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Sulfoxaflor insecticide and azoxystrobin fungicide have no major impact on honeybees in a realistic-exposure semi-field experiment

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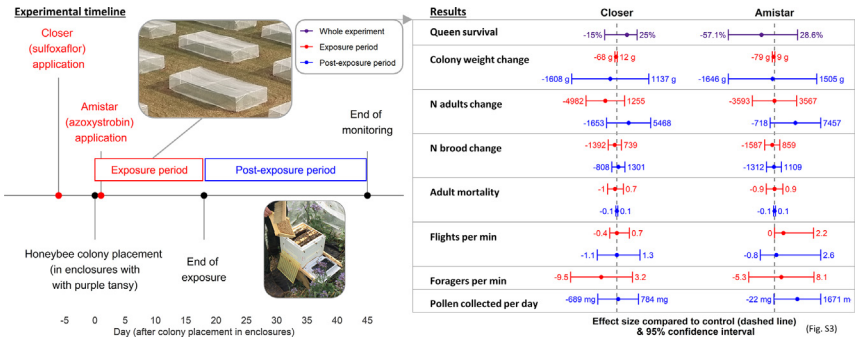
HIGHLIGHTS

- Peer-reviewed field realistic studies on the potential threat of novel insecticides and fungicides for bees are rare
- Sulfoxaflor insecticide (Closer) shows no impact on honeybees when applied in isolation six days before bloom
- Azoxystrobin fungicide (Amistar) sprayed during bloom poses no notable risk to honeybees under semi-field conditions
- Mandatory safety periods between application of sulfoxaflor products and crop bloom may be crucial to limit risk for bees

GRAPHICAL ABSTRACT



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ABSTRACT

Exposure to pesticides is considered a major threat to bees and several neonicotinoid insecticides were recently banned in cropland within the European Union in light of evidence of their potential detrimental effects. Nonetheless, bees remain exposed to many pesticides whose effects are poorly understood. Recent evidence suggests that one of the most prominent replacements of the banned neonicotinoids – the insecticide sulfoxaflor - harms bees and that fungicides may have been overlooked as a driver of bee declines. Realistic-exposure studies are, however, lacking. Here, we assess the impact of the insecticide Closer (active ingredient: sulfoxaflor) and the widely used fungicide Amistar (a.i.: azoxystrobin) on honeybees in a semi-field study (10 flight cages containing a honeybee colony, for each of three treatments: Closer, Amistar, control). The products were applied according to label instructions either before (Closer) or during (Amistar) the bloom of purple tansy. We found no significant effects of Closer or Amistar on honeybee colony development or foraging activity. Our study suggests that these pesticides pose no notable risk to honeybees when applied in isolation, following stringent label instructions. The findings on Closer indicate that a safety-period of 5–6 days between application and bloom, which is only prescribed in a few EU member states, may prevent its impacts on honeybees. However, to conclude whether Closer and Amistar can safely be applied, further realistic-exposure studies should examine their effects in combination with other chemical or biological stressors on various pollinator species.

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1. Introduction

Pollinator declines exacerbate biodiversity losses and threaten global food security (Potts et al., 2016; Tscharntke et al., 2012). The Western honeybee (*Apis mellifera* L.) is widely kept for honey production and is the most important managed crop pollinator (Kleijn et al., 2015; Klein et al., 2007) and often regarded as a representative for other bees in pesticide risk assessments (Quigley et al., 2019; Thompson and Pamminger, 2019). Elevated honeybee colony loss rates (Neumann and Carreck, 2010; Potts et al., 2016) and declines in wild bee abundance (Dupont et al., 2011; Goulson et al., 2015; Mathiasson and Rehan, 2019) and diversity (Biesmeijer et al., 2006; Bommarco et al., 2012; Zattara and Aizen, 2021) have been attributed to a combination of several factors including pesticides, lack of floral food resources and diseases (Goulson et al., 2015; Potts et al., 2016; Mathiasson and Rehan, 2019; Klaus et al., 2021).

Among pesticides, neonicotinoid insecticides have been most thoroughly examined for their impacts on pollinators. Evidence of their detrimental effects on different bee species (Gill et al., 2012; Henry et al., 2012; Rundlöf et al., 2015; Tsvetkov et al., 2017; Whitehorn et al., 2012; Woodcock et al., 2016) resulted in the ban of three neonicotinoids in all outdoor crops in the European Union, leaving a gap that may largely be filled by sulfoximine-based insecticides (Brown et al., 2016). Some authors argue that these neurotoxins should be classified as neonicotinoids (Giorio et al., 2017) as they exploit the same neuron receptors (Sparks et al., 2013). As neonicotinoids, the first commercial active substance of the sulfoximines, sulfoxaflor is a systemic insecticide that spreads throughout treated crops and can contaminate their pollen and nectar (EFSA, 2019; Giorio et al., 2017). Sulfoxaflor is already registered for a wide variety of crops such as wheat, oilseed rape, cotton or tomato in all inhabited continents.

While no impact of sulfoxaflor on bumblebee cognition (Siviter et al., 2019), escape behavior (Parkinson et al., 2020) or honeybee flight activity (Cheng et al., 2018) was detected, detrimental effects on bumblebee reproduction (Siviter et al., 2020, 2018) and honeybee survival (Cheng et al., 2018) were found. The European Food Safety Authority (EFSA) questioned the conclusions of the most prominent study identifying a harm (Siviter et al., 2018), but nonetheless concluded that sulfoxaflor poses a high risk to bees when applied outside of permanent greenhouse structures (EFSA, 2019). France recently banned the substance but it remains authorized in 18 EU member states (European Commission, last accessed 11 October, 2020). Based on industry-provided studies and the high degradability of the substance (Xu et al., 2012), the United States recently authorized even new uses including applications in tank mixtures with other pesticides and applications before and in some cases even during the bloom of bee-attractive crops (EPA, 2019). The disagreement among different experts and regulating bodies calls for further risk assessments of sulfoxaflor applications under realistic conditions.

Pesticides other than insecticides have received less attention (Cullen et al., 2019), although bees are frequently exposed to many different pesticides with fungicides generally being the most abundant ones detected in honeybees and their hive materials (McArt et al., 2017a; Mullin et al., 2010; Pettis et al., 2013). Fungicides are often viewed as relatively non-toxic to bees and harmful impacts on bees have so far mainly been found for ergosterol-biosynthesis inhibitor (EBI) fungicides that can reduce detoxification of other chemicals and magnify their toxicity tremendously (Goulson et al., 2015; McArt et al., 2017a; Sgolastra et al., 2017). However, some experiments identified that also non-EBI fungicides alone (Artz and Pitts-Singer, 2015; Bernauer et al., 2015; Ladurner et al., 2005; Zhu et al., 2014) or in interaction with other pesticides (De Grandi-Hoffman et al., 2013; Zhu et al., 2014) can have negative effects on bees. Besides, fungicide use was linked to reductions in the geographical distribution of declining bumblebee species in the United States and to their *Nosema bombi* infection rate (McArt et al., 2017b). In Belgium, failures and disorders of honeybee colonies were attributed to the presence of fungicide (Simon-Delso et al., 2014). However, information is sparse on the potential effects of common fungicides on bees.

Azoxystrobin is a systemic broad-spectrum fungicide that is widely used in agriculture (Bartlett et al., 2002) and commonly found in bees and bee-collected materials (Genersch et al., 2010; Hladik et al., 2016; Long and Krupke, 2016; Mullin et al., 2010; Sanchez-Bayo and Goka, 2014). There is some evidence that azoxystrobin can increase forager mortality (Fisher et al., 2017) and affect the expression of genes regulating the hormonal system or energy metabolism of honeybees (Christen et al., 2019). However, these effects did either not increase with the dose or were only observed at concentrations above field-realistic levels. In addition, it is unclear whether such effects would scale up to the colony level (Osterman et al., 2019; Wood et al., 2020).

Here, we assessed the impacts of field-realistic exposure of honeybees (*Apis mellifera* L.) to the insecticide Closer (active ingredient: sulfoxaflor, Corteva Agriscience) and the fungicide Amistar (active ingredient: azoxystrobin, Syngenta) in a semi-field enclosure experiment. Honeybee colonies were examined for several colony development and foraging activity parameters while being confined in large flight cages (hereafter '*enclosures*') to treated or untreated purple tansy (*Phacelia tanacetifolia*) as well as in a subsequent post-exposure period where they were allowed to forage freely in the landscape. We hypothesize that Closer and Amistar applied according to label instructions reduce honeybee colony development and foraging activity.

2. Materials & methods

2.1. Study design & study site

The experiment was conducted in 2019 near Winchester in Hampshire, United Kingdom (51.0471°N, 1.4365°W) and consisted of an exposure phase of 18 days and a post-exposure monitoring phase of 27 days where bees were allowed to forage freely in the landscape. The duration of the experiment was delineated in accordance with guidance for semi-field studies (e.g. EFSA, 2013). Honeybee colonies were placed in enclosures with purple tansy (*Phacelia tanacetifolia* Benth.) sprayed with Closer, Amistar, or only water (control group). In total 30 enclosures were used with 10 enclosures per treatment (but see supplementary material for the original experimental setup with the combination treatment). The enclosures (12 m × 5.9 m, height: 2 m) were separated by at least 6 m from each other and at least 10 m from the next field (wheat) and contained only one colony.

The enclosures were erected on a field sown with 5 kg ha^{-1} purple tansy and covered by nets (mesh size = 0.95 mm × 1.35 mm, Howitec). A specialist spray contractor (Oxford Agricultural Trials Ltd) applied the recommended maximum rates for spray applications of 0.4 L ha^{-1} Closer (Dow AgroSciences, 120 g L^{-1} sulfoxaflor, i.e. 48 g a.i. ha^{-1}, product ID: ES-00461) and 1 L ha^{-1} Amistar (Syngenta, 250 g L^{-1} azoxystrobin, i.e. 250 g a.i. ha^{-1}, product ID: A12705B) in the respective enclosures using a portable motorized sprayer equipped with a 3 m long boom with anti-drift spraying nozzles. Applications were performed during days of adequate weather conditions. Wind speed was measured by a vane anemometer, and confirmed to be <2 m s^{-1}. Matching of the nominal and actual application rates were confirmed by measuring discharge volume after application in five cages and calculating the mean application rate based on the volume of applied liquid and the product concentration.

Enclosures were randomly allocated to treatments. Following label instructions, Closer was applied before the bloom of purple tansy (i.e. on 17 July at phenological plant stage BBCH 55 as identified by consultant agronomists Cropfosters Ltd), while Amistar was sprayed one week later during the bloom of purple tansy (BBCH 63). As the label for Amistar permits applications during bee flight, the honeybees were allowed to forage within the enclosure during Amistar spraying. To prevent spray drift, the walls of the sprayed enclosures were covered

with a plastic sheet during application. Enclosures that were not treated with Amistar were sprayed with water only on the same day with the same volume as the Amistar application.

The honeybee colonies were placed in a corner of the enclosures near a zipped entrance on 23 July (i.e. Day 0) at the early stages of flowering (BBCH 61), six days after the application of Closer and 1 day before the application of Amistar (Fig. S1). All colonies faced the same direction, i.e. away from the enclosure entrance towards the opposite end (Fig. S2). At each short end of the enclosure, 5.9 m × 0.5 m of porous fabric was pegged down to facilitate walking and counting of dead bees. In addition, two 0.5 m-wide strips were set centrally parallel to the long sides of the enclosures (Fig. S2), to facilitate the movement of the spray contractor and field technicians within the crop. A water feeder (i.e. a plastic container filled with sawdust and water) was placed inside each enclosure; the water was changed after the Amistar application, and was periodically topped up during the study.

At Day 18 (10 August), the nets of all 30 enclosures were removed to initiate the post-exposure monitoring phase. To prevent continued exposure to the applied pesticides including exposure of bees to a treatment they have not been assigned to, the purple tansy grown within the enclosures was mown and the nets were laid over the mown plants. The available forage in this period comprised the purple tansy in the surrounding 2-ha large field and a variety of wild flowers typical for the region such as *Rubus fruticosus*, *Clematis vitalba*, *Brassica nigra*, *Chenopodium album*, *Taraxacum officinalis*, *Sonchus* spp., *Senecio jacobaea*, etc. The experiment ended on 6 September (Day 45) after more than two brood cycles. During the study period, no extreme air temperatures occurred (minimum temperature 5 °C, maximum temperature: 32 °C) and moderate amounts of rainfall (1–16 mm) were recorded on four days during the exposure period and six days during the post-exposure period. A storm occurred on Day 17 – Day 19. In this period no assessments were conducted since bee activity during this period was largely limited.

2.2. Honeybee colonies

Nucleus Buckfast honeybee colonies headed by closely-related 2-month old queens were acquired from FERA Science Ltd. (UK) in July 2018 and transferred to converter hives containing British Standard frames below a queen excluder and Mini Plus frames above. Each colony was treated against the *Varroa* mite using a strip of Apivar (active substance: Amitraz) before overwintering in an isolated apiary. The colonies were fed with abundant amounts of sugar syrup; small amounts of pollen substitute were also fed to every colony (Fig. S1). Colony growth was limited by removing brood frames. No signs of American Foulbrood, European Foulbrood, or Chalkbrood and only low levels of *Varroa* were found by an inspector from the UK National Bee Unit assessing the colonies approximately one month before the start of the experiment (Fig. S1). At Day -12, the colonies were transferred for ease of management to an untreated clover field and at Day -4/-3 equalized to have approximately 300 g of adult bees (corresponding to about 3000 individuals) and approximately equal amounts of nectar/honey, pollen, open brood, sealed brood, and empty cells/frames. The colonies were established within Mini Plus hives consisting of a bottom brood box and an upper food box (super) each containing six frames (217 mm × 160 mm) with a comb of approximately 1000 cells on each side. The super was prepared in advance using frames of honey stores. Each super weighed 4 kg including the box and the frames. The queen was prevented from moving from the bottom box to the upper box by a queen excluder separating the boxes. The manipulated colonies were randomly assigned to the three treatment groups. For a separate study, 10 adult worker bees per colony were sampled on Day 0 and Day 2.

2.3. Assessed parameters

The colonies were assessed for queen presence, their development (number of adult bees, amount of brood, brood failure, colony weight and adult bee mortality), flight and foraging activity as well as amount of pollen collected. For details regarding the sampling timeline see Fig. S1.

2.3.1. Queen presence

The presence of active (i.e. egg-laying) queens was inferred from the presence of young brood during colony assessments and from examinations of brood photos (see paragraph 2.3.3). If no eggs were found on the photo of one particular date, photos taken earlier or later were inspected to confirm queen failure and to determine the approximate date of occurrence. For instance, if in a colony assessment, young pupae but no eggs were found, queen failure was estimated to have occurred three days earlier. The presence of queen cells was also taken as an indicator of queen failure.

2.3.2. Change in number of adults and brood cells

The number of adult bees and the amount of brood (i.e. number of open and sealed brood cells) were assessed on Days -1, 21, and 44, i.e. before the exposure period, shortly after and at the end of the experiment. The number of adult bees was visually estimated by comparing frames with bees to reference photos with known numbers of bees. The amount of brood was visually assessed by estimating their percentage coverage on each frame side to the nearest 10%. We calculated the change in both number of adult bees and brood cells over the exposure period (difference between Day 21 and Day -1) as well as over the whole experiment (difference between Day 44 and Day -1).

2.3.3. Brood failure

Brood development was evaluated on one side of a study frame per colony, which incorporated a removable queen excluder used for ensuring that the brood on these frames were of the same age. For this, the queen was confined to the frame from Day -4 or -3 to Day -1 when the frame side was photographed for the first time. Subsequent photos were taken on Days -1, 4, 9, 14, 20, 27, 32, 38, and 44 to capture the development of the brood at different key developmental stages in two complete brood cycles. The photos were evaluated using image recognition software able to distinguish between eggs, larvae, capped brood cells and empty cells (HiveAnalyzer by Visionalytics, Stuttgart). We used these data to estimate the final (Day 44) percentage of brood failure for each colony. However, as the recognition of eggs was not reliable on Days 6 and 34, we only considered the number of larvae as reference points for those dates.

2.3.4. Change in colony weight

The colonies including bees as well as food and brood frames were weighed three times (Days 2, 16 and 45). We calculated the difference in weight at the end of both exposure and post-exposure phases compared to the initial one ($Weight_{Day16\ or\ 45} - Weight_{Day2}$).

2.3.5. Adult bee mortality

Mortality of adult worker bees was estimated almost daily during the exposure phase (13 assessments between Days 2–17) by counting and removing the number of dead bees from a trap attached to the hive (c. 30 cm × 20 cm; based on Hendriksma and Härtel, 2010) and fabric placed on the ground. During the post-exposure phase, bee mortality by trap inspection was assessed roughly every second day (9 assessments, between Days 25–42; Fig. S1) while fabric inspection was stopped when colonies were moved at the end of the exposure phase.

2.3.6. Flight and foraging activity

Flight and foraging activity were mostly assessed on the same days as bee mortality (13 and 11 assessments during the exposure and post-exposure phase, respectively; Fig. S1). Flight activity was estimated by counting the number of bees entering the hive within 1 min using a click-counter. Similarly, foraging activity was assessed by counting the number of bees found in a quadrat (1 m × 1 m) placed at

three random locations within the enclosure for 1 min each. All bees inside the quadrat were considered foragers and counts of the three measurements were summed up. Observations were conducted during adequate weather conditions (≥ 13 °C, no rain, wind speed <2 m s^{-1}).

2.3.7. Pollen collection

The amount of pollen collected by foragers was assessed on Day 38 and 42 by weighing pollen from pollen traps incorporated in the hive bottoms that were activated 24 h before by moving a pollen stripping grid in front of the hive entrances.

2.4. Statistical analyses

The analyses presented here explore the differences between control, Closer and Amistar treatments. These treatment groups did not differ in the initial number of bees, brood cells and weight (Table S3, Fig. S5). Six colonies whose queens were found rejected by the colony on Day 1 (one control colony) or still not laying on Day 4 (one control, one Closer and three Amistar colonies) were excluded from all the analyses. All analyses were done in R version 4.0.2.

2.4.1. Queen presence

We first tested whether pesticide exposure affected the presence of active queens (i.e. egg-laying) in the colonies over time. We performed Kaplan-Meier Survival analysis (Klein and Moeschberger, 2006; Wei, 1992), including pesticide treatment as a fixed factor (categorical, three levels). Survival curves were compared using the log-rank test. The analysis was computed with "*survival*" package (Therneau, 2020) considering 24 colonies (8 control, 9 Closer, 7 Amistar).

2.4.2. Colony growth and activity

All the colonies where the queen was found rejected, not laying or only barely laying during the experiment were further removed since queen absence or abnormal behavior greatly influence colony dynamics and activity. We found three of such colonies during the post exposure phase (1 control and 2 Amistar). To better understand short- and longer-term effects of pesticides, we analyzed data regarding exposure and post-exposure phases, separately. Analyses regarding the exposure phase included data from 24 enclosures (8 control, 9 Closer, 7 Amistar), those of the post-exposure phase 21 enclosures (7 control, 9 Closer, 5 Amistar). To test the effects of the pesticides on the change in the number of adults, brood cells, brood failure and change in hive weight we run a total of seven linear models (LMs; one record per enclosure). All models included pesticide treatment as a categorical predictor (three levels). We also included the initial colony strength (i.e. the sum of the initial number of adults and brood cells, continuous) as covariate, in order to account for variations in initial colony size that could have affected colony dynamics. The number of dead adult bees, flight activity, foraging activity and pollen collection were analyzed with a total of six linear mixed-effects models (LMMs; multiple records per enclosure). All models included pesticide treatment, Day (continuous) and their interaction as fixed effects, the initial colony strength as covariate and the enclosure ID as a random factor. The number of dead adult bees and the number of flights were log- and square root- transformed, respectively. Initial colony strength was centred to mean = 0 and standardized to SD = 0.5. Normality and homoscedasticity of the model residuals were validated graphically. Final models were estimated using the REML method in the "lme4" packages (Bates and Mächler, 2014; Pinheiro et al., 2007) implemented in R version 4.0.2 (R Core Team, 2020).

3. Results

The proportion of living egg-laying queens did not differ across pesticide treatments (Fig. 1, $P = 0.250$, log-rank test). Colonies exposed to Amistar presented the lowest proportion of active queens at the end of the experiment (5 out of 7 colonies), compared to the Closer (9 out of 9 colonies) and control (7 out of 8 colonies) treatments. We found no evidence that Closer or Amistar exposure might influence the development (change in the number of adults and brood cells, brood failure), change in hive weight (a proxy for food reserves) or the activity (flight activity, number of dead adult bees and foraging activity) of honeybees during both the exposure and post-exposure phases (Table 1, Figs. 2, S3). Honeybees exposed to Amistar collected more pollen during the post-exposure phase than bees of the other treatments, but the differences were only marginally significant (Tukey multiple comparison test: Amistar vs. control: $P = 0.057$; Closer vs. Amistar: $P = 0.061$; Closer vs. control: $P = 0.985$, Fig. 2h). The number of dead adult bees slightly increased during the exposure phase and decreased during the post-exposure phase, irrespectively of treatments. Flight activity generally increased during the experiment. Moreover, we found the initial colony strength to positively influence foraging activity and pollen collection, whereas it negatively affected the change in colony weight during the post-exposure phase.

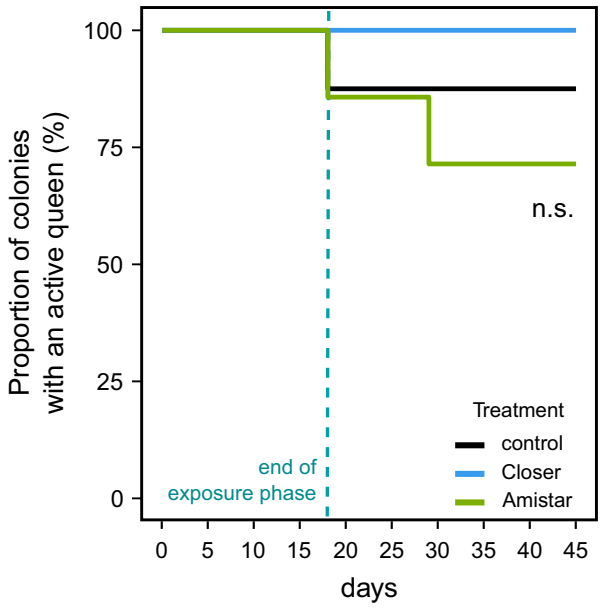

Fig. 1. The Kaplan–Meier survival curves showing the effects of spray application of the product Closer (sulfoxaflor) and Amistar (azoxystrobin) compared to the control treatment on the proportion of active (i.e. egg-laying) honeybee queens during the experiment (both exposure and post-exposure phase) Abbreviation: n.s., not significant. For details on the experimental timeline see Fig. S1.

4. Discussion

We hypothesized that field-realistic exposure (following product label instructions) to two common pesticides, the insecticide Closer (containing sulfoxaflor) and the fungicide Amistar (containing azoxystrobin) would deteriorate honeybee health. But, we did not find any impact on the development or the activity of honeybee colonies.

Our results indicate that Closer poses no substantial risk to honeybees that are exposed to the insecticide six days after spraying. However, honeybees that are exposed to Closer during or shortly after its application may be negatively impacted (EFSA, 2019). High degradation rates of sulfoxaflor imply that at the moment of application residue levels are substantially higher with potential implications for honeybees (Cheng et al., 2018; Siviter and Muth, 2020). In fact, recent semi-field studies in which honeybees were either directly exposed to insecticide spray or visited treated flowers in full bloom one day after application, showed that sulfoxaflor or sulfoxaflor-based products (Transform, Closer) increase honeybee mortality, resource consumption and

Table 1
Results of the linear and linear mixed effects models testing the effects of treatment (sulfoxaflor, azoxystrobin, control) and time (days since colonies were introduced in the enclosures) on colony development (change in number of adult bees and brood cells), hive weight (change in hive weight), adult bee mortality (number of dead bees), brood mortality (proportion of failed brood), flight and foraging activity (number of flights and number of flower visiting bees) and pollen collection during the exposure phase (hives in the enclosures) and during the post exposure phase (hives outside the enclosures). All models also included initial colony strength (number of adults and brood cells) as covariate. Significant ($P < 0.05$) effects are highlighted in bold. For details on the experimental timeline see Fig. S1.

Exposure phase				Post-exposure phase			
Variable	Sum Sq	F-value	*P*-value	Variable	Sum Sq	*F*-value	*P*-value
Change in number of adult bees							
Treatment	540,333.0	0.41	0.671	Treatment	440,407.0	0.34	0.717
Initial colony strength	1,189,736.0	1.80	0.198	Initial colony strength	503,121.0	0.78	0.391
Change in number of brood cells							
Treatment	17,023,290.0	1.50	0.251	Treatment	34,418,420.0	2.33	0.128
Initial colony strength	557,033.0	0.10	0.758	Initial colony strength	12,196.0	0.00	0.968
Proportion of failed brood							
–		–	–	Treatment	0.4	0.37	0.695
–		–	–	Initial colony strength	0.0	0.00	0.639
Change in colony weight							
Treatment	0.0	2.48	0.111	Treatment	0.2	0.10	0.903
Initial colony strength	0.0	2.07	0.166	**Initial colony strength**	**7.4**	**6.71**	**0.019**
Variable		***χ2***	***P*-value**	**Variable**		***χ2***	***P*-value**
Number of dead bees							
Treatment		1.49	0.474	Treatment		0.05	0.974
Day		**7.14**	**0.007**	**Day**		**33.90**	**<0.001**
Treatment:Day		0.26	0.876	Treatment:Day		0.10	0.951
Initial colony strength		1.51	0.219	Initial colony strength		0.01	0.923
Number of flights							
Treatment		4.30	0.117	Treatment		1.35	0.501
Day		38.45	**< 0.001**	**Day**		**8.04**	**0.005**
Treatment:Day		0.37	0.829	Traetment:Day		2.85	0.240
Initial colony strength		**11.08**	**0.001**	Initial colony strength		0.31	0.578
Number of foraging bees							
Treatment		3.35	0.187	–		–	–
Day		1.32	0.250	–		–	–
Treatment:Day		0.11	0.946	–		–	–
Initial colony strength		**10.38**	**0.001**	–		–	–
Pollen collection							
–		–	–	**Treatment**		**7.49**	**0.024**
–		–	–	**Day**		**47.14**	**< 0.001**
–		–	–	Treatment:Day		2.93	0.231
–		–	–	**Initial colony strength**		**4.50**	**0.034**

oxidative stress (Chakrabarti et al., 2020; Cheng et al., 2018; Louque, 2018). These studies used recommended application rates and reflect worst-case scenarios for applications during crop bloom. Interestingly, in two of these studies, honeybee mortality was only increased within the first days after application (Cheng et al., 2018; Louque, 2018).

Our semi-field study was designed to simulate realistic exposure to Closer in those EU member states that prescribe a 5-day safety period between application and crop bloom (Spain, Italy, Croatia and Bulgaria; Corteva, 2020). However, in practice, this safety period may not always be respected due to difficulties in anticipating the onset of flowering. Some labels contain indications about plant development stages (BBCH) during which applications are allowed, but these are often incautious, permitting applications until the immediate stage before flowering (BBCH = 59; Corteva, 2020). Therefore, it is questionable that this indication helps farmers respect the 5-day safety period. Other EU member states that authorized the insecticide prescribe shorter safety periods and Belgium even allows applications during crop bloom (Corteva, last accessed on 1 October 2020). Closer applications during bloom are also permitted in fruiting or cucurbit vegetables and strawberries in the United States or any crops in South Africa (Corteva). In addition, even though in some countries applications should not be done in presence of weeds, contamination of wild flowers can occur, which sometimes poses a larger threat than insecticide exposure through the treated crop (David et al., 2016; Long and Krupke, 2016). Our study adds to the existing evidence that compliance to stringent label instructions regarding the application timing of Closer in flowering crops is important in limiting their impacts on honeybees.

Under the conditions of our experiment, Amistar had no marked adverse effects on colony strength or activity. Our findings, however, do not contradict previous studies that found azoxystrobin to impact honeybees because of the different exposure conditions adopted in those experiments. For example, azoxystrobin (product Quadris) decreased honeybee forager survival in a wind tunnel chamber experiment but only when applied at twice the label dose or at the label dose (213 g active ingredient (a.i.) per hectare) in combination with the fungicide iprodione (product: Iprodione 2SE Select; Fisher et al., 2017).

We observed c. 48% higher pollen collection during the post-exposure phase in colonies exposed to Amistar compared to both the control and the Closer group, but the probability of obtaining differences this large exceeded slightly the conventional significance level α of 0.05 (Fig. 2h). Differences in pollen collection but not in other relevant endpoints measured during the same period (e.g. flight activity, change in colony weight and number of adults) may indicate a potential effect of Amistar on colony resource consumption and use efficiency. However, we are aware that marginally significant results should be interpreted cautiously and the observed differences in pollen collection may be a consequence of the slightly but not statistically significantly higher number of brood cells found during the post-exposure period in the

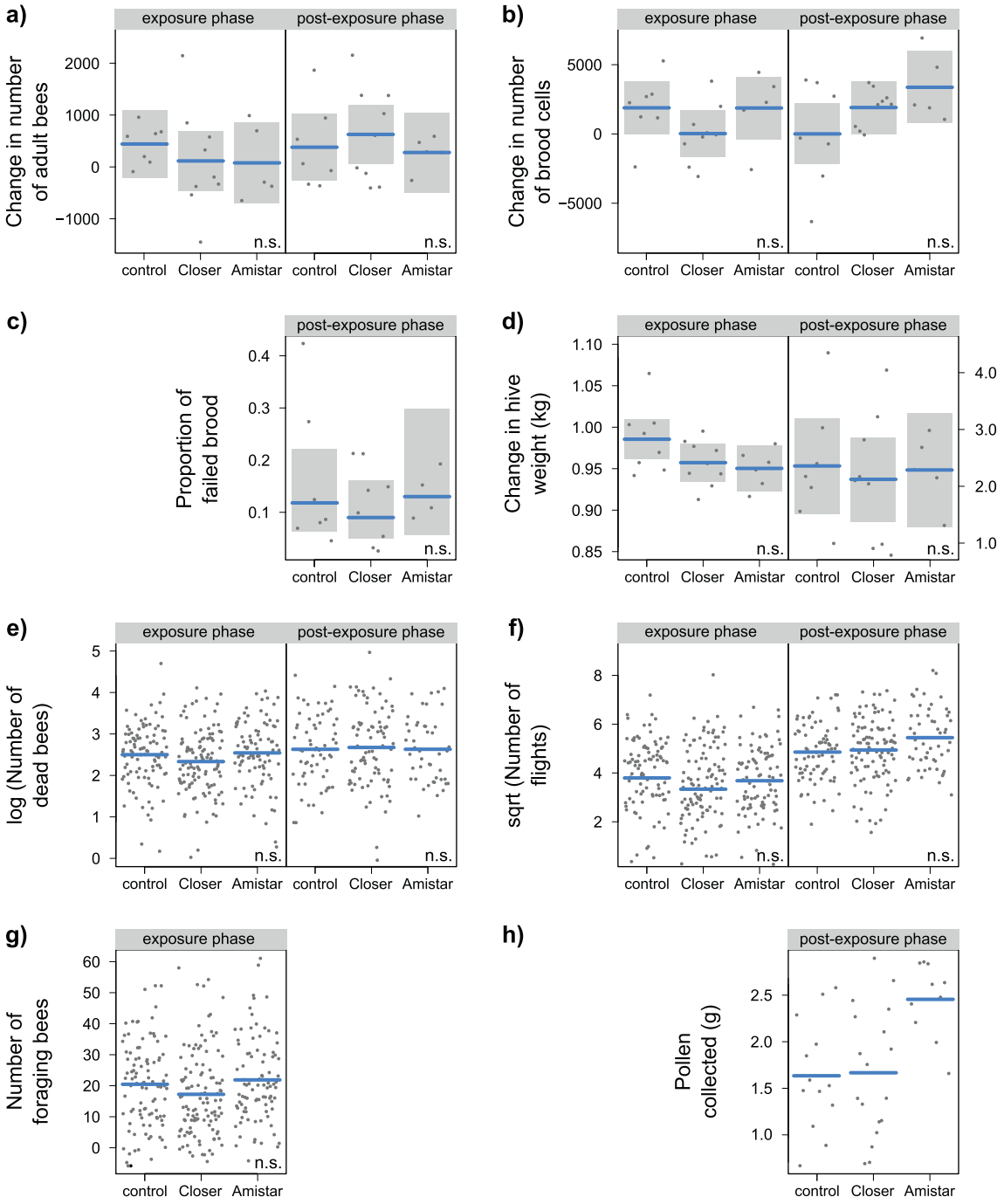

Fig. 2. Effects of spray application of the product Closer (sulfoxaflor) and Amistar (azoxystrobin) compared to the control treatment on honeybees during and after the exposure phase (see Table 1): effects of treatments on change in the number of adult bees (a) and brood cells (b), proportion of failed brood (c), change in colony weight (d), number of dead bees (e), flight activity (f), foraging activity (g) and pollen collection (h). Plots display prediction lines, partial residuals and, for linear model results, confidence bands (95%, a-d). Abbreviation: n.s., not significant. For details on the experimental timeline see Fig. S1.

colonies exposed to Amistar (Fig. 1b; $P = 0.128$). We are also aware that our data on pollen collection cover only two days and that the pollen in the traps reflect only a portion of the total collected pollen. Therefore, more research is needed to further investigate the potential mechanisms underpinning pollen collection in response to treatments.

We found no effects of treatments on the proportion of active queens at the end of the experiment. Recent studies, however, reported evidence for impacts of neonicotinoid insecticides on honeybee queen fecundity and survival by altering their behavior and physiology (Dussaubat et al., 2016; Kairo et al., 2016; Wu-Smart and Spivak, 2016). It has also been suggested that pesticides (miticides and a mixture of insecticide and fungicide) might alter the semiochemicals released by queen mandibular glands, affecting interactions with workers (Walsh et al., 2020). Moreover, studies on queen response to fungicides are scarce. Johnson and Percel (2013) found no alterations in the survival and development of queens reared by nurse bees feeding on pollen contaminated with a fungicide (pyraclostrobin) but they did not test effects on queen-worker interactions. Considering the importance of queen health for the development and survival of honeybee colonies (Winston, 1991), more studies are needed to better understand

queen physiological and behavioral responses to pesticides commonly used in the field.

We found initial colony strength (i.e. the sum of the initial number of adults and brood cells) to be negatively correlated with the change in colony weight in the post-exposure phase ($P = 0.019$). Moreover, initial colony strength increased the number of flights and foraging bees during the exposure phase and the pollen collection in the post exposure phase. These findings suggest that density-dependent mechanisms might have driven colony dynamics (Khoury et al., 2011), with smaller colonies at the beginning of the experiment being able to gain more weight. Available pollen and nectar resources within the enclosures were probably insufficient to properly feed the largest colonies, that did not increase in weight despite the higher foraging activity. However, considering that initial colony strength did not affect how the numbers of brood cells, adult bees and dead bees evolved during the experiment, differences in weight change probably reflect changes in stored resources within the colonies, which buffered pollen and nectar shortages in the enclosures.

Although we found no major impacts of Closer or Amistar on honeybee colonies, we cannot exclude that honeybees exposed to other stressors might respond differently. There is in fact a growing consensus that the increased honeybee colony losses observed in the last decades are driven by the interactive impact of concurrent factors, such as exposure to pesticides, pressure of pests and pathogens, limited resources and poor forage quality (Goulson et al., 2015). For example, poor-quality diets can increase the mortality of honeybees infected with Israeli Acute Paralysis Virus (Dolezal et al., 2019). Pesticides can enhance the impact of common microbial pathogens such the invasive microsporidian *Nosema ceranae*, the Black Queen Cell Virus or the Deformed Wing Virus on honeybee health (De Grandi-Hoffman et al., 2013; Doublet et al., 2015; Fine et al., 2017). Moreover, different pesticides can interact with each other, as was shown for fungicides that are relatively non-toxic by themselves but potentially increase the toxicity of insecticides (Iverson et al., 2019; Sgolastra et al., 2017, 2018; Tosi and Nieh, 2019; Wade et al., 2019). Nevertheless, the majority of the studies investigating interactions among stressors are conducted in highly controlled environments (i.e. laboratory). Our understanding of the effects of multiple stressors on honeybee health under field conditions is still limited and it constitutes an important knowledge gap.

Our results cannot be generalized to other important pollinator groups such as bumblebees and solitary bees. Sensitivity to pesticides varies strongly among bee species with some species exhibiting a much higher sensitivity than honeybees at least when body weight differences are not considered (Arena and Sgolastra, 2014). Honeybees were shown to be better than bumblebees at clearing a neonicotinoid insecticide (Cresswell et al., 2014) and appear to be more resilient than wild bees at the reproductive level (i.e. is the colony level for social bees; Wood et al., 2020). Most notably, a large-scale field experiment in Sweden showed strong effects of a neonicotinoid on solitary bees and bumblebees (Rundlöf et al., 2015; Wintermantel et al., 2018) but not on honeybees (Osterman et al., 2019), even though other studies found no effects of neonicotinoids on wild bees (Thompson et al., 2016; Ruddle et al., 2018). Our results, hence, do not contradict feeding-experiments finding direct effects of sulfoxaflor on bumblebee reproduction (Siviter et al., 2020, 2018) or a meta-analysis showing negative effects of exposure to field-realistic sulfoxaflor doses on bees in general (Siviter and Muth, 2020).

This is one of the first studies to experimentally test the effects of a sulfoximine-based insecticide and a fungicide on honeybees under realistic semi-field conditions. To simulate exposure conditions in the field, we followed label instructions and used commercial formulations rather than only the active ingredients, sulfoxaflor and azoxystrobin, as widely used co-formulants and adjuvants in agrochemicals can be toxic to honeybees (Ciarlo et al., 2012; Zhu et al., 2014; Mullin, 2015). Here, we found no impact of Closer (sulfoxaflor) on the development and activity of honeybee colonies that were exposed to treated plants six days after application. This finding suggests that the period between application and crop bloom was sufficiently long to prevent impacts. A wider implementation of such a safety period may therefore help protecting honeybees. Our semi-field experiment revealed also no marked impacts of Amistar (azoxystrobin) on honeybees, even though there was an indication that the fungicide may affect pollen foraging activity, which deserves attention. The implementation of (semi-)field studies considering longer post-exposure phase and measuring overwintering success might also be useful to better understand the overall impact of these pesticides on honeybees. Finally, more research is needed to explore bee responses to the exposure to multiple pesticides or to pesticides in combination with concurrent stressors such as pathogens or resource limitations that are overwhelmingly ignored in regulatory pesticide risk assessments.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.146084.

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