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Fungicide and insecticide exposure adversely impacts bumblebees and pollination services under semi-field conditions

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ABSTRACT

Sulfoximines, the next generation systemic insecticides developed to replace neonicotinoids, have been shown to negatively impact pollinator development and reproduction. However, field-realistic studies on sulfoximines are few and consequences on pollination services unexplored. Moreover, the impacts of other agrochemicals such as fungicides, and their combined effects with insecticides remain poorly investigated. Here, we show in a full factorial semi-field experiment that spray applications of both the product Closer containing the insecticide sulfoxaflor and the product Amistar containing the fungicide azoxystrobin, negatively affected the individual foraging performance of bumblebees (*Bombus terrestris*). Insecticide exposure further reduced colony growth and size whereas fungicide exposure decreased pollen deposition. We found indications for resource limitation that might have exacerbated pesticide effects on bumblebee colonies. Our work demonstrates that field-realistic exposure to sulfoxaflor can adversely impact bumblebees and that applications before bloom may be insufficient as a mitigation measure to prevent its negative impacts on pollinators. Moreover, fungicide use during bloom could reduce bumblebee foraging performance and pollination services.

1. Introduction

The intensive use of insecticides in agriculture is considered a major driver of pollinator decline worldwide, potentially threatening the pivotal pollination services they provide to crop production and in natural ecosystems (IPBES, 2016; Potts et al., 2016). Systemic chemicals are of particular concern because, contrary to contact insecticides, exposure can occur also through the consumption of contaminated pollen and nectar, as systemic compounds penetrate the plant and reach the flowers (Rortais et al., 2005). Field-realistic studies exploring their effects on pollinators and consequences on the pollination services they provide are urgently needed. Moreover, the impacts of other agrochemicals such as fungicides, and their potential combined effects with insecticides remain poorly explored (Brown et al., 2016; Cullen et al., 2019).

Sulfoximines are a new class of systemic insecticide rapidly growing in the global pesticide market as potential successors to neonicotinoids (Brown et al., 2016). They efficiently control a broad range of sap-feeding pests and act similarly to neonicotinoids as selective agonists of insect nicotinic acetylcholine receptors (Cutler et al., 2013), while exhibiting only a low degree of cross-resistance (Sparks et al., 2013). However, sulfoximines' similarities to neonicotinoids' mode of action (Simon-Delso et al., 2015) and their potential application over vast geographic areas have raised concerns regarding potential adverse effects on non-target organisms (Jiang et al., 2019) and especially on pollinators (Brown et al., 2016). Artificial feeding experiments have recently suggested negative impacts of sulfoxaflor on the reproductive output of bumblebee colonies (Siviter et al., 2018a; Siviter and Muth, 2020). These effects seem to be driven by reduced egg-laying activity and altered larval development (Siviter et al., 2020a, 2020b) rather than impaired bee-foraging behavior and cognition (Siviter et al., 2019, 2018b). However, the impacts of sulfoximine-based insecticides on

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bumblebees when used according to standard application practices under (semi-)field conditions are currently unknown, as well as their potential effects on pollination services. Moreover, such field realistic studies give insights into the effectiveness of mitigation strategies which have been established in several countries to limit exposure of pollinators to sulfoximines (e.g. safety periods between application and crop bloom).

Besides insecticides, pollinators are at risk of exposure to further agrochemicals commonly applied to crop fields globally (Cullen et al., 2019; Mullin et al., 2010). Fungicides, although lacking acute toxicity against insects, may impact bees directly by altering metabolism, reproduction and food consumption (Bernauer et al., 2015; Liao et al., 2017; Mao et al., 2017), and indirectly by increasing insecticide toxicity (Sgolastra et al., 2017; Tosi and Nieh, 2019; Tsvetkov et al., 2017). Fungicides, especially but not exclusively sterol-biosynthesis-inhibitors, can in fact alter metabolic detoxification pathways, decreasing bees' tolerance for insecticides (Carnesecchi et al., 2019; Iwasa et al., 2004). A recent laboratory study found exposure to the fungicide fluxapyroxad, a succinate dehydrogenase inhibitor, to increase the negative impacts of sulfoxaflor on solitary and honeybees, but not on bumblebees (Azpiazu et al., 2021). Combined exposure to multiple pesticides is likely to occur in the field since products are often applied in mixtures and pollinators can forage in different fields, hence being exposed to different agrochemicals in a relatively short window of time. Moreover, co-formulants used in pesticides are generally considered safe for non-target organisms, but can constitute an often overlooked risk for bees (Mullin, 2015). Azoxystrobin is a broad-spectrum systemic fungicide belonging to the strobilurin class, widely used in agriculture (Bartlett et al., 2002) and frequently detected in pollen collected by both managed and wild bees (Hladik et al., 2016; Krupke et al., 2012). This fungicide can be toxic to both terrestrial and aquatic organisms (Leitão et al., 2014; Rodrigues et al., 2013). The few studies exploring pollinator response to azoxystrobin reported alterations of the hormonal regulation system (Christen et al., 2019), but no effects on foraging activity and colony development (Tamburini et al., 2021) and potential for negative synergistic effects when applied with another fungicide (Fisher et al., 2017). Understanding the sublethal effects of commonly used fungicide products and potential synergies with insecticides is of paramount importance to identify drivers of wild pollinator decline in agroecosystems.

Here, we investigate the impacts of two widely used systemic pesticides, the product Closer containing the insecticide sulfoxaflor and Amistar containing the fungicide azoxystrobin, on bumblebee (*Bombus terrestris*) foraging performance and colony growth under field-realistic conditions. We further test the effects of these pesticides on the pollination services provided by bumblebees. We set up forty enclosures in which pesticides were sprayed directly on crop plants (*Phacelia tanacetifolia*) following label instructions. The insecticide was applied before the onset of flowering, in order to test whether application before the bloom effectively limits harm to wild bees. Two days later, when enough flowers had opened, bumblebee colonies were placed inside the enclosures. The fungicide was applied at peak flowering. We hypothesized that both the exposure to the insecticide sulfoxaflor and to the fungicide azoxystrobin may negatively affect bumblebee colony growth, foraging activity and pollination services. We further tested whether exposure to the fungicide would exacerbate insecticide impacts on bumblebees.

2. Methods and materials

2.1. Experimental design

The study was performed in 2019 on an experimental field site of the University of Freiburg (southwest Germany, Freiburg, 48°01′08.5″N 7°49′31.2″E) that had not been cultivated for several years before the onset of the experiment. We tested the direct and combined effects of two broad-spectrum systemic pesticides, the insecticide Closer (Corteva, product ID: 16886, purchased from Ipag, Italy) containing the active ingredient sulfoxaflor, and the fungicide Amistar (Syngenta, product ID: A12705B, purchased from Stähler Suisse AG, Switzerland) containing azoxystrobin on bumblebees (*B. terrestris*). The insecticide further included sulfonated aromatic polymer, sodium salt and 1,2-benzisotiazol3(2H)-one as co-formulants, whereas the fungicide included alcohol ethoxylates, sodium poly (naphthaleneformaldehyde) sulfonate and 1,2-Benzisothiazol-3(2H)-on (Amistar® Safety Data Sheet, 2017; Closer™ Safety Data Sheet, 2021). The simultaneous exposure to both products for pollinators is possible and likely to occur as both pesticides are widely used, they can be applied to the same crops (e.g., oilseed rape, broad beans, peas) and residues can be present within the same time window that includes flowering. We set up a randomized full factorial enclosure experiment, with the two pesticides as crossed factors (four treatments: (i) Closer, (ii) Amistar, (iii) Closer + Amistar and (iv) control, 10 replicates (enclosures) each; Supplementary Fig. 1). The field (0.7 ha) was sown in late April with *Phacelia tanacetifolia* (8 kg seeds/ha), commonly used as model crop in higher-tier ecotoxicological risk assessment studies (Gradish et al., 2016). After crop establishment, we set up 40 enclosures (9 × 6 m, height: 2.5 m; steel frames and fine-mesh nylon net; Howitec Netting b.v) evenly distributed across the field (Supplementary Fig. 1). The enclosures were positioned at least 4 m from the field boundaries (greater than 1000 m distance from the nearest crop) and at least 4 m apart from each other. The net had a vertical zipper on one side that allowed access into the enclosure (all entrances faced in the same direction). The crop was cut in late June at the height of approximately 60 cm with a hedge trimmer as a standard procedure to ensure synchronized and homogenous onset of flowering across enclosures. Enclosures were randomly allocated to treatments (plant cover and the number of closed inflorescences per enclosure estimated before the start of the experiment did not differ between treatments; Supplementary Table 1).

2.2. Bumblebee colonies

We used *B. terrestris* colonies as this species is considered as a key model organism together with *Apis mellifera* and *Osmia bicornis* to investigate pesticide impacts on bees (EFSA, 2013). It is in fact an important pollinator, widespread throughout Europe and in other parts of the world and, along with other *Bombus* species, commercially reared for the pollination of several agricultural and horticultural crops (Velthuis and van Doorn, 2006). Forty *B. terrestris* colonies consisting of a queen and approximately 25 workers were provided by Katz Biotech (Baruth/Mark, Germany) and delivered three days before the start of the experiment (day −3). One day before the start of the experiment (day −1) all colonies were weighed and checked for the presence of the queen and any macroscopic signs of pathogens or parasites (e.g., mites or wax moths) and no visual signs were detected. However, a thorough investigation on the prevalence of pathogens and parasites in the colonies was not carried out before placement, and since commercial colonies can be infested, this could add unexplained noise to our data (Rundlöf et al., 2015). On day −1 pollen provisions were removed and, immediately before releasing bumblebees into enclosures (day 0), access to syrup was closed. On the 17th of July 2019 (day 0), when sufficient open flowers were present in each enclosure to support colony growth (crop stage BBCH 61–63, Meier, 1997), colonies were randomly allocated to treatments and workers were immediately allowed to start foraging inside enclosures. Colonies were placed on stands (15 cm high) and protected from rain and direct sunlight with a wooden roof. The experiment ran for a total of 18 days, until floral resources significantly declined in more than 50% of the enclosures. On the evening of day 18, nest openings were closed and the next morning, 5th of August, colonies were frozen at − 20 °C.

2.3. Pesticide application

We followed label instructions of the products at the time of the

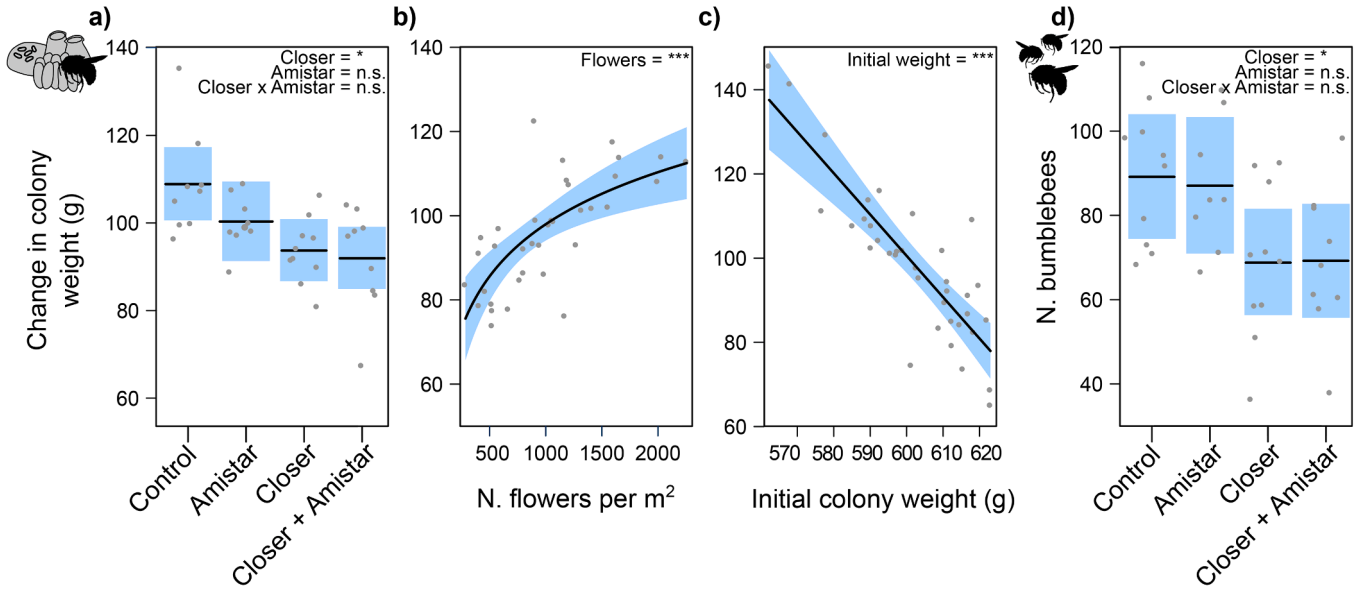

Fig. 1. Effects of (*a*) pesticide exposure, (*b*) flower abundance (average number of flowers m^{-2} per enclosure) and (*c*) initial colony weight on bumblebee (*B. terrestris*) colony growth (colony weight $_{Day15}$ − initial colony weight $_{Day-1}$). (d) Effects of pesticide exposure on colony size (final number of bumblebees per colony). P-values are from linear mixed-effects models (*P < 0.05; ** P < 0.01; *** P < 0.001; n.s., not significant. See Supplementary Table 5). Plots display prediction lines, partial residuals and confidence bands (95%).

experiment in regard to application timing, rate and procedure, in order to ensure field-realistic exposure conditions.

Application timing. The product Closer (sulfoxaflor) was applied in half of the enclosures on the 15th of July, before flowering at BBCH stage 55–59 according to label instructions prescribed in Italy, 2 days before the colonies were placed inside the enclosures (see experiment timeline, Supplementary Fig. 2). Inflorescences presenting open flowers at the time of the application were manually removed in each enclosure before the spraying. The product Amistar (azoxystrobin) was applied in half of the enclosures at full bloom (BBCH 63–65) on the 22nd of July, 7 days after Closer application and 5 days after the colonies were placed inside the enclosures (Supplementary Fig. 2). At each application date, control enclosures were sprayed with a volume of water equal to the volume of diluted product applied to the pesticide-treated enclosures, i.e., each cage was sprayed twice.

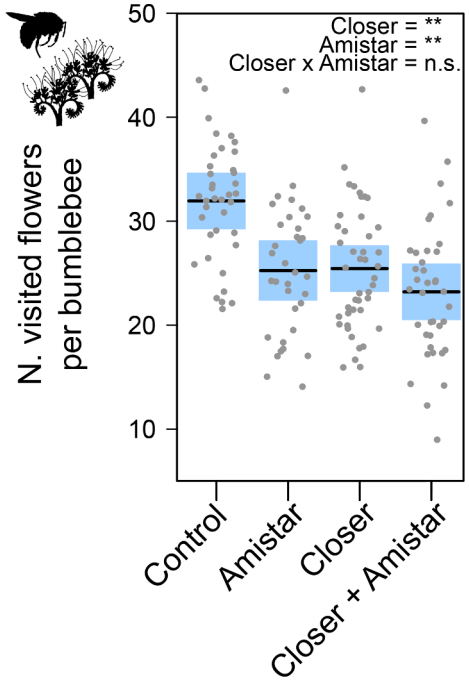

Fig. 2. Effects of pesticide exposure on individual foraging performance (number of visited flowers per bumblebee) after the application of the fungicide. P-values are from linear mixed-effects models (**P < 0.01; n.s., not significant. See Supplementary Table 5). Plots display prediction lines and partial residuals and confidence bands (95%).

Application rate and procedure. Application rates for both pesticides represented the maximum allowed by label instructions for a single application: Closer (sulfoxaflor) was applied at a rate of 48 g a.i. per hectare (=0.4 L/ha of formulated product) and Amistar (azoxystrobin) at a rate of 250 g a.i. per hectare (=1 L/ha of formulated product). Pesticide applications were performed by a "Good Experimental Practice" certified contractor in dry weather days with wind speed lower than 3.0 m/s. To ensure an even application of the products, spraying was performed using a motorized sprayer equipped with a 3 m long bar with anti-drift spraying nozzles. Large plastic sheets covering completely the enclosure walls were attached during spraying to reduce the possibility of spray drift to adjacent enclosures. The application of water and pesticides was performed utilizing different equipment and protective gear to avoid contamination. Although we are confident that our efforts minimized the risk of spray drift to an acceptable level, we cannot rule out the possibility of pesticide contamination between enclosures and by other agrochemicals applied to distant surrounding crop fields. As mentioned before, the simultaneous exposure to multiple pesticides, prevalent condition in agroecosystems (Mullin et al., 2010), has the potential to decrease the detoxification ability in bees, making them more vulnerable to the tested compounds (Carnesecchi et al., 2019). During Amistar (azoxystrobin) application nest openings were kept closed to avoid bumblebee accidental escapes from the enclosure during the spraying operations, and opened few minutes later. This represents a conservative approach compared to the potential exposure risk in the field, since guidelines on product label do not prohibit to spray the fungicide when bees are foraging.

2.4. Colony growth and size

Colony growth was estimated by calculating the difference between the last measure of colony weight (day 15, four days before colony

termination) and the initial one (colony weight $_{Day15}$ − initial colony weight $_{Day-1}$). Initial colony weight did not differ between treatments (Closer = 597.9 ± 17.9, Amistar = 608.0 ± 10.9, Closer + Amistar = 601.2 ± 12.9, control = 598.8 ± 18.0, mean ± SD; ANOVA: $P = 0.458$, $F_3 = 0.89$). Colony size was measured as the total number of bumblebees per colony counted at the end of the experiment (day 15).

2.5. Foraging performance

We assessed foraging performance measuring 1) the number of flowers visited by single bumblebees in a given period of time (i.e., individual foraging performance), 2) the number of flower visiting bumblebees in a given period of time and area (i.e., flower visitation), and 3) the number of flights performed by bumblebees of each colony per day (i.e., number of foraging flights). We additionally measured flower abundance in the enclosures. Individual foraging performance, flower visitation and flower abundance were assessed 7 times during the experiment, every second or third day (Supplementary Fig. 2). Observations were conducted during adequate weather conditions (≥13 °C, no rain, wind speed < 2 m/s) and were randomized across treatments during the day. The number of flights per colony was automatically recorded by bumblebee monitoring devices (see below).

Individual foraging performance. We recorded the number of individual *P. tanacetifolia* flowers visited by an individual worker bumblebee during a period of 2 min. This measure was taken for two different randomly selected bumblebees in each enclosure. If the observed bee was lost by the observer before the two minutes expired (i.e., the bee was not visible anymore or returned to the nest), another bumblebee was immediately selected, and the measure repeated.

Flower visitation. We assessed the number of foraging bumblebees that entered in two randomly selected 3 × 3 m areas (1/6 of the total enclosure area) and that visited flowers, during a period of 3 min. Data were collected by two observers per enclosure (one observer per selected area).

Flower abundance. We estimated the mean number of open flowers per square meter within each of the two 3 × 3 m areas where flower visitation was assessed (two estimates per enclosure). Within a 1 × 1 m sub-plot of the same area, we first counted the total number of inflorescences and then the number of individual open flowers of three representative inflorescences. We then estimated the number of open flowers per square meter as the average number of flowers per inflorescence multiplied by the number of inflorescences.

Number of foraging flights. Colony-level monitoring devices measuring how many bumblebees return to the nest were provided by Atlantic Pollination Ltd and were attached to each colony. The monitoring devices were specifically designed to match the nest box units and to fit its opening system. The monitoring units used infrared sensors to detect arrivals of bumblebees in the nest. A pair of sensor units in each entrance allowed to detect not only the presence of a bee but also the direction of the motion: to count as a valid arrival, the sensors had to correctly report the movement direction, thus ensuring a reliable count (see Supplementary Fig. 3 for details). Bumblebee foraging flights were recorded throughout the period of the study (days 1–18). The daily number of arrivals per colony was used as measure of number of daily foraging flights.

2.6. Pollination services (pollen deposition)

We investigated pesticide effects on pollination services provided by bumblebees by comparing single-visit pollen deposition across treatments (King et al., 2013). To measure single-visit pollen deposition, we randomly selected five inflorescences of *P. tanacetifolia* per enclosure in BBCH stage 59 (right before blooming but before any flower was open) immediately after the fungicide application (day 5). We covered the target inflorescences with air-permeable plastic pollination bags supported by wooden sticks to avoid contact with flowers. The following

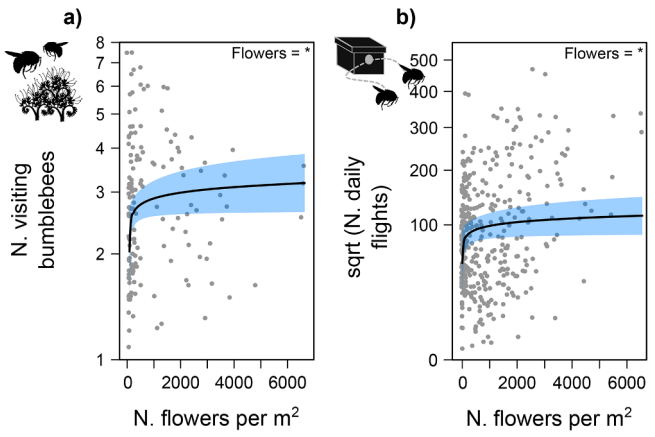

Fig. 3. Effects of flower abundance (average number of flowers m^{-2} per enclosure) on (*a*) flower visitation (number of bumblebees visiting flowers) and on (*b*) the daily number of foraging flights per colony, after the application of the fungicide. P-values are from linear mixed-effects models (*P < 0.05; n.s., not significant. See Supplementary Table 5). Plots display prediction lines, partial residuals and confidence bands (95%).

day (day 6), up to five opening flowers per target inflorescence of which stamens did not yet release pollen were carefully emasculated using scissors to prevent autogamous pollination. Other flowers or buds were removed and the inflorescence was bagged again. Single-visit pollen deposition was subsequently measured on the second, third and fourth day after opening of target flowers (day 7–9) as stigmas of *P. tanacetifolia* are not receptive on the first day after opening of flowers (Williams and Thomson, 2003). Inflorescences were unbagged and offered to bumblebees without disturbing their foraging performance until one or more flowers were visited once (Williams and Thomson, 2003). The two styles with stigmas from each visited flower were then removed with forceps, carefully placed on a microscope slide, immediately fixed with a drop of glycerin jelly and covered with a cover slip. We collected a total of 136 samples (two stigmas per sample), 65 at the first, 47 at the second and 24 at the third day. Sampling was evenly distributed among treatments during each sampling date. We sampled on average 2.3 inflorescences (min = 1, max = 3) and 3.4 flowers (min = 2, max = 6) per enclosure. Moreover, 20 bagged and unvisited flowers (five flowers per treatment combination) were collected to evaluate the efficacy of the emasculation. We counted the number of *P. tanacetifolia* pollen grains present on each slide in the lab under a binocular microscope. We counted the pollen grains found attached both on styles and stigmas and those attached only to stigmas in order to consider both the total pollen deposited by bumblebees and the pollen strictly available for plant reproduction. Unvisited flowers had a lower amount of pollen grains (mean = 0.35, SE = 0.22) than the visited ones (mean = 14.61, SE = 22.31; Welch's test, $P < 0.0001$).

2.7. Statistical analyses

To test the effects of the pesticides on colony growth and size we used two linear models (one record per enclosure). Closer (categorical), Amistar (categorical), flower abundance (continuous; the average number of flowers per enclosure over the whole experiment, log-transformed) and their interactions were included as explanatory variables in the models. We also included the initial colony weight as an additional explanatory variable in the models in order to account for differences in the initial colony size.

Individual foraging performance, flower visitation, and number of flights recorded on different days were analyzed with linear mixed-effects models (LMMs; one record per sampling day per enclosure). All foraging performance and flower abundance data were averaged at the enclosure level for each day to improve model residuals. Data averaging

did not qualitatively affect the results (not presented). Since the two pesticides were applied at different dates, we split the dataset in two, before and after the fungicide application: first period from day 1 to day 4 (two treatments: Closer and control, 20 replicates per treatment) and second period from day 5 to day 18 (four treatments: Closer, Amistar, Closer + Amistar, control; 10 replicates per treatment; Supplementary Fig. 2). Models used to analyze data of the first period included Closer, flower abundance and their interactions as fixed effects. Models used for data analysis of the second period included Closer, Amistar, flower abundance and their interactions as fixed effects. We also included air temperature (data from a weather station positioned 2.5 km away from the experimental site) and the initial colony weight as additional explanatory variables, in order to account for variations in flight performance due to the weather and for differences in the initial colony size that could have affected colony dynamics. All models included enclosure ID as random factor. Time (i.e. days since colonies were placed in the enclosures) was not included in the models because it strongly correlated with flower abundance (Pearson's correlation coefficient r = 0.59 and r = -0.75 for the first and second period, respectively). We therefore decided to only include flower abundance because it is likely to strongly influence bumblebee activity, growth and exposure to pesticide. However, the inclusion of time in the models did not qualitatively affect the results (not presented). In order to match the daily data regarding the number of flights and pollen deposition, we replaced missing flower abundance data interpolating the existing values ("na.approx" function in "zoo" package, (Zeileis and Grothendieck, 2005)). The number of flower-visiting bumblebees (flower visitation) and the number of arrivals per colony (number of foraging flights) were log- and square root-transformed, respectively, to achieve normal distribution of model residuals. We added a first-order autoregressive correlation structure to all models to account for autocorrelation of repeated measures within enclosures.

The number of pollen grains deposited on styles and stigmas and only stigmas was analyzed with two linear mixed-effects models. Pollen deposition data were averaged at the inflorescence level for each enclosure and day when multiple flowers per inflorescence were sampled. Data averaging resulted in simpler models and did not qualitatively affect the results (not presented). Closer, Amistar, flower abundance, and their interactions were included as fixed effects in the model. Air temperature and the initial colony weight were also included in the models as additional explanatory variables. The number of pollen grains was log-transformed to achieve normal distribution of model residuals. Because some cages were sampled in different days, we included both enclosure ID and date in a crossed random structure.

Four colonies were excluded from the analyses because either they failed (i.e., colony collapsed) during the study or workers started building a new nest outside the nest box (three and one colonies, respectively). Two excluded colonies belonged to the Amistar, one to the Closer + Amistar and one to the control group. Nevertheless, results of analyses including those colonies were qualitatively similar to those reported by excluding them (see Supplementary Table 2). On the 26th of July (day 9, fourth foraging performance assessment) individual foraging performance was assessed in 36 enclosures (9 enclosures per treatment) and visitation rate data was assessed in 18 enclosures (four control, five Closer, five Amistar, four Closer + Amistar enclosures). Between day 8 and day 11, high air temperatures decreased bumblebee activity. Nevertheless, results of analyses excluding data after the 24th of July (day 7) were qualitatively similar to those reported by analyzing the whole period (see Supplementary Table 3). Moreover, considering the four pesticide treatments as one categorical factor (i.e. 4 levels) produced qualitatively similar results for all the models (see Supplementary Table 4). To assess potential multicollinearity between the explanatory variables, we calculated the variance inflation factor (VIF) for all the models without interactions. The highest VIF scores were below 2.2, indicating low collinearity in our dataset (Dormann et al., 2013). Normality and homoscedasticity of the model residuals were validated graphically. Final models were estimated using the REML method in the "lme4" and "nlme" packages (Bates et al., 2014; Pinheiro et al., 2019) implemented in R (R Core Team, 2021).

3. Results

Pesticide exposure influenced bumblebee colony growth and size, individual foraging performance and pollen deposition (Supplementary Table 5). No interactive effects between the two pesticides were detected for any of the response variables.

3.1. Colony growth and size

Colonies exposed to Closer gained 11.1% less weight during the experiment compared to those not treated with the insecticide (P = 0.020, Fig. 1a, Supplementary Table 5). Moreover, we found the gain in colony weight to be positively related to flower abundance in the enclosure and negatively to initial colony weight (Fig. 1b and c). The number of bumblebees per colony at the end of the experiment was 21.5% lower under Closer compared to bumblebees not treated with the insecticide (Fig. 1d)

3.2. Foraging performance

The number of flowers that individual bumblebees visited during a period of 2 min was 15% lower under Closer compared to bumblebees not treated with the insecticide (by 14.8% and 15.0% before and after fungicide application, respectively; Fig. 2a and Supplementary Fig. 4a) and 15.7% lower under Amistar (azoxystrobin) application (Fig. 2b, Supplementary Table 5) compared to bumblebees not treated with the fungicide. Moreover, individual foraging performance was positively related to air temperature and negatively to flower abundance before fungicide application (Supplementary Fig. 4b and 4c; Supplementary Table 5). We found no effects of pesticide exposure on flower visitation

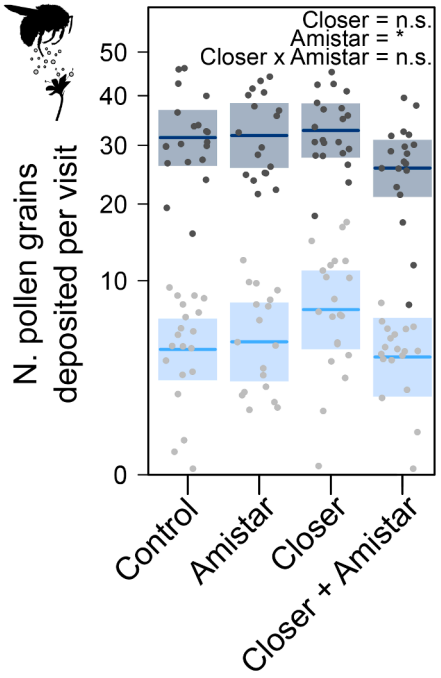

Fig. 4. Effects of pesticide exposure on the number of pollen grains deposited on stigmas (light-tone dots and lines, Amistar, P = 0.072) or styles and stigmas (dark-tone dots and lines, Amistar, P = 0.020) of *Phacelia tanacetifolia* during one visit. P-values are from linear mixed-effects models (*P < 0.05; n.s., not significant. See Supplementary Table 5). Plot displays prediction lines, partial residuals and confidence bands (95%).

(i.e. number of bumblebees visiting flowers) and on the number of daily foraging flights per colony (Supplementary Table 5). Flower visitation was positively related to air temperature before fungicide application (Supplementary Fig. 4d) and to flower abundance after fungicide application (Fig. 3a). The number of daily foraging flights was positively related to flower abundance both before and after fungicide application (Fig. 3b and Supplementary Fig. 4e) and negatively related to the initial colony weight before fungicide application (Supplementary Fig. 4f).

3.3. Pollination services

The number of pollen grains deposited on both styles and stigmas and on stigmas during one visit was 26.0% and 32.0% lower, respectively, under Amistar (azoxystrobin) application (Fig. 4), although the effect was statistically significant only when considering pollen grains deposited on both styles and stigmas ($P = 0.020$, Supplementary Table 5). This pattern was mostly driven by the Closer + Amistar treatment, even though the interaction Closer × Amistar was not significant ($P = 0.239$, Supplementary Table 5). Raw data are presented in the supporting information (Supplementary Figure 5).

4. Discussion

Our study shows that spray applications of two widely used systemic pesticides, the product Closer containing the insecticide sulfoxaflor and the product Amistar containing the fungicide azoxystrobin, can affect bumblebees under semi-field conditions. Both the exposure to the insecticide and to the fungicide reduced individual foraging performance. Insecticide exposure further impaired colony growth and size whereas fungicide exposure decreased pollen deposition. We found indications for resource limitation that might have exacerbated pesticide effects on bumblebee colonies (Tosi et al., 2017). Our results indicate that sulfoxaflor exposure could be hazardous to bumblebees under field conditions (Siviter and Muth, 2020), even when applied two days before crop bloom. This mitigation strategy might thus be inadequate to eliminate the risk for bumblebees. Our study also indicates that the use of fungicides during bloom has the potential to reduce bumblebee activity and the pollination services they provide.

Colonies exposed to Closer (sulfoxaflor) gained less weight during the experiment compared to those not treated with the insecticide. Previous studies found that sulfoxaflor, when administered under laboratory conditions at dosages consistent with potential post-spray field exposure, impacted the reproductive output of bumblebee colonies, reducing the number of eggs, workers and sexuals, i.e. gynes and males, produced (Siviter et al., 2020b, 2018b). The lower weight gain might therefore be explained by lower increase in colony size (Fig. 1d). Moreover, in our study exposure to Closer reduced individual foraging performance, which could have resulted in fewer pollen and nectar stores, contributing to the lower weight gain compared to untreated colonies. We also found colony growth to be positively related to flower abundance and negatively to the initial colony weight. These findings suggest that available pollen and nectar resources within the enclosures were a limiting growth factor and that, consequently, colony dynamics might have been driven by density-dependent mechanisms, with smaller colonies at the beginning of the experiment being able to gain more weight. We cannot rule out that resource limitation in our experiment exacerbated insecticide effects on bumblebee colonies, since lack of (diverse and untreated) resources is expected to increase exposure and susceptibility to pesticides (Klaus et al., 2021; Zaragoza-Trello et al., 2021). Moreover, considering the relatively short period over which colony growth was assessed, we cannot exclude that colonies impacted by Closer would have recovered after exposure. We hence suggest future semi-field studies on bumblebees to use larger enclosures or to use colonies with access to outside resources as reference, and to assess colony growth throughout the colony lifecycle. Our study shows for the first time that sulfoximine-based insecticides can negatively affect bumblebee colonies when products are applied in the field to the crop and when alternative untreated food sources are unavailable.

We found Closer (sulfoxaflor) exposure to impact bumblebee individual foraging performance both before and after fungicide application. Exposure to agonists of nicotinic acetylcholine receptors can reduce foraging efficiency and the resulting decrease in resource acquisition potentially has severe consequences downstream in the colony cycle (Bryden et al., 2013; Feltham et al., 2014; Wintermantel et al., 2018). This type of insecticides can in fact alter the olfactory learning and working memory, fundamental for foragers to efficiently locate suitable unvisited flowers (Gill and Raine, 2014; Siviter et al., 2018b). However, a recent meta-analysis found no evidence for negative effects of sulfoxaflor exposure on bumblebee cognition and behavior (Siviter and Muth, 2020). In particular, bumblebees exposed to sulfoxaflor did not show impaired foraging performance in a field experiment (Siviter et al., 2018a) or altered cognitive abilities under laboratory conditions (Siviter et al., 2019). Nevertheless, studies focusing on sulfoximine-based insecticides are currently limited and methodologies greatly differ across experiments, so results may not be directly comparable (Siviter and Muth, 2020). For example, Siviter et al. (Siviter et al., 2018a) directly fed a sucrose solution containing sulfoxaflor to *B. terrestris* colonies and did not measure foraging activity during the exposure period. Moreover, we cannot exclude that co-formulants and adjuvants in the product Closer might be responsible for the reduced foraging performance observed (Zhu et al., 2014). In this study, in fact, we used commercial formulations rather than only the active ingredients (i.e. sulfoxaflor and azoxystrobin) to simulate realistic exposure conditions in the field. Although this approach is considered important for a better assessment of the total agrochemical exposures on bees, it precludes the identification of clear links between compounds and bee responses to exposure (Mullin, 2015; Straw et al., 2021). Finally, a clear causal relationship between cognitive performance and foraging efficiency has not been established yet (Siviter et al., 2019) and other sublethal impacts beyond cognitive effects such as reduction in foraging motivation might be involved (Lämsä et al., 2018).

Exposure to the fungicide Amistar (azoxystrobin) negatively affected bumblebee individual foraging performance as well. The mechanisms underlying the observed patterns are, however, not evident, as studies exploring azoxystrobin effects on pollinators are currently scarce (but see Christen et al., 2019). Nevertheless, unpublished research supports our results and provides potential explanations for the reduced foraging performance. Straw and Brown (Straw and Brown, under review) found in a laboratory experiment that exposure to Amistar by acute oral dose, severely impacted bumblebee health and that its toxicity was caused by a co-formulant, alcohol ethoxylates. Bumblebees exposed to either the product Amistar or the co-formulant alone, presented damages to their midguts, which likely caused the reduced appetite, loss in weight and increased mortality compared to control bees. Our results indicate that Amistar might negatively impact bumblebees under field conditions as well. Despite the reduced individual foraging performance, we found no detectable effect of Amistar on flower visitation, flight activity or colony growth, suggesting that its impact on single bumblebees did not scale up at the colony level. However, we cannot exclude further sublethal effects in the long term or at higher exposure levels.

We found bumblebees exposed to Amistar (azoxystrobin) to deposit fewer pollen grains on *P. tanacetifolia* flowers than bees not exposed to the fungicide. This might be explained by the reduction in individual foraging performance found under Amistar application: the lower flower visitation frequency might have resulted in lower pollen loads and consequently in decreased pollen transfer to stigmas during flower visitation. However, we did not find a similar effect for bumblebees exposed to Closer, which also showed a decrease in individual foraging performance. Pesticide impacts on pollen deposition might hence be driven by other mechanisms such as altered foraging behavior. Williams and Thomson (Williams and Thomson, 2003) found that bumblebees transferred more pollen during nectar-pollen visits of *P. tanacetifolia*

flowers than during pollen-only visits, because bumblebees collecting nectar have to probe more deeply into the flowers, increasing contact with anthers and stigmas. A reduced appetite under Amistar application (Straw and Brown, under review) could have hence influenced the degree of nectar foraging, limiting pollen deposition. The decrease in pollen deposition was significant only when considering pollen grains deposited on both styles and stigmas, suggesting that a reduction in pollen deposition might not have very strong repercussions for plant reproduction. Our findings highlight the need to further explore the effects of pesticide exposure on foraging behavior and pollination service, aspects that are largely overlooked in the pesticide literature (but see Stanley et al., 2015).

Contrary to our hypothesis, we found no interactive effect of insecticide (Closer) and fungicide (Amistar) exposure on bumblebees. Our findings indicate that azoxystrobin does not increase the toxicity of sulfoxaflor products. Nevertheless, the biochemical processes driving synergism between insecticides and several fungicide classes are not well understood yet (but see Berenbaum and Johnson, 2015), and we cannot exclude that this fungicide might interact with other pesticides. For example, the strobilurin fungicide Pyraclostrobin, which inhibits mitochondrial respiration by blocking electron transport in a similar way as azoxystrobin, can increase the toxicity of tau-fluvalinate, a pyrethroid acaricide, for honeybees (Carnesecchi et al., 2019; Johnson et al., 2013). Moreover, pollinators are exposed to a vast suite of chemical contaminants, including insecticides, acaricides, herbicides and fungicides (Mullin et al., 2010), the combined effects of which remain largely unknown. Despite the enormous number of possible combinations between agrochemicals, studies exploring bee response to compound mixtures are urgently needed (Cullen et al., 2019). Moreover, other stressors can exacerbate the negative impacts of pesticides on pollinators such as pathogens, climate variability and resource limitation, usually ignored in regulatory pesticide risk assessments (Al Naggar and Paxton, 2021; Goulson et al., 2015; Siviter et al., 2020a).

To simulate realistic exposure conditions in the field, we followed label instructions regarding application timing. Countries vary greatly in the legislation of the use of sulfoximine-based insecticides. Spray applications are prohibited during flowering in the EU, where single countries can further apply more stringent regulations. For example, in Italy, Closer spray applications have been recently limited to five days prior to blooming (Corteva Italy, 2021), in Germany they are banned outside of permanent greenhouse structures and France has banned the substance sulfoxaflor completely. The use of sulfoxaflor products during flowering is however not prohibited in many other countries, and mitigation measures often only avoid direct contact exposure (Corteva Australia, 2021; Corteva Canada, 2021; Corteva New Zealand, 2021; Corteva South Africa, 2021; Corteva, 2021). The US Environmental Protection Agency limits sulfoxaflor applications between 3 days prior to bloom and until after petal fall for some crops such as pome fruits, stone fruits, canola and berries, but not for other flowering crops such as citrus, cucurbits, alfalfa and strawberries (EPA, 2019). Our findings indicate that these mitigation strategies might fail in avoiding the risk of sublethal chronic exposure for bumblebees. Despite the high degradability of the substance (Xu et al., 2012), residues can be found in pollen and nectar in the days following applications (Cheng et al., 2018). Recent semi-field studies on the impact of a sulfoxaflor product on honeybees found increased mortality only in the first few days after application (Cheng et al., 2018) or no effects at all when applied six days before bloom (Tamburini et al., 2021), suggesting that mandatory safety periods are important to limit risk for bees. Nevertheless, safety periods might not be sufficient to reduce risks to bees to an acceptable level as wild bees have been shown to be more susceptible to insecticides than honeybees (Azpiazu et al., 2021; Rundlöf et al., 2015) and safety periods may be difficult to implement in practice due to the challenge of predicting flowering onset. In the present study for example, the few inflorescences presenting open flowers at the time of Closer application were manually removed, a procedure that is clearly unfeasible for farmers. Moreover, pre- or post-bloom spraying represents an exposure risk when non-target plants are flowering in the area. Hence, mandatory safety periods can reduce exposure of bees to sulfoxaflor, but they might not be sufficient to eliminate the risk of substantial negative impacts on pollinators. Finally, the impact of sulfoxaflor products under realistic field-conditions on other important pollinator groups such as solitary bees (Sgolastra et al., 2017) is currently unknown, and requires further investigation.

Our study demonstrates that exposure to two widely used pesticides, the product Closer (sulfoxaflor) and the product Amistar (azoxystrobin), can impact bumblebees and the pollination services they provide when used according to standard application practices (i.e. directly sprayed to the crop). Our findings further indicate that first, limited safety periods between spray of sulfoxaflor products and crop bloom may be inadequate for preventing risks for bumblebees, and second, that sublethal impacts of commonly used fungicides might currently be underestimated. Additional field-realistic studies considering the sublethal effects of insecticides and fungicides on bee health and pollination services will provide regulatory bodies with pivotal information to design more sustainable directives for pesticide use in agroecosystems.

CRediT authorship contribution statement

Giovanni Tamburini: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Data curation, Writing - original draft. **Maria-Helena Pereira-Peixoto:** Investigation. **Jonas Borth:** Investigation, Data curation. **Simon Lotz:** Investigation, Data curation. **Dimitry Wintermantel:** Writing - review & editing. **Matthew J. Allan:** Conceptualization, Methodology. **Robin Dean:** Conceptualization, Methodology. **Janine Melanie Schwarz:** Conceptualization, Methodology, Writing - review & editing. **Anina Knauer:** Conceptualization, Methodology, Writing - review & editing. **Matthias Albrecht:** Conceptualization, Methodology, Writing - review & editing. **Alexandra-Maria Klein:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All datasets used in this article will be available in a public database upon acceptance.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106813.

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