Pesticide risk assessment: honeybee workers are not all equal regarding the risk posed by exposure to pesticides

Lena Barascou, Deborah Sene, Yves Le Conte, C. Alaux

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Lena Barascou, Deborah Sene, Yves Le Conte, Cedric Alaux

INRAE, Abeilles et Environnement, Avignon, France

Corresponding authors: lena.barascou@inrae.fr, cedric.alaux@inrae.fr

Abstract

Toxicological studies in honeybees have long shown that a single pesticide dose or concentration does not necessarily induce a single response. Inter-individual differences in pesticide sensitivity and/or the level of exposure (*e.g.* ingestion of pesticide-contaminated matrices) may explain this variability in risk posed by a pesticide. Therefore, to better inform pesticide risk assessment for honeybees, we studied the risk posed by pesticides to two behavioral castes, nurse and forager bees, which are largely represented within colonies and which exhibit large differences in their physiological backgrounds. For that purpose, we determined the sensitivity of nurses and foragers to azoxystrobin (fungicide) and sulfoxaflor (insecticide) upon acute or chronic exposure. Azoxystrobin was found to be weakly toxic to both types of bees. However, foragers were more sensitive to sulfoxaflor than nurses upon acute and chronic exposure. This phenomenon was not explained by better sulfoxaflor metabolization in nurses, but rather by differences in body weight (nurses being 1.6 times heavier than foragers). Foragers consistently consumed more sugar syrup than nurses, and this increased consumption was even more pronounced with pesticide-contaminated syrup (at specific concentrations). Altogether, the stronger susceptibility and exposure of foragers to sulfoxaflor contributed to increases of 2 and 10-fold for the acute and chronic Risk Quotients, respectively, compared to nurses. In conclusion, to increase the safety margin and avoid an under-estimation of the risk posed by insecticides to honeybees, we recommend systematically including forager bees in regulatory tests.

Keywords: *Apis mellifera*, nurse, forager, pesticide sensitivity, pesticide metabolization, risk quotient

Introduction

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). This is the first mandatory step that includes acute toxicity tests after oral or contact exposure in adults (OECD 1998a, b) and a chronic oral toxicity test on adults (OECD 2017). Next, a deterministic approach to characterize risk quantitatively can be used by comparing the pesticide toxicity to environmental exposure. For that purpose, a Risk Quotient (RQ) is generally calculated by dividing a point estimate of exposure by a toxicity

end-point value (*e.g.* LD_{50} or chronic non-observable effect dose - NOED) (Thompson 2021). If RQ values rise above determined levels of concern (*e.g.* 0.4 or 1 for acute and chronic exposure, respectively), then high-risk situations are identified and supplementary tests are required at a higher tier (semi-field and field tests) for regulatory decision making (Thompson 2021).

However, a single pesticide dose or concentration does not necessarily induce a single response, as the level of the measured toxicity endpoint may vary depending on the physiological state of honeybees (Poquet et al. 2016). Investigating this intraspecific variability or modulation of response is therefore important, notably at Tier 1, to better screen the risks posed by pesticides to honeybees. In this regard, some studies have shown that heavier honeybees are less sensitive to pesticides than lighter honeybees (Tahori et al. 1969; Gerig 1991; Nogueira-Couto et al. 1996). In addition, sensitivity may depend on age with younger bees being more sensitive to certain pesticides, but less to others, than older bees (Mayland and Burkhardt 1970; Ladas 1972; Bendahou et al. 1997; Rinkevich et al. 2015; Zhu et al. 2020). This is likely related to the tremendous changes in endocrine and metabolic activity occurring during age-related behavioral maturation (transition from nurse to forager tasks) (Robinson 2002). For instance, foragers can weigh two times less than nurse bees (Vance et al. 2009), and the activity of glutathione S-transferase, an enzyme involved in the detoxification pathways (Claudianos et al. 2006; Berenbaum and Johnson 2015), is significantly higher in forager bees than in nurses (Smirle and Robinson 1989). As a result, pesticide sensitivity might strongly vary depending on the behavioral state of honeybee individuals. Confirmation of this hypothesis was given by Tosi and Nieh (2019) who found that foragers were consistently more susceptible to flupyradifurone (4-fold greater effect) than in-hive bees. Nevertheless, this intraspecific difference in pesticide sensitivity between behavioral castes, as well as the underlying mechanisms, has rarely been studied, although it could better inform pesticide risk assessment for honeybees.

In addition, and as mentioned earlier, the risk posed by pesticide to honeybees not only depends on the pesticide toxicity but also on the level of exposure, which includes both the environmental concentrations of pesticides and the level of ingestion or contact with pesticide-contaminated matrices (*e.g.* nectar). While differential consumption of pollen and nectar between behavioral castes of honeybees (Rortais et al. 2005; Rodney and Purdy 2020) is well-established, with, for instance, forager bees consuming more nectar than nurse bees, the consumption of nectar was also found to be affected by pesticide contamination. Indeed, honeybee foragers strongly preferred sugar solutions containing neonicotinoids (imidacloprid

or thiamethoxam), glyphosate or chlorothalonil at specific concentrations, and avoided prochloraz at high concentrations (Kessler et al. 2015; Liao et al. 2017). The mechanisms underlying this preference or avoidance of contaminated nectars are currently not known, but this phenomenon could potentially trigger an increase or decrease in the exposure to pesticides, which would lead to differences between behavioral castes.

Therefore, in order to better quantify the risks posed by pesticides on honeybees, intraspecific variability in bee sensitivity and exposure (consumption of contaminated food) to these chemicals needs to be considered in risk assessment tests. To investigate to what extent behavioral caste affects the risks posed by pesticides , we exposed nurse and forager bees either to sulfoxaflor, a new neurotoxic insecticide that shares the same mode of action with neonicotinoids, or to azoxystrobin, a fungicide widely used in agriculture and regularly found in bee-foraged food (Mullin et al. 2010; Sanchez-Bayo and Goka 2014; Long and Krupke 2016). We then assessed the sensitivity of nurse and forager bees to both pesticides, as well as their consumption of contaminated food, and calculated the acute and chronic RQs. We finally investigated whether dissimilarities in pesticide sensitivity could be attributed to differences in detoxification capacities by measuring the residual concentrations of pesticides over time in both nurse and forager bees.

Materials and methods

1. Bee sampling

Experiments were conducted with honeybees obtained from a local apiary at the "*Institut National de la Recherche pour l'Agriculture, l'Alimentation et l'Environnement"* (INRAE) in Avignon (France). To determine whether pesticide sensitivity differs between bees of different behavioral castes, nurses and foragers were collected the same day from four different colonies. Nurses were identified by removing brood combs from colonies and detecting bees that dipped their heads into several cells containing larvae. Foragers were identified as bees returning to the colony with pollen loads, thereby discarding bees performing orientation or cleansing flights.

To validate the nurse sampling method, we checked whether bees collected as nurses had more developed hypopharyngeal glands (HPG) than forager bees. Indeed, HPGs, where jelly is produced to feed larvae, the queen and drones (Crailsheim 1992), are consistently more developed in nurse than forager bees (Knecht and Kaatz 1990; Robinson et al. 1992). For that

purpose, 10 bees of each behavioral caste were sampled in all colonies and stored at -20°C. Glands from 10 bees per caste and colony were dissected in distilled water under a binocular magnifier (LEICA MZ 12). Pictures of each gland were taken with a digital camera (Toupcam™) and ToupView image-capturing software (v3.7.5660). Then, the gland development was assessed by measuring the maximum diameter of 10 to 15 randomly chosen ovoid acini per gland using ImageJ v1.53e (http://rsb.info.nih.gov/ij/index.html). The diameters of acini were significantly larger in nurse (80.34 ± 9.66 µm) than in forager bees (53.54 ± 9.45 µm; Kruskal-Wallis test, $\chi^2 = 1274.4$, $p < 0.001$), which validated our sampling method.

After collecting bees from the four different colonies, nurses and foragers were immediately placed in different cages (10.5 cm x 7.5 cm x 11.5 cm) (Pain 1966; Williams et al. 2013) containing a feeding tube with a solution of 50 % (w/v) sucrose and brought back to the lab. They were then placed in an incubator under controlled conditions (28°C and 50-70 % relative humidity).

2. Nurse and forager bee sensitivity to pesticides

Acute toxicity tests

We assessed the sensitivity of nurse and forager bees to sulfoxaflor and azoxystrobin by exposing them to a range of pesticide doses and estimating the dose-response curve. We tested a control dose (0 ng/bee) and a total of five doses of azoxystrobin (1, 10, 20, 40, 60 µg/bee) and six doses of sulfoxaflor (1, 10, 25, 50, 100, 150 ng/bee). Each exposure to azoxystrobin or sulfoxaflor was tested alone following the OECD test guidance for acute oral toxicity tests (OECD 1998a). The doses were chosen to surround the theoretical LD_{50} previously reported by EFSA for each pesticide (LD_{50} EFSA: > 25 µg/bee for azoxystrobin and 146 ng/bee for sulfoxaflor (EFSA 2010, 2014).

Stock solutions of sulfoxaflor (Techlab, France) and azoxystrobin (Sigma Aldrich, France) in acetone were previously aliquoted and conserved at −20°C. Bees were sugar-starved for 2 h and then fed with a solution of 50% (w/v) sucrose and azoxystrobin (10% acetone) or sulfoxaflor (1% acetone). Since a previous study showed that a uniform food distribution is best approached with 10 bees par cage (Brodschneider et al. 2017), for each dose, one cage of 10 bees per colony was used (except for one colony for which we had 2 cages per dose). Each treated cage received 100 µl of the solution laced with pesticides, which was fully consumed within 60 min (giving around 10 µl per bee) Control groups were fed with pesticide-free sucrose solution (50% w/v sucrose, 1 or 10% acetone). The experiment was repeated twice for

each colony (n = 10 cages per dose and behavioral caste). Mortality was recorded at 24 and 48h post-exposure.

To express the toxicity endpoint (median lethal dose – LD_{50}) in ng per g of bees, we determined the weight of nurse and forager bees. We did not weigh test bees but bees from the same colony and collected at the same time as the acute toxicity tests. Bees were frozen at -20°C, then individually placed in glass petri dishes, at room temperature for 1 hour. They were weighed and then dried at 60°C for 72 hours to measure their dry weight.

Chronic toxicity test and pesticide-contaminated syrup consumption

Nurse and forager bees were chronically exposed to a low and a high concentration of pesticides. Groups of 20 nurse or forager bees from the same four colonies were placed in different cages (n = 1 or 2 cages per colony giving n = 6 cages per pesticide concentration and behavioral caste). Bees were provided with a solution of 50 % (w/v) sucrose, 0.1 % acetone and azoxystrobin (0.2 or 2 µg/ml) or sulfoxaflor (0.02 or 0.2 µg/ml). Control groups were fed with pesticide-free sugar solution (50 % w/v sucrose, 0.1 % acetone). The concentrations were chosen based on pesticide residue data found in nectar. Depending on different application rates of sulfoxaflor and the crops, field studies reported levels of the neurotoxin ranging from 0.04 to 2.37 mg/kg in nectar (EPA 2019). Residues of azoxystrobin have been found at high concentrations (up to 1.45 mg/kg) in nectar collected by honeybees, shortly after the application day (Schatz and Wallner 2009). The chronic pesticide treatments were performed over 5 days and the syrup feeders were replaced every day. Since forager lifespan is on average 8 days (Prado et al. 2020), chronic toxicity tests were performed over 5 days to minimize the risk of natural forager mortality. For each cage, individual syrup consumption was assessed daily, by weighing feeders and dividing the consumed food by the number of remaining live bees. Dead bees were counted daily and removed over the 5-day period. The exact concentrations of sulfoxaflor and azoxystrobin were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS, see Barascou et al. 2021), giving low and high concentrations of 0.021 and 0.223 µg/ml for sulfoxaflor and 0.16 µg/ml and 1.46 µg/ml for azoxystrobin.

3. Residual concentrations of pesticides in nurse and forager bees

In order to investigate the potential mechanisms underlying the difference in pesticide sensitivity between nurses and foragers, we compared their residual pesticide concentrations at 2 and 8 h post-exposure. Groups of 60 bees were placed in cages. Bees were starved for 2

hours and then fed with a concentration of 2 µg/ml of azoxystrobin or sulfoxaflor. Control groups were fed a sucrose solution only. Each cage received 600 µl of sucrose solution, giving a theoretical dose of 20 ng of pesticide per bee (19 ng of azoxystrobin and 23.5 ng of sulfoxaflor, based on the exact concentration of the tested solution, see above). Once solutions were all consumed, bees were provided with a 50 % sucrose solution (w/v). At 2 and 8h post-exposure, 50 bees per cage were respectively sampled on dry ice and stored at -80°C for later analysis (n = 5 cages per behavioral caste, pesticide and time point).

Pesticide concentrations were analyzed on pools of 25 bees (2 pools per cage giving n = 10 pools per behavioral caste, pesticide and time point). Each pool of bees was weighed and sulfoxaflor and azoxystrobin content were subsequently analyzed via LC-MS/MS. The QuEChERS method was used for the extraction of the active ingredients from samples, following the European Standard EN 15662 (see Barascou et al. (2021) for further method details). The limit of quantification for azoxystrobin and sulfoxaflor was 0.001 mg/kg and 0.01 mg/kg, respectively.

4. Statistical analysis

Data were analyzed using the statistical software R v3.3.3 (R Core Team 2020). In the acute toxicity test, the LD_{50} values were calculated for each pesticide and each behavioral caste, by fitting a dose-response model to the data (*drm* function of the "drc" package) (Ritz et al. 2015). Different models were compared based on the log likelihood value, Akaike's information criterion, and the estimated residual standard error. For sulfoxaflor and azoxystrobin data, the two-parameter (W1.2) and four-parameter Weibull models (W2.4) were shown to best describe the data analyzed for nurses and foragers, respectively, and were therefore used to calculate all of the dose–concentration response curves in the present study. The toxicity between nurses and foragers was compared by interpooling the 95% CI (confidence interval) limits of the LD_{50} values, considering the LD_{50} as different if the CI values did not overlap.

Variations in body weights between nurses and foragers were analyzed using a Kruskal-Wallis test, followed by Dunn's multiple comparison tests with the Benjamini–Hochberg correction. Syrup consumption between nurses and foragers, and among experimental groups in the chronic toxicity experiment was also analyzed using a Kruskal-Wallis test, followed by Dunn's multiple comparison test with the Benjamini–Hochberg correction. Survival data from the chronic toxicity tests were analyzed with a Cox proportional hazards regression model (*coxph* function of the survival package in R (Cox 1970)).

In order to assess the potential risk posed to nurse and forager bees by an acute exposure to pesticide, a Risk Quotient (RQ) was calculated based on the exposure concentration, the caste-specific sucrose consumption values from the chronic toxicity experiment (mean consumption within 5 days), and acute toxicity data (LD_{50}):

$$RQ = \frac{\text{Exposure concentration (µg/kg)} \times \text{Consumption (kg/day)}}{\text{LD50 (µg/bee)}}$$

We similarly assessed the potential risk posed to nurse and forager bees by a chronic exposure to pesticide, by using the NOED from the chronic toxicity test:

$$RQ = \frac{\text{Exposure concentration (µg/kg)} \times \text{Consumption (kg/day)}}{\text{chronic 5 day oral NOED (µg/bee/day)}}$$

The acute and chronic RQ threshold levels of concern (LOC) are 0.4 and 1, respectively. If the RQ is less than 0.4 or 1, the risk posed by the pesticide is acceptable, but if the RQ is equal or greater than 0.4 or 1, the risk is not acceptable (Thompson 2021).

Residual pesticide concentrations were compared between behavioral castes and time points using Wilcoxon rank test pairwise comparisons and a Bonferroni adjustment.

Results

Acute toxicity for nurse and forager bees

We recorded bee mortality rates at both 24 and 48 h following exposure to the different doses of pesticides (Table S1 and S2). We then calculated the LD_{50} of each pesticide by using the 48 h mortality data since additional mortality occurs between 24 and 48 h post-exposure. Only a slight overlap in the 95% CI values was observed between the dose-response curves of nurse and forager bees, and the calculated sulfoxaflor LD_{50} was lower for foragers (41.04 ng/bee,) than for nurse bees (54.40 ng/bee; Fig.1 and Table 1). Nurses were 1.6 times heavier (134.77 ± 24.24 mg) than foragers (85.81 ± 8.69 mg, Kruskal-Wallis test, $\chi^2 = 69.88$, $p < 0.001$; Table 1), and when the sulfoxaflor toxicity was expressed in ng per g of bees, a strong overlap was observed between the dose-response curves of nurses and foragers (Table 1).

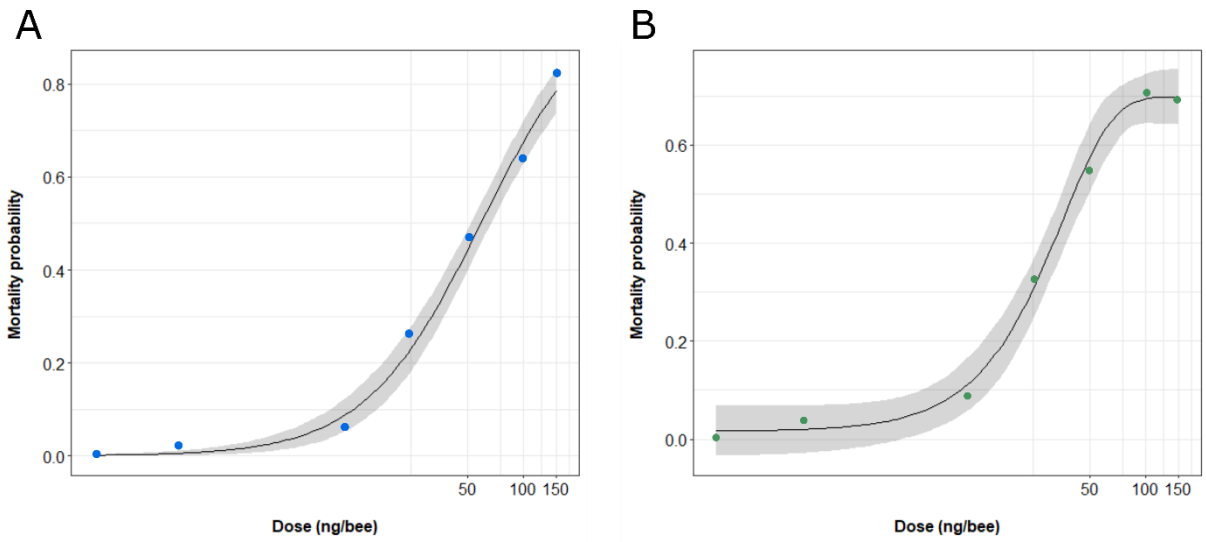

Figure 1. Dose-response relation resulting from oral exposure to sulfoxaflor in (A) nurse and (B) forager bees. Data show bee mortality at 48 h post-exposure (n = 10 bees per cage and 10 cages per dose). Dots represent the mean values of bee mortality for each tested dose. Grey areas show the confidence interval at 95%.

Tested azoxystrobin doses marginally increased bee mortality (up to 20% for the highest dose; Table S2). It was therefore not possible to determine an exact azoxystrobin LD_{50} for both foragers and nurses (LD_{50} values > 60 µg a.i./bee; Table 1).

Table 1. Oral median lethal dose (LD_{50}) of sulfoxaflor and azoxystrobin in nurse and forager bees. N: total number of bees treated with one of the pesticides.

Bee caste	Pesticide	N	LD_{50} (95 % CI), ng/bee	LD_{50} (95 % CI), ng/g
Nurses	Sulfoxaflor	685	54.40 (47.47 – 61.31)	403.65 (352.23 – 454.92)
	Azoxystrobin	605	> 60 µg/bee	> 445.20 µg/g
Foragers	Sulfoxaflor	710	41.04 (33.50 – 49.28)	478.27 (390.40 – 574.29)
	Azoxystrobin	602	> 60 µg/bee	> 699.22 µg/g

Chronic toxicity for nurse and forager bees

Regardless of the pesticide and its concentration, forager bees consistently consumed more sugar solution than nurse bees (sulfoxaflor: Kruskal-Wallis test, $\chi^2 = 31.49$, $p < 0.001$ and azoxystrobin: $\chi^2 = 37.67$, $p < 0.001$; Fig. 2). Overall, forager bees ingested 1.4 times more syrup than nurse bees. Although we did not find any effect of pesticide concentration on sugar consumption by nurse bees (sulfoxaflor: $p = 0.38$ and azoxystrobin: $p = 0.528$), a significant effect was observed in forager bees (sulfoxaflor: $p = 0.024$ and azoxystrobin: $p < 0.01$; Fig. 2). Foragers exposed to 0.021 µg/ml of sulfoxaflor consumed more syrup (68.79 ± 19.76 mg/day) than control foragers (53.90 ± 14.90 mg/day, Dunn's test, $p = 0.022$) but not for

foragers exposed to 0.223 µg/ml of sulfoxaflor (p = 0.07). Similarly, foragers exposed to 1.46 µg/ml of azoxystrobin consumed more syrup (72.16 ± 26.71 mg/day) than bees exposed to 0.16 µg/ml of azoxystrobin (54.16 ± 14.11 mg/day, p < 0.01) and control bees (53.90 ± 14.90 mg/day, p < 0.01).

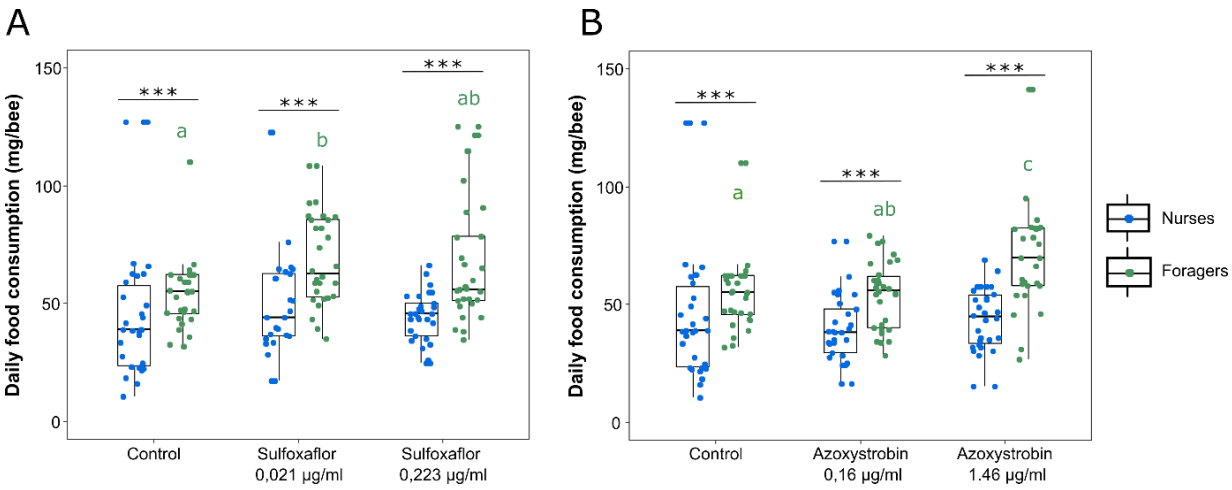

Figure 2. Individual syrup consumption according to pesticide treatments in nurse and forager bees. Daily individual consumption (mg/bee) is shown for foragers and nurses exposed to (A) sulfoxaflor and (B) azoxystrobin (n = 20 bees per cage and 6 cages per pesticide concentration and behavioral caste). Boxes indicate the first and third interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Different letters and number of asterisks indicate significant differences between pesticide concentrations and between nurse and forager bees, respectively (Kruskal–Wallis tests followed by Dunn's multiple comparison test, *** denotes p < 0.001).

Chronic exposure to azoxystrobin (0.16 – 1.46 µg/ml) and sulfoxaflor (0.02 – 0.22 µg/ml) did not affect the survival of nurse bees (Cox model, p = 0.99; Fig. 3A). While we did not find any effect of both azoxystrobin concentrations and the lowest concentration of sulfoxaflor (0.021 µg/ml) on forager mortality, the highest concentration of sulfoxaflor (0.223 µg/ml) reduced their survival probability by around 50% within 5 days (Cox model, p < 0.001, Fig. 3B).

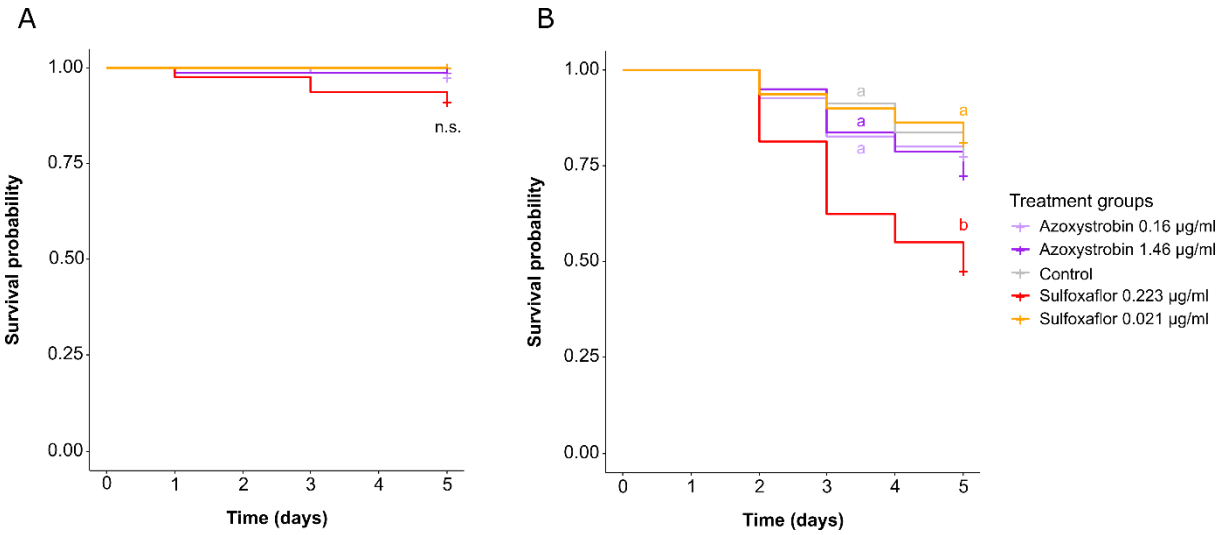

Figure 3. Chronic toxicity of azoxystrobin and sulfoxaflor on (A) nurse and (B) foragers bees. Data represent the survival probabilities of bees (n = 20 bees per cage and 6 cages per pesticide concentration and behavioral caste). Different letters indicate significant differences (Cox model).

Risk quotient for nurse and forager bees

Due to a higher level of exposure (consumption of pesticide-contaminated syrup) and a lower LD_{50}, the acute risk quotient (RQ) upon exposure to sulfoxaflor was twice as high for forager bees than for nurse bees (Table 2). Based on the tested field-relevant concentrations of sulfoxaflor, none of the acute RQ exceeded the theoretical threshold level of concern of 0.4 (Table 2). However, the acute RQ for foragers exposed to 0.223 µg/ml of sulfoxaflor was close to this threshold (RQ = 0.309; Table 2).

The chronic 5-day oral NOED of sulfoxaflor for nurse bees was 8.36 ng/bee/day (NOEC: 0.223 µg/ml). None of the chronic RQ for nurses exceeded the theoretical threshold LOC of 1 (Table 2). However, the chronic 5-day oral NOED for foragers was 1.22 ng/bee/day (NOEC: 0.021 µg/ml), leading to a chronic RQ above the theoretical threshold LOC of 1 (RQ = 10.373) and ten times higher than that of nurse bees (Table 2), when exposed to a sulfoxaflor concentration of 0.223 µg/ml.

The azoxystrobin LD_{50} could not be determined and therefore the acute RQ for both nurse and foragers bees was very low. The chronic RQ based on the higher concentrations of azoxystrobin with no-observed lethal effect (NOEC: 1.46 µg/ml) was calculated for both nurse (NOED: 89.28 ng/bee/day) and forager bees (NOED: 53.81 ng/bee/day). For both concentrations of azoxystrobin, none of the chronic RQ exceeded the theoretical threshold LOC of 1 (Table 2).

Table 2. Acute and chronic RQ for nurse and forager bees under different scenarios of exposure to the tested concentrations of sulfoxaflor and azoxystrobin.

	Nurses			Foragers		
	Consumption (mg/day ± SE)	Acute RQ	Chronic RQ	Consumption (mg/day ± SE)	Acute RQ	Chronic RQ
Sulfoxaflor 0.021 µg/ml	49.38 ± 21.84	0.016	0.105	68.79 ± 19.76	0.030	1
Sulfoxaflor 0.223 µg/ml	44.24 ± 10.68	0.154	1	67.21 ± 25.97	0.309	10.373
Azoxystrobin 0.16 µg/ml	39.52 ± 13.78	< 0.0001	0.099	54.16 ± 14.11	< 0.0001	0.082
Azoxystrobin 1.46 µg/ml	43.49 ± 13.49	< 0.0011	1	72.16 ± 26.71	< 0.0018	1

Residual concentrations of pesticides in nurse and foragers bees

Residues of azoxystrobin were detected at very low concentrations at 2 h post-exposure and could be quantified (above LOQ) in only 3 nurse samples (1.35 ± 0.54 ng/bee) and 6 forager samples (1.26 ± 0.60 ng/bee), demonstrating a high metabolization rate (amount of eliminated pesticide divided by the amount of pesticide to which bees were exposed; nurses: 92.89 ± 4.25 % and foragers: 93.37 ± 3.47 %). At 8h post-exposure, azoxystrobin concentrations were below the LOQ.

Regarding sulfoxaflor, the concentrations of residues found at 2 h post-exposure were lower in forager (metabolization rate = 36.30 ± 13.44 %) than in nurse bees (metabolization rate = 19.59 ± 10.99 %) (Wilcox test, $p < 0.01$; Fig. 4). However, this difference disappeared at 8 h post-exposure (Wilcox test, $p = 0.059$; Fig. 4), likely because sulfoxaflor residual concentrations decreased significantly between 2 and 8 h post-exposure in nurse bees (Wilcox test, $p = 0.036$; metabolization rate at 8 h = 33.31 ± 21.76 %) while it did not in forager bees (Wilcox test, $p = 0.059$; metabolization rate at 8 h = 49.80 ± 10.64 %; Fig. 4).

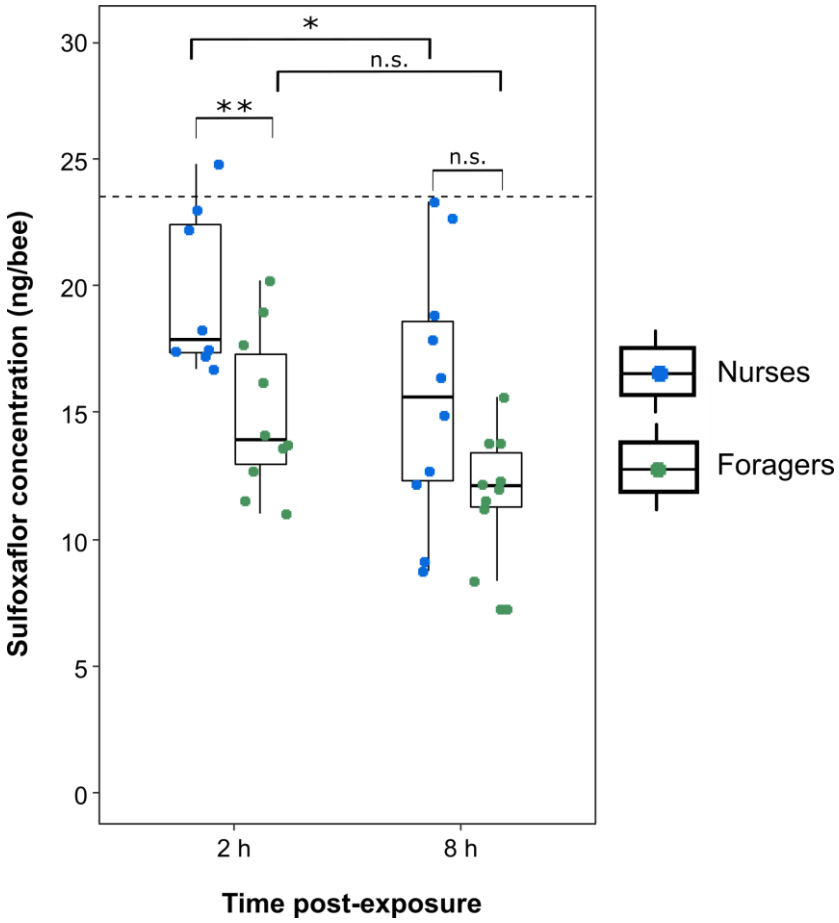

Figure 4. Residual concentrations of sulfoxaflor in nurse and forager bees. Data represent the pesticide concentrations in 10 pools of 25 bees per experimental condition. Boxes indicate the first and third interquartile range with a line denoting the median. Whiskers include 90% of the individuals, beyond which circles represent outliers. Asterisks indicate statistically significant differences between behavioral caste (ns: not significant; *p < 0.05, Wilcoxon test). The dotted lines represent the dose provided to bees (assuming an equal consumption of sugar syrup between bees).

Discussion

Responses to pesticides can be highly variable between species (Uhl et al. 2016; Sgolastra et al. 2017; Spurgeon et al. 2020; Adams et al. 2021; Azpiazu et al. 2021), but also within species (Graves and Mackensen 1965; Calow 1996; Dahlgren et al. 2012; Szabó et al. 2021). This intraspecific variance might add another level of complexity to ecotoxicological studies, but also provides highly relevant information on the risk posed to populations by pesticides (Calow 1996). Such levels of information are especially important for better understanding the risk associated with pesticide exposure in honeybees, which exhibit a high inter-individual variability in their physiological backgrounds.

In the sulfoxaflor acute toxicity test, a slight overlap of the dose-response curves was observed between the two behavioral castes, but in general, foragers were more sensitive to the insecticide as compared to nurse bees. Such results are consistent with a previous study,

which showed that in LD_{50} tests foragers were more susceptible to another insecticide (flupyradifurone) than in-hive bees (Tosi and Nieh 2019). This higher sensitivity of foragers was also confirmed at the chronic level, since exposure to the highest sulfoxaflor concentration significantly reduced the survival of foragers but not of nurse bees. An increase in bee sensitivity to insecticides as bees age has been reported in several studies (Mayland and Burkhardt 1970; Ladas 1972; Bendahou et al. 1997; Zhu et al. 2020). This phenomenon is somewhat in agreement with our results as forager bees are generally older than nurse bees. However, this age-dependent sensitivity is not always consistent as indicated by a Rinkevich et al. (2015) study, which showed that sensitivity to the insecticides naled (organophosphate) and phenothrin (pyrethroid) significantly increased and decreased with bee age, respectively. In addition, one still needs to be cautious with the interpretation of age-dependent effects because honeybee workers may have the same chronological age (i.e. time elapsed since adult emergence) but different biological ages, which refer to the changes in the physiological state that occur throughout its lifespan. For instance, within the colony, bees of the same chronological age might be specialized in different behavioral tasks and therefore have different physiological backgrounds (Robinson 2002; Whitfield et al. 2003). The biological age, determined by the behavioral specialization of bees (e.g. nurse vs forager) might therefore better reflect the biological state of bees and give information on their sensitivity to pesticides.

Further evidence of variation in honeybee sensitivity to pesticides is the comparison of sulfoxaflor LD_{50} values across studies. The oral LD_{50} (48h) reported by EFSA for in-hive bees was of 146 ng/bee (EFSA 2014), which is almost three times the LD_{50} we found for nurse bees. This may be explained by differences in the genetic backgrounds leading to dissimilar responses to pesticides, but also to the large range of ages among the tested in-hive bees given that all age cohorts can normally be found on hive frames (Free 1960; van der Steen et al. 2012). Similarly, some inconsistencies can be observed in the forager LD_{50}, as indicated by the higher LD_{50} reported in a recent study (55.38 ng/bee vs 41.04 ng/bee in our study; (Azpiazu et al. 2021). This difference might find its origin in the sampling procedure since we focused on pollen foragers, while Azpiazu et al. (2021) sampled all bees returning to the hive, which might include young bees performing orientation or cleansing flights in addition to forager bees, and is particularly reflected by the wider range of the LD_{50} 95 % CI (26.34 to 111.46 ng/bee) compared to our study (33.50 – 49.28 ng/bee).

An age-related increase in the sensitivity to herbicide and fungicide has also been previously described by Wahl and Ulm (1983). However, in our study, the fungicide azoxystrobin was

found to be weakly toxic for both nurse and forager bees upon both acute and chronic exposure, confirming data from regulatory tests (> 25 µg/bee; EFSA 2010). This lack of effect might be due to either the tested doses, which were limited to 60 µg/bee, because of the low solubility of azoxystrobin in solvent, or to the rapid metabolization of the fungicide (no trace of azoxystrobin could be found at 8 h post-exposure). Alternatively, azoxystrobin may be non-toxic or weakly toxic to honeybees given its targeting of fungi with a mode of action that might be too specific to affect insects.

How to explain the differences in sulfoxaflor sensitivity between nurse and forager bees? To survive toxic compounds, insects have developed detoxification mechanisms, which prevent their accumulation in organs and tissues (Smith 1955; Panini et al. 2016; Lu et al. 2021). Accordingly, we expected a more efficient elimination of sulfoxaflor by nurse bees as compared to foragers. However, the analysis of sulfoxaflor residues showed that its concentration did not differ between nurse and forager bees at 8 h post-exposure. On the contrary, sulfoxaflor metabolization was stronger in foragers within 2 h post-exposure, suggesting faster sulfoxaflor elimination by forager bees in the very short-term. These results agree with a study that showed that the expression level of three genes encoding cytochrome P450 monooxygenases (involved in the detoxification pathway) was higher in forager bees than in nurses (Vannette et al. 2015). This was later confirmed at the enzymatic level, in which cytochrome P450 monooxygenase activity gradually increased with bee age (Zhu et al. 2020). However, we cannot exclude that in the long-term (> 8 h post-exposure), nurse bees are more efficient in eliminating sulfoxaflor than foragers. This is suggested by the improved ability of bees to metabolize pesticides after the consumption of pollen (Ardalani 2021; Ardalani et al. 2021; Barascou et al. 2021), which is essentially consumed by nurses. Lastly, given that the impact of pesticides depends not only on the fate of the molecule in the body, but also on its interaction with the biological target and consecutive effects on the organism, we could also expect that sulfoxaflor affected nurses and foragers in different manners. A recent study demonstrated that nurse and forager bees were affected in different ways by neonicotinoids at the gene expression level in the brain: while the expression of genes involved in cognition and development was predominantly affected in foragers, the expression of genes involved in metabolism was modified in nurses (Tsvetkov and Zayed 2021). Although, it is not known how such effects might affect honeybee survival or performance, it could help to explain differences in pesticide sensitivity. But perhaps, the most reasonable explanation relies on the body weight difference, because for any given bee species, the heavier the individual bees are the less sensitive they are to a given dose of

pesticide (Tahori et al. 1969; Gerig 1975; Nogueira-Couto et al. 1996; Thompson and Hunt 1999). When converted to ng/g of bee, the sulfoxaflor LD_{50} did not differ between nurses and foragers. However, the fold change in body weight was much stronger in favor of nurses (1.6 times heavier than foragers), which likely explains the higher sensitivity of foragers compared to nurses at the individual level. For a given dose, the concentration of pesticide in the bee body will be higher in foragers than in nurses.

We also noted a significantly higher consumption of sugar syrup by forager bees compared to nurses, and this propensity to consume more syrup was even more pronounced when it was laced with pesticide (at a specific concentration). This phenomenon confirms the often observed preferences of forager bees for sugar solutions containing pesticides (Kessler et al. 2015; Liao et al. 2017), but also has the consequence of intensifying the risk posed by pesticides to foragers. Indeed, the higher consumption of pesticide-contaminated syrup (sulfoxaflor and azoxystrobin) combined with the stronger susceptibility to pesticide (sulfoxaflor) in foragers contributed to an increase by 2 and 10-fold of the acute and chronic RQ, respectively. The magnitude of RQ differences might however be lower in the case of pollen contamination by pesticides given that nurse bees can additionally consume on average 5-10 mg pollen/day (Pernal and Currie 2000; Brodschneider and Crailsheim 2010).

In conclusion, our results show that honeybee workers are not all equal regarding the risk posed by pesticides and that, depending on the honeybee behavioral caste, it might be under or over-estimated. The growing agreement across studies that foragers or old bees are more sensitive to insecticides than nurse or young bees, therefore suggests consistent inclusion of forager bees in regulatory tests should allow for an increase in the safety margin of pesticide risk assessment. However, further studies are needed to determine whether this caste-dependent variation in insecticide sensitivity also occurs and to what extent in response to a range of fungicides and herbicides.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

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

Compliance with Ethical Standards

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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