



## Flowering resources modulate the sensitivity of bumblebees to a common fungicide



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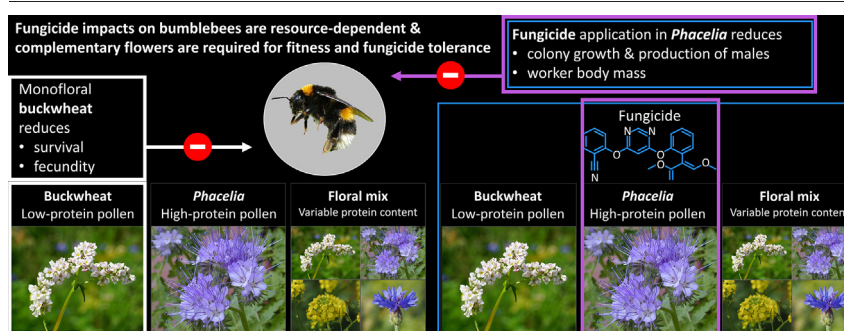
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### HIGHLIGHTS

- Studies testing interactions between flowering resources and pesticides are lacking.
- Impacts of the azoxystrobin fungicide Amistar® on bumblebees are resource-dependent.
- Amistar reduces bumblebee colony and body size in *Phacelia* monocultures.
- Bumblebees require complementary resources for fitness and fungicide tolerance.
- Re-evaluation of Amistar and pesticide risk assessment on bees required.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Bees are exposed to various stressors, including pesticides and lack of flowering resources. Despite potential interactions between these stressors, the impacts of pesticides on bees are generally assumed to be consistent across bee-attractive crops, and regulatory risk assessments of pesticides neglect interactions with flowering resources. Furthermore, impacts of fungicides on bees are rarely examined in peer-reviewed studies, although these are often the pesticides that bees are most exposed to.

In a full-factorial semi-field experiment with 39 large flight cages, we assessed the single and combined impacts of the globally used azoxystrobin-based fungicide Amistar® and three types of flowering resources (*Phacelia*, buckwheat, and a floral mix) on *Bombus terrestris* colonies.

Although Amistar is classified as bee-safe, Amistar exposure through *Phacelia* monocultures reduced adult worker body mass and colony growth (including a 55% decline in workers and an 88% decline in males), while the fungicide had no impact on colonies in buckwheat or the floral mix cages. Furthermore, buckwheat monocultures hampered survival and fecundity irrespective of fungicide exposure. This shows that bumblebees require access to complementary flowering species to gain both fitness and fungicide tolerance and that Amistar impacts are flowering resource-dependent. Our findings call for further research on how different flowering plants affect bees and their pesticide tolerance to improve guidelines for regulatory pesticide risk assessments and inform the choice of plants that are cultivated to safeguard pollinators.

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## 1. Introduction

Declines in bee diversity and distribution (Biesmeijer et al., 2006; Potts et al., 2016; Vanbergen et al., 2013; Zattara and Aizen, 2021) are believed to be driven by a combination of stressors including pesticides, diseases, and loss of flowering resources (Dicks et al., 2021; Goulson et al., 2015; Potts et al., 2016). Research on the effects of pesticides on bees has mostly focused on insecticides due to the taxonomic proximity of target organisms (insects) to bees (Cullen et al., 2019). However, fungicides are often the pesticides that bees are most exposed to (McArt et al., 2017a; Mullin et al., 2010; Pettis et al., 2013) and despite their image of being relatively non-toxic to bees, they can have negative effects on bees. Experiments show that various fungicides can either magnify the toxicity of insecticides (McArt et al., 2017a; Pilling and Jepson, 1993; Sgolastra et al., 2017; Wernecke et al., 2019) or impair bees independently from other agrochemicals (Artz and Pitts-Singer, 2015; Bernauer et al., 2015; Ladurner et al., 2005; Zhu et al., 2014). Additionally, fungicides were linked to accelerated declines of four bumblebee species in the United States (McArt et al., 2017b) and increased honeybee colony failure in Belgium (Simon-Delso et al., 2014) but not in Germany (Genersch et al., 2010).

Azoxystrobin is a systemic broad-spectrum fungicidal substance that was launched in 1996 and became the globally best-selling fungicide within three years with an 8-fold increase in annual global sales in the following twelve years (Bartlett et al., 2002; Leadbeater, 2014). Comprehensive data on recent global sales are unavailable but azoxystrobin use continued to grow in the US in recent years (US Geological Survey, 2019). It is widely used in a broad range of crops including cereals, oilseed rape, corn, vegetables and fruits (FAO, 2020; US Geological Survey, 2019) and is frequently found in bees (max residue = 1776 ppb), pollen (max residue = 1870 ppb), nectar (max residue = 1450 ppb) and honey (max residue = 174 ppb; maximum tolerated residue level (MRL) = 50 ppb) (EFSA, 2012; Mullin et al., 2010; Piechowicz et al., 2018; Rennich et al., 2013; Schatz, 2009). However, only few published studies examined azoxystrobin effects on bees leaving uncertainties about its risk. In honeybees, azoxystrobin affected gene expression but not in a dose-response manner (Christen et al., 2019) and increased forager mortality but only at concentrations above field-realistic levels (Fisher et al., 2017). Semi-field studies with field-realistic exposure found no effects on honeybee fecundity and mortality but a reduction in the foraging performance and pollination services of *Bombus terrestris* colonies (Tamburini et al., 2021a, 2021b). The European food safety authority (EFSA) reported results from limit tests that indicated low acute toxicity (oral exposure: >25 µg active ingredient (a.i.) bee<sup>-1</sup>, contact exposure: >200 µg a.i. bee<sup>-1</sup>). However, azoxystrobin itself has a low water-solubility (6 mg L<sup>-1</sup> at 20 °C, Hazardous Substances Data Bank), which can cause precipitation in syrup and therefore lead to inaccurate results. In addition, a recent study found that surfactants (alcohol ethoxylates) found in Amistar (and other products) rather than the active ingredient (azoxystrobin) damage bumblebee guts and increase their mortality (Straw and Brown, 2021).

Agricultural intensification affects bees not only through increased pesticide exposure but also through altering flowering resource availability. Although mass-flowering crops are an important food source for honeybees and bumblebees (Rollin et al., 2013; Westphal et al., 2009), a lack of flowering plant diversity in the landscape can harm bees through a temporary reduction in the quantity of available resources (as diversity ensures continuous flowering) (Kaluza et al., 2018; Papanikolaou et al., 2017; Requier et al., 2015, 2017; Wintermantel et al., 2019), and through an unbalanced diet (Dance et al., 2017; Di Pasquale et al., 2013; Filipiak et al., 2017; Leza et al., 2018; Sutter et al., 2017). Flowering plants differ in the nutrients they provide and bees, especially bumblebees, can adapt their foraging behavior to their nutritional demands (Kraus et al., 2019; Ruedenauer et al., 2015, 2020; Somme et al., 2015; Vaudo et al., 2015, 2016, 2020). Bumblebees generally prefer protein-rich pollen (Hanley et al., 2008; Leonhardt and Blüthgen, 2012; Ruedenauer et al., 2015; Vaudo et al., 2015), which can foster their development (Baloglu and Gurel, 2015; Kämper et al., 2016; Roger et al., 2017) and resilience to pathogens (Roger et al., 2017).

Both the quantity and quality of food may affect the sensitivity of bees to pesticides. While flower density and insecticide exposure additively impaired *Osmia lignaria* reproduction (Stuligross and Williams, 2020), low-sugar diets and insecticide exposure synergistically decreased food consumption, hemolymph sugar levels, and survival in honeybees (Tosi et al., 2017). The presence of pollen in the diet mitigated pesticide effects on honeybee survival through upregulation of detoxification-related genes and subsequently increased pesticide clearance (Ardalani et al., 2021; Barascou et al., 2021; de Mattos et al., 2018; Schmehl et al., 2014). It is less clear which pollen components confer tolerance to pesticide impacts. High protein content and high pollen diversity have been suggested to decrease pesticide sensitivity but studies investigating interactions between pesticides and either pollen differing in protein content or diversity (polyfloral vs monofloral pollen) found effects ranging from antagonistic over additive to synergistic (Archer et al., 2014; Barascou et al., 2021; Barraud et al., 2020; Crone and Grozinger, 2021; Dance et al., 2017; Leza et al., 2018; Wahl and Ulm, 1983). Flowering plant diversity might, however, reduce pesticide sensitivity particularly when bees can select flowers themselves. Indeed, a semi-field study mimicking the effect of flower strips through planting diverse untreated flowering species close to insecticide-treated oilseed rape found that floral diversification can offset insecticide impacts on *Osmia bicornis* (Klaus et al., 2021). However, it remains unclear whether this buffering effect was caused by a more diverse diet, a reduction in insecticide exposure, or both. A recent meta-analysis found overall no interaction between agrochemicals and nutritional stress (i.e. reduced food quantity or quality) on bee survival, but only three studies, consisting of 19 data-sets of which 10 showed synergistic effects, were investigated. For non-lethal parameters, the sample size was markedly smaller, and especially field-realistic studies are lacking (Siviter et al., 2021).

To study the interactive effects of the azoxystrobin-based fungicide Amistar and different flowering plant resources on *Bombus terrestris*, we conducted a full-factorial semi-field experiment. Thereby, we enclosed bumblebee colonies with untreated or Amistar-treated *Phacelia* (*Phacelia tanacetifolia*), common buckwheat (*Fagopyrum esculentum*), or a floral mix consisting of these two species, and several other planted or spontaneously flowering species. The two monocultural species are commonly used in flower strips and are recommended for semi-field experiments for regulatory pesticide risk assessments (*Phacelia* in both the EU and North America, buckwheat in North America (Franke et al., 2021; Frewin et al., 2019; Gradish et al., 2016; OECD, 2007; OEPP/EPP, 2010; US EPA & Health Canada Pest Management Regulatory Agency Ottawa, 2014)). They provide similar amounts of nectar (Knauer et al., 2022; Petanidou, 2003; Cawoy et al., 2009), but *Phacelia* is regarded as a more valuable resource for bees than buckwheat due to its nearly three times higher crude pollen protein content (Pernal and Currie, 2000; Somerville, 2001). We hypothesize that both Amistar and flowering resource type (hereafter simply 'resource') affect bumblebee foraging performance (flowers visited per time per individual), colony growth (colony weight, number of bees per caste, mortality) and body size (body mass of pupae and adults and intertegular distance of workers). In addition, we expect monocultural buckwheat to have direct negative impacts on colony growth and body size as well as to increase the susceptibility to the fungicide.

## 2. Material and methods

### 2.1. Study design

The experiment was conducted in 2020 and consisted of 39 cages with a ground cover of 53 m<sup>2</sup> (5.9 m × 9 m; height = 2.5 m), which exceeds the minimum recommended size of 30 m<sup>2</sup> (Knäbe et al., 2020). These were erected at a minimum distance of 4 m from each other on a 0.7 ha-large university-owned experimental field in Freiburg, Germany (48°01'08.5"N 7°49'31.2"E). The three resources – buckwheat, *Phacelia*, and the floral mix – were randomly assigned to the cages. For the floral mix, a custom seed mix from Rieger Hofmann (Blaufelden-Raboldshausen, Germany, www.rieger-hofmann.de) was sown that consisted of *F. esculentum* (40%

by weight), *P. tanacetifolia* (10%), *Centaurea cyanus* (20%), *Sinapis arvensis* (10%), *Malva sylvestris* (10%) and *Trifolium resupinatum* (10%; Table S1; Appendix A). The latter two, however, barely germinated. Unlike the monocultures, floral mix cages were not weeded and contained, therefore, also flowering *Achillea millefolium*, *Cirsium arvense*, *Linaria vulgaris*, *Persicaria lapathifolia*, *Plantago lanceolata*, *Verbascum nigrum*, and *Vicia cracca*.

The cages were covered by a nylon net (mesh size =  $0.95 \times 1.35$  mm). Each cage contained one colony during a 1-week pre-exposure period (before Amistar application) and a 10-day exposure period (after Amistar application, Fig. 1). The colonies were additionally examined after a 13-day post-exposure period, in which they were allowed to forage freely outside the cages. During the study period, it rained sporadically and air temperature ranged from 10 to 32 °C (Fig. S1).

Stratified random allocation approaches were used to assign cages to spray treatments (i.e. Amistar or water/control) and colonies to resource-spray combinations with strata being based on flower density and number of adult bees, respectively (Appendix A).

## 2.2. Fungicide application

The fungicide Amistar (a.i. = azoxystrobin) was applied at a rate of 250 g a.i. per hectare (= 1 L ha<sup>-1</sup> of formulated product) in the morning of 4 July 2020 in 6 of 13 *Phacelia* cages, in 6 of 12 buckwheat cages, and 7 of 14 floral mix cages (Appendix A, Fig. S2a) during full bloom of *Phacelia* and buckwheat (BBCH ~63–65). Amistar application was done according to label instructions in EU member states ([www.syngenta.com](http://www.syngenta.com)) by a ‘Good Experimental Practices’-certified spray contractor using a motorized sprayer equipped with a 3 m-long bar with anti-drift spraying nozzles during dry weather with low wind speed (<2 m s<sup>-1</sup>). During application, the sprayed cage was covered with plastic sheets to further reduce the probability of spray drift to adjacent cages. Using different equipment, control cages were sprayed with water of the same volume as the diluted product. As the study goal was to measure impacts of dietary exposure to Amistar in interaction with the nutritional quality of floral resources, contact exposure was minimized by closing the exits of all bumblebee nests early in the morning and opening them again directly after the application.

## 2.3. Experimental bumblebee colonies

Forty-three *B. terrestris terrestris* colonies purchased from Katz Biotech AG were delivered on day - 8 (day 0 = day of Amistar/water application) and assessed for queen presence, disease signs, and colony size. No visual signs of pathogens or parasites were detected. The 39 colonies selected

for the experiment had on average 36.3 living workers (standard deviation (SD) = 7.4) and 60.1 brood cells (SD = 11.5) with no difference between resources or spray treatments in number of living adults (two-way ANOVA,  $P > 0.93$ ) or brood cells (two-way ANOVA,  $P > 0.30$ ) and contained only few dead workers (range = 0–3, mean = 0.9, median = 1; Fig. S3). Colonies assigned to different spray treatments developed also very similarly during the pre-exposure period (Fig. S3). On day - 7, the colonies were placed inside the cages on the short side opposite the entrance, facing South-East (Fig. S2b). Each cage contained a water feeder (a plastic bowl filled with water that contained a stone for the bees to sit on). A straight path without flowers, dividing the cage into two halves, allowed easy access to the colonies. The colonies were housed in the delivered plastic nest boxes placed inside wooden boxes that sheltered the colonies from sunlight and rainfall. These were placed about 20 cm above ground on small wooden stands or bricks. All colonies were delivered with two syrup containers and a pollen supplement. The larger syrup container underneath the nest was closed immediately after delivery, whereas the smaller syrup container and pollen supplement (both within the nest) were removed during the first assessment in the field on day - 5, two days after the placement of colonies inside the cages. One colony (buckwheat-control) died on day - 3 and was replaced by a spare colony.

## 2.4. Azoxystrobin residue analysis

To quantify azoxystrobin exposure, two foragers per cage were collected on the evening of day 1 using sweep nets. However, not from all cages samples were taken as bees stopped foraging due to the approaching sunset before the work was completed (Table S2). Samples taken from the same resource-spray combination were pooled and analyzed by the Research Centre for Agriculture and Environment (CREA-AA, Bologna, Italy) using liquid chromatography/tandem mass spectrometry (LC-MS/MS). The samples were put on dry ice in the field as well as during transport and were stored at -20 °C. The limit of quantification was 0.01 mg kg<sup>-1</sup>.

## 2.5. Assessments before and during exposure

The colonies were assessed once in the laboratory (day - 8), and eight times inside the flight cages (three times before and five times after Amistar application; Fig. 1) for

- (1) colony weight: Colonies (including their nest box) were weighed and the weight of an empty plastic nest box was subtracted;
- (2) cumulative number of dead adults: Dead adult bees inside the nest were

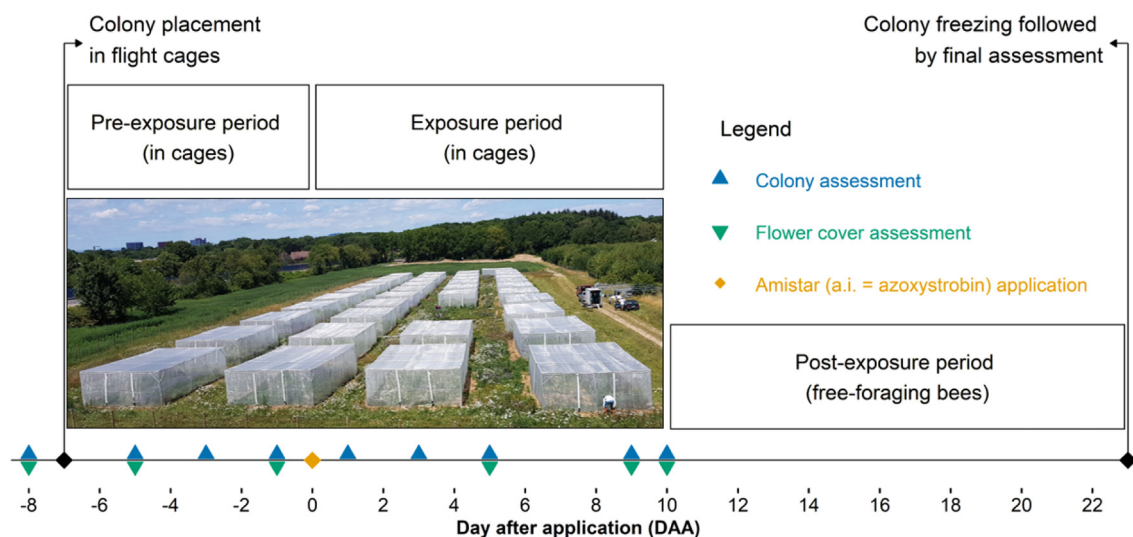


Fig. 1. Experimental timeline. Sequence of bumblebee colony and flower cover assessments in the pre-exposure period (before Amistar application) and exposure period (after Amistar application).

counted without removing them while visually inspecting the colonies through the transparent plastic cover;

- (3) number of living adults: Adult bees were counted from a photo of the nest taken through a transparent acrylic cover. Number of dead adults was subtracted from this count and estimated numbers of bumblebees that left during placement of the cover or were foraging, while the photo was taken, were added.

In addition, individual foraging performance was assessed on days -4, -3, 4, 9, 10. For this, if enough bees were foraging, three foragers per cage were observed for 2 min and the number of flowers visited per bee was recorded. Cages of the same spray treatment were assessed in parallel while alternating between resources.

Flower density was assessed once before and five times after colony placement. For this, the cages were divided into six equally large areas (rectangles; Fig. S2b). During each flower density assessment, one of three rectangles per side of the cage was randomly selected without replacement until all rectangles were assessed (then random selection without replacement re-started). Inside these rectangles, a quadrat (1 m × 1 m) was placed so that it contained a flower cover/composition that appeared representative either for the whole cage (only in the first assessment) or for the selected rectangle. Inside the quadrats, the number of inflorescences per plant species was counted, multiplied by the mean number of flowers of three representative inflorescences, and averaged across the two rectangles. In the case of the floral mix, the process was done for all plant species and summed up.

## 2.6. Final assessment after colony termination

Colonies were freeze-killed on day 23 when all foundress queens had died, possibly because flowering resources declined in the study site and were lacking in the surroundings (Franko et al., 2018). The colonies were afterward examined for

- (1) numbers of adult males and workers: Adult bees were separated by caste and counted. These counts included bees that lived until colony termination or died within the four days before, as dead bees were removed from the colonies on day 19. As only five colonies (2 *Phacelia* - control, 2 *Phacelia* - Amistar, and 1 floral mix - control) produced queens, the number of queens was not analyzed.
- (2) number of worker and/or male cocoons: Closed cocoons (from which no bee had emerged yet) with a diameter < 12 mm were counted. In only one terminated colony a queen cocoon (floral mix-control) was found and not further considered.
- (3) adult worker body mass and intertegular distance: If available, 15 workers that were presumably alive until colony termination were weighed using a high-precision balance with wind-break and measured for the distance between the insertion points of the wings using a digital caliper. Bees that were particularly dry or ridged were assumed to have died already before colony termination and were therefore sorted out. Males and queens were not examined due to their low numbers.
- (4) pupal body mass and developmental stage: Up to 35 cocoons were opened to obtain 20 pupae that presumably were alive until colony termination. The cocoons were sexed, weighed and their developmental stage was rated on a scale from 1 to 6 based on eye color, body color, and presence/absence of wings (Wintermantel et al., 2018, Table S3). Pupae last approximately two days in each developmental stage.

## 2.7. Data analysis

The statistical analyses on bumblebee parameters were done separately for the three assessment phases: pre-exposure period, exposure period, and the final assessment using (generalized) linear mixed-effects models ((G) LMMs; for parameters with multiple observations per colony) with colony identity as a random factor or generalized linear models (GLMs; for colony-level parameters in the final assessment). Square root-transformed flower density was analyzed in a single LMM for both the pre-exposure

and exposure period with colony identity as a random factor and a three-way interaction (including two-way interactions and main effects) between resource (categories: buckwheat, *Phacelia*, floral mix), spray treatment (categories: control and Amistar) and a quadratic term (including the linear term) for day as fixed effects.

The colony that was replaced on day -3 was excluded from the data analyses (but its replacement was included). During the exposure period, the foundress queens of eight colonies died; these queen losses were quite balanced between resources and spray treatments (1-2 queen losses per resource-treatment combination; Table S2). Data collected after the death of the queen were excluded from analyses of the exposure period and only data of colonies whose queens lived throughout the exposure period were considered in the final assessment. For analyses of individual-level measures, bees that showed signs of disease were excluded (parasitism, necrosis, or deformed wings); these were however only few (Table S2). In addition, three adult workers were excluded from analyses of body mass as body parts had fallen off. Sample sizes of all parameters and analyses are listed in Table S4.

All analyses were conducted in R version 3.6.3. Colony weight, body mass, intertegular distance, and flower density were analyzed using LMMs fitted with the function `lmer` of the `lme4` package (Bates et al., 2015). All GLMMs were fitted with the `glmmTMB` function/package (Brooks et al., 2017). Number of dead adults was analyzed using GLMMs with a Poisson distribution. For number of living adults, GLMMs with a quasi-Poisson distribution (specified as `nbinom1`) were used for the pre-exposure period, to account for overdispersion, and GLMMs with Poisson distribution were used for the exposure period. For number of flowers visited per bee and time, GLMMs with a quasi-Poisson distribution (specified as `nbinom1`) were used for the pre-exposure period, and GLMMs with a negative binomial distribution (specified as `nbinom2`) were used for the exposure period. The final number of cocoons and adult males and workers were analyzed using GLMs with a negative binomial distribution to account for overdispersion using the `glm.nb` function of the `MASS` package (Venables and Ripley, 2002). Models with a quasi-Poisson or negative binomial distribution had an `ln`-link function. (G)GLMMs were fit with maximum likelihood during model selection and with restricted maximum likelihood when selected models were evaluated.

Number of flower visits per bee in 2 min were fitted using GLMMs that contained as fixed effects flowering resource, day (continuous variable) and a quadratic term (including the linear term) for time of day in the pre-exposure period and a two-way interaction between flowering resource and spray treatment, day and a quadratic term (including the linear term) for day time in the exposure period. Other models for the pre-exposure period contained an interaction (including main effects) between flowering resource and day (continuous variable) as fixed effects. For the exposure period, models (except for those on number of flower visits per bee and time) contained a three-way interaction (including all two-way interactions and main effects) between flowering resource, spray treatment, and day as fixed effects. Pupal body mass was fitted using an LMM containing a three-way interaction (including all two-way interactions and main effects) between resource, spray treatment, and developmental stage (continuous variable). All other models on the final assessment contained an interaction (including main effects) between flowering resource and spray treatment as fixed effects.

In all of these models, an interaction between flower density and resource (including main effects) was included if a likelihood ratio test showed  $P < 0.05$  and the root-mean-square error decreased. For the pre-exposure and exposure period, flower density was an interpolated variable across assessment days, whereas in the final assessment the mean of all flower density assessments during the period where colonies were encaged was used. Both of these variables were centered to mean = 0 and standardized to standard deviation = 1.

Flower density was interpolated using the `approx` function of the `stats` package and data from all flower density assessments. However, in models on number of living adults or number of dead adults, on day -8, flower density was assumed to be the mean interpolated flower density of all

cages on day  $-7$ , as on day  $-8$  colonies were assessed in the laboratory (and therefore not exposed to flowers). For colony weight, the assessment period started with the first field assessment on day  $-5$  (after pollen and nectar supplies were removed).

Models were evaluated by calculating estimated marginal means (EMMs) using the emmeans (for simple/main effects) and emtrends (for slopes) functions of the emmeans package. A Tukey post-hoc correction was applied when analyzing differences between any pair of resources. In the pre-exposure period, straightforward comparisons between resources were made (pairwise~resource). In the exposure period and final assessment, differences between resources and spray treatments were determined relative to the other of these two factors (pairwise~resource|spray treatment or pairwise~spray treatment|resource). To avoid confounding effects with spray treatment, resource effects in the exposure period and final assessment are only reported for the control group. To compare differences between spray treatments (over time), Amistar and control cages of the same flowering resource were compared (pairwise~spray treatment|resource) using emmeans (main effects) or emtrends (interaction with day).

To determine effects on colony weight change over the pre-exposure period (days  $-5$  to  $-1$ ) or the exposure period (days 0 to 10), regression slopes were compared. For the estimation of effect sizes (and their confidence intervals), models for colony weight were refit with a different time variable (instead of day), where a unit equals the length of the regarded assessment period. As number of living adults and number of dead adults were modeled on the ln-scale, slope coefficients were less meaningful, and therefore back-transformed model estimates on day  $-1$  (for the pre-exposure period) and day 10 (for the exposure period) were compared for the estimation of effect sizes and their confidence intervals.

### 3. Results

#### 3.1. Impact of flowering resources

To avoid confounding effects with spray treatment, flowering resource effects were assessed by comparing colonies from untreated cages (i.e. any in the pre-exposure period and control cages in later assessments). While there were no differences between *Phacelia* and floral mix colonies, monocultural buckwheat adversely impacted several parameters in comparison to monocultural *Phacelia* and/or the floral mix. In the pre-exposure period, buckwheat colonies showed higher mortality than *Phacelia* or floral mix colonies with 3.7 (i.e. >200%) more dead adults (Fig. 2, Fig. S4). Buckwheat colonies ended this period with 9.8 (i.e. 23.6%) fewer living adults than *Phacelia* colonies and decreased 21.4 g (i.e. 22.5%) in weight ( $P < 0.001$ ), while floral mix colonies maintained a stable weight ( $P = 0.57$ , Fig. S4; difference in weight change: 23.3 g; Fig. S4). Colony weight declined also in *Phacelia* ( $-9.2$  g,  $P = 0.039$ ), but no difference to the other resources was determined ( $P > 0.1$ ). Buckwheat colonies ended the exposure period with over 25 (i.e. 30%) fewer living adults than colonies of the other two resources, despite no difference in number of dead adults (Fig. 2). In addition, buckwheat colonies continued to lose weight (15.3 g i.e. 19%) in the exposure period ( $P = 0.04$ ), while *Phacelia* and floral mix colonies gained 58.8 g (i.e. 50%) and 32.4 g (i.e. 28%), respectively ( $P < 0.001$ ). Number of flower visits per bee in 2 min was generally higher in buckwheat than in *Phacelia* (pre-exposure: +25.3 visits i.e. 70.5%, exposure: +26.2 visits i.e. 65.7%,  $P < 0.001$ ) and the floral mix (pre-exposure: +27.8 visits i.e. 82.6%, exposure: +29.8 visits i.e. 82.2%,  $P < 0.001$ ).

At the end of the experiment, buckwheat colonies had over 150 (i.e. 86%) fewer cocoons than *Phacelia* or floral mix colonies (Fig. 2) and 53.0 (i.e. 57%) fewer adult workers than *Phacelia* colonies (Fig. 2).

#### 3.2. Impact of Amistar exposure

Amistar negatively affected bumblebee colonies in *Phacelia* cages, but no effects were found in buckwheat or floral mix colonies (Fig. 3). Colonies

exposed to Amistar through *Phacelia* gained 22.5 g less weight compared to control colonies (Fig. 3, Fig. S4). At the end of the experiment, colonies exposed to Amistar through treated *Phacelia* had 51.5 (i.e. 55%) fewer adult workers, 7.0 (i.e. 88%) fewer adult males and a by 21 mg (i.e. 14%) reduced body mass of adult workers (Fig. 3, Fig. S5). Amistar had no apparent effect on the shape of the distribution of worker body mass but shifted the mean so that Amistar-exposed colonies in *Phacelia* tended to have more light and fewer heavy workers than control colonies (Fig. S6).

Residue analysis confirmed that foragers of the Amistar group were exposed to azoxystrobin during the exposure period (Table 1). Quantifiable levels of azoxystrobin were also found in foragers of the buckwheat control group but these were 76% lower than the azoxystrobin concentration detected in foragers of Amistar-treated buckwheat cages.

#### 3.3. Flower density and its impact

Flower density in all three resources exhibited a non-linear growth pattern (quadratic term:  $P < 0.001$ ) with an increase at the beginning of the experiment and a decline starting within the exposure period (Fig. 4). Already at the start of the experiment (day =  $-7$ ), the floral mix had a higher flower density than the other two resources ( $P < 0.001$ ), and all three resources developed differently over time (differences in linear terms:  $P > 0.07$ , differences in quadratic terms:  $P < 0.009$ ).

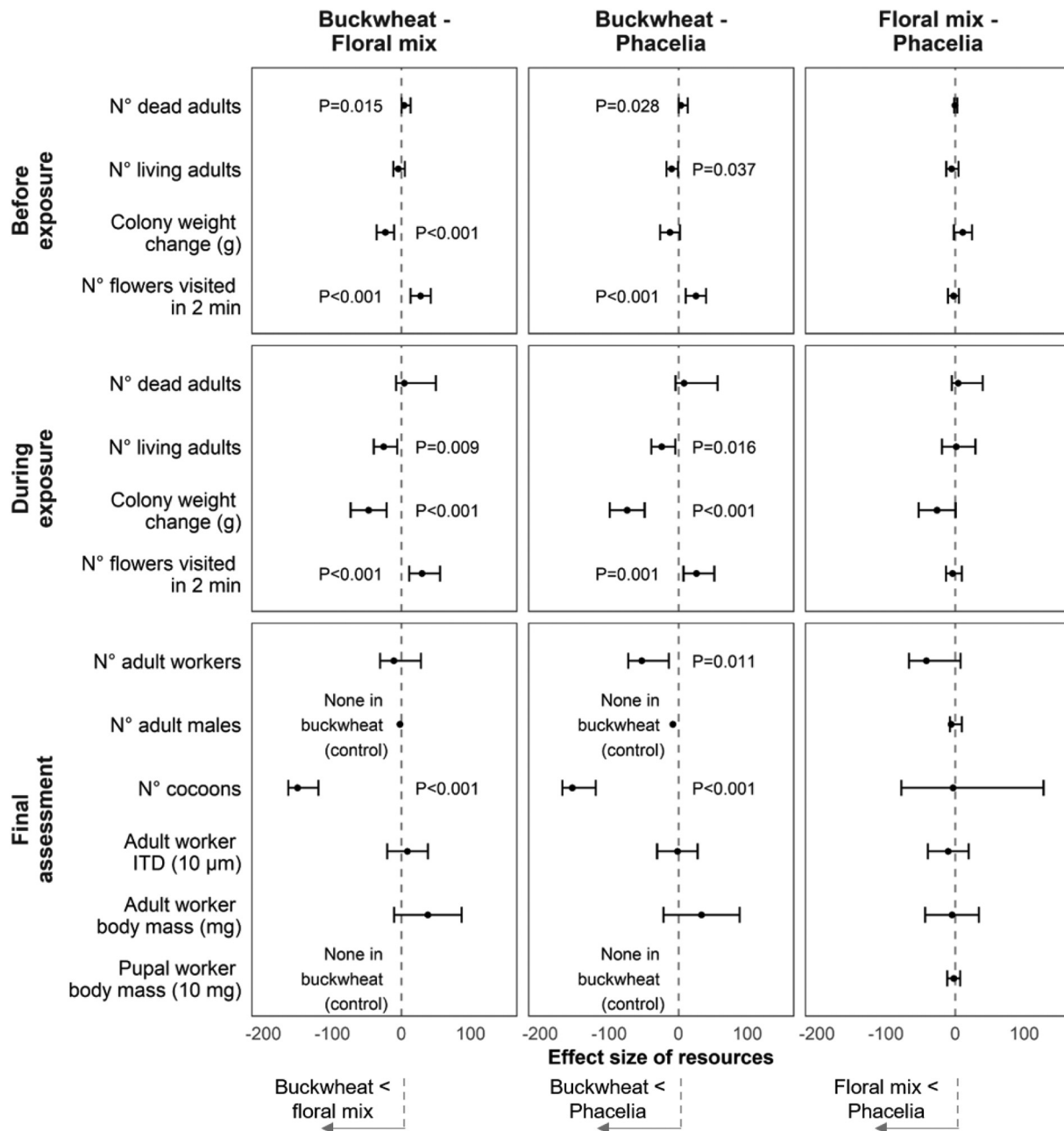
In contrast, flower density did not differ between cages assigned to different spray treatments at the start of the experiment (day  $-7$ ;  $P > 0.35$  in all resources). In addition, spray treatments did not differ in the change of flower density over time (differences in linear and quadratic terms between spray treatments in any resource:  $P > 0.23$ ).

Flower density in *Phacelia* cages positively affected colony weight in both the pre-exposure and the exposure period (Table S5). Flower density in the floral mix positively affected colony weight in the exposure period and negatively affected final number of cocoons (Table S5).

### 4. Discussion

Our semi-field experiment revealed that flowering resource type can strongly impact *B. terrestris* colonies directly and by modulating the effect of the azoxystrobin-based fungicide Amistar. We find that overall fitness and fungicide tolerance are promoted by different plant resources and that *B. terrestris* require, therefore, access to diverse flowering resources.

As hypothesized, colonies confined with untreated buckwheat developed less well than colonies confined with a floral mix or monocultural *Phacelia*. Although buckwheat is an excellent nectar source, similar to *Phacelia* (Knauer et al., 2022; Petanidou, 2003; Cawoy et al., 2009), its pollen is considered of relatively low quality due to its low protein content (11%) compared to *Phacelia* (30%) and the other abundant species of the floral mix (field mustard: 22%, cornflower: 23%) (Baloglu and Gurel, 2015; Pernal and Currie, 2000; Radev, 2018; Somerville, 2001). High protein diets foster bumblebee development (Baloglu and Gurel, 2015; Kämper et al., 2016; Roger et al., 2017) and immunocompetence (Roger et al., 2017). In contrast, buckwheat was shown to increase *Crithidia bombi* infestation (Adler et al., 2020; Giacomini et al., 2018; LoCascio et al., 2019). The availability of diverse flowers should benefit bees through a more balanced diet (Dance et al., 2017; Di Pasquale et al., 2013; Filipiak et al., 2017; Leza et al., 2018) and allow bumblebees, which are particularly selective and exhibit a preference for protein-rich pollen (Hanley et al., 2008; Leonhardt and Blüthgen, 2012; Ruedenauer et al., 2015; Vaudo et al., 2015), to select resources of high nutritional value. Although buckwheat flowers provide about 95% less pollen than *Phacelia* flowers (Knauer et al., 2022) and floral mix cages had a higher flower density, the adverse impacts of buckwheat seem not to be driven by a lack of flowers. Flower density in buckwheat did not affect any bumblebee parameter while flower density in *Phacelia* and the floral mix affected colony weight gain and number of cocoons. We can, however, not exclude that subtle effects of buckwheat flower density remained undetected due to its comparatively low variation between cages.

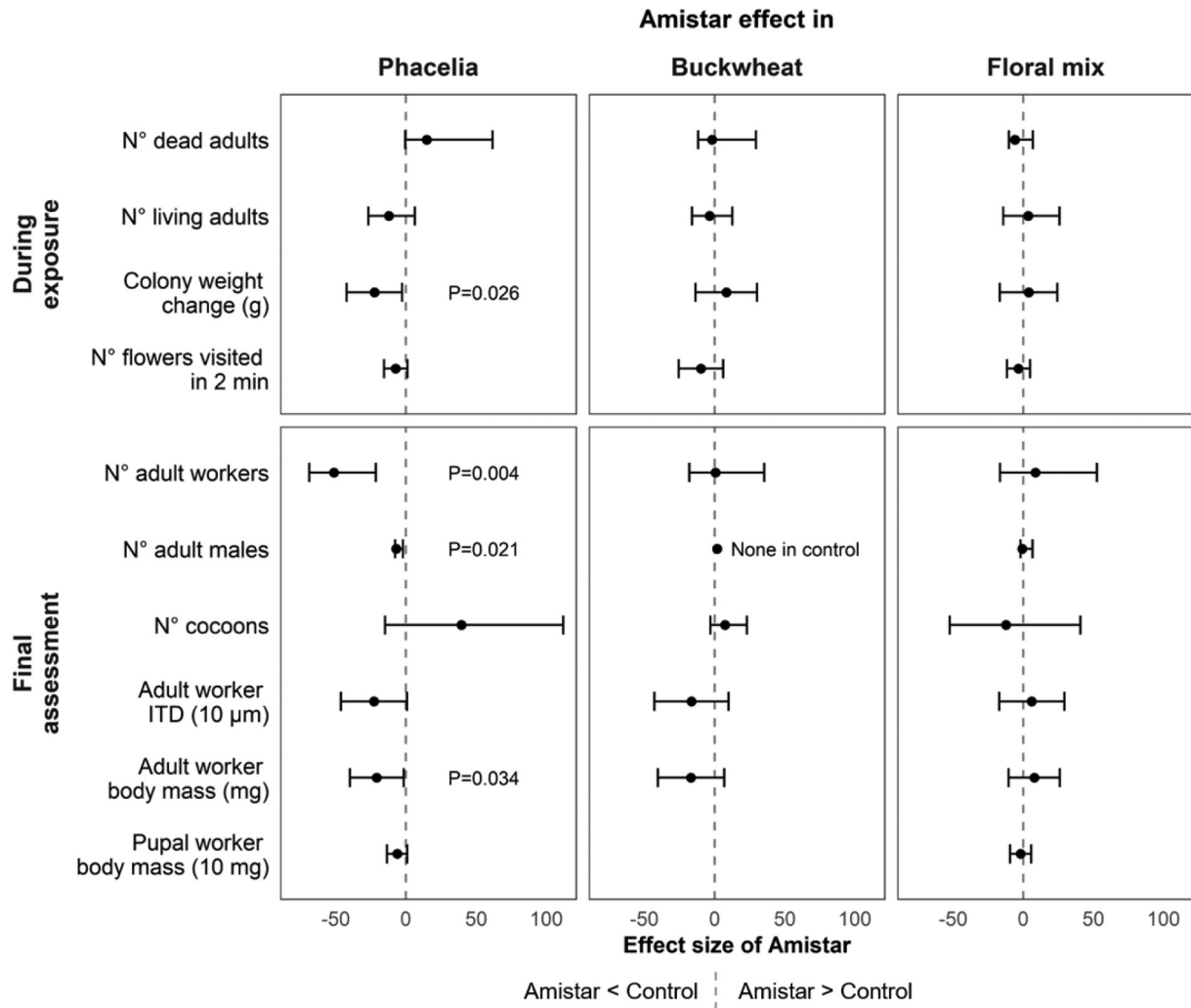


**Fig. 2.** Effect sizes of flowering resources on bumblebees. Differences in estimated marginal means between different types of flowering resources are illustrated as dots. Error bars indicate 95% confidence intervals. P-values <0.05 are shown. To avoid confounding effects with spray treatment (Amistar 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.

Amistar applied on *Phacelia* monocultures negatively affected *B. terrestris* colonies as manifested by reduced colony size, production of males, and body mass of adult workers. Amistar may cause these effects by impairing foraging behavior and metabolism. Azoxystrobin acts on fungi by inhibiting mitochondrial respiration and consequently energy supply. This effect is, however, not limited to fungi, as it was also found in fish (Olsvik et al., 2010). In honeybees, azoxystrobin altered the expression of genes involved in energy generation and hormonal regulation, which may disrupt the development of bees and impair their foraging efficiency (Christen et al., 2019). Indeed, Amistar can reduce the foraging rate of *B. terrestris* (Tamburini et al., 2021a), damage their guts and cause a decline in sucrose consumption, weight gain, and consequently survival rate (Straw and Brown, 2021).

To what extent Amistar affects bumblebee population sizes remains unclear, as we were unable to evaluate impacts on queen production, as almost all colonies failed to produce queens. This may be because confinement itself represents a stressor for bee colonies (Pistorius et al., 2012) and a large number of bumblebee colonies were competing for few flowering resources after they were released from the cages, which can negatively affect queen production (Franke et al., 2018).

Nevertheless, our study shows that the effects of a fungicide on bees are modified by flowering resources, as only colonies foraging exclusively on *Phacelia* were impacted. The absence of Amistar effects on colonies in floral mix cages aligns with the hypothesis that floral diversity can mitigate pesticide effects (Klaus et al., 2021; Wahl and Ulm, 1983). However, contrary to our expectations, we found no Amistar effects in colonies feeding



**Fig. 3.** Effect sizes of Amistar bumblebees. Differences in estimated marginal means between Amistar-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.

exclusively on buckwheat. We detected azoxystrobin residues in foragers from untreated buckwheat cages, but do not think that these explain the absence of Amistar effects in buckwheat colonies. This contamination was likely caused by handling bee samples of different treatments on the same table rather than by spray drift as the walls of the cages where the fungicide was applied were covered with plastic foil during the application. Furthermore, azoxystrobin levels were substantially higher in treated than in untreated cages in all resources with the absolute difference being largest in buckwheat.

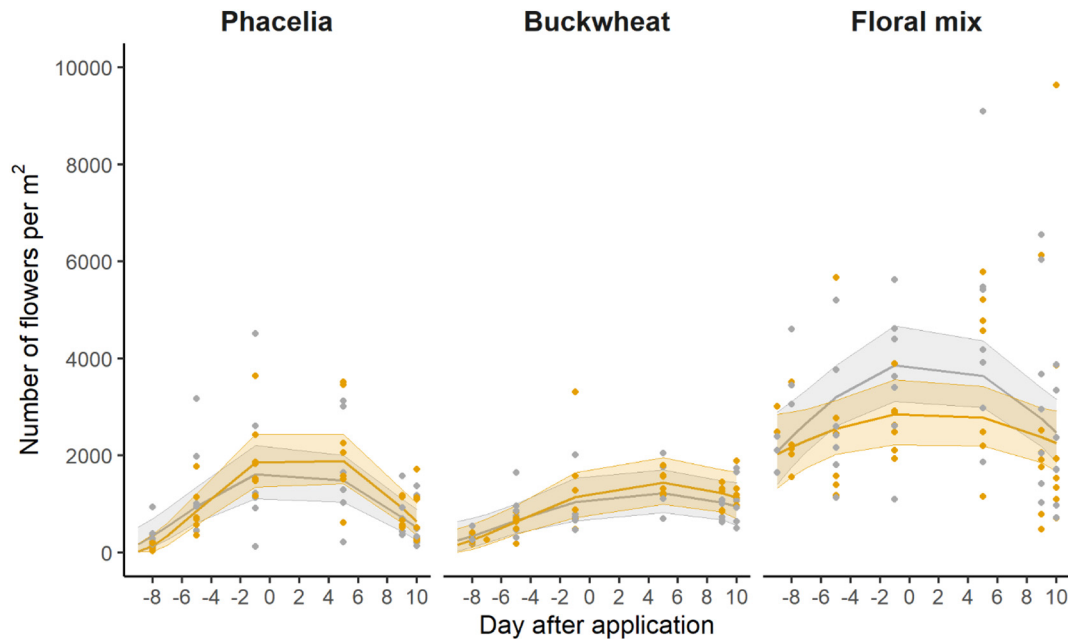
The flowering resources may, however, have differently affected fungicide fate on the plants or fungicide detoxification in the bees. A recent semi-field study found fungicide and insecticide levels in plant material to degrade more slowly in *Phacelia* than in maize and a floral mix (Castle et al.,

**Table 1**

Azoxystrobin residue concentrations (mg kg<sup>-1</sup>) in foraging bumblebees exposed to different spray treatments (control and Amistar) and resources (*Phacelia*, buckwheat, floral mix). From all colonies, 2 bumblebee individuals were taken on either day 1 or 2 and then pooled in the six resource-treatment combinations. The limit of quantification (LoQ) was 0.01 mg kg<sup>-1</sup>.

	<i>Phacelia</i>	Buckwheat	Floral mix
Control	< LoQ	0.0773	< LoQ
Amistar	0.3627	0.5595	0.3013

2022), suggesting that *Phacelia* may inhibit pesticide degradation. However, the activity of detoxification enzymes in bees foraging on *Phacelia* was increased compared to bees foraging on maize (Castle et al., 2022). Pesticide detoxification in bees can be accelerated by feeding on pollen (Ardalani et al., 2021; Barascou et al., 2021; de Mattos et al., 2018; Schmehl et al., 2014) and differ with pollen composition (Ardalani et al., 2021; Barascou et al., 2021). Interestingly, adding *Phacelia* pollen to a syrup diet for honeybees increased detoxification of a neonicotinoid insecticide but not of an acaricide or a fungicide (Ardalani et al., 2021). Earlier studies suggested that a high pollen protein content can decrease pesticide sensitivity (Archer et al., 2014; Wahl and Ulm, 1983), recent studies did not find such an effect in bumblebees (Barraud et al., 2020) or even an increase in pesticide sensitivity in honeybees feeding on pollen with a high protein content (Barascou et al., 2021) or protein-to-lipid ratio (Crone and Grozinger, 2021). This indicates that pollen protein (or its ratio to other nutrients) affects fitness and development differently than fungicide tolerance. In addition, other nutritional components of pollen such as secondary metabolites may be more important for pesticide detoxification and hence pesticide tolerance (Ardalani et al., 2021; Barascou et al., 2021). In our study, azoxystrobin concentrations were measured only once in bees to reduce disturbance of the colonies. Testing pesticide residues on multiple days in bees and additionally in pollen or nectar would have been beneficial to better understand how flowering resources impact pesticide exposure and detoxification.



**Fig. 4.** Flower density. The estimated number of flowers per  $m^2$  is shown in relation to time and spray treatment (grey: control, orange: Amistar). Lines represent estimated marginal means and shaded areas depict 95%-confidence interval, both obtained from an LMM. Dots represent observations.

Amistar may also have affected bees by attacking specific microorganisms present on *Phacelia*. Plant species differ in the microbial (including yeast and other fungal) communities they harbor, which bees can acquire through foraging and feeding (Manirajan et al., 2016; McFrederick and Rehan, 2019). Beneficial microorganisms can alter the durability of nectar and pollen, increase plant attractiveness to pollinators, protect bees from pathogens and promote detoxification (Herrera et al., 2013; Koch and Schmid-Hempel, 2011; Leonard et al., 2020; Pozo et al., 2015; Raymann and Moran, 2018; Vollet-Neto et al., 2017; Zheng et al., 2016). Pesticides can impact microorganisms found on flowering resources or within bee guts. For instance, azoxystrobin reduced yeast growth in nectar with potential implications for nectar chemistry and attractiveness (Bartlewicz et al., 2016).

Lastly, it is conceivable that differences in flower morphology caused the diverging results. *Phacelia* corollae are narrower and deeper compared to buckwheat (Vattala et al., 2006), which impedes access and resulted in longer durations that bumblebees spent on single flowers and over 65% fewer flower visits per bee and time. Hence, foraging on buckwheat was perhaps not challenging enough for potential effects on foraging ability (Tamburini et al., 2021a) to translate into reduced body size and colony growth. However, we did not detect a significant effect of Amistar on individual foraging performance, although we cannot exclude that such effect occurred shortly after the Amistar application as the first post-application assessment was on day 4. Also, we did not distinguish between nectar and pollen foraging, although pollen foraging is considered more challenging and particularly affected by pesticide exposure (Feltham et al., 2014; Gill and Raine, 2014; Stanley et al., 2016).

Our semi-field experiment highlights a potential shortcoming of the legislation on regulatory risk assessments. We found now in this and another semi-field study that Amistar applied on *Phacelia* can negatively affect *B. terrestris* (Tamburini et al., 2021a), even though it is classified in the EU as 'non-hazardous to bees', and is therefore approved for in-bloom applications (as are the comparable azoxystrobin products Abound® and Quadris® in the US). In a semi-field study with honeybees, we found no impacts of Amistar (Tamburini et al., 2021b). This indicates that bumblebees may be more sensitive to Amistar than honeybees, which was previously also found for other pesticides (Cresswell et al., 2014; Osterman et al., 2019; Rundlöf et al., 2015; Wintermantel et al., 2018), but in the EU and

the US, there is no legal requirement to test pesticides in bees other than honeybees (US EPA, 2016; European Commission, 2009). EFSA released in 2013 a guidance document for the risk assessment of plant protection products that involved testing on bumblebees and solitary bees. This was, however, never fully implemented due to the opposition of some EU member states (EFSA, 2013; More et al., 2021). We acclaim that EFSA is currently revising the guidance document and that standardized methods for higher-tier tests with *Osmia* and *Bombus* are being developed (Franke et al., 2021; Klein et al., 2018; Knäbe et al., 2020).

## 5. Conclusions

We found that the impacts of the fungicide Amistar on bumblebees depend on the forage plants it is applied on. Although confinement with buckwheat had direct negative effects on bumblebees, it did not render bees susceptible to the fungicide. In contrast, colonies feeding exclusively on *Phacelia* were negatively affected by Amistar. This would suggest that *Phacelia*, which is most commonly used in regulatory testing is a suitable worst-case crop. However, a recent semi-field study found a fungicide-insecticide tank mixture to affect honeybee survival less in *Phacelia* than in maize, possibly through increased detoxification (Castle et al., 2022).

The fact that fungicide impacts depend on the forage plant it applies on has potential implications for regulatory risk assessment systems of pesticides. While guidelines for the EU and the US require exposure to an active ingredient to be evaluated in multiple crops, the European Commission, EFSA and the US EPA principally request impacts to be tested only in a single plant species or even no plant species (i.e. when Tier 1 tests indicated no potential risk) although the active ingredient is to be registered for a range of crops (EFSA, 2013; US EPA, 2016; European Commission, 2009). For systemic pesticides, EU guidelines encourage (additional) testing in a crop of intended use and extrapolating between crops based on plant metabolism data (EFSA, 2013; OEPP/EPP, 2010), but as Castle et al. (2022) indicated, *Phacelia* can be a worst-case crop in terms of plant metabolism but a best-case crop in terms of detoxification in bees.

Therefore, there is a need to better understand the role that different resources play in mitigating pesticide effects and to identify how plant morphology, as well as pollen macro- and micro-nutrient contents, influence the fitness and pesticide tolerance of bumblebees and other pollinators.



These findings may help identify worst-case crops for regulatory testing and inform the choice of plants used in flower strips to limit pesticide effects on pollinating insects.

### CRedit authorship contribution statement

**Dimitry Wintermantel:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Maria-Helena Pereira-Peixoto:** Investigation. **Nadja Warth:** Investigation, Data curation, Visualization, Writing – review & editing. **Kristin Melcher:** Investigation, Data curation. **Michael Faller:** Investigation, Data curation. **Joachim Feurer:** Investigation. **Matthew J. Allan:** Methodology. **Robin Dean:** Methodology. **Giovanni Tamburini:** Writing – review & editing. **Anina C. Knauer:** Writing – review & editing. **Janine Melanie Schwarz:** Writing – review & editing. **Matthias Albrecht:** Conceptualization, Writing – review & editing. **Alexandra-Maria Klein:** Conceptualization, Writing – review & editing, 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 accessibility statement

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

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2022.154450>.

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