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No evidence for impaired solitary bee fitness following pre-flowering sulfoxaflor application alone or in combination with a common fungicide in a semi-field experiment

Janine Melanie Schwarz [a,b,*], Anina C. Knauer [a], Matthew J. Allan [c], Robin R. Dean [d], Jaboury Ghazoul [b], Giovanni Tamburini [e,f], Dimitry Wintermantel [e], Alexandra-Maria Klein [e], Matthias Albrecht [a]

[a] Agroscope, Agroecology and Environment, Zurich, Switzerland
[b] ETH Zurich, Institute for Terrestrial Ecosystems, Ecosystem Management, Zurich, Switzerland
[c] Atlantic Pollination Ltd, Eastleigh, United Kingdom
[d] Red Beehive Company, Bishops Waltham, United Kingdom
[e] University of Freiburg, Nature Conservation and Landscape Ecology, Freiburg, Germany
[f] University of Bari, Department of Soil, Plant and Food Sciences (DiSSPA - Entomology), Bari, Italy

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ABSTRACT

Pesticide exposure is considered a major driver of pollinator decline and the use of neonicotinoid insecticides has been restricted by regulatory authorities due to their risks for pollinators. Impacts of new alternative sulfoximine-based compounds on solitary bees and their potential interactive effects with other commonly applied pesticides in agriculture remain unclear. Here, we conducted a highly replicated full-factorial semi-field experiment with the solitary bee *Osmia bicornis*, an important pollinator of crops and wild plants in Europe, and *Phacelia tanacetifolia* as a model crop. We show that spray applications of the insecticide sulfoxaflor (product Closer) and the fungicide azoxystrobin (product Amistar), both alone and combined, had no significant negative impacts on adult female survival or the production, mortality, sex ratio and body size of offspring when sulfoxaflor was applied five days before crop flowering. Our results indicate that for *O. bicornis* (1) the risk of adverse impacts of sulfoxaflor (Closer) on fitness is small when applied at least five days before crop flowering and (2) that azoxystrobin (Amistar) has a low potential of exacerbating sulfoxaflor effects under field-realistic conditions.

1. Introduction

Pesticide exposure is considered a major driver of pollinator decline (IPBES, 2016, Potts et al., 2016, Dicks et al., 2021). Neonicotinoid insecticides, for example, can negatively affect bees (Blacquiere et al., 2012, Hopwood et al., 2016, Lu et al., 2020, Siviter et al., 2021b), and the use of four neonicotinoids in outdoor agricultural settings has been banned in the European Union (European Commission, 2018, 2020). The sulfoximine-based insecticide sulfoxaflor is a potential replacement for neonicotinoids, and its global use is increasing (Simon-Delso et al., 2015). Similarly to neonicotinoids, sulfoxaflor acts systemically in the plant, interacts with the nicotinic acetylcholine receptors in the nervous system of invertebrates (Babcock et al., 2011; Zhu et al., 2011; Cutler et al., 2013; Ulens et al., 2019) and targets sap-feeding insect pests (e.g., aphids, whiteflies). Moreover, sulfoxaflor remains effective against pests resistant to neonicotinoids (Sparks et al., 2013). Sulfoxaflor is classified as posing high risks to bees when applied during flowering (EFSA, 2020), and its use is restricted to non-flowering crop stages in the European Union (Corteva Ireland, 2021; Corteva Italy, 2021). Still, potential negative effects of pre-flowering sulfoxaflor applications on solitary bees under realistic conditions remain unknown. Assessing impacts of this relatively new insecticide is a high-priority research topic, particularly given current threats to pollinators and food production (Brown et al., 2016; IPBES, 2016).

Recent studies suggest sub-lethal effects of field-realistic sulfoxaflor exposure (i.e., exposure levels likely encountered by insects in agriculture) on reproduction, larval development, food consumption and foraging performance in social bumblebees (*Bombus terrestris*) and

* Corresponding author at: Agroscope, Agroecology and Environment, Zurich, Switzerland.
E-mail address: janine.schwarz130790@gmail.com (J.M. Schwarz).

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honeybees (*Apis mellifera*) (Siviter and Muth, 2020; Li et al., 2021; Linguadoca et al., 2021; Tamburini et al., 2021a, but see Tamburini et al., 2021b), while impacts on solitary bees, particularly under (semi-)natural conditions, remain largely unexplored (Boff et al., 2021). Since solitary and social bees differ in their physiologies, e.g., detoxification abilities (Hayward et al., 2019), and life-history traits, their sensitivities and levels of pesticide exposure can differ substantially (Arena and Sgolastra, 2014; Sgolastra et al., 2019). Adverse impacts of pesticides are expected to be more severe for solitary bee populations, because they can directly affect the fitness of reproductive females, whereas colonies of social bees may compensate for the impairment of individual workers (Henry et al., 2015; Rundlöf et al., 2015; Straub et al., 2015; Sgolastra et al., 2019).

Bees in agricultural landscapes are often exposed to multiple pesticides (Sanchez-Bayo and Goka, 2014; Tosi et al., 2018), which can result in detrimental synergistic effects on bee health (Siviter et al., 2021a). Fungicides are commonly and widely used (Zhang, 2018), and while they do not target insects, there is evidence that they may negatively affect bees directly (Artz and Pitts-Singer, 2015; Bernauer et al., 2015; Mao et al., 2017) or indirectly by enhancing the toxicity of insecticides (Iwasa et al., 2004; Johnson et al., 2013; Sgolastra et al., 2018; Carnesecchi et al., 2019). Their potential risks to bees might be underestimated and more studies are urgently needed (Cullen et al., 2019). Especially (semi-)field studies investigating interactive effects between fungicides and insecticides on solitary bees are rare (Lehmann and Camp, 2021).

We conducted a highly replicated semi-field experiment to investigate the effects of field-realistic exposure to the insecticide sulfoxaflor (product Closer) and the widely used fungicide azoxystrobin (product Amistar) alone and combined on survival, reproduction, and offspring development of the solitary bee *Osmia bicornis*, a generalist solitary bee and an important pollinator in Central Europe (Westrich, 2019). Azoxystrobin is a globally used broad-spectrum systemic fungicide (Bartlett et al., 2002) with residues frequently detected in bee-collected pollen and nectar (Mullin et al., 2010; Krupke et al., 2012; Böhme et al., 2018). With a full-factorial design with 40 large flight cages (54 m^2; four treatments with ten cages per treatment: 1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor + azoxystrobin (mix), 4) water control) sown with purple tansy (*Phacelia tanacetifolia*), we tested both the individual and combined effects of the two pesticides during a period of 26 days. Sulfoxaflor was applied five days before purple tansy flowering, as demanded by current mitigation measures in many European countries, while azoxystrobin was applied during flowering according to label guidelines. Sulfoxaflor and azoxystrobin are systemic pesticides, thus they can be taken up by the plant, spread through its tissue and be assimilated also into nectar and pollen (Heller et al., 2020). Moreover, as azoxystrobin is sprayed during crop flowering, it can directly contaminate pollen and nectar. We expected that exposure to sulfoxaflor via pollen and nectar feeding on treated plants negatively affects *O. bicornis* fitness components (i.e., survival of adult females, number of offspring produced per cage, larval survival, sex ratio and body size of offspring), and that adverse effects are amplified by simultaneous exposure to azoxystrobin. Specifically, we hypothesized that sulfoxaflor negatively affects the (1) survival of adult females, (2) number of offspring produced per cage, (3) larval survival, (4) sex ratio and (5) body size of the offspring, and that (6) combined exposure to sulfoxaflor and azoxystrobin leads to more adverse effects on these mentioned fitness components.

2. Materials & methods

2.1. Study system and experimental design

We investigated the impacts of the insecticide sulfoxaflor (product Closer, Corteva Agriscience, purchased from Ipag, Italy) and the fungicide azoxystrobin (product Amistar, Syngenta, purchased from Stähler AG, Switzerland) alone and combined on Red mason bees, *Osmia bicornis* (Hymenoptera: Megachilidae). *Osmia bicornis* is a generalist, univoltine solitary bee species mainly distributed across Europe. It is suited for different experimental set-ups due to its easy handling and established rearing methods (Dietzsch et al., 2015) and has been proposed by the European Food Safety Authority (EFSA) as a model solitary bee species for the risk assessment of plant protection products in Europe (EFSA, 2013). *Osmia bicornis* is a cavity-nesting bee species, whose offspring overwinters as cocooned adults inside the nest (Westrich, 2019). A randomized full factorial semi-field experiment consisting of a total of 40 cages with the two pesticides as crossed factors (four treatments: 1) sulfoxaflor (product Closer), 2) azoxystrobin (product Amistar), 3) mix (Closer + Amistar) and 4) control (no pesticides applied, water only), ten replicates (cages) per treatment; Supplementary Fig. 1) was conducted in June/July 2019. The cages (9 m × 6 m, height: 2 m; steel frame covered with transparent nylon netting of ca. 1.15 (0.95–1.35) mm mesh size; Howitec Netting b.v, Netherlands) were erected on an experimental field (approx. 0.9 ha) sown with purple tansy, *Phacelia tanacetifolia* (variety BALO, untreated seeds, sowing rate 8 kg seeds/ha, sowing date: 21 March 2019) at the experimental field site of Agroscope near Zürich, Switzerland (GPS coordinates: 47.440520, 8.499212). *Phacelia tanacetifolia* is commonly used in pesticide risk assessments on bees (EFSA, 2013) and proposed as a model crop by the non-*Apis* working group of the International Commission for Plant-Pollinator Relationships (ICPPR) for higher-tier ecotoxicological risk assessment using *O. bicornis* (Franke et al., 2021). Cages were spaced 5 m apart from each other and from field margins. Each net had a vertical zipper on one side that allowed access into the cage. Two separate custom-made nesting aids (hereafter nesting units; Atlantic Pollination Ltd.) were installed inside each cage at a height of 1.5 m above ground. A nesting unit consisted of ten individually removable plates each offering ten nesting cavities covered with acetate sheets for *O. bicornis* females to nest and build brood cells (i.e., 100 nesting cavities per nesting unit and 200 cavities per cage). A wooden roof was attached to the nesting units to protect them from rain and excessive sunlight (Supplementary Fig. 2a). This system allowed for daily measurements of offspring production during the experiment (see section 2.3.). All cages and nesting units were oriented in the same direction, with openings facing southeast. Additionally, a small hollow was dug into the ground and regularly filled with water to offer females access to moist mud for the construction of brood cell walls.

Cocoons of *O. bicornis* were provided by a local breeding company, Wildbiene + Partner AG, Switzerland, and kept at 4 °C in a ventilated cooling room before hatching them at room temperature. For hatching, cocoons were placed in brown paper bags with a hole on one side (approx. 2 cm diameter), which were then placed in a transparent ventilated plastic box. To synchronize emergence of males and females (males emerge before females under natural conditions), cocoons considered to contain female bees (>6 mm cocoon diameter) were incubated at room temperature four days and those considered to be males two days before the release of bees in the cages. Emerged adults crawled out of the paper bag into the plastic box and could easily be separated from cocoons. Hatching boxes were checked daily and emerged bees were transferred to a ventilated 4 °C cooling room, where they were directly distributed to 80 jars (two jars per cage, one for females, one for males) covered with fine-meshed fabric. Ten days before the start of the exposure phase of the experiment (day −10, 18 June 2019), 50 *O. bicornis* females and 75 males were introduced into each cage (Supplementary Fig. 3).

The exposure phase lasted for 26 days (days 0–25) until only few female *O. bicornis* were still alive (Fig. 1, number of alive females on day 25, mean ± SE, sulfoxaflor: 1.8 ± 0.8, azoxystrobin: 2.7 ± 0.7, mix: 2 ± 0.4, control: 4.1 ± 0.7), and before *P. tanacetifolia* flowering started to markedly drop (approx. 50% of flower abundance compared to full flowering). Immediately after termination of the exposure phase, nesting units (free of adult bees) were covered with a fine mesh to prevent parasitism or predation of offspring and transported to a sheltered place

close to the institute, where the offspring completed their development. For controlled overwintering, the nesting units were transferred into a cool room (2–4 °C) at the end of November 2019, where they remained until emergence of offspring in May of the following year (see section 2.4.).

2.2. Pesticide applications

Following a proposed suitable test design for semi-field risk assessments using *Osmia* spp. by the ICPPR non-*Apis* working group (Franke et al., 2021), the exposure phase started after mating and initiating of nesting activity of *O. bicornis* females in each cage. To (i) ensure that bees had sufficient floral food resources to initiate nesting, and (ii) strictly follow label guidelines for the application of sulfoxaflor five days or earlier before the start of crop flowering and (iii) be able to assess risks for nesting *O. bicornis* females under a worst-case field-realistic scenario, we divided the area in each cage into two parts (A and B). After the onset of flowering, the *P. tanacetifolia* plants were cut at a height of 70 cm on two thirds of the area in each cage (area A, 36 m^2) using electric grass shears and remaining open flowers were removed by hand. In the remaining third of the area (area B, 18 m^2), the plants continued to flower. After cutting, the bees were introduced into the cages (day −10), where they mated and started nesting until the start of the exposure phase of the experiment (day 0) feeding on flowering plants in area B. During the night of day −5, sulfoxaflor was applied to the previously cut plants, which were not yet flowering (area A), at the highest spraying rate recommended by the manufacturer and in agreement with label instructions (48 g a.i./ha, 0.4 L formulated product Closer/ha). No open flowers were present during spraying on the area where the product was applied. During application, it was ensured that roosting *O. bicornis* were not exposed to the product by trapping them inside their nesting units using a fine mesh, which was additionally covered with a plastic sheet. Furthermore, it was ensured that the flowering third of the plants (area B) was strictly prevented from exposure to the product by carefully covering it with plastic sheets during spray application. During the night before azoxystrobin application (day −1), when bees were roosting inside nesting units, the one third of the plants that provided floral food resources until the start exposure phase (area B) was cut at ground level and the cut plants removed from the cages. In the morning of day 0 (start of exposure phase), shortly after onset of bee flight, azoxystrobin was applied to the remaining plants (area A) by spray application at a rate of 250 g a.i./ha (1 L formulated product Amistar/ha) at the start of the flowering period (approx. 10% of flower abundance compared to full flowering) in agreement with label instructions. Both sulfoxaflor and azoxystrobin applications were conducted by Innovative environmental services ltd., Switzerland, in accordance with good experimental practice standards. For spraying, a motorized sprayer equipped with a 3 m long bar with anti-drift spraying nozzles was used. Applications were conducted in good weather conditions with no wind.

2.3. Adult survival, reproduction and offspring mortality

The number of adult females roosting inside the nesting units during the night (after 9:30 pm) was counted as an estimate of the number of females alive inside each cage. We rarely observed females spending the night outside the nesting units. Counts were performed the nights before conducting product applications and every second night after the application of azoxystrobin (day 0) during the first 14 days of the exposure phase. Afterwards, counts were performed at least every fourth night until the end of the experiment (day 25). In total, the number of adult females in each cage was counted once before and at 11 time points during the exposure phase (Supplementary Fig. 3).

The progress of brood cell production was monitored by taking pictures of each nesting layer daily after 4 pm the night before and during the first 11 days of the exposure phase (days −1 to 10, Supplementary Fig. 2b, 3). A brood cell consists of a pollen provision with an egg laid on top, which is sealed with a mud wall. The construction date of each brood cell (i.e. the date when the egg was laid and the cell was sealed) was assessed by visual assessments of pictures after the experiment. Additionally, the status of each brood cell was assessed during overwintering of offspring by visual inspection.

2.4. Offspring size and sex ratio

In the following spring (May 2020), the completed cocoons were hatched separately according to the date they were produced (egg laying date) during days 0–10. Although most offspring reached the cocoon stage, only approx. 10% of the offspring also successfully hatched from the cocoons in the next spring. We therefore refrained from analysing the emergence rate as an endpoint. Furthermore, due to time and workload constraints, we focused on the first days of the exposure phase. Days 0–5 were considered in the analyses assessing effects on offspring body size and sex ratio, as we expected effects to be most pronounced during these first days of the exposure period. Cocoon size was assessed as a proxy of offspring size as both measures are strongly correlated in *Osmia* (Bosch and Vicens, 2002). Length and width of each cocoon produced during days 0–5 of the experiment were measured using a digital caliper and cocoon volume was approximated using the formula for a spheroid. Only un-emerged offspring could be measured. Additionally, cocoons were opened and the sex of all offspring produced during this period was assessed using binoculars. At the pupal stage, females and males can be distinguished by the length of their antennae (longer in males) and characteristic bumps on the faces of females.

2.5. Flower abundance

The abundance of open *P. tanacetifolia* flowers was estimated at least every third day after the start of the experiment until day 14, and at least every fourth day from day 15 until the end of the experiment (day 25) resulting in a total of ten assessments in each cage (Supplementary Fig. 3). The number of individual open *P. tanacetifolia* flowers was estimated in four randomly chosen plots of 1 m^2 in each cage until day 10, and in two randomly chosen plots after day 10. The estimated number of open flowers per m^2 in each cage was calculated as the mean of the individually assessed squares.

2.6. Pesticide residues in bee-collected pollen

Samples for residue analysis of applied products were collected with a spatula from pollen-nectar provisions of two brood cells per cage built during the first four days of the exposure phase (samples were taken on day 3 from brood cells built 0–3 days after azoxystrobin application, corresponding to 5–8 days after sulfoxaflor application) (Supplementary Fig. 3). Only completed brood cells were selected to avoid disturbing nesting females and to minimize the number of brood cell provisions required for residue analysis. Samples from all cages per treatment were pooled and stored at −20 °C. The multi-residue analysis was performed at CREA-AA, Bologna according to standard protocols using QuEChERS solid-phase extraction and liquid chromatography-mass spectrometry (LC-MS/MS) (Tosi et al., 2018). Brood cells from which pollen samples were taken, were excluded from statistical analyses, unless a female provisioned fresh pollen and laid a new egg.

2.7. Statistical analysis

All statistical analyses were conducted in R version 4.1.2 (R Development Core Team, 2021). To directly test for interactive effects of the two pesticides, treatment effects were assessed via the two-level factors sulfoxaflor (sulfoxaflor, product Closer applied (+sulfoxaflor) or not applied (-sulfoxaflor)) and azoxystrobin (azoxystrobin, product Amistar applied (+azoxystrobin) or not applied (-azoxystrobin)) and their interaction term. Results of analyses using a 4-level factor treatment

(levels: sulfoxaflor (product Closer), azoxystrobin (product Amistar), mix (Closer + Amistar), water control) as main fixed effect and Tukey's HSD post-hoc tests (package emmeans (Lenth, 2021)) to test for differences between individual treatment levels yielded qualitatively similar results (Supplementary Tables 1-3).

Survival of *O. bicornis* females during the exposure phase (days 0–25) was visualized with the package *survminer* (Kassambara et al., 2021) and analysed with a mixed-effects cox proportional hazards model including the factors azoxystrobin, sulfoxaflor and their interaction as fixed explanatory variables and cage ID as random factor with the package *coxme* (Therneau, 2020). A total of 1,785 females were included in the survival analysis (control: 450, sulfoxaflor: 438, azoxystrobin: 455, mix: 442).

To analyse the impacts of azoxystrobin, sulfoxaflor and their interaction on the total number of offspring produced during the exposure phase per cage (i.e., total number of brood cells built during days 0–25) and total number of offspring successfully reaching the cocoon stage per cage (40 cages in total, 10 per treatment), linear models (LMs) including sulfoxaflor, azoxystrobin and their interaction as explanatory variables were fitted. The mean flower abundance per m^2 over the whole exposure phase was included as additional co-variate. During the exposure phase, a total of 11,595 offspring (brood cells) were produced (control: 2,811, sulfoxaflor: 2,844, azoxystrobin: 2,957, mix: 2,983) and a total of 7,575 thereof reached the cocoon stage (control: 1,896, sulfoxaflor: 1,755, azoxystrobin: 1,945, mix: 1,979). To further analyse how the number of brood cells produced per day in each cage changed with time after exposure (days 0–10, period for which daily assessments of produced brood cells are available, see methods section 2.3.) a linear mixed-effects model (LMM) including the additional co-variate day (continuous), its interactions with azoxystrobin and sulfoxaflor, the flower abundance per m^2 and the random factor cage ID was fitted using the package *lme4* (Bates et al., 2015). For this model, the flower abundance per m^2 was interpolated for days on which it was not assessed in the cages using the package zoo (Zeileis and Grothendieck, 2005). Because flower abundance was strongly correlated with day (Pearson's correlation = 0.84), we scaled the flower abundance within each day (function scale_by in the package *standardize* (Eager, 2017)) to account for variability between cages. The response variable (offspring per day) was square root transformed to obtain normally distributed residuals. A third order polynomial term was added for day (function poly) to improve model fit. A total of 8,653 offspring (brood cells) were produced during days 0–10 of the exposure phase (control: 2,031, sulfoxaflor: 2,171, azoxystrobin: 2,255, mix: 2,196). For each day (days 0–10), the number of offspring produced was calculated for each cage resulting in 110 data points per treatment condition and 440 data points in total.

Developing offspring mortality (i.e., the proportion of offspring produced per cage and day that died as egg or larva before reaching the cocoon stage) and its temporal change between days 0–10 (period for which daily assessments were made, see section 2.3.) was analysed using a generalized linear mixed-effects model (GLMM) with binomial error distribution using sulfoxaflor, azoxystrobin and egg laying day (continuous) as well as their interactions and the additional co-variate flower abundance per m^2 (interpolated, scaled) as explanatory variables. Cage ID was included as random factor. For each egg laying day (days 0–10), the proportion of dead offspring was calculated for each cage resulting in 110 data points (proportions) per treatment condition and 440 data points in total. Analyses on number of offspring produced and offspring mortality were performed on total numbers per cage, not per individual female.

Differences in offspring cocoon volumes were analysed separately for female and male offspring produced during the first six days of the exposure phase (days 0–5, period for which data were available, see section 2.4.) using LMMs containing sulfoxaflor and azoxystrobin as well as their interaction as explanatory variables and cage ID as random factor. The mean flower abundance per m^2 over days 0–5 was included as additional co-variate. A total of 414 un-emerged females (control: 99, sulfoxaflor: 98, azoxystrobin: 109, mix: 108) and 1,639 un-emerged males (control: 317, sulfoxaflor: 401, azoxystrobin: 500, mix: 421) were included in the cocoon volume analysis. The overall sex ratio of offspring (i.e., the proportion of females) produced during the first six days of the exposure phase (days 0–5, period for which data were available, see section 2.4.) in each cage was analysed with a quasi-binomial generalized linear model (GLM) fitted with sulfoxaflor and azoxystrobin, their interaction as well as the mean flower abundance per m^2 over days 0–5 as fixed effects. A quasi-binomial GLM instead of a binomial GLM was used to account for overdispersion of the data. A total of 2,236 emerged or un-emerged offspring were included in the sex ratio analysis (control: 433, sulfoxaflor: 566, azoxystrobin: 663, mix: 574). One cage (control) was excluded from the sex ratio and cocoon volume analyses, because data were missing for offspring produced on day 5.

Likelihood ratio tests were used for statistical inference of (G)LMMs (Zuur et al., 2009; Luke, 2017). Results were qualitatively similar when using the Kenward-Roger approximations for LMMs (package lmerTest (Kunzetsova et al., 2017)). Model assumptions of homoscedasticity and normality were checked visually and overdispersion was quantitatively assessed for GLM(M)s (Zuur et al., 2009).

3. Results

Contrary to our expectation, we found no significant negative effects of the single and combined exposure to sulfoxaflor and axoystrobin on *O. bicornis* survival, reproduction, offspring mortality, size and sex ratio in our semi-field experiment.

3.1. Adult survival, reproduction and offspring mortality

Adult female survival and reproduction was monitored during the experiment by counting the number of females roosting inside the nests at night and taking pictures of the nesting progress. Explorative analyses showed that the initial number of *O. bicornis* females in cages at the beginning of the exposure phase as well as the flower abundance during the experiment did not significantly differ among treatments (Supplementary Tables 4 and 5). The number of females inside the cages on day −1 was as follows (mean ± SE): sulfoxaflor: 43.8 ± 0.8, azoxystrobin: 45.5 ± 1.5, mix: 44.2 ± 1.3, control: 45 ± 1.2). The survival probability of adult female *O. bicornis* during the exposure phase (days 0–25) was not significantly affected by sulfoxaflor, azoxystrobin or their combination (Fig. 1; Table 1). The pesticides did not affect the total number of offspring produced during days 0–25 (Fig. 2a, b; Table 2) or reduce the number of offspring produced per day during the days 0–10 (Fig. 2c, Table 2, Supplementary Fig. 4). We found, however, a significant interaction between the two pesticides and time (day) during the course of the experiment. Nevertheless, no overall synergistic or antagonistic interaction between the two pesticides was found (Fig. 2c, Table 2, Supplementary Fig. 4).

The offspring in the nests was visually inspected during development and its laying date was assessed using the nesting progress pictures. The proportion of offspring produced during days 0–10 of the exposure phase that died as egg or larva before reaching the cocoon stage was not significantly affected by exposure to sulfoxaflor, azoxystrobin, or their combination (Fig. 2d; Table 2). Irrespective of the pesticide treatment, offspring mortality decreased during this period (Supplementary Fig. 5).

3.2. Offspring size and sex ratio

After overwintering, the size and sex of the offspring were assessed. The size of female and male offspring was not significantly affected by the pesticide treatments (Fig. 3a, b; Table 3). The proportion of female offspring produced during days 0–5 of the exposure phase was not significantly affected by sulfoxaflor, azoxystrobin or their combination, although there was a trend for an antagonistic interactive effect of the two pesticides (Fig. 4; Table 3).

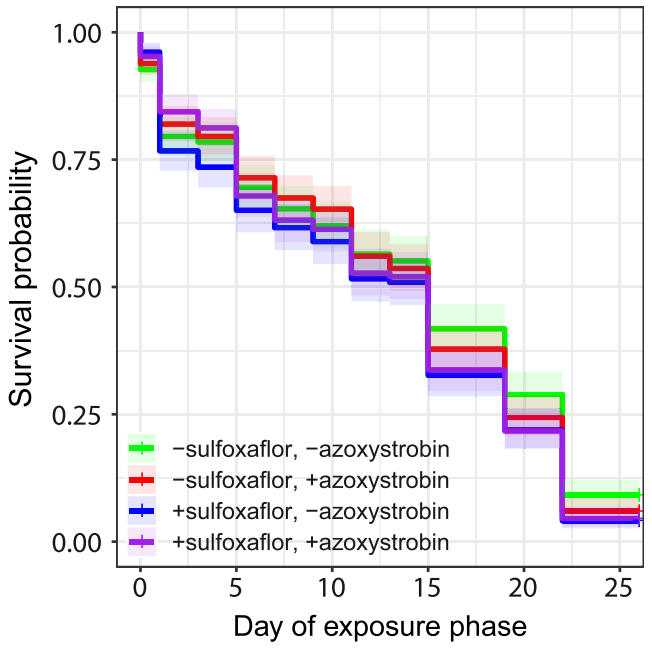

Fig. 1. Survival of adult *O. bicornis* females during the exposure phase. Kaplan-Meier survival curves are shown for each treatment level over the entire exposure phase of the experiment (day 0–25; *green*: control, *blue*: sulfoxaflor (product Closer) *red*: azoxystrobin (product Amistar), *purple*: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

Table 1
Survival of adult female *O. bicornis*. Results of the mixed-effects cox proportional hazard model of adult female *O. bicornis* survival during the whole exposure phase (days 0–25) of the semi-field experiment to test for the impact of sulfoxaflor (product Closer applied or not), azoxystrobin (product Amistar applied or not) as well as their interactive effect. Cage ID was included as random factor.

Survival of adult female *O. bicornis* during the exposure phase					
Coefficients	**Hazard ratio**	**Lower 95% CI**	**Upper 95% CI**	***Z***	***P*-value**
sulfoxaflor	1.31	0.93	1.86	1.58	0.11
azoxystrobin	1.13	0.80	1.60	0.73	0.47
sulfoxaflor×azoxystrobin	0.86	0.53	1.40	−0.63	0.53

3.3. Pesticide residues in bee-collected pollen

Pesticide residues were analysed in pollen-nectar provisions produced at the beginning of the exposure phase, 5–8 days after sulfoxaflor application and 0–3 days after azoxystrobin application. Sulfoxaflor residues were 74 ppb and 129 ppb in the single (sulfoxaflor) and combined (sulfoxaflor + azoxystrobin) treatment respectively, while azoxystrobin residues were 3811 ppb and 4307 ppb for the single (azoxystrobin) and combined treatment. No residues of sulfoxaflor or azoxystrobin were detected in the control treatment (<LOQ).

4. Discussion

To our knowledge, the present study is the first investigating impacts of the novel insecticide sulfoxaflor (product Closer) and the widely used fungicide azoxystrobin (Amistar) alone and combined on a solitary bee species under semi-field conditions. Sulfoxaflor applied in accordance with a mitigation measure restricting its application to five days before crop flowering, azoxystrobin or their combination had no major lethal or sub-lethal effects on *O. bicornis* survival and reproduction.

Field-realistic sulfoxaflor exposure through plants treated five days before flowering did not negatively affect adult female survival or induce sub-lethal effects on *O. bicornis* reproduction comparable to the ones observed in (semi-)field studies on neonicotinoid insecticides, such as negative impacts of clothianidin and thiamethoxam on nesting activity, reproduction, larval development and proportion of female offspring in *Osmia* spp. (Rundlöf et al., 2015; Stuligross and Williams, 2020; Klaus et al., 2021). In fact, a recent meta-analysis concludes that neonicotinoids can impair the reproductive output of non-*Apis* bees, and specifically *Osmia* spp., at field-realistic exposure levels (Siviter et al., 2021b). Our results suggest that sulfoxaflor, despite its similar mode of action to neonicotinoids, might be less harmful to *O. bicornis* provided that it is applied at least five days before crop flowering. Our findings obtained under semi-field conditions are also in agreement with a recent laboratory study showing that acute exposure of bees to sulfoxaflor is less toxic than to the neonicotinoids clothianidin, thiamethoxam and imidacloprid (Azpiazu et al., 2021). Moreover, sulfoxaflor, as opposed to neonicotinoids, is considered to degrade relatively quickly in the environment with observed approximate half-life times of 2–3 days (EPA, 2019).

Other studies, mostly conducted in the laboratory, observed negative impacts of sulfoxaflor on reproduction, larval development, food consumption and foraging behaviour in honeybees and bumblebees (Siviter et al., 2018; Siviter et al., 2020a, Siviter et al., 2020b, Linguadoca et al., 2021; Tamburini et al., 2021a). Recently, negative effects on survival, foraging and flying behaviour have also been reported for *O. bicornis* after field-realistic exposure of sulfoxaflor in the laboratory (Boff et al., 2021). In fact, *O. bicornis* has been found to be even more susceptible to acute sulfoxaflor exposure compared to honeybees and bumblebees according to LD_{50} values (Azpiazu et al., 2021). We therefore expected to find stronger negative impacts on *O. bicornis* in our semi-field study, also as residue analysis of pollen provisions sampled 5–8 days after sulfoxaflor application revealed relatively high levels of 74 and 129 ppb in the sulfoxaflor and the combined treatment, respectively. The laboratory study by Boff and colleagues (Boff et al., 2021) found negative consequences on *O. bicornis* survival at 50 ppb sulfoxaflor in the administered sugar water, while in other studies honeybee and bumblebee survival was affected only at higher doses (Siviter et al., 2020b, Al Naggar and Paxton, 2021; Li et al., 2021), suggesting that *O. bicornis* is more susceptible to sulfoxaflor (Azpiazu et al., 2021). Nonetheless, sulfoxaflor residues in nectar, the main energy supply for adult females, can be up to 10-fold lower than in pollen (EPA, 2016) and while bees were repeatedly exposed to the same dose in the study of Boff et al. (2021), residue levels after single application of sulfoxaflor dropped in the course of our semi-field study. Our semi-field study provides substantially more insights into impacts of fields-realistic exposure of sulfoxaflor and azoxystrobin on bees than standard higher-tier risk assessments, for example by studying not only single but also combined impacts of the two pesticides or by assessing population level impacts of a number of key components of solitary bee fitness. In real intensively managed agricultural landscapes, however, bees might be exposed to further pesticides, or in particular cases, even repeatedly to the same products, which would likely increase exposure levels. This might happen even if only one application of the product in a crop is recommended (as for Closer containing sulfoxaflor), for example, if multiple crop fields located in close proximity might be sprayed on different days during the activity period of bees. Although challenging, such worst-case scenarios should be further investigated in field studies. It is also conceivable that other factors such as high quality and quantity of the offered food resources and less stressful and more natural conditions for *O. bicornis* in our semi-field experiment could have positively affect the bees' ability to cope with sulfoxaflor exposure (see below).

We did not find evidence for reduced size or altered sex ratio of produced offspring, which suggests that the pre-flowering sulfoxaflor application did neither impair the efficiency of females to collect pollen and provision their nests nor affect the development of the offspring

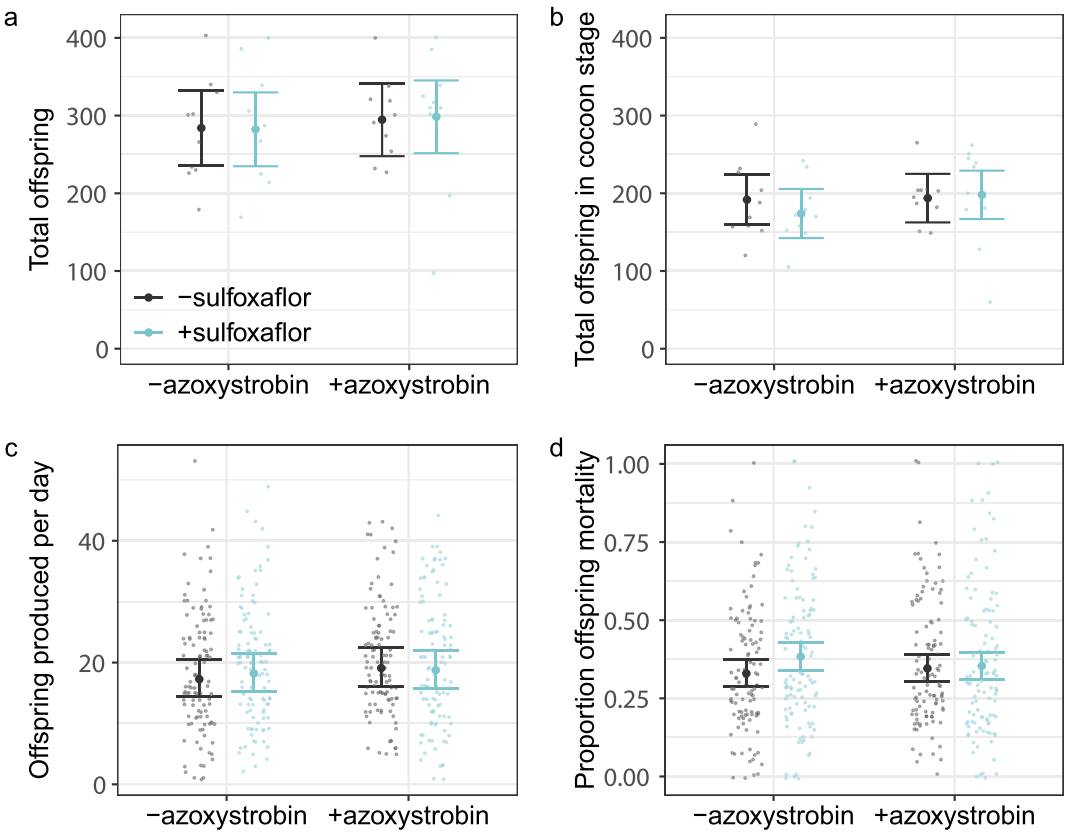

Fig. 2. Reproduction and offspring mortality. (a) Total number of offspring (brood cells) produced per cage during the exposure phase of the experiment (days 0–25), **(b)** total number of offspring produced per cage during the exposure phase (days 0–25) that successfully reached the cocoon stage, **(c)** number of offspring produced per day and cage during days 0–10 of the exposure phase, **(d)** proportions of daily offspring mortality per cage during days 0–10 of the exposure phase (referring to the day the egg was laid). Black bars/dots: no sulfoxaflor (product Closer) applied, blue bars/dots: sulfoxaflor applied. Bars depict model predictions and 95% confidence intervals, dots show the raw data points.

directly. Offspring body size is strongly positively correlated with the amount of pollen provisioned by mothers (Bosch and Vicens, 2002; Radmacher and Strohm, 2010), and it is influenced also by their physical condition (Bosch, 2008; Seidelmann et al., 2010). Impaired foraging efficiency after insecticide exposure may further result in a male biased offspring sex ratio (Sandrock et al., 2014; Stuligross and Williams, 2020), as stressed females might shift their production to less costly males (Bosch, 2008). We did not find such effects. Our results are in line with studies reporting no direct negative effects of field-realistic exposure of neonicotinoid insecticides on larval development in *Osmia* spp. (Abbott et al., 2008; Sandrock et al., 2014; Nicholls et al., 2017; Claus et al., 2021). This suggests that *O. bicornis* offspring appears to be relatively tolerant to field-realistic exposure of neonicotinoid and sulfoximine compounds. In our experiment, in addition to sex ratio and size, we could only assess impacts on offspring mortality during larval development (egg until cocoon spinning stage), but we refrained from analysing offspring hatching rates due to the low percentage (approximately 10%) of hatched offspring. We can therefore not rule out potential single or combined impacts of sulfoxaflor and azoxystrobin on hatching success. Opening of the cocoons revealed that the highest proportion of offspring died at the pupal stage (74.8%), while the remaining part completed their metamorphosis and reached the adult stage (23.1%) and only few died already as cocooned larvae (2.1%). Among the most likely reason for this mortality of offspring, irrespective of treatments, are the often high temperature bees were exposed to, in particular in and right before the cocoon stage in summer (Supplementary Fig. 6). This was a consequence of the relative late timing of the experiment (June/July) compared to the typical activity period of *O. bicornis* in the study region (April - June; Westrich, 2019). Theoretically, also the some weeks longer than typical overwintering period of the *O. bicornis* bees used for the experiment could have affected bees (Bosch and Kemp, 2003). However, as the remaining measured endpoints such as adult female survival, offspring production etc. were in the normal and expected range for semi-field studies with caged *O. bicornis* (Franke et al., 2021), we have no evidence that this factor played a significant role. Even if this would have been the case, bees should have been affected identically across treatments, or alternatively, such a worst-case scenario of long overwintering periods might have acted as an additional stressor, which could have reinforced negative impacts of pesticides. However, our findings do not support such a hypothesis. In general, future studies on pesticide effects on solitary bees would greatly benefit from assessing also potential carryover effects on offspring fitness (Stuligross and Williams, 2021).

The high quantity and quality of readily accessible floral resources in our semi-field experiment with purple tansy might have been an additional factor preventing negative effects of sulfoxaflor on adults and offspring. High quality food such as purple tansy might improve the ability of bees to detoxify xenobiotic compounds (Schmehl et al., 2014; Klaus et al., 2021). If bees are exposed to further stressors, such as nutritional stress, impacts of pesticides such as sulfoxaflor might be augmented (Linguadoca et al., 2021). Further semi-field and field studies would be desired to better explore impacts of potential interactions of sulfoxaflor and azoxystrobin with additional stressors on solitary bee populations. Finally, differences in the findings of our study compared to a recent semi-field study on bumblebees, which found an impaired foraging efficiency and lowered colony growth after sulfoxaflor application, might be partly explained by the shorter time gap of only two days between sulfoxaflor application and exposure of bees in

Table 2
Reproduction and offspring mortality. Results of linear models (LMs) and (generalized) linear mixed-effects models ((G)LMMs) testing for the impact of sulfoxaflor (product Closer applied or not), azoxystrobin (product Amistar applied or not) as well as their interactive effect on offspring production and mortality. Flower abundance per m^2 was included as co-variate. LMs were used to test for differences in total numbers of offspring produced per cage during the entire exposure phase of the experiment and offspring that reached the cocoon stage. An LMM including the additional continuous variable day of exposure phase (day) was used to analyse differences in the numbers of offspring produced per day (square root transformed to obtain normal distribution of residuals). A third order polynomial term was added for day to improve model fit (function poly). Offspring mortality (i.e. the proportion of offspring that died as egg or larva) was analysed with a binomial GLMM and included egg laying day as co-variate. The (G)LMMs included cage ID as random factor. Likelihood ratio tests were used for statistical inference of (G)LMMs. Significant effects ($p \leq 0.05$) are indicated in bold.

Reproduction and offspring mortality			
Variables	**Sum of squares**	***F*-value**	***P*-value**
Total number of offspring produced (days 0–25)			
sulfoxaflor	13	$F_{1,35}$=0.00	0.962
azoxystrobin	1802	$F_{1,35}$=0.34	0.564
mean flower abundance	1366	$F_{1,35}$=0.26	0.615
sulfoxaflor×azoxystrobin	70	$F_{1,35}$=0.01	0.909
Total number of offspring reaching cocoon stage (days 0–25)			
sulfoxaflor	389	$F_{1,35}$=0.17	0.687
azoxystrobin	1741	$F_{1,35}$=0.74	0.395
mean flower abundance	740	$F_{1,35}$=0.31	0.578
sulfoxaflor×azoxystrobin	1135	$F_{1,35}$=0.48	0.492
	λ_{LR}	**df**	***P*-value**
Number of offspring produced per day (days 0–10)			
sulfoxaflor	0.05	1	0.831
azoxystrobin	0.65	1	0.419
day+day^2+day^3	**218.93**	**3**	**<0.001**
flower abundance	**7.05**	**1**	**0.008**
sulfoxaflor×azoxystrobin	0.24	1	0.626
sulfoxaflor×(day+day^2+day^3)	2.15	3	0.541
azoxystrobin×(day+day^2+day^3)	3.36	3	0.340
sulfoxaflor×azoxystrobin×(day+day^2+day^3)	**14.54**	**3**	**0.002**
Offspring mortality (days 0–10)			
sulfoxaflor	1.81	1	0.178
azoxystrobin	0.10	1	0.758
egg laying day	**606.27**	**1**	**<0.001**
flower abundance	**5.15**	**1**	**0.023**
sulfoxaflor×azoxystrobin	1.02	1	0.313
sulfoxaflor×egg laying day	0.37	1	0.543
azoxystrobin×egg laying day	0.73	1	0.392
sulfoxaflor×azoxystrobin×egg laying day	0.00	1	0.948

the latter study (Tamburini et al., 2021a), differences in the level of food limitation or further stressors, or different exposure pathways (e.g., nectar or pollen) and sensitivities of the two bee species under (semi-) field conditions. Additionally, *O. bicornis* and bumblebees vary greatly in their life history traits, and could therefore be differentially affected by sulfoxaflor (Brittain and Potts, 2011). For example, *Osmia* larvae need more than a week to hatch after the eggs are laid on the pollen provisions, and the subsequent consumption of the pollen takes approximately one month (Bosch and Kemp, 2000). This time span may allow for sulfoxaflor residues to drop substantially. In bumblebees, on the other hand, larvae hatch and develop faster (Goulson, 2003), which could result in higher exposure levels to sulfoxaflor.

Our results suggest that restricting the application of sulfoxaflor containing products to at least five days before crop flowering could help to reduce negative effects on bees. While in-flowering applications of sulfoxaflor induced acute lethal effects in honeybees (Cheng et al., 2018), no negative effects were found after a pre-flowering application (Tamburini et al., 2021b). Such a mitigation measure might not be sufficient for other bee species, and compliance with a 5-day rule necessitates that the timing of flowering onset is correctly estimated in the field. Shorter time gaps between application and flowering could lead to a higher sulfoxaflor exposure and induce harmful effects on bees. Bumblebees suffered already from a sulfoxaflor application conducted two days before flowering and showed a reduced colony growth and impaired foraging performance (Tamburini et al., 2021a). Nevertheless, the application of sulfoxaflor products into flowering is currently allowed in many countries and mitigation measures often only aim at avoiding direct contact exposure to wet spray droplets (Corteva Australia, 2021; Corteva Canada, 2021; Corteva New Zealand, 2021; Corteva South Africa, 2021). In the European Union, sulfoxaflor applications are only permitted before crop flowering, and many European countries have adopted more stringent measures by restricting applications to five or six days before flowering onset (Corteva Bulgaria, 2021; Corteva Croatia, 2021; Corteva Ireland, 2021; Corteva Italy, 2021; Corteva Spain, 2021). In the United States, sulfoxaflor can be applied up to three days before flowering, although this mitigation measure is not implemented in all bee-attractive crops (EPA, 2019; Corteva US, 2021). Our findings suggest that a safety period of at least five days between application and flowering should be respected for sulfoxaflor containing products globally, to reduce risks for bees and other flower visiting insects. Nonetheless, further studies including other bee species and crop plants are needed to test this mitigation measure for a range of pollinator species and crop systems.

We found no adverse effects of field-realistic azoxystrobin (product Amistar) exposure on survival and reproduction in *O. bicornis*, which is in agreement with previous studies on honeybees (Fisher et al., 2017; Tamburini et al., 2021b) and bumblebees (Tamburini et al., 2021a). Nevertheless, there is also evidence of possible negative effects of this product on foraging behaviour of bumblebees and consequences on pollination services (Tamburini et al., 2021a). It remains unclear whether the observed adverse effects are caused by azoxystrobin itself or a co-formulant, as such a co-formulant in Amistar has recently been identified to cause lethal and sub-lethal effects in bumblebees (Straw and Brown, 2021). This underpins the importance of not only testing the effects of active ingredients on pollinators, but also commercial formulations of plant protection products (Ciarlo et al., 2012; Zhu et al., 2014; Mullin, 2015). Moreover, azoxystrobin has been shown to induce alterations in the expression of hormone-system-related genes in honeybees at sub-lethal concentrations (Christen et al., 2019) and the related fungicide picoxystrobin can induce cell death in the midgut of Africanized honeybees (Batista et al., 2020). Such effects might become critical if bees are exposed to multiple pesticides simultaneously, which is frequently the case in intensively managed agroecosystems (Sanchez-Bayo and Goka, 2014; Tosi et al., 2018), potentially leading to negative synergistic effects (Siviter et al., 2021a). Recently, a study demonstrated synergistic effects of sulfoxaflor and the fungicide fluxapyroxad (a succinate dehydrogenase inhibitor, i.e., blocking mitochondrial respiration) in *O. bicornis* and *A. mellifera* under laboratory conditions (Azpiazu et al., 2021). Azoxystrobin, similarly, targets mitochondrial respiration (Fernández-Ortuño et al., 2010), offering a potential pathway for synergistic interactions with sulfoxaflor. We did not find significant interactive effects of sulfoxaflor and azoxystrobin on our studied endpoints, and only a trend for an antagonistic interaction on the proportion of female offspring, which deserves further attention. Our results suggest that there is a low potential of azoxystrobin to modulate the effects of sulfoxaflor in a negative synergistic manner. Nevertheless, more studies on interactions between the novel sulfoxaflor and other commonly applied pesticides are needed to better understand potential risks for pollinating insects.

In conclusion, no major negative impacts of sulfoxaflor (product Closer), azoxystrobin (product Amistar) or their combination on

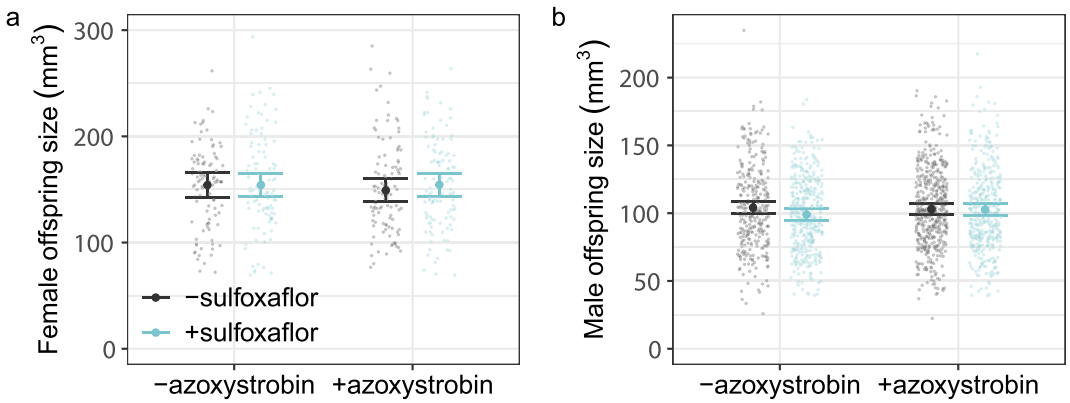

Fig. 3. Offspring size. Cocoon size of female **(a)** and male **(b)** offspring produced during days 0–5 of the exposure phase. Black bars/dots: no sulfoxaflor (product Closer) applied, blue bars/dots: sulfoxaflor applied. Bars depict model predictions and 95% confidence intervals, dots show the raw data points.

Table 3
Offspring size and sex ratio. Results of linear mixed effects models (LMMs) and a generalized linear model (GLM) on offspring size (cocoon volume) and sex ratio (proportion of female offspring) for testing the impact of sulfoxaflor (product Closer applied or not), azoxystrobin (product Amistar applied or not) as well as their interactive effect. Flower abundance per m^2 was included as covariate. The cocoon volumes of un-emerged female and male offspring were separately analysed with LMMs. Cage ID was included as random factor in the LMMs and likelihood ratio tests were used for statistical inference. A quasi-binomial GLM was used to analyse differences in the proportions of female offspring produced.

Offspring size and sex ratio			
Variables	**λ_{LR}**	**df**	***P*-value**
Cocoon volume of female offspring (days 0–5)			
sulfoxaflor	0.34	1	0.557
azoxystrobin	0.20	1	0.656
mean flower abundance	1.51	1	0.219
sulfoxaflor×azoxystrobin	0.22	1	0.637
Cocoon volume of male offspring (days 0–5)			
sulfoxaflor	1.70	1	0.192
azoxystrobin	0.51	1	0.475
mean flower abundance	**4.19**	**1**	**0.041**
sulfoxaflor×azoxystrobin	1.46	1	0.227
	Sum of squares	***F*-value**	***P*-value**
Overall offspring sex ratio (days 0–5)			
sulfoxaflor	0.18	$F_{1,34}$=0.15	0.706
azoxystrobin	1.47	$F_{1,34}$=1.19	0.284
mean flower abundance	0.06	$F_{1,34}$=0.05	0.832
sulfoxaflor×azoxystrobin	4.05	$F_{1,34}$=3.28	0.079

survival or reproductive success of the solitary bee *O. bicornis* were detected in this semi-field study. We were, however, not able to analyse offspring hatching rates, which remains to be explored in future studies. Our results do not contradict previous studies that found negative effects of exposure to field-realistic doses of sulfoxaflor on bees, but rather suggest that pre-flowering applications help to minimize adverse effects on *O. bicornis*. A wider implementation of such a safety period between application and crop flowering could therefore help to reduce exposure and thereby to protect pollinators from negative impacts of sulfoxaflor containing products. Further (semi-)field studies are required to confirm the generality of our findings also for other bee and non-bee pollinator taxa, and to identify most effective mitigation measures. We suggest that sulfoxaflor (Closer) should not have major adverse impacts on *O. bicornis* when applied at least five days before crop flowering alone or in combination with azoxystrobin (Amistar). We caution against generalising our results to other bee species, as some taxa might have higher

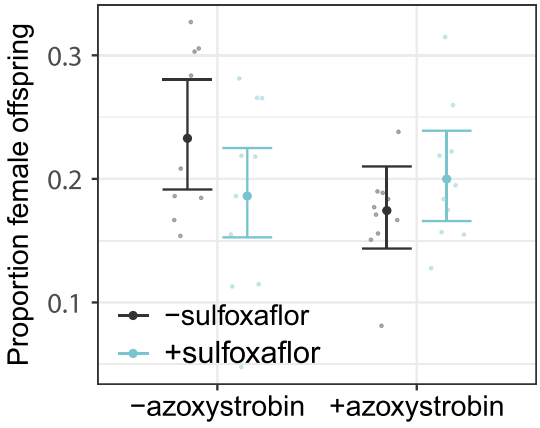

Fig. 4. Offspring sex ratio. Proportion of female offspring produced during days 0–5 of the exposure phase. Black bars/dots: no sulfoxaflor (product Closer) applied, blue bars/dots: sulfoxaflor applied. Bars depict model predictions and 95% confidence intervals, dots show the raw data points.

sensitivities to sulfoxaflor. Finally, more research is needed to better understand potential impacts of sulfoximine insecticides, including also interactions with other pesticides or other types of stressors such as pathogens or food stress, which are currently ignored in regulatory pesticide risk assessment procedures.

CRediT authorship contribution statement

Janine Melanie Schwarz: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Visualization. **Anina C. Knauer:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Visualization. **Matthew J. Allan:** Conceptualization, Methodology, Resources. **Robin R. Dean:** Conceptualization, Methodology, Resources. **Jaboury Ghazoul:** Writing – review & editing. **Giovanni Tamburini:** Conceptualization, Methodology, Writing – review & editing. **Dimitry Wintermantel:** Writing – review & editing. **Alexandra-Maria Klein:** Conceptualization, Methodology, Writing – review & editing, Project administration. **Matthias Albrecht:** Conceptualization, Methodology, Writing – review & editing, Visualization, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

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

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Data Availability

The data used in this study are available at https://doi.org/10.5061/dryad.1jwstqjx5.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107252.

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