Manuscript on single and combined effects of key chemical and other stressors on bees under semi-field conditions

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

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Table of contents

Summary

A series of semi-field experiments were conducted to examine the single and combined impacts of key stressors (different pesticides and nutritional stress) on three model bee species, the Western honey bee (*Apis mellifera*), the buff-tailed bumble bee (*Bombus terrestris*) and the solitary red mason bee (*Osmia bicornis*). In a first set of semi-field experiments, the same full-factorial design was used across all three model bee species to assess the single and combined impact of the insecticide Closer (active ingredient sulfoxaflor) and the fungicide Amistar (active ingredient azoxystrobin). In each experiment, a total of 40 flight cages, planted with purple tansy (*Phacelia tanacetifolia*), were randomly assigned to one of the following four treatments (spray applications): 1) Closer, 2) Amistar, 3) Closer + Amistar (mix), 4) water only control. Closer (sulfoxaflor) significantly reduced colony growth (weight gain, number of bees) and foraging performance of *B. terrestris*; no major impacts were found on colony performance of *A. mellifera* or survival and reproductive success of *O. bicornis* when Closer was applied before crop flowering (≥2-6 days). The fungicide Amistar (azoxystrobin) reduced foraging performance of *B. terrestris*, with negative consequences on pollination service provisioning and tended to impair nest finding performance of *O. bicornis*, but no significant impacts on colony performance or foraging behaviour of *A. mellifera* were found. Moreover, no significant synergistic interactive impacts of the two pesticides were detected in any of the model bee species. However, some measures of foraging performance of *O. bicornis* were affected by antagonistic interactions among the two pesticides. In a further set of semi-field experiments, the role of food stress in shaping impacts of pesticides on bees was investigated. In *O. bicornis* the impact of Sivanto prime (active ingredient flupyradifurone) was assessed for bees foraging on different food plants varying in quantity and quality of floral resources. Buckwheat offered, compared to the other two plant species, purple tansy and wild mustard, low quantity and quality of floral resources, and exacerbated adverse impacts of Sivanto prime. Adult mortality of *O. bicornis* feeding on buckwheat sprayed with Sivanto prime was much higher compared to control bees within the first day of application, while it was similar in exposed and control bees foraging on the other two plant species. Similar negative synergistic impacts of Sivanto exposure and food stress were found on offspring production, flight activity, flight duration and flower visitation frequency of *O. bicornis*. In *B. terrestris*, combined effects of nutritional stress and exposure to Amistar (azoxystrobin) were studied in cages planted with purple tansy, buckwheat and a mix of multiple foraging plants. Exposure to Amistar reduced colony growth of *B. terrestris*, corroborating findings of the previous semi-field experiment, but only in cages planted with monocultures of purple tansy, but not when bumble bees foraged on buckwheat or mixes of multiple foraging plants. Further, in *A. mellifera*, the effect of Amistar was tested for bees foraging on purple tansy and buckwheat. Preliminary results indicate that Amistar tended to reduce foraging performance of *A. mellifera*, irrespective of the floral resource. In a semi-field dose-response experiment in which different doses of the fungicide Ortiva (active ingredient azoxystrobin) were administered to bumble bee (*B. terrestris*) colonies via syrup, a reduced colony weight gain and higher mortality compared to control colonies was found, but only for colonies exposed to a dose representing a four times higher exposure than a field-typical dose.

These findings show that impacts of pesticide products tested under semi-field conditions can vary substantially among different model bee species. Adverse impacts of Closer (sulfoxaflor) were only detectable in bumble bees (*B. terrestris*), which also showed the strongest negative responses to Amistar (azoxystrobin). However, slight differences the experimental procedures, such as the timing of spray applications of Closer, may have contributed to these varying impacts on the three different bee species. Furthermore, the findings of the semi-field studies assessing effects of pesticide-nutrition interactions on *O. bicornis* and *B. terrestris* clearly highlight that impacts of pesticides can vary across different forage plants, and that such differences should be considered in higher-tier risk assessment schemes. Our experiments underpin recommendations of the European Food Safety Authority to evaluate risks of pesticides in multiple crops (EFSA 2013). The results of the semi-field experiment on the impact of Sivanto prime (flupyradifure) on the solitary bee species *O. bicornis* further highlight how

nutritional stress can substantially augment the adverse impacts of pesticides on bee survival, reproductive success and foraging behaviour.

1. Introduction

Pollinators provide essential pollination services to wild plants (Ollerton *et al*. 2011) and many globally important crops (Klein *et al*. 2007; IPBES 2016). Managed and wild bees are the most important pollinators in most parts of the world, including Europe (Kleijn *et al*. 2015). With roughly 20,400 described species, bees are a highly diverse group of insects, encompassing a range of morphological differences, nesting behaviours, life-histories and foraging behaviours (Michener 2000). However, while global demand for pollination services increases (Aizen *et al.* 2019), declines of both wild and managed pollinators are reported in different parts of the globe (Cameron *et al.*, 2011; IPBES 2016; Powney *et al.*, 2019), and a high percentage of wild bees are listed as threatened or endangered species on Red Lists, such as the Red List of bees of Europe (Nieto *et al.* 2014).

Bees are threatened by multiple, potentially interacting stressors, in particular in intensively managed agroecosystems. Stressors include pesticide exposure, loss and degradation of habitat and associated loss of floral food resources, exposure to parasites and pathogens, as well as climate change (e.g., Vanbergen *et al*. 2013; Goulson *et al.* 2015; Potts *et al.* 2016). An improved understanding of the interactive effects of multiple stressors and their consequences for bee health under (semi-)natural conditions is crucial to be able to develop effective policies to protect bee pollinators (Potts *et al.* 2016).

Pesticide exposure is considered a major threat to bees (IPBES 2016; Potts *et al.* 2016; Dicks *et al.* 2021). For example, neonicotinoid insecticides have been shown to have manifold harmful effects on the behaviour and fitness of both managed and wild bees (Henry *et al.* 2012; Godfray *et al.* 2014; Sanchez-Bayo and Goka 2014; Pisa *et al.* 2015; Rundlöf *et al.* 2015; Woodcock *et al.* 2016; Siviter *et al.* 2021a). While this resulted in a ban of three neonicotinoids for outdoor use in the EU, these systemic insecticides are still widely used around the world. At the same time, new insecticides have been introduced and there is concern about potentially similar adverse impacts on bee health (Brown *et al.* 2016; Siviter *et al.* 2018; Siviter & Muth 2020). Yet, insecticides are not the only group of agrochemicals that may impair bee health. Herbicides and fungicides are heavily used around the globe, and evidence is increasing that some of them can also have negative impacts on bees (e.g., Artz and Pitts-Singer 2015; Bernauer *et al.* 2015; Mao *et al.* 2017; Cullen *et al.* 2019; Belsky and Joshi 2020); not only the active ingredients themselves, but also co-formulants contained along with the active ingredients in these products may be responsible for these impacts (e.g., Straw and Brown 2021).

In fact, bees are typically exposed to multiple pesticides in agricultural landscapes (Sanchez-Bayo and Goka 2014; Tosi *et al.* 2018), and evidence is increasing that different pesticides, such as insecticides and fungicides, can additively or even synergistically interact with each other, thereby reinforcing negative impacts on bee health (Iwasa *et al.* 2004; Johnson *et al.* 2013; Sgolastra *et al.* 2018; Carnesecchi *et al.* 2019; Siviter *et al.* 2021).

Moreover, the loss of appropriate floral resources is considered a major driver of reported bee declines (Biesmeijer *et al.* 2006; Scheper *et al.* 2014; Goulson *et al.* 2015; IPBES 2016). Bees are fully dependent on nectar and pollen from flowering plants (Michener 2000). Nectar is the main source of energy in the form of carbohydrates, whereas pollen offers essential micro- and macronutrients, e.g., proteins, lipids, vitamins, sterols and minerals (Brodschneider and Crailsheim 2010; Nicolson 2011, Filipiak 2018; Wright *et al.* 2018). Suitable nutrition is key for bees' immunocompetence and their ability to cope with pesticide exposure (e.g., Alaux *et al.* 2010; Foley *et al.* 2012; Di Pasquale *et al.* 2013; Schmehl *et al.* 2014; Barascou *et al.* 2021; Crone and Grozinger 2021; Linguadoca et al. 2021). In fact, laboratory studies have provided evidence that poor nutrition and pesticide exposure may synergistically impair bee health (Siviter *et al.* 2021b). For example, honey bees with limited access to carbohydrates or pollen showed an increased susceptibility towards insecticides, while bees that fed on a diet with a low protein to lipid ratio were more resilient to pesticide exposure (Schmehl *et al.*, 2014; Tosi *et al.*, 2017). Further, the detoxification of pesticides from a bee's body is energetically

costly, making high quality nutrition crucial to support this process (Berenbaum and Johnson 2015). Indeed, diets of suitable quality and quantity of proteins and lipids, as well as secondary metabolites, can enhance the expression of detoxification genes and may increase the survival and performance of insecticide-exposed bees (Mao *et al.* 2011; Johnson *et al.* 2012; Schmehl *et al.* 2014; Liao *et al*. 2017; Ardalani *et al.* 2021; Crone and Grozinger 2021). Pesticides, in turn, can affect food consumption rates and foraging success of bees, which may reinforce negative impacts of nutritional stress (Thompson *et al.* 2015; Stanley and Raine 2016; Sgolastra *et al.* 2018; Barraud *et al.* 2020; Vodovnik *et al.* 2021).

However, most of the evidence outlined above for interactive effects of different pesticides, such as between insecticides and fungicides, or between pesticide exposure and nutritional stress on bees, comes from studies conducted under laboratory conditions (Siviter *et al.* 2021b; Straub *et al.* 2022), while studies examining such interactive effects under (semi-)field conditions are scarce, but urgently needed (Lehmann and Camp 2021). Laboratory studies are essential to examine mechanistic relationships of interactive effects of specific stressors on bee health under controlled conditions (Medrzycki *et al.* 2013). Consequently, laboratory assessments using standard protocols have traditionally been the cornerstone of regulatory risk assessments processes (e.g., EFSA 2014; OECD 1998). However, the advantages of reducing complexity and excluding variation comes at a high price. Ignoring factors that characterise real-world systems and uncertainty about field-realism of pesticide exposure levels used in laboratory experiments may lead to unrealistic effect sizes and potentially incorrect conclusions about the existence and magnitude of impacts of multiple-stressor interactions on bees (Sgolastra *et al.* 2020; Van Oystaeyen *et al.* 2020; Topping *et al.* 2021). Moreover, certain key response variables can only be reliably assessed through (semi-)field studies. In particular, it is crucial to examine impacts of interactions of stressors on the reproductive success and fitness of bees, and thus their likely consequences on colony or population dynamics (Straub *et al.* 2020). This requires experimental settings, typically (semi-)field settings, in which bees can nest, forage and provision their offspring (Sgolastra *et al.* 2020; Van Oystaeyen *et al.* 2020).

A better understanding of field-realistic impacts of pesticides on bees has also been hampered by the fact that higher-tier (semi-)field assessments have largely focused on only one bee species, the Western honey bee, *Apis mellifera* (EFSA 2013; EPA 2014). However, levels and pathways of exposure to individual and combined stressors, as well as a bee's sensitivity to them, may strongly depend on specific life-history traits (Arena and Sgolastra 2014; Uhl *et al.* 2016; Kopit and Pitts-Singer 2018; Kopit *et al.* 2021). For example, solitary and social bee species differ in physiology (e.g. detoxification ability; Hayward *et al.* 2019), activity period, nesting duration, body size, foraging range, food plant preference and specialisation, level of pollen and nectar consumption or mode of nesting, which likely results in different levels of exposure and impacts of pesticides on bees (Sgolastra *et al.* 2019; Uhl and Brühl 2019). Furthermore, amplification of potential negative impacts of agrochemicals by other stressors (e.g., mixtures of multiple pesticides, interactive effects of pesticides and food stress) are currently not considered in higher-tier risk assessment schemes (Sgolastra *et al.* 2020; Topping *et al.* 2020). We therefore urgently need more studies of their potentially interactive effects on different bee species under (semi-)field conditions. The semi-field experiments performed in this task were conducted to address these knowledge gaps.

2. Overview of semi-field experiments conducted

2.1. Tested agrochemicals

Insecticide sulfoxaflor (tested as the commercial product Closer)

Sulfoxaflor is the first commercially developed compound of a new class of systemic insecticides called sulfoximines. It has already been registered for use in 81 countries around the world (Siviter *et al*. 2020). It is used primarily as a foliar spray on a wide variety of crops, such as leafy vegetables, cereal crops, potatoes, citrus and pome fruits, brassicas and nuts. Its mode of action is similar to that of neonicotinoids; it interacts with the nicotinic acetylcholine receptor in the nervous system of invertebrates and targets sap-feeding insects (Babcock *et al.* 2011; Zhu *et al.* 2011; Cutler *et al.* 2013;

Ulens *et al.* 2019). Sulfoxaflor is also effective against insects resistant to other neonicotinoids (Sparks *et al.* 2013). It has a half-life of 2-3 days in soil (EPA 2019) and it is reported to be generally less persistent in the environment than neonicotinoid insecticides. Because of the rising occurrence of pests resistant to the heavily used neonicotinoids (Bass *et al.* 2015), sulfoxaflor is expected to be widely used as a replacement of neonicotionoids in the future (Simon-Delso *et al.* 2015). Sulfoxaflor was registered for use in the EU in 2015. Due to evidence for negative effects on pollinators, it has recently been banned from outdoor uses in the EU (European Commission 2022). It remains, however, widely used in other parts of the world.

Insecticide flupyradifurone (tested as the commercial product Sivanto prime)

Flupyradifurone is a relatively new compound and belongs to the systemic butenolid insecticides, which share similar modes of action to neonicotinoids by binding to the nicotinic acetylcholine receptors of invertebrates (Nauen *et al.* 2014: Jeschke 2015; Giorio *et al.* 2017). It is used against sucking insect pests for a wide variety of crops (e.g., citrus, cotton, pome fruits, grapes; Nauen *et al.* 2014). Similarly to sulfoxaflor, flupyradifurone is a likely successor of neonicotinoid insecticides (Siviter and Muth 2020). It can be applied via spray application, drench or seed treatment. The acute oral toxicity of flupyradifurone to honey bees (acute oral LD_{50}: 1.2 µg/bee) suggests that it may be less toxic to honey bees than sulfoxaflor (LD_{50}: 0.146 µg/bee) or the neonicotinoids imidacloprid, clothianidin or thiamethoxam (LD_{50}: 0.0037, 0.004 and 0.005 µg/bee, respectively; Lewis *et al.* 2016).

Fungicide azoxystrobin (tested as the commercial products Amistar or Ortiva)

Azoxystrobin is a broad-spectrum systemic fungicide which acts by inhibiting fungal respiration pathways and belongs to the group of methoxy-acrylates, which are derived from naturally occurring strobilurins and inhibit mitochondrial respiration in fungi. It is currently widely used in agriculture and approved for use on 84 different crops in 72 countries worldwide. Azoxystrobin is effective against the four main types of fungi: *Ascomycota*, *Deuteromycota*, *Basidiomycota* and *Oomycota*. It exhibits curative action (after infection but before symptoms occur) and can have eradicant (after symptoms occur) and antisporulant activity (reduced sporulation; Bartlett *et al.* 2002). Strobilurins are the world's bestselling types of fungicides, with a market share of 20% in 2016 (see Wang *et al.* 2020). Residues of azoxystrobin have frequently been detected in bees, bee-collected pollen and nectar, bee wax, and honey (Mullin *et al.* 2010; Krupke et al. 2012; Sanchez-Bayo and Goka 2014: Hladik *et al.* 2016, Roszko *et al.* 2016; Böhme *et al.* 2018). Azoxystrobin-based fungicides will likely continue to be widely used in the future.

An overview of agrochemicals used in the different semi-field experiments is given in Table 1 below.

2.2. Model bee species

Three bee species were used as model species in the semi-field experiments on single and combined effects of stressors (Table 1): the social Western honey bee (*Apis mellifera*), the social buff-tailed bumble bee (*Bombus terrestris*), and the solitary red mason bee (*Osmia bicornis*). *Apis mellifera* has been extensively used as a model species in risk assessment schemes for plant protection products (EFSA 2013). However, since life history traits that are associated with different sensitivities and exposure routes in bees vary substantially across species (Arena and Sgolastra 2014), it is crucial to investigate effects of pesticides and other stressors on other model bee species. The bumble bee species *B. terrestris* is an important pollinator of wild plants and crops, and colonies are commercially produced. Recently, it has been used as a further model bee species, e.g., in European risk assessment of plant protection products (EFSA 2013). *Osmia bicornis* is an abundant solitary bee species and increasingly reared and managed as a pollinator of top fruit and berry crops in Central Europe and Northern Europe. The species is univoltine and polylectic, readily nesting in artificial nesting aids (Westrich 2019). It has therefore been proposed as a solitary bee model species for the risk assessment of plant protection products in Europe (EFSA 2013).

2.3. Overview of stressors and their interactions in conducted semi-field experiments

Table 1. Overview of stressors and their interactions studied in various semi-field experiments in 2019, 2020 and 2021.

Partner(s)	Year	Study species	Type of stressor interactions	Stressors
ATPOLL, RBH	2019	*A. mellifera*	insecticide × fungicide	Sulfoxaflor (product Closer), azoxystrobin (product Amistar)
ALU-FR	2019	*B. terrestris*	insecticide × fungicide	Sulfoxaflor (product Closer), azoxystrobin (product Amistar)
WBF-Agroscope	2019	*O. bicornis*	insecticide × fungicide	Sulfoxaflor (product Closer), azoxystrobin (product Amistar)
ALU-FR	2020	*B. terrestris*	fungicide × nutrition	Azoxystrobin (product Amistar), nutritional stress: varying nutritional value: purple tansy, buckwheat, mix
WBF-Agroscope	2020	*O. bicornis*	insecticide × nutrition	Flupyradifurone (product Sivanto), nutritional stress: varying nutritional value: purple tansy, field mustard, buckwheat, mix
ATPOLL, RBH	2021	*A. mellifera*	fungicide × nutrition	Azoxystrobin (product Amistar), nutritional stress: high vs. low nutritional value: purple tansy or buckwheat
ALU-FR	2021	*B. terrestris*	Fungicide (different doses)	Dose-response azoxystrobin (product Ortiva)
WBF-Agroscope	2021	*O. bicornis*	Insecticide (different doses)	Dose-response flupyradifurone

3. Material and methods

3.1. Sulfoxaflor-azoxystrobin interaction experiments

The single and combined impacts of sulfoxaflor (product Closer) and azoxystrobin (product Amistar) were assessed using the same full-factorial design under semi-field conditions for all three model bee species (*A. mellifera*, *B. terrestris* and *O. bicornis*). The same application rates according to label instructions were used in all three semi-field experiments. However, it is important to note that due to differences in the life histories of the three bee species, slightly different timings of spray applications and measurements of endpoints across experiments were required (e.g., sulfoxaflor application was roughly 2 days before the start of crop flowering and colony placement in the experiment with *B. terrestris*, while sulfoxaflor was applied at least 5 days before crop flowering in the experiment with *A. mellifera*, and at least 5 days before crop flowering in the experiment with *O. bicornis*). Furthermore, flower availability and the ratio of flowers available to the number of bees

inside cages, as well as weather conditions, were different to some extent among experiments. This needs to be considered when comparing and interpreting results of the three experiments with the three different model bee species.

3.1.1. Impacts on honey bees (*Apis mellifera*)

Study design

The effects of the insecticide sulfoxaflor (product Closer) and the fungicide azoxystrobin (product Amistar) on honey bees (*A. mellifera*) were tested in a semi-field experiment by ATPOLL and RBH in Hampshire (UK) in summer 2019. In total, 40 flight cages (12 m × 6 m, height: 2 m) planted with purple tansy (*P. tanacetifolia*) were used in the experiment. Ten cages were randomly assigned to each of four spray treatments: 1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor + azoxystrobin (mix), 4) water only control. Due to unequal colony sizes of *A. mellifera* at the beginning of the experiment and damages due to a storm, the sulfoxaflor + azoxystrobin treatment could, however, not be used for the analysis of the data. Spray application of sulfoxaflor was conducted before the onset of *P. tanacetifolia* flowering at BBCH 55 (application rate: 48 g a.i./ha), while azoxystrobin was sprayed one week later at the beginning of flowering (BBCH 63; application rate: 250 g a.i./ha). One honey bee colony was placed inside each cage six days after the application of sulfoxaflor and one day before the application of azoxystrobin. The exposure phase inside the cages lasted for 18 days. Subsequently, the nets of the cages were removed and bees were free to forage on surrounding wild flowers for the following 27 days post-exposure phase (access to sprayed *P. tanacetifolia* was prevented; Fig. 5). A detailed description of the study design and methods can be found in Tamburini *et al*. (2021a).

Data collection and statistical analyses

The following assessments were conducted on the honey bee colonies: queen presence, number of adult bees, amount of brood, brood failure, colony weight, adult bee mortality, flight and foraging activity, amount of pollen collected. The exact timing of the assessments during the exposure and post-exposure phases of the experiment is shown in Fig. 6. The effect of sulfoxaflor and azoxystrobin on queen presence was analysed using Kaplan-Meier survival analysis. For the analyses of colony growth and activity, data from exposure and post-exposure phases of the experiment were analysed separately (excluding cages in which queens were absent or did not lay eggs). These endpoints were analysed using linear (mixed-effects) models using treatment as the explanatory variable (factor with three levels: sulfoxaflor, azoxystrobin, control). Cage ID was included as random effect in the mixed-effects models.

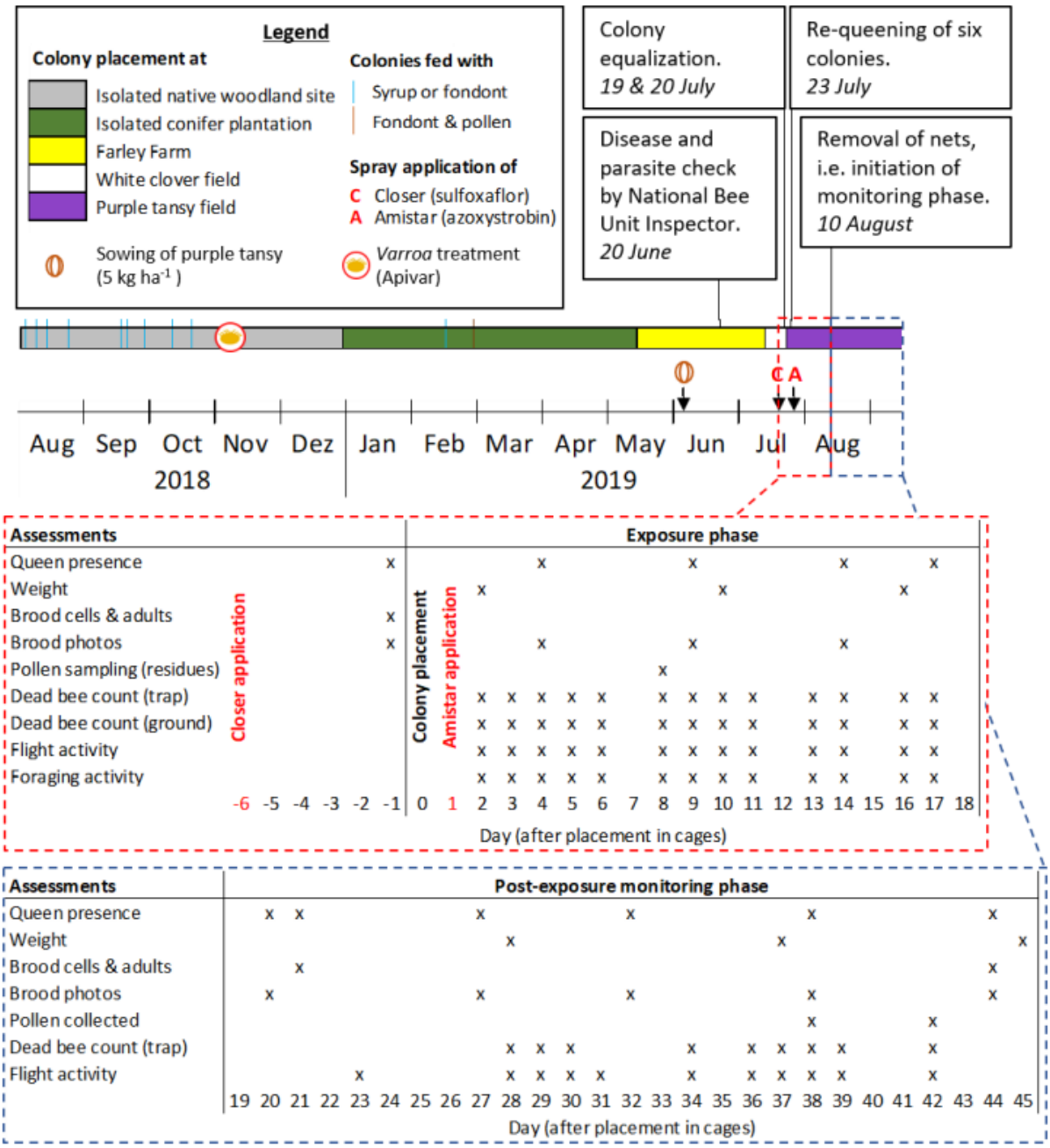

Figure 1: Experimental timeline and overview of actions taken in preparation and during the experiment: honey bee colony inspection, equalization, feeding, moving, treatment against the *Varroa* mite, and re-queening as well as sowing of purple tansy on the study site, as well as treatment applications. The more detailed timelines of the exposure and post-exposure monitoring phase show dates of honey bee colony assessments. On the days noted for 'queen presence' the colonies were inspected or brood photos were taken. If no eggs were found, all brood photos were inspected to estimate the date of queen failure (i.e. absence of an egg-laying queen) based on the presence or absence of eggs and young larvae.

3.1.2. Impacts on bumble bees (*Bombus terrestris*)

Study design

The effects of sulfoxaflor (product Closer), azoxystrobin (product Amistar) and their combination on bumble bees (*B. terrestris*) were investigated by ALU-FR in a semi-field study in Freiburg (Germany) in 2019. A full-factorial design was implemented and ten cages (9 m × 6 m, height: 2.5 m) were assigned to one of the following four treatment groups: 1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor +

azoxystrobin (mix), 4) water only control. As a model crop, purple tansy (*P. tanacetifolia*) was planted inside the cages. One bumble bee colony consisting of a queen and approximately 25 workers was placed into each cage (Fig. 6). Sulfoxaflor (Closer) was sprayed in the designated cages before crop flowering (BBCH 55-59, as required by label instructions in Italy at the time of the experiment, application rate: 48 g a.i./ha) and two days before the placement of the bumble bees inside the enclosures. Azoxystrobin (Amistar) application rate: 250 g a.i./ha) was sprayed at full flowering (BBCH 63-65) (Fig. 7). The exposure phase of the experiment lasted for 18 days. After termination of the experiment, colonies were frozen at -20 °C. A detailed description of the study design and methods can be found in Tamburini *et al*. (2021b).

Data collection and statistical analyses

The total change in colony weight was calculated by weighing the colony before and after the exposure phase. Additionally, the number of individual bees in each colony was counted at the end of the experiment. Multiple assessments were conducted to measure the foraging performance of bumble bees: 1) the individual foraging performance (i.e., the number of flowers visited by an individual bumble bee during a certain time), 2) flower visitation (i.e., the number of flower visiting bumble bees in a given period of time and area), and 3) the number of foraging flights performed by each colony in one day. Additionally, floral abundance inside the cages was monitored. To assess the pollination service provided by bumble bees, single-visit pollen deposition (Kings *et al.* 2013) on *P. tanacetifolia* styles and stigmas was assessed on multiple days following the application of azoxystrobin (Amistar). An overview of all assessments performed during the experiment is shown in Fig. 7. Statistical analyses were performed using the statistical software R (R Development Core Team 2022) by fitting linear (mixed-effects) models. The data set was analysed in two parts: before and after the application of the fungicide azoxystrobin (Amistar; Fig. 7). The effects of sulfoxaflor (Closer), azoxystrobin (Amistar) and their interactions were assessed by including both pesticides as 2-level factors (categorical, present or absent) as explanatory variables. Mixed-effects models included cage ID as a random factor.

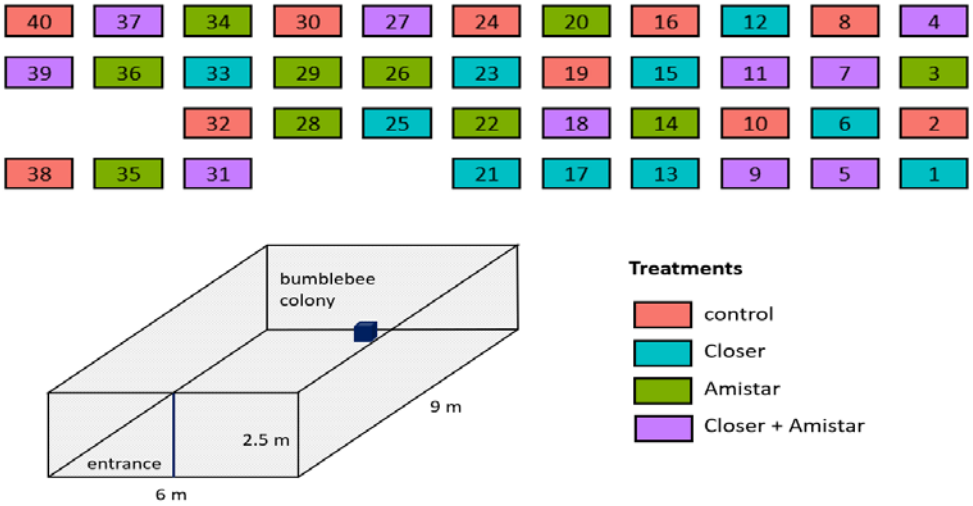

Figure 2: Illustration of the 40 cages with the distribution of the treatments across cages of the experimental field site. Cages were positioned at least 4 m from the field boundaries (>1000 m from the nearest crop) and at least 4 m apart from each other.

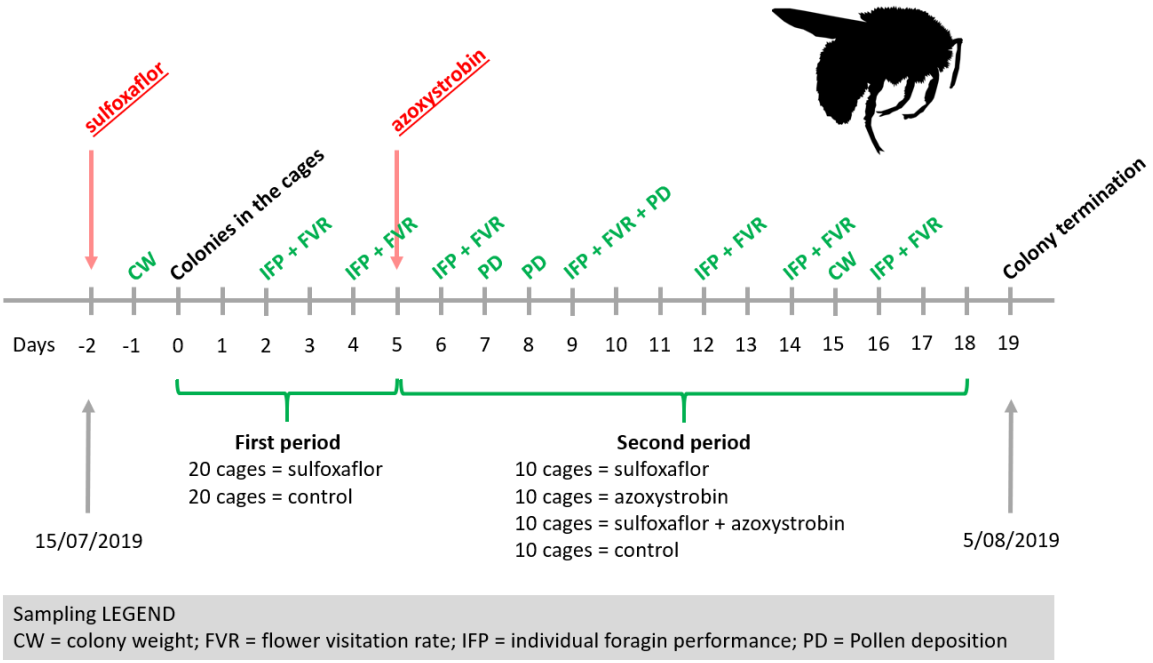

Figure 3: Timeline of the semi-field experiment. Red: pesticide applications; black: start and end of the study; green: different types of data collection (see legend). The experiment was divided in two different periods with respect to data analysis (see Data collection and statistical analyses).

3.1.3. Impacts on solitary bees (*Osmia bicornis*)

Study design

The impacts of sulfoxaflor (product Closer) and azoxystrobin (product Amistar) alone and combined on the solitary bee species *O. bicornis* were tested by WBF-Agroscope in Zürich (Switzerland) in 2019. A full-factorial semi-field experiment using 40 cages (9 m × 6 m, height: 2 m) planted with purple tansy (*P. tanacetifolia*) was conducted. Ten cages were randomly assigned to one of four treatment combinations: 1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor + azoxystrobin (mix), 4) water only control (Fig. 8). Each cage was equipped with two custom-made nesting units (Atlantic Pollination Ltd.) offering a total of 200 cavities for *O. bicornis* females to build their nests (Fig. 8). Per cage, 50 freshly hatched adult females and 75 males were released. Sulfoxaflor (Closer) was sprayed 5 days before crop flowering, according to label guidelines in several European countries at the time of the study, at a rate of 48 g a.i./ha (0.4 L formulated product Closer/ha). Azoxystrobin (Amistar) was sprayed at the beginning of flowering at a rate of 250 g a.i./ha (1 L formulated product Amistar/ha) and marked the start of the exposure phase, which lasted 26 days in total (day 0 – day 25). After termination of the experiment, remaining adult bees were released from the cages and nesting units (containing produced offspring) and were carefully covered with fine mesh and transported to a sheltered place outdoors. At the end of November 2019, the nesting units were transferred to a cool room (2-4 °C) for overwintering. In the following spring 2020, offspring were hatched at room temperature. For a detailed description of the study design and methods see Schwarz *et al*. (2022).

Data collection and statistical analyses

Data on survival, reproduction, offspring size, sex ratio, foraging performance and nest recognition ability of *O. bicornis*, as well as pollination service provisioning was collected. Additionally, floral abundance during the exposure phase was monitored. An overview of all assessments and their timing during the experiment is shown in Fig. 9. The survival of adult female *O. bicornis* was monitored by counting the number of roosting individuals in the nesting units at night. The production of brood cells (i.e., a cell containing a pollen provision with an egg laid on top, sealed with a mud wall) and offspring was followed by repeatedly taking pictures of each individual nesting layer of the nesting units. During

the first ten days of the experiment, pictures of nests were taken daily, in order to be able to calculate the number of offspring produced per day. Also the total number of offspring produced per cage, as well as the mortality of the produced offspring until reaching the cocoon stage, were assessed. Moreover, the size of cocoons of offspring produced between days 0-5 of the exposure phase and the sex of the produced offspring were assessed. The foraging performance of *O. bicornis* females on five days during the exposure phase (days 0, 1, 4, 7, 10) was assessed: to this end, flower visits of individual foraging bees during a given time period (ca. 2 minutes; individual foraging performance) as well as flower visitation (i.e., the total number of flower visits in a plot in a given time period) and foraging activity (i.e., number of female bees foraging in the plot during a time period) in randomly selected 1 m^2 plots. The nest recognition ability of foraging bees was assessed 1 and 7 days after the start of the exposure phase. For this purpose, the cavity entrances of one nesting unit per cage were video recorded for approximately 20 minutes. The number of cavity entrances a bee probed before finding its own nest was counted. Pollination service delivery was assessed on days 1 and 7 of the exposure phase. Styles and stigmas from *P. tanacetifolia* flowers, which had opened on the same day, were collected and the number of pollen grains deposited was quantified. The collected data were analysed using (generalised) linear mixed-effects models or linear models, where possible. Sulfoxaflor (Closer; applied or not), azoxystrobin (Amistar; applied or not) and their interaction were included in the models as explanatory variables. Mixed-effects models included cage ID as a random effect. The survival data were analysed using a mixed-effects Cox proportional hazards model.

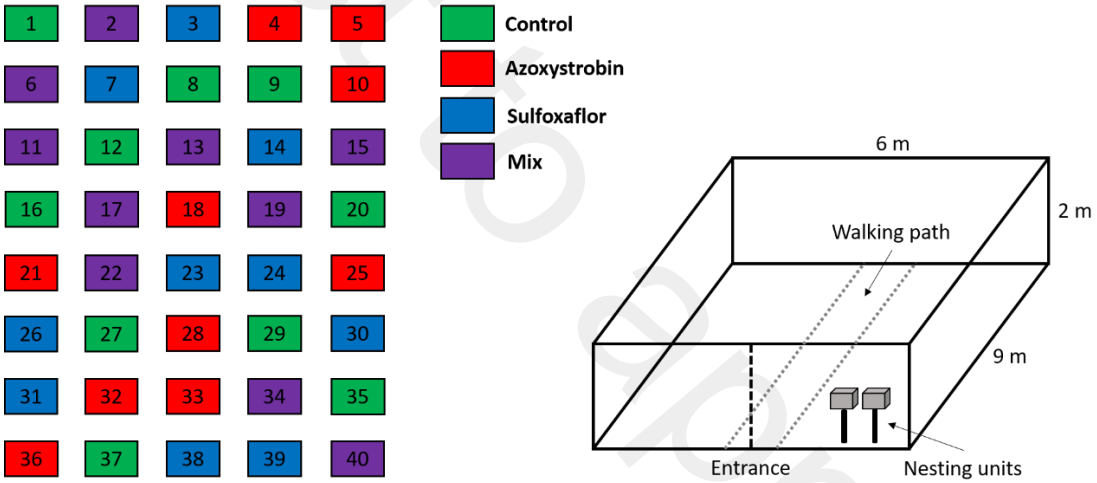

Figure 4: Random distribution of cages on the experimental field site and illustration of an individual flight cage used in the semi-field experiment to test for effects of sulfoxaflor (product Closer) and azoxystrobin (product Amistar) alone and combined on the solitary bee model species *O. bicornis*.

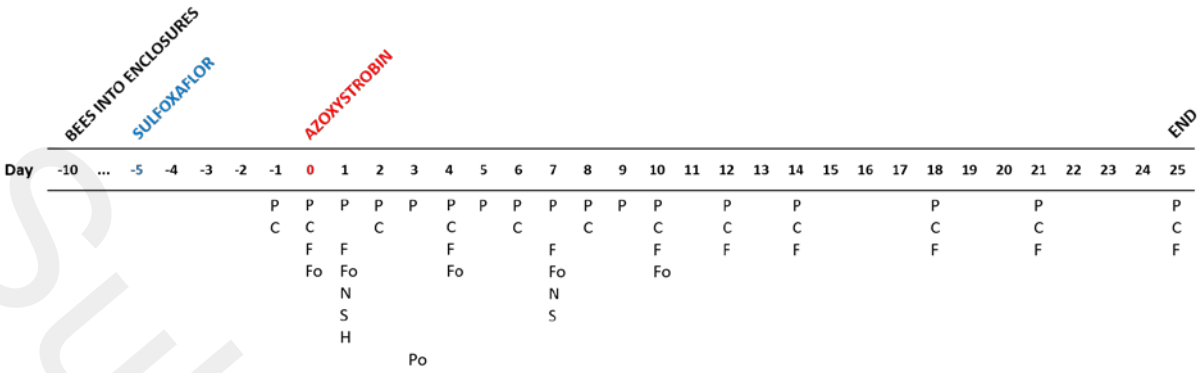

Figure 5: Timeline of the semi-field experiment. Assessments: P: pictures of nests to monitor reproduction; C: counting of *O. bicornis* females in nests at night for monitoring survival; F: assessment of flower abundance in cages; Fo: observations of foraging performance of *O. bicornis*; N: video recording of nesting unit entrances to assess nest recognition ability; S: collection of *P. tanacetifolia* stigmas to assess pollination service delivery; H: collection of haemolymph samples from three females and three males for a separate study; Po: collection of pollen-nectar provisions from brood cells to analyse residue levels of sulfoxaflor and azoxystrobin.

3.2. Pesticide-nutrition interaction experiments

3.2.1. Interactive azoxystrobin-nutrition effects on honey bees (*A. mellifera*)

Study design

A full-factorial semi-field experiment was carried out by ATPOLL and RBH in 2021. In this study the effects of exposure to a fungicide azoxystrobin (product Amistar) on honey bees (*A. mellifera*) feeding on two different forage plant species differing in nutritional quality (with respect to amount of pollen and nectar offered, as well as protein content of the pollen): purple tansy (*P. tanacetifolia*; high nutritional value) and buckwheat (*Fagopyrum esculentum*; low nutritional value) was tested. A total of 40 flight cages (12 m × 6 m, height: 2 m) were established on plots sown with either *P. tanacetifolia* or *F. esculentum* on an experimental field. Ten cages were randomly assigned to one of four treatment combinations: 1) azoxystrobin (Amistar) sprayed on *P. tanacetifolia*, 2) azoxystrobin (Amistar) sprayed on *F. esculentum*, 3) water only sprayed on *P. tanacetifolia* (no fungicide), 4) water only sprayed on *F. esculentum* (no fungicide). Application rate of azoxystrobin (product Amistar) was 250 g a.i./ha (1 L formulated product Amistar/ha).

Data collection and preliminary statistical analyses

The study was divided into (a) a pre-exposure phase (before spray applications), (b) the exposure phase (after spray applications; colonies inside enclosures, and (c) a post-exposure phase after the removal of the enclosures. The endpoints collected included colony weight, number of alive bees, foraging activity (i.e., number of bees entering hives for 2 min), estimated number of foraging honey bees on flowers, number and stage of dead bees on the ground, the number and stage of dead bees in hives and pollen collection rate (assessed through pollen traps). Additionally, flower density was assessed. (Generalized) linear mixed-effects models were used with colony ID included as a random effect. Type of resource (buckwheat or purple tansy), azoxystrobin treatment (azoxystrobin (Amistar) applied or not) and the interaction of these two factors were used as fixed explanatory variables. Data for each phase of the experiment (pre-exposure, exposure and post-exposure) were considered separately and the experimental phase and its interaction with resource type, azoxystrobin treatment and their interaction were included as fixed effects.

3.2.2. Interactive azoxystrobin-nutrition effects on bumble bees (*B. terrestris*)

Study design

The interactive effects of azoxystrobin exposure (product Amistar) and three different types of flowering resources (monocultures of purple tansy (*P. tanacetifolia*), buckwheat (*F. esculentum*) and a floral mix) on bumble bees (*B. terrestris*) were investigated by ALU-FR in a semi-field study in Freiburg (Germany) in 2020. The floral mix consisted of *F. esculentum* (40% by weight), *P. tanacetifolia* (10%), *Centaurea cyanus* (20%), *Sinapis arvensis* (10%), *Malva sylvestris* (10%) and *Trifolium resupinatum* (10%). In total, 39 cages (6 m × 9 m, height: 2.5 m) were used in this study and floral resources were randomly assigned to them. One bumble bee colony containing ca. 36 workers was placed inside each cage seven days before the application of the fungicide azoxystrobin (pre-exposure period). Azoxystrobin (Amistar) was sprayed at a rate of 250 g a.i./ha during full bloom of the crop (BBCH 63-65) (Fig. 10). The exposure phase lasted for ten days following the application of the fungicide. After termination of the exposure phase, colonies were left to forage freely outside the cages and afterwards freeze-killed and examined after 13 days. A detailed description of the methods can be found in Wintermantel *et al*. (2022).

Data collection and statistical analyses

The following assessments were conducted on the bumble bee colonies once in the laboratory (eight days before the start of the exposure phase, one day before placement inside the cages, respectively) and eight times inside the cages (three times before and five times after application of azoxystrobin (Amistar): 1) colony weight, 2) cumulative number of dead adults, 3) number of living adults (Fig. 11). The individual foraging performance (*i.e.*, the number of flowers visited by an individual bumble bee forager during three minutes) was assessed for three bees per cage on days -4, -3, 4, 9 and 10. Additionally, flower density was monitored during the experiment (Fig. 11). After colony termination (day 23, after the 13-day post-exposure phase), the colonies were inspected for 1) the number of adult males and workers, 2) the number of worker and/or male cocoons, 3) adult worker body mass and intertegular distance, and 4) pupal body mass and developmental stages. The data set was analysed separately according to the assessment periods: pre-exposure period, exposure period and final assessment. (Generalized) linear mixed-effects models were used with colony ID included as a random effect. Type of resource (factor, three levels), azoxystrobin treatment (azoxystrobin (Amistar) applied or not) and the interaction of these two factors were used as explanatory variables.

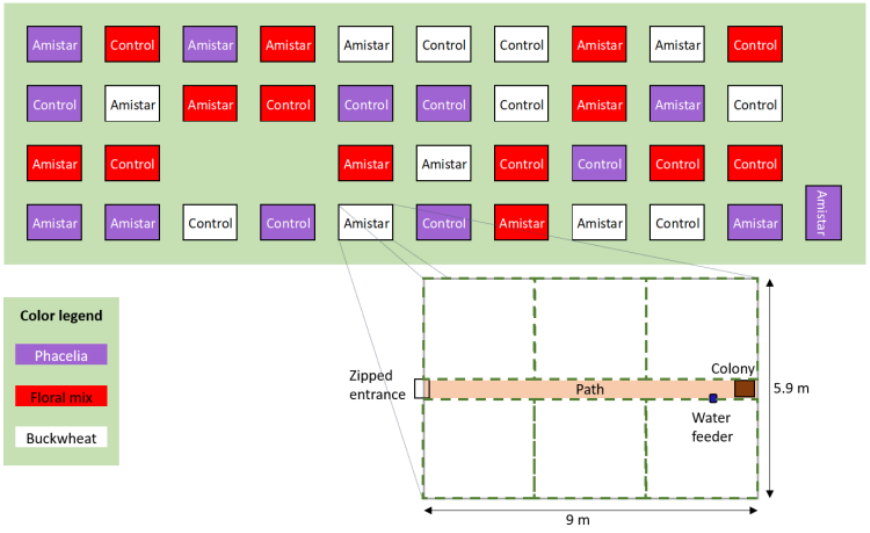

Figure 6: Illustration of the study site and of an individual flight cage. Flight cages are colour-coded according to flowering resource type. Green dashed rectangles within the flight cage are separations based on the poles of the cage and were used for random sampling of plots where flower density was determined.

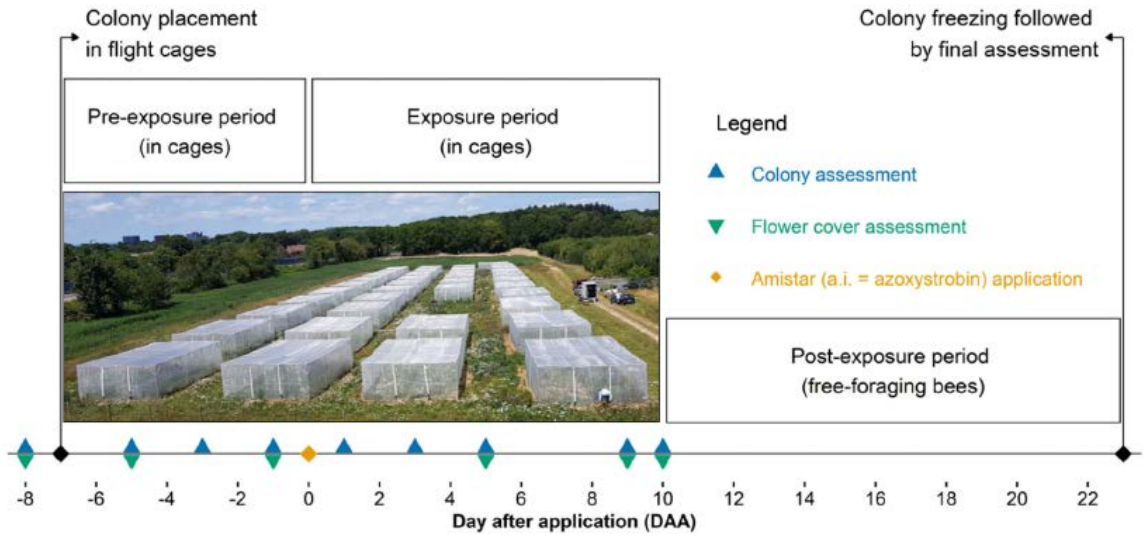

Figure 7: Experimental timeline. Sequence of bumble bee colony and flower cover assessments in the pre-exposure period (before azoxystrobin (Amistar) application) and exposure period (after azoxystrobin application).

3.2.3. Interactive flupyradifurone-nutrition effects on solitary bees (*O. bicornis*)

Study design

The interactive effects of the insecticide flupyradifurone (product Sivanto prime) and three different types of floral resources (monocultures of purple tansy (*P. tanacetifolia*), buckwheat (*F. esculentum*) or wild mustard (*S. arvensis*) on the solitary bee *O. bicornis* were investigated by WBF-Agroscope in a semi-field study in 2020 near Zürich (Switzerland); the nutritional value for bees was high in purple tansy, intermediate in wild mustard and low in buckwheat (based on foraging preferences, food-plant dependent clearance of flupyradifurone from the bees' bodies after a single exposure in the laboratory and food plant-dependent gene expression level of vitellogenin, Fig. 12). In total, 18 cages (9 m × 6 m, height: 2 m) were established on an experimental field and three cages were assigned to each treatment combination in a crossed block design (Fig. 13 a-d). In each cage, 24 individually marked female bees (Fig. 13e) and 36 males were released. A nesting unit offering 120 nesting cavities was installed in each cage (Fig. 13f). Spray application of flupyradifurone (Sivanto prime) was conducted at a rate of 205 g a.i./ha according to label guidelines during crop flowering and after the majority of females had started nesting. Application was done before the onset of full bee flight in the early morning.

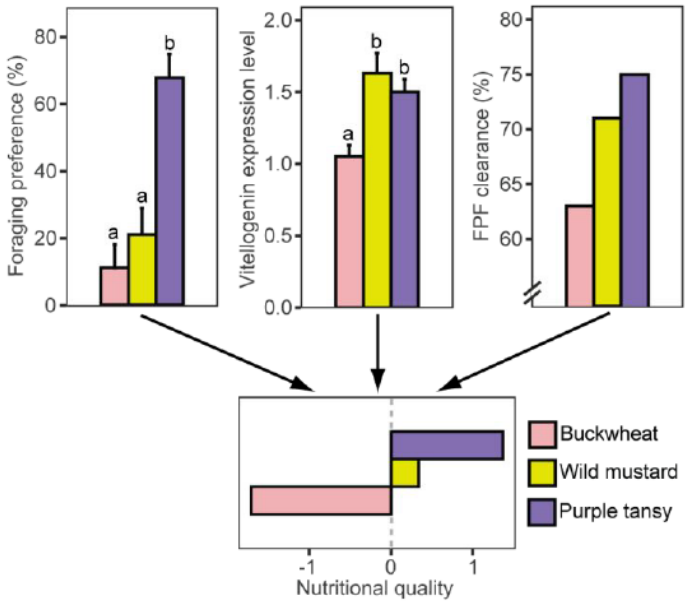

Figure 8: Mean values (+ SE) of the measurements used to assess the food plants' nutritional value for *O. bicornis*. Significant differences in foraging preferences and the gene expression levels of vitellogenin are indicated with different letters.

Data collection and statistical analyses

Assessments of *O. bicornis* fitness proxies were performed on three days: day 1 (day on which flupyradifurone was applied), day 2 and day 9. Survival of adult females and reproduction were monitored by taking pictures of nesting unit layers and counting the number of roosting females inside the nests at night. Flower visitation frequency (i.e., the number of flower visits per individual female within two minutes) was observed for five females per cage. Additionally, flight activity, flight duration and nest recognition ability of females were analysed using video recordings of the nesting units taken for several hours on each assessment day and analysing them with the machine learning software "Bee Tracker" (Knauer *et al.* 2022). The software is able to identify each individual bee by its number tag and assigns it to the nest the bee constructed to be able to calculate per female fitness proxies. Additionally, samples of pollen-nectar provisions from *O. bicornis* nests were taken the night after application of flupyradifurone (Sivanto prime) to assess residue levels of the substance in bee-collected pollen. (Generalized) linear mixed-effects models were used to analyse impacts of flupyradifurone exposure (flupyradifurone applied or not), nutritional value (continuous, see Fig. 12) and their interaction as explanatory variables. Separate models were fitted for days 0, 1 (short-term effects) and 9 (longer-term effects). Cage ID was included as a random effect.

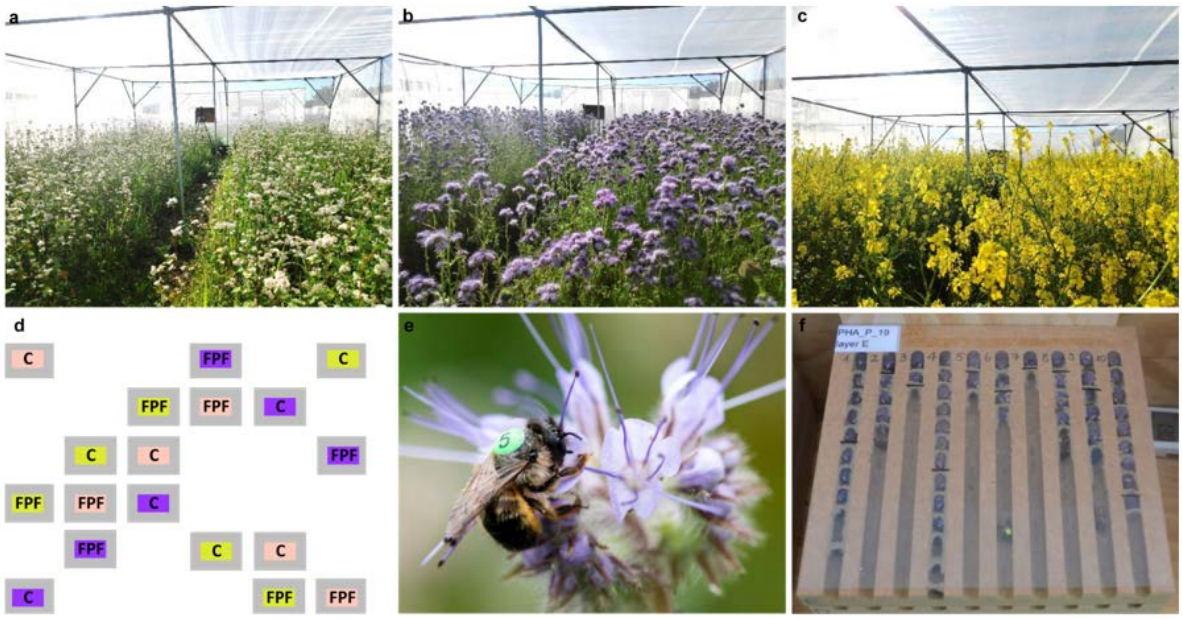

Figure 9: Nutrition treatments and experimental methods of the semi-field experiment. a) Buckwheat cage on assessment day 1; b) Purple tansy cage on assessment day 1; c) Wild mustard cage on assessment day 1; d) Arrangement of flight cages on experimental field. Rose: buckwheat, yellow: wild mustard, purple: purple tansy. FPF: Flupyradifurone application, C: control treatment; e) *O. bicornis* female with a marker tag (unique colour-digit combination) on its thorax; f) Layer of a nesting unit: wooden board (MDF) with nest cavities covered with a plastic foil for documentation of nesting progress.

3.3. Dose-response experiments

3.3.1. Dose-response experiment on the impact of azoxystrobin on bumble bees (*B. terrestris*)

Study design

The impact of five different doses of azoxystrobin (product Ortiva) was tested under semi-field conditions by ALU-FR in Freiburg (Germany) in 2021. The experiment was initially planned to be conducted with the azoxystrobin-containing product Amistar. However, shortly before the start of the experiment, Syngenta decided not to sell Amistar in Germany anymore; only Amistar products such as Amistar max with additional active ingredients are still sold. Therefore, we used the product Ortiva for the experiment, which is identical in composition to Amistar (only the crops it is authorised for and the recommended dose differ). In total, 42 bumble bee colonies consisting of a queen and approximately 56 workers were placed on an experimental field sown with plots of monocultures of purple tansy (*Phacelia tanacetifolia*), buckwheat (*Fagopyrum esculentum*), or a mixture of flowering plant species including blueberry (*Vaccinium myrtillus*), different clover species (*Trifolium* spp.) and white mustard (*Sinapis alba*)(Fig. 1). Four days after placement of the colonies in the field, exposure to azoxystrobin started: azoxystrobin (Ortiva) was diluted to the desired concentrations in 50% (w/w) sugar solution and fed to the colonies through feeders. The exposure lasted for ten days. To mimic pesticide degradation, the provisioned concentrations of Ortiva declined in the first 4 days; subsequently, a constant dose was provided (Fig. 2). One set of colonies received a field-typical sequence of doses (1170 ppb, 656 ppb, 70 ppb, 16 ppb on days 0-3, respectively, and 5 ppb on days 4-9). Per dosing factor, seven *B. terrestris* colonies were used. Additional sets of colonies received either a multiple or a fraction of these doses (factors: 0, 0.5, 1, 2, 4, 8; Fig. 2). After the exposure period, the colonies were left on the field site for the next 22 days (post-exposure phase).

Additionally, a choice-experiment was conducted in the laboratory to examine a potential preference for or against syrup spiked with Ortiva. Twenty micro-colonies of 5 workers each were created using spare colonies from the main experiment and were given access to syrup free of Ortiva and syrup spiked with Ortiva (300 ppb azoxystrobin) and the consumption was monitored over 4 days.

Assessments and statistical analyses

The following parameters were assessed regularly during the exposure and post-exposure phase: number of larvae and pupae, number of dead/alive adults, founder queen presence, presence of new queens, colony weight, syrup consumption, flight activity.

Figure 10: Experimental field-site in Freiburg (Germany) to test the effects of azoxystrobin (Ortiva) on bumble bees (*B. terrestris*).

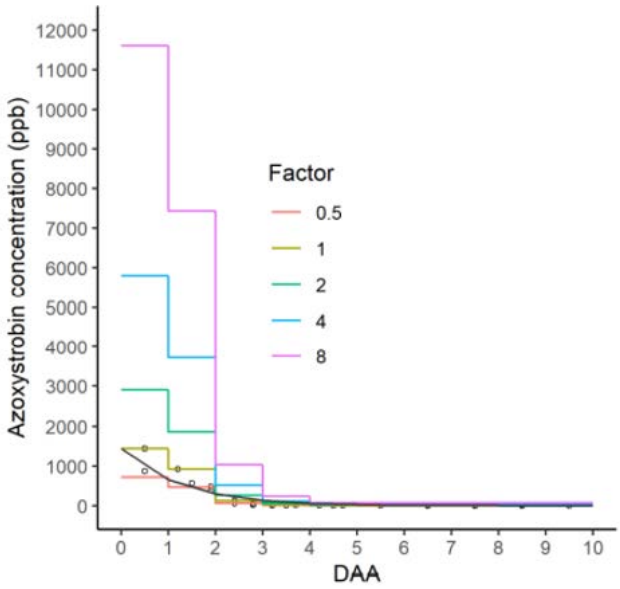

Figure 11: Azoxystrobin (product Ortiva) concentrations administered to syrup samples across 10 days. Circles and triangles indicate measured azoxystrobin concentrations in nectar collected from honey bees of two different colonies foraging on oilseed rape treated with Ortiva from the literature (Schatz 2009). Factor 1 represents a field-typical dose that was determined by taking the daily mean azoxystrobin concentration of these observed values within the first 4 days. Afterwards a constant dose of 5 ppb was used until day 9. All other doses are multiples of this set of field-typical doses as indicated by the factor.

3.3.2. Dose-response experiment on the impact of flupyradifurone on *solitary bees (O. bicornis)*

Study design

The effects of different doses of flupyradifurone on *O. bicornis* were investigated by WBF-Agroscope in Zürich (Switzerland) in 2021. A total of eight flight cages (9 x 6 m; height 2.5 m) were established on a field sown with purple tansy (*P. tanacetifolia;* Fig. 3). The flight cages were equipped with a nesting unit specifically developed for ecotoxicological semi-field assessments using *O. bicornis*, which allows for daily measurements of brood cell and offspring production. A total of 24 females were individually marked with colour-digit tags (three sets of numbers from 1 to 8 in the colours green, yellow and white; 24 unique ID tags) glued on the bee's thorax. This enabled measurement of the response variables (e.g. offspring production) per individual nesting female bee. Together with 36 male bees, females were released into the flight cages. During the acclimation of around one week, bees mated and started nesting. After initiation of nesting, all bees from an enclosure were captured and exposed to five different concentrations of flupyradifurone in Nicot cages (0 µg/ml, 35 µg/ml, 70 µg/ml, 105 µg/ml, 140 µg/ml; oral exposure through spiked syrup; Fig. 4). To obtain good feeding rates during flupyradifurone exposure, females were previously starved for 36 hours. In each flight cage, the same number of bees exposed to each of the five concentrations was used in order to rule out confounding effects between treatments and flight cage identity (such as potential variation in flower availability). After exposure, the females were released into the flight cages again and acute effects on foraging behaviour and nesting progress were monitored.

After 30 minutes of acclimatization, a video camera was placed in front of the nesting unit to record nesting and foraging activity of bees. The videos were then analysed using a machine-learning based software ("BeeTracker"; Knauer *et al.* 2022) specifically developed for such purposes. The software is able to link each nest to the marked female bee that has constructed it, and thereby enables, in combination with assessments of brood cell and offspring production of nests, calculation of endpoints such as the number of offspring produced and other fitness-related variables for each individual female bee. The software is also able to record a bee's arrival and departure from the nest. Each of these events provides an exact time stamp and therefore further measurements, such as foraging trip (flight) duration and the number of falsely entered cavities before finding the correct nesting cavity can be calculated.

At the same time, bees were visually observed during their foraging trips. Across the total observation time of four hours, each bee was observed twice for two observation periods, for up to 30 minutes. The bees were followed for 1 to 2 min and the number of visited flowers was counted per observed time period. Inactive bees outside the nest box were recorded as well. In the evening of the same day, the nesting units were photographed again to assess the nesting progress after the experimental treatment (flupyradifurone dose).

Figure 12: Flight cage at field site of Agroscope near Zürich (Switzerland) with sown flowering purple tansy (*P. tanacetifolia*) and a nesting unit.

Figure 13: Nicot cage system to administer different doses of flupyradifurone to *O. bicornis* via syrup.

4. Results

4.1. Sulfoxaflor-azoxystrobin interaction experiments

4.1.1. Impacts on honey bees (*A. mellifera*)

Sulfoxaflor (product Closer) or azoxystrobin (product Amistar) did not significantly affect the proportion of living egg-laying queens in the experiment. Further, there was no evidence that the two pesticides negatively affected the development (number of adults and brood cells, brood failure), colony weight changes or the activity (flight activity, foraging activity, number of dead adult bees) of honey bees during the exposure or the post-exposure phase of the experiment (Fig. 15a-g). Colonies exposed to azoxystrobin tended to collect more pollen during the post-exposure phase compared to control or sulfoxaflor-exposed colonies (Tukey multiple comparison test: Amistar vs. control: $P = 0.057$; Closer vs. Amistar: $P = 0.061$; Closer vs. control: $P = 0.985$; Fig. 15h).

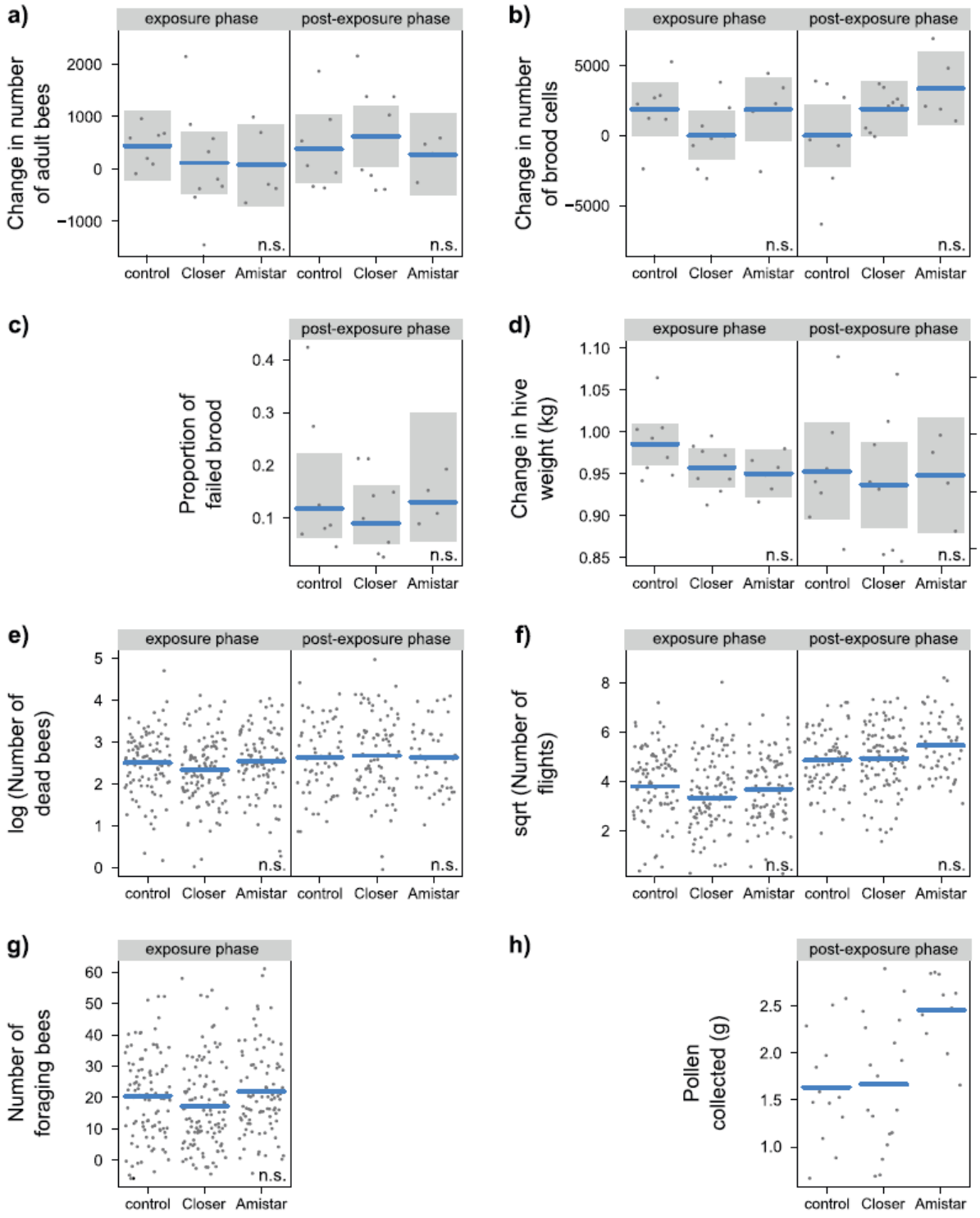

Figure 14: Effects of spray application of the sulfoxaflor (product Closer) and azoxystrobin (product Amistar) compared to the control treatment on honey bees during and after the exposure phase. Shown are effects of treatments on (a) change in the number of adult bees and (b) brood cells, (c) proportion of failed brood, (d) change in colony weight, (e) number of dead bees, (h) flight activity, (g) foraging activity and (h) pollen collection. Plots display prediction lines, partial residuals and, for linear model results, confidence bands (95%, a-d). Abbreviation: n.s., not significant.

4.1.2. Impacts on bumble bees (*B. terrestris*)

Exposure to sulfoxaflor (product Closer) reduced colony growth by 11.1% (P = 0.020) when compared to the growth of non-exposed colonies (Fig. 16a). Additionally, the final number of bumble bees at the end of the experiment was 21.5% lower in colonies exposed to Closer (P = 0.014; Fig. 16b). Both exposure to Closer and Amistar impaired the individual foraging performance of bees (Fig. 16c). Bees exposed to Closer visited 15% fewer flowers across 2 minutes compared to non-exposed bees (before Amistar application: P = 0.006; after Amistar application: P = 0.002). Exposure to Amistar lowered the number of visited flowers by 15.7% (P = 0.003). Flower visitation and the number of daily foraging flights per colony were, however, not affected by exposure to the pesticides. Exposure to Amistar lowered the number of pollen grains deposited on both styles and stigmas (-26%, P = 0.020) and tended to lower the number of deposited pollen grains on stigmas (-32%, P = 0.072) when compared to pollen deposition without exposure to Amistar (Fig. 16d).

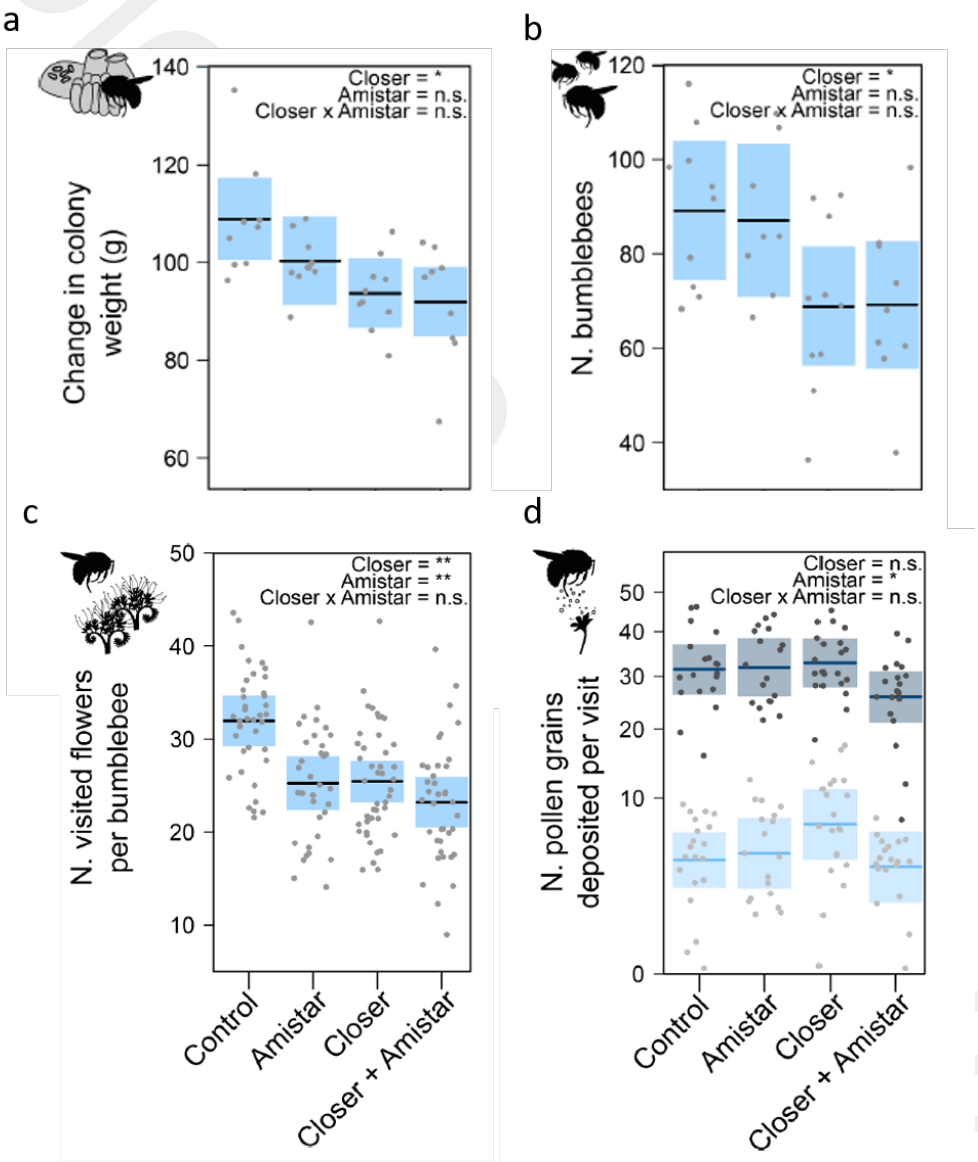

Figure 15: Effects of pesticide exposure on bumble bee (*B. terrestris*) (a) colony growth (b) colony size, (c) individual foraging performance and (d) pollen deposition on stigmas (light blue) or styles and stigmas (dark blue). *P*-values are from linear mixed-effects models (P* < 0.05; ** *P* < 0.01; *** P < 0.001; n.s., not significant. Plots display prediction lines, partial residuals and confidence bands (95%).**

4.1.3. Impacts on solitary bees (*O. bicornis*)

No significant adverse effects of sulfoxaflor (product Closer), azoxystrobin (product Amistar) or their combination on *O. bicornis* adult survival (Fig. 17), reproduction (Fig. 18a,b), offspring mortality (Fig. 18c) or offspring size and sex ratio (proportion of female offspring) were detected. There was however, a tendency for an antagonistic interaction of Closer and Amistar on sex ratio (P = 0.079; Fig. 19).

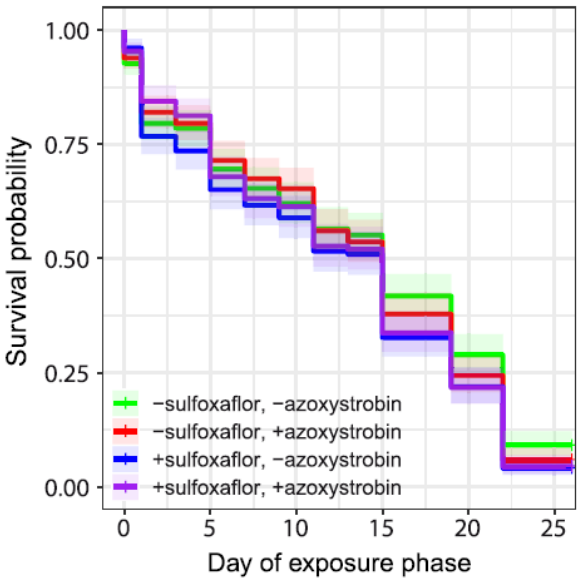

Figure 16: Survival of adult *O. bicornis* females during the exposure phase. Kaplan-Meier survival curves are shown for each treatment level over the entire exposure phase of the experiment (day 0–25; green: control, blue: sulfoxaflor (product Closer) red: azoxystrobin (product Amistar), purple: mix (products Closer + Amistar)). Shaded areas depict 95% confidence intervals. On day 1, three females per cage were sampled for analysis of haemolymph (WP9).

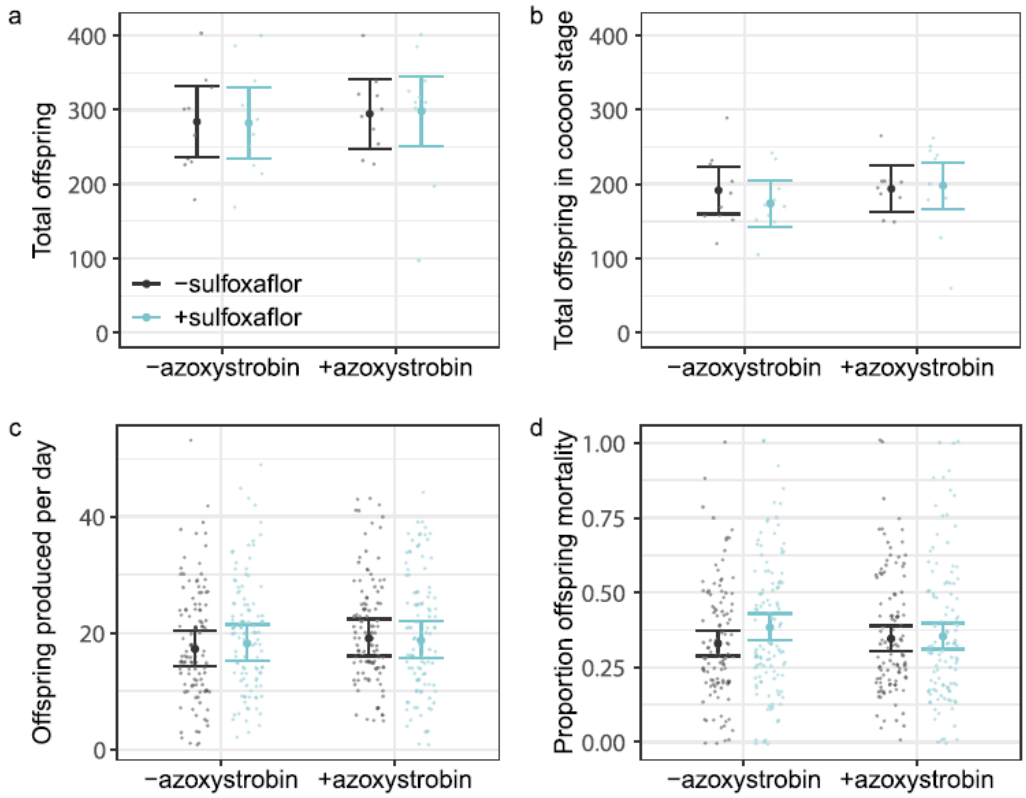

Figure 17: Single and combined effects of sulfoxaflor (product Closer) and azoxystrobin (product Amistar) on different aspects of *O. bicornis* fitness: (a) total number of offspring (brood cells) produced per cage during the exposure phase of the experiment (days 0–25), (b) total number of offspring produced per cage during the exposure phase (days 0–25) that successfully reached the cocoon stage, (c) number of offspring produced per day and cage during days 0–10 of the exposure phase, (d) proportions of daily offspring mortality per cage during days 0–10 of the exposure phase (referring to the day the egg was laid). Black bars/dots: no sulfoxaflor (Closer) applied, blue bars/dots: sulfoxaflor (Closer) applied. Bars depict model predictions and 95% confidence intervals, dots show the raw data points.

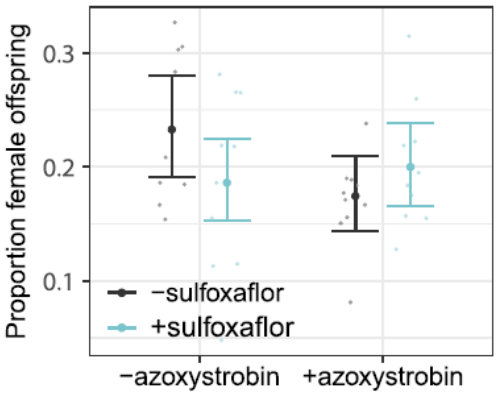

Figure 18: Single and combined effects of sulfoxaflor (product Closer) and azoxystrobin (product Amistar) on offspring sex ratio of *O. bicornis*. Proportion of female offspring produced during days 0–5 of the exposure phase. Black bars/dots: no sulfoxaflor (Closer) applied, blue bars/dots: sulfoxaflor (Closer) applied. Bars depict model predictions and 95% confidence intervals, dots show the raw data points.

No significant main effects of sulfoxaflor (Closer) or azoxystrobin (Amistar) were found on individual foraging performance, flower visitation or foraging activity. However, we found 3-way interactions among Closer, Amistar and time after exposure for individual foraging performance and flower visitation. On day 1, there was an antagonistic interaction of Closer and Amistar on the individual foraging performance. Foraging activity was not affected by the pesticides. Nest recognition ability of *O. bicornis* females tended to be affected by the pesticides, but only at day 7: exposure to Amistar tended to lower the probability of bees to directly find their own nest at the first trial.

4.2. Pesticide-nutrition interactions

4.2.1. Interactive azoxystrobin-nutrition effects on honey bees (*A. mellifera*)

Preliminary results indicate no significant main or interactive effects of Amistar and floral resource plant species on colony weight or the number of alive honey bees (*A. mellifera*) during the pre-exposure, exposure or post-exposure phase of the experiment (Fig. 20a, b). Foraging activity, estimated as the number of bees returning to the colony for 2 min, was also not affected during the pre- and post-exposure phase, but Amistar tended to reduce foraging activity during the exposure phase, irrespective of the floral food resource plant (Fig. 20c; marginally significant Amistar effect: P = 0.077). Please note, however, that data analysis is ongoing, and that these results represent preliminary findings.

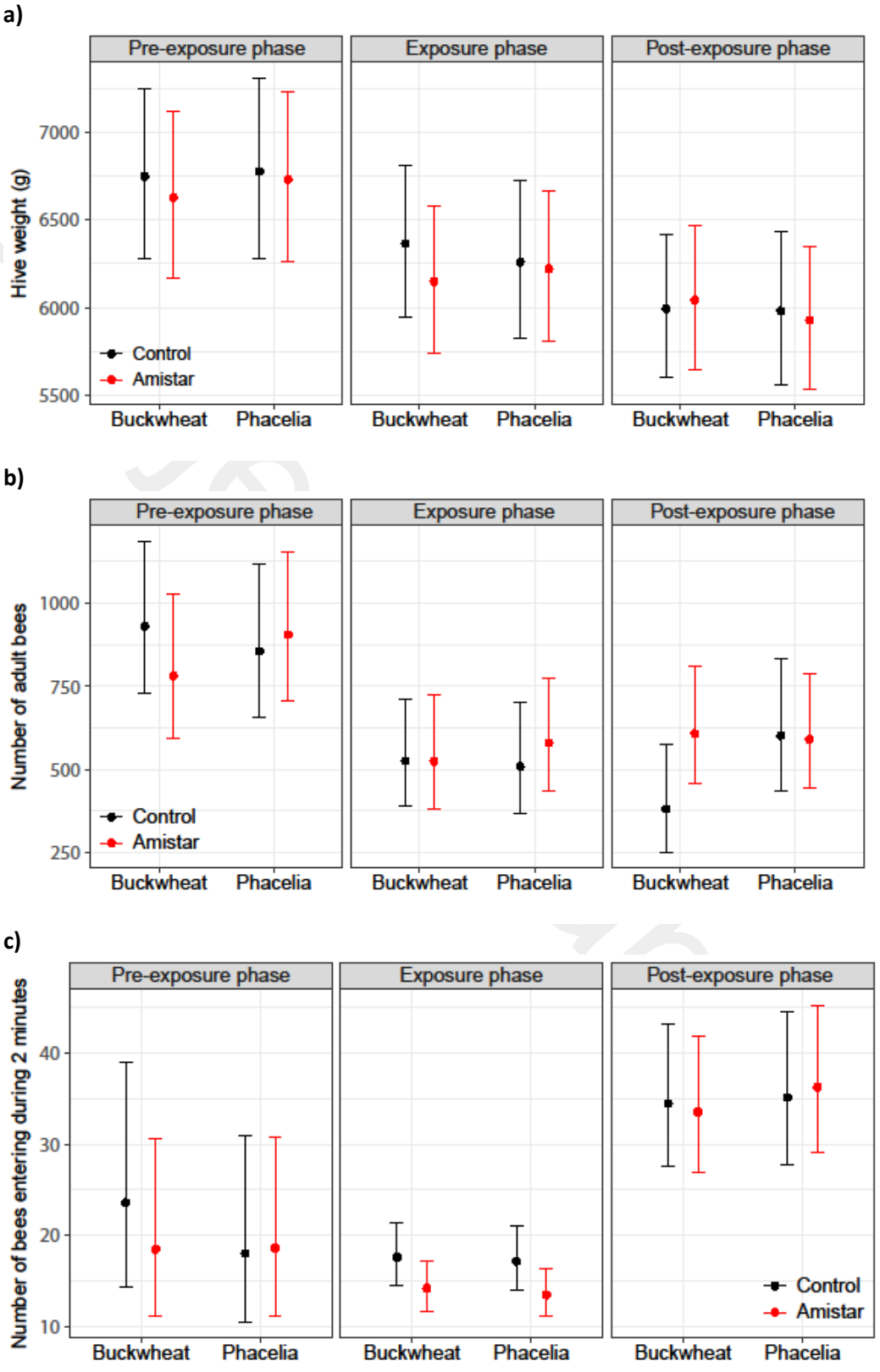

Figure 19: Estimated mean (± 95% confidence intervals) a) colony weight, b) number of alive adult bees and c) foraging activity of honey bees (*A. mellifera*) in enclosures sown with buckwheat (*Fagopyrum esculentum*) or purple tansy (*Phacilia tanacetifolia*) sprayed with the fungicide Amistar (containing azoxystrobin, red colour) or a water-only control (black colour) during the pre-exposure, exposure and post-exposure phase of the semi-field experiment.

4.2.2. Interactive azoxystrobin-nutrition effects on bumble bees (*B. terrestris*)

Effects of flower resource type

Monocultures of buckwheat (*F. esculentum*) had several negative effects compared to monocultures of *P. tanacetifolia* or the floral mix (Fig. 21) on *B. terrestris* colonies. During the pre-exposure phase, mortality in buckwheat colonies was higher and after termination of the exposure phase, buckwheat colonies contained 30% fewer living adults. Additionally, during the exposure period, buckwheat colonies lost weight, while colonies in *P. tanacetifolia* and floral mix cages gained weight. After termination of the experiment, colonies from buckwheat cages had 86% fewer cocoons than colonies from *P. tanacetifolia* or floral mix cages, and 57% fewer adult workers than colonies from *P. tanacetifolia* cages.

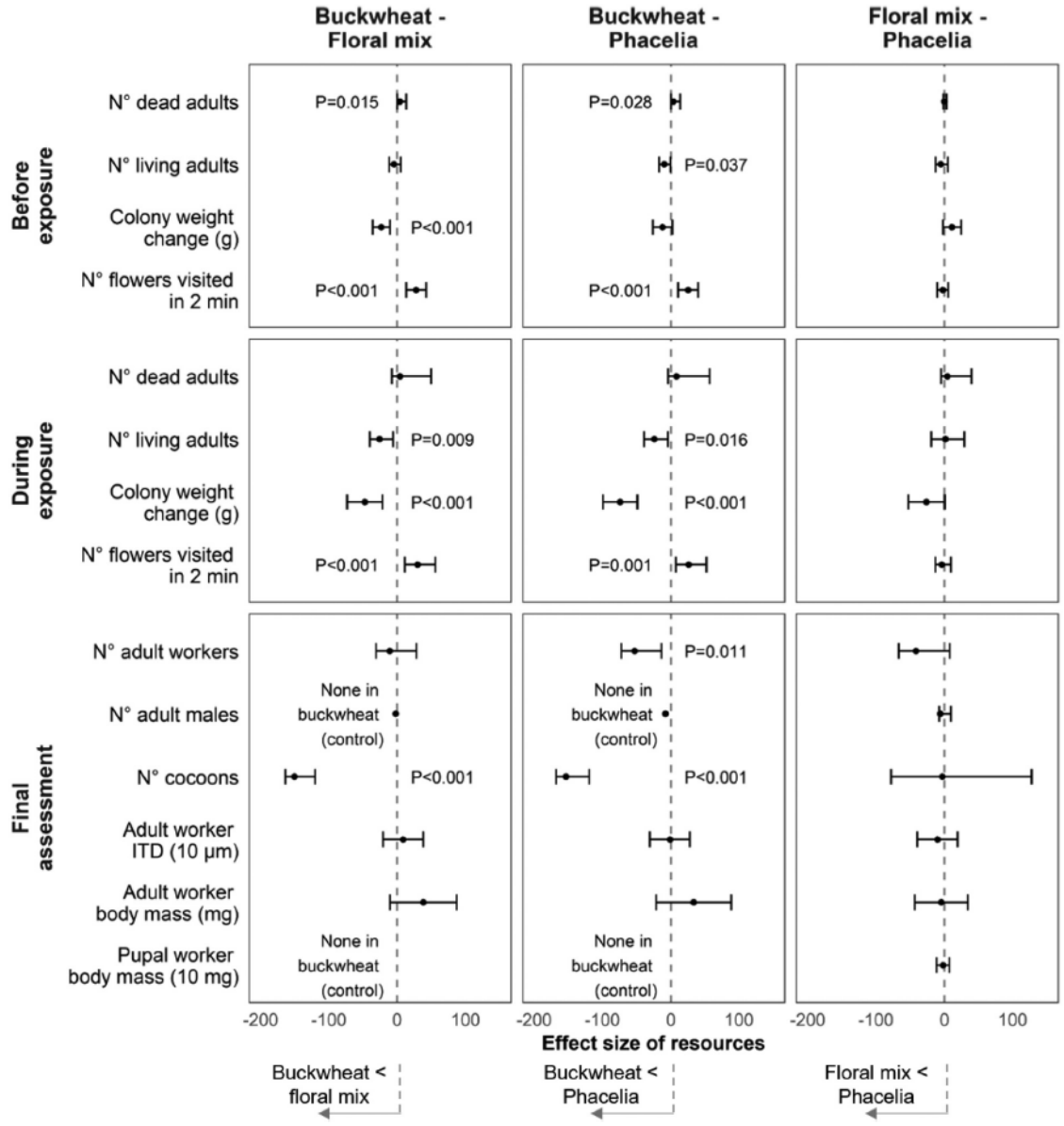

Figure 20: Effect sizes of different floral resource types on *B. terrestris*. Differences in estimated marginal means between different types of flowering resources are illustrated as dots. Error bars indicate 95% confidence intervals. *P*-values < 0.05 are shown. To avoid confounding effects with spray treatment (Amistar or control) only the control group was considered for the exposure period and the final assessment. No confidence intervals for the number of males for comparisons with buckwheat are shown as there were no adult males found in the control colonies placed in buckwheat cages.

Effects of azoxystrobin exposure across floral resource types

Exposure to azoxystrobin (Amistar) caused negative effects on bumble bee colonies in cages planted with monocultures of *P. tanacetifolia*, but no such effects were found for buckwheat or floral mix colonies (Fig. 22). In *P. tanacetifolia* cages, azoxystrobin-exposed colonies gained less weight compared to control colonies. Additionally, azoxystrobin-exposed colonies had 55% fewer adult workers, 88% fewer adult males and a 14% reduced adult worker body mass compared to control colonies at the end of the experiment.

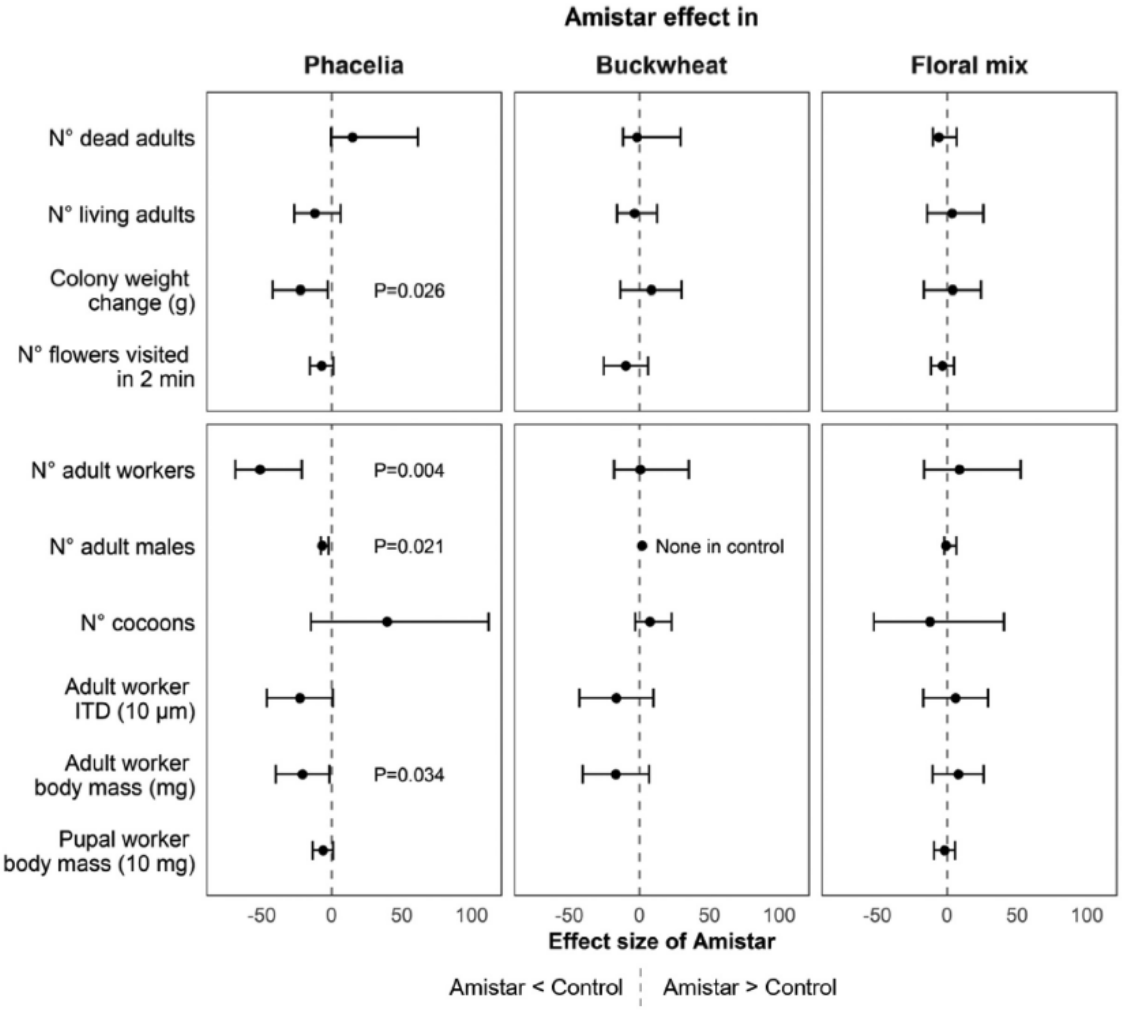

Figure 21: Effect sizes of azoxystrobin (Amistar) exposure across different floral resource types on *B. terrestris*. Differences in estimated marginal means between azoxystrobin-exposed and control colonies, error bars indicating 95% confidence intervals, and *P*-values < 0.05 are shown. No confidence intervals for the number of males in buckwheat are shown as there were no adult males found in the control colonies placed in buckwheat cages.

4.2.3. Interactive flupyradifurone-nutrition effects on solitary bees (*O. bicornis*)

Effects of flupyradifurone and nutritional quality on *O. bicornis*

A synergistic interaction of flupyradifurone (Sivanto prime) exposure and nutritional stress on adult *O. bicornis* survival was found on day 1 of the exposure phase of the experiment (P = 0.010). Flupyradifurone exposure adversely affected *O. bicornis* survival on buckwheat, resulting in a 43% reduced survival in bees treated with flupyradifurone (Sivanto prime) compared to non-treated bees, while there was no significant effect of flupyradifurone in purple tansy or wild mustard (Fig. 23a). There was, moreover, a synergistic interaction between flupyradifurone exposure and nutritional stress on

offspring production on day 1 and 2 (P = 0.007 and P = 0.042, respectively). Offspring production was reduced by 76% and 67% on day 1 and 2, respectively, in buckwheat cages after exposure to flupyradifurone, while there was no significant effect of the insecticide in cages of purple tansy or wild mustard (Fig. 23b). Flupyradifurone exposure and nutritional stress also synergistically affected flight activity (P = 0.001, Figure 22c), flight duration (P = 0.008, Fig. 23d) and flower visitation frequency (P = 0.036, Fig. 23e) on day 1. On day 2, flupyradifurone negatively affected flower visitation frequency (-20%) independent of the type of floral resource (Fig. 24). Nest recognition ability was reduced by 11% and 14% on days 1 and 2, respectively, independent of nutritional stress (Fig. 26). Flupyradifurone residue levels in bee-collected pollen provisions approximately twelve hours after insecticide application were 41.7 ppm in purple tansy, 21.1 ppm in wild mustard and 7.9 ppm in buckwheat.

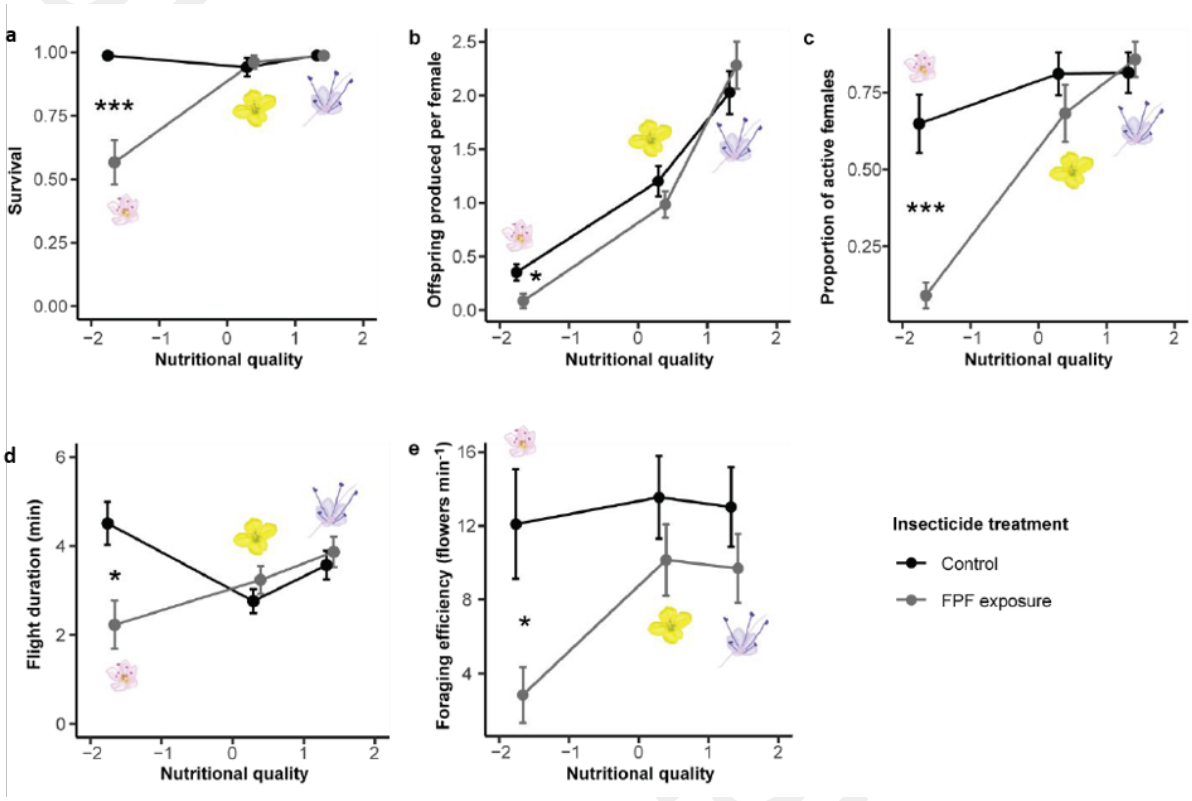

Figure 22: Synergistic interactions between flupyradifurone (FPF) exposure (product Sivanto prime) and nutritional stress on *O. bicornis*. During day 1 after FPF (and water control) application, FPF and nutritional stress (low nutritional quality values) synergistically impacted: a) adult female survival; b) number of offspring (brood cells) a female produced; c) proportion of active females (flight activity); d) flight duration; e) flower visitation frequency. Bars represent estimated marginal means (± SE). Asterisks indicate significant differences between insecticide treatments (FPF application or control) within plant species (*: $P < 0.05$; ***: $P < 0.001$). From left to right: buckwheat (*Fagopyrum esculentum*), wild mustard (*Sinapis arvensis*) and purple tansy (*Phacelia tanacetifolia*).

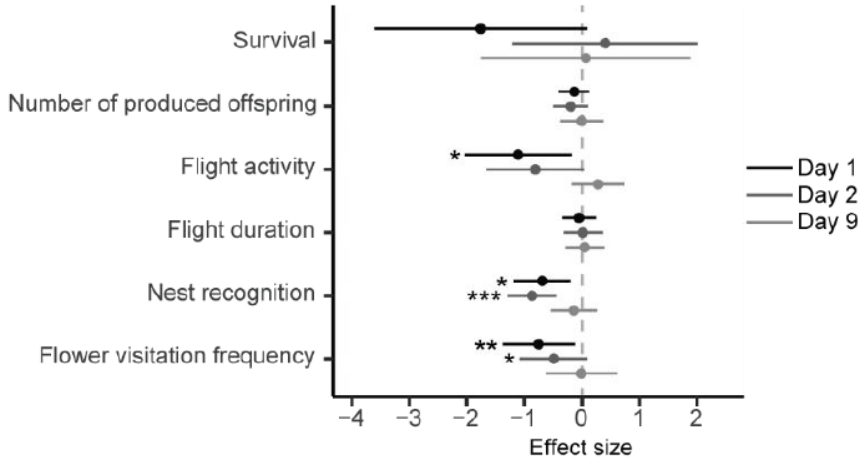

Figure 23: Standardized effect size of flupyradifurone exposure (product Sivanto prime) across the three food plants on *O. bicornis*. Estimates are reported separately for the different assessment periods after flupyradifurone (and water control) application. Error bars represent 95% confidence intervals. Asterisks indicate significant effects (*: *P* < 0.05; **: *P* < 0.01; *: *P* > 0.001).**

4.3. Semi-field dose-response experiments

4.3.1. Dose-response experiment on the impact of azoxystrobin on *B. terrestris*

Preliminary analyses indicate that in the control group (dosing factor = 0), daily syrup consumption per bee increased over time (*P* = 0.032; Fig. 25a), whereas for all other dosing factors, no change in daily syrup consumption per bee was observed (*P* > 0.11; Fig. 25a). This was, however, because the control group started with a lower consumption than other groups and no difference in overall syrup consumption between dosing groups was found after post-hoc correction (linear mixed-effect model (LMM), Šidák correction, *P* > 0.37; Fig. 25a). The colonies exposed to 4 times the field-typical dose experienced a reduced colony weight gain compared to control colonies (Fig. 25b, LMM, Šidák-correction, *P* = 0.01). Similarly, this group experienced higher mortality than the control group (Fig. 25c, LMM, Šidák-correction, *P* = 0.013). However, no dosing group differed from control colonies in the number of living adults (Fig. 25d).

In the additional choice experiment conducted in the laboratory, no differences in syrup consumption per bee were detected (syrup spiked with Ortiva (300 ppb azoxystrobin) vs. control syrup without fungicide).

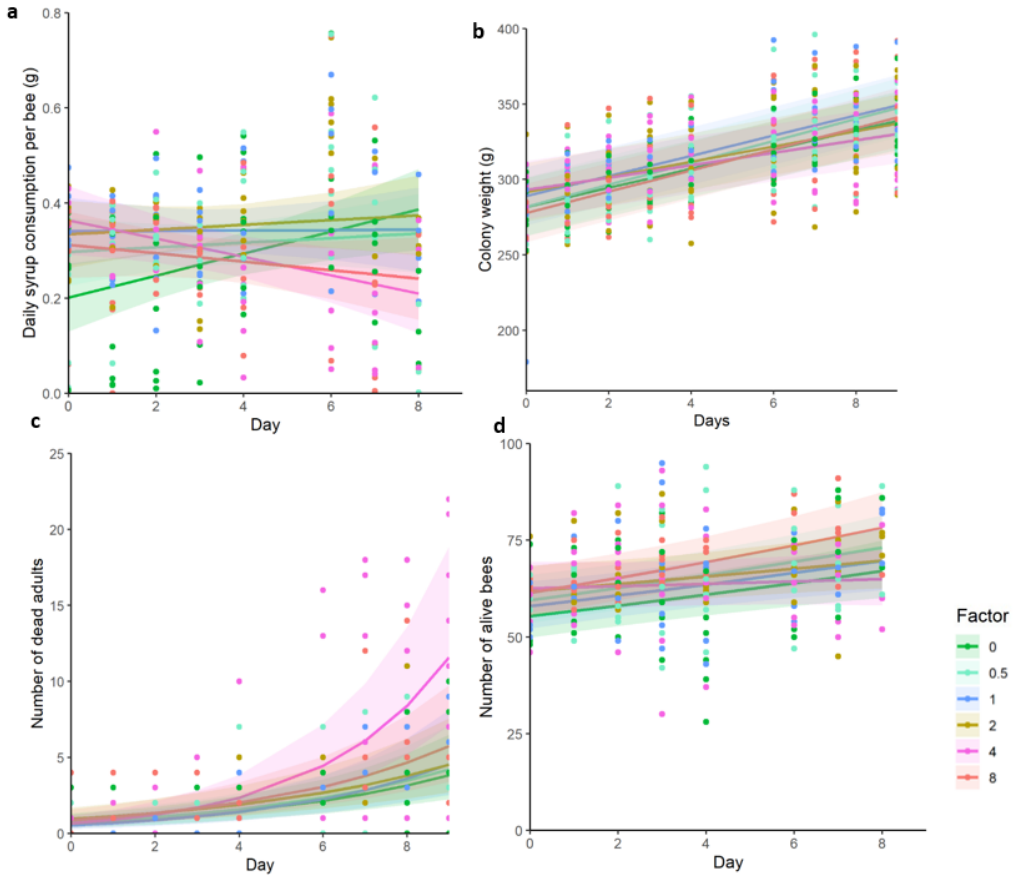

Figure 24: a) Daily syrup consumption per bee, b) colony weight, c) cumulative number of dead adults in relation to time, and d) the number of alive bees per colony in regard to the dosing factor (multiple of a field typical fungicide degradation curve). Dots indicate observations, lines show estimated marginal means obtained from (generalized) linear mixed-effects models and shaded areas depict 95% confidence intervals.

4.3.2. Dose-response experiment on the impact of flupyradifurone on *O. bicornis*

In total, only 47 females nested and 41 of them produced offspring. Results suggest that flupyradifurone concentration had no significant effect on the number of offspring (brood cells; N = 7-9 bees per dose), the number of attempts of a female to find its own nesting cavity or a bee's activity (N = 9-12 bees per dose). Moreover, flupyradifurone concentration did not significantly affect a female bee's foraging trip duration (N = 10-15 bees per dose). However, these results have to be interpreted with great caution considering the low sample sizes and challenges for example in the form of extreme weather conditions during the experiment. Furthermore, the methodology of single exposure to flupyradifurone via syrup in Nicot cages and re-introducing bees into flight cages may have caused considerable stress to the bees, masking any potential treatment effects. In conclusion, these preliminary results should not be considered as evidence for weak effects of flupyradifurone on *O. bicornis*, and clearly more studies under field-realistic conditions are required to assess potential risks associated with different exposure levels of this pesticide for *O. bicornis*.

5. Overall discussion and implications

5.1. Impacts of studied agrochemicals on bees under semi-field conditions

To our knowledge for the first time, the single and combined impacts of the systemically acting insecticide sulfoxaflor (product Closer) and the widely used fungicide azoxystrobin (product Amistar)

were assessed using the same full-factorial design and application rates according to label instructions under field-realistic conditions in highly replicated semi-field experiments across three different model bee species, *A. mellifera*, *B. terrestris* and *O. bicornis*. Interestingly, detected impacts of the two products and their combination differed substantially between the three bee species. No major impacts of Closer or Amistar could be found on the performance of mini colonies of honey bees (*A. mellifera*). Similarly, no major impacts of Closer or Amistar were found on various aspects of fitness and reproductive success of solitary bees (*O. bicornis*) in the semi-field experiment, in which Closer was applied five days before the crop (*P. tanacetifolia*) started to flower, according to label guidelines in different European countries at the time the experiment was conducted (2019). In contrast, pronounced negative impacts of the insecticide Closer (active ingredient sulfoxaflor) were found on different fitness related endpoints of bumble bee (*B. terrestris*) colonies.

Moreover, the fungicide Amistar (active ingredient azoxystrobin) had significant negative impacts on bumble bee (*B. terrestris*) colony development when sprayed onto purple tansy in the semi-field experiment performed in 2021, but not in the experiment performed in 2020. Interestingly, the fungicide Ortiva, having the same composition with azoxystrobin as the active ingredient, had no significant negative impacts on bumble bee colony performance under field-realistic exposure levels when administered via syrup in the dose-response experiment; only at four times higher doses of Ortiva were negative impacts found. It is therefore conceivable that different co-formulants contained in the two products along with the active ingredient azoxystrobin have different impacts on bumble bees. This hypothesis is also supported by the findings of a recent laboratory study that found negative impacts of co-formulants in Amistar on bumble bees (Straw & Brown 2021). Beyond direct effects on bees, fungicides such as azoxystrobin or any co-formulants may also act on (fungal) microorganisms in pollen, thereby indirectly affecting bee health. An alternative explanation could be that the route of exposure determines whether or not azoxystrobin affects bumble bees. In the dose-response experiment, contact exposure during flight was excluded, and it is conceivable that dietary exposure to azoxystrobin through pollen is more harmful than through nectar. Irrespective of the mechanistic pathways underlying our findings, it should be noted that the potential risks of the products Amistar and Ortiva cannot be directly compared based on our studies.

Furthermore, both the insecticide Closer (sulfoxaflor) and the fungicide Amistar (azoxystrobin) had substantial negative consequences on foraging performance of bumble bees, which resulted in impaired pollination service provisioning when bumble bees were exposed to the fungicide Amistar. Yet, during the first days after application of the fungicide Amistar (applied according to label guidelines during crop flowering) antagonistic interactive effects with Closer were found on foraging performance of *O. bicornis*, and a trend for negative impacts of Amistar on nest recognition, but only at day 7 after Amistar application. Moreover, foraging activity of honey bees exposed to Amistar tended to be reduced, irrespective of the food resource in the semi-field experiment performed in 2021.

It is important to note that due to differences in the life histories of the three bee species, which had to be considered in the timing and set up of the semi-field experiments, the exact timing of spray applications slightly varied across experiments (e.g., sulfoxaflor application was 2 days before the start of crop (*P. tanacetifolia*) flowering and colony placement in the experiment with *B. terrestris*, while sulfoxaflor was applied at least 5 days before *P. tanacetifolia* in the experiments with *A. mellifera* and *O. bicornis)*. Furthermore, flower availability and the ratio of flowers available to the number of bees inside cages, as well as weather conditions differed to some extent among experiments. This needs to be considered when comparing and interpreting results of these experiments across the three model bee species.

In addition to the semi-field experiments on the effects of Closer containing sulfoxaflor and Amistar and Ortiva (containing azoxystrobin), impacts of Sivanto prime (containing flupyradifurone) on the solitary bee *O. bicornis* were assessed. Spray applications of Sivanto prime according to label guidelines had significant negative impacts on adult survival, offspring production and foraging performance of *O. bicornis* applied to buckwheat, a plant species offering poor amounts and quality of floral resources.

In fact, Sivanto prime caused a mean mortality of 43% of adult female *O. bicornis*, which, together with several negative sub-lethal effects observed, could cause reductions of the total reproductive output above 40% when exposure occurs early during the reproductive season under such poor nutrition. Thus, although the observed adverse effects were relatively short-lasting they might substantially reduce population development of food stressed *O. bicornis*.

5.2. Interactive effects of multiple stressors on bees

Bees are typically exposed to multiple agrochemicals, such as different insecticides and fungicides, particularly in intensively managed agricultural landscapes. A number of laboratory and a few semi-field studies have demonstrated that certain insecticides and fungicides can additively or synergistically interact with each other to negatively impact bee health (Iwasa *et al.* 2004: Johnson *et al.* 2013, Sgolastra *et al.* 2018, Carnesecchi *et al.* 2019; Siviter *et al.* 2021b). Our findings provide no evidence for major synergistic impacts of the insecticide Closer (sulfoxaflor) and the fungicide Amistar (azoxystrobin) under semi-field conditions, if Closer is applied before crop flowering. We found, in contrast, an antagonistic interactive effect on solitary bees' (*O. bicornis*) foraging performance. The cause of this antagonistic interactive effect remains unclear and deserves further investigation.

The loss of appropriate floral resources is, along with exposure to pesticides, considered a key threat to bees (IPBES 2016), and evidence is accumulating that poor nutrition and pesticide exposure may synergistically impair bee health (Siviter *et al.* 2021b). However, most studies, that have studied interactive effects of pesticides and nutritional stress on bees, have been conducted under laboratory conditions (Siviter *et al.* 2021b; Straub *et al.* 2022). In the semi-field experiments assessing effects of the fungicide Amistar (azoxystrobin) on the bumble bee species *B. terrestris* and the insecticide Sivanto prime (flupyradifurone) on the solitary bee species *O. bicornis*, impacts on bee mortality and fitness related endpoints strongly depended on the floral resources of plants the bees were foraging on. Interestingly, negative effects of Sivanto prime on *O. bicornis* were synergistically reinforced by food stress (i.e., reduced quantity and quality of floral resources), while adverse effects of Amistar on *B. terrestis* colony performance were only observed for bees foraging on purple tansy, offering high quantity and quality of floral resources, but not for bees foraging on buckwheat, a plant species offering poor floral resources.

These differences in effects of the studied pesticides on bees across the different foraging plant species or between monocultures or mixtures of forage plants were not simply driven by differences in exposure levels, but rather by distinct nutritional value (*O. bicornis* experiment) or possibly a combination of nutritional quality and plant morphology potentially affecting foraging performance (*B. terrestris* experiment). In the experiment with *O. bicornis*, for example, the strongest adverse effects of Sivanto prime containing flupyradifurone were observed when applied to buckwheat, which was the plant species used in the experiment with the lowest residue levels in bee-collected pollen. Instead, the additional detoxification experiment, along with analyses of vitellogenin gene expression and various nutritional aspects of the pollen and nectar composition of the different forage plant species used in this experiment, indicate that poor nutrition and associated impaired detoxification were the principal drivers of these synergistic interactive impacts of Sivanto prime on *O. bicornis*. A low protein-lipid ratio and a high protein content of the diet can increase tolerance towards pesticides (Hýbl *et al.* 2021, Linguadoca et al. 2021). Additionally, secondary metabolites, such as phenolic compounds or glucosinolates, can upregulate detoxification and increase bees' resilience to insecticide exposure (Mao *et al.* 2013; Ardalani *et al.* 2021; Hýbl *et al.* 2021). The higher pollen protein content of purple tansy and the lower protein-lipid ratio in combination with the presence of glucosinolates in wild mustard could therefore have contributed to the increased resilience of *O. bicornis* bees towards Sivanto prime (flupyradifurone) compared to buckwheat, with the latter also offering lower quantities of floral resources.

5.3. Implications for risk assessment

Beyond an improved understanding of the potential risks associated with the specific pesticides studied under semi-field conditions outlined above, our findings have broader implications for higher-tier risk assessment of pesticides and contribute to an improved understanding of interactive effects among pesticides and between pesticides and nutrition.

First, our results show that impacts of pesticide products tested under semi-field conditions can substantially vary among different model bee species. Adverse impacts of Closer (sulfoxaflor) and Amistar (azoxystrobin) were strongest in the bumble bee species *B. terrestris*, while for honey bees and the solitary bee species *O. bicornis* impacts were weaker or not detectable for the studied endpoints. This underpins the necessity to consider multiple species in pesticide risk assessments for bees.

Second, several observed negative sub-lethal impacts on endpoints that cannot be assessed in lower-tier laboratory studies, such as impacts on key endpoints such as colony growth or reproductive success of solitary bees highlight the importance of (semi-)field studies in pesticide risk assessments. Results highlight that this is very relevant not only for insecticides, but also fungicides, as demonstrated by the significant negative impacts of the studied fungicide on several endpoints of bumble bee performance and pollination service under semi-field conditions, which would have remained undetected in lower-tier assessments.

Third, while higher tier (semi-)field studies in current risk assessments do not consider potentially distinct impacts of pesticides applied to multiple crops (EFSA 2013; EPA 2014), our findings of the semi-field studies assessing pesticide-nutrition interactions on *O. bicornis* and *B. terrestris* clearly highlight that floral resource differences should be considered in risk assessments, and underpin recommendations of the European food safety authority to evaluate risks of pesticides in multiple crops (EFSA 2013). The results of the semi-field experiment on the impact of Sivanto prime (flupyradifurone) on the solitary bee species *O. bicornis* demonstrates how nutritional stress can substantially augment the adverse impact of pesticides on bee survival, reproductive success and foraging behaviour, which is currently not considered in higher-tier risk assessments that typically use model crops offering high amounts and quality of floral resources, such as purple tansy, *P. tanacetifolia*, which may potentially lead to underestimating the risks of evaluated pesticides on bee health.

Fourth, our results show that pesticides may exert interactive impacts on bees under semi-field conditions. Although we did not find significant synergistic interactions among Closer (sulfoxaflor) and Amistar (azoxystrobin), we found antagonistic interactions for several measured endpoints in different bee species, which need further investigation. These interactions point to the potential for various interactive effects among pesticides to which bees are typically co-exposed, which are currently not considered in higher-tier risk assessment.

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