**Sulfoxaflor exposure reduces bumblebee reproductive success**

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**Abstract**

**Intensive agriculture currently relies on pesticides to maximise crop yield1,2. Neonicotinoids are the most widely used insecticides worldwide3, but increasing evidence of negative impacts on important pollinators**4–9 **and other non-target organisms**10 **has led to legislative re-assessment and created demand for the development of alternative products. Sulfoximine-based insecticides are the most likely successor**11**, and are either licensed for use or under consideration for licensing in several worldwide markets3, including within the European Union**12 **where certain neonicotinoids (imidacloprid, clothianidin & thiamethoxam) are now banned for agricultural usage outside of permanent greenhouse structures. There is an urgent need to pre-emptively evaluate the potential sub-lethal effects of sulfoximine-based pesticides on pollinators**11**, because such effects are rarely detected by standard ecotoxicological assessments, but can have major impacts at larger ecological scales**13–15**. Here, we show that chronic exposure to sulfoxaflor (a sulfoximine-based insecticide), at dosages consistent with potential post-spray field exposure, has severe sub-lethal impacts on bumblebee *(Bombus terrestris*) colonies. Field-based colonies that were exposed to sulfoxaflor during the early growth phase produced significantly fewer workers than unexposed controls, and ultimately produced fewer reproductive offspring. Differences between the life-history trajectories of treated and control colonies first became manifest when individuals exposed as larvae began to emerge, suggesting that direct or indirect effects on a small cohort may have cumulative long-term consequences for colony fitness. Our results caution against the use of sulfoximines as a direct replacement for neonicotinoids. To avoid continuing cycles of novel pesticide release and removal, with concomitant impacts on the environment, a broad evidence base needs to be assessed prior to the development of policy and regulation.**

**Main text**

The widespread global use of highly effective neonicotinoid-based pesticides has led to the evolution of resistance amongst several insect crop pests16, and generated worldwide interest in emerging sulfoximine-based alternatives that have been shown to be effective in targeting some neonicotinoid-resistant species17–19. This potential lack of cross-resistance may reflect differences in three-dimensional molecular structure that preclude the breakdown of sulfoximines by enzymes involved in neonicotinoid metabolism20, supporting the claim that sulfoximines and neonicotinoids are chemically distinct17. However, as selective agonists of insect nicotinic acetylcholine receptors (NAChRs)17, the two pesticide groups share a common biological mode of action. This raises major concerns about potential effects on non-target species, and particularly bees. Neonicotinoids, while not lethal to bees at field realistic levels, have severe sub-lethal effects on both social and solitary bees, influencing cognition, foraging ability, homing ability, reproductive output, colony initiation 5,7,8,15,21–25, and, potentially, pollination services26. Mathematical modelling has shown that these sub-lethal stressors can have drastic negative consequences for colony fitness downstream in the colony cycle14,15.

To assess whether sulfoxaflor, the first marketed sulfoximine-based pesticide, has similar negative effects on bees, we fed either untreated sucrose solution (1.8M), or sucrose solution containing 5μg/dm3 (5 ppb) ofsulfoxaflor, to nascent *Bombus terrestris* colonies reared from wild-caught queens. We based this concentration on available estimates for sulfoxaflor residues in forager-collected nectar post-spray27 (Extended Data Fig.1A), because spray application is currently the most common application procedure (although products containing sulfoxaflor have also been developed for seed-treatments and are already available for use on bee-pollinated crops within some markets28). After two weeks of laboratory-based exposure, size-matched colonies were placed in the field around a university parkland campus following a paired design, and no longer provided with additional resources. Staggered weekly nocturnal censuses revealed a clear difference in colony demographics between control and experimental colonies. The bumblebee colony cycle is characterised by an early growth phase in which worker numbers increase rapidly to create a large workforce, followed by a switch to production of reproductive brood later in the season. Between 2 and 3 weeks post-exposure, detectable differences in worker numbers between treated and control colonies began to emerge, persisting until close to the end of the colony cycle (Figure 1A; Table S2D; glmer: treatment parameter estimate = -0.28, 95% CI: -0.48 to -0.01; treatment:week parameter estimate = -0.06, 95% CI: -0.11 to -0.01, treatment:week2 parameter estimate: 0.11, 95% CI: 0.05 to 0.16).

As the colony cycle progressed, negative impacts upon colony reproductive output became apparent. Treated and control colonies were equally likely to produce male reproductive offspring, but treated colonies produced significantly fewer males in total (Zero-inflated count model, binomial section, treatment parameter estimate = 0.71, 95% CI = -0.67 to 2.09; count section, treatment parameter estimate = -0.54, 95% CI: -0.72 to -0.37; Figure 2). This difference became apparent from approximately week 9 onwards (Figure 1B). The dry mass of these males was no different to those produced by control colonies (*wi* (null model) = 0.974), indicating that our results cannot be explained by differential investment in reproductive biomass. Neither treated nor control colonies produced an abundance of queens, but control colonies produced more than treated colonies (total: 36 new gynes from 3 of 26 control colonies, none in any of 25 treated colonies) and so our findings hold when the total number of sexual offspring is analysed (Zero-inflated count model, binomial section, treatment parameter estimate = 0.71, 95% CI = -0.67 to 2.09; count section, treatment parameter estimate = -0.64, 95% CI: -0.81 to -0.46). The timing of reproductive onset, queen longevity and colony survival did not differ between control and treated colonies (Extended Data Fig. 2; Survival analyses, treatment parameter estimate (reproductive onset) = -0.05, 95% CI: -0.41 to 0.31; (colony longevity) = -0.03, -0.43 to 0.38); (queen survival) = -0.07, -0.47 to 0.33).

Based on the neonicotinoid literature, we considered the explanation that this difference in the production of sexual offspring might be mediated through poor provisioning of larvae by foraging workers9,21, at the time when sexual offspring were developing. However, daytime foraging censuses revealed no significant differences in the relative number of bees returning to control and treated colonies (generalized linear mixed model, treatment, parameter estimate = -0.07, 95% CI: -0.32 to 0.19). Similarly, although visual inspection of the data was suggestive of a lower proportion of workers returning with pollen to pesticide-treated vs. control colonies from week eight onwards (Fig. 1C), this effect did not receive statistical support (glm, week:treatment, parameter estimate = -0.14, CL: -0.29 to 0.001; treatment, parameter estimate = 0.46, CL:-0.38 to 1.31) and furthermore occurred too late in the colony cycle to explain differences in male production, which became apparent at approximately the same time. We also found no significant differences in the size of pollen loads collected between control and pesticide-treated colonies (Extended Data Fig.3). Instead, consideration of the timing of differences between control and treated colonies suggests that impacts of sulfoxaflor exposure on reproductive output were mediated by the early drop in worker numbers that began at weeks 2-3 post-exposure. Bumblebee worker pupae take approximately 14 days to develop29, so the onset of deceleration of colony workforce growth corresponds to the eclosion of individuals that had encountered maximum exposure as larvae (Figure 1D). It remains unclear whether this failure to eclose was driven by direct effects on exposed larvae30 , or indirect effects, perhaps mediated by poor provisioning9,21 by exposed workers (although note that colonies were provided with pollen and sucrose in the laboratory during this time). In either case, the resultant drop in worker numbers led to deviation in the life-history trajectories of control and sulfoxaflor-treated colonies, with consequent effects on colony reproductive output14. These knock-on effects of early exposure to a small cohort of colony members are entirely consistent with the results of mathematical explorations of stress impacts on bee colonies, which predict that chronic stress at an early stage can push bee colonies beyond a ‘tipping point’, increasing the likelihood of colony failure14.

Sulfoxaflor is a systemic pesticide that is soluble in water and thus is transported around plant tissues following foliar or seed application. The likely exposure trajectory of pesticide crop treatments differs between seed treatments, which deliver prolonged exposure, and spray applications, which deliver a short-term dose that is initially high but typically declines rapidly. Sulfoxaflor, like neonicotinoid-based pesticides, can be administered using both methods, and sulfoxaflor-based products that are used as a seed-treatment have recently been developed for bee-attractive crops (including oilseed crops)31. However, most currently marketed preparations are spray applications. The dosage used in this study is below US EPA estimates for field-realistic immediate post-spray concentrations of sulfoxaflor in forager-collected nectar, and remains below residual concentrations estimated at 10 days post-spray (the maximum period for which data are available; concentration range over whole period: 5.41 to 46.97µg a.i./kg, application rate: 2 x 0.045 lb active ingredient /A; Extended Data Fig.1A & B)27. Note that our treatment protocol is particularly conservative in that our nascent colonies were fed untreated pollen in addition to the syrup provided, potentially producing significant underestimates of effects on larvae. Post-spray sulfoxaflor residues in pollen have been documented to be more than tenfold higher than those in forager-collected nectar (Extended data Fig. 1A & 1B), ranging from 510.95 to 50.12 µg a.i./kg over the same post-spray period27 . Mitigation measures can be used to reduce bee exposure to sulfoxaflor when used as spray treatments (for example, spray application to bee-attractive crops during bloom is precluded by law in the USA) 32, but globally, under current usage such measures are often either absent33 or limited to product label recommendations to avoid spray 6 days prior to bloom34. No such measures are possible for those products that have been developed as a seed treatment31.

The impact of sulfoxaflor identified here can be compared with previous experiments that focused upon exposure to neonicotinoids. For example, bumblebee colonies placed next to oil seed rape fields that were treated with neonicotinoids exhibited a 71% reduction in the mean number of queen cocoons found within the nest6 and a 32-36% reduction in the mean number of males/workers produced7. Similarly, colonies foraging next to thiacloprid-treated raspberry crops had a 46% reduction in reproductive output35 and commercial bumblebee colonies exposed to imidacloprid for a period of two weeks had an 85% reduction in the number of new queens produced8. Here, we found that sulfoxaflor exposed colonies had a 54% reduction in the total number of sexual offspring produced compared with control colonies, suggesting that from the perspective of wild pollinators, sulfoxaflor exposure could lead to similar environmental impacts to neonicotinoids if used on bee-attractive crops in the absence of evidence-based legislation.

 Sulfoximine-based pesticides are a newly-emerging class of product, but are already licensed in many countries worldwide, including China3, Canada28 and Australia36. Within the European Union, where the use of certain neonicotinoids is now banned for open-field crops, substances containing sulfoxaflor as an active ingredient have been assessed by the European Food Safety Authority37 and approval has been granted for use in 5 member states, with applications from seven more member states currently in progress38. Our results provide pre-emptive evidence that, if exposure at equivalent dosages to those used in our study occurs via bee-attractive crops pre- or during bloom, either through spray or seed treatment applications, these products could pose a significant risk to pollinators. The effects that we identified were the longer-term outcome of initial short-term exposure, and were only detected by monitoring of the full colony cycle. Bans and restrictions on neonicotinoid-based pesticides have largely been implemented to protect important pollinators such as bees, following years of widespread use with potential long-term population-level consequences. To avoid a situation whereby pesticides such as neonicotinoids are replaced with products that are similarly contentious, regulatory bodies should move towards an evidence-based approach that assesses both the lethal and sub-lethal consequences of novel insecticides such as sulfoxaflor on non-target organisms, and incentivises integrated pest management approaches, before products are licenced for use39.

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**Figure 1: The impact of sulfoxaflor exposure on bumblebee colony life-history trajectories. Week-by-week colony field census data for (a) Number of workers, for treated (n = 26) and control colonies (n = 26) (b) Number of sexual offspring, and (c) Proportion of workers returning to the colony with pollen, for treated and control colonies (n = 25 and 26 respectively; reduced sample size for treated colonies reflects the death of one queen at week 2, see methods). Data presented show means ± SE. Demographic timeline (d) indicates the time points at which (i) laboratory-based exposure started (exposure period indicated in red), ii) colonies were moved into the field (iii) adults that encountered maximum exposure as larvae should begin to emerge**29 **and iv) maximum colony lifespan.**

**Figure 2 Male offspring production. Mean (±SE) number of male sexual offspring produced by sulfoxaflor-treated (n=25) and control (n=26) colonies.**

**Methodology**

*Exposure regime*

Sulfoxaflor-based preparations have been developed for use on a wide range of bee-attractive crops that flower at varying times of the year. The regime used in our study most closely mimics spring-flowering crops in temperate environments, allowing comparison with similar neonicotinoid-based studies6,7,15 that also exposed colonies for a short period during the early growth phase.

Preparations containing sulfoxaflor as an active ingredient are currently most commonly applied as a foliar spray. We thus based our pesticide concentrations on the best available information from a realistic and bee-relevant spray experiment reported by the USA EPA, in which sulfoxaflor was applied to a cotton crop at an application rate of 2 x 0.045 pounds of active ingredient per acre. Under this application regime, mean sulfoxaflor residue levels in honeybee-collected nectar did not drop below 5μg a.i./kg over an 11-day period (the maximum period for which data are available; Extended Data Fig.1A)27. We are confident that our exposure is conservative because a) in the same experiment, pollen residue levels did not drop below 50μg a.i./kg3 27 (Extended Data Fig.1B), while we provided all colonies with untreated pollen *ab libitum*, andb) this application rate is similar to label recommendations for at least some sulfoxaflor-based products33. A second study has also measured residues (in cucumber), but application rates were 1.5 times above recommended usage, and the relevance of this experiment for bees is unclear as the cucumber tissue sprayed and sampled was not described40.

In terms of current usage, our data are most relevant to sulfoxaflor preparations when sprayed on crops immediately prior to or during bloom (note that this practice has recently been reviewed and prohibited in the USA27). While some product labels recommend avoidance of spraying 6 days before bloom34, this ignores experimental data showing that residues could remain present in pollen at levels which we show to have sub-lethal impacts after this 6-day period27 (Extended Data Fig.1D). Other labels allow spraying during bloom at night33. To the best of our knowledge, no data are currently available on field-realistic residues for seed-treatment preparations that have been developed for use on oilseed crops and are already available in some markets28.

*Queen rearing*

332 bumblebee (*Bombus terrestris audax*) queens were caught between the 28th of February and the 23rd of March 2017, from Windsor Great Park, Surrey, UK. Chilled queens were transported to the laboratory, where their faeces were microscopically examined for parasites (*Nosema* spp, *Apicystis bombi, Sphaerularia bombi* and *Crithidia bombi*; x400 magnification). Parasitized individuals (N = 54) were removed from the experiment. A second parasite screening was repeated after one week (29 further queens removed, remaining N=249).

Queens were placed in rearing boxes (W 67 x L 127 x D 50; Allied Plastics, Kingston, UK) and were provided with a gravity feeder containing an *ad libitum* supply of 1.8M sucrose solution (changed weekly; Thorne, Windsor, UK) and a pollen ball (changed twice-weekly, unless the queen was laying eggs in which case more pollen was added; Biobest, Westerlo, Belgium). Each queen was housed in a dark/red-lit room maintained at 26°C and 50-60% relative humidity. Queens that did not produce eggs after eight weeks were removed from the experiment (N=107). Once a queen had produced at least 6 workers, the colony was moved into a wooden nest box (W 280 x L 320 x D 160mm) and randomly assigned to a treatment group (see below). The time taken to reach this stage varied but on average was 7.2 weeks (± sd: 1.5 weeks). On transfer, the queens underwent a final parasite screening (2 queens removed). 2 queens died prior to transfer, thus 52 colonies reached this stage. The use of colonies from wild-caught queens is a key feature of our experimental design that enabled us to a) have a complete overview of the lifecycle of these colonies (both in the laboratory and the field, see below), and b) use colonies with a life-history that was adapted to the local environment.

*Pesticide exposure*

Prior to pesticide exposure, colonies were allocated to control and treatment groups and paired for size according to the number of workers present (mean = 8.43 ± SD 1.87). Each colony was then provided with an *ab libitum* supply of either 1.8M sucrose solution containing 5µg/dm3 (5ppb) sulfoxaflor (derived from a stock solution of 1g/dm3 in acetone; Greyhound chromatography and allied chemical, Merseyside, UK), or 1.8M sucrose containing equivalent acetone concentration but no sulfoxaflor, for a two-week period. Sucrose solution was weighed on placement in and removal from the colony; no differences in consumption were found between treatment groups (*wi*(null model) = 0.985). During the exposure period we recorded the number of workers produced, colony mass and the number of dead workers, on a weekly basis. One queen died during the exposure period, thus 51 colonies were present at the start of the field experiment (control N = 26 & pesticide N = 25).

*Field placement*

After two weeks of exposure in the laboratory, colonies were moved into the field. Nest boxes were placed within plastic field boxes (W 440 x L 710 x D 310mm; Really Useful Box, Kingston, UK) containing insulation wrap (Thermawrap, Creswell, UK) and aluminium foil, and placed at locations around the Royal Holloway University of London campus, Egham, UK (45ha; Extended Data Fig.4). Paired colonies were matched for location within the campus, and were positioned at least 20 metres from one another to reduce drifting. Each colony entrance was demarcated by a distinctive visual pattern. Colonies were placed in discreet, shaded and south-east facing locations, and secured with a ratchet strap to avoid badger damage. To prevent usurpation attempts from other queens and social parasite species *(Bombus vestalis*)*,* queen excluders were placed on each colony. Upon initial placement in the field the colonies were supplied with a gravity feeder containing 46g 1.8M sucrose solution, after which they received no further food supplements. The process of field placement was staggered over six weeks (10/04/2017- 21/05/2017) owing to variation in the date at which queens were initially caught. The week of placement was included as a predictor in each statistical analysis (see below).

*Data collection*

We combined methodological approaches from previous studies of the effects of neonicotinoids on bumblebees8,21 , as well as studies of bumblebee life-history41 to maximise our measurement of both impacts and potential mechanisms. We conducted censuses every night such that each colony was visited once per week, between the hours of 21:30-04:00. Using a red-light torch, we recorded the number of live workers (average of three counts), dead workers, males, and new queens. We also recorded the state of the original queen (dead or alive), the presence of gyne larvae and/or pupae, the presence of worker larvae and/or pupae, the number of pollen and nectar pots containing stores, and the mass of the colony (average of three recordings; EM-30KAM balance, A&D instruments). In cases where the wax covering prevented observation, we peeled it back in order to conduct the count. Weekly censuses continued until moribundity, defined as either a live queen and 3 or fewer workers, or no queen and 10 workers or fewer42. After the experiment, all sexual offspring that had been found in the colonies (N = 600) were dried for 72 hours and weighed (accuracy = + 0.001g).

All 51 colonies were also visited during daylight hours twice per week. Colony traffic (number of bees entering and leaving the nest) was recorded during 10-minute counts, once between 9:00 & 13:00 and once between 14:00 & 18:00. We also recorded whether returning workers had large (pollen basket was over-flowing) or small (pollen enclosed within pollen basket) pollen loads relative to their body size. Control and pesticide pairs were always observed directly after one another, in a random order. The average daily temperature, humidity and total rainfall were obtained from a local weather station (www.wunderground.com).

**Statistical analyses**

We employed an information theoretic model selection approach. For each response variable, the initial candidate set included a full model and all subsets, including a null model. Reported parameter estimates and confidence intervals are based upon full-set averaging of the 95% confidence set (i.e. the set of models with cumulative Akaike weight > 0.95). Model types, error structuring, a list of parameters included within each model and parameter estimates are provided in tables S1 & S2. Briefly, to analyse the number of workers produced per week, we used a generalized linear model (glmer; Poisson error structure) with colony nested within pair as a random factor, and the week of initial field placement (week started), treatment, week of experiment and a two-way interaction between treatment and week of experiment as fixed factors. Since the number of workers increased to a maximum and then decreased for each colony, “week of experiment” was modelled as a quadratic factor (ΔAIC between full linear and full quadratic model: 1206.40). Many colonies did not produce sexual offspring, so we used zero-inflated generalized linear models (zeroinfl) to analyse differences in both the overall number of sexual offspring and the number of males produced by colonies, with the week of initial field placement, treatment and their interaction as predictors. The number of workers returning to the nest was analysed using a zero-inflated generalised linear model (glmmadmb; negative binomial error structure) in which treatment, week started, colony week and temperature were included as fixed factors and colony as a random factor. The proportion of workers returning with pollen was also analysed using a generalised linear model (glmmadmb: binomial error structure) with treatment, colony week and their interaction, week started, temperature, and time of day included as fixed factors and colony/pair included as a random factor. Week of reproductive onset and queen survival were analysed using a Cox proportional hazards survival analysis that contained treatment and week started as fixed factors. All analyses were conducted in R studio (Version 1.0.136) using the r packages*, pscl*43*, lme4* 44, *glmm*45*, MuMin*46 *survival* 47and *glmmadmb*48

**Data availability:** The full data set is available as an open science framework project.

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**Extended data Fig.1: Concentrations of sulfoxaflor in forager-collected resources from a USA EPA cotton study**27**. Mean µg of active ingredient (a.i.)/kg (± SE) found in the (A, C, E) nectar and (B, D, F) pollen of honeybees foraging on cotton crops sprayed with sulfoxaflor. Note the differences in y-axis scale between graphs, due to considerably higher concentrations in pollen. Red lines indicate spray application. Dosage: (A & B) twice over ten days at 0.045 lb a.i. per acre; (C & D) once over ten days at 0.045 lb a.i. per acre; (E & F) twice over ten days at 0.089 lb a.i. per acre. The black horizontal line indicates the equivalent amount of sulfoxaflor (5 ppb) that was fed to sulfoxaflor-treated colonies in sucrose, within our experiment. Data are means from two hives; number of individual bees sampled is not published.**

**Extended data Fig.2: Timing of colony life-history events. The probability of (a) reproductive onset, (b) queen survival and (c) colony survival for control (n = 26) and sulfoxaflor-treated (n = 25) colonies (± confidence intervals).**

**Extended data Fig.3: Pollen foraging. The mean proportion (± SE) of foragers returning to the nest with large pollen loads, for control (n = 25) and pesticide-treated (n = 22) colonies (note that not all of the colonies in the experiment had pollen foragers).**

**Extended data Fig.4) Distribution of colonies across the Royal Holloway Campus. Blue dots indicate control colonies, red dots indicate treated colonies (grid reference; TQ000706; Imagery ©Google, Map data©2018 Google).**