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Survival rate and changes in foraging performances of solitary bees exposed to a novel insecticide

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ABSTRACT

Solitary bees are among the most important pollinators worldwide however population declines especially in croplands has been noticed. The novel pesticide sulfoxaflor is a competitive modulator of nicotinic acetylcholine receptors (nAChR) in insects. While there is evidence of a negative impact of neonicotinoids on bees of several social organization levels, our overall knowledge on the impact of sulfoxaflor on bees is poor. Here we present for the first time a study showing effects of field realistic doses of sulfoxaflor on solitary bees. Bees submitted to long term exposure of field realistic doses of sulfoxaflor (5 μg dm^{-3}, 10 μg dm^{-3}, 50 μg dm^{-3}) and control were observed regarding their survival rate. Moreover, we recorded metrics related to flower visitation and flight performance. We discover that the highest field realistic dose is lethal to *Osmia bicornis* along five days of exposure. The effect of sulfoxaflor reduces the outcome of foraging, important features for fruit and seed production of cross-pollinated plant species. Bees exposed to pesticide visited flowers mostly walking rather than flying. Flight performance was also impaired by the pesticide.

1. Introduction

About 85% of the angiosperms are visited by animals (Ollerton et al., 2011), especially by bees (Klein et al., 2007), which are responsible for pollination and stability of terrestrial food webs. Worldwide insect pollinators, including bees, account for 35% of global crop pollination (Garibaldi et al., 2014). Although the importance of insects as pollinators has been generally recognized, bees and other insects are still on the decline (Biesmeijer et al., 2006; Powney et al., 2019). Bee decline has been linked to anthropogenic activities including landscape management, flower loss and pesticide use (Goulson et al., 2015; Potts et al., 2010; Rundlöf et al., 2015; Tsvetkov et al., 2017; Woodcock et al., 2017). Therefore, lack of pollinators may lead to a reduction of food production and ecosystem services, which can reflect on economic and social issues (Winfree, 2008; Potts et al., 2016).

Recent studies have helped to understand that pesticides such as neonicotinoids binding to nicotinic acetylcholine receptors of insects can lead bees to reduced survival and abnormal behaviours when applied in sub-lethal doses. This impact was reported, for example, through impairment in social communication (Boff et al., 2018a), learning (Stanley et al., 2015), reproduction (Sandrock et al., 2014), discrimination of floral scents (Mustard et al., 2020), foraging behaviour and performance (Gill and Raine, 2014), which has been found to impair the pollination system (Whitehorn et al., 2017). Novel classes of neurotoxic pesticides binding to acetylcholine receptors (nAChR) in the neuro-systems of insects have been released to the market. Sulfoxaflor, for example, was reported to negatively impact egg laying in the social bumble bees (Siviter et al., 2018) but did not significantly affect olfactory conditioning and memory (Siviter et al., 2019). The effect caused by this pesticide was recently reviewed for multiple insect species (Siviter and Muth, 2020). However, studies covering the effect of sulfoxaflor on solitary bees seems rare or absent. Sulfoxaflor has an active compound indicated for use in apples, citrus, cotton, cucurbits, grapes, pear, peaches, strawberries, tomatoes and other crops. This novel pesticide is a systemic insecticide belonging to the class of sulfoximines and a competitive modulator of the nicotinic acetylcholine receptors in insects which are known to play a role in central nervous system responses (Watson et al., 2011).

The solitary bee *Osmia bicornis* is distributed widely throughout Europe and known as important pollinators in agriculture (Westrich,

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2019). The females actively forage and provision from the beginning of the spring season and can remain visiting flowers till summer (Bertrand et al., 2019). Due to their foraging performance in numerous plants species, they are considered generalists among solitary bees (Gresty et al., 2018; Westrich, 2019). They are important pollinators of cross-pollinated crops as well as of crops that benefit from cross pollination (Gruber et al., 2011; Hansted et al., 2014; Klatt et al., 2013; Ryder et al., 2020; Steffan-Dewenter, 2003). High pollination efficiency of crop species with *O. bicornis* in a cage environment has shown their suitability to be used in green houses (Herrmann et al., 2019; Steffan-Dewenter, 2003).

Here we test the long-term exposure effect of different doses of sulfoxaflor on survival of the solitary bee *Osmia bicornis* (Linnaeus, 1758). In addition, we study the consequences of sulfoxaflor exposure for foraging outcomes as well as for behaviour-flight performances. In this paper we hypothesize higher doses of sulfoxaflor impose a higher risk on survival and on foraging outcomes and flight performance.

2. Material and methods

2.1. Sulfoxaflor

The doses of sulfoxaflor (Greyhound Chromatography and Allied Chemicals) used here were based on a study obtained from the United States Environmental Protection Agency (2016), which is based on an application rate of 20 g of active compound per acre applied twice. It resulted in 5.41–46.97 μg dm^{-3} of sulfoxaflor in nectar of cotton plants. Here a stock solution was prepared in which sulfoxaflor was dissolved in acetone (0.01 mg mL^{-1}) and combined with 25% sucrose solution. The three following treatments were applied [mass (μg) of sulfoxaflor per volume (dm^{-3}) of acetone]: 5 μg dm^{-3}, 10 μg dm^{-3}, 50 μg dm^{-3}, resembling field realistic doses, in addition to a control: acetone (0.01 mg mL^{-1}) combined with 25% sucrose solution.

2.2. Experimental set up

A total of 85 newly emerged females of *O. bicornis* were maintained for at least 12 h in individual survival cages with a feeder. Twenty-three control bees were maintained in the cages with the feeder filled with sucrose solution 25% ad libitum. Eighteen bees were exposed to 5 μg dm^{-3} of sulfoxaflor with 25% sucrose solution, while 22 bees were exposed to 10 μg dm^{-3} and 22 other females were exposed to 50 μg dm^{-3} of sulfoxaflor with 25% sucrose solution. Sucrose solution uptake (pure or with pesticide) was assessed visually by direct observation of consumption in the feeders. Bees were allowed to feed ad libitum and feeders were renewed once a day to account for long-term exposure (see Supplementary material for pesticide exposure set up details).

2.3. Survival experiment

All bees were monitored and censored twice a day for five days. Dead bees were removed and the information regarding treatment was recorded.

2.4. Foraging behaviour of bees

Foraging observations were performed in the same five days of survival experiment with all survivals of a given day. Two flight cages (60 × 60 × 60 cm) were used side by side simultaneously in the laboratory. To avoid risk of contamination with pesticide residues one of the cages was restricted to control bees. Twelve artificial and colored flowers were placed inside the flight cages at the bottom of the arena (Supplementary material Figs. S1 and S2). All flowers except blue were filled with 20 μL of pure 25% sucrose solution from which bees could feed; that was because the experimental set up was initially designed to test different hypotheses that are not part of the central aim of the current study. Foraging outcomes were recorded in two daily rounds after 1 h of starvation per round per bee (Boff et al., 2020). The bees were observed for 10 min each round (20 min observations for a maximum of five days). The observation of the foraging behaviour of the bees was registered during 5250 min (525 video recordings) through individual observation (1360 min for controls, 1270 min for 5 μg dm^{-3}, 1570 min for 10 μg dm^{-3} and 1050 min for bees exposed to 50 μg dm^{-3}). The experiment was performed in June of 2019 in lab conditions with the temperature set to 26 °C in the Entomological laboratory of the Department of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy.

2.5. Visitation rate

We recorded the number of visits and the number of flowers visited.

2.6. Locomotion to flowers and flight performance

Bees accessed flowers displaying three different foraging behaviour: by flying, flying and walking or only by walking. The flying velocity of controls and treated bees was measured with the free software Tracker® 5.14 (Brown, 2020) available at: https://physlets.org/tracker/ (see Supplementary material for data collection). Since the angle of the recordings did not cover the entire flight cages, we selected 29 ($n_{5\ \mu g\ dm\text{-}3} = 5$, $n_{10\ \mu g\ dm\text{-}3} = 10$, $n_{50\ \mu g\ dm\text{-}3} = 4$, $n_{control} = 10$) of the 525 videos from which we were able to track the entire foraging bout (bee taking off, flight displacement and flower visit).

2.7. Statistical analyses

Statistical analyses were conducted in R computing environment (R Core Team, 2018). Analysis of survival was performed with Kaplan-Meier with the package survival (Therneau, 2020) and survminer (Kassambara and Kosinski, 2020). Pairwise comparisons with correction for multiple test (Log-Rank Test) among survivals was performed with the function pairwise_survdiff (Therneau, 2020) with p value adjusted for multiple comparison.

Due to the inherent correlation between the number of visits and number of flowers visited, we performed analysis of covariance (ANCOVA) to test if the different doses of sulfoxaflor affect the number of visits in relation to the number of flowers visited. Conversely, we tested if the concentration of the pesticide affected the number of visited flowers in respect of the number of visits. Pairwise comparisons from estimated marginal means (least-square means) with the R package emmeans (Russell, 2019), following a Tukey's correction, was used to compare the number of visits between groups in ANCOVA model.

We recorded the frequency of flower approach types (flying vs. walking and flying vs. walking) performed per bee during events of flower visitation. To test if these approaches varied across treatment, first we performed a non-metric multidimensional scaling (NMDS) to ordinate bees by differences (Bray-Curtis distances) of these three response variables. Then we performed a free permutation test (observed plus 9999 permutations) to test treatment correlation on NMDS bidimensional ordination with function enfvit from R package vegan (Oksanen et al., 2019).

To test the effect of treatment on flight performance we conducted a path analysis (SEM-Structural Equation Model) to access direct and indirect effects on flight.

3. Results

3.1. Survival analysis

From 85 bees at the beginning of the experiment, 44 died during the five days of the experiment. No difference in survival was recorded between controls and bees treated with five or 10 μg dm^{-3} of sulfoxaflor.

Bees exposed to 50 μg dm^{-3} of sulfoxaflor displayed a much higher proportion of death (82%). Kaplan-Meier survival analysis showed that the probability of survival was circa ¼ of the original sample size for bees exposed to 50 μg dm^{-3} of sulfoxaflor (Fig. 1). A significant reduction of survival probability was only registered at 50 μg dm^{-3} compared to all of the other groups (log-rank test: $\chi^2 = 12.1$, $p_{50\text{–}0\ \mu g\ dm\text{-}3\ (control)} = 0.001$; $\chi^2 = 12$, $p_{50\text{–}5\ \mu g\ dm\text{-}3} = 0.001$, $\chi^2 = 5.7$, $p_{50\text{–}10\ \mu g\ dm\text{-}3} = 0.033$, Supplementary material, Table S1).

3.2. Impact on foraging behaviour

Flower visitation occurred in 34% of the rounds (177 out of 525). All control bees visited flowers and a total of eight bees exposed to sulfoxaflor never visited a flower ($n_{5\ \mu g\ dm\text{-}3} = 3$, $n_{10\ \mu g\ dm\text{-}3} = 3$, $n_{50\ \mu g\ dm\text{-}3} = 2$).

The number of flowers visited explained the number of visits irrespective of sulfoxaflor concentration ($F_{1,70} = 107.39$, $p < 0.001$) in an ANCOVA model ($R^2 = 0.6$, $F_{4,70} = 28.94$, $p < 0.001$). Sulfoxaflor reduced the number of visits in relation to the number of flowers visited (Fig. 2a). Number of flower visits was (mean ± SD) 13.31 ± 8.77 bee^{-1} in the control group, 8.38 ± 5.9 in the 5 μg dm^{-3} group, 6.3 ± 4.66 in the10 μg dm^{-3} group and 6.52 ± 3.96 in the 50 μg dm^{-3} group. Pairwise comparisons show that 50 μg dm^{-3} sulfoxaflor significantly reduced the number of visits to flowers in comparison to the control group (t ratio = 2.7, p = 0.046, see Fig. 2b). Additionally, the concentration of sulfoxaflor did not affect the number of flowers visited in respect to the number of visits (ANCOVA, $F_{3,70} = 0.355$, $p = 0.786$).

3.3. Effect on flower visitation and flight performance

The behavioural approach displayed by 65 bees ($n_{control} = 17$, $n_{5\ \mu g\ dm\text{-}3} = 14$, $n_{10\ \mu g\ dm\text{-}3} = 16$, $n_{50\ \mu g\ dm\text{-}3} = 18$) during events of flower visitation was significantly affected by pesticide treatment. We found a gradient of variation with increasing sulfoxaflor concentration leading the bees to preferentially visit flowers walking rather than flying (Goodness of fit: $r^2 = 0.16$, $p = 0.003$, Fig. 3).

We found direct and indirect effects of sulfoxaflor on flight performances of bees (Fig. 4). Sulfoxaflor concentration increased time of flying preceding flower visitation ($z = 2.21$, $p = 0.027$) which resulted in a significant enhancement in flight distance ($z = 3.95$, $p < 0.001$). Sulfoxaflor had a marginal effect on velocity ($z = -1.89$, $p = 0.059$). The other variables were not significant different from zero (Supplementary material Table S2).

4. Discussion

In the current study, we investigated the impact of different field realistic concentrations of sulfoxaflor on the survival and foraging behaviour of the solitary bee, *Osmia bicornis*. We found that long-term exposure to the highest field realistic dose (50 μg dm^{-3}) of sulfoxaflor reduced more than 50% of the bee population within five days. Foraging behaviour was reduced in all doses tested, however, bees exposed to 50 μg dm^{-3} were more heavily affected. Flower visitation and flying behaviour were negatively affected. Direct and indirect effects of the pesticide were found to change flight performance of bees drastically.

4.1. Survival of Osmia bicornis

We found that long-term exposure to the highest dose (50 μg dm^{-3}) of sulfoxaflor decreased the probability of survival to 25% in 120 h. Sulfoxaflor binds to the nicotinic acetylcholine receptor (nAChR) in neuronal associations of insects similarly to neonicotinoids (Matsuda et al., 2001) but with distinct actions (Watson et al., 2011). Several studies on neonicotinoids showed that the probability of bee survival decreases by long-term exposure in social bees (Moncharmont et al., 2003) and solitary bees (Anderson and Harmon-Threatt, 2019; Sgolastra et al., 2018).

Chronic exposure of systemic neonicotinoid pesticides (acetamiprid, imidacloprid) with a fungicide (myclobutanil) did not reveal a reduction in longevity in *O. bicornis* (Azpiazu et al., 2019). However, the results have pointed to an apparent high toxicity of imidacloprid for bees in the first days of exposure (Azpiazu et al., 2019). A high susceptibility was therefore registered along the first three days when newly emerged were

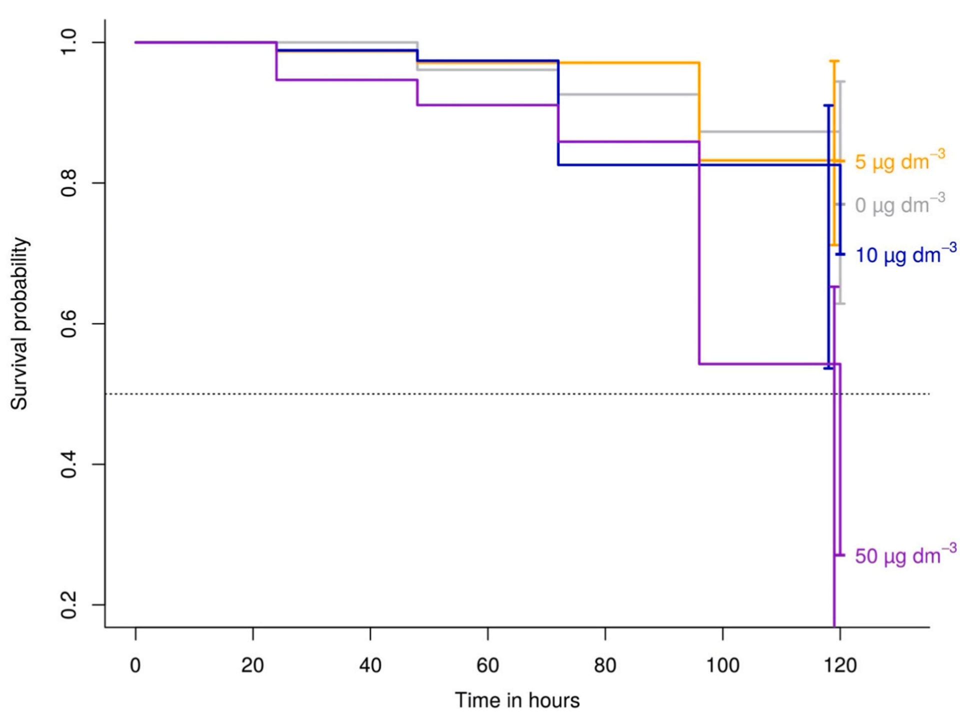

Fig. 1. Survival probability (full line represents the median) of *Osmia bicornis* bees fed with pure sucrose solution (control bees) and bees treated with three different doses of sulfoxaflor. Dashed line represents the lethal dose (LD_{50}). The highest dose of sulfoxaflor (50 μg dm^{-3}) decreased the original population size to a number smaller than 50% (dashed line) on the fourth day of observation. Lethal doses (LD_{50}) were observed on the 5th day of bees being exposed to 50 μg dm^{-3}.

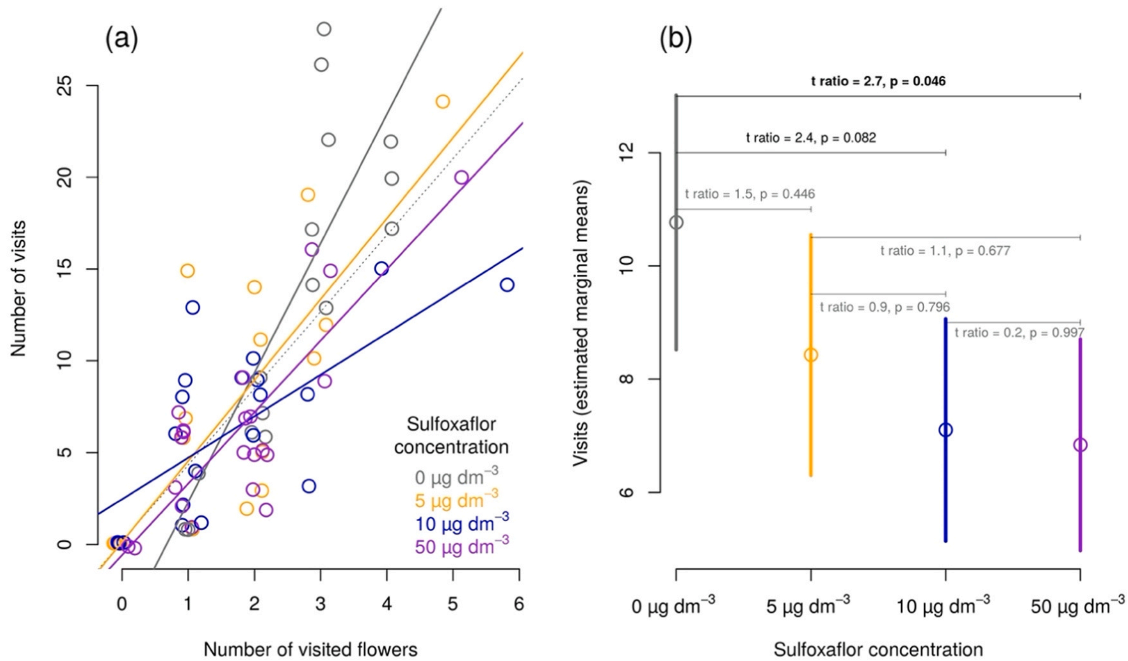

Fig. 2. (a) Sulfoxaflor concentration significantly decreases the number of visits in relation to the number of flowers visited. Lines indicate regressions adjusting to experimental groups. Dotted line represents the global linear model. (b) The visitation rate of *Osmia bicornis* is reduced when exposed to increasing doses of sulfoxaflor (partial effect in ANCOVA model with number of visited flowers, as shown in Fig. 2a).

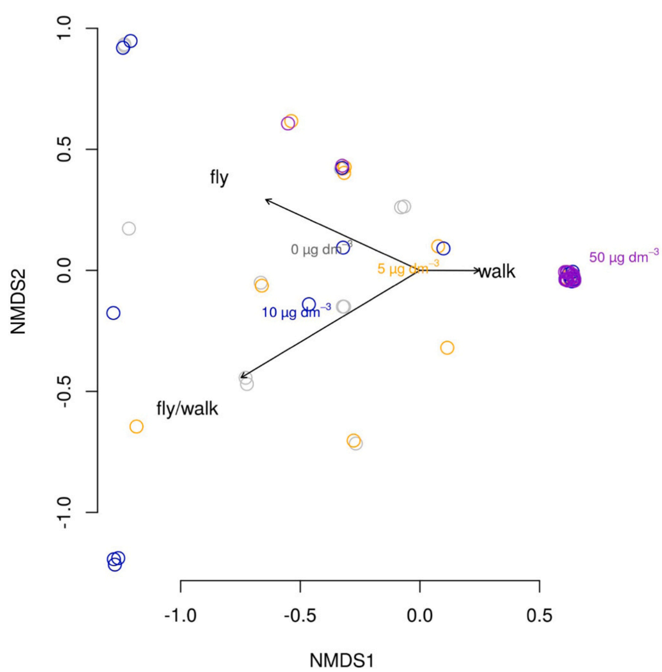

Fig. 3. Bees' ordination by non-metric multidimensional scaling (NMDS in two dimensions: stress = 0.002 and $R^2 = 1$) to relative differences (Bray-Curtis) in behaviour to access flowers in an experimental setup for sulfoxaflor concentration effect (control, 5, 10, and 50 µg dm^{-3}). Arrows indicate loading of each three attributes: fly, fly/walk, and walk. Position of experimental groups indicate correlations of them with ordination plan (free permutation).

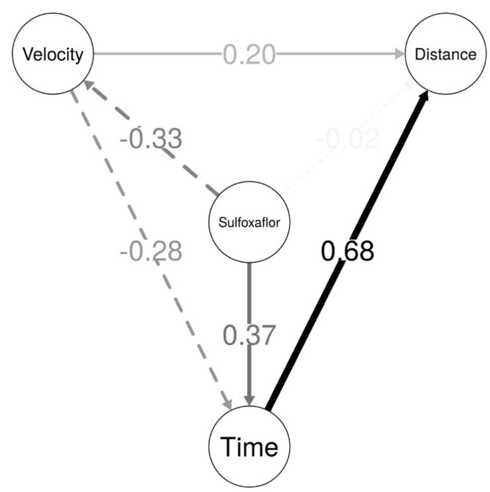

Fig. 4. Path diagram from effects of sulfoxaflor on flight performance of *Osmia bicornis* (standardized path coefficients are shown on arrows). Sulfoxaflor has a significant effect on flying time. Flying time was a significant determinant of distance. Sulfoxaflor impaired velocity but the effect was marginal; all other effects were not significant. Dashed arrows indicate negative effects.

exposed to a mix of clothianidin and propiconazole (Sgolastra et al., 2018). In our study with observations limited to five days and daily exposure to sulfoxaflor, the toxicity observed through survival analysis was significantly different between control and bees exposed to the highest dose (50 µg dm^{-3}) of sulfoxaflor with increased mortality from the 3rd to 5th days of exposure.

Bees inserted in the flight cages participated in a scenario where they had the availability of artificial flowers to drink sucrose solution free of pesticide. However, we cannot compare with the current experimental setup whether the amount of nectar ingested differed among groups, if lower doses are less prone to kill the bees or if ingestion of sucrose solution had led to a reduction of stress (Mayack and Naug, 2009). If this was the case, sucrose solution might have helped to enhance life expectation of bees exposed to five and 10 µg dm^{-3} sulfoxaflor, but this effect might have not been strong enough to avoid a high mortality of bees exposed to 50 µg dm^{-3}. Moreover, high doses of pesticides might have a stronger impact on the immune system of these bees (Brandt et al., 2020).

Although we interrupted our observation on the fifth day, previous studies with *Osmia* spp. reported survival for a longer period, around 17 days in lab conditions (Sgolastra et al., 2018). If our control bees had survived for a similar period, it would probably have indicated that bees exposed to 50 μg dm^{-3} of sulfoxaflor had a reduced life expectancy of more than 10 days.

4.2. Visit to flowers

Pesticide treated groups seemed to have a lower flower perception with significantly reduced flower visitation when they had been treated with the highest field realistic dose. This may reflect a reduction in the motivation to forage, as has been observed in bumble bees exposed to neonicotinoids (Lämsä et al., 2018). Several studies report the impact of systemic pesticides on the proboscides extension reflex and an impairment in olfactory learning (Démares et al., 2018; Hesselbach and Scheiner, 2018). In our study, responses to sucrose solutions were not mechanically stimulated by direct contact with bees' antennae. Instead, bees were free to feed on artificial flowers after a period of starvation known to stimulate bees to forage (Boff et al., 2020). Use of (artificial) flowers may emerge as a suitable alternative to test learning and nectar intake on non-proboscides extension reflex bees such as *O. bicornis* (Vorel and Pitts-Singer, 2010).

Despite the fact that flight capacity was harmed in bees exposed to sulfoxaflor (see below), we cannot exclude the possibility that treated bees showed a reduced responsiveness to sucrose, similar to when bees were exposed to flupyradifurone (Hesselbach and Scheiner, 2018) and were therefore less motivated to perform foraging flights. Individual responsiveness to sucrose, which depends on neuronal signaling cascades (Scheiner et al., 2003), had earlier been shown to affect the motivation to learn associations between flower characteristics and a sugar water reward in honeybees (Scheiner et al., 2005). The same mechanisms might work in *Osmia*, too.

Osmia bicornis is a generalist visitor of ruderal (Westrich, 2019) and crop flowers (Klatt et al., 2013) and the enhancement of pollination with this species was shown to be positive in reaching the optimum quality of a crop species (Herrmann et al., 2019; Ryder et al., 2020). Overall, *O. bicornis* exposed to sulfoxaflor decrease their visitation rate to 65% on average in comparison to control bees. If our findings are transferred to outdoor and indoor pollination systems, pollination could decrease to an alarming degree, because pollination efficiency seems to increase with high visitation rate, especially for cross pollinated plant species (Boff et al., 2018b; van Gils et al., 2016). Thus, decreasing the number of visits could trigger a reduction in yield (Fijen et al., 2020) by affecting fruit and seed production (Aizen et al., 2009; Classen et al., 2014).

The offspring production of wild solitary bees such as *Osmia* also relies heavily on the foraging activities of a single female. This requires the mother bee to perform a high number of foraging bouts every day (Gathmann and Tscharntke, 2002; Vicens and Bosch, 2000). If sulfoxaflor reduces the foraging activities in natural conditions to a similar extent as observed in our experiments, *O. bicornis* population outputs might be reduced as observed in the social bumble bees (Siviter et al., 2018). The impact on foraging observed here may also explain the reason why the number of brood cells decreased when *O. bicornis* were exposed to neonicotinoids (Sandrock et al., 2014), since neonicotinoids are known to impair foraging in bees (Gill and Raine, 2014).

4.3. Flight performance

Sulfoxaflor is a highly competitive agonist of the nicotinic acetylcholine receptors (nAChR), which play a role in the central nervous system and therefore impact insect locomotion. Indeed, we found that bees exposed to sulfoxaflor were less likely to fly or rarely flew when exposed to 50 μg dm^{-3}. The bees exposed to sulfoxaflor visited flowers mostly by walking, which is possible but unrealistic to be efficient as a flying bee in field conditions.

We hypothesized that the concentration of sulfoxaflor directly affects all parameters of flight performance, i.e. flight distance, time, and velocity. Time has effects on distance and velocity of flight, while velocity affects distance. Thus, sulfoxaflor indirectly affects flight velocity and distance, once it affected time. Treated bees spent 2.5 s on average to perform a foraging bout before they landed on a flower. Yet controls needed only 1.2 s on average to visit a flower. Tracking the movement of bees in space and time provided evidence for a changed flight performance between control bees and bees exposed to sulfoxaflor. All bees had been equally deprived of food before being inserted in the flight arena. Delays of nectar uptake may impose several risks to bees treated with sulfoxaflor, including a reduction in power of competition. Unexposed bees might perform foraging visits faster than treated bees, which may enforce starvation of the latter in situations when resources (e.g. nectar and pollen) are not produced continuously.

Negative effects of pesticides on foraging performance have been registered in social bees (Hesselbach et al., 2020; Kenna et al., 2019; Tong et al., 2019). In bumble bees, imidacloprid exposure led to hyperactivity and consequently higher velocity (Kenna et al., 2019). Nonetheless, hypoactivity, which was more likely to occur in *O. bicornis* exposed to sulfoxaflor, has been registered in other bees exposed to pesticides (Suchail et al., 2001; Tosi et al., 2017). Bees flying longer, at space and time scales, may be at higher risk of biotic and abiotic injuries such as predation and wind-dissection.

5. Conclusions

Sulfoxaflor reduces survival when bees are long-term exposed to the field realistic dose of 50 μg dm^{-3}. This pesticide further impairs foraging performance and flower visitation rate. Thus, sulfoxaflor may not only impact *Osmia* mortality, foraging behaviour and consequently reproduction, but may further have negative impacts on pollination outputs even at indoor environments.

CRediT authorship contribution statement

SB, JR, DL designed the study; SB performed the experiments; SB, JR performed statistical analysis; SB drafted the manuscript; SB, RS, JR, DL participated in discussions and contributed with the final version of the manuscript.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111869.

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