Scientific Reports

https://doi.org/10.1038/s41598-026-55827-9

Article in Press

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Received: 13 March 2026

Accepted: 27 May 2026

Published online: 03 June 2026

Cite this article as: Schwarz J.M., Arnet N.L., Knauer A.C. *et al.* Sulfoxaflor reduces food intake and learning efficiency of solitary *Osmia bicornis* bees. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-55827-9

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Sulfoxaflor reduces food intake and learning efficiency of solitary *Osmia bicornis* bees

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Abstract

Bees in agroecosystems can be exposed to multiple pesticides, such as insecticides and fungicides, but potential sub-lethal consequences on fitness-relevant endpoints like food intake, foraging performance and learning efficiency remain poorly understood. The insecticide sulfoxaflor is used as alternative to neonicotinoid insecticides, however, its impacts alone or in combination with other pesticides on foraging and learning of solitary bees are largely unknown. We conducted experiments with solitary *Osmia bicornis* bees to investigate the effects of field-realistic doses of sulfoxaflor alone or in combination with the fungicide azoxystrobin on female bees' food intake in the laboratory and their learning efficiency and foraging performance on artificial flowers. We found that sulfoxaflor lowered the intake of sugar solution on average by 23.8% and interacted antagonistically with azoxystrobin. Moreover, bees exposed to sulfoxaflor learned to discriminate between rewarding and non-rewarding flower colours less efficiently. However, other aspects of foraging performance were not negatively affected by the investigated pesticides. These findings suggest that field-realistic exposure to sulfoxaflor can impair food intake and learning efficiency of *O. bicornis*, thereby pointing out mechanistic sub-lethal pathways that may lead to a reduced fitness and harmful consequences on population dynamics of solitary bees.

Keywords

interactive effects, mason bees, pesticide hazard, pollinators, risk assessment, sulfoximines

1. Introduction

Bees play a critical role as pollinators of entomophilous wild plants and crops in terrestrial ecosystems, with wide implications for the maintenance of biodiversity and food security [1, 2]. However, they are threatened by the intensification of agriculture and concurrent loss and degradation of habitat as well as high pesticide inputs, climate change and anthropogenically facilitated spread of pathogens and diseases [3-6]. In agricultural landscapes, bees are commonly exposed to multiple pesticides [7], which might lead to detrimental synergistic interactions on bee health [8]. Learning and memory abilities are essential for bees to efficiently forage on flowers and navigate between flowers and their nests. However, they might be impaired after exposure to pesticides [9], with potential negative consequences for reproductive success [10]. A better understanding of the impacts of novel or less-studied pesticides, or on mixtures of them, on sub-lethal endpoints of bees is therefore crucial for effective risk mitigation and protection strategies for pollinators. In particular, studies on solitary bees are still largely lacking.

Foraging of bees may for example be affected by neonicotinoid [9, 11] or other related neurotoxic insecticides [12]. They bind to nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects [13] and can thereby interfere with brain development and function [14, 15]. In order to forage efficiently, bees assess the quality of floral resources [16] and memorize them by association to visual and olfactory cues [17, 18]. They need to learn the handling of complex flowers [19] and be able to navigate well between flowers and their nest [20]. Potential pesticide-driven impairments of locomotion [21], orientation [22, 23], learning and memory [24, 25] may lead to a reduced foraging efficiency [26, 27] and resource intake [28, 29], which can ultimately lead to a lowered offspring provisioning and reproductive output [10, 30]. Moreover, as the detoxification of pesticides from the bee body is energetically costly [31], a lowered food intake might reinforce negative impacts of pesticides on bees, and finally aggravate potential negative impacts on reproductive success and fitness [32, 33]. Additionally, insecticides acting as nAChR-agonists have been found to lower the bees' responsiveness to sucrose [15] and motivation to forage [34], which might further reduce their ability of learning to associate floral cues to sugar rewards [35].

While the impacts of neonicotinoids on bees, and particularly on honey bees, are well documented and have led to bans of their use [36, 37], less is known about other pesticides, such as newly emerged insecticides or also fungicides in general, which do not target insects and are therefore generally assumed to be relatively safe for them. Sulfoximines are a novel class of insecticides exhibiting a similar mode of action as neonicotinoids [38]. The systemic insecticide sulfoxaflor has been developed against sap-feeding insect pests (e.g., aphids) on a variety of crops [39]. Yet, increasing evidence for potentially harmful impacts of sulfoxaflor on bees and other non-target organisms [40-44] have recently led to its ban with respect to outdoor use in Europe [45]. However, sulfoxaflor is still widely used worldwide (see current product labels), posing risks to pollinators. Moreover, non-lethal impacts of fungicides on bees have gained more scientific attention recently [46, 47]. Yet, their impacts on learning abilities and orientation in bees, particularly in solitary bees, remain poorly understood [48, 49]. Certain fungicides may also synergistically reinforce negative impacts of insecticides, e.g., by interfering with detoxification pathways in bees, thereby increasing the toxicity of insecticides by several factors of magnitude [50]. Up to date, studies on the combined exposure of insecticides and fungicides on solitary bees are, however, rare [51, 52].

The effects of pesticides on learning abilities in honey- and bumblebees are typically tested using the so-called proboscis extension reflex (PER). Bees learn to couple a conditioned stimulus (e.g., an odour) with a sugar reward, which leads them to extend their proboscis upon its registration [25]. This method is, however, not applicable to solitary *Osmia* bees, as they do not show this reflex in laboratory conditions [53]. Moreover, PER experiments seem a rather simple way of testing learning and memory in bees since they do not reflect the complex behaviour that bees show when foraging on flowers. Therefore, several studies have attempted to test pesticide effects on foraging and learning under more realistic settings using flight cages and (artificial) flowers [e.g., 24, 26]. However, such studies have rarely been conducted with free-flying solitary bees, such as *Osmia* spp. [54, 55] and, to our knowledge, this experimental approach has not yet been used to assess the impacts of single and combined pesticides on learning efficiency of solitary bees.

In this study, we therefore investigated the effects of exposure to the insecticide sulfoxaflor, the fungicide azoxystrobin and their combination on (1) the intake of sugar solution under laboratory conditions and (2) the foraging performance and learning efficiency of female *O. bicornis* on artificial flowers of different colours in the greenhouse. For both experiments, bees were exposed to an acute field-realistic oral dose of either sulfoxaflor, azoxystrobin, their combination, or a control solution (no pesticide). We hypothesized that sulfoxaflor (1) reduces the amount of sugar solution consumed by the bees, (2) impairs foraging performance and learning efficiency of *O. bicornis* and (3) that effects of sulfoxaflor would be enhanced by simultaneous exposure to azoxystrobin.

2. Material & Methods

2.1. Experimental designs

2.1.1. Food intake experiment

The food intake experiment was conducted in May 2021 under controlled laboratory conditions at Agroscope, Zürich. Approximately 200 *O. bicornis* females were hatched from cocoons purchased from Wildbiene + Partner AG (Switzerland) and subsequently introduced into a flight cage (1.4 m × 1.4 m × 1.4 m, model “Grünhaus M”, Aerarium Nets GmbH, Switzerland) located in a greenhouse. The flight cage was equipped with a wooden nesting aid for the bees serving as a shelter, a shallow bowl filled with water containing stones for the bees to sit on, a dish offering fine ground organic apple pollen as pollen food resource (honeybee-collected, purchased from L’Abeille heureuse, France), and an array of artificial flowers (hereafter flower meadow) with white flowers offering 33% (w/w) sugar solution. Even though *O. bicornis* females typically collect mixtures of pollen from different plants [56], we consider providing mono-floral pollen suitable in this experiment, as the bees were already fully developed adults and they were not provisioning offspring. The pollen was merely provided to offer them a source of protein and complement the sugar solution. The artificial flower meadow was constructed from a styrofoam plate covered with green crepe paper to mimic a grassy ground (Fig. 1a). On top of the crepe paper, artificial flowers were placed. These were made of wooden cylinders with a small hole in the middle on which round paper disks were attached (Fig. 1b). Modified 0.5 mL Eppendorf tubes (cut in half) filled with 33% sugar solution were placed into the small holes of the cylinders in the middle of the paper disks and refilled daily with fresh sugar solution. After one and a half days inside the cage, the artificial flowers (offering sugar solution) and the pollen were removed from the cage and bees were starved for 24h before the pesticide exposure. These steps (i.e., spending time inside the flight cage and overnight starvation) were conducted because in pre-experiments the proportion of *O. bicornis* females consuming sugar solution during the period of the food intake experiment (see next paragraph) directly after hatching was low.

Figure 1: Artificial flower meadow consisting of green crepe paper and wooden "flowers" (setup used for the foraging and learning experiment) (a), *Osmia bicornis* female sitting on an artificial paper flower (b), *O. bicornis* females placed inside NICOT systems containing small cups with pesticide-treated sugar solution (c) and *O. bicornis* female marked with a numbered plate on its thorax for identification (d).

On the subsequent experiment day, 45 bees were randomly assigned to one of four treatment conditions in a full-factorial design 1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor + azoxystrobin (mix) or 4) water-acetone control (see 2.2 for doses of pesticides). The bees were individually caged into NICOT systems in a cooled room of 4 °C and 5 µL of treatment solution (see section 2.2.) was pipetted into a small cup inside the system (Fig. 1c). The bees were then placed at room temperature and left to consume the solution for one hour. It was visually checked whether the bees consumed the entire treatment solution (feeder) or only part of it (non-feeder) (the exact number of feeders can be found in section 2.3.1.). Only feeders were considered for the data analysis and therefore taken back to the 4 °C room immediately after pesticide exposure and then transferred to new NICOT systems, in which cups were filled with 150 µL 33% sugar solution. We deliberately provided a large volume of sugar solution to ensure *ad libitum* availability. After the transfer, the bees were directly placed at room temperature in the laboratory and left to consume the sugar solution for three hours. Nine additional identical NICOT systems with no bees inside treated exactly the same (same laboratory, temperature conditions, observation time) served as control to measure the amount of solution that evaporated during the time of the experiment. After the feeding time, each cup was weighed once before and once after removing the remaining sugar solution. The volume of consumed solution was then calculated using the weight of the consumed solution and its density.

2.1.2. Foraging performance and learning efficiency experiment

This experiment was conducted from 27 April to 7 June 2021 during 14 separate rounds in a greenhouse at Agroscope in Zürich. The following section describes the procedure conducted within one experimental round. It was repeated 14 times and contained the following steps: 1) hatching and marking of *O. bicornis* females, 2) 1-week acclimatisation of females in cages in the greenhouse on white artificial paper flowers, 3) 24h starvation period, 4) pesticide exposure inside small NICOT cages and 5) foraging and learning assessment in cages in the greenhouse (approx. 3 h) on coloured artificial flowers (yellow and pink), of which one colour was rewarding. The rewarding flower colour was randomly chosen for each experimental round, resulting in six and eight rounds for pink and yellow, respectively.

For each round, approximately 85 cocoons purchased form Wildbiene + Partner AG (Switzerland) containing female *O. bicornis* bees were hatched, taken to the 4 °C-room and individually marked with numbered plates (usually used for queen rearing in honeybees, Imkereibedarf Wespi GmbH, Switzerland). For marking, a small droplet of glue was placed on the thorax and the number plate carefully pressed onto the bee using a toothpick (Fig. 1d). The marked females were then released into a flight cage located inside the greenhouse containing an artificial flower meadow with exclusively white flowers offering sugar solution ad libitum (see section 2.1.1.). The females spent one week inside this cage in order to get used to foraging on the artificial flowers (acclimatisation phase). The sugar solution in the artificial flowers was re-filled daily. Water and pollen were provided as well (see above). During this week, we deliberately used white paper flowers instead of coloured ones to prevent females from already developing preferences for a specific flower colour.

Assessment of foraging performance and learning efficiency. One day before the assessment of the foraging performance and learning efficiency, the females inside the cages were deprived of the artificial flowers and the pollen, i.e., they did not have access to food during this day, but only to water (24 h starvation period). This was done in order to enhance their motivation for foraging on the artificial flowers in the experiment on the following day. In the evening prior to an experimental trial, when the females were already roosting inside cavities of the nesting aid, the nesting aid was covered with a fine mesh and placed into the 4 °C-room overnight.

In the following morning (experimental assessment day), each female was randomly assigned to one of four pesticide treatment conditions in a full-factorial design: 1) sulfoxaflor only, 2) azoxystrobin only, 3) sulfoxaflor + azoxystrobin (mix) or 4) water-acetone control (see 2.2. for doses of pesticides). For the pesticide exposure, the females were individually caged in small NICOT systems, in which they had access to a small cup filled with 5 μL of treatment solution (see section 2.2.). The solution was pipetted into the cups and the females were placed into the systems inside the 4 °C-room (this procedure took approx. 20 minutes). They were then directly placed at room temperature in the laboratory and left for one hour to consume the solution. Afterwards, all females in the NICOT systems were released simultaneously in the flight cage containing the flower meadow (Fig. 1a), in which assessments were subsequently conducted. For each female, it was visually checked whether the whole amount of treatment solution was consumed (feeder) or not (non-feeder). Only feeders were included in the statistical analyses (sample sizes are given in section 2.3.2.).

The flower meadow in the flight cage during the observations consisted of 15 yellow and 15 pink artificial flowers randomly arranged on a green ground. The position of each flower was identifiable via the use of a grid painted on the green ground (Fig. 1a). Flowers of one colour were filled with 0.5 µl of 33% sugar solution (rewarding), whereas the flowers of the other colour were filled with 0.5 µl water (non-rewarding). Such a small volume of solution was chosen with the intention to increase the number of flowers visited by the bees. The assessments of foraging performance and learning efficiency were performed by two experimenters per experimental round during approximately three hours. During each approximately three-hours experiment, the position of the flowers on the grid was re-arranged every hour during the observations by randomly changing their position. For this, the experimenter removed all flowers at once, shuffled them by hand and then re-positioned them on the grid. The coloured paper disks were not changed during this process. This was done to prevent bees from memorizing the position rather than the colour of the rewarding flowers. Every single flower visit of each marked female was noted. A flower visit was defined as a bee landing on a flower and sticking its tongue into the Eppendorf tube. For this purpose, a voice recorder was used by the observer, on which the bee ID, the colour of the visited flower and the exact flower position on the grid was recorded. Using the voice recorder, it was also possible to determine the exact time point of each visit during the observation session. After each visit of a bee on a flower, the respective Eppendorf tube was re-filled with either sugar solution or water, depending on the flower colour and experimental round. For each experimental round, a new set of artificial flowers was used (i.e., the paper discs were exchanged).

We assessed the following measures for describing the foraging performance of individual bees: (i) the time elapsed until the first flower was visited (time to first flower visit, min), (ii) the number of flowers visited per hour, (iii) the total distance flown by a bee per hour as well as (iv) the mean distance flown between individual flower visits (mean distance to next flower). The distance was measured as the grade of neighbourhood using the position of the flowers on the grid (see Fig. 1a). If a bee visited a flower directly next to the previous one, this corresponded to a distance of 1 unit. If it visited a flower three positions next to the previous one, this corresponded to a distance of 3 units etc. (Supplementary Fig. 1). The flown distance per time period (per hour) was calculated by adding all the distances flown between flower visits and dividing the sum by the total observation time for the respective experimental round (in hours). The mean distance to the next flower was calculated by dividing the total distance by the total number of flower visits. The assessed measures are relevant in terms of pollination efficiency. The time until the first flower visit can be interpreted as the motivation of a bee to forage in the first place. The number of flowers visited per hour (visitation rate) is a common measure for assessing foraging efficiency, as a higher number of visits results in higher pollination rates [e.g., 57]. The distance between individual flowers and the total distance can be important aspects in systems where cross-pollination is important, or, might enhance gene-flow between more distant flowers [57]. It can, however, also be more efficient for a bee to visit flowers close by, in order to maximise visitation rates and minimize energy demands [58].

2.2. Pesticide treatments

The bees were exposed to one acute oral dose of one of four pesticide treatments in a fully crossed experiment 1) sulfoxaflor, 2) azoxystrobin, 3) sulfoxaflor + azoxystrobin (mix) and 4) water-acetone control). The doses used were field-realistic determined based on residue levels detected in nectar of treated plants shortly after application (worst-case scenario). For sulfoxaflor, we tested a scenario where females would be exposed to flower nectar contaminated with 0.1 ppm sulfoxaflor, which corresponds approximately to a scenario of bees foraging on apple or oilseed rape one to two days after application of sulfoxaflor to flowering crops [59]. For azoxystrobin, we tested a scenario of bees being exposed to nectar containing 2 ppm azoxystrobin based on residue levels found in nectar of sprayed oilseed rape [60] as well as on residue levels found in bee-collected pollen in France (Observatory of Pesticide Residue, ITSAP – Institute de l'Abeille 2014, personal communication). The amount of treatment solution fed to the bees needed to be relatively small, representing only a fraction of their daily nectar consumption requirements to maximise the probability that feeders continued foraging during the subsequent experimental trails in the artificial flower arenas. Therefore, we orally exposed bees to only 5 µL of pesticide or control solution (treatment solution). To simulate a worst-case intake of pesticides, we exposed bees to a dose of pesticides expected to be ingested during one day during foraging: Nectar intake in *O. bicornis* under laboratory conditions has been shown to be approximately 30 µL per day [61]. Therefore, to simulate a 0.1 ppm and 2 ppm exposition scenario for sulfoxaflor and azoxystrobin, respectively, we fed the bees with a 6-times higher dose dissolved in 5 µL of solution. This resulted in an acute oral exposure of bees to 5 µL of 0.6 ppm sulfoxaflor, 12 ppm azoxystrobin, their mix, or a water-acetone control.

The pesticides were purchased as pure substances (reference standards, no co-formulants). Sulfoxaflor (10 mg) was purchased from Greyhound Chromatography and Allied Chemicals Ltd, UK and azoxystrobin from Sigma Aldrich® (PESTANAL™ analytical standard, 100 mg). The substances were dissolved in acetone directly inside the vials they were delivered in. Subsequently, the solutions were diluted to the desired concentrations using 33% (w/w) sugar solution. The proportion of acetone in the solutions was adjusted, so that each treatment solution contained identical proportions of acetone (< 1%). The substances were handled using appropriate protective measures. The treatment solutions were filled into 4 mL glass vials and stored at -20 °C until they were used in the experiment.

2.3. Statistical analysis

The statistical analysis of the data was performed in R version 4.5.1 [62] using the packages lme4 [63], nlme [64] and glmmTMB [65]. Statistical inferences of linear mixed-effects models were calculated via *F*-tests with Kenward-Roger approximation and that of generalized linear mixed-effects models using likelihood ratio tests [66]. For linear models, type II ANOVA was used. Model assumptions of homoscedasticity and normality were checked visually [67] or with the package DHARMa [68]. Post-hoc tests were run in emmeans [69].

2.3.1. Food intake experiment

The volume of consumed sugar solution in the food intake experiment was analysed using a generalized least-squares fitted linear model (GLS) to account for non-homogeneous variances in the different treatment groups. The model included sulfoxaflor (present or absent), azoxystrobin (present or absent) and their interaction as explanatory variables. The average volume of sugar solution that had evaporated during the time of the experiment was subtracted from the calculated volume of solution consumed by the bees. Of the 180 female *O. bicornis* bees included in the experiment, 134 (74.4%) were feeders (i.e., they consumed the entire treatment solution; feeders per treatment: sulfoxaflor: 33 (18.3%), azoxystrobin: 34 (18.9%), sulfoxaflor + azoxystrobin (mix): 37 (20.5%), control: 30 (16.7%)). During the subsequent handling, five bees escaped, resulting in the following sample sizes in the experimental treatment groups in the statistical analyses: sulfoxaflor: 31, azoxystrobin: 32, mix: 37, control: 29. All feeders subsequently consumed a part of the offered sugar solution and no spilling of solution by bees was observed.

2.3.2. Foraging performance and learning **efficiency** *experiment*

In total, 652 female *O. bicornis* bees were used in this experiment (sulfoxaflor: 165, azoxystrobin: 163, mix: 163, control: 161) of which 542 were feeders (83.1%; sulfoxaflor: 131 (20.1%), azoxystrobin: 132 (20.2%), mix: 135 (20.7%), control: 144 (22.1%)) and 196 subsequently participated in the study by visiting at least one artificial flower (30.1%; sulfoxaflor: 42 (6.4%), azoxystrobin: 50 (7.7%), mix: 44 (6.7%), control: 60 (9.2%)). Only participating feeders were included in the statistical analyses. Of the participating bees, 100 participated in experimental rounds in which pink was the rewarding flower colour, and 96 in rounds where yellow was rewarding.

Foraging performance. Different aspects of foraging performance (time to first flower visit, flower visits per hour, distance per hour and mean distance to next flower) were analysed with linear mixed-effects models (LMMs) using the package lme4 [63]. The models included sulfoxaflor (present or absent), azoxystrobin (present or absent) as well as their interaction as explanatory variables. The experimental round was included as random intercept. For the analysis of the mean distance to the next flower, only bees which visited more than one flower were included (i.e., 177 individuals). The response variables time to first flower visit, flower visits per hour and distance per hour were square root transformed to obtain normal distribution of residuals.

Learning efficiency. The learning efficiency of *O. bicornis* was analysed with a generalized linear mixed-effects model with binomial error distribution. Every flower visit performed by a bee was categorized as “rewarding” (1) or “non-rewarding” (0) (binary response variable). The model included sulfoxaflor (present or absent), azoxystrobin (present or absent), flower visit number (continuous) as well as their two- and three-way interactions as explanatory variables. Additionally, we included the rewarding colour (categorical, two levels) and the rank of the bee (i.e., the sequence in which bees performed their first visit) as explanatory variables. The unique ID of each bee nested in the experimental round was included as random intercept and random slopes were included to account for the variability of learning curves of individual bees. We only analysed data up to the 46th flower visit, as (1) visual exploration indicated that the learning curves reached a plateau approximately at this number of visits, and (2) at least 15 bees per treatment completed the 46th flower visit and thus represent an adequate sample size of bees across treatments for the statistical analysis, while increasingly less bees completed higher number of visits. In total, 5,603 flower visits performed by 196 bees were included in the analysis.

3. Results

3.1. Food intake

The volume of sugar solution ingested by *O. bicornis* was negatively affected by exposure to sulfoxaflor ($F_{1,125}$ = 37.26, $P < 0.001$, Fig. 2, Supplementary Table 1). On average, consumption was reduced by 23.8% compared to bees not exposed to sulfoxaflor. Moreover, we found an antagonistic interaction between sulfoxaflor and azoxystrobin on the amount of sugar solution consumed (sulfoxaflor × azoxystrobin, $F_{1,125}$ = 8.10, $P = 0.005$, Fig. 2, Supplementary Table 1). In absence of azoxystrobin, sulfoxaflor lowered the amount of consumed solution significantly (control vs. sulfoxaflor only, t-ratio = 6.60, $P < 0.001$, Bonferroni-corrected for two tests), whereas the reduction in presence of azoxystrobin (i.e., azoxystrobin only vs. mix) was not significant ($P = 0.370$). On the other hand, azoxystrobin tended to reduce the intake of sugar solution in absence, but not in presence of sulfoxaflor (t-ratio = 2.18, P = 0.068 and P = 0.143, respectively).

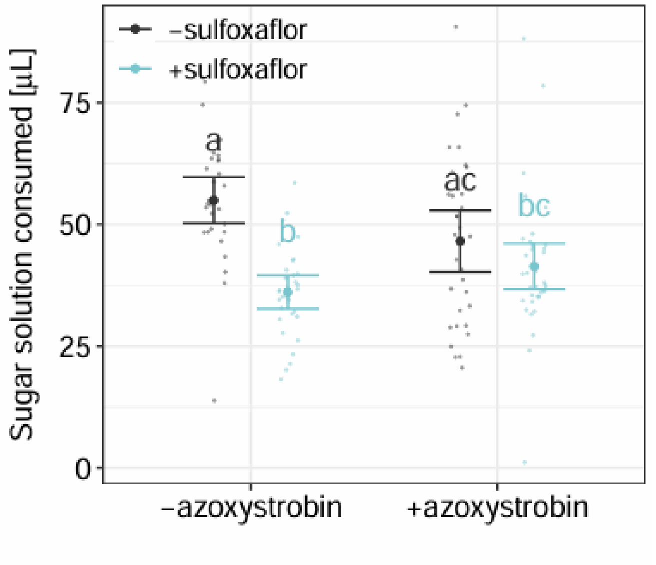

Figure 2: Effect of sulfoxaflor and azoxystrobin on the volume of 33% (w/w) sugar solution consumed by female *O. bicornis* during three hours after exposure to a single acute oral dose of the pesticides. Bars depict model predictions (emmeans) and 95% confidence intervals. Raw data points are shown as dots. *Black bars/dots*: no sulfoxaflor exposure, *blue bars/dots*: sulfoxaflor exposure.

3.2. Foraging performance and learning efficiency experiment

3.2.1. Foraging performance

We found no significant single or interactive effects of sulfoxaflor and/or azoxystrobin on any of the measured aspects of foraging performance of *O. bicornis* (Fig. 3a-d, Supplementary Table 2). There was, however, a trend for sulfoxaflor-exposed bees to visit 28.4% more flowers within one hour compared to bees not exposed to sulfoxaflor (approximately three flower visits more; λ_{LR} = 3.23, P = 0.074; Fig. 3b, Supplementary Table 2). Most often, the bees flew to neighbouring flowers after a visit (29.9%), followed by visits to the next but one flower (14.4%) and flowers further away (Supplementary Fig. 5). In a few cases, bees visited the same flower again directly after leaving it (3.6%; Supplementary Fig. 5).

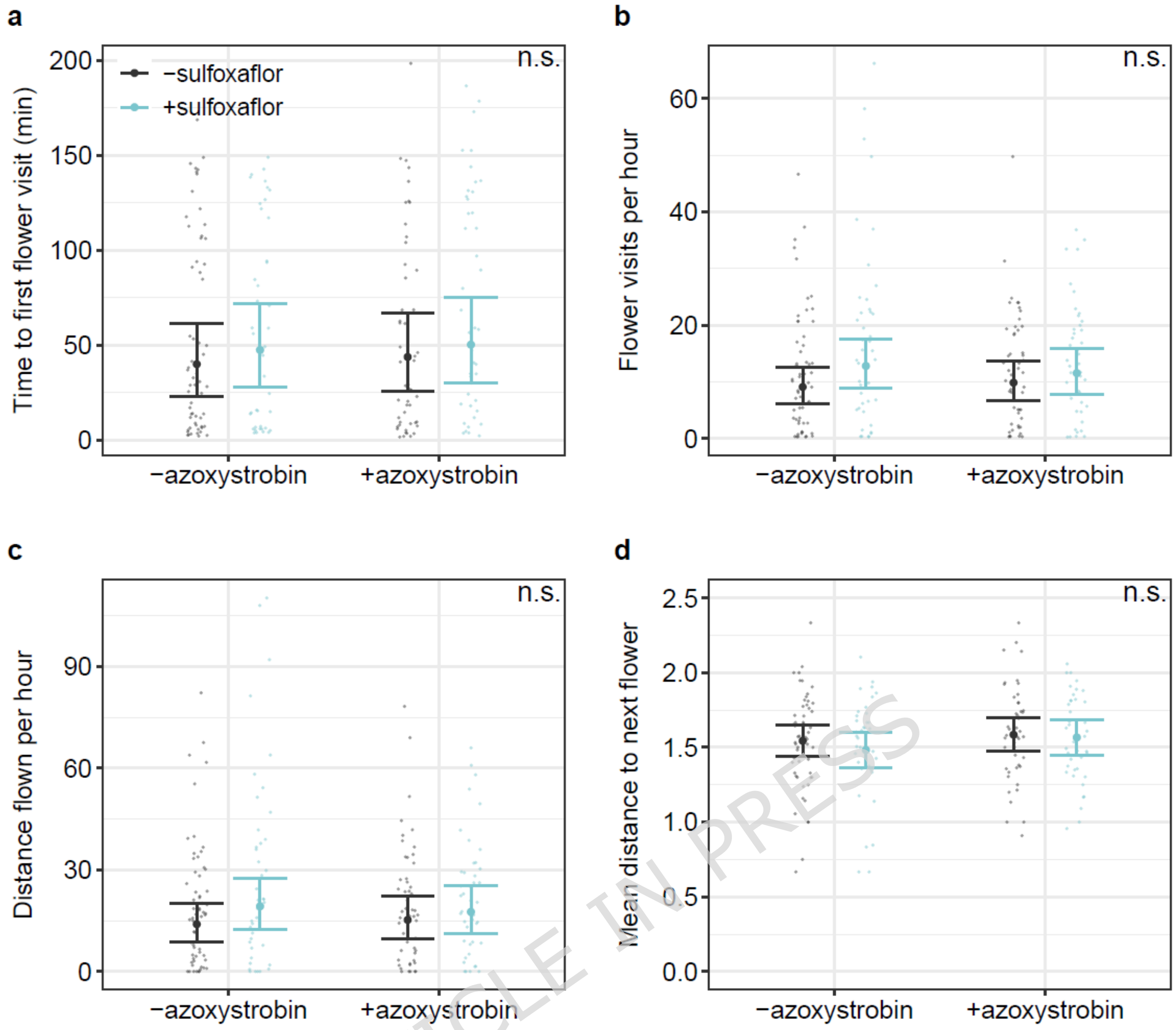

Figure 3: Effects of sulfoxaflor and azoxystrobin exposure on **a)** time elapsed until an individual bee's first flower visit, **b)** the number of flowers visited per individual bee per hour, **c)** the distance flown per individual bee per hour and **d)** the mean distance an individual bee flew between two consecutive flower visits. Bars depict model predictions (emmeans) and 95% confidence intervals. Raw data points are shown as dots. *Black bars/dots*: no sulfoxaflor exposure, *blue bars/dots*: sulfoxaflor exposure, n.s.: not significant.

3.2.2. Learning efficiency

Overall, the probability of choosing the rewarding flower colour increased with every flower visit a bee conducted (λ_{LR} = 21.65, $P < 0.001$, Fig. 4, Supplementary Table 3). This demonstrates that the ability of bees to distinguish the rewarding from the non-rewarding flower colour improved over the time of the experiment (learning). We did not find significant negative main or interactive effects of sulfoxaflor or azoxystrobin on the overall probability of choosing the correct flower throughout the whole observation time (main effects: P = 0.172 and P = 0.697, respectively; interaction: P = 0.178). Bees exposed to sulfoxaflor showed, however, a lower increase of correctly selecting rewarding flowers with progressing flower visits (interaction term sulfoxaflor × flower visit number, λ_{LR} = 4.76, P = 0.029), showing that the slopes of the learning curves were flatter for sulfoxaflor-exposed compared to non-exposed bees (Fig. 4, Supplementary Table 3). Further, bees showed a tendency to perform better when yellow was the rewarding colour (marginal effect, P = 0.050), and individuals that began foraging later in an experimental round (higher rank) tended to choose the correct flower colour more often (marginal effect, P = 0.060). It cannot be ruled out that bees developed a slight tendency for yellow flowers due to their acclimatisation phase on exclusively white flowers. To bees, yellow paper might appear more similar to white paper compared to what we see as humans. Importantly, however, removing these variables from the model did not qualitatively change the significant results reported above.

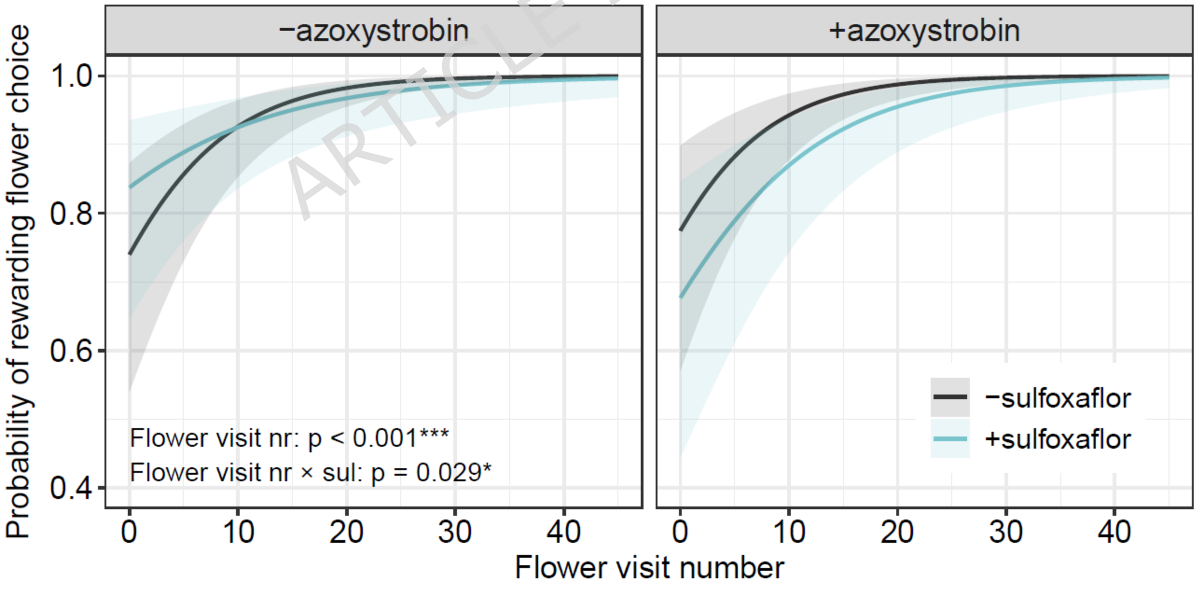

Figure 4: Learning curves of *O. bicornis* exposed to sulfoxaflor (*blue* lines) or not (*grey* lines), in absence (*left*) and presence (*right*) of azoxystrobin. The bees' probability of choosing the rewarding flower colour is shown in dependence of the flower visit number. Curves depict model predictions

(emmeans) and 95% confidence intervals (shaded areas). *Black lines*: no sulfoxaflor exposure, *blue lines*: sulfoxaflor exposure.

4. Discussion

In this study, we tested the single and combined effects of the insecticide sulfoxaflor and the fungicide azoxystrobin on the intake of sugar solution as well as on foraging performance and learning efficiency of solitary *O. bicornis* bees. We found that a field-realistic acute oral exposure to sulfoxaflor lowered the subsequent intake of untreated sugar solution by 23.8% compared to non-exposed bees. Moreover, we found an antagonistic interaction between sulfoxaflor and azoxystrobin on sugar solution consumption even though sulfoxaflor alone as well as the mix (sulfoxaflor and azoxystrobin) had a negative effect when compared to the control group. In addition, we found that, when bees were able to forage freely in a flight cage equipped with artificial flowers, sulfoxaflor-exposed individuals learned the discrimination of rewarding and non-rewarding flowers less efficiently than non-exposed bees. On the other hand, we did not find a negative effect of sulfoxaflor or an interactive effect between sulfoxaflor and azoxystrobin on foraging performance.

Sulfoxaflor substantially lowered the intake of sugar solution in the laboratory after bees were exposed to a single acute oral dose of the insecticide. Our result mirrors findings of studies on honeybees and bumblebees reporting reduced feeding rates after exposure to neurotoxic insecticides like neonicotinoids [29, 70], sulfoxaflor [71] or flupyradifurone [72, 73]. *Osmia bicornis* exposed chronically to 100 ppb sulfoxaflor also showed reduced daily feeding rates in a recent study, further corroborating our findings [74]. The reduction of daily sugar solution consumption in the latter study was ca. 9%, whereas we found a reduction of 23.8%. However, direct comparisons among studies are difficult, as methods differ (e.g., acute vs. chronic exposure, pesticide concentrations, housing of bees). Here, we exposed bees to one acute dose of sulfoxaflor before letting them feed on pesticide-free sugar solution, therefore the observed negative impact was likely driven by impaired locomotor abilities [75], a reduced responsiveness to sucrose [76], or a lowered motivation to forage or feed [34, 77]. Bees in our study consumed a higher amount of sulfoxaflor in a shorter period, potentially explaining the stronger effect on food intake as compared to the study of Azpiazu et al., 2022 [74]. A reduced food intake can induce nutritional stress in bees, potentially causing negative effects on fitness-related aspects such as immune function, foraging performance and reproduction [78-80]. Combined stress from low food intake and pesticide exposure might further lead to additive or synergistic negative impacts on bees, ultimately potentially even leading to population declines [8, 44, 71, 81, 82].

When foraging on artificial flowers, bees clearly learned to associate the sugar reward to a specific colour and the probability of a rewarding choice increased in course of the experiment, which was also demonstrated in previous studies [17]. However, the probability of a rewarding choice increased at a slower rate in sulfoxaflor-exposed bees compared to non-exposed bees (Fig. 4). Previous studies investigating impacts of sulfoxaflor on learning related endpoints in bees focused on honeybees or bumblebees [83-87], while, to the best of our knowledge, our study is the first assessing the single and combined impact of sulfoxaflor and azoxystrobin on learning in foraging solitary bees. So far, no common protocol for performing studies assessing pesticide risks and their impacts on foraging and learning of free-flying solitary bees using artificial flower arrays has been available [but see 55, 88]. Our study might therefore help in guiding future studies adopting and potentially further developing the experimental set-up presented here.

Most of the mentioned studies on honeybees and bumblebees have tested the relatively simple proboscis extension reflex (PER), which is not reflecting a realistic situation for bees and from which results might not be translatable to foraging bees under a real-world scenario. The study by Capela et al. (2022) [85] discovered that sulfoxaflor can impair the homing ability of honeybees when they were exposed to an acute oral dose of 10 µL sugar solution spiked with 2600 ppb sulfoxaflor. Although this dose used by Capela and colleagues was approximately 9 times higher than the dose we used (i.e., 5 µL of sugar solution with 600 ppb sulfoxaflor), the negative effect on learning in *O. bicornis* we observed in our study is plausible, since *O. bicornis* is about four times more susceptible to sulfoxaflor than honeybees (according to 24h LD_{50} values [89]). Additionally, a recent study by Cartereau et al. (2022) [86] found that honeybee learning and memory retrieval was negatively affected after a single acute oral dose of sulfoxaflor corresponding to the no-observed effect level (NOEL, i.e., the exposure level at which no negative effects were previously observed on honeybees), further corroborating evidence for potential adverse effects of field-realistic exposure of bees to sulfoxaflor on learning and memory retrieval.

Interestingly, we observed that the *O. bicornis* bees in our experiment already had an unexpectedly high probability of choosing the rewarding flower colour at their first flower visit (Fig. 4). As dissolved sugar is not volatile at room temperature, the increased attractivity of rewarding flowers might have been associated with scent marks left by *O. bicornis* females on the flowers when visiting them [90, 91]. These could induce either a repulsive or an attractive effect for bees that started foraging on the flowers later during an experimental round. However, when the rank of each bee (i.e., the series in which bees started to participate in the experiment) was considered in the statistical model, the obtained results did neither change nor was the probability of choosing a rewarding flower at the first flower visit increased for bees that started foraging later (i.e., bees with a higher rank), and thus these findings do not provide support for this hypothesis (Supplementary Table 4). Another potential way for bees to distinguish between rewarding and non-rewarding flowers could be visual discrimination of sugar solution and water. However, in our experiment, the rewards (or water only) were hidden inside Eppendorf tubes and not visible to the bees from a distance. A previous study showed that in such a setting (in absence of any additional cues such as colour or odour), bumblebees were not able to distinguish between sugar solution and water only, suggesting they are neither able to see or smell a difference if rewards are hidden inside a tube [92]. To our knowledge, there are no additional peer-reviewed studies investigating whether bees might be able to visually discriminate sugar solution from water (but see [93]). However, this might be an interesting and highly relevant question to investigate further. The exact mechanism driving the observed high probability for a rewarding flower choice at the first flower visit remains therefore unclear.

Even though we detected a negative effect of sulfoxaflor on learning efficiency, we found no significant impacts on other aspects of foraging performance, (i.e., the time until the first flower visit, the number of flower visits per hour as well as the total distance flown and the mean distance between visited flowers). Our results contrast findings of a recent laboratory study that found impaired foraging performance of *O. bicornis* after repeated exposure to 50 ppb sulfoxaflor in sugar solution [55]. The authors found that sulfoxaflor exposure reduced flower visitation but increased the flight distance. Differences in the obtained results might be due to the different pesticide exposure regimes. While in our study, bees received only a single sulfoxaflor dose, bees in the latter study were repeatedly exposed to the pesticide over five days. For the estimation of a daily dose, we used mean sugar solution consumption rates of *O. bicornis* in the laboratory [61]. Therefore, we created a worst-case field-realistic scenario of a single acute oral exposure. In the field, however, foraging bees would likely consume such doses more continuously over the course of the day, allowing them additional time to detoxify the ingested pesticide. On the other hand, the actual intake of sulfoxaflor under real-world conditions might be higher, as foraging *O. bicornis* do not only consume nectar and pollen for themselves, but repeatedly collect these resources to provision their offspring with pollen-nectar provisions and also have an increased energy demand during flying. Moreover, different bee species can vary greatly in their sensitivity to pesticides [94, 95]. In a more realistic semi-field setting inside large flight cages, bumblebees exposed to sulfoxaflor on *Phacelia tanacetifolia* (sprayed two days before flowering) were negatively affected in their foraging performance [40]. As *O. bicornis* is more susceptible to acute sulfoxaflor exposure based on LD_{50} values [89], it is surprising that we did not detect significant negative effects. On the other hand, the bumblebees were also exposed to pesticide residues in pollen, which was not the case in our study. Given the chronic exposure and the fact that contamination values in pollen can be up to tenfold higher than in nectar [96], the overall exposure was likely substantially higher in the study conducted by Tamburini et al. (2021) [40].

We found no significant negative effects of the fungicide azoxystrobin alone or in combination with sulfoxaflor on food intake, foraging performance or learning efficiency of *O. bicornis* in our study. However, there was a trend ($0.05 < P \leq 0.01$) for reduced food intake after exposure to azoxystrobin, but only in absence of sulfoxaflor, suggesting that this fungicide might still negatively affect food consumption. Previous semi-field studies on azoxystrobin effects on bumblebees suggested negative effects of azoxystrobin on foraging performance and colony development [40, 97]. However, in these studies, azoxystrobin was applied in form of the commercial product Amistar, whose co-formulants have been shown to adversely affect bumblebees [98]. Therefore, it is conceivable that the negative effects observed in these studies might actually not be due to the active ingredient azoxystrobin itself but may have rather been caused by co-formulants. Yet, a related fungicide, picoxystrobin, is apparently capable of inducing damage to the midgut of bees [99]. It is, however, unlikely that such an impairment would affect bees already directly after the ingestion of a pesticide, as tested in our study, and manifest in reduced feeding rates. Accordingly, a recent study did not find altered feeding rates in honeybees exposed to azoxystrobin [100].

Interestingly, however, we found that azoxystrobin interacted antagonistically with sulfoxaflor on the consumption of sugar solution. While sulfoxaflor alone exhibited a pronounced negative effect, mixing sulfoxaflor and azoxystrobin did not negatively affect feeding anymore. This suggests that sulfoxaflor and azoxystrobin might directly interact with each other on a molecular level. In a previous semi-field study on *O. bicornis*, indications for similar antagonistic interactions of the two substances (commercial products Amistar and Closer) on the sex ratio of produced offspring were found [51], while no such antagonistic interaction was found in bumblebees [40]. Interestingly, a recent laboratory assay has similarly found an antagonistic interaction between sulfoxaflor and azoxystrobin when the bumblebees were exposed via contact exposure at certain doses [101]. Using measurements of respiratory rates, Jürison et al. (2025) [101] have shown a relieving effect of azoxystrobin on the overexcitation caused by sulfoxaflor. This could be attributable to azoxystrobin inhibiting mitochondrial respiration and thereby lowering of the elevated metabolic rate [101, 102]. However, the exact mechanism is not yet fully understood.

In conclusion, our study demonstrates a negative effect of field-realistic acute sulfoxaflor exposure on food intake and learning efficiency of foraging solitary *O. bicornis* bees. Such impaired learning could, under real-world conditions, translate into a lowered capability of collecting floral food resources and, potentially result in negative impacts on the fitness of these solitary bees and their populations in agricultural landscapes. Additionally, we found an interesting antagonistic interaction of sulfoxaflor and azoxystrobin, which highlights that the effect of pesticide mixtures can be highly complex and unpredictable. A better understanding of pesticide interactions is needed to effectively protect bees in agricultural landscapes, where they are commonly exposed to cocktails of substances.

Data Availability

The data used in this study are available at https://doi.org/10.5281/zenodo.18621357.

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Acknowledgements

We thank Moana Castiello, Janine Hitz, Sebastian Schaffner and Tobias Ulrich for their help while conducting the observations in the greenhouse.

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Funding

The research leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 773921, PoshBee Project (www.poshbee.eu).

Competing Interests

The authors declare no competing interests.

Figure Legends

Figure 1: Artificial flower meadow consisting of green crepe paper and wooden “flowers” (setup used for the foraging and learning experiment) (a), *Osmia bicornis* female sitting on an artificial paper flower (b), *O. bicornis* females placed inside NICOT systems containing small cups with pesticide-treated sugar solution (c) and *O. bicornis* female marked with a numbered plate on its thorax for identification (d).

Figure 2: Effect of sulfoxaflor and azoxystrobin on the volume of 33% (w/w) sugar solution consumed by female *O. bicornis* during three hours after exposure to a single acute oral dose of the pesticides. Bars depict model predictions (emmeans) and 95% confidence intervals. Raw data points are shown as dots. *Black bars/dots*: no sulfoxaflor exposure, *blue bars/dots*: sulfoxaflor exposure.

Figure 3: Effects of sulfoxaflor and azoxystrobin exposure on **a)** time elapsed until an individual bee’s first flower visit, **b)** the number of flowers visited per individual bee per hour, **c)** the distance flown per individual bee per hour and **d)** the mean distance an individual bee flew between two consecutive flower visits. Bars depict model predictions (emmeans) and 95% confidence intervals. Raw data points are shown as dots. *Black bars/dots*: no sulfoxaflor exposure, *blue bars/dots*: sulfoxaflor exposure, n.s.: not significant.