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

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1 **Abstract**

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

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

23

24 **Introduction**

25 Within the current regulatory framework for pesticide risk assessment, the effects of
26 pesticides on honeybees are assessed by standard tests in a stepwise approach. In Tier 1,
27 active substances or formulated products are tested on honeybees at different life stages
28 (larvae and adults). This is the first mandatory step that includes acute toxicity tests after oral
29 or contact exposure in adults (OECD 1998a, b) and a chronic oral toxicity test on adults
30 (OECD 2017). Next, a deterministic approach to characterize risk quantitatively can be used
31 by comparing the pesticide toxicity to environmental exposure. For that purpose, a Risk
32 Quotient (RQ) is generally calculated by dividing a point estimate of exposure by a toxicity

33 end-point value (*e.g.* LD₅₀ or chronic non-observable effect dose - NOED) (Thompson 2021).
34 If RQ values rise above determined levels of concern (*e.g.* 0.4 or 1 for acute and chronic
35 exposure, respectively), then high-risk situations are identified and supplementary tests are
36 required at a higher tier (semi-field and field tests) for regulatory decision making (Thompson
37 2021).

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

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

67 or thiamethoxam), glyphosate or chlorothalonil at specific concentrations, and avoided
68 prochloraz at high concentrations (Kessler et al. 2015; Liao et al. 2017). The mechanisms
69 underlying this preference or avoidance of contaminated nectars are currently not known, but
70 this phenomenon could potentially trigger an increase or decrease in the exposure to
71 pesticides, which would lead to differences between behavioral castes.
72 Therefore, in order to better quantify the risks posed by pesticides on honeybees, intraspecific
73 variability in bee sensitivity and exposure (consumption of contaminated food) to these
74 chemicals needs to be considered in risk assessment tests. To investigate to what extent
75 behavioral caste affects the risks posed by pesticides, we exposed nurse and forager bees
76 either to sulfoxaflor, a new neurotoxic insecticide that shares the same mode of action with
77 neonicotinoids, or to azoxystrobin, a fungicide widely used in agriculture and regularly found
78 in bee-foraged food (Mullin et al. 2010; Sanchez-Bayo and Goka 2014; Long and Krupke
79 2016). We then assessed the sensitivity of nurse and forager bees to both pesticides, as well as
80 their consumption of contaminated food, and calculated the acute and chronic RQs. We
81 finally investigated whether dissimilarities in pesticide sensitivity could be attributed to
82 differences in detoxification capacities by measuring the residual concentrations of pesticides
83 over time in both nurse and forager bees.

84

85 **Materials and methods**

86 **1. Bee sampling**

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

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

99 purpose, 10 bees of each behavioral caste were sampled in all colonies and stored at -20°C.
100 Glands from 10 bees per caste and colony were dissected in distilled water under a binocular
101 magnifier (LEICA MZ 12). Pictures of each gland were taken with a digital camera
102 (Toupcam™) and ToupView image-capturing software (v3.7.5660). Then, the gland
103 development was assessed by measuring the maximum diameter of 10 to 15 randomly chosen
104 ovoid acini per gland using ImageJ v1.53e (<http://rsb.info.nih.gov/ij/index.html>). The
105 diameters of acini were significantly larger in nurse ($80.34 \pm 9.66 \mu\text{m}$) than in forager bees
106 ($53.54 \pm 9.45 \mu\text{m}$; Kruskal-Wallis test, $\chi^2 = 1274.4$, $p < 0.001$), which validated our sampling
107 method.

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

113 **2. Nurse and forager bee sensitivity to pesticides**

114 Acute toxicity tests

115 We assessed the sensitivity of nurse and forager bees to sulfoxaflor and azoxystrobin by
116 exposing them to a range of pesticide doses and estimating the dose-response curve. We
117 tested a control dose (0 ng/bee) and a total of five doses of azoxystrobin (1, 10, 20, 40, 60
118 $\mu\text{g}/\text{bee}$) and six doses of sulfoxaflor (1, 10, 25, 50, 100, 150 ng/bee). Each exposure to
119 azoxystrobin or sulfoxaflor was tested alone following the OECD test guidance for acute oral
120 toxicity tests (OECD 1998a). The doses were chosen to surround the theoretical LD_{50}
121 previously reported by EFSA for each pesticide (LD_{50} EFSA: $> 25 \mu\text{g}/\text{bee}$ for azoxystrobin
122 and 146 ng/bee for sulfoxaflor (EFSA 2010, 2014).

123 Stock solutions of sulfoxaflor (Techlab, France) and azoxystrobin (Sigma Aldrich, France) in
124 acetone were previously aliquoted and conserved at -20°C. Bees were sugar-starved for 2 h
125 and then fed with a solution of 50% (w/v) sucrose and azoxystrobin (10% acetone) or
126 sulfoxaflor (1% acetone). Since a previous study showed that a uniform food distribution is
127 best approached with 10 bees par cage (Brodschneider et al. 2017), for each dose, one cage of
128 10 bees per colony was used (except for one colony for which we had 2 cages per dose). Each
129 treated cage received 100 μl of the solution laced with pesticides, which was fully consumed
130 within 60 min (giving around 10 μl per bee) Control groups were fed with pesticide-free
131 sucrose solution (50% w/v sucrose, 1 or 10% acetone). The experiment was repeated twice for

132 each colony (n = 10 cages per dose and behavioral caste). Mortality was recorded at 24 and
133 48h post-exposure.

134 To express the toxicity endpoint (median lethal dose – LD₅₀) in ng per g of bees, we
135 determined the weight of nurse and forager bees. We did not weigh test bees but bees from
136 the same colony and collected at the same time as the acute toxicity tests. Bees were frozen at
137 -20°C, then individually placed in glass petri dishes, at room temperature for 1 hour. They
138 were weighed and then dried at 60°C for 72 hours to measure their dry weight.

139 Chronic toxicity test and pesticide-contaminated syrup consumption

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

160 **3. Residual concentrations of pesticides in nurse and forager bees**

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

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

171 Pesticide concentrations were analyzed on pools of 25 bees (2 pools per cage giving n = 10
172 pools per behavioral caste, pesticide and time point). Each pool of bees was weighed and
173 sulfoxaflor and azoxystrobin content were subsequently analyzed via LC-MS/MS. The
174 QuEChERS method was used for the extraction of the active ingredients from samples,
175 following the European Standard EN 15662 (see Barascou et al. (2021) for further method
176 details). The limit of quantification for azoxystrobin and sulfoxaflor was 0.001 mg/kg and
177 0.01 mg/kg, respectively.

178 **4. Statistical analysis**

179 Data were analyzed using the statistical software R v3.3.3 (R Core Team 2020). In the acute
180 toxicity test, the LD₅₀ values were calculated for each pesticide and each behavioral caste, by
181 fitting a dose-response model to the data (*drm* function of the “drc” package) (Ritz et al.
182 2015). Different models were compared based on the log likelihood value, Akaike's
183 information criterion, and the estimated residual standard error. For sulfoxaflor and
184 azoxystrobin data, the two-parameter (W1.2) and four-parameter Weibull models (W2.4)
185 were shown to best describe the data analyzed for nurses and foragers, respectively, and were
186 therefore used to calculate all of the dose–concentration response curves in the present study.
187 The toxicity between nurses and foragers was compared by interpooling the 95% CI
188 (confidence interval) limits of the LD₅₀ values, considering the LD₅₀ as different if the
189 CI values did not overlap.

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

197 In order to assess the potential risk posed to nurse and forager bees by an acute exposure to
198 pesticide, a Risk Quotient (RQ) was calculated based on the exposure concentration, the
199 caste-specific sucrose consumption values from the chronic toxicity experiment (mean
200 consumption within 5 days), and acute toxicity data (LD₅₀):

$$201 \quad RQ = \frac{\text{Exposure concentration } (\mu\text{g/kg}) \times \text{Consumption (kg/day)}}{\text{LD}_{50} \text{ } (\mu\text{g/bee})}$$

202
203 We similarly assessed the potential risk posed to nurse and forager bees by a chronic exposure
204 to pesticide, by using the NOED from the chronic toxicity test:

$$205 \quad RQ = \frac{\text{Exposure concentration } (\mu\text{g/kg}) \times \text{Consumption (kg/day)}}{\text{chronic 5 day oral NOED } (\mu\text{g/bee/day})}$$

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

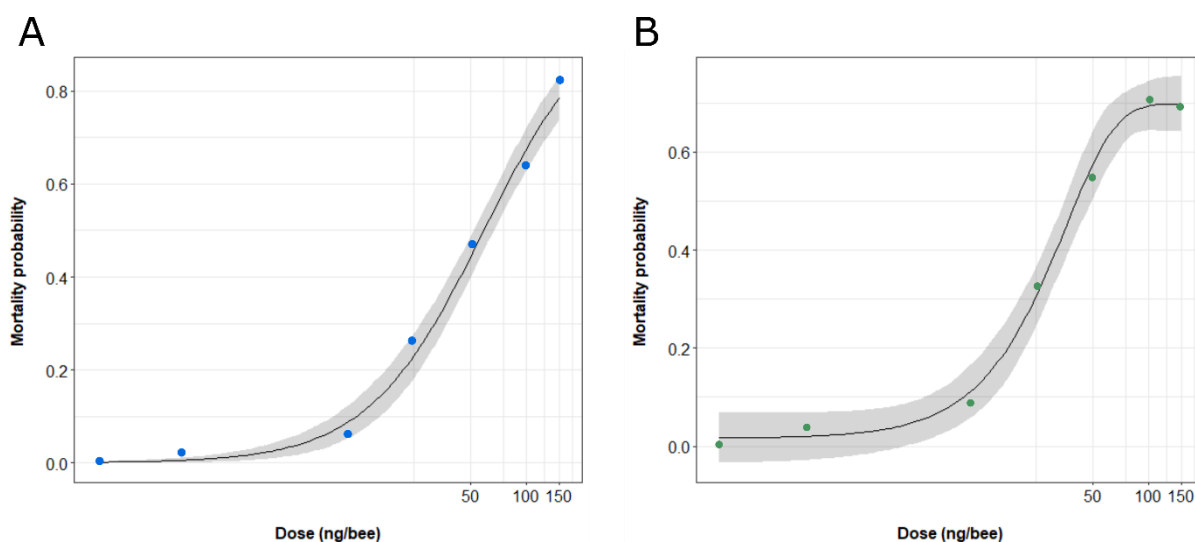
209 Residual pesticide concentrations were compared between behavioral castes and time points
210 using Wilcoxon rank test pairwise comparisons and a Bonferroni adjustment.

211 **Results**

212 *Acute toxicity for nurse and forager bees*

213 We recorded bee mortality rates at both 24 and 48 h following exposure to the different doses
214 of pesticides (Table S1 and S2). We then calculated the LD₅₀ of each pesticide by using the 48
215 h mortality data since additional mortality occurs between 24 and 48 h post-exposure. Only a
216 slight overlap in the 95% CI values was observed between the dose-response curves of nurse
217 and forager bees, and the calculated sulfoxaflor LD₅₀ was lower for foragers (41.04 ng/bee,
218 than for nurse bees (54.40 ng/bee; Fig.1 and Table 1). Nurses were 1.6 times heavier (134.77
219 ± 24.24 mg) than foragers (85.81 ± 8.69 mg, Kruskal-Wallis test, $\chi^2 = 69.88$, $p < 0.001$; Table
220 1), and when the sulfoxaflor toxicity was expressed in ng per g of bees, a strong overlap was
221 observed between the dose-response curves of nurses and foragers (Table 1).

222



223
 224 **Figure 1. Dose-response relation resulting from oral exposure to sulfoxaflor in (A) nurse and (B)**
 225 **forager bees.** Data show bee mortality at 48 h post-exposure (n = 10 bees per cage and 10 cages per
 226 dose). Dots represent the mean values of bee mortality for each tested dose. Grey areas show the
 227 confidence interval at 95%.

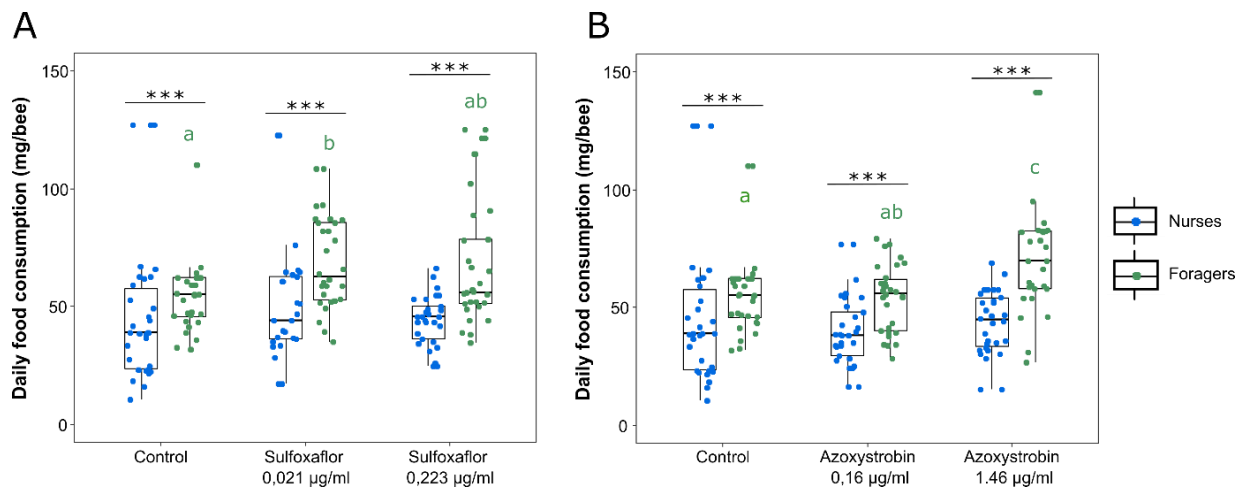
228 Tested azoxystrobin doses marginally increased bee mortality (up to 20% for the highest
 229 dose; Table S2). It was therefore not possible to determine an exact azoxystrobin LD₅₀ for
 230 both foragers and nurses (LD₅₀ values > 60 µg a.i./bee; Table 1).

231 **Table 1. Oral median lethal dose (LD₅₀) of sulfoxaflor and azoxystrobin in nurse and forager**
 232 **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

233
 234 *Chronic toxicity for nurse and forager bees*
 235 Regardless of the pesticide and its concentration, forager bees consistently consumed more
 236 sugar solution than nurse bees (sulfoxaflor: Kruskal-Wallis test, $\chi^2 = 31.49$, $p < 0.001$ and
 237 azoxystrobin: $\chi^2 = 37.67$, $p < 0.001$; Fig. 2). Overall, forager bees ingested 1.4 times more
 238 syrup than nurse bees. Although we did not find any effect of pesticide concentration on sugar
 239 consumption by nurse bees (sulfoxaflor: $p = 0.38$ and azoxystrobin: $p = 0.528$), a significant
 240 effect was observed in forager bees (sulfoxaflor: $p = 0.024$ and azoxystrobin: $p < 0.01$; Fig.
 241 2). Foragers exposed to 0.021 µg/ml of sulfoxaflor consumed more syrup (68.79 ± 19.76
 242 mg/day) than control foragers (53.90 ± 14.90 mg/day, Dunn's test, $p = 0.022$) but not for

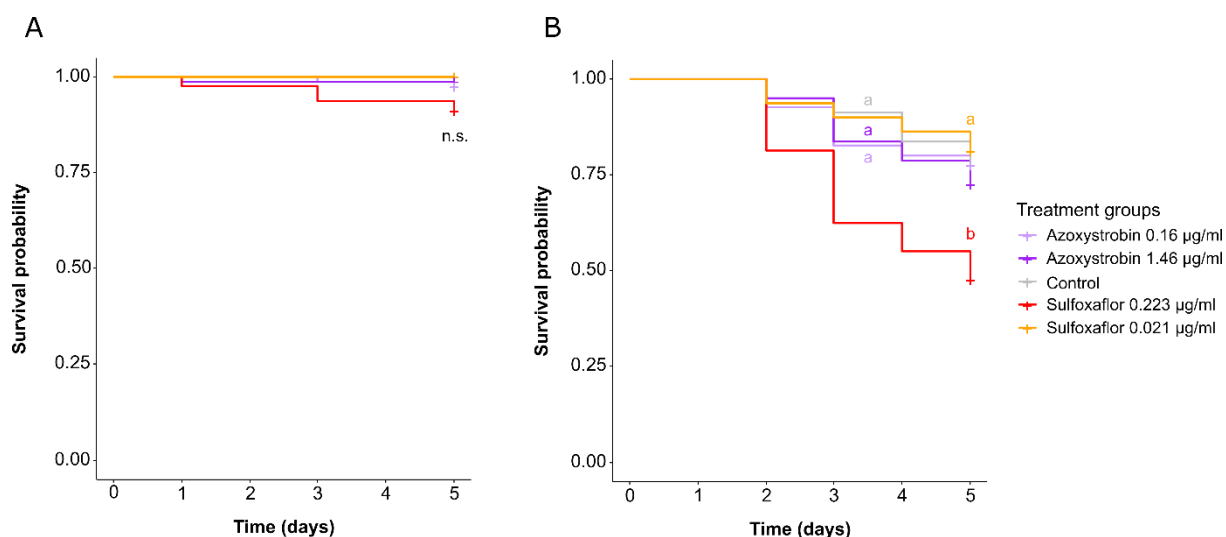
243 foragers exposed to 0.223 $\mu\text{g}/\text{ml}$ of sulfoxaflor ($p = 0.07$). Similarly, foragers exposed to 1.46
 244 $\mu\text{g}/\text{ml}$ of azoxystrobin consumed more syrup (72.16 ± 26.71 mg/day) than bees exposed to
 245 0.16 $\mu\text{g}/\text{ml}$ of azoxystrobin (54.16 ± 14.11 mg/day, $p < 0.01$) and control bees (53.90 ± 14.90
 246 mg/day, $p < 0.01$).



247

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

256 Chronic exposure to azoxystrobin (0.16 – 1.46 $\mu\text{g}/\text{ml}$) and sulfoxaflor (0.02 – 0.22 $\mu\text{g}/\text{ml}$) did
 257 not affect the survival of nurse bees (Cox model, $p = 0.99$; Fig. 3A). While we did not find
 258 any effect of both azoxystrobin concentrations and the lowest concentration of sulfoxaflor
 259 (0.021 $\mu\text{g}/\text{ml}$) on forager mortality, the highest concentration of sulfoxaflor (0.223 $\mu\text{g}/\text{ml}$)
 260 reduced their survival probability by around 50% within 5 days (Cox model, $p < 0.001$, Fig.
 261 3B).



262

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

266

267 Risk quotient for nurse and forager bees

268 Due to a higher level of exposure (consumption of pesticide-contaminated syrup) and a lower
 269 LD₅₀, the acute risk quotient (RQ) upon exposure to sulfoxaflor was twice as high for forager
 270 bees than for nurse bees (Table 2). Based on the tested field-relevant concentrations of
 271 sulfoxaflor, none of the acute RQ exceeded the theoretical threshold level of concern of 0.4
 272 (Table 2). However, the acute RQ for foragers exposed to 0.223 µg/ml of sulfoxaflor was
 273 close to this threshold (RQ = 0.309; Table 2).

274 The chronic 5-day oral NOED of sulfoxaflor for nurse bees was 8.36 ng/bee/day (NOEC:
 275 0.223 µg/ml). None of the chronic RQ for nurses exceeded the theoretical threshold LOC of 1
 276 (Table 2). However, the chronic 5-day oral NOED for foragers was 1.22 ng/bee/day (NOEC:
 277 0.021 µg/ml), leading to a chronic RQ above the theoretical threshold LOC of 1 (RQ =
 278 10.373) and ten times higher than that of nurse bees (Table 2), when exposed to a sulfoxaflor
 279 concentration of 0.223 µg/ml.

280 The azoxystrobin LD₅₀ could not be determined and therefore the acute RQ for both nurse and
 281 foragers bees was very low. The chronic RQ based on the higher concentrations of
 282 azoxystrobin with no-observed lethal effect (NOEC: 1.46 µg/ml) was calculated for both
 283 nurse (NOED: 89.28 ng/bee/day) and forager bees (NOED: 53.81 ng/bee/day). For both
 284 concentrations of azoxystrobin, none of the chronic RQ exceeded the theoretical threshold
 285 LOC of 1 (Table 2).

286

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

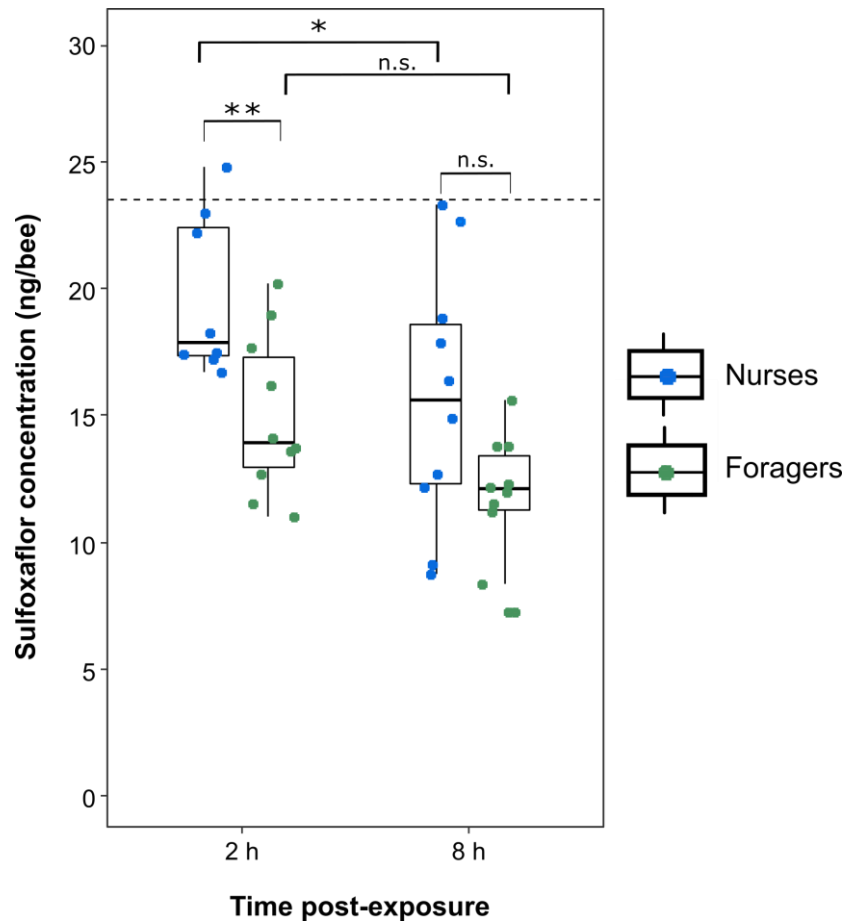
	Nurses			Foragers		
	Consumption (mg/day \pm SE)	Acute RQ	Chronic RQ	Consumption (mg/day \pm SE)	Acute RQ	Chronic RQ
Sulfoxaflor 0.021 μ g/ml	49.38 \pm 21.84	0.016	0.105	68.79 \pm 19.76	0.030	1
Sulfoxaflor 0.223 μ g/ml	44.24 \pm 10.68	0.154	1	67.21 \pm 25.97	0.309	10.373
Azoxystrobin 0.16 μ g/ml	39.52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

289

290 *Residual concentrations of pesticides in nurse and forager bees*

291 Residues of azoxystrobin were detected at very low concentrations at 2 h post-exposure and
 292 could be quantified (above LOQ) in only 3 nurse samples (1.35 ± 0.54 ng/bee) and 6 forager
 293 samples (1.26 ± 0.60 ng/bee), demonstrating a high metabolization rate (amount of eliminated
 294 pesticide divided by the amount of pesticide to which bees were exposed; nurses: 92.89 ± 4.25
 295 % and foragers: 93.37 ± 3.47 %). At 8h post-exposure, azoxystrobin concentrations were
 296 below the LOQ.

297 Regarding sulfoxaflor, the concentrations of residues found at 2 h post-exposure were lower
 298 in forager (metabolization rate = 36.30 ± 13.44 %) than in nurse bees (metabolization rate =
 299 19.59 ± 10.99 %) (Wilcox test, $p < 0.01$; Fig. 4). However, this difference disappeared at 8 h
 300 post-exposure (Wilcox test, $p = 0.059$; Fig. 4), likely because sulfoxaflor residual
 301 concentrations decreased significantly between 2 and 8 h post-exposure in nurse bees (Wilcox
 302 test, $p = 0.036$; metabolization rate at 8 h = 33.31 ± 21.76 %) while it did not in forager bees
 303 (Wilcox test, $p = 0.059$; metabolization rate at 8 h = 49.80 ± 10.64 %; Fig. 4).



304

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

311

312 Discussion

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

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

324 which showed that in LD₅₀ tests foragers were more susceptible to another insecticide
325 (flupyradifurone) than in-hive bees (Tosi and Nieh 2019). This higher sensitivity of foragers
326 was also confirmed at the chronic level, since exposure to the highest sulfoxaflor
327 concentration significantly reduced the survival of foragers but not of nurse bees. An increase
328 in bee sensitivity to insecticides as bees age has been reported in several studies (Mayland and
329 Burkhardt 1970; Ladas 1972; Bendahou et al. 1997; Zhu et al. 2020). This phenomenon is
330 somewhat in agreement with our results as forager bees are generally older than nurse bees.
331 However, this age-dependent sensitivity is not always consistent as indicated by a Rinkevich
332 et al. (2015) study, which showed that sensitivity to the insecticides naled (organophosphate)
333 and phenothrin (pyrethroid) significantly increased and decreased with bee age, respectively.
334 In addition, one still needs to be cautious with the interpretation of age-dependent effects
335 because honeybee workers may have the same chronological age (i.e. time elapsed since adult
336 emergence) but different biological ages, which refer to the changes in the physiological state
337 that occur throughout its lifespan. For instance, within the colony, bees of the same
338 chronological age might be specialized in different behavioral tasks and therefore have
339 different physiological backgrounds (Robinson 2002; Whitfield et al. 2003). The biological
340 age, determined by the behavioral specialization of bees (e.g. nurse vs forager) might
341 therefore better reflect the biological state of bees and give information on their sensitivity to
342 pesticides.

343 Further evidence of variation in honeybee sensitivity to pesticides is the comparison of
344 sulfoxaflor LD₅₀ values across studies. The oral LD₅₀ (48h) reported by EFSA for in-hive
345 bees was of 146 ng/bee (EFSA 2014), which is almost three times the LD₅₀ we found for
346 nurse bees. This may be explained by differences in the genetic backgrounds leading to
347 dissimilar responses to pesticides, but also to the large range of ages among the tested in-hive
348 bees given that all age cohorts can normally be found on hive frames (Free 1960; van der
349 Steen et al. 2012). Similarly, some inconsistencies can be observed in the forager LD₅₀, as
350 indicated by the higher LD₅₀ reported in a recent study (55.38 ng/bee vs 41.04 ng/bee in our
351 study; (Azpiazu et al. 2021). This difference might find its origin in the sampling procedure
352 since we focused on pollen foragers, while Azpiazu et al. (2021) sampled all bees returning to
353 the hive, which might include young bees performing orientation or cleansing flights in
354 addition to forager bees, and is particularly reflected by the wider range of the LD₅₀ 95 % CI
355 (26.34 to 111.46 ng/bee) compared to our study (33.50 – 49.28 ng/bee).

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

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

365 How to explain the differences in sulfoxaflor sensitivity between nurse and forager bees? To
366 survive toxic compounds, insects have developed detoxification mechanisms, which prevent
367 their accumulation in organs and tissues (Smith 1955; Panini et al. 2016; Lu et al. 2021).
368 Accordingly, we expected a more efficient elimination of sulfoxaflor by nurse bees as
369 compared to foragers. However, the analysis of sulfoxaflor residues showed that its
370 concentration did not differ between nurse and forager bees at 8 h post-exposure. On the
371 contrary, sulfoxaflor metabolization was stronger in foragers within 2 h post-exposure,
372 suggesting faster sulfoxaflor elimination by forager bees in the very short-term. These results
373 agree with a study that showed that the expression level of three genes encoding cytochrome
374 P450 monooxygenases (involved in the detoxification pathway) was higher in forager bees
375 than in nurses (Vannette et al. 2015). This was later confirmed at the enzymatic level, in
376 which cytochrome P450 monooxygenase activity gradually increased with bee age (Zhu et al.
377 2020). However, we cannot exclude that in the long-term ($> 8 \text{ h}$ post-exposure), nurse bees
378 are more efficient in eliminating sulfoxaflor than foragers. This is suggested by the improved
379 ability of bees to metabolize pesticides after the consumption of pollen (Ardalani 2021;
380 Ardalani et al. 2021; Barascou et al. 2021), which is essentially consumed by nurses. Lastly,
381 given that the impact of pesticides depends not only on the fate of the molecule in the body,
382 but also on its interaction with the biological target and consecutive effects on the organism,
383 we could also expect that sulfoxaflor affected nurses and foragers in different manners. A
384 recent study demonstrated that nurse and forager bees were affected in different ways by
385 neonicotinoids at the gene expression level in the brain: while the expression of genes
386 involved in cognition and development was predominantly affected in foragers, the
387 expression of genes involved in metabolism was modified in nurses (Tsvetkov and Zayed
388 2021). Although, it is not known how such effects might affect honeybee survival or
389 performance, it could help to explain differences in pesticide sensitivity. But perhaps, the
390 most reasonable explanation relies on the body weight difference, because for any given bee
391 species, the heavier the individual bees are the less sensitive they are to a given dose of

392 pesticide (Tahori et al. 1969; Gerig 1975; Nogueira-Couto et al. 1996; Thompson and Hunt
393 1999). When converted to ng/g of bee, the sulfoxaflor LD₅₀ did not differ between nurses and
394 foragers. However, the fold change in body weight was much stronger in favor of nurses (1.6
395 times heavier than foragers), which likely explains the higher sensitivity of foragers compared
396 to nurses at the individual level. For a given dose, the concentration of pesticide in the bee
397 body will be higher in foragers than in nurses.

398 We also noted a significantly higher consumption of sugar syrup by forager bees compared to
399 nurses, and this propensity to consume more syrup was even more pronounced when it was
400 laced with pesticide (at a specific concentration). This phenomenon confirms the often
401 observed preferences of forager bees for sugar solutions containing pesticides (Kessler et al.
402 2015; Liao et al. 2017), but also has the consequence of intensifying the risk posed by
403 pesticides to foragers. Indeed, the higher consumption of pesticide-contaminated syrup
404 (sulfoxaflor and azoxystrobin) combined with the stronger susceptibility to pesticide
405 (sulfoxaflor) in foragers contributed to an increase by 2 and 10-fold of the acute and chronic
406 RQ, respectively. The magnitude of RQ differences might however be lower in the case of
407 pollen contamination by pesticides given that nurse bees can additionally consume on average
408 5-10 mg pollen/day (Pernal and Currie 2000; Brodschneider and Crailsheim 2010).

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

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420 **Availability of data and materials**

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

423 **Author Contributions**

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

427 **Compliance with Ethical Standards**

428 *Ethics approval and consent to participate*

429 Not applicable.

430 *Consent for publication*

431 Not applicable.

432 *Competing interests*

433 The authors declare no competing interests.

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