Flowering resources modulate sensitivity to a common fungicide in Bombus terrestris

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April 16, 2024

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. We assessed the interactive impacts of the globally used azoxystrobin-based fungicide Amistar and three types of flowering resources (purple tansy, buckwheat, and a floral mix) on Bombus terrestris colonies in a full-factorial semi-field experiment with 39 large flight cages. Fungicide exposure through purple tansy monocultures reduced population (colony) growth, production of males, and adult worker body mass, while Amistar had no impact on colonies in buckwheat or floral mix cages. Furthermore, buckwheat monocultures hampered survival and fecundity irrespective of fungicide exposure. This shows that flowering resources modulate pesticide impacts and that B. terrestris requires access to complementary flowers to gain both fitness and fungicide tolerance.

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Running title : Flowering resources alter fungicide impacts

Keywords: *Bombus terrestris* , azoxystrobin, nutrition, pesticide sensitivity, pollen quality, floral diversity, flowering resource quality, agrochemical, plant protection product, semi-field study

Type of article: Letter

Number of

- words in the abstract: 150
- words in the main text: 5000
- references: 86
- figures: 4
- tables: 1
- text boxes: 0

Authorship: AMK, DW, MJA and MA designed the experiment. DW, MHPP, NW, KM, MF, and JF conducted the field and lab work. DW analysed the data and wrote the first draft of the manuscript. All authors commented and edited the draft and approved the final manuscript.

Data accessibility statement : The data supporting the results will be archived in an appropriate public repository and the data DOI will be included at the end of the article.

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.

We assessed the interactive impacts of the globally used azoxystrobin-based fungicide Amistar and three types of flowering resources (purple tansy, buckwheat, and a floral mix) on *Bombus terrestris* colonies in a full-factorial semi-field experiment with 39 large flight cages.

Fungicide exposure through purple tansy monocultures reduced population (colony) growth, production of males, and adult worker body mass, while Amistar had no impact on colonies in buckwheat or floral mix cages. Furthermore, buckwheat monocultures hampered survival and fecundity irrespective of fungicide exposure. This shows that flowering resources modulate pesticide impacts and that *B. terrestris* requires access to complementary flowers to gain both fitness and fungicide tolerance.

Introduction

Declines in bee diversity and distribution (Biesmeijer *et al.*2006; Vanbergen *et al.* 2013; Potts *et al.* 2016) are believed to be driven by a combination of stressors including pesticides, diseases, and loss of flowering resources (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 pests (insects) to bees (Cullen *et al.* 2019). However, fungicides are often the pesticides that bees are most exposed to (Mullin *et al.* 2010; Pettis *et al.* 2013; McArt *et al.* 2017a) 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 (Goulson*et al.* 2015; McArt *et al.* 2017a; Sgolastra *et al.*2017) or impair bees independently from other agrochemicals (Ladurner*et al.* 2005; Zhu *et al.* 2014; Artz & Pitts-Singer 2015; Bernauer *et al.* 2015). Additionally, correlational evidence indicates that fungicides promoted honeybee colony failure

in Belgium (Simon-Delso *et al.* 2014) and accelerated the declines of four bumblebee species in the United States (McArt *et al.*2017b).

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). It is frequently found in bees, pollen, and honey (Mullin *et al.*2010; Krupke *et al.* 2012; Piechowicz *et al.* 2018), but 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 several honeybee fecundity and mortality but a reduction in the foraging performance and pollination services of *Bombus terrestris* colonies (Tamburini*et al.* 2021a, b).

Agricultural intensification affects bees not only through increased pesticide exposure but also through altering flowering resource availability. Reduced flowering plant diversity harms bees through a temporary reduction in the quantity of available resources, as diversity ensures continuous flowering, (Papanikolaou et al. 2017; Requier et al. 2017; Kaluza et al. 2018) and an unbalanced diet (Di Pasquale et al. 2013; Dance et al. 2017; Filipiak et al. 2017; Sutter et al. 2017; Leza et al. 2018). Flowering plants differ in the nutrients they provide and bees, especially bumblebees, can adapt their foraging behaviour to their nutritional demands (Ruedenauer *et al.* 2015, 2020; Somme *et al.* 2015; Vaudo *et al.* 2015, 2016, 2020; Kraus *et al.*2019). Bumblebees generally prefer protein-rich pollen (Hanley *et al.* 2008; Leonhardt & Blüthgen 2012; Ruedenauer *et al.* 2015; Vaudo *et al.* 2015), which can foster their development (Baloglu & 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 & Williams 2020), low-sugar diets and insecticide exposure synergistically decreased food consumption, haemolymph 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 (Schmehl *et al.* 2014; de Mattos *et al.* 2018). It is less clear what 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 (Wahl & Ulm 1983; Archer*et al.* 2014; Dance *et al.* 2017; Leza *et al.* 2018; Barraud *et al.* 2020; Crone & Grozinger 2021). Perhaps, flowering plant diversity reduces 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 endpoints, 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 purple tansy (*Phacelia tanacetifolia*), common buckwheat (*Fagopyrum esculentum*), or a floral mix consisting of several planted and spontaneously flowering species. The two monocultural species are commonly used in semi-field experiments on pesticides (Gradish *et al.* 2016; Scott-Dupree *et al.* 2017; Frewin *et al.* 2019; Knäbe *et al.* 2020; Franke *et al.* 2021) and provide similar amounts of nectar (Knauer *et al.*unpublished data; Petanidou 2003; Cawoy *et al.* 2009) but purple tansy 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 (1) both Ami-

star and flowering resource type (hereafter simply 'resource') affect bumblebees and (2) colony development and pesticide tolerance are lowest in colonies feeding exclusively on buckwheat due to the lack of flowering plant diversity and pollen protein.

Methods

Study design

The experiment was conducted in 2020 and consisted of 39 cages with a ground cover of 53 m^2 (5.9 m × 9 m; height = 2.5 m) that 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, purple tansy, 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 lanceolate* , *Verbascum nigrum,* and *Vicia cracca* .

The cages were covered by a nylon net (mesh size = 0.95 × 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. 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).

Fungicide application

The fungicide Amistar (active ingredient (a.i.) = azoxystrobin) was applied at a rate of 250 g a.i. per hectare (=1 L $ha^{-1}$of formulated product) in the morning of 4 July 2020 in 6 of 13 purple tansy cages, in 6 of 12 buckwheat cages, and 7 of 14 floral mix cages (Appendix A, Fig. S1a). Amistar application was done according to label instructions in EU member states (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^{-1}). 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. To prevent direct exposition during bee flight, the exits of all bumblebee nests were closed early in the morning and opened again directly after the application.

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 = 7.4) with no difference between resources or spray treatments (two-way ANOVA, $P > 0.93$) and contained only few dead workers (range = 0-3, mean = 0.9, median = 1). On day -7, the colonies were placed inside the cages on the short side opposite the entrance, facing South-East (Fig. S1b). 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 within wooden boxes 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.

Azoxystrobin residue analysis

To quantify azoxystrobin exposure, two foragers from each cage were collected on either day 1 or 2 using sweep nets and pooled for each of the six resource-spray combinations and analysed 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^{-1}.

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 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.

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. S1b). 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.

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. The colonies were afterwards 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 purple tansy – control, 2 purple tansy – Amistar, and 1 floral mix – control) produced queens, the number of queens was not analysed.
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 calliper. 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 1-6 based on eye colour, body colour, and presence/absence of wings (Wintermantel et al., 2018, Table S2). Pupae last approximately two days in each developmental stage.

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 analysed 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, purple tansy, 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 S3). 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 S3). In addition, three adult workers were excluded from analyses of body mass as body parts had fallen off. Sample sizes of all endpoints 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 analysed 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 analysed 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. The final number of cocoons and adult males and workers were analysed using GLMs with a negative binomial distribution to account for overdispersion using the glm.nb function of the MASS package (Venables & Ripley 2002). Models with a quasi-Poisson or negative binomial distribution had an ln-link function. (G)LMMs were fit with maximum likelihood during model selection and with restricted maximum likelihood when selected models were evaluated.

Models for the pre-exposure period contained an interaction (including main effects) between resource and day (continuous variable) as fixed effects. For the exposure period, models contained a three-way interaction (including all two-way interactions and main effects) between 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 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 standardised 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 sim-

ple/main effects) and emtrends (for slopes) functions of the emmeans package. A Tukey post-hoc correction was applied when analysing 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 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 modelled 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.

Results

Impact of flowering resources

To avoid confounding effects with spray treatment, 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 purple tansy and floral mix colonies, monofloral buckwheat adversely impacted several parameters in comparison to monofloral purple tansy and/or the floral mix. In the pre-exposure period, buckwheat colonies showed higher mortality than purple tansy or floral mix colonies with 3.7 (i.e. >200%) more dead adults (Fig. 2, Fig. S2). Buckwheat colonies ended this period with 9.8 (i.e. 23.6%) fewer living adults than purple tansy 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. S2; difference in weight change: 23.3 g; Fig. S2)). Colony weight declined also in purple tansy (-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 purple tansy and floral mix colonies gained 58.8 g (i.e. 50%) and 32.4 g (i.e. 28%), respectively (P <0.001). At the end of the experiment, buckwheat colonies had over 150 (i.e. 86%) fewer cocoons than purple tansy or floral mix colonies (Fig. 2) and 53.0 (i.e. 57%) fewer adult workers than purple tansy colonies (Fig. 2).

Impact of Amistar exposure

Amistar negatively affected bumblebee colonies in purple tansy cages, but no effects were found in buckwheat or floral mix colonies (Fig. 3). Colonies exposed to Amistar through purple tansy gained 22.5 g less weight compared to control colonies (Fig. 3, Fig. S2). At the end of the experiment, colonies exposed to Amistar through treated purple tansy 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. S3). 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 purple tansy tended to have more light and fewer heavy workers than control colonies (Fig. S4).

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.

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 purple tansy 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).

Discussion

Our semi-field experiment reveals 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 resources.

As hypothesized, colonies confined with untreated buckwheat developed less well than colonies confined with a floral mix or monocultural purple tansy. Although buckwheat is an excellent nectar source, similar to purple tansy (Knauer *et al.* unpublished data; Petanidou 2003; Cawoy *et al.* 2009), its pollen is considered of relatively low quality due to its low protein content (11%) compared to purple tansy (30%) and the other abundant species of the floral mix (field mustard: 22%, cornflower: 23%) (Pernal & Currie 2000; Somerville 2001; Baloglu & Gurel 2015; Radev 2018). High protein diets foster bumblebee development (Baloglu & Gurel 2015; Kamper *et al.* 2016; Roger *et al.* 2017) and immunocompetence (Roger *et al.* 2017). The availability of diverse flowers should benefit bees through a more balanced diet (Di Pasquale *et al.* 2013; Dance *et al.* 2017; 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 & Bluthgen 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 purple tansy flowers (Knauer *et al.* unpublished data) 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 purple tansy and the floral mix affected colony weight gain and number of cocoons.

Amistar applied on purple tansy monocultures negatively affected *B. terrestris* colonies as manifested by reduced population size, production of males, and body mass of adult workers. Amistar may cause these effects by impairing foraging behaviour 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. 2021), damage their guts and cause a decline in sucrose consumption, weight gain, and consequently survival rate (Straw & Brown 2021).

Our study shows for the first time that the effects of a fungicide on bees are modified by flowering resources, as only colonies foraging exclusively on purple tansy were impacted. The absence of Amistar effects on colonies in floral mix cages aligns with the hypothesis that floral diversity can mitigate pesticide effects (Wahl & Ulm 1983; Klaus *et al.* 2021). However, contrary to our expectations, we found no Amistar effects in colonies feeding 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. The contamination likely occurred during the handling of bee samples on the same table. Furthermore, azoxystrobin levels were substantially higher in treated than in untreated cages in all resources with the absolute difference being largest in buckwheat.

Differences in flower morphology may explain the diverging results. Purple tansy corollae are narrower and deeper compared to buckwheat (Vattala *et al.* 2006), which impedes access and resulted in noticeably longer durations that bumblebees spent on single flowers. Hence, foraging on buckwheat was perhaps not chllenging enough for potential effects on foraging ability to translate into reduced body size and population growth.

Amistar may also have affected bees by attacking specific microorganisms present on purple tansy. Plant species differ in the microbial (including yeast and other fungal) communities they harbour, which bees can acquire through foraging and feeding (Manirajan *et al.* 2016; McFrederick & Rehan 2019). Beneficial microorganisms can alter the durability of nectar and pollen, increase plant attractiveness to pollinators, protect bees from pathogens and promote detoxification (Koch & Schmid-Hempel 2011; Herrera *et al.* 2013; Pozo *et al.* 2015; Zheng *et al.* 2016; Vollet-Neto *et al.* 2017; Raymann & Moran 2018; Leonard *et al.* 2020). Pesticides can impact microorganisms found on floral 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, pollen protein (or its ratio to other nutrients) may affect fitness and development differently than fungicide tolerance. Although earlier studies suggested that a high pollen protein content can decrease pesticide sensitivity (Wahl & Ulm 1983; Archer *et al.* 2014), 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-to-lipid ratio (Crone & Grozinger 2021).

Our semi-field experiment indicates that the risk of Amistar, which is characterized as 'non-hazardous to bees', should be re-evaluated. Interestingly, we found now in two semi-field experiments that Amistar applied on purple tansy can affect *B. terrestris* , while we found no effects in a similar study on honeybees (Tamburini *et al.*2021a, b). This indicates that *B. terrestris* may be more sensitive to Amistar than honeybees, which was previously also found for other pesticides (Cresswell *et al.* 2014; Rundlof *et al.*2015; Wintermantel *et al.* 2018; Osterman *et al.* 2019). The active ingredient azoxystrobin was first approved at a time when risk assessments on pollinators were exclusively done with honeybees and we are unaware of any regulatory testing of azoxystrobin on bumblebees that was done since then. Besides, the guidance document on the risk assessment of plant protection products in the EU that includes testing on bumblebees was never fully implemented due to the opposition of some member states (EFSA 2013; More *et al.* 2021). In addition, a recent study indicates that surfactants (alcohol ethoxylates) found in Amistar (and other products) rather than the active ingredient (azoxystrobin) damage bumblebee guts and increase their mortality (Straw & Brown 2021). The risk of such co-formulants is, however, not assessed in regulatory pesticide risk assessments of the EU (EFSA 2013).

Our study has shown that the effects of a pesticide on bees can depend on the forage plants it is applied on. This has potential implications for the regulatory assessment of pesticides. The European food safety authority recommends that exposure to an active ingredient is evaluated in multiple crops, but requests impacts to be tested only in a single plant species (and only if lower-tier testing indicated a potential risk) although the active ingredient is to be registered for a range of crops (EFSA 2013). However, in our experiment, differences in effects between plant species/mixture did not seem to be driven by differences in exposure levels but rather by nutrition or plant morphology. Therefore, resource differences should arguably be considered more strongly in the risk assessment of pesticides on bees.

Our findings call for further research to evaluate 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 resilience of bumblebees and other pollinators. Agricultural landscapes may then be modified accordingly, for instance by cultivating flower strips containing beneficial species, to limit pesticide

effects on pollinating insects.

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Tables

Table 1. Azoxystrobin residue concentrations (mg kg^{-1}) in foraging bees exposed to different spray treatments (control and Amistar) and resources (purple tansy, buckwheat, floral mix). From all colonies, 2 bees 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^{-1}.

	Purple tansy	**Buckwheat**	**Floral mix**
Control	< LoQ	0.0773	< LoQ
Amistar	0.3627	0.5595	0.3013

Figures

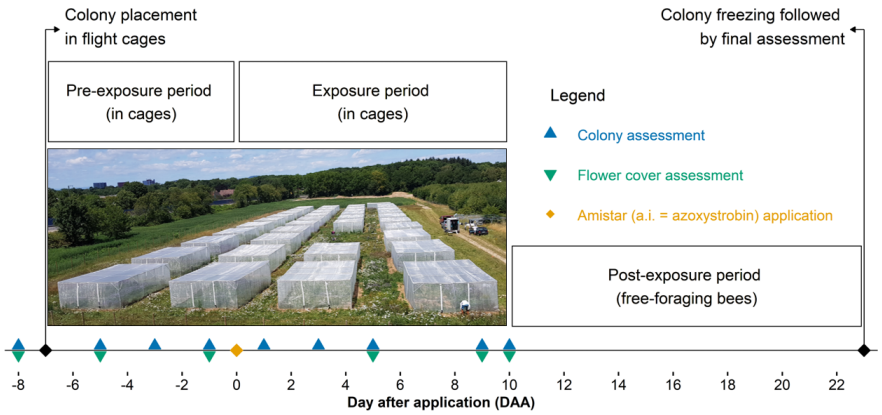

Figure 1. Experimental timeline. Sequence of colony and flower cover assessments in the pre-exposure period (before Amistar application) and exposure period (after Amistar application).

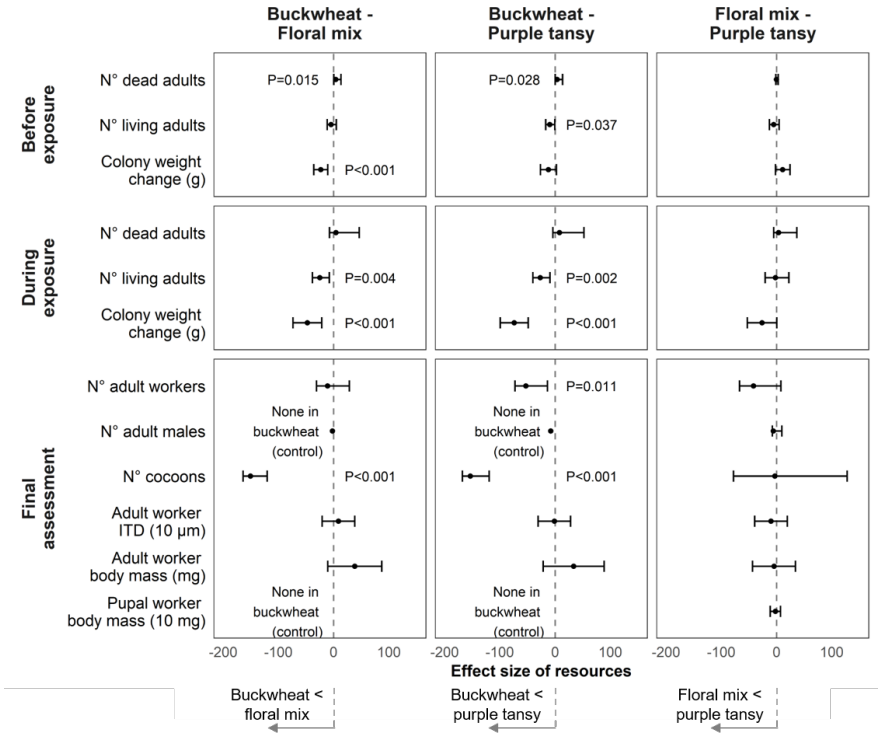

Figure 2. Effect sizes of floral resource effects. Differences in estimated marginal means between different types of floral resources are illustrated as dots. Error bars indicate 95% confidence intervals. P -values < 0.05 are shown. To avoid confounding effects with spray treatment (Amistar or control) 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.

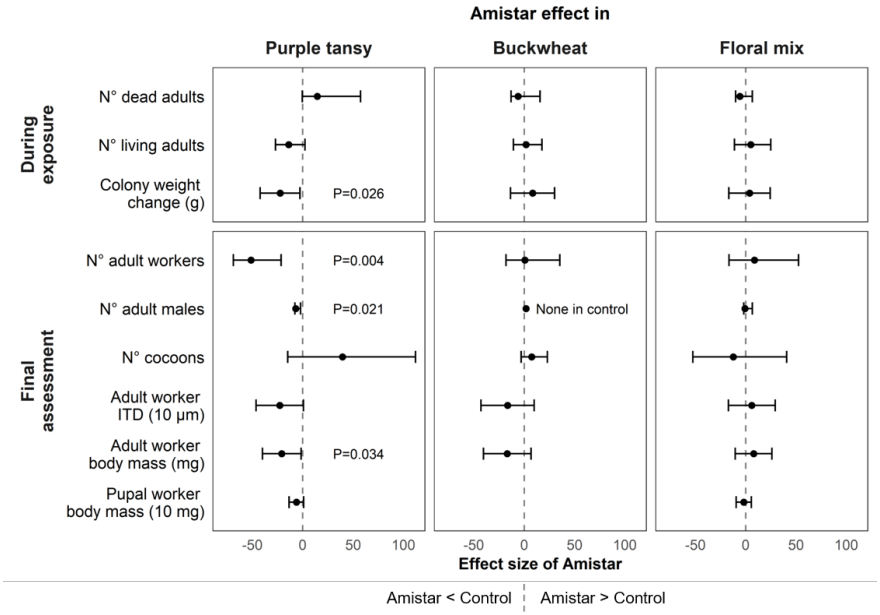

Figure 3. Effect sizes of Amistar effects. Differences in estimated marginal means between Amistar-exposed and control colonies, error bars indicating 95% confidence intervals, and P -values < 0.05 are shown. No confidence intervals for the number of males in buckwheat are shown as there were no adult males found in the control colonies placed in buckwheat cages.

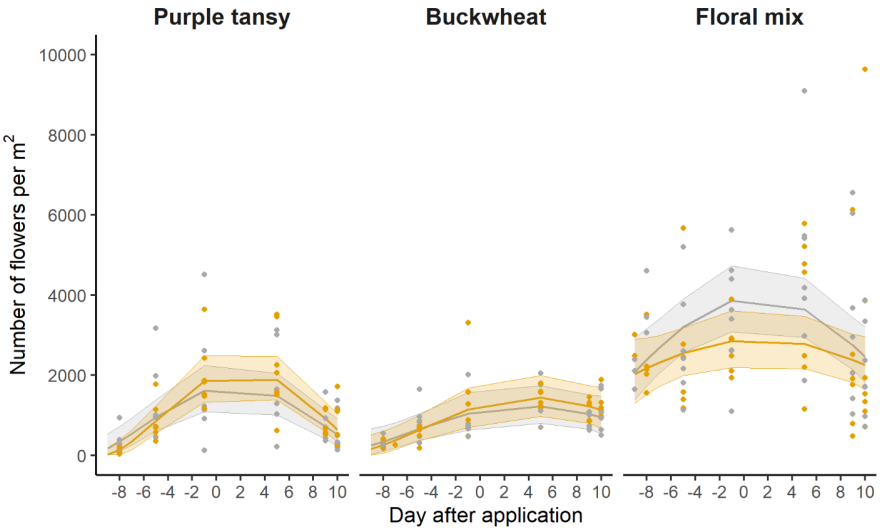

Figure 4. Flower density. The estimated number of flowers per m^2 is shown in relation to time and spray treatment (grey: control, orange: Amistar). Lines represent estimated marginal means and shaded areas depict 95%-confidence interval, both obtained from an LMM. Dots represent observations.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The study was funded by the European Horizon 2020 research and innovation programme under grant agreement No 773921. We thank Angela Gronert, Laura-Sophia Ruppert, Maiara da Silva Gonçalves, Felix Fornoff, Björn Henrik Törnqvist, Johanna Niermann and Semjon Krassovitski for their assistance during field and/or lab work.