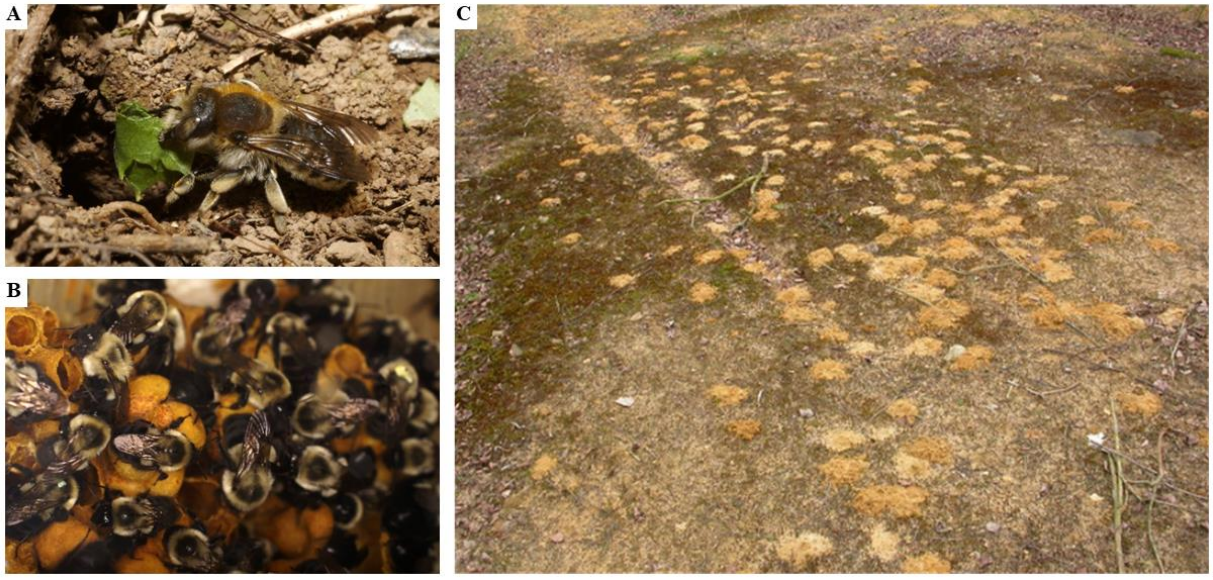


Fig. 2 – Examples of wild bee sociality. A) A solitary bee, *Trachusa byssina* collecting nesting material (picture credits: Nicolas Vereecken), B) a primitively eusocial colony of *Bombus impatiens* (picture credits: Rob Cruickshank), and C) a nest aggregation of the solitary bee, *Andrena vaga* (picture credits: Nicolas Vereecken)

The first characteristic, the social behaviour, varies from solitary individuals to truly eusocial colonies (Michener, 1969). While only 6% of the species are social, the solitary species account for a major part of this group. The solitary females fill individual cells with nectar and pollen to entirely support the needs of their larva, until emergence. They nest on their own (Fig. 2-A), either isolated from individuals of the same species, or in aggregation. If the conditions are suitable, it can lead to areas with hundreds of nests (Fig. 2-C). When females live together, in the same nest, but provide resources for their own progeny, they are described as communal species. Lastly, several different systems are found under the social organisation type (Fig. 2-B). The quasi-social colonies are composed of females living

together, but provisioning for more than one progeny. In the semi-social species, females of two different generations, or with truly defined social groups (castes), can be found in the same nest. Finally, the eusocial organization with two types of complexity, namely primitive and highly social. In both types, the colony comprises individuals from several generations, and is divided into true castes (i.e. egg-layers and workerlikes), with some of them progressively feeding the larva. First, the primitive organization presents either no morphological differentiation between social groups, or a shallow differentiation. However, in the highly eusocial colonies, the workers differ morphologically and physiologically from the queen. The colony also presents some complex communication systems to indicate food resources, or nesting sites (Michener, 1969).

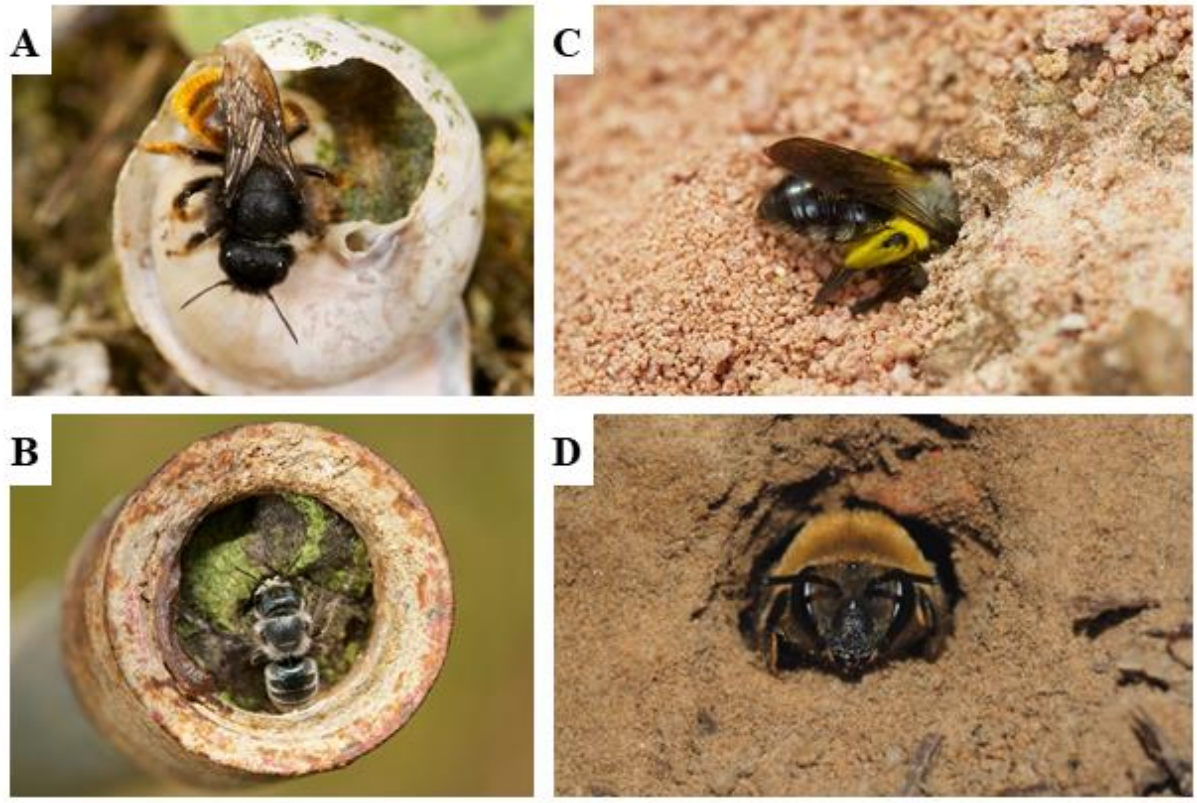


Fig. 3 – Examples of nesting strategies among wild bees. Some cavity nesters: A) *Osmia bicolor* nesting inside an empty snail shell, and B) *Osmia caerulescens* closing its nest, made inside a metallic pipe. Some ground nesters: C) *Andrena vaga* digging its nest, and D) *Colletes hederae* at the entrance of its nest (picture credits: Nicolas Vereecken).

The nesting strategy is also a characteristic that varies among the wild bee species, and two main techniques can be distinguished (Michener, 2007). The first group is the ground nesters that characterize more than 55% of the bee species (Cane & Neff, 2011). Some bees of this group, called the fossorial bees, dig their own nests into the soil (Fig. 3-C and D). The choice of substrate to dig can be very species-specific, with some species nesting into loess

soil, and other into sandy soil. Some other species will use abandoned rodent burrows, or other cavities already dug. The second group comprises the cavity nesters that can use for example, empty snail shells, birdhouses, or holes found into vertical walls (Fig. 3-A and B; Michener, 2007). These cavities can be filled with plant parts, and can be closed by resin, or mud depending on the species (Benton & Fremlin, 2018; Horne, 1995; Maciel De Almeida Correia, 1977; Michener, 2007).

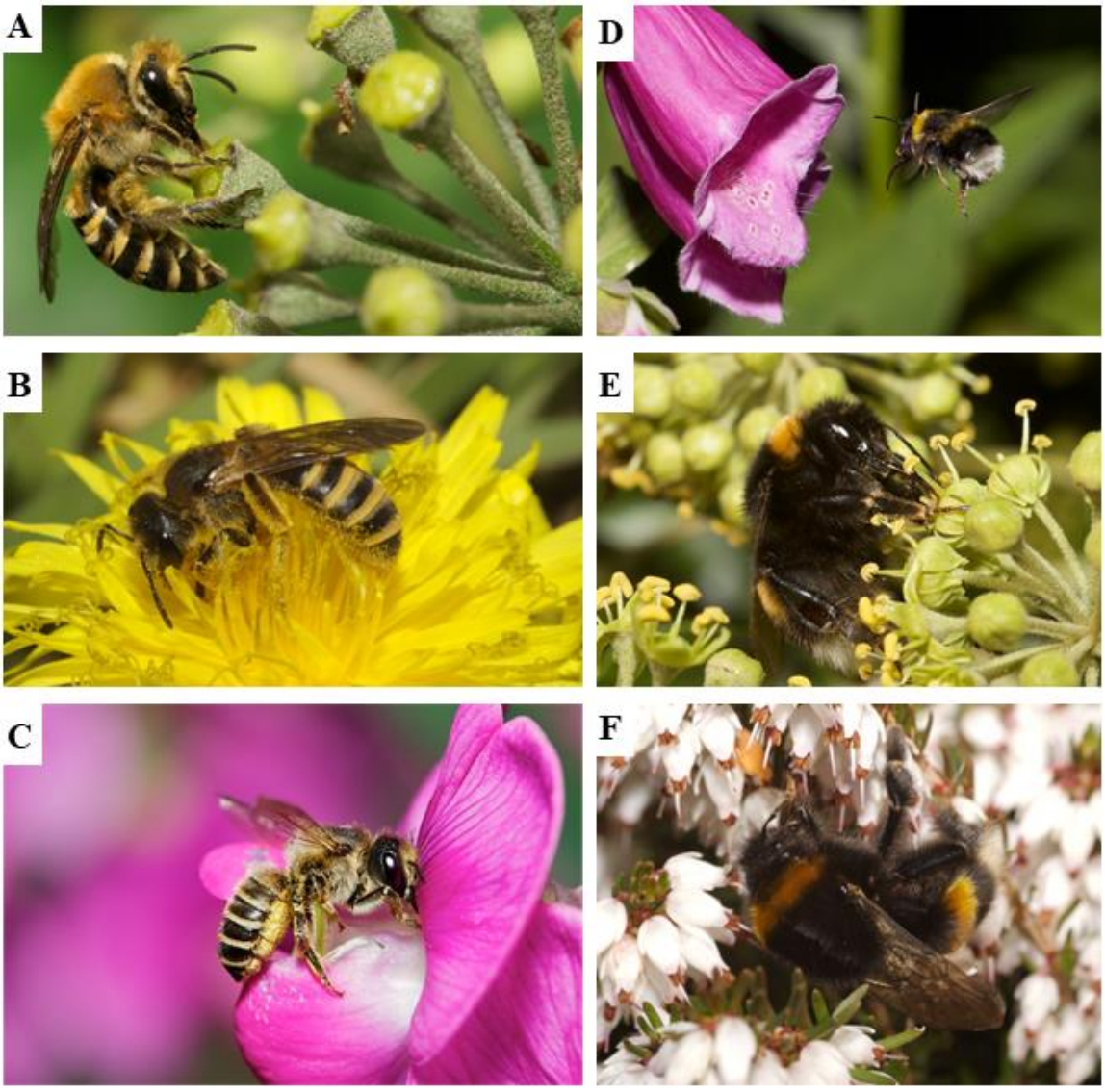


Fig. 4 – Oligolectic and polylectic bees. A) *Colletes hederae*, an oligolectic bee on *Hedera helix* (Araliaceae), B) *Halictus scabiosae*, an oligolectic species on Asteraceae, here foraging on *Taraxacum* sp., C) *Megachile ericetorum*, an oligolectic species on Fabaceae, here foraging on *Lathyrus odorata*, and *Bombus terrestris*, a polylectic species found foraging on several different plant species, D) *Digitalis purpurea* (Scrophulariaceae), E) *Hedera helix*, and F) *Erica carnea* (Ericaceae). (Picture credits: Nicolas Vereecken)

Finally, both adults and larvaeof bees rely on floral resources such as pollen and nectar, sources of proteins, lipids and energy (Michener, 2007). Some bees, called polylectic bees, can use resources from a broad range of plant species (Fig. 4-D, E and F). Other bee

species, the oligolectic ones, specialized themselves for some particular families, genus, or even species of plants (Fig. 4-A, B, and C; Michener, 2007; Peeters *et al.*, 2012; Westerkamp, 1997). This specialisation can be behavioural with specific skills developed to handle complex floral labyrinth protecting the pollen (Westerkamp, 1997), or physiological to deal with toxic secondary compounds occurring in nectar and pollen of some plant species, such as species from the Asteraceae Family (Müller & Kuhlmann, 2008; Praz *et al.*, 2008).

1.2. The bee decline

1.2.1. Importance of wild bees

In temperate and tropical zones, animal-pollinated plants represent respectively 78 and 94% of the plant species (Ollerton *et al.*, 2011), including a majority of the domesticated plants. Hence, the economic value of animal pollination is estimated at 150 billion euro per year (Gallai *et al.*, 2009; Potts *et al.*, 2016). While the majority of animal pollinators are insects, a big part of the crop pollination is performed by bees (Geslin *et al.*, 2016; Klein *et al.*, 2007). They are necessary for the pollination of more than 30% of the main agricultural crops. Moreover, some of these crops provide needed macro- and micro-nutrients, e.g. spinach producing vitamin A (Chaplin-Kramer *et al.*, 2014; Klein *et al.*, 2007). To enhance pollination, honeybee hives are frequently put near crops. Yet, many studies highlighted that wild bees also enhance the fruit set, quality and therefore, the economic value of cultures, such as strawberries, and apples (Garibaldi *et al.*, 2013; Garratt *et al.*, 2014; Klatt *et al.*, 2014; Klein *et al.*, 2007). Besides, wild bees are needed for several plants that need specific pollination, e.g. buzz-pollination for tomatoes, and potatoes. This consists into a particular handling of the anther by the bee, what honeybees are not able to perform (Goulson, 2003).

1.2.2.Threats on bees

Bees are affected by global change (Fig. 5; Nieto *et al.*, 2014)), such as several other insect pollinator groups, e.g. butterflies (Parmesan *et al.*, 1999) and hoverflies (Miller-Struttmann *et al.*, 2015). Since the last century, modifications in wild bee distribution, ecology and diversity have been recorded from all around the world (Biesmeijer, 2006; Cameron *et al.*, 2011; Duchenne *et al.*, 2020; Rasmont *et al.*, 2005; Zattara & Aizen, 2021). In Europe, almost ten percent of the bee species are actually threatened. Moreover, due to the gap of data about more than fifty percent of the European bee species, this number could be underestimated (Fig. 5-B; Nieto *et al.*, 2014). As a matter of fact, national trends and

conservation status showed higher values in United Kingdom and Belgium (Drossart *et al.*, 2019; Powney *et al.*, 2019). For example, 61% of the Belgian wild bee species are shown to be in decline since the past 70 years (Duchenne *et al.*, 2020). Nowadays, the causes of bee decline seem to be mainly anthropogenic (Fig. 5-A). These are the climatic change, habitat losses, and agricultural intensification (Goulson *et al.*, 2015; Miličić *et al.*, 2018).

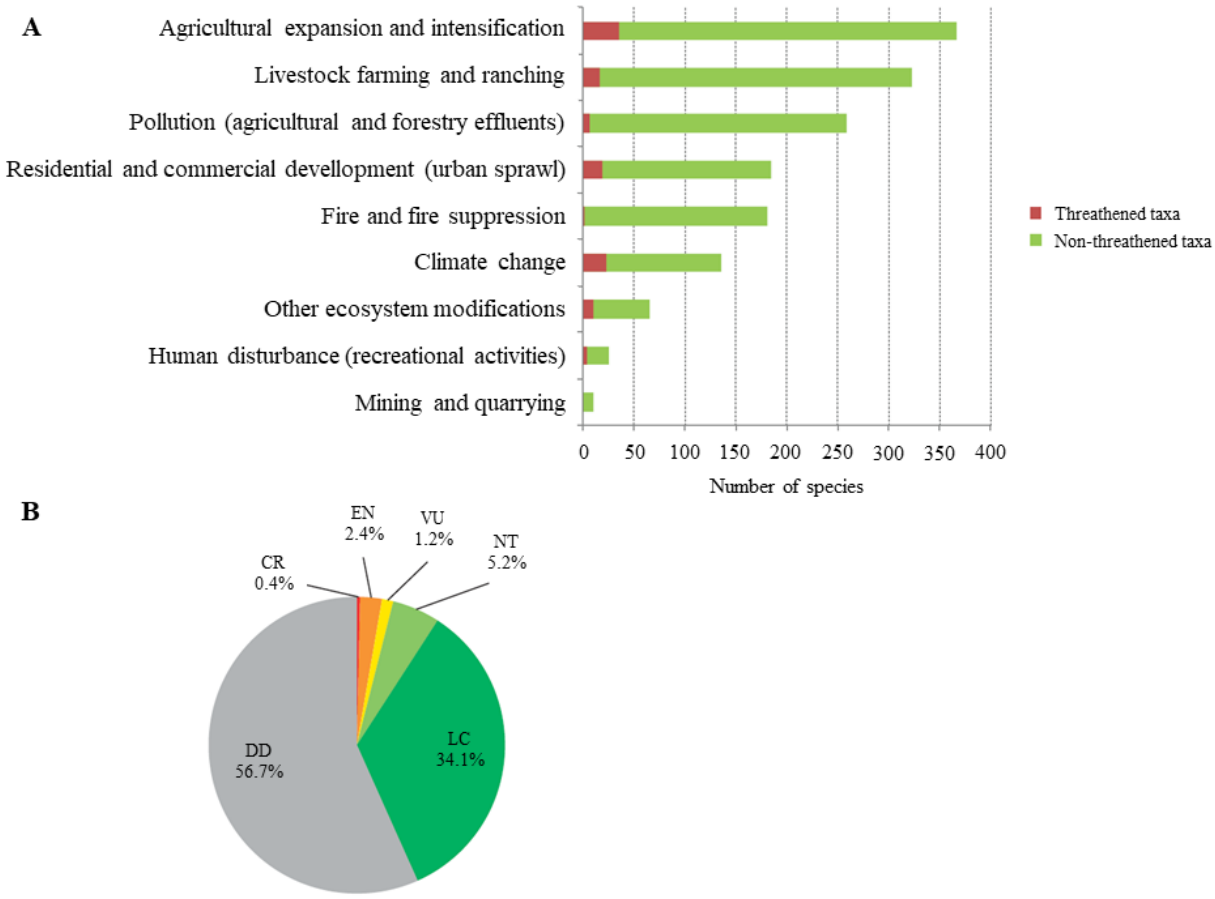


Fig. 5 – Threats on bees. A) Major factors threatening bees in Europe, and B) IUCN Red List status of bees in Europe. DD = Data deficient, CR = Critically endangered, EN = Endangered, VU = Vulnerable, NT = Near threatened, LC = Least concern (adapted from Nieto *et al.*, 2014)

One of these human-mediated threats against wild bees is climate change (Fig. 5-A). For example, 36% of the European bumblebee species are adapted to forage at temperate and cold climates. It was shown that these would meet the limit of their suitable area by 2100. Indeed, helped by the increased of the global temperatures, they are currently expanding their distribution range northward (Kerr *et al.*, 2015; Rasmont *et al.*, 2015). In addition to the distribution changes of wild species, potential mismatches between bees and their floral resources have been brought out (Fig. 6; Gérard *et al.*, 2020). For example, global warming is leading to a decrease in insect body size, and, particularly, pollinator tongue length. However, the flower corolla seems to become deeper with the increase of temperature. Therefore, a risk

of morphological mismatch between the bee and its host plant could disrupt some plant-pollinator interactions (Anderson *et al.*, 2014; Gerard *et al.*, 2018).

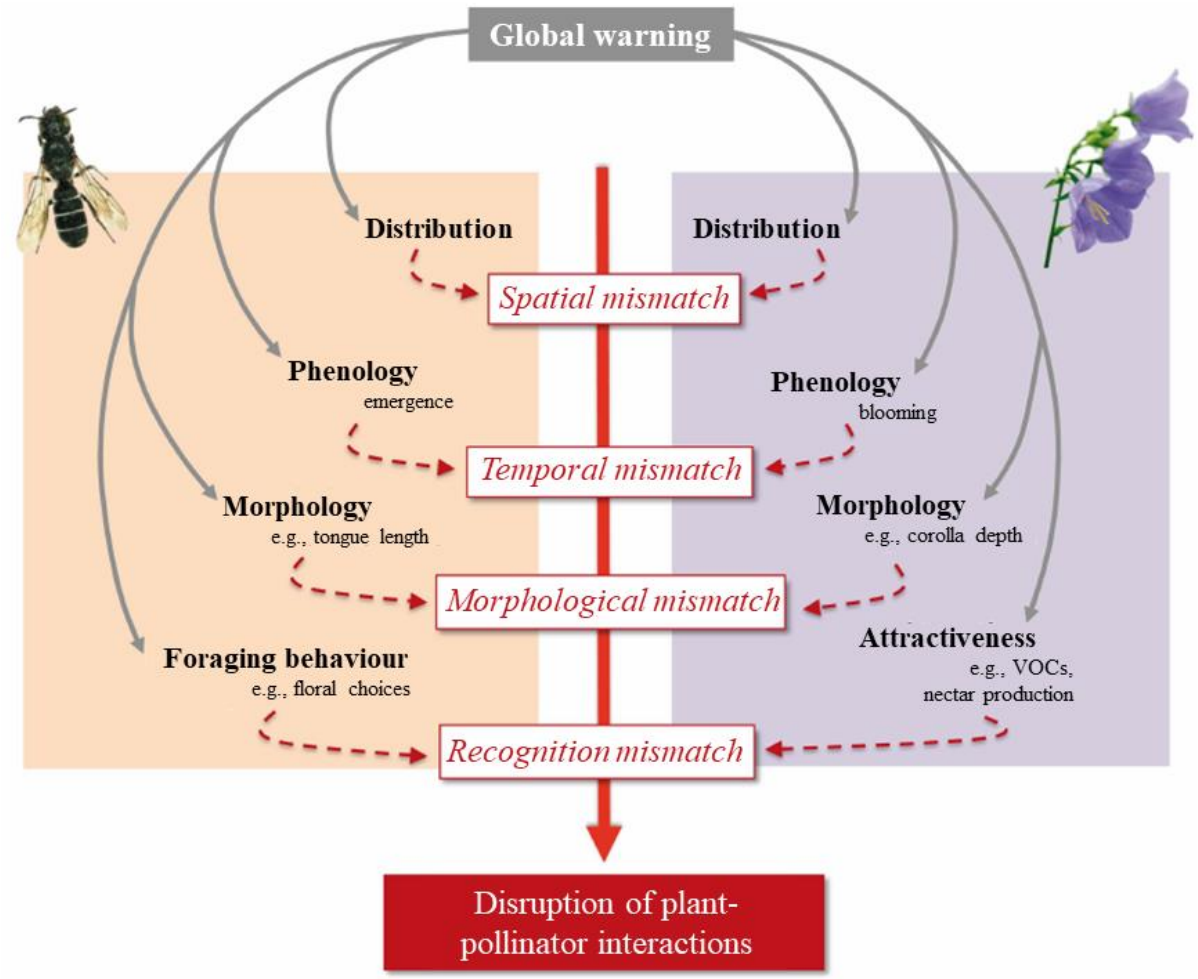


Fig. 6 – Potential mismatches plant-pollinators that could appear due to climate warning (adapted from Gérard *et al.*, 2020).

In addition to climate warming, bees are also affected by the increasing urbanization and the intensification of agricultural land use (Fig. 5-A; Aguilar *et al.*, 2006; Fortel *et al.*, 2014; Vray *et al.*, 2019). These are leading to habitat losses and fragmentation, and are negatively affecting wild plant communities along with pollinator population sizes (Hunter, 2002). Besides its consequences on habitat changes, agricultural intensification is impacting wild bees in many other ways. The use of agrochemical products, including pesticides, and synthetic fertilizers, seem to play a role in the bee decline. Indeed, these are affecting the targeted crops, as well as the surrounding areas (Carvalheiro *et al.*, 2020; Goulson *et al.*, 2015). Fertilizers use leads to water and soil eutrophication, affecting the plant composition and wild bee communities (Carvalheiro *et al.*, 2020). Meanwhile, lethal and sublethal effects

of pesticides seem to directly affect wild bee communities (Goulson *et al.*, 2015; Heard *et al.*, 2017).

Yet, all bee species do not respond to anthropogenic threats in the same way (Bartomeus, Park, *et al.*, 2013; Rader *et al.*, 2013; Tuell & Isaacs, 2010). The high variability of their ecological traits can help understanding this response variation (Cariveau & Winfree, 2015). Indeed, species-specific population trends over time, and differential responses to the same human-mediated change have been shown (Arena & Sgolastra, 2014; Bartomeus, Ascher, *et al.*, 2013; Cariveau *et al.*, 2013). While several species are under high climatic risk, other ones are taking advantage of the current changes to increase their occupancy, their flight period, and their distribution (Duchenne *et al.*, 2020; Ghisbain *et al.*, 2021). Polylectic bees could for example be less affected by this change than oligolectic bees. Hence, their ability to forage on several different species could buffer the risk linked to the appearance of mismatches (Bartomeus *et al.*, 2011). Besides climate change, oligolectic bees are strongly affected by land-use (Cane *et al.*, 2006; Williams *et al.*, 2010). However, this effect appears to be species-specific, as it will depend on the host plant. While the increase of specialist bees is linked to the planting frequency of their crop (Bischoff *et al.*, 2009), some others specialized to forage on Fabaceae for example are in decline. As a matter of fact, this trend follows the decrease of the plant host use as cover crops (Scheper *et al.*, 2014).

1.3. The pesticide use

1.3.1. Generalities

Worldwide, the food demand is growing, and the agricultural sector tries to enhance its productivity to keep up with this demand (Deguine *et al.*, 2014). This agricultural intensification led to the emergence of monocultural crops, in order to maximize food production in terms of both quality and quantity. Since the last century, agrochemicals of which synthetic fertilizers and pesticides are applied on fields to maximize the crop yields (Boardman, 1986). These respectively supply the crops with directly available nutrients, and eradicate weeds, pathogenic organisms, and herbivorous predators (Deguine *et al.*, 2014). Indeed, the aggregation of thousands of plant individuals from the same species in a limited area, as well as the use of limited genetic diversity stand for the optimal conditions of pest outbreaks. Pesticides are therefore needed to control their populations (Botías *et al.*, 2015; Godfray *et al.*, 2014; Hillocks, 2012). Nowadays, pesticides comprise a broad molecule variety targeting different organisms, of which (i) herbicides which target unwanted plants

(e.g. glyphosate), (ii) fungicides which parasitic fungi, (e.g. strobilurins), and, finally, (iii) insecticides which target insect pests,(e.g. neo-nicotinoids and sulfoximins).

During and after the Second World War, while organic compounds were more commonly used until then, synthetic pesticide production peaked. The dichlorodiphenyltrichloroethane, commonly known as DDT, was the first widespread synthetic insecticide. It was successfully used to stop several epidemies, during the war, as the typhus epidemy in Naples, and later to eradicate malaria (Boardman, 1986). After that, a great variety of chemical compounds were produced, or were discovered as having pesticide properties (Deguine *et al.*, 2014). However, while the diversity of new developed chemicals was rising up, two main issues were encountered: (i) concerns about health and environmental consequences of the massive pesticide use started to be brought up, and (ii) the development of pest resistances constrained the agrochemical firms to developed new compounds. Therefore, in 1991, the Pesticide Authorization Directive 91/414/EEC was initiated. The objective was to review the plant protection products available on the market, and establish a list of authorized active molecules in Europe. In 2012, 50% of the active ingredients used in Europe before 1991 were consequently withdrawn from sale (Boardman, 1986; Botías *et al.*, 2015; Deguine *et al.*, 2014; Godfray *et al.*, 2014).

1.3.2. Bee exposure to pesticide

Concerns about the pesticide effects on human health were early brought up (Hayes, 1956). Rachel Carson (1962), in her book “Silent Spring”, originally started to raise the public awareness about the consequences of agrochemical use on the health and environment. However, their role in the decline of wild bees remains mitigated. By their reliance on floral resources (Michener, 2007), and their high presence on flowering crops and surrounding areas, bees are frequently exposed to pesticides (Godfray *et al.*, 2014). Therefore, several different routes of pesticide exposure can be detected for the wild bees (Fig. 7).

Firstly, some pesticides have a systemic mode of action. This means that they are transported through the plant, and can be found in pollen and nectar. Adult bees can thus ingest those molecules while they are foraging, and bring them back to the nest (Krupke *et al.*, 2012). The exposure can be different between solitary and social species. Indeed, reproductive females of social species are not foraging, or at least not during their whole life. They are thus less likely to be exposed. In contrast, reproductive individuals of solitary species that forage alone to provide resources to their own progeny, are way more at risks (Sgolastra *et al.*,

2019). Moreover, the larval feeding differs greatly between social and solitary species (Michener, 1969). Solitary species make a stock with unprocessed pollen to ensure the whole development of their progeny. Larvaeof solitary species can therefore be exposed to high quantities of accumulated pesticides. However, larvaeof social species benefit from a colonial detoxification as they are fed by trophallaxis with the glandular secretions of workers (Sgolastra *et al.*, 2019).

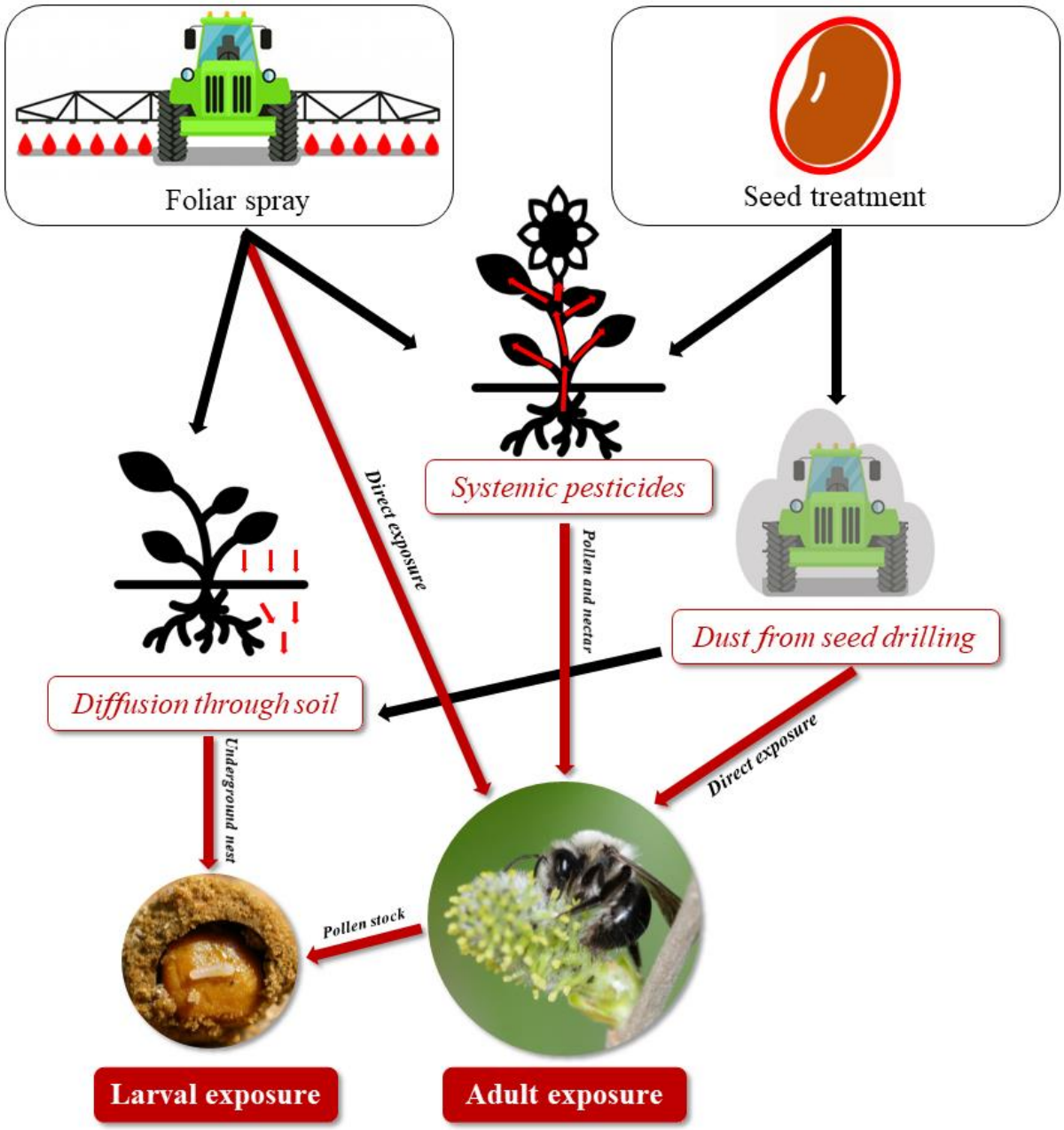


Fig. 7 – Summary of the main routes of pesticide exposure for wild bees. The grey-backed mining bee, *Andrena vaga*, was chosen to illustrate the multiple routes of exposure, because it can be exposed during all its lifecycle through each of the routes presented (picture credits: Sophie Gierens for the adult *Andrena vaga* foraging on *Salix* sp.; Nicolas Vereecken for the *Andrena vaga* egg on its pollen ball; IYIKON on thenounproject.com for the plant and root icons).

While being sprayed, the pesticide can either directly be in contact with the foraging bees, or diffuse in the soil and surrounding waters. Moreover, the 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 (Brown *et al.*, 2016; Goulson *et al.*, 2015; Krupke *et al.*, 2012; Sgolastra *et al.*, 2019). The ground-nesting bees 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, applied as seed treatments, e.g. neonicotinoid estimated half-lives of the order 15-300 days (Willis Chan *et al.*, 2019). Therefore, they are sometimes found in the soil in greater quantities than in nectar and pollen. Moreover, it is important to note that, in Belgium, a majority of the bee species are ground nesters, and can therefore be affected through this route of exposure (Drossart *et al.*, 2019).

Furthermore, exposure to mixture of pesticide that could present 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 activity, to delay the appearance of resistant strains by minimizing the selection strength, or use the synergistic effects (Bliss, 1939; Koziol & Witkowski, 1982; Macht, 1929). Therefore, the risk of exposure to mixture for a bee is the same as the risk of exposure to pesticides used alone, and the adverse effect can be enhanced by synergisms (Siviter *et al.*, 2021).

1.3.3. Pesticide impacts on bees

1.3.3.1. Mechanisms

Nowadays, a wide variety of insecticidal and fungicidal molecules are used in the agricultural sector. They present different mode of action as for example by being an enzyme inhibitor, or the agonists of several chemical signal systems.

For example, neonicotinoids is a family of insecticidal molecules that are structurally close to nicotine, and act as nicotinic acetylcholine receptor agonists (Fig. 8-B). They cause damages in the central neuronal system of its target, i.e. piercing and sucking insects (Gross, 2013; Tomizawa & Casida, 2003). In the early time of their use, pesticides were designed to kill pests, without making discrimination between targeted and non-targeted organisms.

Therefore, in the 90s, neonicotinoids were produced with the primary idea of achieving a greater specificity (Bass & Field, 2018).

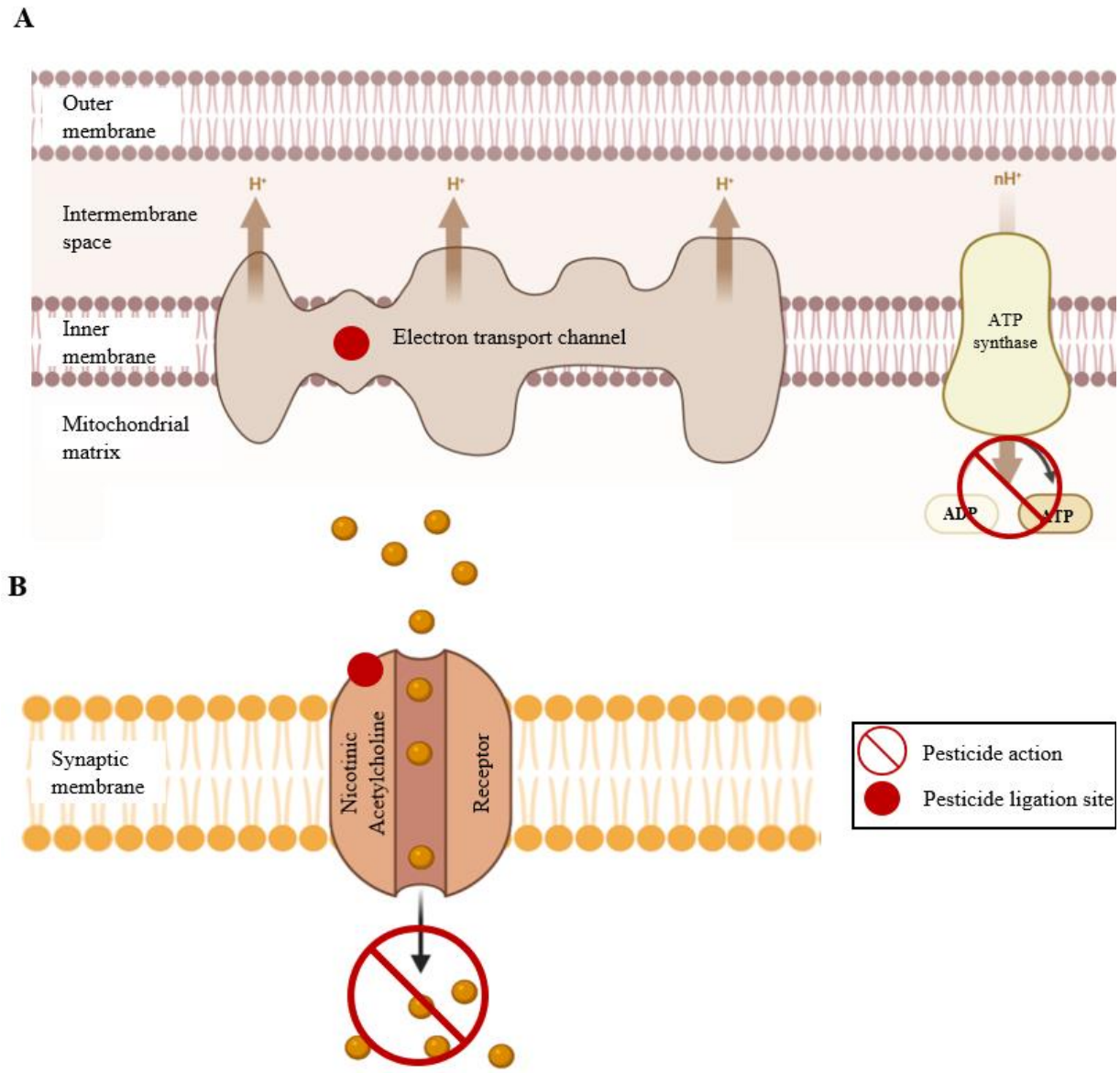


Fig. 8 – Modes of action of strobilurin fungicides, and neonicotinoid-like insecticides. A) The azoxystrobin molecule binds at the Q_o site of cytochrome b from cyt bc_1 complex located in the inner mitochondrial membrane. It blocks the electron transfer between cytochrome b and cytochrome c_1, and disrupt the energy cycle to halt the ATP production (based on Bartlewicz *et al.*, 2016). B) The neonicotinoids, and sulfoxaflor act as agonists of the nicotinic acetyl-choline receptors (based on Tomizawa & Casida, 2003) (both illustrations are created with BioRender.com)

While manufacturers were strongly claiming that used field concentrations were not toxic for bees and other pollinators, the scientific community investigated the neonicotinoid use, as main cause of several beehive deserting events, known as "Colony Collapse Disorder" that happened during the winter 2006-2007 in the United States. In 2013, European Food Safety Authority (EFSA) banned the use of neonicotinoid on flowering crops, and started a

two-years review of neonicotinoid insecticide risks (Bass & Field, 2018). In 2018, three main used insecticides of this family, i.e. thiamethoxam, clothianidin and imidacloprid, were banned from all field crops (Siviter *et al.*, 2020). Around ten years ago, a new insecticide, now registered for use in 81 countries, has been discovered by Dow Agroscience (Babcock *et al.*, 2011; Zhu *et al.*, 2011). Sulfoxaflor (Fig. 9-B), the only sulfoximin-based insecticide, acts through a similar mechanism than the neonicotinoids, i.e. acting as nAChRs agonists (Sparks *et al.*, 2013). This molecule has an equal to higher activity level on the economically important species already targeted by the neonicotinoid insecticides, such as the cotton and green peach aphids. Moreover, the overexpression of the monooxygenase enzymes implied in the resistance mechanism against neonicotinoids seems to have no effect on Sulfoxaflor (Babcock *et al.*, 2011).

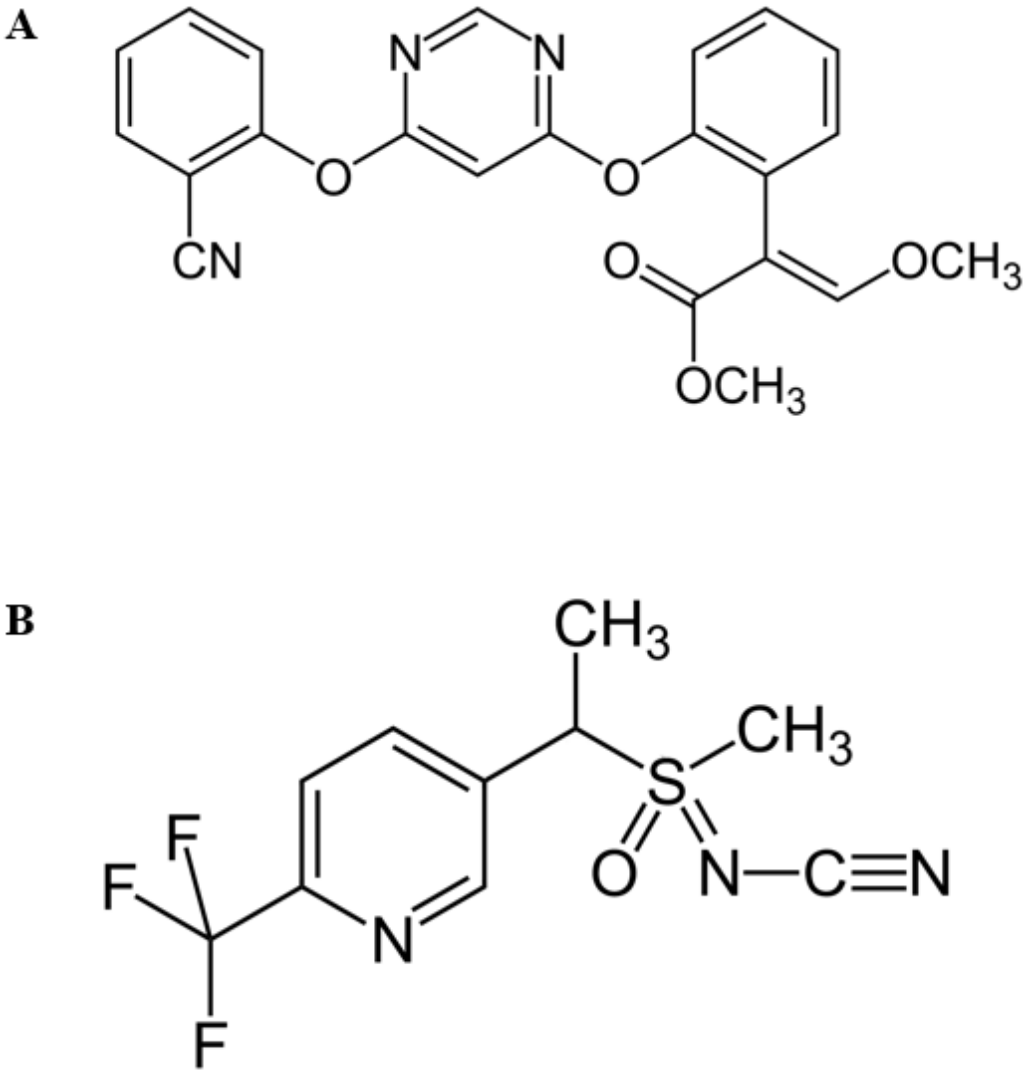


Fig. 9 – Chemical structures of A) the azoxystrobin molecule, a strobilurin fungicide, and B) Chemical structure of the sulfoxaflor molecule, a sulfoximin pesticide with a similar mechanism than neonicotinoid.

Another family of widely used pesticides is the strobilurin family. This important class of fungicides is globally used on crops such as cereals, turfgrass, potatoes, grapevines, of which the sells represent 10 % of the fungicide market. They are inspired by natural fungicidal derivatives of β-methoxy-acrylic acid, produced by basidiomycete wood-rotting fungi, and the gliding bacterium, *Myxococcus fulvus*. It inhibits mitochondrial respiration by binding at the Q_o site of cytochrome b from cyt bc_1 complex located in the inner mitochondrial membrane of most eukaryota. It therefore blocks the electron transfer between cytochrome b and cytochrome c_1, and disrupts the energy cycle to halt the ATP production (Fig. 8-A). These molecules were interesting for scientists because this mode of action was newly discovered for the use in agriculture, and there was therefore no cross-resistance towards them. However, the natural substances were not specific enough, and new synthetic molecules were manufactured to only target fungus species. As an example of this class, in 1966, azoxystrobin (Fig. 9-A) was the first strobilurin molecule to be sold, and its sells were worth to 415 million dollars making it world's biggest selling fungicide in 1999. This molecule is now registered for use on 84 crops of 72 countries, and acts on more than 400 crop-disease systems. It can be applied either by foliar application, seed treatment, or directly in furrow (Bartlewicz *et al.*, 2016).

1.3.3.2. Lethal and sub-lethal effects on bees

Great concerns about neonicotinoid responsibility in the current bee species decline through negative effects on mortality, on reproductive success, and sublethal effects on behaviour have already been highlighted in several studies (Barraud *et al.*, 2020; Cresswell *et al.*, 2012; Feltham *et al.*, 2014; Gill & Raine, 2014; Laycock *et al.*, 2012; Phelps *et al.*, 2018). Despite the substitution potential of sulfoxaflor over neonicotinoids on the market, the fact that they share a same mode of action raises concerns about the potential similar sub-lethal effects of the molecule on pollinators (Centner *et al.*, 2018). Indeed, in several field-laboratory combined studies, (Siviter, Brown, *et al.*, 2018) highlighted negative impacts on bumblebee colony fitness through lowered reproductive performances. However, in follow-up studies, no impacts of Sulfoxaflor on bee cognition, but a reduced egg-laying in microcolonies experiment was found (Siviter, Brown, *et al.*, 2018; Siviter *et al.*, 2019, 2020). An hypothesis about this can be linked to the nutritional intake (Pettis *et al.*, 2013; Siviter *et al.*, 2020). Therefore, besides data on wild bee species, more studies are needed to understand how sulfoxaflor exposure can impact bee behaviour, food intake, cognition, and reproductive capacity.

While a lot of studies have focused their efforts on insecticide adverse effects on non-targeted arthropods, such as bees, fungicides have received far less attention. However, 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 were also 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 some strobilurin adverse effects 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). Besides that, it was highlighted that picoxystrobin inhibits the ATP production by mitochondria of bee thorax cells *in vitro* (Domingues *et al.*, 2017), induces changes in morpho-physiology of hepato-nephrotic system, reduces the survival time (Batista *et al.*, 2020), and causes cytotoxic effects on bee midgut epithelial cells which may lead to malnutrition and bad nutrient absorption (Degrandi-Hoffman *et al.*, 2015). Therefore, as already shown with picoxystrobin, azoxystrobin could interfere with nutrition, and food intake (Tadei *et al.*, 2019).

Synergisms are known since before the late 1930's, and have been studied for their capacity of modifying the LD50 (i.e. the dose at which mortality in the sample reaches 50%) of the components alone. However, their sub-lethal effects on bees were not investigated until 1992, with the synergy between Deltamethrin, a pyrethroid insecticide, and Prochloraz, an imidazole fungicides (Colin & Belzunces, 1992). Since then, decreases of bee LD50 of at least one of the mixture components due synergisms between several widely used insecticides and fungicides were observed (Robinson *et al.*, 2017; Schmuck *et al.*, 2003; Sgolastra *et al.*, 2017; Thompson *et al.*, 2014; Thompson & Wilkins, 2003; Wade *et al.*, 2019). The appearance of sub-lethal effects was also highlighted (Brittain *et al.*, 2013). For example, larval exposure of Africanized honeybee to a neonicotinoid insecticide, and a strobilurin fungicide together were found to impacts the behaviour and the survival of emerged bees (Tadei *et al.*, 2019). Studying the synergistic effects of pesticide exposure on bees is as important as studying the impacts of each molecule alone. Indeed, even if one of the applied molecules is declared safe for bees, mixture with other agrochemicals can enhance its activity,

and decrease the bee LD50, or cause sub-lethal effects. Recently, it has been shown that, while sulfoxaflor had lower bee LD50 than the remaining authorized neonicotinoids, this value decreases when exposed at the same time as fluxapyroxad fungicide, a succinate dehydrogenase inhibitor (Azpiazu *et al.*, 2021).

While the evaluation of these effects on bees are mainly done on the honeybee, species-specific sensitivity is being highlighted (Arena & Sgolastra, 2014). Indeed, all bee species differ in their ecological, physiological and morphological traits. For example, they can present lower haemolymph pH that could help with the detoxification process (Ahmad & Johansen, 1973). In addition, oligolectic bees might show higher LD50 than the species mainly used in risk assessment. Model species (i.e. *A. mellifera*, *B. terrestris*, and *Osmia bicornis*) are generalist species, and hence could present less detoxification pathways than some wild bee species that are specialised to feed on plants, already providing toxic secondary metabolites (Praz *et al.*, 2008). For example, the sweat bee, *Halictus scabiosae*, mainly feeds on Asteraceae (Peeters *et al.*, 2012) which are known to produce pollen and nectar that are rich in toxic secondary metabolites (Vanderplanck et al., 2020). Finally, the specific size and body weight of bees can differ greatly between species, and with it, their sensitivity to pesticides. Besides the fact that the sensitivity has been shown to increase with the surface-to-volume ratio (Johansen, 1972), it has recently been highlighted that the specific bee body weight seemed to be a good predictor of the sensitivity towards acute contact pesticide exposure (Pamminger, 2021).

1.3.4. Methods and models currently used in pesticide risk assessments

The methods currently used to assess the toxicity of pesticide exposure on bees mainly rely on tier-based toxicity tests, a method developed by the International Organisation of Biological Control (IOBC). The tier I tests consist into an evaluation of the toxicity of a chemical compound on mortality and performances under laboratory conditions. During this step, the LD50 is calculated. If the molecule successfully pass through this step, no more tests are needed before placing it on the market (Boller et al., 2006). However, the evaluation of LD50 usually fails to detect sub-lethal effects. Indeed, a wide range of toxic effects due to pesticide exposure can affect the survival of an organism on the long run without instantly affecting the mortality. Moreover, current pesticide risk assessments mostly rely on the domesticated honeybees (Fig. 10-A), because this well-known species is commercially available (Franklin & Raine, 2019).

Fig. 10 – Model species currently recommended in the risk assessment protocols for the testing of chemicals on bees. A) The domesticated honeybee, *Apis mellifera*, B) the red mason bee, *Osmia bicornis*, and C) the buff-tailed bumblebee, *Bombus terrestris*. (Picture credits: Nicolas Vereecken).

By using this species and predicting the results onto wild bees, and bumblebees, the adverse effects on a species could be severely underestimated. Indeed, the impacts and exposure to pesticides on a species in which the reproductive individuals are actually foraging can be different than for the honeybee (Franklin & Raine, 2019). In 2013, the European Food Safety Authority (EFSA) suggested to include two other surrogate species than honeybee: the buff-tailed bumblebee, *Bombus terrestris* (Fig. 10-C) and solitary bees from the Osmia genus (Fig. 10-B; Spurgeon *et al.*, 2016).

2. Research questions and aim of the study

Current knowledge about bee sensitivity to pesticides is mainly based on mortality test performed on a few species. There is a need to gather more data about the lethal and sub-lethal effect of pesticides on wild bees. Therefore, the present work aims (i) to compare the effects of oral and topical acute sulfoxaflor exposure on the individual mortality of several wild bee species with *B. terrestris*, and (ii) to evaluate the impacts of oral exposure to sub-lethal doses of sulfoxaflor and azoxystrobin, as well as their mixture, on *B. terrestris* feeding behaviour.

Species-specific trends are expected towards acute individual sulfoxaflor exposure (Arena & Sgolastra, 2014). Indeed, all bee species differ in terms of ecology, physiology and morphology. The specific haemolymph pH, the detoxification mechanisms probably possessed by some oligolectic bees, or the surface-to-volume ratio and specific mean body size could influence the specific sensitivity (Ahmad & Johansen, 1973; Johansen, 1972; Pamminger, 2021; Vanderplanck *et al.*, 2020).

Concerning the second part of this work, pesticide impacts on the feeding behaviour of *B. terrestris* are expected. Sulfoxaflor presents a similar mode of action than neonicotinoid insecticides (Siviter *et al.*, 2020), and adverse effects on nutritional intake of *B. terrestris* due to neonicotinoid exposure were already brought up (Bartlewicz *et al.*, 2016; Domingues *et al.*, 2017; Pettis *et al.*, 2013). Azoxystrobin might also interfere with the feeding behaviour because it was highlighted that fungicides from the strobilurin family can have adverse effects on the midgut cells, and digestive microbiota of bees (Tadei *et al.*, 2019). Finally, adverse synergistic effects on bees have already been highlighted with mixtures between other strobilurin fungicides and neonicotinoid insecticides, e.g. pyraclostrobin-clothianidin mixture on Honeybee (Tadei *et al.*, 2019).

3. Material and methods

3.1. Pesticides

During the experiments, we used two types of pesticides: the active ingredient of a new type of insecticide, i.e. Sulfoxaflor, and the commercial formula of the fungicidal molecule azoxystrobin, i.e. Amistar®. While these have been showed to be non-toxic for honeybees in semi-field conditions (Tamburini *et al.*, 2021), very little is known about their mode of action, as well as the sub-lethal and lethal effects on wild bees.

Firstly, the sulfoxaflor, is a new type of insecticidal molecule applied on a broad range of crops (Babcock *et al.*, 2011; Zhu *et al.*, 2011). Residue levels of this systemic insecticide can be found in nectar and pollen of sprayed plants (USEPA, 2019). Bees are therefore orally exposed to this molecule. While still little is known about its lethal and sub-lethal effects on wild bee species, adverse effects on the reproductive success of bumblebees were already brought up (Siviter, Brown, *et al.*, 2018). Therefore, we used it in the acute oral and topical exposure experiments, as well as during the feeding behaviour experiments. All sulfoxaflor treatments used in the experiments were derived from a sulfoxaflor (Greyhound Chromatography and Allied Chemicals) solution of 1 mg/mL in acetone.

Then, the azoxystrobin was used during a feeding behaviour experiments alone, as well as in mixture with sulfoxaflor, to evaluate its sub-lethal effects on *B. terrestris*. This systemic fungicidal molecule have already been found on bee-material and on the bees themselves in high quantities (Hladik *et al.*, 2016; Long & Krupke, 2016). However, like for the sulfoxaflor molecule, very little is known about the sub-lethal effects of azoxystrobin and its commercial formulas on wild bees. During the experiments, we used Amistar®, an azoxystrobin-based commercial formula, rather than the active ingredient alone, because previous results showed that, in contrast to sulfoxaflor-based insecticides, the active ingredient was not the only component of the formula to affect the honeybee (Unpublished results). The Amistar® treatments used in the experiments were derived from the 250g/L commercial solutions (Syngenta).

When a positive control was required, a Dimethoate treatment was used. Indeed, a dose of 10 µg/bee dose ensure a mortality rate of more than fifty percent in the treated group. The Dimethoate treatments were derived from a 10 µg/µL solution in distilled water. This broad spectrum acaricidal, and insecticidal molecule was suggested as reference compound in

toxicity test for bees in the 90's. Indeed, it presented a low variability in terms of times, seasonal activity, and strains of honeybee used. Moreover, the similarity between its oral and topical LD50s on honeybee and the solubility in water and other organic compounds make it an easy molecule to use as positive control (Gough *et al.*, 1994).

3.2. Individual acute sulfoxaflor exposure

3.2.1. Bee species selection, and sampling

First, for the preliminary tests, the buff-tailed bumblebee, *Bombus terrestris*, was used. Since 2016, toxicity test protocols for this species have been added by the international ICPPR and OECD ring-test groups for pesticide risk assessments. Before that, only the honeybee was taken into account for ecotoxicological tests (OECD, 2017b, 2017a). The yearly colonies contain a great number of mature workers, and are commercially available. This allows toxicity tests that require a high number of individuals, such as the determination of LD50. To perform the experiment, queen-right colonies of 100 *Bombus terrestris* workers were purchased from Biobest NV[1] (Waterloo, Belgium). At their arrival in the lab, they were maintained in a controlled room at 25 ± 5°C and 60 ± 5% humidity. They were fed with *ad libitum* biogluc syrup (65% sugar, 35% water), and once a week 10 g of dried freeze *Salix* spp. pollen was given to the colony.

Then, females of eight bee species were taken from the wild, and used to compare the sulfoxaflor sensitivity with *B. terrestris*: two bumblebee species, the tree bumblebee, *Bombus hypnorum* and the common carder bee, *Bombus pascuorum*; one Andrenidae species, the grey-backed mining bee, *Andrena vaga*; one Halictidae species, the sweat bee, *Halictus scabiosae*; and four Megachilidae species, the European orchard bee, *Osmia cornuta*, the blue mason bee, *Osmia caerulescens*, the Orange-vented mason bee, *Osmia leaiana*, and finally, the large-headed resin bee, *Heriades truncorum*. The bombus species, *Osmia cornuta* and *Andrena vaga* were kept in the same conditions than the *Bombus terrestris* colonies, while the other bees were kept at room temperature.

Four other species were also caught, and brought back to the laboratory. However, *Anthidium manicatum* (Megachilidae), *Dasypoda hirtipes* (Melittidae), *Anthophora plumipes* and *Anthophora quadrimaculata* (Apidae) did not survived to laboratory conditions.

[1] https://www.biobestgroup.com/fr/lutte-biologique-pollinisation-par-les-bourdons

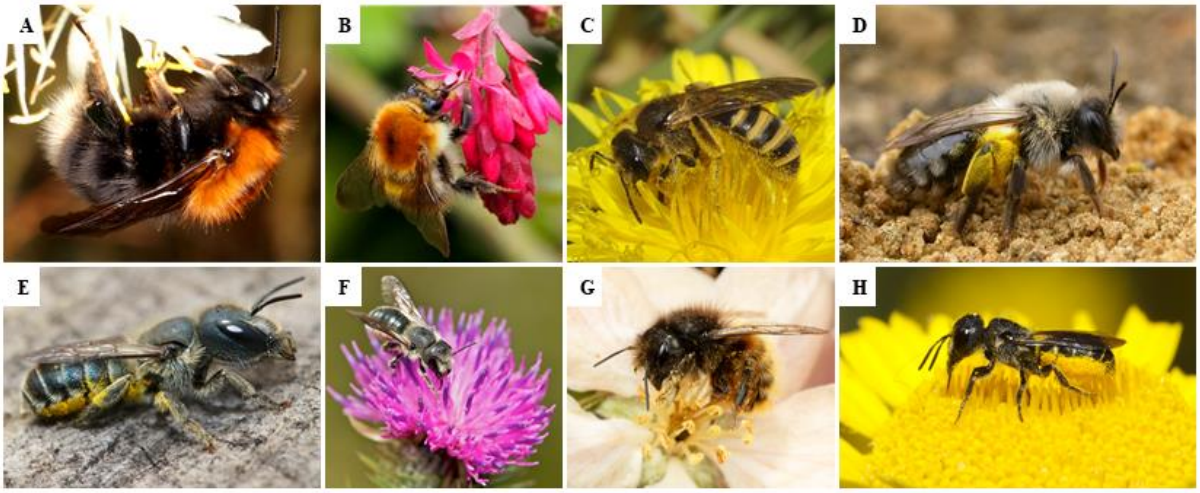


Fig. 11 – Model species for the acute sulfoxaflor exposure experiments. A) *Bombus hypnorum*, B) *Bombus pascuorum*, C) *Halictus* scabiosae, D) *Andrena vaga*, E) *Osmia caerulescens*, F) *Osmia leaiana*, G) *Osmia cornuta*, and H) *Heriades truncorum* (picture credits: bombus species from Andrew Green; other bees from Nicolas Vereecken).

The tested bee species captured in Mons (Belgium) and surroundings were selected their abundance in the region and their survival capacities towards laboratory conditions. Thanks to the implemented "zero-phyto" policy, no plant protection product is used in the city of Mons[2]. Therefore, we can assume that these caught bees were not exposed to pesticides before the experiments.

3.2.1.1. Bombus species

Although primitively eusocial, the lifecycle of *Bombus* species includes a solitary new-born queen that, once mated, will enter in the diapause phase to overwinter in a suitable hibernation place, such as disturbed soil (Goulson, 2010). At the end of the diapause period, the queen will start searching for a suitable nest. In the case of *Bombus terrestris*, it is usually an abandoned rodent burrow, while *Bombus hypnorum* is frequently found nesting into bird houses, or natural cavities dug in the wood (Kells & Goulson, 2003; Rasmont *et al.*, 2015). Once the nest found, the bumblebee queen will start its new colony by laying the first batch of eggs, and incubating the ball made from mixed wax and pollen where the eggs are located. Until the emergence of the first worker bees, 4 days after the laying, she will entirely depend on the resources that she provisioned after she found the nest. Once the workers hatched, they will help the queen to support the larvae by foraging for her, and regurgitating pollen-nectar mixture. At the end a species-specific time of the season, from April to August, males and

[2] https://www.mons.be/vivre-a-mons/territoire/environnement/biodiversite/zero-phyto

new bumblebee queens are reared. After their emergence, newly born queens will accumulate fat, follow the pheromone-traced route of some males, and mate (Goulson, 2010). Concerning their lectism, bumblebee species are generalist pollinators. However, their floral choice range may be different between species (Goulson & Darvill, 2004). Indeed, species from *Bombus* genus (Hymenoptera: Apidae, Bombini) present great similarities in terms of morphology and lifecycle, differing almost only by their size, their tongue length, and the emergence date of their queens (Goulson, 2010; Goulson & Darvill, 2004). Their choice will therefore be driven by the availability of the resources, their morphology, but also by their capacity to choose a plant over another, by gauging both the pollen and nectar load and quality that the species can provide to them (Goulson, 2010; Somme *et al.*, 2015). To perform the experiment, a colony of the tree bumblebee, *Bombus hypnorum*, a smaller species compared to *B. terrestris*, containing 50 workers was sent from a bee breeding garden in Luxemburg, maintained by Jean Habay. At its arrival at the laboratory, the colony was kept in the exact same conditions as the *B. terrestris* colonies. In addition, workers of *Bombus pascuorum* were caught while foraging on *Viscia* spp. at the "Parc de la Cascade", Hyon, Belgium (N50°26'15.2", E 3°58'04.4").

3.2.1.2. Halictus scabiosae

Another species that was used in the experiment is the sweat bee, *Halictus scabiosae*. Individuals of this species were captured in two different locations of Mons, at "Campus de la Plaine de Nimy", UMons (N50°27'48.5", E3°57'19.2"), and at the "Parc de la Cascade", Hyon, Belgium. They were usually caught while foraging on flowers from the Asteraceae family, such as *Centaurea scabiosae*. Indeed, this sweat bee is an oligolectic bee on this family. The species presents a particular social organization, and an annual lifecycle, that starts in Spring, when mated females, namely the foundresses, emerge (Batra, 1966; Brand & Chapuisat, 2012). They either overwinter in small groups of sisters, or alone (Ulrich *et al.*, 2009). Once emerged, they, alone or in group of sisters, search for a nesting site, dig a nest up to 30 cm deep, and provision it. The nests of this species can be found in aggregation (Batra, 1966; Bischoff, 2003; Lienhard *et al.*, 2010; Ulrich *et al.*, 2009). Then, the biggest female becomes the dominant egg-layer. Before the first batch of eggs emerges (June-July), she drives the other females out of her nest (Brand & Chapuisat, 2012; Ulrich *et al.*, 2009). The first brood is almost exclusively composed of females that will play the role of workers for their mother, guarding the nest, foraging for her and helping her to raise the second brood (Batra, 1966; Brand & Chapuisat, 2012). At this time, a high level of drifting to other nests by

the foundresses can be observed (Gonzalez *et al.*, 2018). Indeed, unlike bumblebee workers that are not allowed to product other females and future queens (Goulson, 2010), first brood females of *H. scabiosae* colonies do not completely lose the capability of producing females, if they are mated by early emerged males. These mated females can either lay eggs in their natal nests or in a neighbour nest, or found a new one (Brand & Chapuisat, 2012). This phenomenon gives a great flexibility to this species social organization, through frequent foundress turnover, and high tolerance between non-nestmates. This phenomenon probably increases the colony survival and productivity, e.g. in small colonies (Brand & Chapuisat, 2014). The second brood comprises both males and females that emerge during the months of August to September. The females will stay in the nest and continue to forage as workers, but once mated, will enter in diapause to overwinter, and start their own colony the next year (Brand & Chapuisat, 2012). Although Batra (1966) suggested that this honeybee-sized species did not present any difference in terms of size between the egg-layers and the worker-like females, a more recent study highlighted that the size differs clearly between castes (Brand & Chapuisat, 2012), in contrast to other primitively eusocial species that remain morphologically indistinguishable (Michener, 1969).

3.2.1.3. Andrena vaga

Adult females of the grey-backed mining bee species, *Andrena vaga*, from the Andrenidae family, were captured directly at the entrance of their nests found in aggregation in Erbisoeul (N50°30'15.1", E3°52'50.7"), Belgium. *Andrena vaga* measures between 13 to 15mm. This species is an oligolectic bee that feeds only on *Salix* pollen, but is exceptionally found foraging on *Taraxacum* sp. or *Crataegus* sp. The nests are usually found in aggregation. *A. vaga* females dig 25 to 60 cm deep burrows that are irregularly verticals, with two lateral burrows at the bottom of the nest where the egg cells are located. The nests are dug into sandy soils, poorly vegetated. At the end of March, the males emerge first and start patrolling around the nest. Then, the female emerges, only mate with one male, and will reject other mating attempts. The female digs a nest, provisions it, and lays her eggs. The larvaewill develop and reach the nymphal stage in the middle of Summer, to hibernate as imago until next Spring (Peeters *et al.*, 2012).

3.2.1.4. Megachilidae species

Species from the Megachilidae family, i.e. the European orchard bee, *Osmia cornuta*, the orange-vented mason bee, *Osmia leaiana*, the blue mason bee, *Osmia caerulescens*, and

the large-headed resin bee, *Heriades truncorum*, were also used. *O. leaiana* and *O. caerulescens* were purchased from Wildbienen + Partner AG[3] (Zürich Switzerland). We received them in bamboo sticks, and placed those in a greenhouse tent on the "Campus de la Plaine de Nimy", UMons. *O. cornuta* and *H. truncorum* were captured in insect hotels placed around the same location. The female individuals were caught directly at the entrance of the nests. Indeed, these species are cavity nesters that lay eggs inside wall holes, or hollow stems. *O. cornuta* and *O. caerulescens* are both polylectic species. On the other hand, *O. leaiana*, and *H. truncorum* are both oligolectic bees that are mostly found foraging on Asteraceae flowers, such as *Centaurea scabiosae*, or *Picris* spp. The females of *O. cornuta* can measure between 11 and 15mm, both other *Osmia* species between 8 and 10 mm, while *H. truncorum* is a small bee species measuring between 5 and 7 mm. They all have a similar lifecycle. The males emerge first, followed by the females. O. cornuta is the earliest bee of the considered species as it starts its lifecycle at the end of February. *O. leaiana* is active from April to August, and *O. caerulescens* and *H. truncorum* start their lifecycles in March and finish it in August. They mate at the beginning of the season, and start provisioning their nest. Mated females will align nine to 12 cells, separate either by mud, as *O. cornuta*, by chewed leaves, as the both other Osmia species, or one to ten cells, separate by resin collected mostly on pine trees, as *H. truncorum*. Then, they close the last cell with a mix of loam and saliva for the Osmia species, or a mix of resin and small gravel for *H. truncorum*. The larvae will develop, and hibernate as imago, excepted for *H. truncorum* which overwinters as pre-pupae (Peeters *et al.*, 2012).

3.2.2. Experimental setup

The experimental setups of topical and oral exposure (for schematic timelines, see Appendix 1 – Pesticide acute exposure of the wild bees) were adapted from the OECD guidelines (OECD, 2017b, 2017a), and the improved protocols for testing agrochemicals in bees (Medrzyck *et al.*, 2021). Although the OECD guidelines recommended the use of at least 30 individuals for each treatment group, due to the fact that the majority of the wild bee species are not commercially available, like *Bombus terrestris* colonies, less individuals were used. All the species were exposed to the oral and topical LD50 values previously found for the model species, *Bombus terrestris* (oral LD50: 0.563µg/g b.w.; topical LD50: 43.9 µg/g b.w.; Unpublished data).

[3] https://wildbieneundpartner.ch/

Preliminary tests on the model species were performed following the same protocols as the ones we used with wild bees. During these test experiments to confirm the topical LD50 values, a positive control was used, and consisted into a 10µg/bee dimethoate treatment. However, the sulfoxaflor topical and oral mortality rates values that we found for *B. terrestris* were not equal to 50%. Therefore, we decided to use the obtained mortality rates, and not the value of 50% to compare with other bee species.

3.2.2.1. Acclimation

Before the beginning of the experiment, each individual was weighted individually, placed under a see-through plastic glass, and fed with *ad libitum* 50% w/w sugar solution through soak capillaries (Fig. 12A; Fig. 13A). Then, they were left at least eight 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 stayed in these rooms during the whole duration of the experiment. For each species, the individuals were randomly assigned to treatment groups of same mean weight. At the end of the experiments, a sample of haemolymph from each individual that was still alive was collected.

3.2.2.2. Sulfoxaflor exposure

3.2.2.2.1. Oral exposure

After the 8 hours period of acclimation, the capillaries were removed from the see-through plastic glasses before a four-hour starvation period (Fig. 12-B). Then, for each treatment group, spectrophotometer cuvettes were filled with 20µL of either treatment or control solution, placed under the glasses.

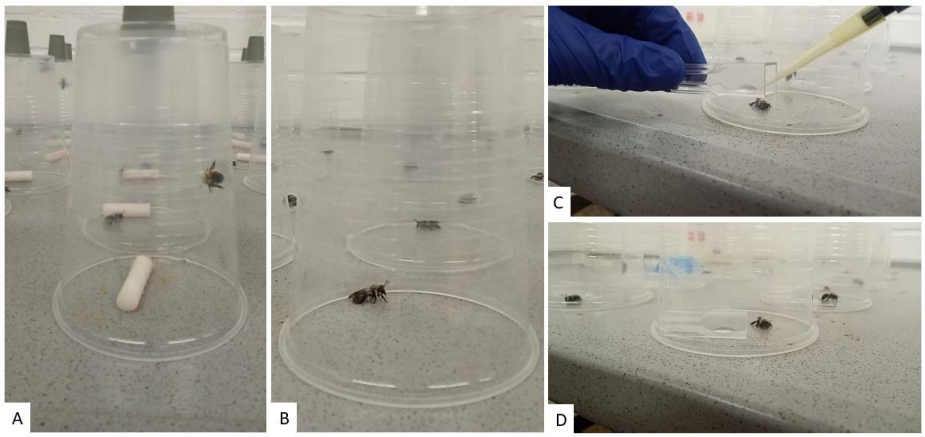


Fig. 12 – Example of acute oral exposure to sulfoxaflor with *Andrena vaga*. A) Acclimation period under a see-through plastic glass with a soaked capillary as food source, B) starvation period with the soak capillary removed from the glass, C) positioning of the 20µL droplet inside the spectrophotometer cuvette, and D) exposure period with the cuvettes under the glass (picture credits: Justine Dewaele).

The cuvettes were left there during the four-hour exposition period during which the consumption was observed every 30 minutes (Fig. 12 – C, D). The negative control solution consisted into a 50% w/w sugar-water solution with 0.05% of acetone. Once a bee consumed at least 80% of the droplet, it was included in the test, and a new capillary soaked with a 50% w/w sugar-water solution was placed back under the glass for the observation period.

To control the evaporation rate during the exposure period, five additional doses have been placed into spectrophotometer cuvettes, under empty glasses. These cuvettes were weighted before and after the three hours of exposure time.

3.2.2.2.2. Topical exposure

For each treatment group, the bees were chilled until fainting (max. ten minutes for the biggest species) before handling. They were then exposed by applying 2µL droplets with a micro-pipette on the dorsal side of the thorax. The negative control individuals were treated with 2µL of distilled water with 0.05% acetone. To ensure a homogenic dispersal of the treatment and control solutions on the bee thorax, octylphenolethoxylate (Triton X-100) 0.05% was used as surfactant. Once the 2µL droplet was applied, the individual was placed in a petri dish until the awakening, and then placed back under the see-through plastic glasses of the controlled room for the observation period.

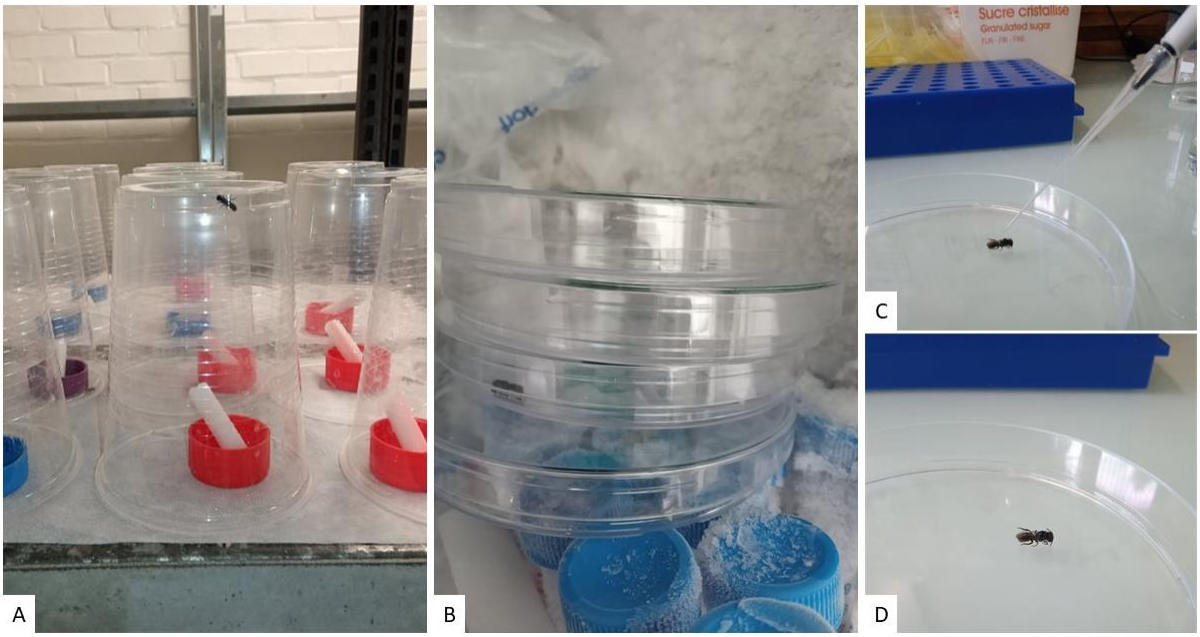


Fig. 13 – Example of acute topical exposure to sulfoxaflor with *Heriades truncorum*. A) Acclimation period under a see-through plastic glass with a soaked capillary as food source, B) chilling process into petri dishes, C) topical exposure by positioning a 2µL droplet on the bee thorax, and D) recovery period into the petri dish after chilling and before going back under the see-through plastic glass (picture credits: Justine Dewaele).

3.2.2.3. Observation period

After the exposition period, the sub-lethal effects and mortality values were observed, and recorded under red light every 24 hours during maximum 48 hours. At the end, the final mortality rate was recorded.

3.3. Effects of sulfoxaflor on feeding behaviour

3.3.1. Bee species selection

For this experiment, we considered only the model species, *Bombus terrestris*. In total, three colonies of *Bombus terrestris* with a queen purchased from Biobest (Westerloo, Belgium) were used in the experiments. This species was used because it is easily available on the market, and could therefore be used without discontinuing during the three months of the experiments. The colonies were kept in the same conditions, and fed in the same way as the colony used for the acute exposure experiments (see section "1.3.2. Bee species selection and sampling").

3.3.2. Equipment description

Fig. 14 – The ten automatic food dispensers used in the experiments. (Picture credits: Justine Dewaele)

For this experiment, ten automatic food dispensers, built by Michel Sokolowski, were used (Fig. 14), and placed in a room with monitored temperature and humidity (for temperature and humidity data, see Appendix 3 – Monitoring of the automatic dispenser

room), under a 12 hours cycle of artificial light. One machine consists into three main parts (Fig. 15-A). The first compartment includes two pump systems (distribution and purge), all the necessary connections to the recording computer (Fig. 15-B), as well as three 30mL tanks, of which one contains the feeding solution, the second one contains distilled water, and the last one is used to gather waste fluids (Fig. 15-C). This part also contains the lower part of an artificial flower (Fig. 15-F), equipped with a motion sensor and the connections with the computer. The second compartment of the box is a cage where the bees were placed. This is where the artificial flower is allowing the bees to feed (Fig. 15-F). The third part of the box contains a video recording system (Fig. 15-E).

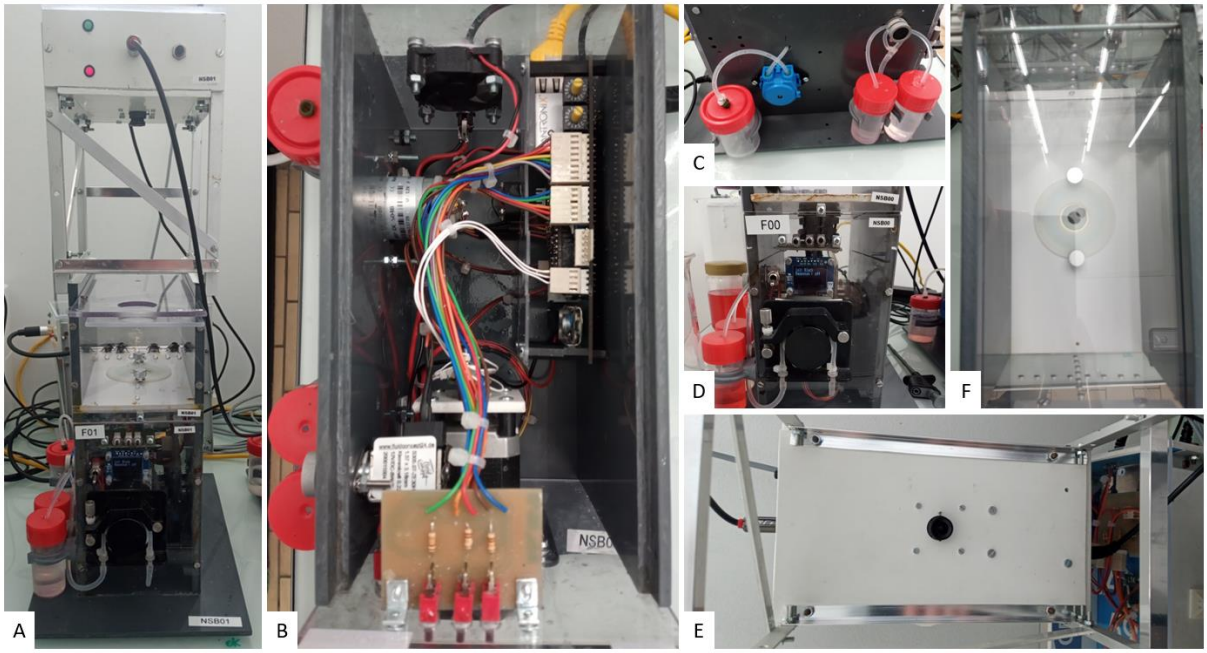


Fig. 15 – Automatic food dispenser description. A) Automatic food dispenser, B) connection compartment, C) the three tanks, form right to left: waste, distilled water, and syrup tanks, D) front side of the first compartment, E) camera, F) empty cage compartment with the artificial flower in the middle (picture credits: Justine Dewaele).

When a bee comes inside the flower, it is detected by the motion sensor, and the distribution pump delivers 1µL of syrup. Once the bee comes out of the flower, the purge pump drains the remaining fluids. To avoid crystallisation of the syrup in the capillaries, a purge program with distilled water was activated at least six times a day. Measurements of the room temperature and humidity were recorded every 10 minutes during the whole time of the experiment and the video recording device took pictures of each box once an hour to check for mortality.

3.3.3. Experimental setup

Four randomly chosen *B. terrestris* workers from the same colony were placed inside of each box, and were exposed during seven whole days. The frequency of feeding movements (responses) was measured, starting eight hours after the bees were placed inside the boxes. Each week, two replicates for each treatment were made, and each month the dose was increased. In total, eight replicates by doses were made.

We administrated, through the artificial flowers, three increasing doses of either sulfoxaflor, Amistar®, or a mixture of both molecules, diluted into 50% w/w sugar-water solutions. The mixture treatments were made from corresponding doses of both treatments alone (Tab. 1). The lowest doses were based on field realistic residual quantities found in pollen and nectar (Piechowicz *et al.*, 2018; USEPA, 2016). Then, these doses were increased two and four times.

Tab. 1 - Pesticide treatments and doses used in the feeding behaviour experiment.

Pesticide / Period	Sulfoxaflor	Amistar	Sulfoxaflor x Amistar
Month 1	150 ppb	2000 ppb	150 ppb x 2000 ppb
Month 2	300 ppb	4000 ppb	300 ppb x 4000 ppb
Month 3	600 ppb	8000 ppb	600 ppb x 8000 ppb

The boxes and flowers were cleaned up with soap and rinsed with distilled water each week, and the tubes and tanks each month at the dose shift.

3.4. Statistical analyses

All the statistical analyses were carried out in the R environment v4.1.0 (R Core Team, 2021). The generalized linear mixed models were built using lme4 package v1.1-27 (Bates *et al.*, 2015). The Chi-squared and Fisher's tests omnibus and pairwise were performed using the stats package v4.1.0 (R Core Team, 2021). All the graphs were produced using ggplot2 package v3.3.5 (Wickham, 2016), associated either with ggmosaic v0.3.3 (Jepson *et al.*, 2021), or ggeffects v1.1.0 (Lüdecke, 2018).

3.4.1. Individual acute sulfoxaflor exposure

To evaluate the effect of acute sulfoxaflor exposure on the mortality of wild bees, Chi-squared test 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 the 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

$$Corrected\ \% = \frac{mortality\ (\%)\ in\ treatment + change\ (\%)\ in\ control}{100 + change\ (\%)\ in\ control} x100$$

Then, in order to compare the sensitivity of wild bees with *B. terrestris* the model species, we used either chi-squared test followed by a pairwise proportion comparison test, 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 adjust the p-value.

3.4.2. Effects of sulfoxaflor on feeding behaviour

3.4.2.1. Data treatment

The recording started at 8 AM, the second day of exposure. In order to take into account the glitches, and short circuits that appeared inside the flower connections during the experiments, only the responses lasting more than one second, and less than ten seconds were taken into account in the analyses.

For each replicate separately, the number of responses per flower was first aggregated for each hour, to make it correspond with the mortality data that were recorded by camera.

3.4.2.2. Data analysis

The day and night response frequencies were analysed separately to avoid autocorrelation caused by the 12-hour cycle of light exposition applied in the experiment room. Because we worked with count data, slightly over-dispersed, we built generalized linear models with negative binomial distribution. In order to consider the non-independence of the data recorded on one flower, we used a random structure corresponding to each hour. Moreover, an offset with the number of alive individuals was used allowing us to work with integer count data while including the mortality. To determine the effect of each treatment on the response frequency, Tukey's post-hoc tests were applied following the models.

4. Results

4.1. Individual acute sulfoxaflor exposure

4.1.1. Laboratory condition survival

While we did not manage to keep several species alive under adapted laboratory conditions, namely *A. manicatum*, *D. hirtipes*, *C. daviesanus*, *A. plumipes*, and *A. quadrimaculata*, ten wild bee species were tested to be treated either orally, topically, or both with acute Sulfoxaflor doses besides *B. terrestris*. In total, individuals of five spring bee species, and five summer species were caught, placed under adapted laboratory conditions, and exposed to acute Sulfoxaflor doses (Tab. 2).

Tab. 2 – Morphological and ecological characteristics of the species used in the experiments. Average mass ± SE (g) was calculated directly on the living individuals then exposed to the treatments.

Species	Average mass ± SE (g)	Phenology	Lectism	Sociality
Bombus terrestris (model species)	0.259 ± 0.003	Summer Spring	Polylectic	Primitively social
Bombus hypnorum	0.150 ± 0.004	Summer	Polylectic	Primitively social
Bombus pascuorum	0.153 ± 0.003	Summer	Polylectic	Primitively social
Halictus scabiosae	0.087 ± 0.004	Summer	Oligolectic	Primitively social
Adrena vaga	0.146 ± 0.002	Spring	Oligolectic	Solitary
Heriades truncorum	0.014 ± 0.000	Summer	Oligolectic	Solitary
Osmia caerulescens	0.041 ± 0.001	Spring	Polylectic	Solitary
Osmia cornuta	0.122 ± 0.002	Spring	Polylectic	Solitary
Osmia leaiana	0.066 ± 0.005	Spring	Oligolectic	Solitary

However, among the species used in the experiments, the survival rates after 48H of the control differed significatively (Pearson's chi-square test, χ^2=51.95, df=6, p-value= 1.904689e-09, n=393). The species that survived the most were *B. terrestris*, the model species, *B. hypnorum*, and *H. truncorum* (Fig. 16). Despite the low survival rates of *A. vaga*, *O. cornuta* and *O. caerulescens*, they were yet used in the analysis when the number of caught individuals compensated for the high mortality rate. Finally, the survival of *H. scabiosae*, *C. florisomme* and *O. leaina* could not be tested, as they did not feed on the solution, and were therefore not maintain in captivity any longer.

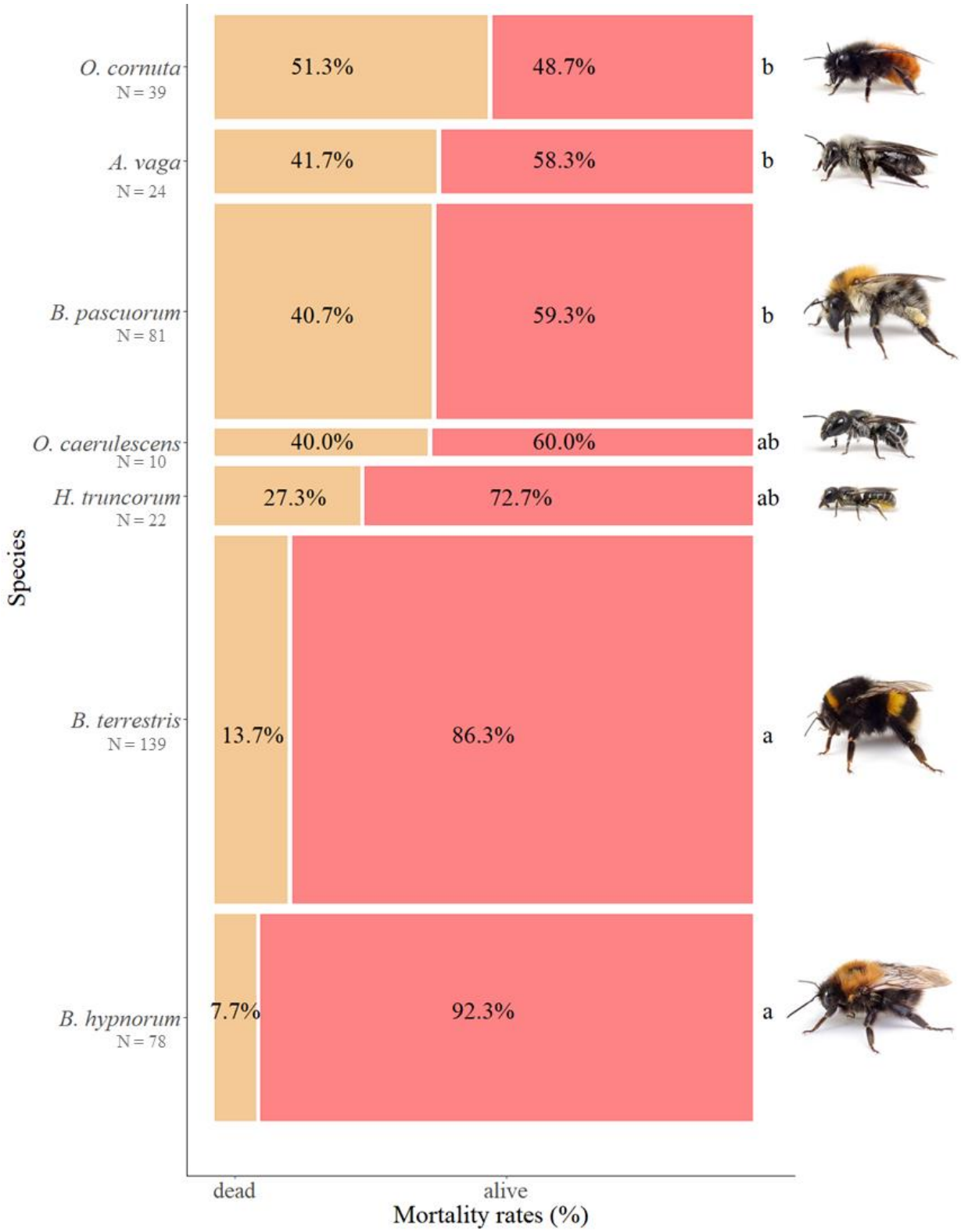


Fig. 16 – Mortality rates (%) towards laboratory conditions of the treated species. The width of the boxes is proportional to the sample size (N). Species that do not share the same letter have significantly different proportions of dead and alive control individuals after 48 hours under adapted laboratory conditions at p-value<0.05 (Post-hoc, Pairwise comparisons using Pairwise comparison of proportions (Fisher) with Holm correction).

4.1.2. Oral acute sulfoxaflor exposure

Besides the model species, *B. terrestris*, we managed to expose 7 species to an acute sulfoxaflor dose of 0.563 µg/g b.w. These included two Bombus species, *B. hypnorum*, and *B. pascuorum*; three Megachilidae species, *O. cornuta*, *O. caerulescens*, and *C. florisomme*; as well as *H. scabiosae*, and *A. vaga* (Tab. 3).

Tab. 3 – Average mass ± SE (g) and sample size (n) of the species used during the oral exposure experiment.

Species	**Average mass ± SE (g)**	
	Control	**Sulfoxaflor**
Bombus terrestris (model species)	0.260 ± 0.006 n=63	0.259 ± 0.008 n=63
Bombus hypnorum	0.146 ± 0.005 n=60	0.142 ± 0.005 n=54
Bombus pascuorum	0.170 ± 0.006 n=54	0.172 ± 0.006 n =56
Osmia cornuta	0.123 ± 0.003 n=39	0.122 ± 0.003 n=39
Osmia caerulescens	0.039 ± 0.003 n=12	0.039 ± 0.002 n=12
Chelostoma florisomme	0.033 ± 0.002 n=6	0.034 ± 0.003 n=6
Halictus scabiosae	0.086 ± 0.005 n=14	0.088 ± 0.005 n=14
Adrena vaga	0.151 ± 0.003 n=60	0.142 ± 0.003 n=92

However, it appeared that the proportion of individual that fed on the solutions significantly differs between species (Fisher's exact test for count data with simulated p-value based on 2000 replicates, p-value=0.0005). Since no individual of *O. caerulescens*, and *C. florisomme* fed on the control and treatment solutions, only *B. terrestris* (the model species), *B. hypnorum*, *B. pascuorum*, *O. cornuta*, *H. scabiosae*, and *A. vaga* were used for the sensitivity comparison analyses (Fig. 17).

Finally, out of the six species selected for the analysis, only two presented a significant sensitivity to acute sulfoxaflor exposure. The species were *B. terrestris* (the model species), and *O. cornuta* with respectively 18 (90.3%) and 28 (30%) individuals that died 48 hours after feeding on a sulfoxaflor solution, compared to 0 and 11 (36.7%) individuals from the control group (Pearson's chi-square test, *B. terrestris*: χ^2=20.86, df=1, p-value=4.9554e-06, n=119; *O. cornuta*: χ^2=19.04, df=1, p-value= 1.283196e-05, n=61).

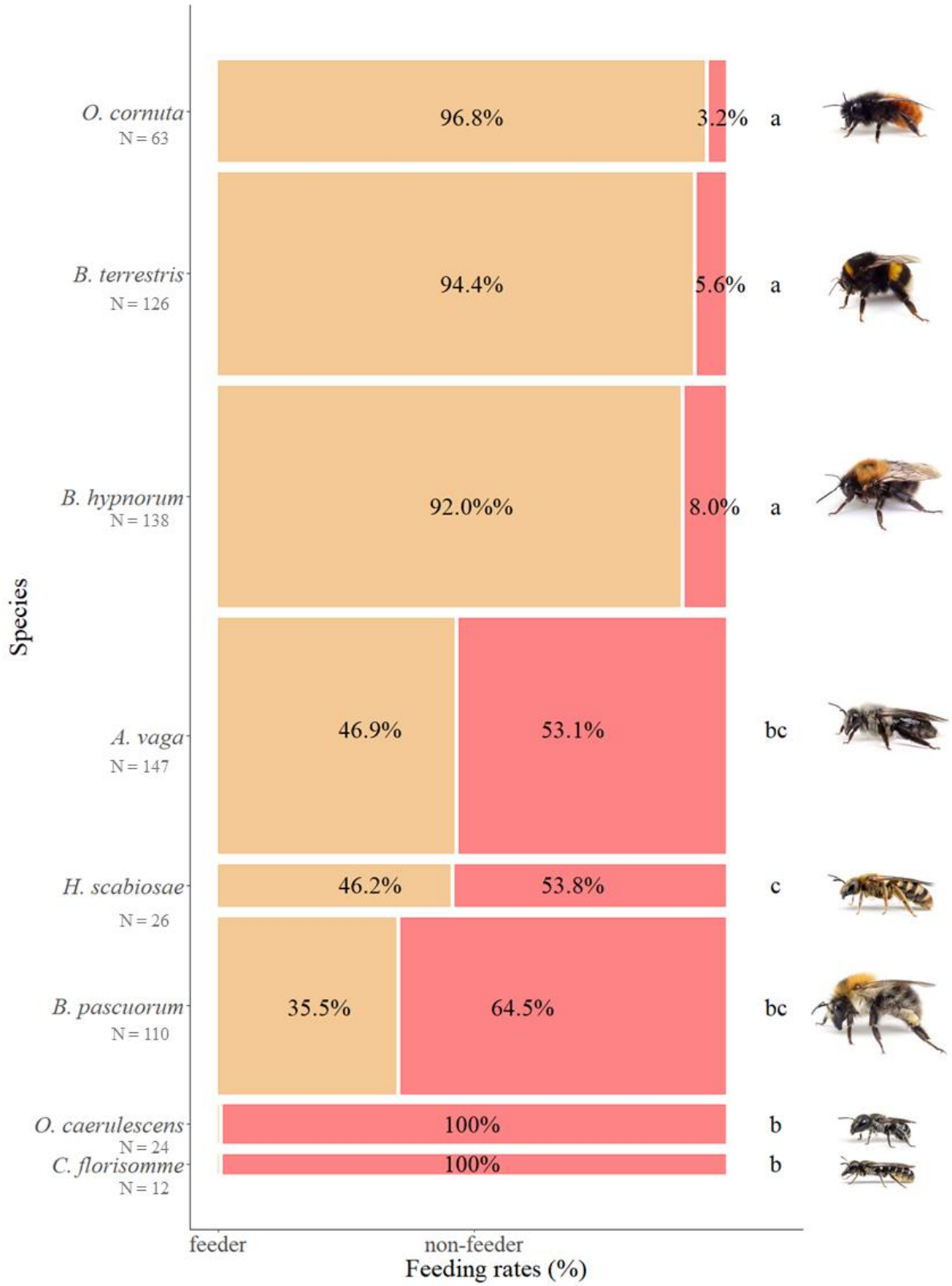


Fig. 17 – Feeder proportion (%) in each species used in the experiment. The width of the boxes is proportional to the sample size. Species that do not share the same letter have significantly different proportions of feeders at p-value<0.05 (Post-hoc, Fisher's pairwise comparison of proportions with Holm correction; picture Copyright © Mandy & Michael Fritzsche).

Tab. 4 – Effects of oral exposure to acute sulfoxaflor dose (0.563µg/g b.w.) on the mortality rates of each species selected for the sensitivity analyses. χ^2 statistic, degrees of freedom and p-values are reported when a chi-square test was used, and 95%CI and p-values when a Fisher's exact test for count data was used. (Legend: Number of individuals; *expected number of individuals*; **proportion of individuals**)

Species	Treatments	Dead	Alive	Statistic	df	p-value
B. terrestris	Control	0	59	χ^2=20.86	1	4.9554e-06***
(model species)		*8.92*	*50.08*			
		0%	**100%**			
	Sulfoxaflor	18	42			
		9.08	*50.92*			
		30.0%	**70.0%**			
B. hypnorum	Control	2	56	95%CI=[0.0093, 11.1180]	/	1
		1.60	*56.40*			
		3.4%	**96.6%**			
	Sulfoxaflor	1	50			
		1.404	*49.60*			
		2.0%	**98.0%**			
B. pascuorum	Control	4	16	χ^2=3.28	1	0.0699
		6.67	*13.33*			
		20%	**80%**			
	Sulfoxaflor	9	10			
		6.33	*12.67*			
		47.4%	**52.6%**			
A. vaga	Control	7	14	χ^2=1.26	1	0.2608
		9.13	*11.87*			
		33.3%	**66.7%**			
	Sulfoxaflor	23	25			
		20.87	*27.13*			
		47.9%	**52.1%**			
H. scabiosae	Control	0	6	95%CI=[0.4734, Inf.]	/	0.1818
		1.5	*4.5*			
		0%	**100%**			
	Sulfoxaflor	3	3			
		1.5	*1.5*			
		50%	**50%**			
O. cornuta	Control	11	19	χ^2=19.04	1	1.2832e-05***
		19.18	*10.82*			
		36.7%	**63%**			
	Sulfoxaflor	28	3			
		19.82	*11.18*			
		90.3%	**9.7%**			

Significance level: ***p<0.001

The Sun-Shepard's corrected proportions of *B. terrestris* and *O. cornuta* individuals that died 48 hours after the exposure showed that *O. cornuta* was more sensitive than *B. terrestris* towards acute oral Sulfoxaflor exposure. Indeed, while only 30 % of the *B. terrestris* individuals died after being exposed to a sulfoxaflor dose of 0.563 µg/g b.w., this proportion was higher in the *O. cornuta* treated group, with 93.5% of mortality (Fig. 18; chi-square test, χ^2=33.05, df=1, p-value=8.9674e-09, n=91).

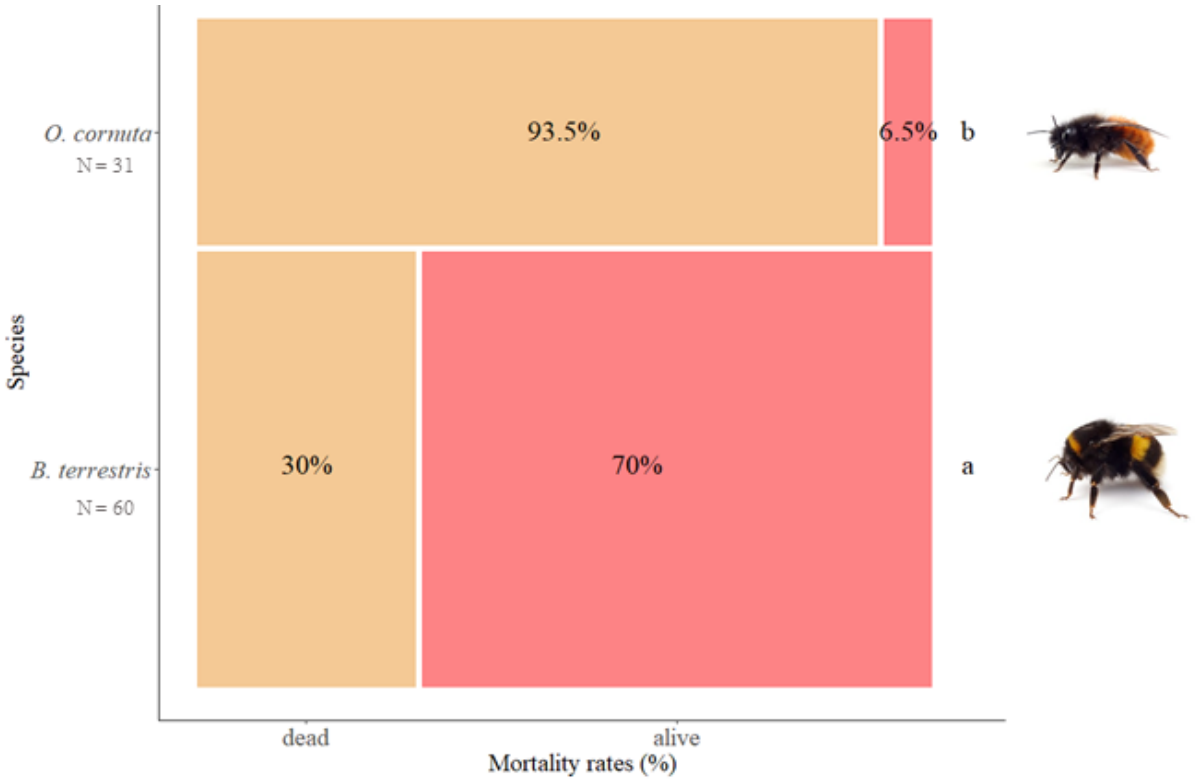


Fig. 18 – Mortality rates (%) of treated individuals 48H after ingestion of Sulfoxaflor (0.563 µg/g b.w.) for each species corrected with Sun-Shepard's formula. The width of the boxes is proportional to the sample size. Species that do not share the same letter have significantly different proportion of dead and alive individual 48 hours after the exposure at p-value<0.05 (Post-hoc, chi-square pairwise comparisons with Holm correction; picture Copyright © Mandy & Michael Fritzsche).

4.1.3. Topical acute sulfoxaflor exposure

Due to a lack of caught individuals, *O. cornuta*, *C. florisomme*, and *H. scabiosae* could not be topically exposed. However, besides the model species, *B. terrestris*, five species were topically exposed to acute sulfoxaflor dose of 43.9 µg/g b.w. (Tab. 5).

Tab. 5 – Average mass ± SE (g) and sample size (n) of the individuals topically treated and their control for the four species used and the *B. terrestris* model.

Species	Average mass ± SE (g)	
	Control	Sulfoxaflor
Bombus terrestris (model species)	0.255 ± 0.007 n=68	0.261 ± 0.007 n=67
Bombus hypnorum	0.165 ± 0.023 n=10	0.163 ± 0.017 n=12
Bombus pascuorum	0.134 ± 0.004 n=53	0.135 ± 0.004 n=57
Heriades truncorum	0.014 ± 0.001 n=19	0.014 ±0.001 n=18
Osmia leaiana	0.070 ± 0.008 n=4	0.067 ± 0.013 n=4
Osmia caerulescens	0.043 ± 0.003 n=10	0.042 ± 0.003 n=11

Two Bombus species, *B. hypnorum* and *B. pascuorum*; and three Megachilidae species, *H. truncorum*, *O. leaiana* and *O. caerulescens* were therefore topically exposed, and showed a significative effect of acute Sulfoxaflor exposure on their mortality (Tab. 6).

Tab. 6 – Effects of topical exposure to acute sulfoxaflor dose (43.9 µg/g b.w.) on the mortality rates of each species. χ^2 statistic, degrees of freedom and p-values are reported when a chi-square test was used, and 95%CI and p-values when a Fisher's exact test for count data was used. (Legend: Number of individuals; *expected number of individuals*; **proportion of individuals**)

Species	Treatments	Dead	Alive	Statistic	df	p-value
B. terrestris (model species)	Control	7	61	χ^2=46.09	1	1.1296e-11***
		26.19	*41.81*			
		10.3%	**89.7%**			
	Sulfoxaflor	45	22			
		25.81	*41.19*			
		67.2%	**32.8%**			
B. hypnorum	Control	2	8	95%CI=[0.8588, 101.7337]	/	0.0427*
		4.55	*5.46*			
		20%	**80%**			
	Sulfoxaflor	8	4			
		5.46	*6.55*			
		66.7%	**33.3%**			
B. pascuorum	Control	21	32	χ^2=44.94	1	2.0272***
		37.1	*15.90*			
		39.6%	**60.4%**			
	Sulfoxaflor	56	1			
		39.90	*17.100*			
		98.2%	**1.8%**			
O. caerulescens	Control	4	6	χ^2=9.24	1	0.0024**
		7.14	*2.86*			
		40%	**60%**			
	Sulfoxaflor	11	0			
		7.86	*3.14*			
		100%	**0%**			
O. leaiana	Control	0	4	95%CI=[1.3391, Inf.]	/	0.0286*
		2	*2*			
		0%	**100%**			
	Sulfoxaflor	4	0			
		2	*2*			
		100%	**0%**			
H. truncorum	Control	3	16	χ^2=26.71	1	2.3679e-07***
		10.78	*8.22*			
		15.8%	**84.2%**			
	Sulfoxaflor	18	0			
		10.22	*7.78*			
		100%	**0%**			

Significance level: *p<0.05; **p<0.01; ***p<0.001.

The Sun-shepard's corrected proportions of dead individuals 48 hours after the exposure for each species were then compared to *B. terrestris* (Fig. 19). Only *B. pascuorum* showed a significatively greater sensitivity with 98.2% (n_{tot}=57) of mortality 48 hours after the exposure (Fig. 19; Post-hoc, Fisher's pairwise comparison of proportions with Holm correction, p=0.0003).

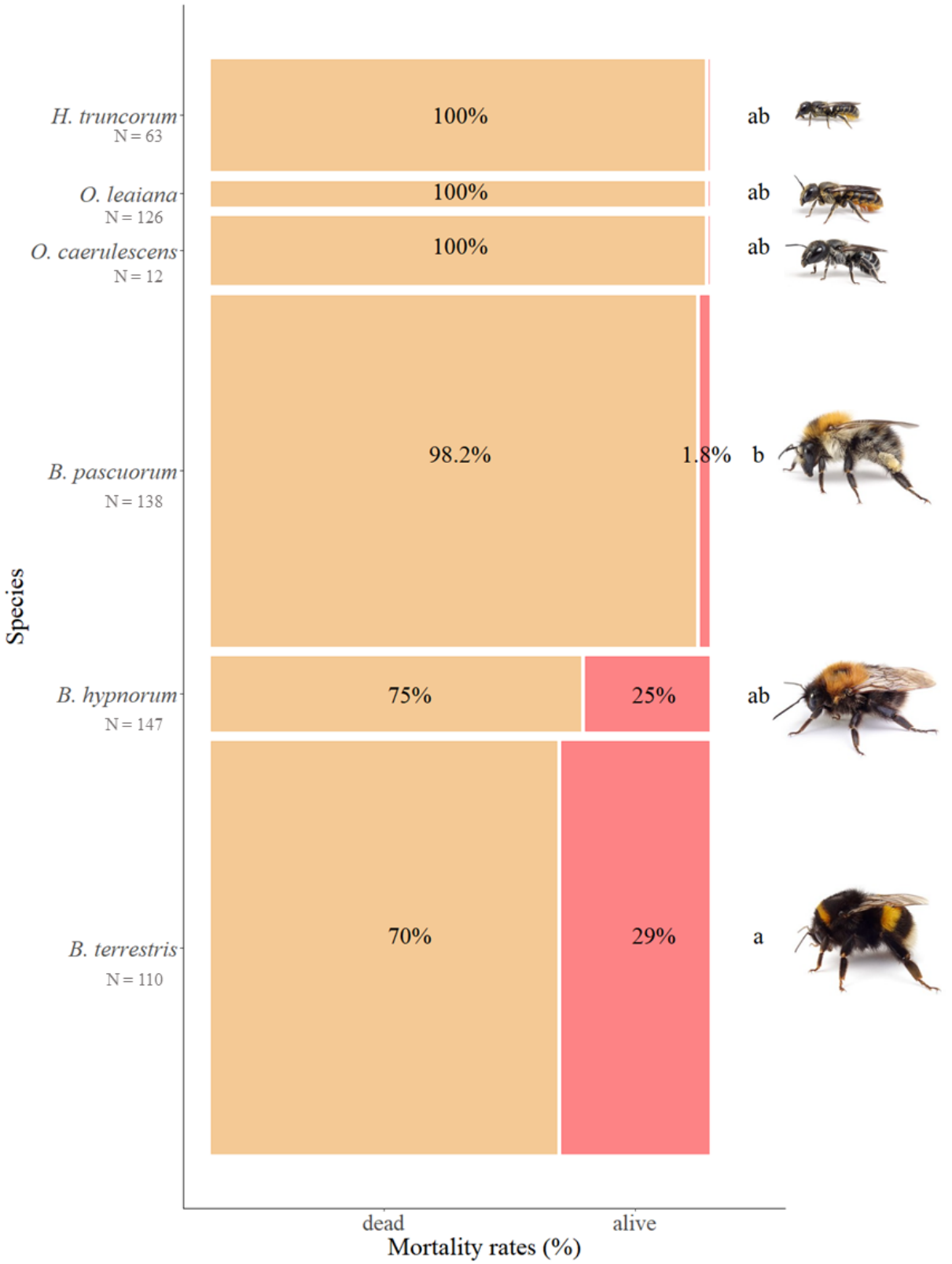


Fig. 19 – Mortality rates (%) of treated individuals 48H after exposure to sulfoxaflor (43.9 µg/g b.w.) for each species corrected with Sun-Shepard's formula. The width of the boxes is proportional to the sample size. Species that do not share the same letter have significantly different proportions of dead and alive individuals 48 hours after the exposure at p-value<0.05 (Post-hoc, Fisher's pairwise comparisons with Holm correction; picture credits: Mandy & Michael Fritzsche).

4.2. Effects of pesticides on feeding behaviour

Concerning all three doses, there was a significant global effect of the treatments on the response frequency (Tab. 7). Firstly, during the exposure to the first dose, the Amistar® and mixture treatment seemed to greatly increase the response frequency (Fig. 20A), more than the sulfoxaflor treatment did during the night (Fig. 20A; GLM negative binomial, Tukey's post-hoc test, p-values<0.001). Indeed, during the day, while the sulfoxaflor and Amistar® treatments significantly increased the response frequency in the same manner compared to the control treatment, the mixture increased the response frequency even more than both pesticides alone (Fig. 20A; GLM negative binomial, Tukey's post-hoc test, p-values<0.001).

Tab. 7 – Model coefficients for the effects of chronic exposure to Sulfoxaflor, Amistar® and a mixture between on the *B. terrestris* response frequency (response number per hour) during the night, and the day periods (GLM, negative binomial family with hour as random effect, and the number of alive worker in the box as offset).

Dose	Considered period	Treatments as levels	Estimates ± SE	χ^2	df	p-value
Dose 1	night	Amistar®=intercept	-1.06675 ± 0.04294	208.35	3	< 2.2e-16 ***
		control	-0.74243 ± 0.05535			
		mixture	-0.13267 ± 0.05314			
		sulfoxaflor	-0.32394 ± 0.05651			
	day	Amistar®=intercept	-0.81740 ± 0.02943	129.45	3	< 2.2e-16 ***
		control	-0.31595 ± 0.04056			
		mixture	0.10338 ± 0.03941			
		sulfoxaflor	-0.01325 ± 0.04171			
Dose 2	night	Amistar®=intercept	-0.92161 ± 0.03195	57.864	3	1.68e-12 ***
		control	-0.19971 ± 0.04544			
		mixture	-0.18342 ± 0.04200			
		sulfoxaflor	0.07341 ± 0.04117			
	day	Amistar®=intercept	-0.30818 ± 0.02420	300.91	3	< 2.2e-16 ***
		control	-0.11201 ± 0.03366			
		mixture	-0.16966 ± 0.03145			
		sulfoxaflor	0.31891 ± 0.03034			
Dose 3	night	Amistar®=intercept	-1.25087 ± 0.04357	295.85	3	< 2.2e-16 ***
		control	0.30737 ± 0.05324			
		mixture	-0.10843 ± 0.06450			
		sulfoxaflor	-0.58606 ± 0.05726			
	day	Amistar®=intercept	-0.595536± 0.035077	575.34	3	< 2.2e-16 ***
		control	0.007461± 0.044125			
		mixture	-0.594330± 0.054182			
		sulfoxaflor	-0.885370± 0.047179			

Significance level: ***p<0.001

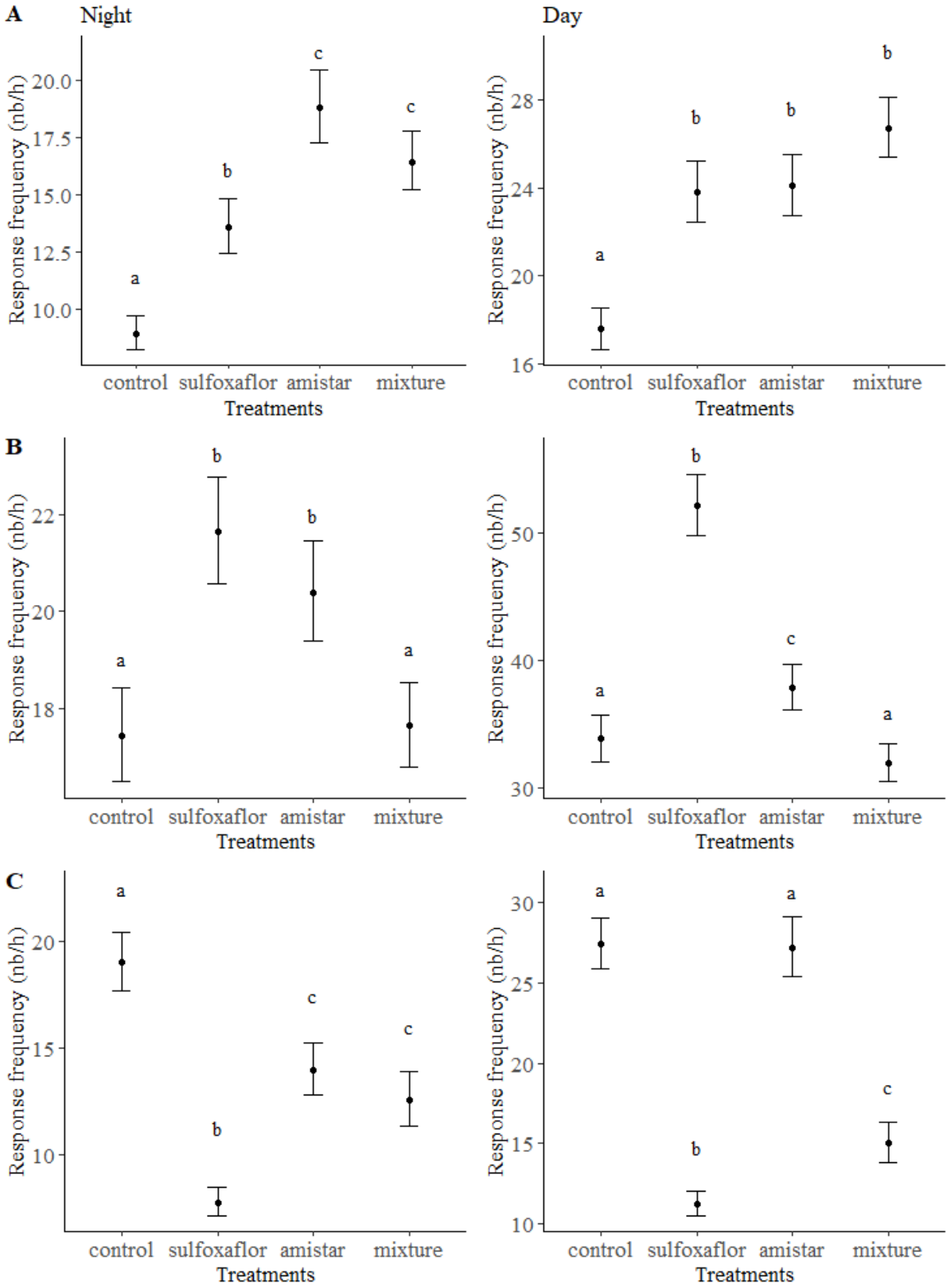


Fig. 20 – Effects of chronic exposure to Sulfoxaflor, Amistar® and a mixture of both pesticides on the *B. terrestris* response frequency (response number per hour) during the night, and the day periods for each dose. A) First set of doses: Sulfoxaflor 150ppb, Amistar® 2000ppb, and mixture; B) Second set of doses: Sulfoxaflor 300ppb, Amistar® 4000 ppb, and mixture; C) Third set of doses: Sulfoxaflor 600ppb, Amistar® 8000 ppb, and mixture. Raw values and error bars (95% CI) that are not sharing the same letter are significantly different at p-value <0.05 (GLM negative binomial family, Tukey post-hoc tests).

Then, concerning the second dose, while the mixture seemed to not have any significant effect on the response frequency (Fig. 20B; GLM negative binomial, Tukey's post-hoc test, $p\text{-value}_{night}=0.9850$, $p\text{-value}_{day}=0.3180$), Sulfoxaflor and Amistar® enhanced it, Sulfoxaflor increasing even more the response frequency (Fig. 20B; GLM negative binomial, Tukey's post-hoc test, p-values < 0.05).

During the exposure to the third and highest dose, all treatments significantly decreased the response frequency (Fig. 20C; GLM negative binomial, Tukey's post-hoc test, p-values > 0.05), apart from the group exposed to the Amistar® treatments during the day (Fig. 20C; GLM negative binomial, Tukey's post-hoc test, p-values=0.9980). In both case, Sulfoxaflor that has the strongest effect on the response frequency by decreasing it both during the night and the day (Fig. 20C; GLM negative binomial, Tukey's post-hoc test, p-values > 0.05).

5. Discussion

Our results confirmed that wild bees can present a higher sensitivity towards acute sulfoxaflor exposure than the currently used model species. Moreover, we showed that chronic exposure to field-realistic, and higher doses of pesticides alone and in a mixture can have sub-lethal impacts on the feeding behaviour of bees.

5.1. Impact of acute sulfoxaflor exposure

Most of the orally treated species were not affected by acute sulfoxaflor exposure, but *O. cornuta* still presented a higher sensitivity than the model species *B. terrestris*. Then, it also appeared that an acute dose of sulfoxaflor had a negative impact on all the species that were topically treated, except for *B. pascuorum* that presented a higher sensitivity than *B. terrestris*.

A meta-analysis of Arena & Sgolastra (2014), performed on 19 bee species commonly used in toxicological studies, showed that the sensitivity of most of those bees was lower than the sensitivity of the model species, *Apis mellifera*. However, to cover up a maximum of the wild bee variability in terms of sensitivity, they advised to multiply by 10 the LD50 values found for *Apis mellifera* (Arena & Sgolastra, 2014). Our results with other bee species seem to confirm this trend. Hence, a majority of the tested wild bee species were either not impacted by sulfoxaflor exposure, or were less affected than *B. terrestris*, the second model species. However, *O. cornuta* and *B. pascuorum* showed a sensitivity that was higher than *B. terrestris* to the oral and topical treatments.

Bees differ greatly in their ecology, and it could have been expected that these species, similar in terms of lectism, would be equally to less sensitive than *B. terrestris*. Indeed, *O. cornuta* is a polylectic spring bee that presents a solitary lifestyle (Peeters *et al.*, 2012). Therefore, it was expected that this solitary bee would be more robust than a social species. Hence, social species, such as *B. terrestris*, benefit from a colonial immunity. Complex behavioural, physiological and spatial mechanisms thus prevent the colony from being exposed to xenobiotics. This would probably make isolated individual more sensitive (Cremer *et al.*, 2007). However, in this study, a solitary species, *O. cornuta*, was more sensitive than the social one, *B. terrestris*. Moreover, another social and polylectic species, *B. pascuorum*, was more sensitive than *B. terrestris* to sulfoxaflor topical exposure.

The high variability in terms of pesticide sensitivity found amongst bees is complicated to explain. Indeed, several intra- and inter-specific differences in the physiological, and morphological traits can influence the sensitivity of a bee. First, at the intra-specific level, van der Steen (1994) showed an intra-specific correlation between the body weight and the sensitivity towards pesticides. It was also shown that the inter-specific sensitivity was 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, concerning the topical applications of pesticides, the sensitivity can vary as the cuticle composition varies. Indeed, the first barrier protecting the insect body from external perturbation is the chitinous cuticle that covers its whole body (Reece & Campbell, 2011). However, the insecticides are designed with the aim of easily penetrating this barrier and killing the insect pests. The effect of an insecticide depends on the rate at which it will enter the insect body. Therefore, the surface-to-volume ratio, thickness, cellular and molecular components of the cuticle can play a determining role in the sensitivity of an insects towards pesticides (Lewis, 1980). *O. cornuta* and *B. pascuorum* were more sensitive than *B. terrestris* while sharing some ecological traits. However, their differences in terms of size, and cuticle composition could explain their higher sensitivity.

Another characteristic that can influence the sensitivity is directly related to the detoxification mechanisms. Indeed, some species have the physiological ability to detoxify toxins, such as alkaloids contained in the floral resources of some plant species. It could therefore help with the detoxification of some pesticides, such as the neonicotinoid (Cresswell *et al.*, 2012). The sensitivity of *H. scabiosae*, *H. truncorum*, and *O. leaiana* were not higher than the sensitivity of *B. terrestris*. Indeed, these are oligolectic on the Asteraceae family, that has been shown to provide toxic secondary compounds through their pollen and nectar (Vanderplanck *et al.*, 2020).

Finally, once inside the bee body, some pesticides can already be degraded thanks to the pH of the haemolymph that can differ among bees. For example, while the honeybee pH was measured at 6.0, the *Megachile rotundata* pH was measured at 6.8. They can therefore detoxify some pesticides at different rate depending on the species (Ahmad & Johansen, 1973). Therefore, analysing the haemolymph of the species that were less sensitive than *B. terrestris*, such as *B. hypnorum*, *B. pascuorum* (oral exposure only), and *A. vaga*, could give

more explanations about the underlying mechanisms of the detoxification efficiency, and variation in terms of sensitivity.

While a majority of the treated bee species were shown as being less sensitive than *B. terrestris*, these results need to be analysed carefully. Indeed, for some species such as *H. scabiosae*, *H. truncorum*, *O. leaiana* or *O. caerulescens*, the sample size was way lower than recommended by the OECD guidelines (OECD, 2017b, 2017a). Increasing the sample size in those cases could probably make some additional effects appear.

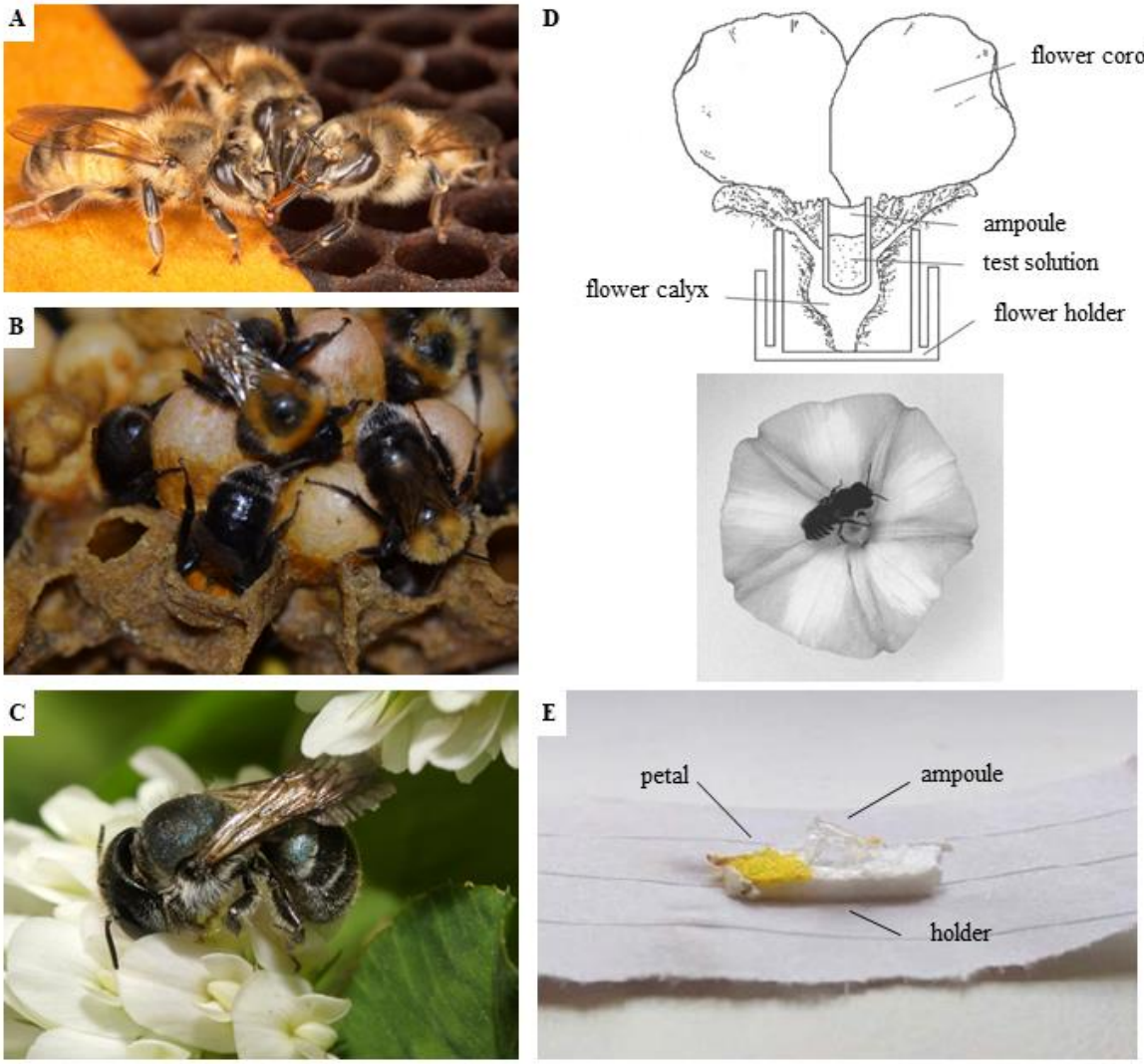


Fig. 21 – Feeding behaviour of bees, and laboratory feeding methods for wild bees. Bee feeding behaviour: A) A group of *Apis mellifera* workers performing group feeding, i.e. trophallaxis (picture credits: U.S. Department of Agriculture), B) workers of *Bombus hypnorum* filling/feeding on nectar stocks of the colony (picture credits: Simon Merrifield), and C) *Osmia caerulescens* foraging on *Trifolium* sp. (picture credits: Nicolas Vereecken). Two laboratory feeding methods for solitary bees: D) replacing the reproductive column of a flower with an ampoule filled with the solution that needs to be tested (cross-section drawing and photography of *Megachile rotundata* on the artificial flower adapted from Ladurner *et al.*, 2003), and E) adding a petal as visual clue ahead of the ampoule containing the test solution (photography adapted from Medrzyck *et al.*, 2021).

As shown by the laboratory condition survival tests, wild bees are difficult to maintain in captivity. Therefore, a high number of individuals needs to be taken from the wild to compensate, which is not always possible for small bee communities, and bees that do not nest in aggregation. Moreover, the high percentage of non-feeders found during the oral exposure experiment, and the high mortality rate found in the control groups further diminished the sample size, and showed that the laboratory conditions must be improved to allow toxicological tests on a larger spectrum of wild bee species. For further experiments, in order to increase the sample size, some methods that have been developed to adapt the feeding of bees to their natural feeding behaviour (Fig. 21-A, B, and C) 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* (Fig. 21-D; Ladurner *et al.*, 2003). Difficult to install, and to adapt to specialist bees, this process has been improved by the "petal method" that seemed to increase the feeding success, and to be easily set up. This method consists of using a single petal as visual clues to indicate the treatment solution and facilitate the feeding of solitary species (Fig. 21-E; Hinarejos *et al.*, 2014). To increase the survival under laboratory conditions, the stress caused by the catching in the wild could be avoided by rearing the species under laboratory conditions, from the first larval stages until the emergence (Eeraerts *et al.*, 2020). However, these methods are only known for a few species (Peterson & Artz, 2014), and would have taken more than a year, what could not be done for this project.

5.2.Impact of pesticides on feeding behaviour

Our results highlighted that a chronic oral exposure to sulfoxaflor, and azoxystrobin (Amistar®) alone, as well as in a mixture can impact the feeding behaviour of *Bombus terrestris*. At field-realistic doses, and when increasing these doses twice, the pesticides and mixtures boosted the feeding behaviour by increasing the frequency of responses (nb/hour). In contrast, at the highest doses tested (four times the field-realistic doses), the response frequency was reduced, and adverse effects on the feeding behaviour of the bees appeared. Sulfoxaflor presented stronger effects than Amistar®. However, when mixed together, the effects were either equal or weaker than the effects of sulfoxaflor alone, without cancelling these effects.

Siviter and colleagues (2020) hypothesized that the decrease in egg-laying found during micro-colony exposure to sulfoxaflor was caused by a diminution of the syrup

consumption. Therefore, the repeated exposure to sulfoxaflor could have adverse effects on the colony fitness, and reproductive performances (Siviter, Brown, *et al.*, 2018). Our results concerning sulfoxaflor exposure are in line with this hypothesis, as sulfoxaflor exposure can actually impair the syrup consumption of *B. terrestris*. Sulfoxaflor presents a similar mode of action than neonicotinoids, acting as nAChRs agonists (Zhu *et al.*, 2011). In fact, neonicotinoid exposure showed inactivation effects on the mushroom body of bee brain, a crucial part for their learning and memory (Menzel, 2012; Palmer *et al.*, 2013). For example, Acetamiprid, an insecticidal molecule from the family of neonicotinoids, was shown to increase the water-triggered reflex when applied topically (El Hassani *et al.*, 2008). El Hassani and colleagues (2008) then hypothesized that this molecule could have effects on bee thirst by inhibiting the satiety feeling. Despite the fact that the exposure to sulfoxaflor was oral, a similar effect could have appeared during our experiments. However, when the doses were increased four times, adverse effects of sulfoxaflor exposure appeared. This confirms that sulfoxaflor exposure has an impact on the behaviour of bees probably through an impairment of the bee cognitive system. Similar results have been found for molecule of the neonicotinoid family. For example, proboscis extension reflex assays and analogue tests of radial-arm maze on bees already revealed that acute and chronic neonicotinoid, thiamethoxam, exposure negatively impacts the cognition of bees (Samuelson *et al.*, 2016; Stanley *et al.*, 2015). In contrast, for acute sulfoxaflor exposure, the same tests were made and no impairment of the bumblebee learning and memory abilities were highlighted (Siviter *et al.*, 2019). Nevertheless, these tests were only performed for acute exposure, but chronic exposure have been shown to induce larger effects on bee cognition (Siviter, Koricheva, *et al.*, 2018).

Concerning the adverse effects on the feeding behaviour of the bees treated with Amistar®, other studies showed that molecules from the strobilurin family can negatively impact the digestive system of bees (Bartlewicz *et al.*, 2016; Campbell *et al.*, 2016; Degrandi-Hoffman *et al.*, 2015). Indeed, these molecules have shown adverse effects on digestive organs and cells, as they areknown to halt the ATP production in mitochondria of fungicidal cells (Bartlewicz *et al.*, 2016). As a matter of fact, Piraclostrobin, another strobilurin molecule, was shown to cause cytotoxic effects on bee midgut epithelial cells which may lead to malnutrition and bad nutrient absorption (Degrandi-Hoffman *et al.*, 2015). Therefore, our results confirm that the feeding behaviour of the bees is impacted by the commercial formula of the fungicide, azoxystrobin. Indeed, the increase in response frequency observed with low

doses reinforces the hypothesis of a digestion impairment caused by the fungicide. At low doses, the bee would need to absorb higher quantity of food than usual to balance the adverse effect. However, at high doses, if the adverse effects become too strong, it would be impossible to compensate for the lack of food absorption.

A last hypothesis about the increasing effect encountered at field realistic doses is that, as it already has been demonstrated with Imidacloprid and Thiamethoxam, bees could prefer food containing sulfoxaflor, or Amistar® (Kessler *et al.*, 2015). Therefore, at low doses, they would ask for food more frequently, resulting in the observed increase in the response frequencies. However, at too high doses, the adverse effects of pesticides would take over the taste, and be detected, explaining the decrease in response frequency at high pesticide doses. However, this hypothesis would need to be tested in experiments during which bees can choose between a contaminated, and a non-contaminated food source. Nevertheless, as highlighted by Kessler and his team (2015), if it was found out that bees prefer food with these pesticides, it would mean that in nature, they could not avoid feeding on treated flowers by themselves, even though it would be toxic for them.

Finally, a great number of studies highlighted synergistic effects of mixtures between insecticides and fungicides (Iverson *et al.*, 2019; Pettis *et al.*, 2013; Robinson *et al.*, 2017; Sgolastra *et al.*, 2017; H. M. Thompson *et al.*, 2014; Wade *et al.*, 2019). However, our results actually showed that the presence of Amistar® in the mixture lowered the effects of sulfoxaflor on the feeding behaviour of bumblebees.

However, our understanding of the underlying mechanisms of the impairment of the bee food intake is here limited to the observation of bee behaviour. In order to assess of the exact effects and mode of action of those molecules on bee cognition and digestive system, more studies are needed. However, the effects already highlighted in this study could be deleterious on the long run not only for the individual, but also for bee communities.

6. Conclusion and perspectives

While the current pesticide risk assessments mainly focus their efforts on mortality tests performed on a small number of bee species, of which *B. terrestris*, this study firstly showed that wild bees can present a higher sensitivity towards acute pesticide exposure. The use of automatic food dispensers also confirmed that the chronic oral exposure to field realistic doses of pesticides can affect the feeding behaviour of bees. Therefore, whereas more wild bee models are needed to better cover toxicity risks of pesticides, the sub-lethal effects should also be taken into account. Indeed, those can impact not only the individual, but also the species on the long-run.

This kind of project opens a great variety of further analyses. Indeed, *haemolymph* analyses were not included in these experiments, but molecular, proteomic and immunological analysis could help in understanding the mechanisms underlying the variation of the sensitivity amongst bee species, as well as the effects of a chronic exposure. Moreover, once the laboratory conditions for the captivity of other wild bee species will be known, more comparison could be done.

In this study, we managed to monitor the feeding behaviour of *B. terrestris* through automatic food dispensers and artificial flowers. Therefore, this method could be applied to several different Bombus species at first, and be extended to semi-social and non-territorial solitary bees. A great variety of chemicals, and mixtures could also be tested, and their effects on bee feeding behaviour assessed.

A big gap of knowledge about the pesticide lethal and sub-lethal effects on bees remains to be filled. Indeed, while the diversity of plant protection products is increasing due to the emergence of pest resistances, their toxic effects are mainly studied on a single species, *Apis mellifera*. Fortunately, the risk assessments recently began to include non-Apis species, such as *B. terrestris* and *O. bicornis*. However, the high ecological, morphological and physiological variability found amongst the 20,000 species urge the development of more tests.

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8. Appendices

8.1. Appendix 1 – Pesticide acute exposure of the wild bees

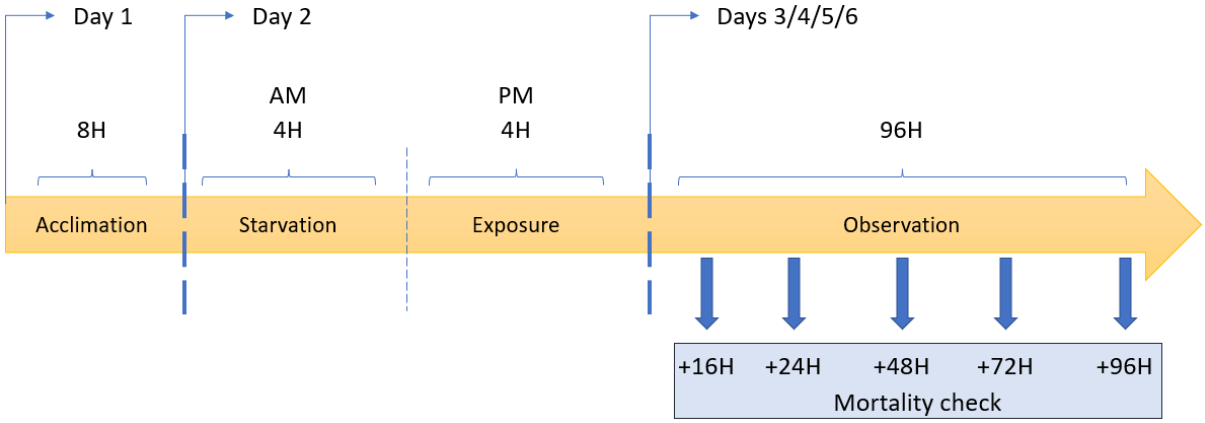


Figure 1 – Timeline of the acute oral exposure experiment.

Figure 2 – Syrup consumption by an *Andrena vaga* female during an acute oral exposure experiment.

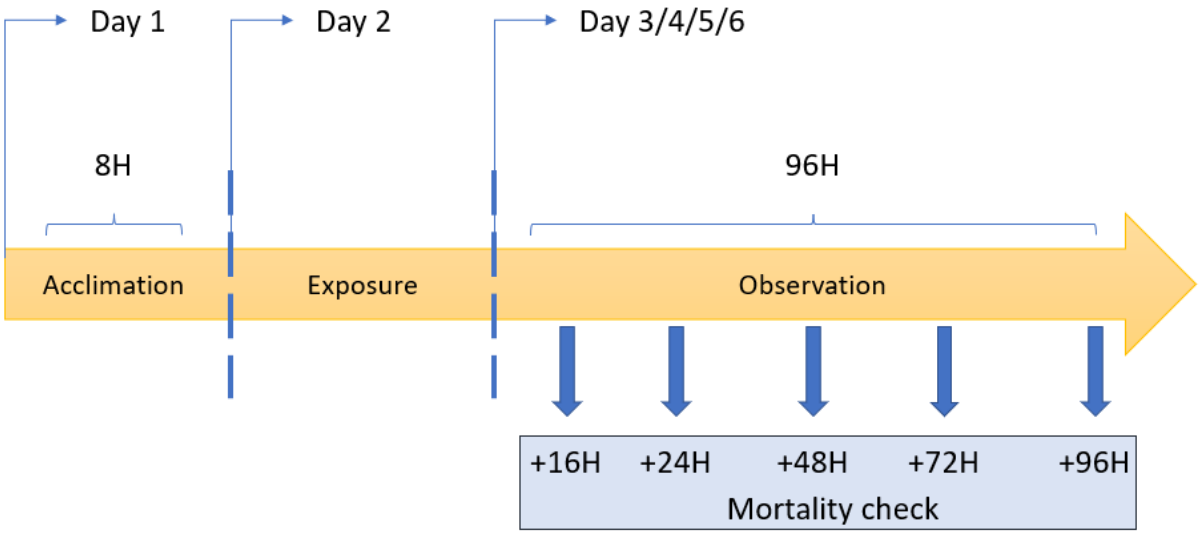


Figure 3 – Timeline of the acute topical exposure experiment.

8.2. Appendix 2 – Complete results of *Bombus terrestris* exposure

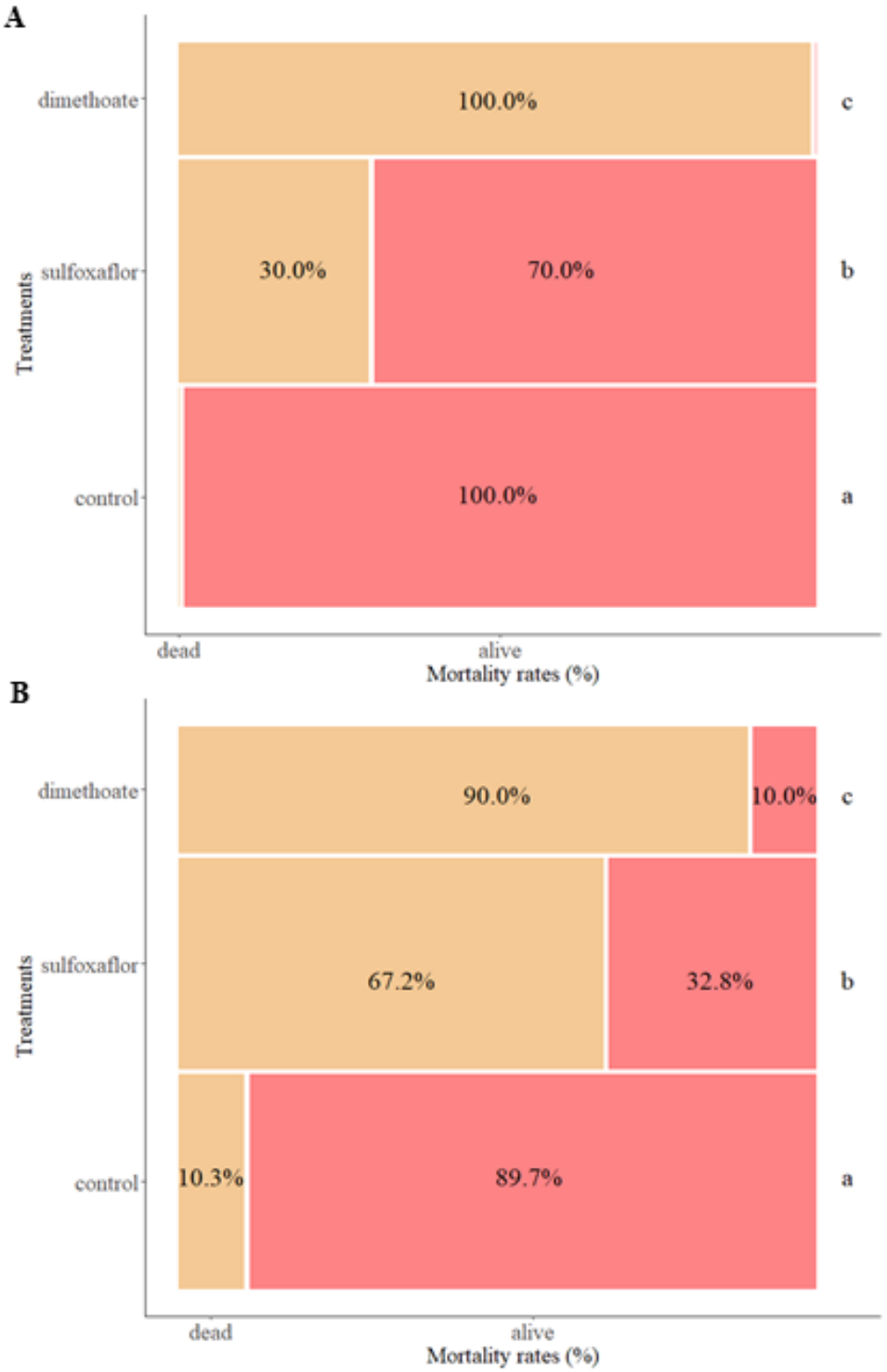


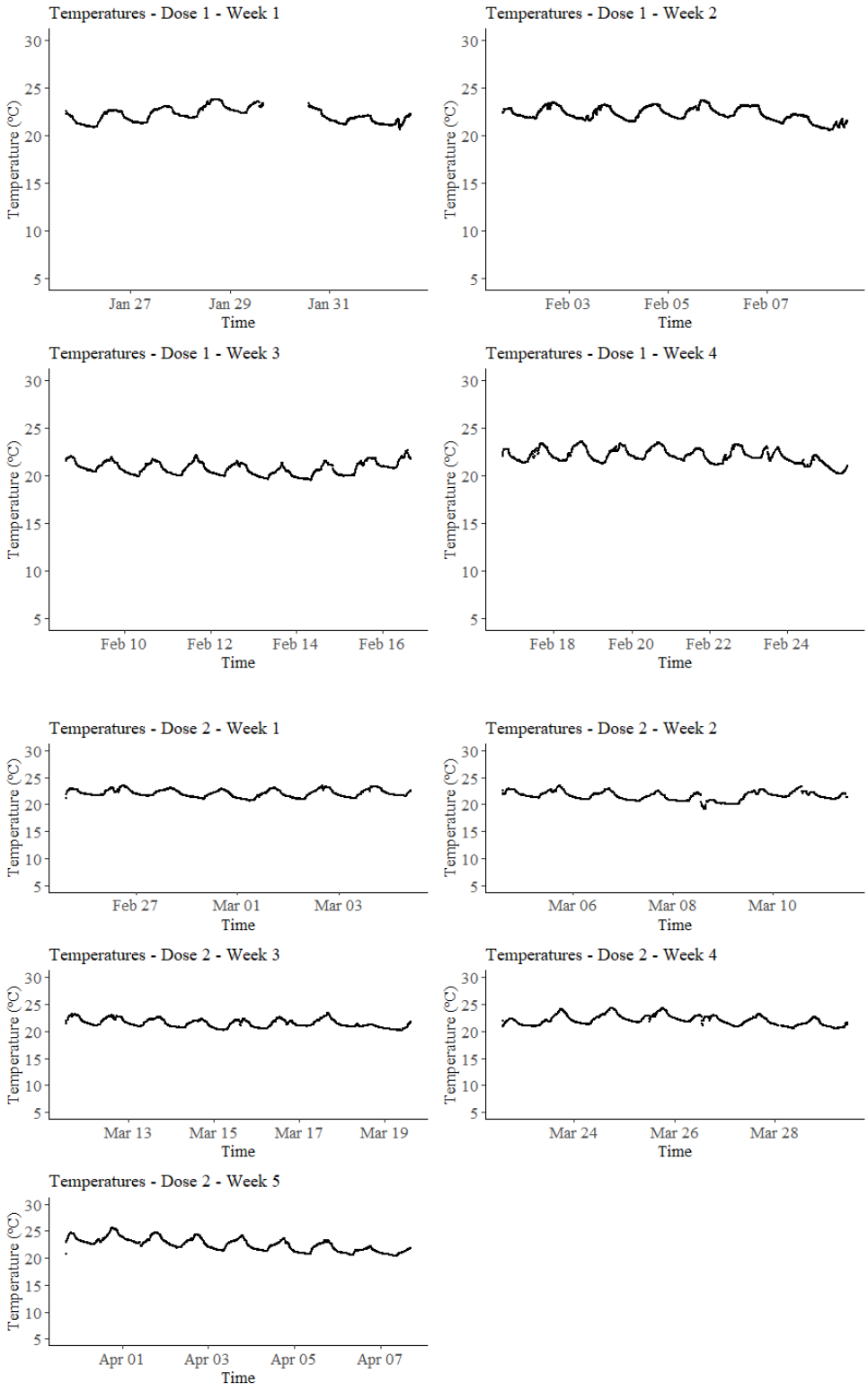
Figure 4 – Acute exposure sulfoxaflor of *B. terrestris*, the model species. A) Oral exposure results, and B) topical exposure results for *B. terrestris*. Dimethoate was used as positive control, and the negative control consisted into a 50% v/v water sugar solution with 0.05% acetone. Within each graph, the treatments that do not share the same letter are different at p-value<0.005 (Post-hoc, Pairwise comparisons using Pairwise comparison of proportions (chi-squared) with Holm correction).

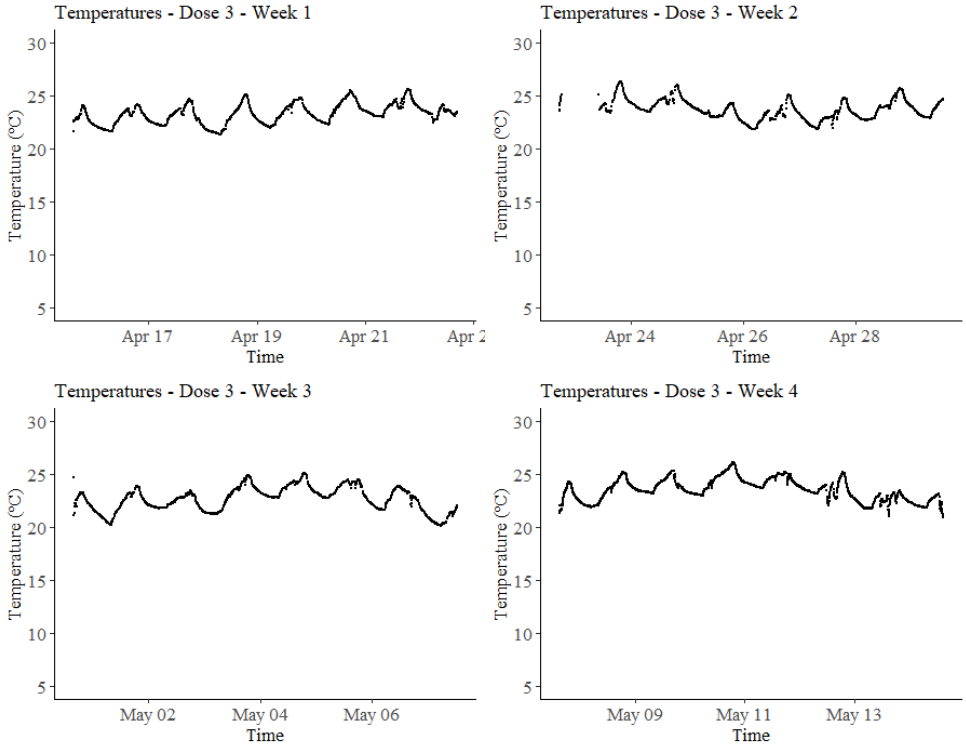
Table 1 – Effects of acute sulfoxaflor exposure on the mortality rates of *B. terrestris*. (Legend: Number of individuals; *expected number of individuals*; proportion of individuals)

Exposure	Treatments	Dead	Alive	χ^2	df	p-value
Oral	Control	0	59	91.299	2	1.494828e-20***
		19.01	*39.99*			
		0%	**100%**			
	Sulfoxaflor	18	42			
		19.33	*40.67*			
		30.0%	**70.0%**			
	Dimetoate	30	0			
		9.66	*20.34*			
		100.0%	**0.0%**			
Topical	Control	7	61	76.375	2	2.602882e-17***
		34.19	*33.80*			
		10.3%	**89.7%**			
	Sulfoxaflor	45	18			
		33.69	*33.31*			
		67.2%	**32.8%**			
	Dimetoate	36	4			
		20.11	*19.89*			
		90.0%	**10.0%**			

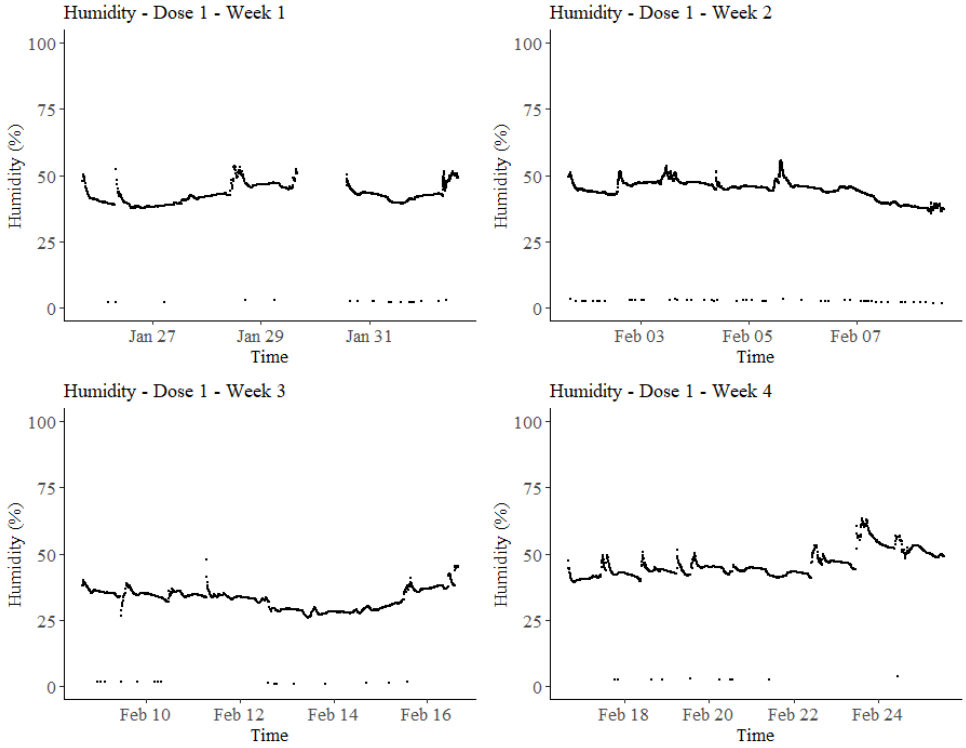
8.3.Appendix 3 – Monitoring of the automatic dispenser room

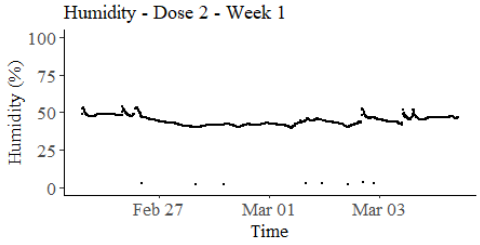
8.3.1. Temperatures

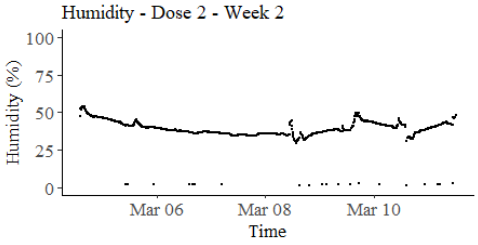


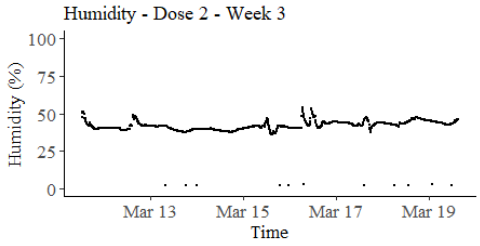


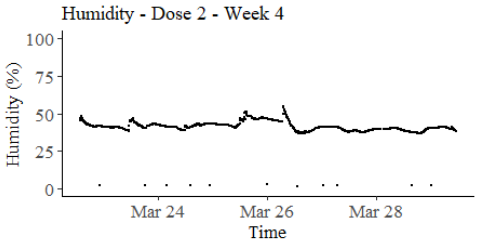
8.3.2. Humidity

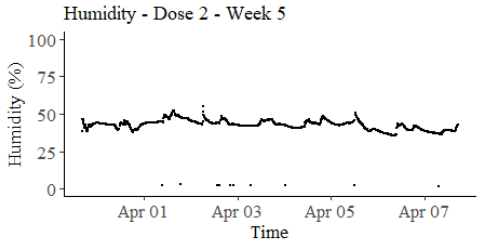


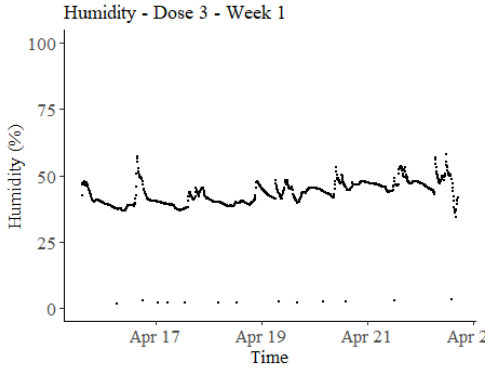

Humidity - Dose 2 - Week 1
Humidity (%)
100
75
50
25
0
Feb 27
Mar 01
Mar 03
Time

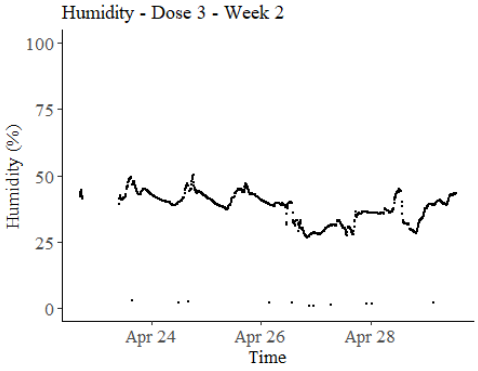

Humidity - Dose 2 - Week 2
Humidity (%)
100
75
50
25
0
Mar 06
Mar 08
Mar 10
Time

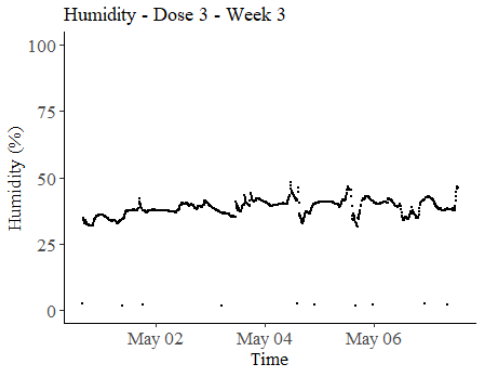

Humidity - Dose 2 - Week 3
Humidity (%)
100
75
50
25
0
Mar 13
Mar 15
Mar 17
Mar 19
Time

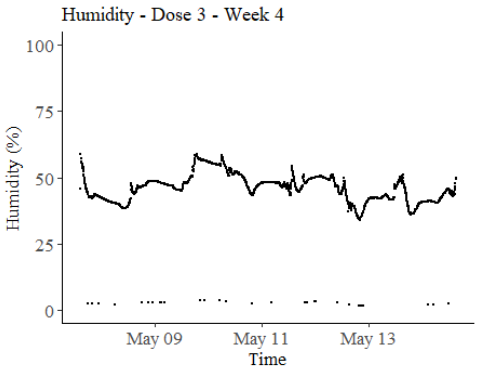

Humidity - Dose 2 - Week 4
Humidity (%)
100
75
50
25
0
Mar 24
Mar 26
Mar 28
Time


Humidity - Dose 2 - Week 5
Humidity (%)
100
75
50
25
0
Apr 01
Apr 03
Apr 05
Apr 07
Time


Humidity - Dose 3 - Week 1
Humidity (%)
100
75
50
25
0
Apr 17
Apr 19
Apr 21
Time


Humidity - Dose 3 - Week 2
Humidity (%)
100
75
50
25
0
Apr 24
Apr 26
Apr 28
Time


Humidity - Dose 3 - Week 3
Humidity (%)
100
75
50
25
0
May 02
May 04
May 06
Time


Humidity - Dose 3 - Week 4
Humidity (%)
100
75
50
25
0
May 09
May 11
May 13
Time