



Sulfoxaflor and nutritional deficiency synergistically reduce survival and fecundity in bumblebees

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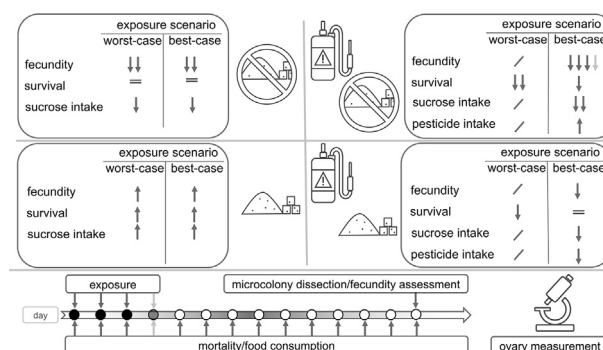
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HIGHLIGHTS

- We modelled exposure as a dynamic process, mimicking field degradation.
- Decaying sulfoxaflor exposure caused lethal and sublethal effects in bumblebees.
- These effects were exacerbated by nutritional stress.
- Dynamics of sugar consumption had a major influence on pesticide intake.

GRAPHICAL ABSTRACT



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ABSTRACT

A range of anthropogenic factors are causing unprecedented bee declines. Among these drivers the usage of pesticides is believed to be crucial. While the use of key bee-harming insecticides, such as the neonicotinoids, has been reduced by regulatory authorities, novel, less studied substances have occupied their market niche. Understanding the threat of these chemicals to bees is, therefore, crucial to their conservation.

Here we focus on sulfoxaflor, a novel insecticide, targeting the same neural receptor as the neonicotinoids. In stark contrast to the growing concerns around its negative impacts on bee health, a recent assessment has resulted in the extension of its authorisations across the USA. However, such assessments may underestimate risks by overlooking interactive impacts of multiple stressors. Here we investigated co-occurring, lethal and sublethal risks of sulfoxaflor and a dietary stress for bumblebees (*Bombus terrestris*), a key pollinator. Specifically, we employed a novel microcolony design, where, for the first time in bees, pesticide exposure mimicked natural degradation. We orally exposed workers to sulfoxaflor and a sugar-deficient diet in a fully factorial design.

Field realistic, worst-case sulfoxaflor exposure caused a sharp increase in bee mortality. At sublethal concentrations, sulfoxaflor negatively affected bee fecundity, but not survival. Nutritional stress reduced bee fecundity and synergistically or additively aggravated impacts of sulfoxaflor on bee survival, egg laying and larval production. Our data show that non-mitigated label uses of sulfoxaflor may have major, yet severely neglected effects on bumblebee health, which may be exacerbated by nutritional stress.

By unravelling mechanistic interactions of synergistic risks, our study highlights the need to overcome inherent limitations of Environmental Risk Assessment schemes, which, being based on a "single stressor paradigm", may fail to inform policymakers of the real risks of pesticide use.

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1. Introduction

Bees are vital ecosystem service providers (Klein et al., 2007). They enable reproduction in a significant proportion of wild and cultivated plants (Klein et al., 2007; Ollerton et al., 2011; Winfree, 2008) for a total value of 235–577 billion US dollars (IPBES, 2016). However, wild bees, including bumblebees, are undergoing widespread decline (Goulson et al., 2008; IPBES, 2016; Nieto et al., 2014), which may result in a shortfall in pollination services, with downstream effects on food production (Gallai et al., 2009; Garibaldi et al., 2011) and plant diversity (Biesmeijer et al., 2006) on a global scale. Agrochemical pesticides are believed to be one of the key anthropogenic drivers behind this decline (Potts et al., 2016). Initially, research aimed at characterising the risks of pesticides to bees was focused on neonicotinoid insecticides (Godfray et al., 2015; Wood and Goulson, 2017), which consequently went through a unique cycle of regulatory reassessments (Auteri et al., 2017). This resulted in a European ban of three of these substances (European Commission, 2018a, 2018b, 2018c). However, at the same time, new, potentially threatening systemic insecticides targeting the same family of pests have already been introduced to the market. Among these, the systemic insecticide sulfoxaflor has – despite claims of distinct chemistry and site interaction (Watson et al., 2011) – been suggested to have homologous site-specific activity to neonicotinoids (Ulens et al., 2019). Surprisingly, despite resultant concerns that this novel pesticide poses a potential threat to bee health (Brown et al., 2016; Siviter et al., 2019, 2018a), a recent regulatory decision led to the extension of field uses of this substance on bee attractive crops in the US (EPA, 2019). This is particularly concerning given that a recent European regulatory review highlighted knowledge gaps around chronic exposure of bumblebees to sulfoxaflor (EFSA, 2020, 2019). Nevertheless, sulfoxaflor-based products are still marketed across European Member States, awaiting further evidence to support risk management decisions.

Understanding the threat of novel chemicals like sulfoxaflor requires knowledge of likely exposure. Routes, levels and timing of bee exposure to pesticides are functions of a variety of behavioural and environmental factors, whose interactions remain unclear (Boyle et al., 2018; Gradish et al., 2018). Among the least investigated aspects of bee exposure is how it changes over time, as a function of pesticide degradation. Ecotoxicological studies of bees, either in the laboratory or under semi-field conditions, have used methodologies that model pesticide concentrations as static. However, real-world field exposure decreases over time (Kyriakopoulou et al., 2017), suggesting that incorporating time-dependent dynamics may be crucial if we are to understand real-world risks to bees. As static exposure ignores spikes, and may overestimate exposure duration, this exposure paradigm is prone to mischaracterisation of risks. We are not aware of published studies that simulate temporal patterns of exposure in bee laboratory studies. Thus, in order to explore time-dependent exposure dynamics of sulfoxaflor, here we use a conceptual framework adopted in other areas of ecotoxicology (Boesten et al., 2007) to reproduce field degradation in the laboratory.

While pesticides may be important drivers of bee declines, they do not act in isolation, and a range of other hazardous stressors, including poor nutrition (Brown et al., 2000), are likely to work in combination with pesticides to impact bee health in agricultural ecosystems (Goulson et al., 2015; Potts et al., 2016; Vanbergen and Insect Pollinators Initiative, 2013). Because Environmental Risk Assessment (ERA) schemes are based on the premise of a single stressor, single crop paradigm, they largely overlook how interactions between stressors impact bee health at the landscape level (Sgolastra et al., 2020; Topping et al., 2019).

Research on bee nutrition has largely focused on pollen consumption (Alaux et al., 2010; Schmehl et al., 2014). However, while pollen nutrition is essential for wild bee health and fitness, carbohydrates, which are largely derived from nectar, are key for energetic requirements (Leach and Drummond, 2018). Nectar foraging in social bees is regulated by a complex balance between individual and social energy

requirements (Heinrich, 1975; Leach and Drummond, 2018), as a function of which bees are required to maximise their foraging efficiency. In resource rich habitats, maximising foraging efficiency means choosing better quality nectar, while budgeting energetic and time costs (Cnaani et al., 2006; Harder and Real, 1987; Konzmann and Lunau, 2014). However, agricultural intensification may constrain the ability of bees to make optimal choices, by limiting floral diversity and, consequently, the availability of resources in the environment (Tscharrntke et al., 2005). In these conditions, where one or very few flowering species are likely to dominate the landscape, bees may settle for the more abundant flowering species and consume more nectar if its quality is low (EFSA, 2013). Consistent with this hypothesis, bumblebee males were shown to maximise nectar consumption when its sugar content was in the range of 10% to 20% (Brown and Brown, 2020). Nectar quality, per se, affects nutritional status and survival in bumblebee queens (Woodard et al., 2019) and exacerbates pesticide toxicity (Cecala et al., 2020; Tong et al., 2019; Tosi et al., 2017). However, an unexplored but key aspect of nectar quality is that it may modulate pesticide exposure by regulating nectar consumption rates (Cecala et al., 2020). Therefore, to understand how sulfoxaflor exposure and limits on carbohydrate nutrition interact to impact bumblebee health, we used a microcolony design (Laycock et al., 2012) to test the following hypotheses: 1) that time-decaying, realistic exposure to sulfoxaflor will impact bumblebee health; and 2) that poor nectar quality (hereafter defined as sugar deficiency) will exacerbate sulfoxaflor toxicity by a combination of physiological stress and increased pesticide intake.

2. Materials and methods

In this study we tested effects of pesticide exposure, nectar quality, and their interaction in a fully factorial microcolony design, both with the aim of mechanistically exploring this interaction and quantifying the possible confounding effect that sugar concentration may have in microcolony designs.

2.1. Pesticide exposure regimes

We re-analysed a residue dataset published in a recent European update on the ERA of Sulfoxaflor (EFSA, 2019) (full references to the individual study reports are included in the supplementary information, appendix A, S1) to design time-decaying realistic exposure regimes of sulfoxaflor in nectar. This dataset – which underwent evaluation by two regulatory authorities (EFSA, 2019; EPA, 2019) – consisted of 16 trials, each investigating residue levels in bee collected matrices at multiple time points after controlled, worst-case application of sulfoxaflor in flowering crops (i.e., pumpkin, strawberry, oilseed rape and apple).

We adapted the principles behind conceptual models linking exposure and effects in aquatic ecotoxicology (Boesten et al., 2007) to characterise risks to bumblebees from time-dependent exposure to sulfoxaflor. Specifically, we designed time-decaying exposure regimes in a 4-step process, which included i) the extraction of residue data from publicly available regulatory reports; ii) the modelling and iii) assessment of degradation kinetics (FOCUS, 2014) in nectar and iv) the quantification of daily exposure concentrations and the selection of exposure scenarios. We developed a repeatable methodology for the design of time-variable exposure in laboratory-based studies. Using this methodology we identified, across the investigated scenarios, pumpkin and strawberry uses to represent worst- and best- case exposure conditions respectively.

Further details are included in the supplementary information (Appendix A, 2.2 and 2.3).

2.2. Microcolonies

We obtained 14 queen-right captive colonies (*Bombus terrestris audax*, Agralan UK) of approximately 130 individuals with brood at

various stages of development. Upon arrival, five bees per colony were screened for the most prevalent gut parasites (*Apicystis bombi*, *Crithidia* spp., and *Nosema* spp.) through microscopic examination of faecal samples using a Nikon eclipse (50i) compound microscope at 400× magnification. No infections were detected.

Across experiments, 1344 medium-sized workers (OECD, 2017a) were allocated to 336 microcolonies by weight and colony of origin, with workers belonging to the same queen-right colony being housed in groups of 4.

Given the considerable number of bees, the allocation process took a total of four days. Prior to allocation, workers were weighed and housed individually in plastic cages (nicot system, Nicotplast SAS, FR). During this phase, workers were fed a 1:1 dilution of ambrosia syrup (Thornes, UK) through a two ml syringe (BD emerald, Becton Dickinson, USA) with the tip removed. This intermediate step, which lasted two days, was followed by two additional days where workers were transferred from nicot cages into microcolony boxes according to the allocation scheme described above. A small number of bees who died for unknown reasons or escaped (worst-case exposure, $N = 12$; best-case exposure, $N = 9$) during this process were replaced with individuals belonging to the same queen-right colony of origin. At the start of the test, colonies were distributed equally across treatment groups and there was no difference in the mean bodyweight across treatments (supplementary information, Appendix A, 2.4, mean (g) \pm SD; worst-case exposure, sucrose 15% = 0.25 (± 0.03), sucrose 50% = 0.25 (± 0.03), sulfoxaflor + sucrose 15% = 0.25 (± 0.04), sulfoxaflor + sucrose 50% = 0.25 (± 0.03); best-case exposure, sucrose 15% = 0.23 (± 0.04), sucrose 50% = 0.23 (± 0.04), sulfoxaflor + sucrose 15% = 0.23 (± 0.04), sulfoxaflor + sucrose 50% = 0.23 (± 0.04)).

Microcolonies were set-up in custom-made acrylic boxes (width: 50 mm; length: 115 mm; height: 65 mm) with a hole in one side to accommodate a horizontally positioned 15 ml centrifuge tube (Starstedt AG & Co. KG, DE), which had a 2 mm hole drilled at the end to function

as syrup feeder. A 35 mm diameter polystyrene petri dish base was used as a pollen feeder in each box. Over the course of the test, microcolonies were kept in darkness at controlled temperature and humidity conditions (supplementary information, appendix A, 2.4, average temperature \pm SD: worst-case = 24.4 \pm 0.7 °C; best-case = 24.5 \pm 0.7 °C; average humidity \pm SD: worst-case = 67.5 \pm 3.7%; best-case = 60.5 \pm 4.5%). Microcolonies were initially provided with fresh sucrose and pollen balls (\approx 1.5 g, not weighed) prepared by mixing fresh-ground pollen to distilled water in a 4:1 ratio. Consequently, fresh pollen pellets were provided four additional times over the course of the test (i.e., on day 2, 3, 9, and 12 or 1, 4, 9, 12 of exposure in the worst- and best-case exposure trials respectively). All pollen used across experiments was sourced from a local supplier (Agralan, UK). Screening of this pollen source found only low levels of mite control products used in honeybee hives, but not other agrochemicals (data not shown). Consequently, it is highly unlikely that this pollen contained residues of our focal insecticide, and any residues from veterinary chemicals will have been consistent across treatments. Pesticide exposure began one day after allocation, when untreated syrup was replaced with sucrose solutions (15% or 50% w/w) spiked with either distilled water (as the control) or sulfoxaflor (Table 1, Fig. 1, mean measured concentration (mg. a.s/kg \pm S.D.); worst-case exposure: day 1 = 1.37 \pm 0.009; day 2 = 0.146 \pm 0.004; day 3 = 0.013 \pm 0.003; best-case exposure: day 1 = 0.16 \pm 0.011; day 2 = 0.052 \pm 0.002; day 3 = 0.014 \pm 0.002; day 4 = 0.004 \pm 0.0003). Following exposure, treated syrups were replaced with untreated solutions with matching sucrose concentration, which were changed daily until day 14, when the experiment ended. Mortality was recorded daily and bees who died over the course of the exposure and post-exposure phases were not replaced.

Four bees were excluded, because they were unintentionally starved on day 1. Two additional bees escaped during the best-case experiment. Sucrose consumption (Fig. 2) was measured each day by weighing feeders to the nearest milligram (Scout® STX, Ohaus, CH). Daily

Table 1
The chemical analysis of sulfoxaflor treated syrups and stock solutions (mean of three samples).

Experiment	Day of exposure	Time (hh:mm)	Solution	Sugar concentration (% w/w)	Nominal concentration of sulfoxaflor	Measured concentration of sulfoxaflor (mg/kg) (mean, S.D.)	
N. 1: worst-case, pumpkin exposure	-	-	Stock	-	400	417 (7.693)	
			Syrup	50	1.36	1.37 (0.009)	
	1	00:00	24:00	Syrup	15	0.13	1.39 (0.058)
					15	0.13	1.35 (0.036)
		24:00	00:00	Syrup	50	0.013	1.32 (0.077)
					15	0.013	0.146 (0.004)
		24:00	00:00	Syrup	50	0.012	0.147 (0.001)
					15	0.012	0.131 (0.004)
	2	00:00	24:00	Syrup	50	0.012	0.14 (0.004)
					15	0.012	0.013 (0.003)
		24:00	00:00	Syrup	50	0.012	0.012 (0.0002)
					15	0.012	0.014 (0.0002)
24:00		00:00	Syrup	50	0.012	0.014 (0.0002)	
				15	0.012	0.014 (0.0002)	
N.2: best-case, strawberry exposure	-	-	Stock	-	400	407 (5.796)	
			Syrup	50	0.161	0.16 (0.011)	
	1	00:00	24:00	Syrup	15	0.047	0.17 (0.005)
					15	0.047	0.16 (0.013)
		24:00	00:00	Syrup	50	0.047	0.16 (0.004)
					15	0.047	0.052 (0.002)
		24:00	00:00	Syrup	50	0.014	0.052 (0.003)
					15	0.014	0.053 (0.002)
	2	00:00	24:00	Syrup	50	0.014	0.041 (0.002)
					15	0.014	0.014 (0.002)
		24:00	00:00	Syrup	50	0.014	0.012 (0.001)
					15	0.014	0.014 (0.0009)
24:00		00:00	Syrup	50	0.004	0.011 (0.0008)	
				15	0.004	0.004 (0.0003)	
3	00:00	24:00	Syrup	50	0.004	0.004 (0.0005)	
				15	0.004	0.004 (0.0004)	
	24:00	00:00	Syrup	50	0.004	0.005 (0.0009)	
				15	0.004	0.005 (0.0009)	

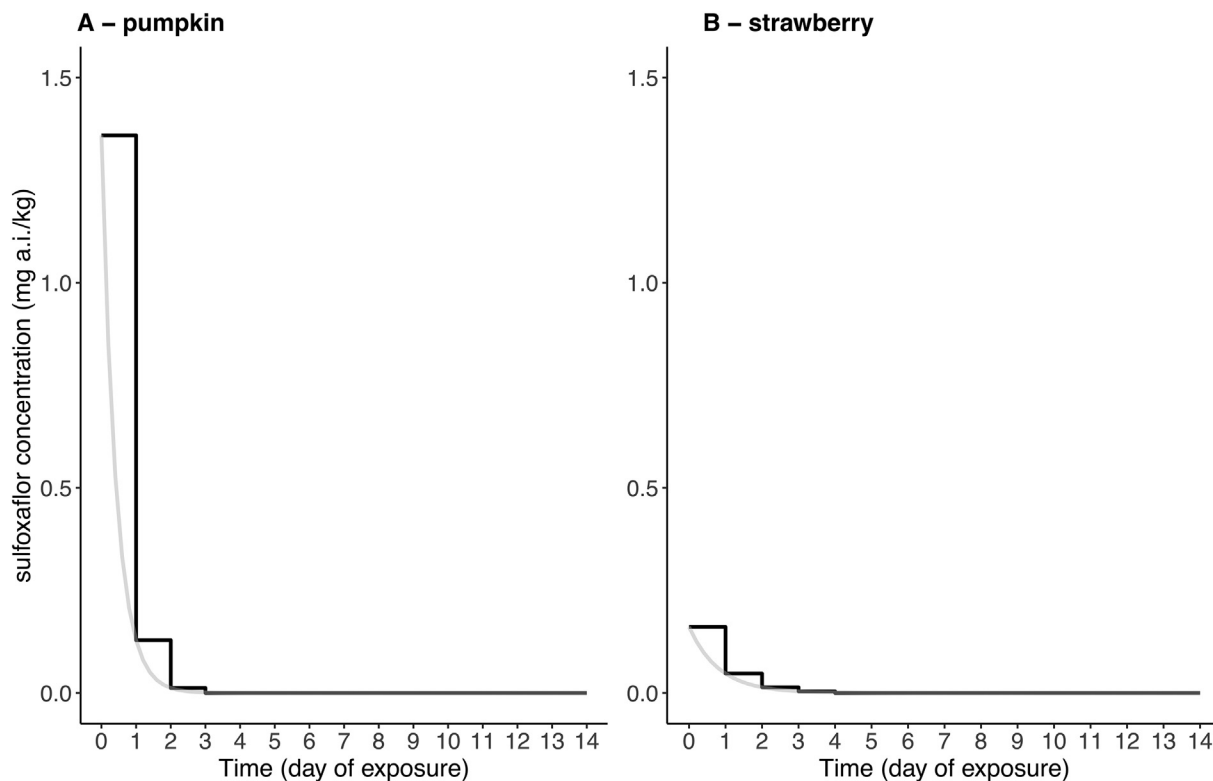


Fig. 1. The worst- (A) and best-case (B) short-term exposure scenarios. Black step lines graphically represent the tested exposure regimes (i.e., the daily nominal concentrations of sulfoxaflor in test solutions), matching the modelled exposure. The grey curve represents the SFO kinetic model (supplementary information, appendix A, 2.2 and 2.3) describing sulfoxaflor levels in nectar starting one day after the spray application of sulfoxaflor in pumpkin (A) and strawberry (B) (re-analysis of EFSA (2019), see Appendix A, S1).

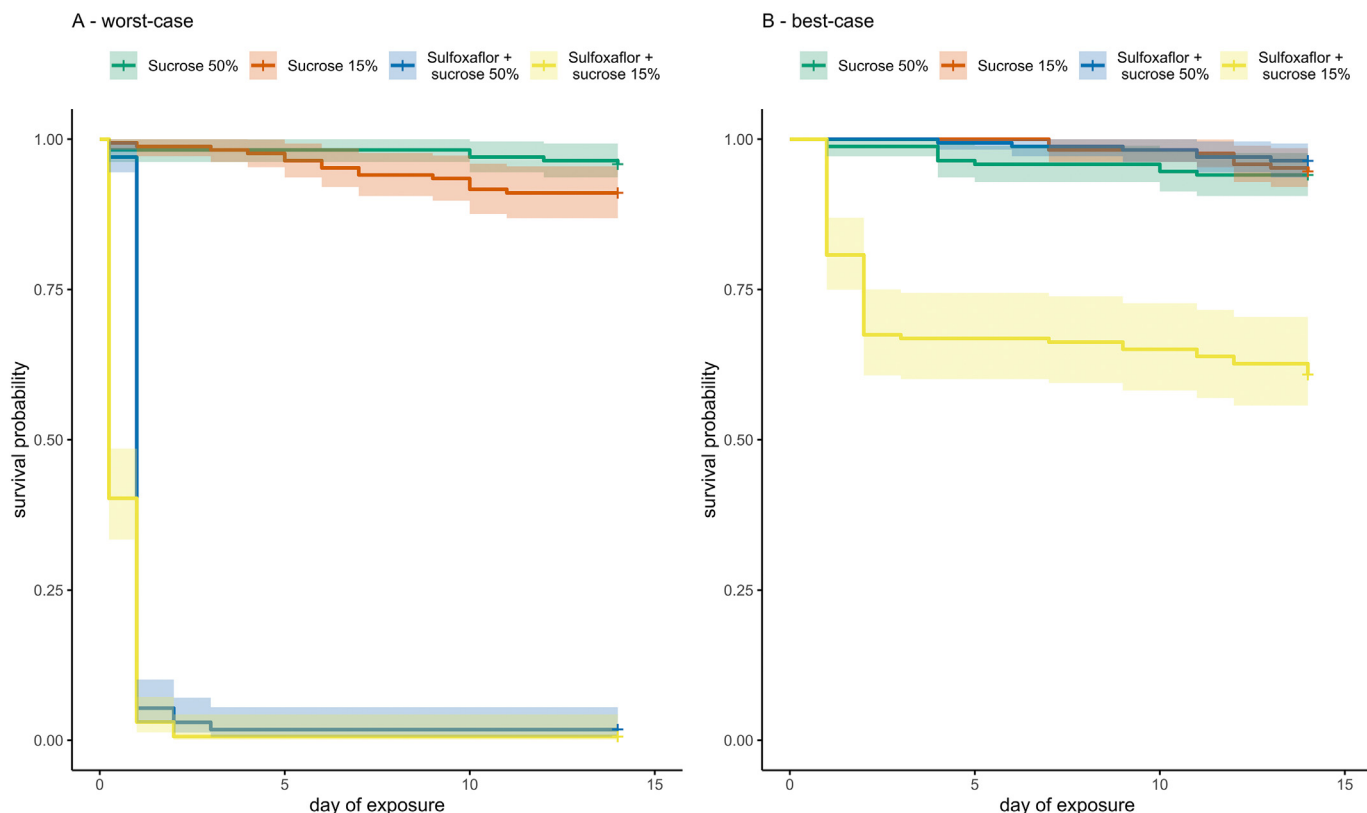


Fig. 2. The Kaplan-Meier curves ($\pm 95\%$ CI) for the cumulative probability of survival over time of bumblebee workers under worst- (A) and best-case (B) exposure conditions.

consumption was adjusted by evaporation, measured as weight change of four syrup feeders per sugar concentration ($n = 16$) kept in empty microcolony boxes under the same experimental conditions.

2.3. Assessment of fertility and fecundity

At the end of day 14, live bees were individually frozen at -20°C for later quantification of their ovary development and measurement of their thoracic width. At the same time eggs and larvae were removed from each microcolony and counted.

Workers were dissected in Ringer's solution (Sigma-Aldrich, UK) using a stereo microscope (Nikon SM2800) at 10–30 X magnification to determine the length of their terminal oocytes (Brown et al., 2000). Following previous studies (Baron et al., 2017; Laycock et al., 2012) we quantified terminal oocyte size as the mean length of all intact terminal oocytes. All ovary dissections were carried out blind to treatment. The inter-tegular span was measured as a proxy for bee size (Cane, 1987) using digital callipers (Mitutoyo, UK). 11 size measurements were incorrectly recorded, and, therefore, discarded from the statistical analysis.

2.4. Probabilistic risk assessment

Probabilistic approaches make use of hazardous doses from species sensitivity distributions (Uhl et al., 2016), exposure concentration distributions (Chan et al., 2019) or both (Van Sprang et al., 2004; Verdonck et al., 2003) to characterise risks and related uncertainty to non-target organisms. Normally, these methods are used to quantify the likelihood of toxicity-exposure combinations exceeding acceptable risk thresholds (Van Sprang et al., 2004; Verdonck et al., 2003). Here we use exposure concentration distributions to identify the likelihood of bees experiencing equal or higher exposure levels – hence, risks – than those identified in our experiments. We collated EU (EFSA, 2019) and USA (EPA, 2019) regulatory datasets (full references provided in the supplementary information, appendix A, S1). As both datasets aimed to maximise the potential uptake of residues in nectar and pollen in agricultural fields, our assessment complies with a realistic-worst case paradigm. Using the methodology described in Chan et al. (2019), we modelled the cumulative probabilities of sulfoxaflor nectar concentrations in the field, where sulfoxaflor is sprayed at (or close to) flowering with little or no mitigation measures implemented. We derived the Exposure Concentration Distribution (ECD, see Fig. 5) for sulfoxaflor in nectar by fitting several distributions (i.e., log-normal, log-logistic, gamma, Weibull, normal) to the residue data via Maximum Likelihood. The best fitting model was chosen via comparison of the Akaike information criterion (AIC). Since the log-normal distribution was identified as the best fitting model with a large delta margin (i.e., $\Delta \text{AIC} > 22$, Appendix A, 2.6), model averaging was not used. 95% confidence intervals around the cumulative distribution were calculated by parametric bootstrapping (1000 iterations). Non-quantifiable residue data points (i.e., below the limit of quantification or determination) were fitted as censored data.

2.5. Pesticide treatments

Sulfoxaflor treated syrups were freshly prepared each day of exposure from the same batch of water-based stock solution (CAS n° 946578-00-3, technical grade active ingredient, 99.4% purity, Chemservice Inc., USA). Frozen-stored aliquots of the stock were defrosted each day of exposure for pesticide preparation. A fraction of the concentrated solution was resuspended in distilled water before mixing it into sucrose syrups.

Fresh and aged samples of sulfoxaflor solutions were sampled to analyse their pesticide content and to quantify possible degradation. Solutions were sampled immediately before administration ($N = 14$) and at the end of each exposure day ($N = 14$). Aged solutions were randomly sampled across experimental blocks directly from feeding tubes. In addition to treated syrups, aliquots of the stock solution ($N = 2$) were

sampled to verify sulfoxaflor concentration and storage stability. All samples were stored at -20°C immediately after collection and analysed within one month from collection.

Treated syrups and samples of the stock solution were analysed using Ultra-Performance Liquid Chromatography (UPLC) with positive ion Atmospheric Pressure Chemical Ionisation (APCI) Mass Spectrometry (MS). All analyses were carried out using the Acquity UPLC H-class system coupled with a QDa detector (Waters, USA; further details on UPLC methods and MS setup are provided in the supplementary information, appendix A, 2.5). Briefly, for chemical quantification, sulfoxaflor calibration curves were derived using a range of pesticide solutions spanning from 5 to 20 ng a.i./ml. These solutions were made by diluting a methanol-based stock solution of sulfoxaflor (99.4% purity, Chemservice Inc., USA) in acetonitrile/Milli-Q water (10v/90v). Additionally, prior to the analysis, atrazine d-5 (CAS n° 163165-75-1, PESTANAL®, Sigma-Aldrich) was added to both calibration standards and treatment solutions at the concentration of 1 ng/ml, as an internal standard. Analyses were carried out using 10 μl injection volumes. When necessary, particularly concentrated samples were diluted prior to being analysed. The limits of detection (LOD) and quantification (LOQ) of our analytical methodology were identified at 0.003 and 0.01 mg a.i./kg respectively.

Each solution was analysed in three replicates, with each replicate analysis being initially repeated twice. Because recoveries were consistent across analyses of the worst-case exposure experiment, we did not repeat analyses of individual replicates during the best-case exposure experiment.

2.6. Sugar diet treatments

We defined nutritional stress as sugar deficiency. Recent analyses (Pamminger et al., 2019), consistent with earlier estimates (Heinrich, 1975), have shown nectar concentration in wildflowers and crops to vary from 10% w/w to 80% w/w, with some of the lower quality nectars being identified as crop species. Similarly, a recent review (Knopper et al., 2016) reported nectar concentration of strawberry and cucurbits (i.e., the botanical family of pumpkin) to vary from 26% w/w to 36% w/w and 20% w/w to 50% w/w, respectively. Other crops are reported to typically produce nectar with a sugar content below 20% (e.g., pear (Benedek et al., 2000)). Based on these findings and aiming to explore the widest possible realistic variation of nectar quality, in this study we defined low- and high-quality diets as containing 15% w/w and 50% w/w sucrose respectively. Despite laboratory studies showing that bumblebees prefer slightly more concentrated nectars than 50% w/w (Bailes et al., 2018; Brown and Brown, 2020), we set this concentration as the upper limit of our assessment, as this value is the most commonly used across official lab and semi-field bee testing methodologies (OECD, 2017a; Oomen et al., 1992). As yet, there has been no harmonisation of types and sugar content of treated syrups in the micro-colony paradigm we use in this study (Klinger et al., 2019). Syrups were prepared by dissolving sucrose in distilled water proportionally to its nominal concentration. Upon preparation, solutions were tested with a refractometer (Eclipse, Bellingham Stanley™) to verify their sugar content.

2.7. Analyses

We used an information-theoretic approach to model selection based on AIC (Richards et al., 2011). For each analysis we built a set of candidate models, including a full model, all biologically meaningful subsets of the full model and a null model only containing the intercept and random effect. We then used Akaike weights to choose the best fitting model or set of models across candidates. Where a model could not be rejected with 95% confidence, model averaging was used, and parameter estimates, and confidence limits were based on full-set averaging of the 95% confidence set. Details on model selection and parameter estimates for each model are provided in Tables 2.6-1 and 2.6-2 of the supplementary information (Appendix A, 2.6).

Food consumption was analysed using a generalised linear mixed effect model with treatment, day and their interaction as fixed effects and microcolony as a random effect. We defined consumption as the mean daily amount of syrup or sugar processed (i.e., both the amount consumed and that stored into nectar pots) by a single bee over a period of 13 days. Specifically, nectar consumption was calculated as the daily amount of syrup consumed by a microcolony divided by the number of live workers in that microcolony. While the experiment ran for 14 days, we could not measure consumption on the last day, as microcolony dissections were done in parallel. Therefore, we analysed data only up to the 13th day of the experiment. Where feeding holes were found to be clogged up with wax residues due to bee activity ($N = 88$), or where data recording errors were identified ($N = 50$), datapoints were removed from the analysis.

We initially analysed the mortality risk of bees using a Cox proportional hazard model using treatment, bee size and their interaction as fixed effect. However, the inspection of Schoenfeld residuals against time revealed that the assumption of proportionality of hazard was violated. Therefore, we analysed survival using non-parametric, pairwise log-rank tests, adjusting the resulting p -values using the Bonferroni correction. One drawback of this approach is that log-rank tests do not allow the testing of multiple covariates. However, our allocation scheme was designed to control for colony and size effects. Therefore, we are confident that, if size was to be identified as a meaningful explanatory variable during model selection, its effects would not vary across treatments.

Fecundity, defined as mean production of viable larvae or eggs per microcolony, was analysed using a hurdle model. Hurdle models are two-part models used for the analysis of count dataset with an excess of zeros. This analysis first models the binary likelihood that a 0 value is observed, and, second, models the non-zero observations using a truncated Poisson or negative binomial model. In other words, hurdle models analyse two separate processes, one causing the potential absence of brood in a microcolony and a separate one influencing the abundance of eggs and larvae in the microcolonies where brood is present. For analyses of egg and larval production we used a zero-altered Poisson model using treatment as a fixed effect and colony of origin as a random effect.

We carried out a post-hoc analysis on the fecundity dataset (Siviter et al., 2020) to determine whether the sulfoxaflor exposure and sugar deficiency interacted antagonistically, additively or synergistically. We ran the same zero-altered Poisson model and used pesticide exposure, sugar deficit and their interaction as fixed effect and colony of origin as a random effect (supplementary information, Appendix A, 2.6).

Ovary development was defined as the proportion of workers who successfully developed measurable terminal oocytes. It was analysed using a generalised model with a binomial error structure and a logit link function, with treatment, size and their interaction as fixed effects (supplementary information, appendix A, 2.6 and 3.1). Mean terminal oocyte length was analysed using a generalised linear model with treatment, size and their interaction as fixed effects (supplementary information, appendix A, 2.6 and 3.1).

Degradation kinetics were modelled using CAKE v3.3 (Tessella Technology and Consulting, 2020), which was recently validated (Ranke et al., 2018) for the study of standard kinetic models and up to three metabolites in a single compartment. All other analyses were performed in R (3.6.1 R Core Team, 2019) using lme4 (Bates et al., 2015), survival (Therneau, 2020), survminer (Alboukadel et al., 2020), ggplot2 (Wickham, 2016), glmmTMB (Brooks et al., 2017), MuMIn (Barton, 2019) and ssdtools (Thorley and Schwarz, 2018).

3. Results

3.1. Sulfoxaflor and low-sugar diet negatively impact survival

Worst-case sulfoxaflor exposure caused a sharp decrease in survival, when compared to the untreated high-sugar diet (Fig. 2A, log-rank test, Bonferroni correction, $p < 0.001$; sulfoxaflor + high-sugar diet: $p <$

0.001 ; sulfoxaflor + low-sugar diet: $p < 0.001$). In addition, the low-sugar diet further reduced the likelihood of survival of bees exposed to sulfoxaflor in the worst-case scenario (Fig. 2A, log-rank test, Bonferroni correction, $p < 0.001$). However, survival was not reduced, in comparison to the untreated high-sugar diet, when bees were fed the untreated low-sugar diet (Fig. 2A, log-rank test, Bonferroni correction, $p = 0.46$).

In the best-case scenario, mortality was not influenced by sulfoxaflor alone, when exposure occurred through a high-sugar diet (Fig. 2B, log-rank test, Bonferroni correction, $p = 1$). However, when sulfoxaflor was combined with the low-sugar diet, it significantly increased mortality (Fig. 2B, log-rank test, Bonferroni correction, $p < 0.001$). Consistent with results from the worst-case scenario, the low-sugar diet alone did not affect survival when compared to the untreated high-sugar diet in the best-case scenario (Fig. 2B, log-rank test, Bonferroni correction, $p = 1$).

3.2. Low-sugar diets and sulfoxaflor reduce fecundity

The low-sugar diet significantly reduced the likelihood of bees laying eggs in the worst-case scenario, but not in the best-case scenario (Fig. 3 A-B, Hurdle zero count (Hzc), worst-case scenario: PE = 0.92, CI = 0.01 to 1.83; best-case scenario: PE = 0.85, CI = -0.06 to 1.76). Conversely, the proportion of microcolonies with viable larvae was only reduced by the low-sugar diet under the best-case exposure scenario (Fig. 3 C-D, best-case scenario: Hzc: PE = 1.17, CI = 0.22 to 2.13; worst-case scenario: Hzc: PE = 1.15, CI = -1.16 to 3.46). However, across successful microcolonies, the low-sugar diet always caused a reduction in the number of eggs and larvae (Fig. 3 A-B-C-D, Hurdle truncated Poisson (HtP), worst-case exposure, eggs: PE = -0.41, CI = -0.058 to -0.23; worst-case exposure, larvae: PE = -0.51, CI = -0.61 to -0.4; best-case exposure, eggs: PE = -0.28, CI = -0.43 to -0.13; best-case exposure, larvae: PE = -0.37, CI = -0.52 to -0.21).

Sulfoxaflor exposure alone did not affect the likelihood of bees laying eggs (Fig. 3 B, Hzc, best-case exposure: PE = -0.19, CI = -1.14 to 0.76) or their abundance if laid (Fig. 3 B, HtP, best-case exposure: PE = -0.10, CI = -0.23 to -0.36), and nor did it affect the proportion of microcolonies with larvae (Fig. 3 D, Hzc, best-case exposure: PE = -0.35, CI = -0.65 to 1.35). However, sulfoxaflor exposure significantly reduced the number of larvae produced across successful microcolonies (Fig. 3 D, HtP, best-case exposure: PE = -0.32, CI = -0.45 to -0.18).

Interestingly, when paired with a low-sugar diet, sulfoxaflor exposure caused a significant reduction in the likelihood of laying eggs and producing larvae (Fig. 3 B-D, Hzc, best-case exposure, eggs: PE = 0.99, CI = 0.046 to 1.92; best-case exposure, larvae: PE = 2.17, CI = 1.15 to 3.18). Similarly, simultaneous exposure to the two stressors significantly reduced the total number of eggs and larvae per microcolony (Fig. 3 B-D, HtP, best-case exposure, eggs: PE = -0.91, CI = -1.12 to -0.71; best-case exposure, larvae: PE = -0.43, CI = -0.63 to -0.22).

When combined, sulfoxaflor and sugar deficit additively impacted the likelihood of laying eggs and synergistically reduced their abundance if laid (Fig. 3 B, Hzc, best-case exposure, eggs: PE = 0.33, CI = -0.99 to 1.64; HtP, best-case exposure: PE = -0.54, CI = -0.80 to -0.28). As with the likelihood of egg-laying, the impact on larval production of the combined effects of the two stressors was additive (Fig. 3 B, Hzc, best-case exposure, eggs: PE = 0.31, CI = -0.83 to 1.44; HtP, best-case exposure: PE = 0.12, CI = -0.19 to 0.43).

3.3. Low sugar diets increase food consumption

For the worst-case exposure experiment, due to the exceptionally high levels of mortality observed in the sulfoxaflor groups, we could only analyse food consumption of the untreated syrup groups. Across experiments, bees maintained on a low-sugar diet processed higher amounts of syrup (Fig. 4 A-B, Linear Mixed-Effects Model (LMEM), worst-case exposure: parameter estimate (PE) = 1, 95% CI = 0.92 to

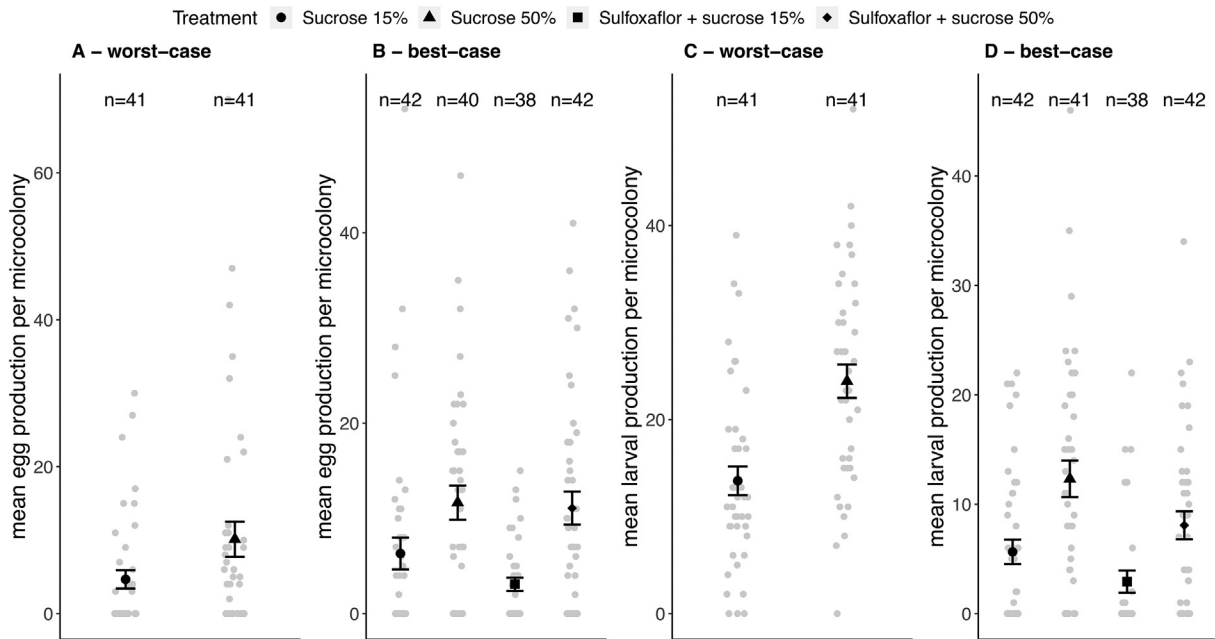


Fig. 3. The mean (\pm SE) number of eggs (A, B) and larvae (C, D) produced per microcolony.

1.09; best-case exposure: parameter estimate (PE) = 0.85, 95% CI = 0.72 to 0.98). However, despite higher consumption rates, their sucrose intake was still significantly lower than bees fed on the high-sugar diet (Fig. 4 C–D, LMEM, worst-case exposure: PE = -0.06, 95% CI = -0.08 to -0.04; best-case exposure: PE = -0.09, 95% CI = -0.12 to -0.07). Interestingly, best-case sulfoxaflor exposure caused a significant

reduction of sucrose consumption (Fig. 4 D, LMEM, high-sugar diet: PE = -0.03, 95% CI = -0.06 to -0.01; low-sugar diet: PE = -0.1, 95% CI = -0.19 to -0.13). However, we found no statistical support that bees exposed to sulfoxaflor alone processed lower volumes of syrup than bees in the control group (Fig. 4 B, LMEM, PE = -0.07, 95% CI = -0.20 to 0.06). Bees exposed to sulfoxaflor through the

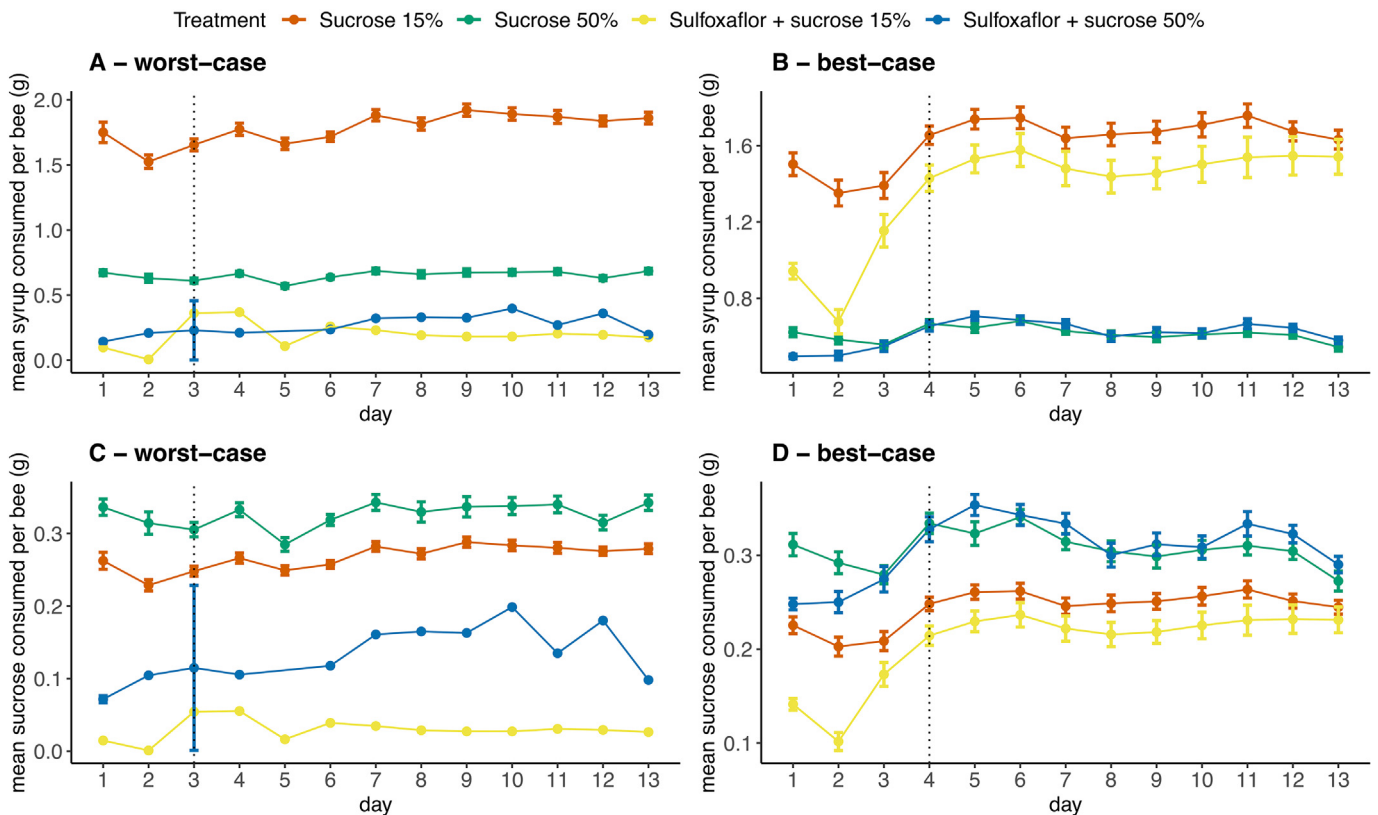


Fig. 4. The mean (\pm SE) consumption of syrup (A, B) and sucrose (C, D). Dotted lines graphically separate days of exposure (left) from the post-exposure phase (right).

sugar-poor diet were still able to volumetrically adjust food consumption relative to the control (Fig. 4 B, LMEM, PE = 0.40, 95% CI = 0.27 to 0.53).

3.4. Field exposure may exceed the tested scenarios

The exposure concentration distribution (ECD) was best described by a lognormal distribution (Fig. 5, parameter estimate (mean, mg a.i./kg) = -3.0 , S.D. = 2.25; lower quartile, PE = 0.0108, CI = 0.00687 to 0.0167; median, PE = 0.0497, CI = 0.0328 to 0.0756; upper quartile, PE = 0.229, CI = 0.140 to 0.370). The illustrative risk assessment was produced by plotting the gradient of concentrations tested in our worst- and best-case exposure regimes against the ECD. The probability of field exposure exceeding the exposure regimes tested in these experiments was quantified as 7.2% and 30% for the worst- and best-case scenarios respectively. In other words, where sulfoxaflor is used without mitigation measures in place (i.e., during or close to flowering), 7.2% and 30% of exposure situations might lead to higher risks than those we quantified in the worst- and best- case exposure scenarios.

3.5. Chemical analyses

Chemical analyses (Table 1) showed that the actual concentrations of test solutions matched the nominal values with negligible deviations. Indeed, the few observed mismatches were well within minimum acceptable ranges proposed by official methodologies for bee testing (OECD, 2015). Additionally, over the course of each day of exposure, no obvious degradation was detected.

4. Discussion

Field-realistic time-decaying sulfoxaflor exposure caused dramatic lethal and sublethal impacts to bumblebees. In addition, under both worst- and best-case time-variable exposure profiles, poor nectar

quality synergistically exacerbated sulfoxaflor risks by simultaneously inducing physiological stress and increasing pesticide intake.

When we mimicked an unrestricted pesticide use scenario on cucurbits, sulfoxaflor exposure, but not sugar deficit, caused a sharp increase in mortality, which was further aggravated by sugar deficiency. Conversely, when we mimicked a less severe application scenario on strawberry survival was not affected by sulfoxaflor alone (i.e., administered through a high-sugar diet). However, the same pesticide exposure regime resulted in a significant decrease in survival when bees were also fed a sugar deficient diet. Together, these data show that the effects of field-realistic sulfoxaflor use on bumblebee populations go beyond sublethal (Siviter et al., 2018a) to lethal effects. Our results show that bee-safe uses of sulfoxaflor, if possible at all, cannot overlook the crucial importance of in-bloom mitigation measures. Use restrictions should allow the substance to degrade to safe levels before flowering of the treated crop or weeds in the field, which may also be contaminated by pesticide drift. Both the effectiveness of such measures, and their appropriateness as farming practice should be urgently investigated.

In our study, sulfoxaflor exposure and sugar deficiency affected fecundity, but not fertility in bumblebee workers, both individually and interactively. Sugar deficiency, but not sulfoxaflor exposure alone, resulted in a reduction of egg laying. However, when sulfoxaflor was administered through a sugar deficient diet, its effects on egg laying were significantly more severe than those caused by the sugar deficit alone. Siviter et al. (Siviter et al., 2019, 2018a) showed that a 2-week long exposure to 0.005 mg/l of sulfoxaflor impacted egg laying, larval production and reproductive success in bumblebees. Recent evidence (EFSA, 2019; EPA, 2019) suggests that transient spikes, rather than prolonged steady concentrations, better describe sulfoxaflor exposure patterns in agricultural fields (see C_0 and DT_{90} values in the supplementary information, Appendix A, 2.3). While testing a best-case time-decaying exposure regime we did not find egg laying to be reduced by sulfoxaflor exposure alone. However, we observed a severe reduction in egg laying when the same exposure regime was administered

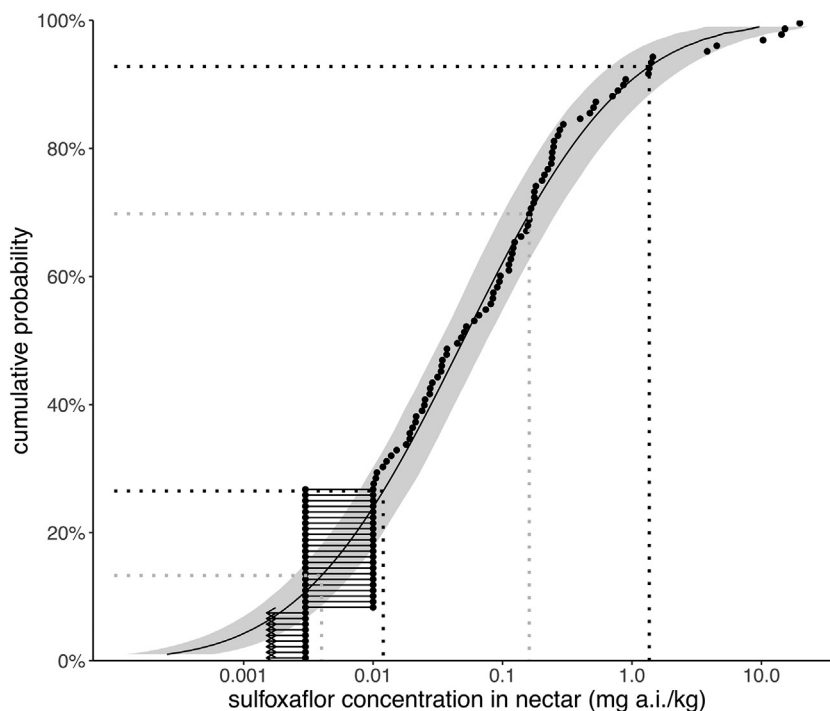


Fig. 5. The exposure concentration distribution ($\pm 95\%$ CI) of sulfoxaflor in nectar of agricultural crops (i.e., apple, strawberry, pumpkin, oilseed rape, alfalfa, peach and sunflower – (EFSA, 2019; EPA, 2019), see Appendix A, S1). Dotted lines show the upper (right) and lower (left) limits of the worst- (black) and best-case (grey) exposure regimes. Non quantified residue data points (i.e., below the limits of detection or quantification) were plotted as censored values (horizontal segments).

through a sugar-poor diet (Hedge's $d = -0.98$ relative to the control). This effect was higher in magnitude than that driven by the longer, steady exposure regime described above (Hedge's $d = -0.37$) (Siviter et al., 2019). Best-case sulfoxaflor exposure caused a more severe reduction in larval production per microcolony (Hedge's $d = -0.441$) than has been observed in previous studies (Hedge's $d = -0.36$) (Siviter et al., 2019). Overall, our study demonstrates that spike, decaying, short-term exposure causes more severe health risks to both survival and fertility than a steady, longer regime (Siviter et al., 2019).

Sulfoxaflor and sugar deficiency were found to affect larval production both individually and interactively. We believe the effect driven by sulfoxaflor alone on larvae was likely a consequence of reduced egg laying (Siviter et al., 2019) and nursing behaviour (Crall et al., 2018). It is unlikely that sulfoxaflor exposure had direct lethal effects on larvae as, due to the dynamics of exposure in our experiments, larvae were unlikely to have been directly exposed to sulfoxaflor at any point in time.

A field-realistic laboratory exposure to the neonicotinoid imidacloprid in microcolony designs has been shown to cause sublethal, but not lethal, effects on bumblebees, reducing their fecundity, but not fertility, by one third relative to the control (Laycock et al., 2012). The reduction of larval production observed in our study for sulfoxaflor has a comparable magnitude to that described above for imidacloprid. Laycock and Cresswell (2013) also demonstrated that the reduction in fecundity observed for imidacloprid may be reversible once the imidacloprid exposure is ended. In contrast with these findings, we found no evidence that microcolonies recovered in the post-exposure phase. This means bee recovery from sulfoxaflor exposure – if possible at all – may require longer timeframes than two weeks. This is crucially important, as sulfoxaflor based products can be applied multiple times with an interval of less than 14 days (e.g., closer SC in the USA can be applied during flowering of strawberry and cucurbits up to four times – two of which may be consecutive – with a minimum interval of 7 days, specimen label revision 07/25/19 downloaded from <https://www.corteva.us/products-and-solutions/crop-protection/closer-sc.html>). Therefore, real-use exposure scenarios of sulfoxaflor consist of consecutive pulses, whose risks for bumblebee populations are likely to add up over time.

Our results clearly demonstrate the importance of time-dependent exposure, and thus have significant implications for the definition of field realism in bee toxicity studies. The lack of standardised approaches in exposure assessment has sparked criticism that laboratory studies are not field-realistic and expose bees to higher doses than would be seen in the field (Carreck and Ratnieks, 2014). This resulted in suggestions that the use of field-realistic laboratory designs should be discouraged and replaced by field-scale experiments (Eisenstein, 2015). However, currently adopted “higher-tier” field designs have been shown both to be overly focussed on honeybees and lacking in the power needed to detect colony-level effects (Franklin and Raine, 2019). Consequently, there is a growing consensus that microcolony studies could become key tools for risk characterisation in a regulatory context (Babendreier et al., 2008; EFSA, 2013; EFSA PPR, 2012; Klinger et al., 2019). However, current definitions of field-realism are highly contentious and prone to subjectivity (Siviter et al., 2018b). The current lack of standardisation in exposure assessments may therefore jeopardize our ability to properly assess the risk of pesticides to bees, and other beneficial organisms, in the context of ERA. Consequently, we believe that our approach, which objectively quantifies the dynamics of field exposure to pesticides, can provide a key tool in bee toxicology.

Sucrose consumption was also reduced by sulfoxaflor exposure. Additionally, syrups with lower sucrose concentration resulted in lower sucrose intakes, despite higher syrup consumption rates. This confirms that workers' ability to volumetrically increase food consumption as a response to the reduced sugar content is limited by their capacity to process water, similar to the situation in males (Brown and Brown, 2020). These data emphasise the importance of nectar quality as a predictor of pesticide toxicity, hence demonstrating how the lack of

harmonisation of sugar content in the treated solutions (Klinger et al., 2019) may undermine the reliability of the microcolony paradigm. This is particularly relevant, as bumblebees do not perform trophallaxis (the act of sharing of food between individuals (Moritz and Hallmen, 1986)), which indirectly enables honey bees to even out the amount of processed food across individuals (OECD, 2017b). Within a group of bumblebees, each bee is likely to process a different amount of food, as a function of individual energy requirements. Uneven exposure of group-housed bumblebees may hinder our ability to reliably infer the pesticide dose received by an individual bumblebee in microcolony designs. Since we cannot reliably estimate doses, microcolony studies have quantified risks as function of exposure concentrations. However, our results show that the exclusive reliance on the concept of field realistic concentration is likely to bias the characterisation of risks, if the critical role of sugar concentration is overlooked.

Our time-variable exposure estimates reflect a worst-case realistic paradigm, given that residues were measured shortly after in-bloom spray applications (EFSA, 2019). However, we still believe that our exposure regimes are conservative. Residues in pollen, which were not included in our assessment or experimental paradigm, were often found to be higher than nectar (EFSA, 2019; EPA, 2019) (Appendix A, S1, the highest residue levels were 1.9 to 4.3-fold higher in pollen than nectar for pumpkin and strawberry respectively). Consequently, as bees are likely to be exposed to both contaminated pollen and nectar in the field, our exposure levels in these experiments are conservative. Additionally, as shown in the ECD we demonstrated that exposure levels in the field may exceed those tested in our experiment, if farmers do not implement in-bloom mitigation measures while spraying sulfoxaflor. While in Europe, for instance, it is recommended that sulfoxaflor-based applications are stopped five days before flowering of the crop to reduce exposure peaks, such restrictions – whose effectiveness for non-*Apis* bees is yet to be demonstrated (Azpiazu et al., 2021; Tamburini et al., 2021) – are often deemed impractical outside the EU, (e.g., for crops with indeterminate bloom, like pumpkin and strawberry in the USA). One of the arguments for the safety of such unrestricted use is the quick degradation of sulfoxaflor in pollen and nectar (EPA, 2019). However, our experiment shows that this characteristic is insufficient to ensure the protection of bees, since peak exposure alone can cause significant impacts on mortality and fitness.

In conclusion, we emphasise the urgent need for a deeper understanding of field exposure (i.e., including routes other than via the treated crop) and effects of sulfoxaflor across a wider range of worst-case real-use scenarios, to ensure the safety and survival of bees in agro-environments.

CRediT authorship contribution statement

Conceptualization: A.L., M.J.F.B.; data collection, curation, analysis, visualisation, writing - original draft: A.L.; chemical analyses: C.R., S.V.; writing - review & editing: M.J.F.B., C.R., S.V.; supervision: M.J.F.B., S.V.; funding acquisition: M.J.F.B.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.148680>.

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