Real-time monitoring of honeybee colony daily activity and bee loss rates can highlight the risk posed by a pesticide

Lena Barascou, Ugoline Godeau, Maryline Pioz, Olivier Martin, Deborah Sené, Didier Crauser, Yves Le Conte, Cedric Alaux

Real-time monitoring of honeybee colony daily activity and bee loss rates can highlight the risk posed by a pesticide

Lena Barascou[1*], Ugoline Godeau[1], Maryline Pioz[1], Olivier Martin[2], Deborah Sené[1], Didier Crauser[1], Yves Le Conte[1], Cedric Alaux[1]

[1]INRAE, Abeilles et Environnement, Avignon, France

[2]INRAE, Biostatistique et processus Spatiaux, Avignon, France

* Corresponding author: lena.barascou@inrae.fr

Abstract

Information on honeybee foraging performance and especially bee loss rates at the colony level are crucial for evaluating the magnitude of effects due to pesticide exposure, thereby ensuring that protection goals for honeybee colonies are met (*i.e.* threshold of acceptable effects). However, current methods for monitoring honeybee foraging activity and mortality are very approximate (visual records) or are time-limited and mostly based on single cohort analysis. We therefore assess the potential of bee counters, that enable a colony-level and continuous monitoring of bee flight activity and mortality, in pesticide risk assessment.

After assessing the background activity and bee loss rates, we exposed colonies to two concentrations of sulfoxaflor (a neurotoxic insecticide) in sugar syrup: a concentration that was considered to be field realistic (0.59 µg/ml) and a higher concentration (2.36 µg/ml) representing a worst-case exposure scenario. We did not find any effect of the field-realistic concentration on flight activity and bee loss rates. However, a two-fold decrease in daily flight activity and a 10-fold increase in daily bee losses were detected in colonies exposed to the highest sulfoxaflor concentration as compared to before exposure. When compared to the theoretical trigger values associated with the specific protection goal of 7% colony-size reduction, the observed fold changes in daily bee losses were often found to be at risk for colonies. In conclusion, the real-time and colony-level monitoring of bee loss rates, combined with threshold values indicating at which levels bee loss rates threaten the colony, have great potential for improving regulatory pesticide risk assessments for honeybees under field conditions.

Keywords: pesticide risk assessment, specific protection goal, bee counter, mortality rates, foraging activity, *Apis mellifera*.

Introduction

Plant protection products, also referred to as pesticides, have been developed to protect crops against harmful organisms (*e.g.* insects, fungi) or prevent the growth of undesirable plants (herbicides). However, the yield and/or quality of around 75% of globally important crop types also depend on pollination mediated by animals, especially bees, which visit more than 90% of the leading 107 crop types (Klein et al. 2007). As a consequence, bees, including the honeybee (*Apis mellifera*), can be exposed to several pesticides recovered from pollen and nectar (Mullin et al. 2010, Pohorecka et al. 2012, Sanchez-Bayo and Goka 2014, Botías et al. 2015, Zioga et al. 2020). There is therefore a clear need to provide an assessment of the risk presented by pesticides and to determine to what extent they are safe for bees. For that purpose, OECD (Organization for Economic Co-operation and Development) and EPPO (European and Mediterranean Plant Protection Organization) test guidelines require toxicological data on honeybees (*Apis mellifera*). Indeed, the honeybee is relatively easy to rear, its

biology has been well-studied, and it is one of the most important pollinators worldwide (Klein et al. 2007, Hung et al. 2018).

Within the current regulatory framework for pesticide risk assessment, the effects of pesticides on honeybees are assessed by standard tests in a stepwise approach. In Tier 1, active substances or formulated products are tested on honeybees at different life stages (larvae and adults)(OECD 1998a, 1998b, 2013, 2017). Then, a deterministic approach to characterize risk quantitatively can be used by comparing the pesticide toxicity to environmental exposure (Hazard quotient - HQ). When the results of this first step identify a risk (for ex. HQ > 50 for foliar spray), semi-field and field tests on colonies (Tier 2 and 3) are required for marketing authorization (MA) (see also Thompson 2021 for risk identification upon oral exposure: Risk quotient > 0.4 and 1 in acute and chronic toxicity tests, respectively). Higher tier tests can also be performed in post-MA studies monitoring adverse effects linked to the use of pesticides.

Major issues are that the test guidance for the conduct of such semi-field and field studies (EPPO 2010) relies on measurements of several key colony parameters that are very approximate (Wang et al. 2020) or do not overly account for chronic and/or sub-lethal effects. For instance, the estimation of flight activity is based on the count for a few seconds of bees foraging on flowers near the hive, which represents a small snapshot of what happens over a day that does not take into account the daily changes in foraging activity according to the weather and others environmental factors (*e.g.* discovery of new food sources)(Winston 1987). In addition, mortality estimates rely mainly on the number of bees in dead bee traps in front of the hive. It excludes bees that have died outside of the traps, but also suchtrap cannot be used for long time periods since its precision decline over time due for instance to predation. Therefore, such method does not provide robust information on the background mortality. Counting the number of bees entering and exiting the hive can also be used to assess forager activity and bee losses given that worker bees die in the field or are removed from the hive if found dead inside. To accurately determine these assessment endpoints, researchers in the past years have relied on individual tagging and detection of bees at the hive entrance. Notably, radiofrequency identification (RFID) tags coupled with detectors at the hive entrance and bee counters recording the activity of bees marked with data-matrix barcodes have been successfully used in several studies to automatically record individual bee activity and mortality in response to pesticide exposure (Bortolotti et al. 2003, Schneider et al. 2012, Henry et al. 2012, Prado et al. 2019, Monchanin et al. 2019, Colin et al. 2019, Hesselbach et al. 2020, Shi et al. 2020, Barascou et al. 2021a). While individual bee tracking is highly effective, it provides time-limited data on honeybee flight activity and mortality, based on a small portion of the bee population, or on a single age-cohort, ignoring the fact that pesticide sensitivity may depend on age with older bees being more sensitive than younger bees to certain pesticides, and less to others (Mayland and Burkhardt 1970, Ladas 1972, Bendahou et al. 1997, Rinkevich et al. 2015, Zhu et al. 2020, Barascou et al. 2022).

These methodological gaps of current semi-field and field regulatory tests combined with the lack of tools and methods for the field assessment of bee activity and mortality at the colony level represent a major gap for pesticide risk assessment, especially since the measurement of bee mortality has emerged as a benchmark for evaluating the magnitude of effects due to pesticide exposure (EFSA 2013, EFSA et al. 2020). Indeed, specific protection goals (SPGs) have been established as the maximum permitted levels of colony size reduction resulting from exposure to pesticides, and the levels and duration of bee losses that would cause this specific decline in colony size are defined as trigger values. For instance, a reduction in colony size that does not exceed 7 % has been considered by EFSA (European Food Safety Authority) as negligible and therefore allowable for proper honeybee colony strength and development (EFSA 2013). Accordingly, it was determined by using a quantitative model of colony population dynamics (Khoury et al. 2011), that for a healthy colony forager mortality should not increase, compared with controls, by more than a factor of 1.5 for 6 days, a factor of 2 for 3 days or a factor of 3 for 2 days (EFSA 2013).

In this context, tools, such as bee counters, that allow a colony-level and continuous monitoring of bee flight activity and mortality, should pave the way toward a more reliable pesticide risk assessment (Odemer 2022). First attempts to evaluate the potential of bee counters in pesticide risk assessment have been performed (Struye et al. 1994, Ngo et al. 2019). However, a relatively low number of replicates was used and effects were not always statistically analyzed. We therefore aimed to further provide a proof-of-concept for the use of bee counters in pesticide risk assessment in honeybees by assessing *i)* the effect of a pesticide on the colony-level daily activity and bee loss rates and *ii)* the consecutive risk for colonies. For that purpose, we performed real-time monitoring of colony population flight activity with video-based bee counters. We first identified the background activity and mortality and then chronically exposed colonies either to a field realistic concentration of the insecticide sulfoxaflor or a higher concentration representing a worst-case exposure scenario. Finally, to assess the risk posed by sulfoxaflor to honeybee colonies, we compared the level and duration of bee losses to the theoretical trigger values associated with the SPG of 7% colony-size reduction. Sulfoxaflor is a sulfoximine-based insecticide that shares a mode of action with neonicotinoids as selective agonists of nicotinic acetyl choline receptors (nAChRs) (Sparks et al. 2013). It is increasingly used as an alternative to neonicotinoid insecticides for controlling sap-feeding insect pests since several neonicotinoids are now banned in the European Union (Stokstad 2018) and there are numerous cases of resistance to this class of insecticides, contrary to sulfoxaflor so far (Watson et al. 2021). Sulfoxaflor was used for assessing the potential of bee counter in pesticide risk assessment under chronic exposure (even though its formulation product Closer is registered as a foliar spray) since we previously characterized its concentration-dependent toxicity in laboratory experiments (Barascou et al. 2021b, Barascou et al. 2022). These laboratory tests and concentrations could then be used as landmarks for the toxicity tests performed with bee counters.

Materials and methods

Experimental setup

Experiments were performed in 2021 in a peri-urban area near Avignon (France, 43°540N-4°-520E) with honeybee colonies (*Apis mellifera*) from INRAE (Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement) livestock. All colonies were treated yearly in August with Apistan® (tau-fluvalinate) against the parasite *Varroa destructor*. Information on the landscape characteristics, within a 1.5 km radius area around the apiaries (mean foraging range; Steffan-Dewenter and Kuhn, 2003), was obtained from the French national remote sensing database on vegetation layers (Institut Géographique National) via BeeGIS (https://appli.itsap.asso.fr/app/01-beegis). The landscape was predominantly surrounded by urban areas (49%), cropping systems, including meadows, vegetables and cereal crops (32%), dunes and sand (10.5%), orchards (4.5%) and forests (4%).

Each colony, composed of five Dadant frames, was selected and then randomly assigned to one of the three treatments (see below 'Exposure to sulfoxaflor'). The day before exposure to sulfoxaflor, we assessed the demographic state and food storage (pollen and honey) of each colony. For that purpose, each side of each frame, was visually inspected and the area covered by adult bees, open and closed brood, as well as honey and bee bread, were reported in percentages (one full side = 100%). Percentages were then converted into numbers of adult bees, open and capped brood cells, and area in dm^2 of honey and pollen based on a previously determined coefficient for Dadant frames (Hernandez et al. 2020). Colonies were visually inspected again at the end of exposure and 10 days post-exposure to sulfoxaflor by following the same methodology (Hernandez et al. 2020). Finally, colony overwintering survival was checked the following year (March 2022).

Exposure to sulfoxaflor

Colonies were left untreated for four days and then exposed to one of the two sulfoxaflor concentrations, depending on their randomly assigned treatment groups. The sulfoxaflor concentrations were chosen based on pesticide residue data found in pollen and nectar. Depending on the application rates and the crops, field residue studies reported levels of sulfoxaflor ranging from 0.04 to 2.37 mg/kg (e.g. 0.047 to 2.80 µg/ml) in nectar collected by bees and from 0.00447 to 31.8 mg/kg (e.g. 0.005 to 37.52 µg/ml) in the nectar of flowers (EPA 2019). Thereby, colonies were provided with a solution of 50 % (w/v) sucrose, 0.1 % acetone and sulfoxaflor at a field-realistic concentration (0.5 µg/ml) or a higher concentration representing a worst-case exposure scenario (2 µg/ml). Similar concentrations were previously found to be non-toxic (0.02 µg/ml) or to significantly decrease bee survival (2.35 µg/m) in laboratory tests (Barascou et al. 2021b). Control groups were fed with pesticide-free sugar solutions (50 % w/v sucrose, 0.1 % acetone) and were used to determine whether differences in colony activity could be attributed to variation in field conditions (*e.g.* climate, resources). Stock solutions of sulfoxaflor (Techlab, France) were diluted in acetone, aliquoted and conserved at -20°C. The exact concentrations

were checked with LC-MS/MS (Barascou et al. 2021b) and resulted in 0.59 µg/ml and 2.36 µg/ml for the prepared sulfoxaflor concentrations. Colonies were given 500 ml of pesticide-free or sulfoxaflor-contaminated sugar solutions every two days, over a 10-day exposure period (totaling 2,500 ml of syrup). The amount of sugar solution was chosen to optimize its full consumption by colonies. The sugar solutions were added in an internal feeder box placed above the hive frames. The experiment was repeated with new colonies, each month between April and September 2021 (except in August due to elevated environmental temperatures and therefore low colony activity). Each month was composed of one colony per experimental treatment, except in April (no colony was exposed to the 0.59 µg/ml sulfoxaflor treatment) and June and July (two colonies were exposed to the 0.59 µg/ml sulfoxaflor treatment); giving a total of n = 5 colonies for the control and 2.36 µg/ml sulfoxaflor treatments, and n = 6 colonies for the 0.59 µg/ml sulfoxaflor treatment.

Colony-level monitoring of daily flight activity and homing flight rates

To assess the effect of sulfoxaflor on the daily flight activity and homing flight rates, we equipped each colony with an video-based bee counter (patent IDDN.FR.001.130013.000.R.P.2010.000.31235) (Crauser and Le Conte 2010). For each replicate, bee counters were assigned to a different treatment group, so that each bee counter monitored the activity of colonies exposed, or not, to sulfoxaflor (0.59 and 2.36 µg/ml).

The bee counter device consists of a camera that monitors the hive entrance and image-analysis software that detects and registers in real time the activity of all bees by counting the number of out-going and in-coming bees (Fig. 1). The hive entrance was modified and composed of eight passages narrow enough so that only one bee can circulate in each tunnel and with only the back of the thorax facing the camera (see Dussaubat *et al.*, 2013 for more details). The software runs in the LabView environment for graphic programming and adjusts the frequency of images captured per second in order to reduce the chance of missing bees passing in front of the camera. Validation tests to assess the bee counter error rates were performed in 2007 and 2010 by comparing the numbers of bees recorded by the counter to the ones recorded by a human observer. Tests were done through 93 sessions spanning the bee season (May to October) and giving 27 855 bee counts (see the Excel supplementary file for raw data). The average error rate was of -2.26% and -1.3% in 2007 and 2010, meaning that the counter is slightly underestimating the flight activity by 1.3 to 2.26%. Most importantly, in 2007 we assessed the error rates for entries and exits separately, and found similar error rates for both type of flights (-2.23% for entries and -2.29 for exits). This indicates that bee loss rate (or gain) values are relatively little affected by the error rates.

The cumulated activity was recorded every 5 minutes and automatically saved with the time and date. From these bee entry and exit data, we determined the daily: *i)* flight activity (sum of the number of bee exits during a day) and *ii)* homing flight rates, calculated as follows: (number of entries - number of

exits) / number of exits for each day (Odemer 2022). When negative, the homing flight rate was assimilated to a loss rate. Positive values corresponded to a gain of bees, likely from drifting behavior because experimental colonies were located in the same apiary (two meters away from each other).

The colony-level monitoring with bee counters started 4 days before the exposure to sulfoxaflor and ended at 10 days post-exposure. We therefore had data on the daily flight activity and homing flight rates for three time periods: pre-exposure period (4 days), during exposure (10 days), and post-exposure (10 days).

Data analysis

Raw flight activity data are shown in the Excel supplementary file. Data were analyzed using the statistical software R v4.0.3 (R Core Team 2020). Statistical significance was set at $\alpha = 0.05$ for all tests. Since data were not normally distributed (Shapiro-Wilk test), variations among treatment groups in the number of honeybees, open and capped brood cells, and the amount of honey and pollen before exposure to sulfoxaflor were analyzed using Kruskal-Wallis tests. The influence of treatment groups on the cumulated syrup consumption was also analyzed using a Kruskal-Wallis test.

The effects of sulfoxaflor were determined on both the daily flight activity of bees and the homing flight rates, using a Linear Mixed Model (LMM) fitted by maximum likelihood using the *lme4* package (Bates et al. 2015). *P*-values for the t-test of fixed terms of the LMM were obtained through the *lmer* function of the *lmerTest* package (Kuznetsova et al. 2017). For each model, we selected the best one based on the second-order Akaike's Information Criterion (AICc) (Burnham and Anderson 2003) by using backward model selection from the full model. Variations in the daily flight activity and homing flight rates were analyzed with treatment, pesticide exposure period and months as fixed factors (with a treatment x exposure period interaction), and colony replicates as a random factor. For homing flight rate analysis, due to a heavy tailed distribution of the response variable and non-normal residues of the LMM, we used a bootstrap procedure to assess the significance of variables. The 95% confidence intervals for model parameters were estimated using the *lme4* R-package (*bootMer* function). We generated 10,000 bootstrapped samples from the model and refitted the model to these samples to obtain bootstrapped parameter estimates. If bootstrapped confidence intervals around the parameter estimate in that model did not overlap with zero, we concluded a significant effect.

In addition, we assessed for each treatment group the magnitude of sulfoxaflor effects on the daily flight activity and homing flight rates. For each pesticide treatment exposure periods (before vs during exposure; during vs after exposure; before vs after exposure), we calculated Hedges's g (g) or Glass's delta (Δ) as a measure of effect size (*hedges_g* or *glass_delta* functions of the *effectsize* package (Ben-Shachar et al. 2020)). Glass's delta was used when the standard deviations were significantly different between groups, as it uses only the second group's standard deviation. The interpretation values for both measures were as follows: $0.2 < g$ or Δ: very small effect, $0.2 \leq g$ or $\Delta < 0.5$: small effect, $0.5 \leq g$ or $\Delta < 0.8$: medium effect, and g or $\Delta \geq 0.8$: large effect (Ben-Shachar et al. 2020). An effect size (g or Δ)

was considered significant if the 95% confidence intervals of effect size did not overlap 0.0, and as negative or positive when it was below or above 0.0, respectively (Ben-Shachar et al. 2020).

For each treatment group, variations in the number of honeybees, open and capped brood cells, and the amount of honey and pollen between the pre-exposure period and the post-exposure periods were analyzed using Wilcoxon rank test pairwise comparisons.

Finally, we determined whether or not an increase in mortality rates (only with negative homing flight rates) exceeded the theoretical trigger values associated with the SPG for honeybees (i.e. 7% colony size reduction) (EFSA 2013). For that purpose, we calculated the fold-changes in bee loss rates between the pre-exposure and exposure periods to sulfoxaflor. These values were determined for exposure lasting 2, 3 or 6 days and then compared to the theoretical trigger values (EFSA 2013).

Results

We did not find any difference between treatment groups in the colony demographic state and food storage before exposure to sulfoxaflor (number of bees: Kruskal-Wallis, $p = 0.417$, open brood cells: $p = 0.309$, capped brood cells: $p = 0.284$, honey: $p = 0.803$ and pollen: $p = 0.564$; Fig. S1). The cumulated syrup consumption also did not differ between treatment groups (control: 1 949 ± 874 ml, 0.59 µg/ml of sulfoxaflor: 2 235 ± 757 and 2.36 µg/ml of sulfoxaflor: 2 140 ± 492 ml, Kruskal-Wallis: $p = 0.40$; Fig. S2).

Colony-level effect of sulfoxaflor on the daily flight activity

The results of model selection regarding the effect of sulfoxaflor treatment on the daily flight activity are presented in Table S1 (LMM analysis). The best model selected based on the AICc criterion included the month and an interaction between treatment and exposure period as predictors.

The overall daily flight activity before exposure to sulfoxaflor did not differ between the three treatment modalities (Table 1). Similarly, no statistically significant difference in the daily flight activity was observed between months. The daily flight activity in control colonies was significantly higher after exposure than before exposure (Table 1, Fig. 2 and Fig. S3). In colonies exposed to 0.59 µg/ml, the daily activity during and after exposure did not differ from the activity before exposure (GLMM: $p = 0.812$ and $p = 0.475$, respectively). However, honeybee colonies exposed to 2.36 µg/ml of sulfoxaflor made consistently (each month) fewer flights per day during and after exposure than before exposure (GLMM: $p < 0.001$; Fig. 2 and Fig S3).

In fact, the daily flight activity was reduced by half during the period of exposure to 2.36 µg/ml of sulfoxaflor as compared to before exposure (DE vs BE: $\Delta = -1.76$ – large effect; Fig. 3 and Table S2). Additionally, colonies made around 5 000 more flights after exposure to sulfoxaflor than during the treatment period (AE vs DE: $\Delta = 0.59$ – medium effect; Fig. 3 and Table S2).

Colony-level effect of sulfoxaflor on the daily homing flight rates

The results of model selection regarding the effect of sulfoxaflor treatment on the homing flight rates are presented in Table S2 (LMM analysis). The best model selected based on the AICc criterion included the month and an interaction between treatment and exposure period as predictors.

The overall homing flight rates before exposure to sulfoxaflor did not differ between the three treatment modalities, but were lower in May, June and July as compared to April (Table 2). No statistically significant difference in the homing flight rates of control colonies was observed between periods of exposure (Table 2, Fig. 4 and Fig. S3). In colonies exposed to 0.59 µg/ml, the daily homing flight rates during and after exposure did not differ from the activity before exposure (GLMM: $p = 0.801$ and $p = 0.811$, respectively). However, honeybee colonies exposed to 2.36 µg/ml of sulfoxaflor lost consistently (each month) more bees per day during exposure than before exposure (GLMM: $p < 0.001$; Fig. 4 and Fig. S3).

A 10-fold increase in the loss rate was found between the period of exposure to 2.36 µg/ml of sulfoxaflor (-5.97 ± 9.33 %) and the pre-exposure period (-0.54 ± 3.98 %; DE vs BE: $\Delta = -1.36$ – large effect; Fig. 3 and Table S3). After exposure to this sulfoxaflor concentration, the bee loss rate decreased and was significantly lower (-1.14 ± 5.07 %) than during the treatment period (AE vs DE: $\Delta = 0.52$ – medium effect), and was comparable to that observed before exposure to the insecticide (Fig. 3 and Table S3).

Finally, to determine whether this increase in bee losses caused by the exposure to 2.36 µg/ml of sulfoxaflor could be considered a risk for colonies, we compared the fold changes in bee losses to the theoretical trigger values considered to be safe for colonies, *i.e.* that cause a negligible reduction in colony size (EFSA 2013). We found that the fold changes in bee mortality were predominantly above these trigger values (Table 3). In some cases, homing flight rates before exposure to sulfoxaflor were positive or close to 0, but strongly negative afterwards, indicating a major effect of sulfoxaflor (*i.e.* above the theoretical trigger values).

Colony development and survival

The number of adult bees, open and capped brood cells, as well as the amount of honey and pollen storage, measured before and after exposure to sulfoxaflor are shown for each month in Fig.S2. We did not find any effect of treatment (control, 0.59 µg/ml and 2.36 µg/ml of sulfoxaflor) on the number of adult bees and open brood cells, and on the amount of honey and pollen storage between the different periods of exposure (before vs during and before vs post-exposure; Table S4 and Table S5). However, the number of capped brood cells in honeybee colonies exposed to 2.36 µg/ml of sulfoxaflor was significantly lower 10 days post-exposure than before exposure (Wilcox test: $p = 0.032$; Table S5).

All colonies provided with sugar syrup only (n = 5 colonies) or with sugar syrup laced with 0.59 µg/ml of sulfoxaflor (n = 6 colonies) survived throughout the winter. However, three out of the five colonies exposed to 2.36 µg/ml of sulfoxaflor were found dead at the end of winter; these three colonies were exposed to sulfoxaflor in April, May and June.

Discussion

Within the framework of pesticide risk assessment procedures for honeybees, data on effects on colonies are required if some risks have been identified on individual bees. Such Tier 2 and 3 studies are considered more environmentally realistic but demand greater resources and can be more difficult to interpret. In fact, the high uncertainty and variability of ecotoxicological data under semi-field and field tests may have contributed to some failures in detecting the risks associated with pesticides, such as neonicotinoids (Franklin and Raine 2019, Sgolastra et al. 2020). However, such issues could be partly resolved thanks to the recent technological advances in the monitoring of honeybee behaviors (Bromenshenk et al. 2015, Meikle and Holst 2015, Marchal et al. 2020). Indeed, by performing an *in situ* and real-time monitoring of colony daily flight activity, we were able to determine *i)* the background activity and consecutive homing rates, and then *ii)* to what extent they were affected by exposure to the neurotoxic insecticide sulfoxaflor for identifying risks to colonies.

We first assessed the effect of sulfoxaflor on the colony daily flight activity. Only control colonies, which were used as an environmental control, exhibited an increase in the daily flight activity between the pre- and post-exposure periods. This phenomenon might result from colony growth and/or a higher resource need over the course of the experiment. However, we did not observe any increase in the number of adult bees or brood production between these two periods. This increase in colony activity might rather be a direct consequence of sugar-syrup feeding, given that forager bees have high sugar requirements for performing foraging flights (Rodney and Purdy 2020), and providing sugar syrup to a colony generally may affect its foraging efforts (Free 1965). While some monthly tendencies in the increase of activity were observed for colonies exposed to the lower concentration of sulfoxaflor, the magnitude of effects was not significant. This suggests that at this concentration, sulfoxaflor prevented colonies from having the same dynamic as control colonies, and therefore triggered some minor but sublethal effects on colony activity. A stronger effect was however found at the higher sulfoxaflor concentration, with a reduction by half of the colony daily flight activity, likely due to the impairment of locomotion and foraging behaviors as previously described with neonicotinoids (Schneider et al. 2012, Alkassab and Kirchner 2018). This confirms a recent study, which found lower activity in colonies fed for 21 days with sugar solutions contaminated by the formulated product Closer® (active ingredient: sulfoxaflor, 0.0003 µg/ml) (El-Din et al. 2022). However, no change in flight activity was reported when colonies were not directly exposed to the pesticide- (Tamburini et al. 2021). In this latter study, floral resources were treated with the formulated product at the recommended maximum rate for spray applications (*i.e.* 0.4 L/ha), and effects started to be monitored 6 days after the application. This lack of effect might therefore reflect a lower level of exposure and corresponds to our lower concentration treatment. Another difference in the experimental setup was that flight activity was estimated by counting the number of bees entering the hive within one minute each day, which likely gives a partial

estimate of colony activity since flight activity is strongly governed by environmental factors and can exhibit daily changes (Winston 1987).

The colony-level assessment of bee losses is a key parameter used for evaluating the magnitude of effects due to pesticide exposure and setting protection goals for honeybee colonies (EFSA 2013, EFSA et al. 2020). Indeed, colony demographics and dynamics, like for any population, are strongly governed by the death rate of individuals. By monitoring the colony homing flight rates of all worker bees, we identified a bee loss rate of 2.77 ± 3.18 % for control colonies and of 1.90 ± 2.99 % for colonies exposed to the lower concentration of sulfoxaflor. These values are lower but rather in accordance with a systematic review of the literature, which reported median values of daily background mortality rates of 3.5-4% for all honeybee workers (covering all behavioral roles; dataset based on visual and RFID records); foragers presenting the highest daily mortality rates, with median values at around 10–12% (EFSA et al. 2020). In addition, we observed a monthly change in the overall mortality rates, with higher losses in May, June, and July as compared to April. Despite minor changes in colony-level activity, this change may be a consequence of a higher individual foraging activity (*e.g.* longer foraging trips) during the spring since the more time bees spend foraging, the lower their survival probability is (Prado et al. 2020).

We then detected a significant decrease in the homing flight rates upon exposure to the higher concentration of sulfoxaflor, while no effect was found in control and colonies exposed to the lower pesticide concentration. Several studies have shown that the effects of pesticides could be modulated by others factors such as the quality of nutrition and co-exposure to pathogens or others pesticides (Doublet et al. 2015, O'Neal et al. 2018, Tosi and Nieh 2019, Barascou et al. 2021b, Siviter et al. 2021, Castle et al. 2022). However, the decrease in the homing flight rates was highly consistent across each replicate (month, Fig .4), indicating a robust effect of the higher concentration of sulfoxaflor. Interestingly, the effect on bee mortality, as for the flight activity, was restricted to the period of exposure, which might be explained by the relatively rapid elimination of sulfoxaflor in honeybees (Barascou et al. 2021b, 2022). Such an increase in bee mortality is consistent with previous semi-field studies, which showed adverse effects of sulfoxaflor on bee mortality after two spray applications of a crop (residues in flowers ranging from 5 mg/kg on the first day of application to 0.1 mg/kg 7 days after application) (Cheng et al. 2018). In addition, if no effect of sulfoxaflor on learning and memory has been reported at doses ranging from 0.024 to 2.5 ng (Siviter et al. 2019), a recent study showed that honeybees fed with 26 ng of sulfoxaflor exhibited poor homing flight abilities: only 28% of them successfully returned to the colony compared to 75% for control bees (Capela et al. 2022). We could therefore reasonably assume that the higher concentration of sulfoxaflor impaired the homing ability as well as their flight or foraging capacities as often observed with neonicotinoids (Bortolotti et al. 2003, Matsumoto 2013, Ma et al. 2019, Monchanin et al. 2019).

In a 10-day chronic toxicity experiment performed with honeybees reared in cages, we previously found no lethal effects of sulfoxaflor at a concentration of 0.02 µg/ml, while at 2.35 µg/ml bee survival

significantly decreased (Barascou et al. 2021b). A preliminary experiment also showed that 0.1 µg/ml of sulfoxaflor was not toxic to bees (unpublished data). Altogether, these studies suggest a similar chronic toxicity in Tier 1 and Tier 2 toxicity tests. One difference might be the fold change in bee mortality given that in laboratory conditions the hazard ratio for bees exposed to the higher sulfoxaflor concentration was close to five (Barascou et al. 2021b), while in our current experiment the same concentration triggers a 10-fold increase in bee losses. However, if mortality rates (for all worker bees) upon exposure to the higher sulfoxaflor concentration rose to 21.8 % within a day, it rarely exceeded 13 % (8 times on 50 records). This is in contradiction with a recent study suggesting that daily loss rates less than 13 % could be classified as normal (Ngo et al. 2021). Besides a few exceptions, our data suggest that mortality rates below 5 % could rather be considered as normal, which is in line with the background mortality rates of 3.5-4% (for all honeybee workers) identified from a large dataset including studies carried out in different countries and environmental conditions (EFSA et al. 2020). Finally, it is interesting to note that in some cases (days), the homing flight rates were positive indicating a gain of bees for the colony. This was especially noticed during exposure to the higher concentration of sulfoxaflor in the month of April. Such rates were quite high but were associated with very low flight activity, which shows that the colony gained very few bees. This might be due to some drifting between colonies or to some bees that stayed outside overnight and entered the hive the next day.

Despite an increase in bee mortality, colony size was not impacted in the short-term. This could be explained by the lack of precision of the method used to evaluate the number of bees, since bees foraging in the field could not be included in the colony assessment. However, a significant decrease in the number of capped brood cells was found 10 days post-exposure in colonies exposed to 2.36 µg/ml of sulfoxaflor. Since the capped brood stage lasts between 11 and 12 days, most of these individuals were in the larval stage during exposure to sulfoxaflor. The impact of sulfoxaflor might therefore be an indirect consequence of reduced foraging efforts (lower flight activity combined with a higher bee loss) and therefore less food resources for larval nutrition. A reduction in the storage of pollen, which is used by nurse bees to provision larvae with jelly, was notably found after 21 days of exposure to sulfoxaflor (El-Din et al. 2022). Although not significant, we could observe a similar trend with some colonies having no pollen storage at the end of the treatment with the higher sulfoxaflor concentration. Finally, a non-mutually exclusive hypothesis would be a direct effect of sulfoxaflor on brood development due to the role of acetylcholine and its receptor on larval development (Grünewald and Siefert 2019).

In order to have natural variations in colony state, we made the choice to not artificially homogenize their population size but rather to perform a random assignment of colonies to the different treatment groups. Although, there was no difference in colony state among these groups, colonies exposed to the higher sulfoxaflor concentration tended to be initially smaller than the others. This was not associated with differences in the colony flight activity and daily bee losses, but we cannot exclude a reinforcement of pesticide effects due to a potentially smaller population, which will need to be confirmed by experimentally testing pesticide effects according to colony size.

Lastly, to determine whether the specific protection goals (SPGs) for honeybees were satisfied for the higher sulfoxaflor concentration, we compared the observed fold changes in daily bee losses to the theoretical trigger values associated with the SPG of 7% colony-size reduction. Such fold-changes were often found to be above the SPG and therefore a risk for colonies. Some of them could not be determined due to positive homing flight rates before exposure to the pesticide, but the range of negative values during exposure to sulfoxaflor reflected even larger fold-changes. The risk posed by the bee loss rates was somewhat confirmed by the winter loss of three colonies out of the five. In fact, by using model simulations of colony dynamics, Rumkee *et al.* (2015) found that an increase in adult bee loss rates had a much greater impact on colony survival than mortality of bee larvae or reduction in egg-laying rate. In addition, bees might have stored some contaminated syrup, thereby prolonging the exposure and causing sublethal, rather than lethal, effects in the long-term, as evidenced by the absence of increased mortality once the exposure was over. For instance, a reduction in pollen foraging activity has often been described upon exposure to pesticides in honeybees and bumblebees (Gill et al. 2012, Feltham et al. 2014, Prado et al. 2019), which might lead to carryover effects on colony survival (Requier et al. 2017). The fact that only colonies exposed in the spring died over the winter, but not the ones exposed later in July and September, tends to support this hypothesis of carryover effects. Furthermore, a significant loss of foragers can lead to the early recruitment of nurse bees for foraging, and such precocious foragers were found to be less efficient than normal-age foragers; they have a lower lifespan, make fewer foraging trips and are much more likely to die during their first flights outside the hive (Barron 2015).

Although chronic exposure to sulfoxaflor might be an unrealistic scenario for honeybee colonies, our experiment with bee counters showed that, real-time and longitudinal data on bee mortality, combined with threshold values indicating which fold-changes in mortality rates represent a risk for colonies, can improve pesticide risk assessment under field conditions. Indeed, while we did not find any major effects on the colony demographic state, bee loss rates highlighted a risk for colonies. In addition, bee counters can significantly reduce the measurement complexity of mortality rates in the field and are relatively time-effective for use, which is essential for the regulatory assessments of pesticides. However, further efforts are needed to assess and improve bee counters precision for risk assessment (i.e. lower the error rates) and avoid erroneous interpretation (Borlinghaus et al. 2022, Odemer 2022). Last but not least, since the *in situ* monitoring of daily bee losses can be connected to consequences for colonies (the ultimate protection goal), this measured endpoint has a strong ecological relevance in pesticide risk assessment. All of these criteria are important for test methods in chemical regulation. However, the trigger values for protecting bee colonies have been determined via *in silico* approaches (EFSA 2013), and therefore, future efforts should be made to empirically link mortality rates to effects on colonies and better specify the mortality rates that pose a risk for colonies.

Supporting Information

Supplementary figures detailing colony monitoring upon pesticide exposure (Figure S1, S2 and S3). Supplementary tables detailing statistical analyses of pesticide effects (Table S1, S4 and S5) and effect size of pesticide exposure (Table S2 and S3).

Author Contributions

L.B., Y.L.C and C.A. conceived the study. L.B., D.S., D.C. and C.A. conducted the experiments. L.B., U.G., M.P., O.M. and C.A. analyzed the data. Y.L.C. and C.A. contributed to reagents. L.B, U.G. and C.A. wrote the manuscript. All authors read and reviewed the manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This project received funding from the European Horizon 2020 research and innovation programs under grant agreements no. 773921 (POSHBEE) (LB, DS, YLC and CA). UG was supported by the European Horizon 2020 research and innovation program under grant agreement no. 817622 (B-GOOD).

References

Alkassab AT, Kirchner WH. 2018. Assessment of acute sublethal effects of clothianidin on motor function of honeybee workers using video-tracking analysis. Ecotoxicology and Environmental Safety 147: 200–205.

Barascou L, Requier F, Sené D, Crauser D, Le Conte Y, Alaux C. 2021a. Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honeybee foraging activity. Science of The Total Environment 805: 150351.

Barascou L, Sene D, Barraud A, Michez D, Lefebvre V, Medrzycki P, Di Prisco G, Strobl V, Yañez O, Neumann P, Le Conte Y, Alaux C. 2021b. Pollen nutrition fosters honeybee tolerance to pesticides. Royal Society Open Science 8: 210818.

Barascou L, Sene D, Le Conte Y, Alaux C. 2022. Pesticide risk assessment: honeybee workers are not all equal regarding the risk posed by exposure to pesticides. Environmental Science and Pollution Research.

Barron AB. 2015. Death of the bee hive: understanding the failure of an insect society. Current Opinion in Insect Science 10: 45–50.

Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67.

Bendahou N, Bounias M, Fleche C. 1997. Acute toxicity of cypermethrin and fenitrothion on honeybees (*Apis mellifera mellifera*) according to age, formulations and (chronic paralysis virus) insecticide interaction. J. Environ. Biol. 18: 55–65.

Ben-Shachar M, Lüdecke D, Makowski D. 2020. effectsize: Estimation of Effect Size Indices and Standardized Parameters. Journal of Open Source Software 5: 2815.

Borlinghaus P, Odemer R, Tausch F, Schmidt K, Grothe O. 2022. Honey bee counter evaluation – Introducing a novel protocol for measuring daily loss accuracy. Computers and Electronics in Agriculture 197: 106957.

Bortolotti L, Montanari R, Marcelino J, Medrzycki P, Maini S, Porrini C. 2003. Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. Bulletin of Insectology 56: 63–67.

Botías C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E, Goulson D. 2015. Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. Environmental Science & Technology 49: 12731–12740.

Bromenshenk J, Henderson C, Seccomb R, Welch P, Debnam S, Firth D. 2015. Bees as Biosensors: Chemosensory Ability, Honey Bee Monitoring Systems, and Emergent Sensor Technologies Derived from the Pollinator Syndrome. Biosensors 5: 678–711.

Burnham KP, Anderson DR. 2003. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. Page 655 in. The Journal of Wildlife Management, vol. 67.

Capela N, Sarmento A, Simoes S, Azevedo-Pereira HMVS, Sousa JP. 2022. Sub-lethal doses of Sulfoxaflor impair honey bee homing ability. Science of The Total Environment.

Castle D, Alkassab AT, Bischoff G, Steffan-Dewenter I, Pistorius J. 2022. High nutritional status promotes vitality of honey bees and mitigates negative effects of pesticides. Science of The Total Environment 806: 151280.

Cheng Y, Bu Y, Tan L, Wu W, Li J, Zhou J, Zhai A, Shan Z. 2018. A semi-field study to evaluate effects of sulfoxaflor on honey bee (*Apis mellifera*). Bulletin of Insectology 71: 225–233.

Colin T, Meikle WG, Wu X, Barron AB. 2019. Traces of a neonicotinoid induce precocious foraging and reduce foraging performance in honey bees. Environmental Science & Technology 53: 8252–8261.

Crauser D, Le Conte Y. 2010. Compteur d'entrées et sorties d'abeilles. Agence pour la Protection des Programmes, France.

Doublet V, Labarussias M, de Miranda JR, Moritz RFA, Paxton RJ. 2015. Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle: Pesticide-pathogen interactions in honey bees. Environmental Microbiology 17: 969–983.

Dussaubat C, Maisonnasse A, Crauser D, Beslay D, Costagliola G, Soubeyrand S, Kretzchmar A, Le Conte Y. 2013. Flight behavior and pheromone changes associated to *Nosema ceranae* infection of honey bee workers (*Apis mellifera*) in field conditions. Journal of Invertebrate Pathology 113: 42–51.

EFSA. 2013. EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). EFSA Journal 11(7): 268 pp.

EFSA, Ippolito A, Aguila M del, Aiassa E, Guajardo IM, Neri FM, Alvarez F, Mosbach-Schulz O, Szentes C. 2020. Review of the evidence on bee background mortality. EFSA Supporting Publications 17.

El-Din HS, Helmy WS, Al Naggar Y, Ahmed FS. 2022. Chronic exposure to a field-realistic concentration of Closer® SC (24% sulfoxaflor) insecticide impacted the growth and foraging activity of honey bee colonies. Apidologie 53: 22.

EPA. 2019. Sulfoxaflor: Ecological Risk Assessment for Section 3 Registration for Various Proposed New Uses.

EPPO. 2010. PP 3/10 (3) Environmental risk assessment scheme for plant protection products, Chapter 10: Honeybees. EPPO Bulletin 40: 323–331.

Feltham H, Park K, Goulson D. 2014. Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. Ecotoxicology 23: 317–323.

Franklin EL, Raine NE. 2019. Moving beyond honeybee-centric pesticide risk assessments to protect all pollinators. Nature Ecology & Evolution 3: 1373–1375.

Free JB. 1965. The Behaviour of Honeybee Foragers when their Colonies are Fed Sugar Syrup. Journal of Apicultural Research 4: 85–88.

Gill RJ, Ramos-Rodriguez O, Raine NE. 2012. Combined pesticide exposure severely affects individual- and colony-level traits in bees. Nature 491: 105–108.

Grünewald B, Siefert P. 2019. Acetylcholine and Its Receptors in Honeybees: Involvement in Development and Impairments by Neonicotinoids. Insects 10: 420.

Henry M, Beguin M, Requier F, Rollin O, Odoux J-F, Aupinel P, Aptel J, Tchamitchian S, Decourtye A. 2012. A common pesticide decreases foraging success and survival in honey bees. Science 336: 348–350.

Hernandez J, Maisonnasse A, Cousin M, Beri C, Le Quintrec C, Bouetard A, Castex D, Decante D, Servel E, Buchwalder G, Brunet F, Feschet-Destrella E, de Bellescize K, Kairo G, Frontero L, Pédehontaa-Hiaa M, Buisson R, Pouderoux T, Aebi A, Kretzschmar A. 2020. ColEval: Honeybee COLony Structure EVALuation for Field Surveys. Insects 11: 41.

Hesselbach H, Seeger J, Schilcher F, Ankenbrand M, Scheiner R. 2020. Chronic exposure to the pesticide flupyradifurone can lead to premature onset of foraging in honeybees *Apis mellifera*. Journal of Applied Ecology 57(3): 609–618.

Hung K-LJ, Kingston JM, Albrecht M, Holway DA, Kohn JR. 2018. The worldwide importance of honey bees as pollinators in natural habitats. Proceedings of the Royal Society B: Biological Sciences 285: 20172140.

Khoury DS, Myerscough MR, Barron AB. 2011. A quantitative model of honey bee colony population dynamics. PLoS ONE 6: e18491.

Klein A-M, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274: 303–313.

Kuznetsova A, Brockhoff PB, Christensen RHB. 2017. **lmerTest** Package: Tests in Linear Mixed Effects Models. Journal of Statistical Software 82.

Ladas A. 1972. The influence of some internal and external factors upon the insecticide resistance of honeybee. Apidologie 3: 55–78.

Ma C, Zhang Y, Sun J, Imran M, Yang H, Wu J, Zou Y, Li-Byarlay H, Luo S. 2019. Impact of acute oral exposure to thiamethoxam on the homing, flight, learning acquisition and short-term retention of *Apis cerana*. Pest Management Science 75: 2975–2980.

Marchal P, Buatois A, Kraus S, Klein S, Gomez-Moracho T, Lihoreau M. 2020. Automated monitoring of bee behaviour using connected hives: Towards a computational apidology. Apidologie 51: 356–368.

Matsumoto T. 2013. Reduction in homing flights in the honey bee *Apis mellifera* after a sublethal dose of neonicotinoid insecticides. Bulletin of Insectology 66: 1–9.

Mayland PG, Burkhardt CC. 1970. Honey Bee Mortality As Related To Insecticide-Treated Surfaces and Bee Age12. Journal of Economic Entomology 63: 1437–1439.

Meikle WG, Holst N. 2015. Application of continuous monitoring of honeybee colonies. Apidologie 46: 10–22.

Monchanin C, Henry M, Decourtye A, Dalmon A, Fortini D, Bœuf E, Dubuisson L, Aupinel P, Chevallereau C, Petit J, Fourrier J. 2019. Hazard of a neonicotinoid insecticide on the homing flight of the honeybee depends on climatic conditions and Varroa infestation. Chemosphere 224: 360–368.

Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, Pettis JS. 2010. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLoS ONE 5: e9754.

Ngo T-N, Rustia DJA, Yang E-C, Lin T-T. 2021. Honey Bee Colony Population Daily Loss Rate Forecasting and an Early Warning Method Using Temporal Convolutional Networks. Sensors 21: 3900.

Ngo TN, Wu K-C, Yang E-C, Lin T-T. 2019. A real-time imaging system for multiple honey bee tracking and activity monitoring. Computers and Electronics in Agriculture 163: 104841.

Odemer R. 2022. Approaches, challenges and recent advances in automated bee counting devices: A review. Annals of Applied Biology 180: 73–89.

OECD. 1998a. Test No. 213: Honeybees, acute oral toxicity test. OECD ed.

OECD. 1998b. Test No. 214: honeybees, acute contact toxicity test. OECD ed.

OECD. 2013. Test No. 237: honey bee (*Apis mellifera*) larval toxicity test, single exposure. OECD ed.

OECD. 2017. Test No. 245: honey bee (*Apis mellifera* L.), chronic oral toxicity test (10-day feeding). OECD ed.

O'Neal ST, Anderson TD, Wu-Smart JY. 2018. Interactions between pesticides and pathogen susceptibility in honey bees. Current Opinion in Insect Science 26: 57–62.

Pohorecka K, Skubida P, Miszczak A, Semkiw P, Sikorski P, Zagibajło K, Teper D, Kołtowski Z, Skubida M, Zdańska D, Bober A. 2012. Residues of Neonicotinoid Insecticides in Bee Collected Plant Materials from Oilseed Rape Crops and their Effect on Bee Colonies. Journal of Apicultural Science 56: 115–134.

Prado A, Pioz M, Vidau C, Requier F, Jury M, Crauser D, Brunet J-L, Le Conte Y, Alaux C. 2019. Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient honey bees. Science of The Total Environment 650: 1250–1260.

Prado A, Requier F, Crauser D, Le Conte Y, Bretagnolle V, Alaux C. 2020. Honeybee lifespan: the critical role of pre-foraging stage. Royal Society Open Science 7: 200998.

R Core Team. 2020. R: A language and environment for statistical computing. Vienna, Austria:R Foundation for statistical Computing.

Requier F, Odoux J-F, Henry M, Bretagnolle V. 2017. The carry-over effects of pollen shortage decrease the survival of honeybee colonies in farmlands. Journal of Applied Ecology 54: 1161–1170.

Rinkevich FD, Margotta JW, Pittman JM, Danka RG, Tarver MR, Ottea JA, Healy KB. 2015. Genetics, synergists, and age affect insecticide sensitivity of the honey bee, *Apis mellifera*. PLOS ONE 10: e0139841.

Rodney S, Purdy J. 2020. Dietary requirements of individual nectar foragers, and colony-level pollen and nectar consumption: a review to support pesticide exposure assessment for honey bees. Apidologie.

Rumkee JCO, Becher MA, Thorbek P, Kennedy PJ, Osborne JL. 2015. Predicting Honeybee Colony Failure: Using the BEEHAVE Model to Simulate Colony Responses to Pesticides. Environmental Science & Technology 49: 12879–12887.

Sanchez-Bayo F, Goka K. 2014. Pesticide Residues and Bees – A Risk Assessment. PLoS ONE 9: e94482.

Schneider CW, Tautz J, Grünewald B, Fuchs S. 2012. RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera*. PLoS ONE 7: e30023.

Sgolastra F, Medrzycki P, Bortolotti L, Maini S, Porrini C, Simon-Delso N, Bosch J. 2020. Bees and pesticide regulation: Lessons from the neonicotinoid experience. Biological Conservation 241: 108356.

Shi J, Yang H, Yu L, Liao C, Liu Y, Jin M, Yan W, Wu XB. 2020. Sublethal acetamiprid doses negatively affect the lifespans and foraging behaviors of honey bee (*Apis mellifera* L.) workers. Science of The Total Environment 738: 139924.

Siviter H, Bailes EJ, Martin CD, Oliver TR, Koricheva J, Leadbeater E, Brown MJF. 2021. Agrochemicals interact synergistically to increase bee mortality. Nature 596: 389–392.

Siviter H, Scott A, Pasquier G, Pull CD, Brown MJF, Leadbeater E. 2019. No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. PeerJ 7: e7208.

Sparks TC, Watson GB, Loso MR, Geng C, Babcock JM, Thomas JD. 2013. Sulfoxaflor and the sulfoximine insecticides: Chemistry, mode of action and basis for efficacy on resistant insects. Pesticide Biochemistry and Physiology 107: 1–7.

Steffan-Dewenter I, Kuhn A. 2003. Honeybee foraging in differentially structured landscapes. Proceedings of the Royal Society of London. Series B: Biological Sciences 270: 569–575.

Stokstad E. 2018. European Union expands ban of three neonicotinoid pesticides. Science.

Struye MH, Mortier HJ, Arnold G, Miniggio C, Borneck R. 1994. Microprocessor-controlled monitoring of honeybee flight activity at the hive entrance. Apidologie 25: 384–395.

Tamburini G, Pereira-Peixoto M-H, Borth J, Lotz S, Wintermantel D, Allan MJ, Dean R, Schwarz JM, Knauer A, Albrecht M, Klein A. 2021. Fungicide and insecticide exposure adversely impacts bumblebees and pollination services under semi-field conditions. Environment International 157: 106813.

Thompson HM. 2021. The use of the Hazard Quotient approach to assess the potential risk to honeybees (*Apis mellifera*) posed by pesticide residues detected in bee-relevant matrices is not appropriate. Pest Management Science 77: 3934–3941.

Tosi S, Nieh JC. 2019. Lethal and sublethal synergistic effects of a new systemic pesticide, flupyradifurone (Sivanto®), on honeybees. Proceedings of the Royal Society B: Biological Sciences 286: 20190433.

Wang M, Braasch T, Dietrich C. 2020. Reduction of variability for the assessment of side effects of toxicants on honeybees and understanding drivers for colony development. PLOS ONE 15: e0229295.

Watson GB, Siebert MW, Wang NX, Loso MR, Sparks TC. 2021. Sulfoxaflor – A sulfoximine insecticide: Review and analysis of mode of action, resistance and cross-resistance. Pesticide Biochemistry and Physiology 178: 104924.

Winston ML. 1987. THe Biology of the Honey Bee. Havard University Press ed.

Zhu YC, Caren J, Reddy GVP, Li W, Yao J. 2020. Effect of age on insecticide susceptibility and enzymatic activities of three detoxification enzymes and one invertase in honey bee workers (*Apis mellifera*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 238: 108844.

Zioga E, Kelly R, White B, Stout JC. 2020. Plant protection product residues in plant pollen and nectar: a review of current knowledge. Environmental Research 109873.

Figure legends

Figure 1. Video-based bee counters in the field measuring the activity of a honeybee colony. (A) The video-based bee counter with bee channels and the camera is at the entrance of the hive. The camera recording bee activity is directly connected to a computer and a program analyzing in real time bee traffic. (B) Number of bees exiting and entering the hive over a day during the experiment. During this day, the colony lost 849 bees, corresponding to a loss rate of 3.3 %.

Figure 2. Daily flight activity in response to sulfoxaflor treatments. The experiment was repeated 5 times between April and September. Each plot represents the daily activity of a colony exposed to sulfoxaflor or not. The flight activity was recorded before, during and after exposure to sulfoxaflor. Each colored dot represents a day of flight activity measurement. Box plots show the 1st and 3rd interquartile range with a line denoting the median. Whiskers encompass 90% of the individuals.

Figure 3. Effect size (hedges's g) and 95% confidence intervals of treatment effects on the daily (A) flight activity and (B) homing flight rates between the different periods of exposure. Asterisks indicate significant effect of treatment.

Figure 4. Daily homing flight rates in response to sulfoxaflor treatments. The homing flight rate was calculated as (Number of entries – Number of exits) / Number of exits) x 100. Values below and above the dashed line represent a loss and gain of bees, respectively. The experiment was repeated 5 times between April and September. Each plot represents the daily homing flight rates in a colony exposed to sulfoxaflor or not. Data were recorded before, during and after exposure to sulfoxaflor. Each colored dot represents a day of homing flight rate measurement. Box plots show the 1st and 3rd interquartile range with a line denoting the median. Whiskers encompass 90% of the individuals.

Table 1. Results of the linear mixed model assessing the effects of pesticide treatments, period of exposure and month on the daily flight activity of honeybee colonies. Intercept represents the control group and the period before exposure to sulfoxaflor in April.

Parameters	Estimate	Standard error	*t*-value	*p*-value
Intercept	**26 464.899**	**6 182.069**	**4.281**	**< 0.001**
0.59 µg/ml sulfoxaflor	7 001.899	5 449.945	1.285	0.219
2.36 µg/ml sulfoxaflor	-4 248.850	5 487.602	-0.774	0.450
During exposure	3 526.610	2 416.930	1.459	0.145
After exposure	**6 912.470**	**2 416.930**	**2.860**	**< 0.01**
May	2 047.631	7 084.529	0.289	0.779
June	-3 541.382	6 912.618	-0.512	0.621
July	5 532.833	6 911.513	0.801	0.444
September	74.674	7 083.792	0.011	0.991
0.59 µg/ml sulfoxaflor × During	-2 993.225	3 296.384	-0.908	0.364
2.36 µg/ml sulfoxaflor × During	**-16 989.990**	**3 418.055**	**-4.971**	**< 0.001**
0.59 µg/ml sulfoxaflor × After exposure	-5 311.219	360.014	-1.611	0.108
2.36 µg/ml sulfoxaflor × After exposure	**-15 653.744**	**360.002**	**-4.573**	**< 0.001**
Random effect: Colony	*7 377.1*	*9 135.1*	**-**	**-**

Table 2. Parameter estimates and bootstrapped 95% confidence intervals of the linear mixed effect model assessing the effects of pesticide treatments, the period of exposure and month on the daily flight homing rates. Intercept represents the control group and the period before exposure to sulfoxaflor in April. Data in bold indicate a significant effect when the bootstrapped confidence intervals (CI) around the parameter estimate did not overlap with zero.

Parameters	Original estimate	Bootstrapped standard error	Lower CI	Upper CI
Intercept	-0.069	1.424	-2.958	2.746
0.59 µg/ml sulfoxaflor	1.357	1.533	-1.648	4.421
2.36 µg/ml sulfoxaflor	1.932	1.564	-1.074	5.050
During exposure	-0.767	1.175	-3.028	1.551
After exposure	0.077	1.175	-2.210	2.376
May	**-3.341**	**1.320**	**-5.917**	**-0.765**
June	**-2.534**	**1.289**	**-5.094**	**-0.005**
July	**-4.516**	**1.287**	**-7.031**	**-1.993**
September	-1.669	1.319	-4.282	0.863
0.59 µg/ml sulfoxaflor × During	0.492	1.602	-2.688	3.626
2.36 µg/ml sulfoxaflor × During	**-4.654**	**1.662**	**-7.956**	**-1.385**
0.59 µg/ml sulfoxaflor × After exposure	0.184	1.602	-2.992	3.303
2.36 µg/ml sulfoxaflor × After exposure	-0.695	1.664	-3.990	2.496
Random effect: Colony	*1.186*	*1.089*	*0.000*	*3.543*

Table 3. Fold changes in daily homing flight rates between the pre-exposure and exposure periods to 2.36 µg/ml of sulfoxaflor. Fold changes were determined for exposure lasting 2, 3 and 6 days. Values in bold represent the fold changes that exceeded the theoretical trigger values above which a non-negligible risk of colony size reduction has been identified (*i.e.* forager mortality should not be increased by a factor of 1.5 for 6 days or a factor of 2 for 3 days or a factor of 3 for 2 days). NA represents uncalculated values because some colonies, prior to pesticide exposure, exhibited positive homing flight rates (gain of bees).

Duration of effects (maximum protective fold-change)	Month	Homing flight rates - Before exposure	Homing flight rates - During exposure	Fold-change in bee losses
2 days (x3)	April	-4.07	-8.58	x2.11
	May	1.04	-3.63	NA
	June	0.03	-9.39	NA
	July	-1.42	-4.44	**x3.13**
	September	1.67	-3.59	NA
3 days (x2)	April	-4.07	-9.84	**x2.42**
	May	1.04	-3.08	NA
	June	0.03	-12.05	NA
	July	-1.42	-4.13	**x2.91**
	September	1.67	-4.90	NA
6 days (x1.5)	April	-4.07	-5.43	x1.33
	May	1.04	-6.12	NA
	June	0.03	-10.98	NA
	July	-1.42	-4.67	**x3.29**
	September	1.67	-7.56	NA

Figure 1

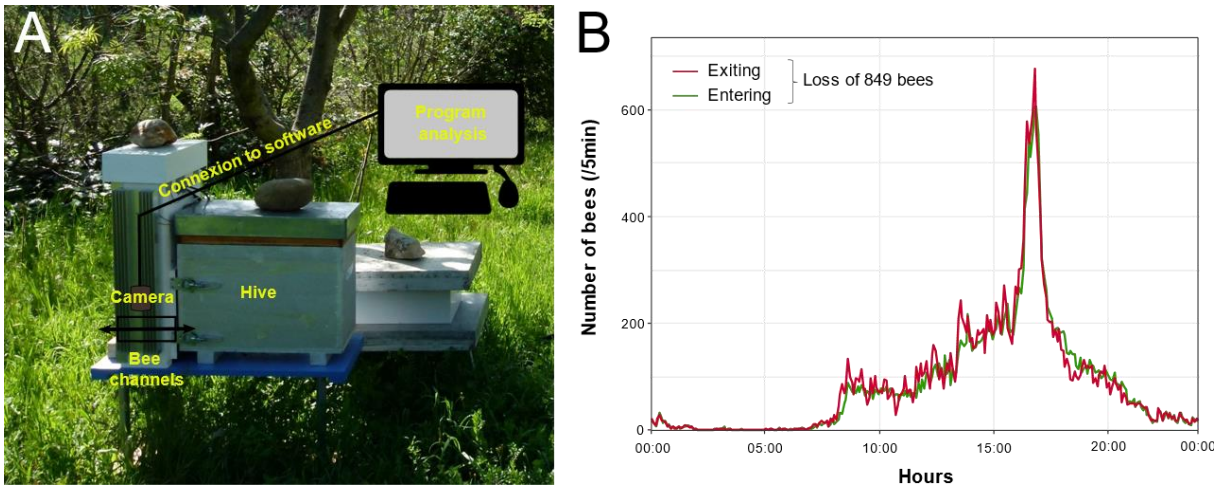

A
Program analysis
Connexion to software
Camera
Hive
Bee channels
B
Exiting
Entering
Loss of 849 bees
Number of bees (/5min)
600
400
200
0
00:00
05:00
10:00
15:00
20:00
00:00
Hours

Figure 2

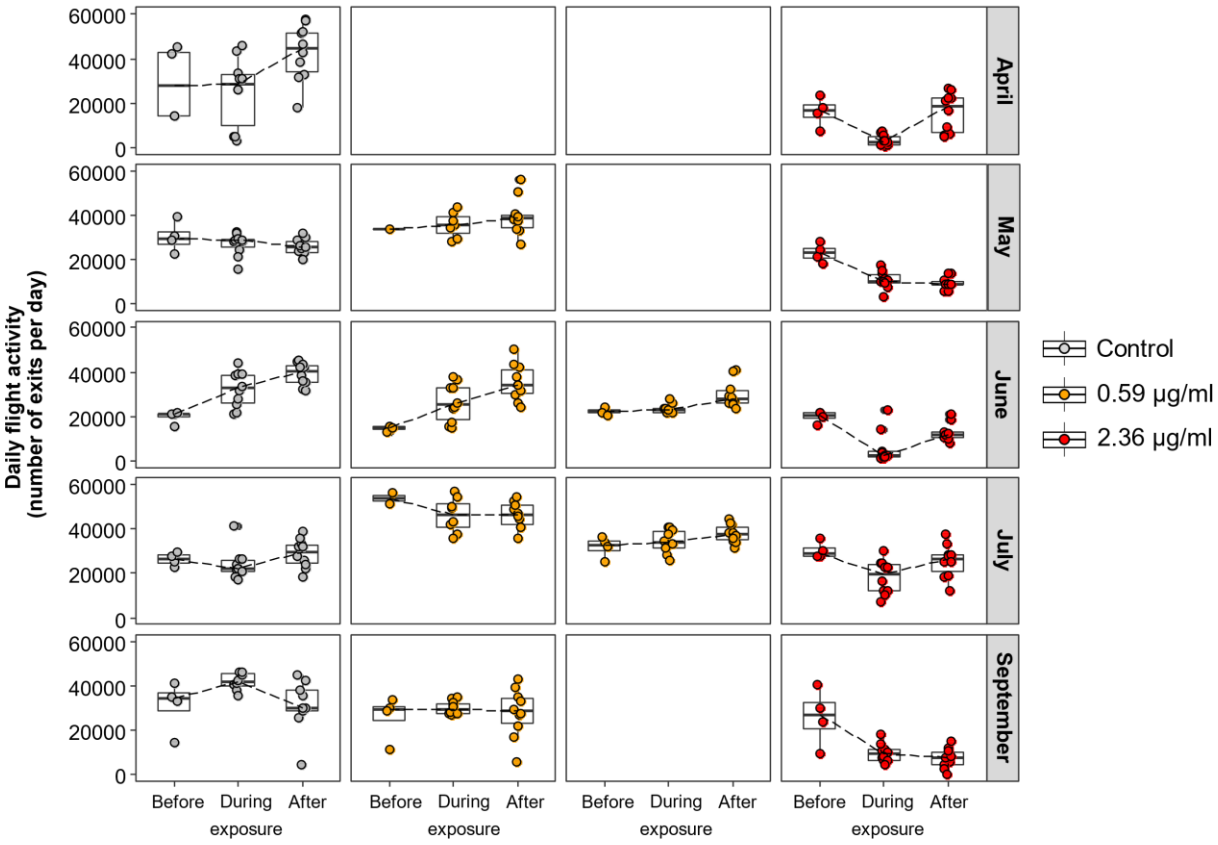
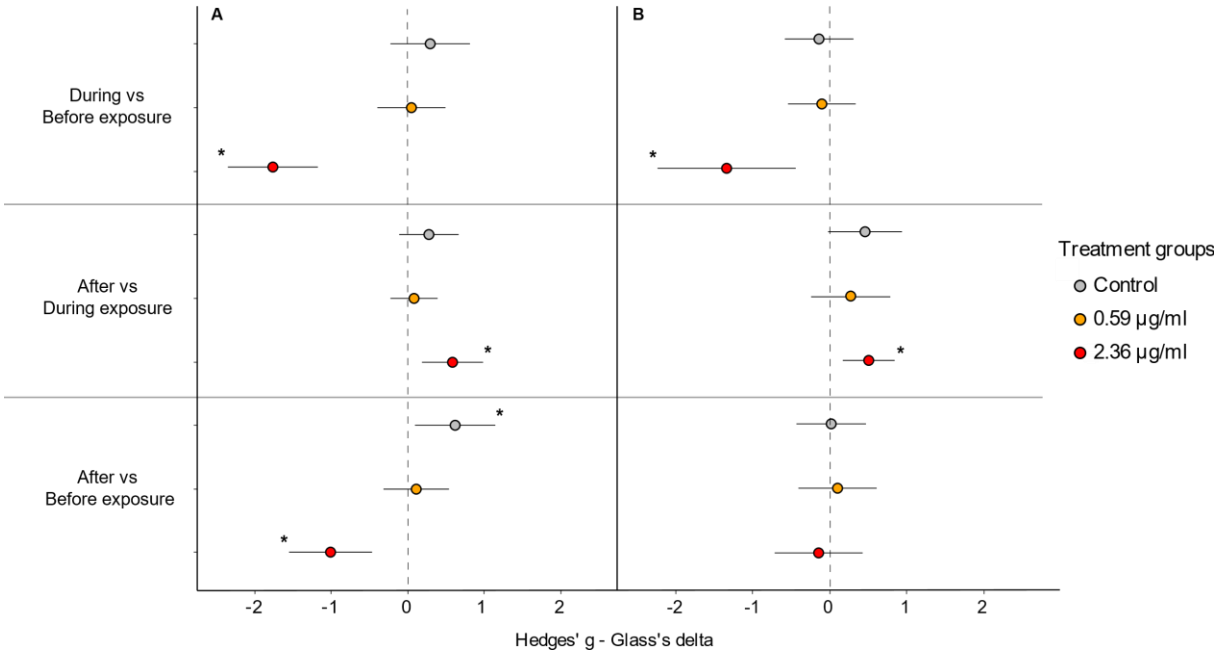
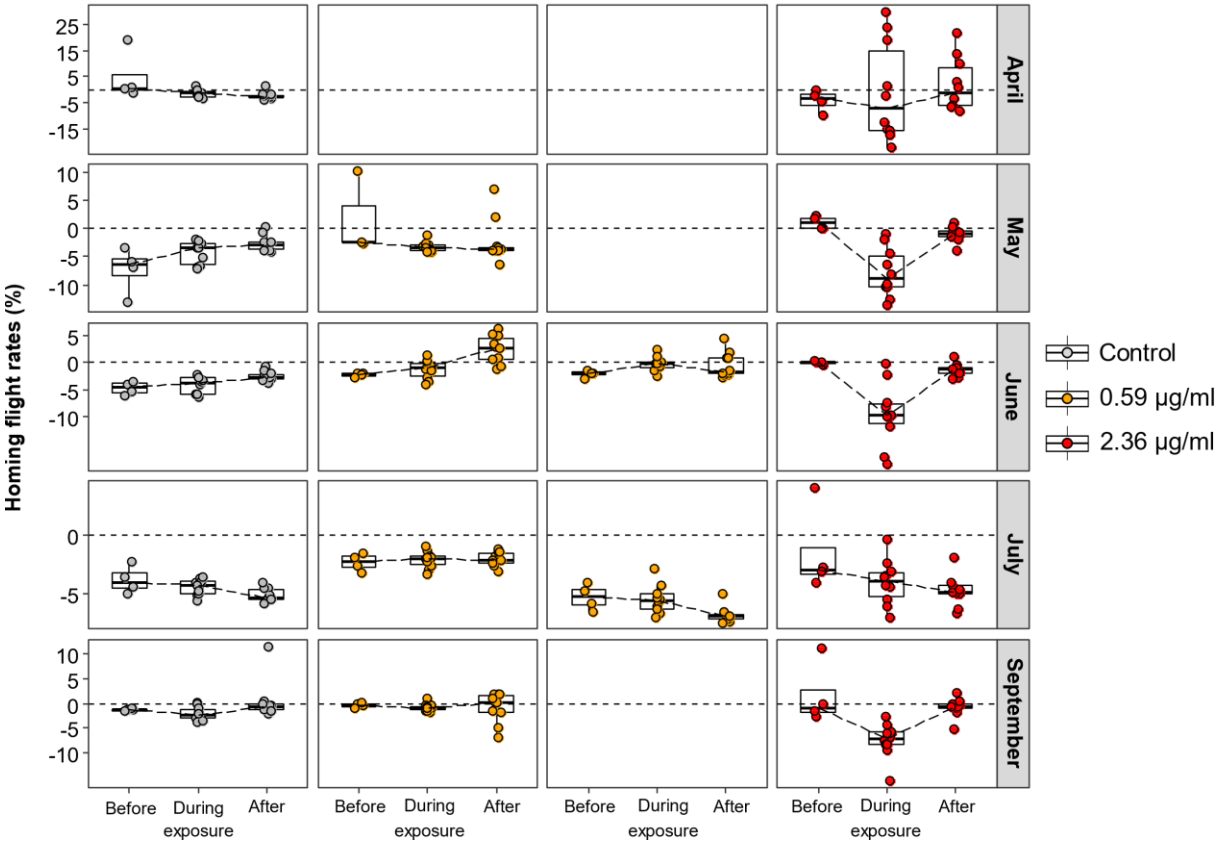

Daily flight activity
(number of exits per day)
60000
40000
20000
0
April
May
June
July
September
Before
During
After
exposure
Control
0.59 µg/ml
2.36 µg/ml

Figure 3


A
B
During vs
Before exposure
After vs
During exposure
After vs
Before exposure
*
-2
-1
0
1
2
Hedges' g - Glass's delta
Treatment groups
Control
0.59 µg/ml
2.36 µg/ml

815

Figure 4


Homing flight rates (%)
April
May
June
July
September
Before
During exposure
After
Control
0.59 µg/ml
2.36 µg/ml