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▶ **To cite this version:**

Lena Barascou, Fabrice Requier, Deborah Sené, Didier Crauser, Yves Le Conte, et al.. Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honeybee foraging activity. Science of the Total Environment, In press, 805, 10.1016/j.scitotenv.2021.150351 . hal-03359304

HAL Id: hal-03359304
https://hal.inrae.fr/hal-03359304

Submitted on 30 Sep 2021

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Delayed effects of a single dose of a neurotoxic pesticide (sulfoxaflor) on honeybee foraging activity

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Highlights

- Young honeybees were exposed to a sublethal dose of sulfoxaflor (16 or 60 ng).
- Both doses of sulfoxaflor reduced the daily flight activity and the total number of flights.
- Sulfoxaflor effects were delayed and emerged when bees transitioned to foraging activity.
- Time-to-effect measurements are needed to evaluate pesticide toxicity in honeybees.

Abstract

Pesticide risk-assessment guidelines for honeybees (*Apis mellifera*) generally require determining the acute toxicity of a chemical over the short-term through fix-duration tests. However, potential long-lasting or delayed effects resulting from an acute exposure (e.g. a single dose) are often overlooked, although the modification of a developmental process may have life-long consequences. To investigate this question, we exposed young honeybee workers to a single sublethal field-realistic dose of a neurotoxic pesticide, sulfoxaflor, at one of two amounts (16 or 60 ng), at the moment when they initiated orientation flights (preceding foraging activity). We then tracked in the field their flight activity and lifespan with automated life-long monitoring devices. Both amounts of sulfoxaflor administered reduced the total number of flights but did not affect bee survival and flight duration. When looking at the time series of flight activity, effects were not immediate but delayed until foraging activity with a decrease in the daily number of foraging flights and consequently in their total number (24 and 33 % less for the 16 and 60 ng doses, respectively). The results of our study therefore blur the general assumption in honeybee toxicology that acute exposure results in immediate and rapid effects and call for long-term recording and/or time-to-effect measurements, even upon exposure to a single dose of pesticide.

Keywords: Ecotoxicology, bees, acute exposure, sublethal effects, pesticide risk assessment

Introduction

Within the framework of the pesticide risk assessment procedure, test guidelines require toxicological data on honeybees (*Apis mellifera*), a major pollinator of crops (Garibaldi et al. 2013) and wild plants (Hung et al. 2018). The first tier assessment relies on acute and chronic toxicity tests measuring the survival rate of bees over the duration of the tests, i.e. 48 hours and 10 days for acute and chronic exposure, respectively (OECD 1998a, b, 2013; OECD 2017). Thus, long-term mortality risks are often neglected in pesticide risk assessment, especially when effects on bee mortality occur not during but after chronic exposure to pesticides (i.e. after 10 days) (Dechaume Moncharmont et al. 2003; Rondeau et al. 2015). In addition, there is growing evidence for long-term sublethal effects upon chronic

exposure to pesticides, such as the impairment of bee behavior, physiology or colony performance (Faucon et al. 2005; Sandrock et al. 2014; Prado et al. 2019; Colin et al. 2019; Al Naggar and Baer 2019; Hesselbach et al. 2020; Traynor et al. 2021). These data therefore show that time-to-effect experiments rather than fixed-duration tests are required for evaluating chronic toxicity (Rondeau et al. 2015; Simon-Delso et al. 2018; Bommuraj et al. 2021).

Similarly, long-lasting or delayed effects induced by an acute exposure have often been neglected. For instance, in the acute toxicity tests, mortality rate is measured over 48 hours and to a maximum of 96 h if the mortality increases by more than 10% after the first 24 h (OECD 1998a, b). Moreover, sublethal effects have been generally assayed immediately or over the days following acute exposure to pesticides (Barascou et al. 2021). In fact, relatively little is known about the occurrence of long-lasting or delayed effects upon acute exposure (Schneider et al. 2012; Shi et al. 2020). After exposure to a single dose, a rapid reduction in pesticide concentration is generally observed in honeybees, which contributes to shortening the exposure to the toxic compound (Ardalani 2021; Barascou et al. 2021b). Relatively short-term effects can thus be expected; however, some pesticides may cause in-depth changes in bee physiology. For instance, neurotoxic insecticides, by targeting the neurotransmission pathways of insects, impact the neural plasticity, and brain function and structure of bees (Cabirol and Haase 2019). Thus, single pesticide exposure may lead to life-long impacts if they inhibit or modify a physiological and/or developmental process in the bee brain.

In honeybees, foraging activity is preceded by orientation flights, during which individuals develop highly complex cognitive capacities essential to navigation and homing (Capaldi and Dyer 1999; Degen et al. 2016). The experience accumulated during this pre-foraging stage then positively influences their foraging capacities and lifespan (Prado et al. 2020). We therefore hypothesized that a perturbation of this neurocognitive process by a neurotoxin could trigger chronic effects. To test this hypothesis we exposed bees at 7-days old, the median age of first orientation flights (Requier et al. 2020; Prado et al. 2020), to two sublethal doses of sulfoxaflor and tracked their flight activity and lifespan with automated life-long monitoring devices (optic bee counters) (Prado et al. 2019, 2020). We expected a modification of bee behavioral performance, given that sulfoxaflor is a new sulfoximine-based insecticide that shares a mode of action with neonicotinoids as selective agonists of nicotinic acetyl choline receptors (nAChRs) (Zhu et al. 2011; Sparks et al. 2013), and nAChRs play a central role in honeybee cognition (Gauthier and Grünewald 2012) .

Materials and methods

Experimental setup

Experiments were performed in a peri-urban area (Avignon, France, 43°540N-4°-520E) with honeybees (*Apis mellifera*) from INRAE livestock. Newly emerged bees were collected from 3 colonies and marked with a data-matrix barcode (3 mm of diameter) glued on the thorax (Sader®).

They were then released into a colony equipped with a bee counter, which consists of a camera that monitors the hive entrance and image analysis software that detects and registers the activity of bees (direction: in or out of the hive, and time of activity: day, hour, minute, and second) (Prado et al. 2020). The experiment was repeated five times in 2019 (2 in May, 2 in June and 1 in July) using three different colonies equipped with bee counters (2 colonies were used twice). Each time, between 500 and 800 bees were introduced into colonies (see Table S1 for details). Colonies consisted of five-frames with similar population size (around 10 000 adult worker bees) and resource storage (honey and pollen). They were all treated against the parasite *Varroa destructor* the previous year (Apistan).

Exposure to sulfoxaflor

Tagged bees were allowed to develop normally for 7 days after their introduction into colonies. They were then collected and randomly assigned to experimental groups (n = 64-126 bees per experimental group; Table S1). Bees were individually fed with 2 µL of a solution of 30% (w/v) sucrose, 0.1 % acetone and sulfoxaflor at 5 µg/ml or 25 µg/ml, which corresponded to a theoretic exposure of 10 and 50 ng of sulfoxaflor/bee and to the ~LD_{50}/15 and LD_{50}/3 reported by EFSA for in-hive (i.e. young) bees (146 ng/bee) (EFSA 2014). Control bees were individually fed with pesticide-free syrup (30% (w/v) sucrose and 0.1% acetone).

The doses 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 and from 0.15 to 2.78 mg/kg respectively in nectar and pollen collected by bees (U.S. EPA 2019). Considering that young bees consume between 35 and 60 mg of nectar and 6 and 10 mg of pollen per day (Rortais et al. 2005; Rodney and Purdy 2020), we could reasonably assume an exposure to sulfoxaflor between 1.4 and 142 ng in nectar and between 0.9 and 27.8 ng in pollen.

After exposure, bees were placed in plastic cages (10.5 cm × 7.5 cm × 11.5 cm) (Pain 1966) and kept for 60 min in the dark to allow them to ingest the sucrose solution before releasing them into their host colony. Individual flight behavior was recorded continuously from day 1 (introduction date) to day 45.

Stock solutions of sulfoxaflor (Techlab, France) were prepared with acetone, aliquoted and conserved at -20°C. The exact concentrations were checked with LC-MS/MS (European Standard EN 15662:2018 procedure) and resulted in 8 µg/ml and 30 µg/ml for the prepared sulfoxaflor concentrations, which corresponded to a real exposure of 16 ng/bee and 60 ng/bee, respectively.

Measuring bee life-history traits

After exposure to the treatments, we tracked 1521 bees overall (3 experimental groups x 5 replicates). Bees without at least one exit and entrance sequence (i.e. a flight sequence) were excluded from analyses, thus longevity and behavioral data were successfully recorded for 1108 bees. This loss was attributable to the loss of the tag number, the ejection of some tagged bees by nestmates, or an early death of individuals (just after re-introduction). Among the 1108 tracked bees, we obtained data for

407 control bees, and 376 and 325 bees exposed to 16 and 60 ng of sulfoxaflor, respectively (Table S1).

For the analysis of flight activity, exit-entrance sequences shorter than 2 sec or longer than 180 min were excluded (Requier et al. 2020; Prado et al. 2020). The last detection for each barcoded bee was used to calculate bee survival over the 45 days of the experiment. The age at onset of foraging (AOF) was computed for each bee using the *aof* function developed in the *aof* R-package (Requier and Rebaudo 2020).

Data analysis

Data were analyzed using the statistical software R v4.0.3 (R Core Team 2020). Survival analyses were performed with the Kaplan-Meier method, using the *survival* package (Therneau 2021) and followed by a log-rank test for the comparison of survival between treatments. Data were transformed in a survival table and the remaining bees were considered alive at day 45.

Variations in flight activity in response to treatments, flight experience (flight activity before pesticide exposure) and age (fixed factors) were analyzed using GLMM fitted by Penalized Quasi-Likelihood (*glmmPQL* function), using the *MASS* R-package (Venables and Ripley 2002). The total number of flights, the daily number of flights and the daily duration of flights over the 45 days were fitted with a Quasi-poisson error distribution due to over-dispersion in the data. The total duration of flight activity (log_{10} transformed) and the AOF were fitted with a Gaussian error distribution, using the *glmmPQL* function. We introduced a quadratic term (age^2) into our daily flight activity models to account for a non-linear pattern of the observed relationship between daily flight activity and bee age. Multiple comparisons between levels of treatment were performed using the Tukey method in the *glht* function of the *multcomp* package (Hothorn et al. 2008). Variations in the total number and duration of flights were analyzed with treatment and flight experience as fixed factors, and replicate as a random factor. Variations in the daily activity (number and duration of flights) of each bee were analyzed with treatment and bee age as fixed factors, and replicate and bee identity as random factors. Effects of treatment on the AOF were investigated with replicate as a random factor. The replicate variable was considered as a random factor in the models given that this variable accounted for seasonal variability between bee cohort introductions performed between May and July.

Results

Bee survival

Bee survival did not significantly differ between experimental groups, demonstrating that the tested doses were non-lethal (Kaplan–Meier: p = 0.1; log Rank test, control *vs.* dose 16 ng: p = 0.56; control *vs.* dose 60 ng: p = 0.19, and dose 16 ng *vs.* dose 60 ng: p = 0.12; Fig. S1).

Total flight activity

At day 7 (day of exposure to sulfoxaflor), the percentage of bees that had already performed their first flight did not differ between experimental groups (19.10 ± 0.01 % control bees, 18.74 ± 0.02 % and 14.81 ± 0.001 % bees exposed to 16 or 60 ng of sulfoxaflor, respectively; $\chi 2 = 0.0012$, $df = 2$, $P = 0.999$). As expected, the total flight activity of bees before pesticide exposure did not differ between experimental groups (Table S2 and S3).

However, the total number of flights was significantly affected by the exposure to sulfoxaflor; bees exposed to 16 ng and 60 ng of sulfoxaflor made significantly fewer trips over the 45 days (28.06 ± 40.21 days and 21.25 ± 24.88 days, respectively) than control bees (33.23 ± 64.22 days; Table 1; Fig. 1A). No difference in the number of flights was observed between the two pesticides doses (Table S4). The total number of flights after exposure to the pesticide treatment was not affected by flight experience (Table 1; Fig. 1A). The total duration of flight activity was not affected by exposure to sulfoxaflor (16 ng: 53 166.8 ± 6 3681.79 sec, 60 ng: 47 441.21 ± 58 916.81 sec and control: 54 117 ± 65 037.63 sec), but it was negatively related to flight experience (Table 1; Fig 1B). The sulfoxaflor exposure of 16 ng balanced out this negative relationship, although the size effect of this interaction was rather small (Table 1; Fig. 1B).

Time series of flight activity

Overall, the daily number of flights was significantly lower in bees exposed to the lower or higher dose of sulfoxaflor compared to control bees (Table 2; Fig. 2A), and differed between the sulfoxaflor doses, with bees exposed to the higher dose performing less daily flights than bees exposed to the lower dose (Table S5). The daily number of flights also changed according to the age of bees. This relationship was significantly affected by both doses of sulfoxaflor, although the effect was less pronounced for the lower dose (Table 2; Fig. 2A). Young bees belonging to the different treatment groups performed a similar number of daily flights, but their daily activity started to differentiate once they reached the AOF (age at onset of foraging). This latter did not differ between treatment groups (14.71 ± 5.27 for control bees, 14.92 ± 5.02 and 14.11 ± 4.47 for bees exposed to 16 or 60 ng of sulfoxaflor, respectively; GLMM, dose 16 ng: $p = 0.703$ and dose 60 ng: $p = 0.261$; Table S6). Consequently, the number of foraging flights (i.e. flights performed after the AOF) was lower in sulfoxaflor-exposed bees as compared to control bees (32.60 ± 51.19) but did not differ between the two doses (24.75 ± 27.93 and 21.62 ± 22.29, respectively for bees exposed to 16 or 60 ng of sulfoxaflor; GLMM, dose 16 ng: $p < 0.01$ and dose 60 ng: $p < 0.005$; Table S7).

The daily duration of flights was not affected by treatments (Table 2; Fig 2B). Only bee age had an effect. Similarly, to the daily number of flights, the duration increased with age up to the age of 30-days old and then declined (Table 2; Fig 2B).

Discussion

In the present study, we showed that acute exposure in honeybees does not necessarily cause short-term effects but can generate delayed effects, as previously found in others insects (Beketov and Liess 2008; Wolz et al. 2021). Long-term effects following exposure to a single dose of pesticide have been previously described in honeybees. However, contrary to our study, latency effects were not investigated and effects were only reported at doses that significantly increased honeybee mortality (Schneider et al. 2012; Shi et al. 2020). The sulfoxaflor doses we tested (16 and 60 ng/bee) were below the LD50 determined for in-hive bees (146 ng/bee) (EFSA 2014) and proved to be non-lethal since no mortality increase was found as compared to control groups over the 45 days of monitoring. Recently, a lower LD50 has been reported for forager bees (55 ng/bee) (Azpiazu et al. 2021). The lack of toxicity of a similar dose in our experiment seems to confirm that in-hive bees are less sensitive to insecticides than forager bees (Tosi and Nieh 2019), although this might not be a general rule as it could depend on the pesticide type (Rinkevich et al. 2015). Another possible explanation for the non-toxicity of the doses in our study is that we monitored bee survival in field conditions. Control honeybees live less long in field conditions than in the laboratory, and therefore the risk of death of experimental groups relative to control groups tend to be reduced in field conditions as compared to the laboratory (Alaux et al. 2014).

Although both doses of sulfoxaflor significantly reduced the number of flights performed by bees, the total duration of flight activity was not affected, as would be expected from a lower number of flights but of similar duration. This might be explained by the natural variation in the duration of flights combined with the fact that the effect of sulfoxaflor on daily activity only emerged at about the time bees transitioned to foraging activity, i.e. a week after exposure to the pesticide. Interestingly, bees that had longer flight activity before exposure had shorter activity afterwards, suggesting that bees have a defined time-budget for their flight activity. We are cautious in our interpretation, because bees were experimentally manipulated during their activity; this work remains to be investigated with non-manipulated free-flying bees. This relationship between flight experience and the duration of flight activity after treatment was affected by the lower sulfoxaflor dose, which resulted in bees flying for a rather fixed amount of time regardless their flight experience. The underlying mechanisms are not known but the effect was quite marginal and driven by a few bees.

An increase in oxidative stress and cell apoptosis, as well as a modification of immunocompetence, have been described upon sublethal exposure to sulfoxaflor (Chakrabarti et al. 2020; Al Naggar and Paxton 2021; Li et al. 2021). However, the behavioral impairments we observed are more in line with an impact on bee cognition. Since sulfoxaflor and neonicotinoids have a similar mode of action (agonist of nAChRs), and neonicotinoids are known to negatively influence bee behavior through impact on cognition (Belzunces et al. 2012; Siviter et al. 2018), we can reasonably assume that sulfoxaflor also affected bee behavioral performance through cognitive impairments. In our experiments, bees were exposed to sulfoxaflor when they initiated their orientation flights. It is therefore possible that sulfoxaflor affected their learning abilities, explaining why bees made later and

fewer foraging flights. By testing the olfactory conditioning performances of honeybee foragers, Siviter et al. (2019) did not find any negative effects of acute sulfoxaflor exposure on learning and memory. However, besides the lower doses (0.05 to 2.5 ng), the tests were performed immediately after exposure to sulfoxaflor. The response to sulfoxaflor we found took more than a few days to develop and could therefore be considered delayed. The latency of response to pesticide may depend on many factors, such as the rates of absorption and distribution and the speed of action at the target site. But it may also depend on the physiological process that is affected. Interestingly, by analyzing the effect of imidacloprid (neonicotinoid) on habituation of the proboscis extension reflex, Guez et al. (2001) found a contrasting effect between 7- and 8-day-old bees. This effect seemed to be associated with a differential expression of two subtypes of nAChRs during bee behavioral maturation i.e. transition from nurse to forager tasks, (Guez et al. 2003). Later, it was found that during their behavioral maturation, young bees exhibit massive changes in brain gene expression, which are essentially completed at 8 days old (Whitfield et al. 2006) and coincide with structural brain changes (Farris et al. 2001). Altogether these studies provide strong evidence for developmental change in the neurophysiological state of 1-week old bees which might explain the effects of sulfoxaflor. By affecting this neurodevelopmental process essential to behavioral maturation, a single dose of neurotoxin might cause intrinsic changes in the bee brain and therefore impair future foraging performances.

Whilst much work remains to investigate the occurrence of long-term effects after acute exposure, our findings blur the general assumption in honeybee toxicology that acute exposure results in immediate and short-term effects. Therefore, long-term recording and/or time-to-effect measurements are needed, even upon exposure to a single dose of pesticide, to avoid a potential underestimation of pesticide toxicity.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This project received funding from the European Horizon 2020 research and innovation program under grant agreement no. 773921 (LB, YLC and CA).

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., C.A. and F.R. analysed the data, Y.L.C. and C.A. contributed to reagents. L.B, C.A and F.R. wrote the manuscript. All authors read and reviewed the manuscript.

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Figure legends

Figure 1. Total flight activity in response to pesticide treatments and flight experience. (A) Total number of flights and (B) total duration of flight activity (sec.) over 45 days (n = 407 control bees, n = 376 and 325 bees exposed to 16 or 60 ng of sulfoxaflor, respectively). Thick lines represent the model predictions with shaded areas indicating ½ standard error. Colored marks show the distribution of raw data along the horizontal axis.

Figure 2. Time series of flight activity in response to pesticide treatments. (A) Number and (B) duration of daily flights (sec.) over bee lifetime (n = 407 control bees, n = 376 and 325 bees exposed to 16 or 60 ng of sulfoxaflor, respectively). Thick lines represent the model predictions with shaded areas indicating ½ standard error and the dashed line represents the day of exposure to pesticides. The transition to foraging activity started on average at 14.5 days. Colored marks show the distribution of raw data along the horizontal axis.

Table 1. Results of the generalized mixed effect models assessing the effects of pesticide treatments and flight experience on the total flight activity. Intercept represents the control bees. Flight experience corresponds to the flight activity of bees before exposure to pesticide.

Parameters	Estimate	Standard error	*p*-value
Total number of flights			
Intercept	3.5824	0.1938	**< 0.001**
Flight experience (number of flights before pesticide exposure)	-0.1401	0.0736	0.0572
Dose 16 ng	-0.3391	0.1262	**< 0.01**
Dose 60 ng	-0.5145	0.1509	**< 0.001**
Flight experience × Dose 16 ng	0.1510	0.0840	0.0725
Flight experience × Dose 60 ng	0.1345	0.1178	0.2537
Total duration of flight activity (sec.)			
Intercept	4.2666	0.1120	**< 0.001**
Flight experience (duration of flight activity before pesticide exposure)	-0.0001	0.00003	**< 0.01**
Dose 16 ng	0.00334	0.06016	0.9568
Dose 60 ng	-0.0138	0.06050	0.832
Flight experience × Dose 16 ng	0.0001	0.00004	**0.0339**
Flight experience × Dose 60 ng	0.0001	0.00005	0.2898

445 **Table 2. Results of the generalized mixed effect models assessing the effects of pesticide treatments and age on the time series of flight activity.** Intercept represents the control bees. Age^2 represents a quadratic term to take into account a non-linear pattern of the observed relationship between daily flight activity and bee age.

Parameters	**Estimate**	**Standard error**	***p*-value**
Time series of flight number			
Intercept	-1.9041	0.1516	**< 0.001**
Age (days)	0.3471	0.0168	**< 0.001**
Dose 16 ng	0.4730	0.2033	**0.0202**
Dose 60 ng	1.0872	0.2170	**< 0.001**
Age × Dose 16 ng	-0.0545	0.0230	**0.018**
Age × Dose 60 ng	-0.1393	0.0248	**< 0.001**
Age^2	-0.0083	0.0004	**< 0.001**
Age^2 × Dose 16 ng	0.0012	0.0006	0.0555
Age^2 × Dose 60 ng	0.0036	0.0007	**< 0.001**
Time series of flight duration (sec.)			
Intercept	3.9608	0.1836	**< 0.001**
Age (days)	0.2996	0.0141	**< 0.001**
Dose 16 ng	-0.0811	0.2047	0.6920
Dose 60 ng	0.3517	0.2128	0.0987
Age × Dose 16 ng	0.0140	0.0201	0.4861
Age × Dose 60 ng	-0.0235	0.0212	0.2691
Age^2	-0.0047	0.0003	**< 0.001**
Age^2 × Dose 16 ng	-0.0005	0.0005	0.2859
Age^2 × Dose 60 ng	0.0004	0.0005	0.3779

Figure 1

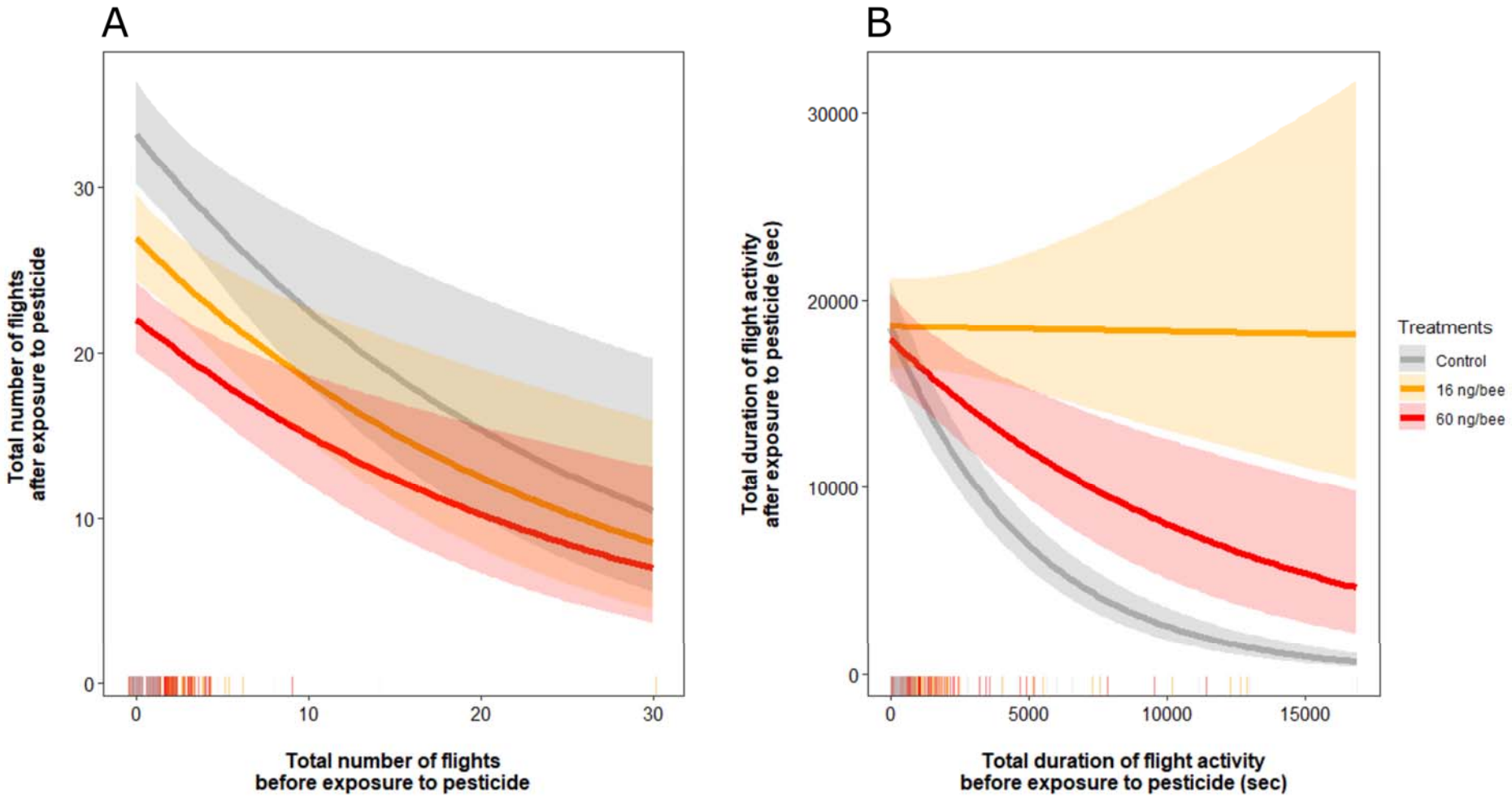
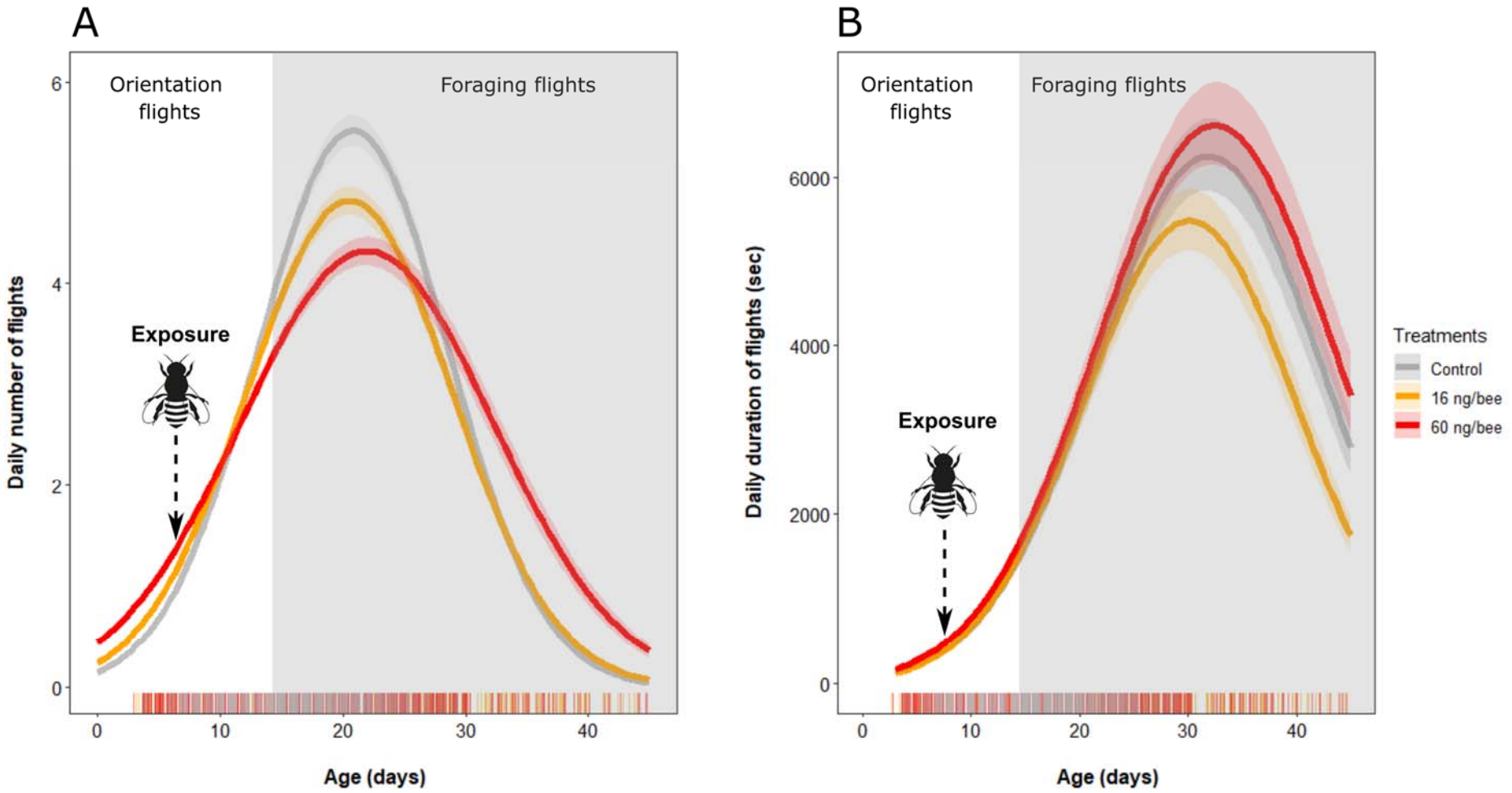

A
Total number of flights after exposure to pesticide
0
10
20
30
Total number of flights before exposure to pesticide
B
Total duration of flight activity after exposure to pesticide (sec)
0
10000
20000
30000
0
5000
10000
15000
Total duration of flight activity before exposure to pesticide (sec)
Treatments
Control
16 ng/bee
60 ng/bee

Figure 2


A
Orientation flights
Foraging flights
Exposure
Daily number of flights
0
2
4
6
0
10
20
30
40
Age (days)
B
Orientation flights
Foraging flights
Exposure
Daily duration of flights (sec)
0
2000
4000
6000
0
10
20
30
40
Age (days)
Treatments
Control
16 ng/bee
60 ng/bee

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