

# The novel butenolide pesticide flupyradifurone does not alter responsiveness to sucrose at either acute or chronic short-term field-realistic doses in the honey bee, *Apis mellifera*

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## Abstract

**BACKGROUND:** Sublethal exposure to neonicotinoids, a popular class of agricultural pesticides, can lead to behavioral effects that impact the health of pollinators. Therefore, new compounds, such as flupyradifurone (FPF), have recently been developed as 'safer' alternatives. FPF is an excitotoxic nicotinic acetylcholine receptor agonist, similar to neonicotinoids. Given the novelty of FPF, what data exist are focused mostly on assessing the effect of FPF on pollinator mortality. One important avenue for investigation is the potential effect of FPF on the sensitivity of nectar foragers, such as *Apis mellifera*, to sucrose concentrations. Neonicotinoids can alter this sucrose responsiveness and disrupt foraging. Compounding this effect, neonicotinoid-containing solutions are preferred by *A. mellifera* over pure sucrose solutions. We therefore conducted four studies, administering FPF under both acute and chronic conditions, and at field-realistic and higher than field-realistic doses, to assess the influence of FPF exposure on sucrose responsiveness and sucrose solutions with FPF in *A. mellifera* nectar foragers.

**RESULTS:** We found no evidence that FPF exposure under acute or chronic field-realistic conditions significantly altered sucrose responsiveness, and we did not find that bees exposed to FPF consumed more of the solution. However, at the much higher median lethal dose (48 h), among bees that survived, FPF-exposed foragers responded to significantly lower concentrations of sucrose than controls and responded at significantly higher rates to all concentrations of sucrose than controls.

**CONCLUSION:** We found no evidence that FPF alters the sucrose responsiveness of nectar foragers at field-realistic doses during winter or early spring, but caution and further investigation are warranted, particularly on the effects of FPF in conjunction with other stressors.

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**Keywords:** sucrose response threshold; field-realistic; proboscis extension response; neonicotinoid

## 1 INTRODUCTION

Neonicotinoid pesticides, modeled on the molecular structure of the insecticide chemical nicotine found in the Solanaceae (nightshade), were developed beginning in the 1980s as safer alternatives to earlier types of agricultural pesticides (e.g. organophosphates, carbamates). However, concern about neonicotinoids has grown because of their detrimental effects on non-target organisms, such as insect pollinators.<sup>1–4</sup> With respect to honey bees, sublethal exposure to neonicotinoids has been associated with impairments in foraging,<sup>5</sup> thermoregulation,<sup>6</sup> olfactory learning,<sup>7</sup> aversive learning,<sup>8</sup> and motor behavior.<sup>9,10</sup> In addition, sublethal neonicotinoid exposure has been shown to interact with other chemical agents<sup>11</sup> and pathogens<sup>12</sup> to increase mortality. In light of these findings, several governments, including the province of Ontario and the European Union, have restricted the use of neonicotinoids.<sup>13,14</sup>

In response, manufacturers have begun to develop novel compounds for use in agriculture. Bayer has recently registered a new molecule, flupyradifurone (FPF) (Sivanto™), based on the molecular structure of the phyto-derived compound, stemofoline, from *Stemona japonica*.<sup>15</sup> Although classified as a butenolide, FPF is an

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excitotoxic nicotinic acetylcholine receptor (nAChR) agonist like the neonicotinoids.<sup>15–17</sup>

Pesticide exposure in *Apis mellifera* can alter the sensitivity of foragers to sucrose concentrations and thereby potentially alter division of labor, with implications for colony fitness.<sup>18</sup> Sucrose responsiveness varies substantially across individuals in *A. mellifera* colonies, and is linked to the division of foraging labor: bees with high thresholds typically become nectar foragers and those with low thresholds become water or pollen foragers.<sup>19–21</sup> Sucrose responsiveness has a significant genetic component<sup>22,23</sup> but individual sensitivities can be modified by the environment.<sup>24,25</sup> Exposure to neonicotinoids can also alter sucrose responsiveness: sublethal exposure to imidacloprid increases sucrose response thresholds (SRTs).<sup>18</sup> Exposure to a different neonicotinoid, acetamiprid, had the opposite effect (SRTs were lowered), and thiamethoxam had no effect.<sup>26</sup> However, Démares *et al.*<sup>27</sup> found that sublethal doses of thiamethoxam, clothianidin, and imidacloprid all decreased responsiveness to high sucrose concentrations. Interestingly, given the choice, both *A. mellifera* and *Bombus terrestris* preferred to collect sucrose solution containing either thiamethoxam or imidacloprid over pure sucrose solution, but did not prefer sucrose solution containing clothianidin.<sup>28</sup>

To date, the limited studies on FPF suggest that its impact on *A. mellifera* may be relatively low.<sup>29</sup> Recently, it has been reported that high acute doses of FPF (1.2 µg a.i. per bee) diminished sucrose responsiveness and impaired performance on associative conditioning tasks.<sup>30</sup> However, these doses may not be field realistic. At field-realistic doses, a significant reduction in olfactory associative learning performance has been observed in the closely related species, *Apis cerana*.<sup>31</sup> Recently, more subtle effects of FPF on honey bees, in combination with factors such as age, seasonality, and exposure to other chemicals, have been demonstrated.<sup>32,33</sup> This is not surprising, given that pesticides have multiple, sometimes subtle detrimental effects and these effects may be magnified in combination with other stressors.<sup>34</sup>

To address the lack of data on the behavioral effects of sublethal FPF exposure alone, we conducted blind experiments and tested FPF at acute or chronic doses (both field realistic and higher than field realistic) to assess the influence of FPF exposure on sucrose responsiveness and on the consumption of solutions containing FPF in *A. mellifera* nectar foragers during winter and early spring.

## 2 MATERIALS AND METHODS

### 2.1 Subjects

We used *A. mellifera ligustica* nectar foragers collected from 20 colonies at the University of California San Diego apiary between February 2016 and January 2018. Bees were housed in standard square Langstroth colony boxes (each colony consisting of ten combs) and were healthy based upon standard inspection procedures.<sup>35</sup>

When collecting bees, we targeted nectar foragers, who are more sensitive to sucrose<sup>19,23</sup> by placing feeders containing 1.8 M sucrose near the entrance of a colony. Once a bee landed at the feeder, we gently placed a small clear plastic vial over it, and allowed it to feed to satiation, after which the bee attempted to fly up inside the vial. We then removed the vial, capped it with a vented cap (four small holes per cap), placed each vial in an insulated cooler at ambient air temperature to avoid stressing the bees with light and excess heat, and transported them back to the laboratory where they were either harnessed (described below) and fed FPF (Sivanto™) in solution (experiments 1 and 4) or put

into cages and incubated for 2 or 4 days while consuming FPF chronically (experiments 2 and 3). We collected between 20 and 100 bees per day, usually from two different colonies.

After excluding bees that died during incubation or following harnessing, or did not respond appropriately to sucrose during the assay, our final analyses included a total of 377 bees across four experiments (experiment 1: 37 control and 40 FPF from three colonies; experiment 2: 55 control and 46 FPF from four colonies; experiment 3: 30 control and 29 FPF from seven colonies; experiment 4: 88 control and 52 FPF from 15 colonies).

### 2.2 Dosages and concentrations of FPF

Before each study, one of the authors (HB) mixed both the control (1.8 M sucrose) and experimental solutions, and aliquoted them into 2 mL plastic Eppendorf vials. Vials were placed in foil-covered plastic boxes to prevent light degradation and stored in a standard freezer (–10 °C). Before use, vials were defrosted in light-proof containers at room temperature. For experiments performed by another member of the laboratory, HB numbered the vials, and all experimenters remained blind to conditions. For experiments performed by HB, another member of the laboratory numbered the vials, and group identity was not known until conclusion of the experiment.

For experiment 1, following Glaberman and White,<sup>29</sup> we used a dose of 0.44 µg FPF (Sigma Aldrich, St. Louis, MO, USA) in 7 µL of 1.8 M sucrose solution, a moderately high field-realistic acute dose. For experiments 2 and 3 (chronic exposure), we aimed for a dose between 0.44 and 0.52 µg per bee per day, also in the moderate to high field-realistic range reported by Glaberman and White.<sup>29</sup> Our experimental solutions contained 9.63 mg/L of FPF in 1.8 M sucrose, which we fed chronically throughout the incubation period, and our recorded consumption was very close to our target range (see results below).

For experiment 4, we administered an acute LD<sub>50</sub> (48 h) dose of 2.2 µg FPF in 7 µL 1.8 M sucrose. Although Glaberman and White<sup>29</sup> report the LD<sub>50</sub> dose as 1.2 µg/bee for pure FPF and 3.4 µg/bee for the seed treatment formulation, we conducted an independent assay in our laboratory using colonies from our apiary from the same supplier stock, and established that 2.2 µg FPF per bee is required to obtain 50% mortality in 48 h.

### 2.3 Experiments 1 and 4: acute FPF exposure

#### 2.3.1 Harnessing

Upon return to the laboratory, we cold anaesthetized the bees by placing each in a separate vial in a bucket of ice. Once the bee had mostly ceased moving, the vial was removed from the ice bucket and gently inserted, using blunt tweezers, head-first into a 2 mL Eppendorf tube with the tip removed. A small ~1 cm section of a drinking straw with a notch cut out of it was then pushed into the tube behind the bee such that the un-notched side slid under the bee's wings.<sup>36</sup> When properly harnessed, the head of each bee was completely free of the tube, and the bee was prevented from backing out by the straw and closing the lid of the Eppendorf tube. All bees were then placed on a plastic tray.

#### 2.3.2 Feeding

Once harnessed, bees were allowed to rest for 2 h (experiment 1) or 1 h (experiment 4) in an incubator (36 °C and 70% humidity). Each bee was fed 7 µL of pre-made FPF in 2.0 M (experiment 1) or 1.8 M (experiment 4) solution, or 7 µL control (no FPF) solution using a pipette. The antennae of each bee was stimulated with the

tip of a pipette, causing the bee to extend its proboscis to feed. Although time-consuming, this approach is preferable to placing bees in vials with the substance and waiting for them to ingest it on their own because we were able to verify that each bee actually received the full dose. After feeding, bees were incubated for a further 1 h, after which the SRT assay was performed (below).

#### 2.4 Experiments 2 and 3: chronic FPF exposure

Each day, two cages containing 10–20 bees each were obtained from the UCSD apiary (above). Once bees were at the laboratory, previously prepared vials of solution in Eppendorf tubes were removed from the freezer, defrosted in the dark, vortexed and drawn into a syringe. The syringe was weighed and then carefully introduced into cages. Each cage was labeled with its respective solution number and the identity of the colony from which the bees were drawn.

Cages were placed in an incubator in the dark at 36 °C and 70% humidity. After 24 h, cages were removed from the incubators, and the syringes were removed and weighed. Each cage was given a new syringe containing the same solution type. At the same time, a separate 'control' syringe filled with 1.8 M sucrose was prepared. This syringe was weighed and placed inside an empty cage in the incubator. Every time syringes were removed from cages with bees and weighed, the control syringe was also weighed. The weight of the control syringe was subtracted from the weight difference obtained in the cages containing bees to control for evaporation. Typically, evaporation rates of < 1% the weight of the syringe were observed. When the syringes were replaced, dead bees were also removed and recorded. This procedure was repeated such that the total duration of incubation was either 2 (experiment 2) or 4 days (experiment 3).

Following the incubation period, bees were harnessed using the harnessing procedure outlined above; bees were allowed to rest in the incubator for 1 h before the SRT assay.

#### 2.5 Sucrose response threshold (SRT) assay

The SRT assay was begun by immersing a capillary tube into an Eppendorf tube of double-distilled water and presenting it to both antennae of one bee simultaneously for 3 s. If the bee fully extended its proboscis (proboscis extension reflex, PER), it was scored as 1; all other responses were scored as 0. This was repeated for all bees per trial (10–20 bees). When 2 min had elapsed since water presentation to the first bee, the procedure was repeated with the lowest concentration of sucrose solution, 0.1%. In total, six concentrations of sucrose (0.1, 0.3, 1.0, 3.0, 10 and 30%) were presented in an ascending series, with a water presentation between each. The intertrial interval was 2 min. Water presentation served two purposes: (1) to prevent build-up of sucrose on the antenna, and (2) to allow the identification and removal of non-nectar foragers (e.g. pollen or water foragers), who extend their proboscises preferentially to water<sup>22,37</sup> from our analyses. Upon completion of the assay (testing with 30% sucrose solution), all bees were killed by freezing.

#### 2.6 Statistical analyses

All analyses were performed using R studio (v 1.0.153) with the R programming language (v 3.4.4).<sup>38</sup>

Owing to unequal bee attrition, there were unequal group sizes in the final analyses. For factorial analysis of variance (ANOVA), unequal group sizes can affect homogeneity of variance; however, ANOVA is relatively robust to small departures with respect to this

assumption.<sup>39</sup> For this reason, analyses were run with both the full data sets and fully balanced subsets of data in which cases were discarded at random. The results for both sets of analyses were identical, and only the data for experiment 4 are substantially unbalanced (52 in the FPF condition *versus* 88 controls). Only analyses on the full data sets are reported here.

Before analysis, any bee that either did not exhibit PER on any trial or exhibited PER for more than two of the water trials was removed from the data set. These data were analyzed separately to see if the proportion of inappropriate responses differed as a function of condition. Abnormal responses (all experiments), as well as mortality during the incubation period, and mortality following harnessing (experiments 2 and 3 only) were analyzed using a 2 × 2 Fisher exact test.

Between-subjects ANOVAs were used to assess the effects of FPF on SRT. Each analysis included colony and condition as fixed categorical effects, and the log-transformed<sup>18</sup> value of the lowest concentration of sucrose to which each bee responded, as the dependent measure.

For all experiments, the number and pattern of PER observed in response to the ascending concentrations of sucrose were considered using repeated-measures ANOVAs with PER (binary) as the response variable, colony and condition as fixed, categorical factors, and trial as a repeated measures factor. The analysis was run twice for each experiment: once for all trials (including the water trials), and once for only the trials on the various concentrations of sucrose solutions.

To assess the effects of FPF on consumption of the sucrose solution during the incubation period (experiments 2 and 3), repeated measures ANOVAs were used with the total weight of sucrose in g/bee as the response, condition, day and colony as fixed as a within-subjects factors (multiple cages were taken from each colony, and exposed to both control and FPF conditions). Because the bees were group-housed, and consumption was recorded for each cage rather than for individuals, cage was the experimental unit for the consumption analyses.

For the chronic experiments (Experiments 2 and 3), the average consumption of FPF is reported ± SD.

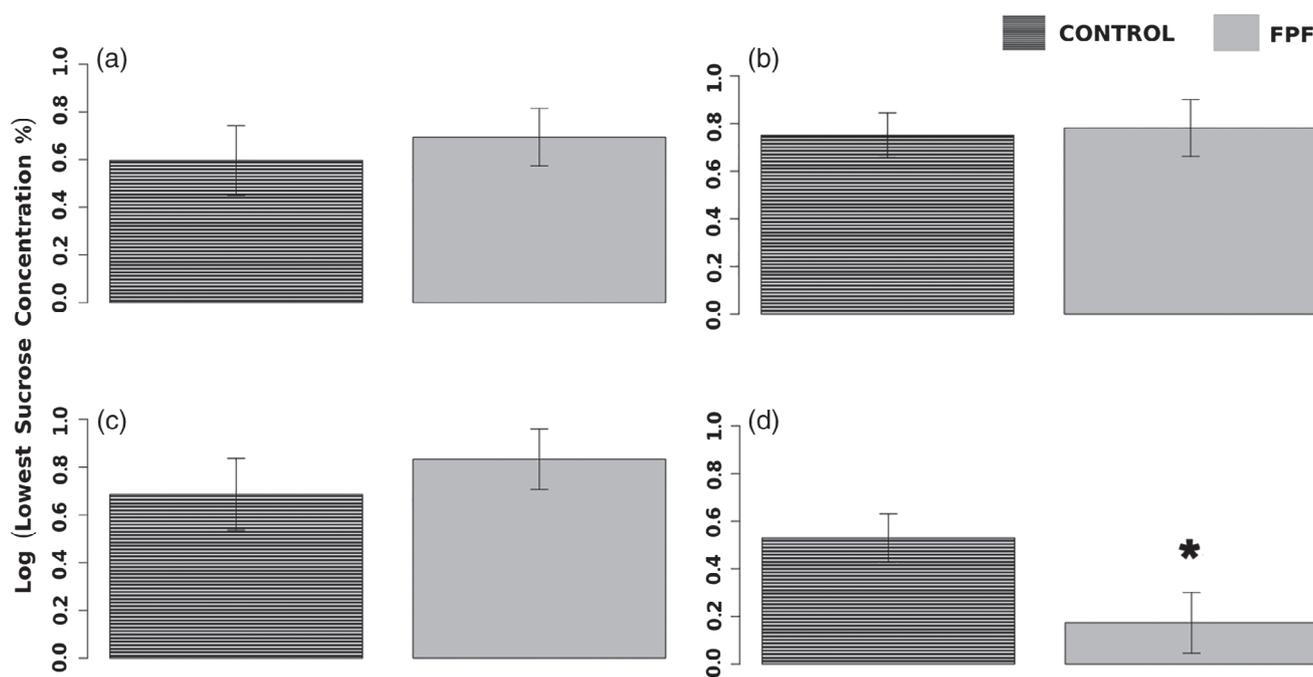
## 3 RESULTS

### 3.1 Experiment 1: acute field-realistic FPF

No bees in either condition died following feeding (Fisher exact test  $P = 1.0$ ), and there was no difference in the proportion of abnormally responding bees (56.0% for controls and 53.9% for FPF; Fisher exact test  $P = 0.879$ ).

When considering the log-transformed value of the lowest concentration of sucrose to which the bees responded, there was a significant effect of colony ( $F_{2,74} = 3.779$ ,  $MSE = 0.642$ ,  $P = 0.028$ ), but no effect of condition ( $F_{1,74} = 0.485$ ,  $MSE = 0.642$ ,  $P > 0.05$ ) (Figure 1a). A Tukey's honestly significant difference (HSD) post-hoc test revealed that bees from one of the three colonies responded to significantly lower concentrations of sucrose than bees from the other two colonies ( $P < 0.05$ ). However, experimental and control animals were balanced across the colonies, and there was no colony × condition interaction.

When the pattern of responses across trials was analyzed, if water trials were included, then there was no significant effect of either colony ( $F_{2,74} = 3.08$ ,  $MSE = 0.146$ ,  $P > 0.05$ ) or condition ( $F_{1,74} = 0.23$ ,  $MSE = 0.146$ ,  $P > 0.05$ ), and no trial × condition interaction ( $F_{11,836} = 0.618$ ,  $MSE = 0.082$ ,  $P > 0.05$ ); however, there was a significant effect of trial ( $F_{11,836} = 71.556$ ,  $MSE = 0.082$ ,  $P < 0.001$ ),



**Figure 1.** Mean log-transformed lowest sucrose concentration to which bees responded. (a) Acute field-realistic FPF, (b) 2-day chronic field-realistic FPF, (c) 4-day chronic field-realistic FPF and (d) acute LD<sub>50</sub> (48 h) FPF. Error bars are 95% confidence intervals. \**P* < 0.05.

as expected, because sucrose concentrations were presented in an increasing series. When only trials during which sucrose was presented were analyzed, a significant effect of colony ( $F_{2,74} = 4.749$ ,  $MSE = 0.230$ ,  $P = 0.012$ ) and trial ( $F_{5,380} = 59.26$ ,  $MSE = 0.130$ ,  $P < 0.001$ ) was found, but neither the condition effect ( $F_{1,74} = 0.104$ ,  $MSE = 0.230$ ,  $P > 0.05$ ), nor the trial  $\times$  condition interaction ( $F_{5,380} = 0.603$ ,  $MSE = 0.130$ ,  $P > 0.05$ ) was significant (Figure 2a). A Tukey's HSD post-hoc analysis revealed that the same colony that responded to lower concentrations of sucrose in the SRT analysis also responded significantly more often across all trials ( $P < 0.05$ ). Given that bees were drawn equally from all colonies, this colony should not have biased our results.

### 3.2 Experiment 2: chronic 2-day field-realistic FPF

Over the 2-day incubation period, bees in the FPF condition consumed an average of  $1.06 \pm 0.37 \mu\text{g a.i. per bee}$  ( $0.53 \pm 0.19 \mu\text{g a.i. per bee per day}$ ). Bees in the control condition consumed an average of  $116.02 \pm 40.72 \mu\text{L}$  of sucrose solution per bee per day, whereas bees in the FPF condition consumed an average of  $87.24 \pm 29.4 \mu\text{L}$  of sucrose solution per bee per day (Table 1).

During the incubation period, significantly more bees in the FPF condition died compared with the control condition (26.5% for FPF and 9.1% for control; Fisher exact test  $P < 0.001$ ) (Table 1). However, it is worth noting that, of the 41 bees that died in the FPF condition, a disproportionate proportion (68%) came from 2 of 11 cages, and thus mortality from the FPF treatment was not equally distributed among the treatment bees. Mortality following harnessing (19.5% for control and 16.1% for FPF) did not differ significantly between controls and bees exposed to FPF (Fisher exact test  $P = 0.547$  and  $P = 0.518$ , respectively). There was no difference with respect to the rate of bees exhibiting abnormal responses (40.4% for controls and 43.3% for FPF; Fisher exact test  $P = 0.689$ ).

There was no effect of colony ( $F_{3,35} = 2.084$ ,  $MSE = 0.0009$ ,  $P > 0.05$ ), condition ( $F_{1,35} = 3.101$ ,  $MSE = 0.0009$ ,  $P > 0.05$ ), or day

( $F_{1,35} = 1.397$ ,  $MSE = 0.0009$ ,  $P > 0.05$ ) on the consumption of sucrose solution during the incubation period.

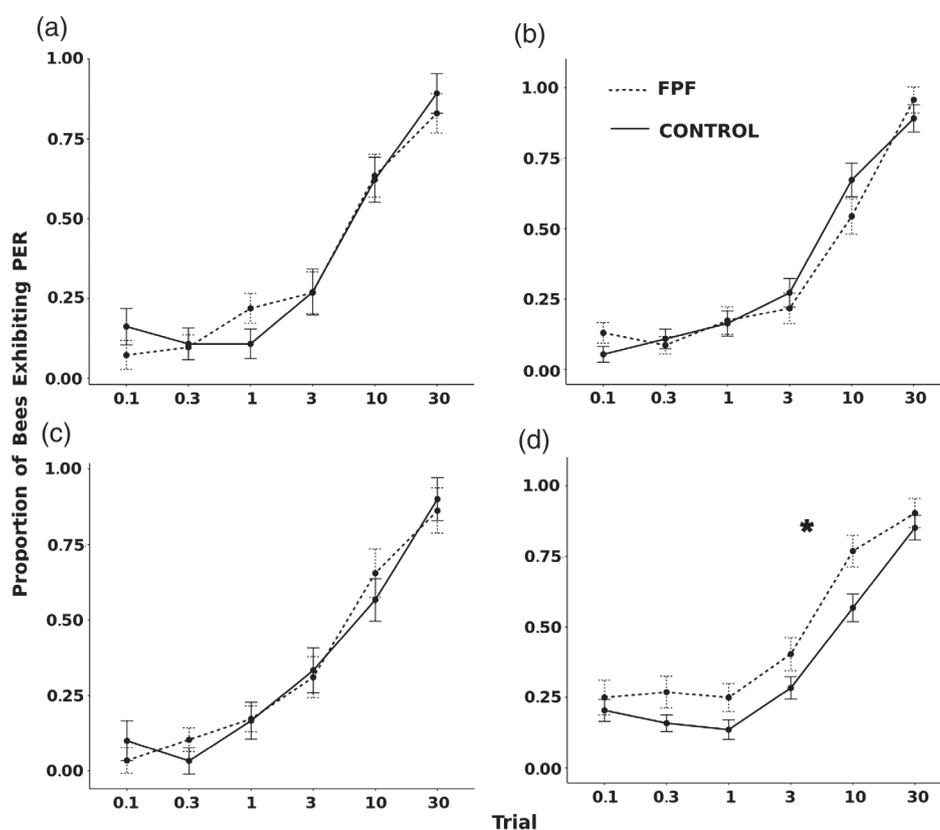
No effect of colony ( $F_{3,96} = 1.50$ ,  $MSE = 0.548$ ,  $P > 0.05$ ) or condition ( $F_{1,96} = 0.04$ ,  $MSE = 0.548$ ,  $P > 0.05$ ) on the log-transformed value of the lowest concentration to which the bee exhibited PERs was found (Figure 1b).

With respect to the pattern of responses across the trials, when all trials were considered, including water-only trials, there was no significant effect of colony ( $F_{3,96} = 1.036$ ,  $MSE = 0.169$ ,  $P < 0.05$ ) or condition ( $F_{1,95} = 0.147$ ,  $MSE = 0.169$ ,  $P > 0.05$ ) on PER. However, a significant effect of trial was found ( $F_{11,1089} = 115.67$ ,  $MSE = 0.072$ ,  $P < 0.001$ ), as expected. There was no trial  $\times$  condition interaction ( $F_{11,1089} = 1.045$ ,  $MSE = 0.072$ ,  $P > 0.05$ ). When water-only trials were removed from the analysis, we also failed to find an effect of either colony ( $F_{3,96} = 1.37$ ,  $MSE = 0.267$ ,  $P > 0.05$ ) or condition ( $F_{1,96} = 0.02$ ,  $MSE = 0.267$ ,  $P > 0.05$ ), but once again, there was a significant effect of trial ( $F_{5,495} = 105.42$ ,  $MSE = 0.109$ ,  $P < 0.001$ ). The condition  $\times$  trial interaction was not significant ( $F_{5,495} = 1.373$ ,  $MSE = 0.109$ ,  $P > 0.05$ ) (Figure 2b).

### 3.3 Experiment 3: chronic 4-day field-realistic FPF

Over the 4-day incubation period, the bees in the FPF group consumed an average of  $1.702 \pm 0.32 \mu\text{g a.i. per bee}$  ( $0.43 \pm 0.08 \mu\text{g a.i. per bee per day}$ ). Bees in the control condition consumed an average of  $81.25 \pm 15.01 \mu\text{L}$  of sucrose solution per bee per day, whereas bees in the FPF condition consumed an average of  $96.19 \pm 15.08 \mu\text{L}$  of sucrose solution per bee per day over the entire incubation period. Over the first 48 h, FPF bees consumed an average of  $83.38 \pm 29.06 \mu\text{L}$  of sucrose solution per bee per day, whereas control bees consumed an average of  $85.84 \pm 21.53 \mu\text{L}$  of sucrose solution per bee per day (Table 1).

There was no difference in mortality rate during the entire incubation period for control and FPF individuals (39.6% and 36.4% respectively, Fisher exact test  $P = 0.6199$ ). Additionally, mortality following 48 h of incubation was analyzed and compared with that



**Figure 2.** Proportion of proboscis extension reflex (PER) observed across ascending series of sucrose concentrations. (a) Acute field-realistic FPF, (b) 2-day chronic FPF, (c) 4-day chronic FPF and (d) acute LD<sub>50</sub> (48 h) FPF. Error bars show within-cell standard error of the mean. \* $P < 0.05$ .

**Table 1.** Consumption and mortality after 48 h

	Chronic 2-day	Chronic 4-day
Consumption (mean $\mu\text{L}$ per bee/day)		
Experimental	87.24 $\pm$ 29.4	83.38 $\pm$ 29.06
Control	116.02 $\pm$ 40.72	85.84 $\pm$ 21.53
Mortality (%)		
Experimental	26.5	12.1
Control	9.1	16.4
Error is standard deviation of the mean.		

in experiment 2. No significant difference was found between the conditions (12.1% for FPF and 16.4% for controls; Fisher exact test  $P = 0.387$ ) (Table 1). Also no difference in mortality was found following harnessing across the groups (20.2% for controls and 21.3% for FPF; Fisher exact test  $P = 1.0$ ). There was no difference in the proportion of bees expressing abnormal responses (51.6% for controls, 56.9% for FPF, Fisher exact test  $P = 0.595$ ).

There was no effect of colony ( $F_{1,42} = 1.56$ ,  $\text{MSE} = 0.0003$ ,  $P > 0.05$ ), condition ( $F_{1,42} = 3.355$ ,  $\text{MSE} = 0.0003$ ,  $P > 0.05$ ), or day ( $F_{3,42} = 2.196$ ,  $\text{MSE} = 0.0003$ ,  $P > 0.05$ ) on consumption of sucrose during the incubation period.

There was no effect of colony ( $F_{6,51} = 1.919$ ,  $\text{MSE} = 0.522$ ,  $P > 0.05$ ) or condition ( $F_{1,51} = 0.350$ ,  $\text{MSE} = 0.522$ ,  $P > 0.05$ ) on the log-transformed value of the lowest concentration of sucrose for which the bees exhibited PERs (Figure 1c).

When considering the pattern of responses over the trials, if water trials were included, a significant effect of colony

( $F_{6,51} = 2.439$ ,  $\text{MSE} = 0.132$ ,  $P = 0.038$ ) and trial ( $F_{11,627}$ ,  $\text{MSE} = 0.072$ ,  $P < 0.001$ ) was found, but no effect of condition ( $F_{1,51} = 0.049$ ,  $\text{MSE} = 0.123$ ,  $P > 0.05$ ), and the trial  $\times$  condition interaction was not significant ( $F_{11,627} = 0.543$ ,  $\text{MSE} = 0.072$ ,  $P > 0.05$ ). If water trials were removed, the pattern of the results was identical, with colony ( $F_{6,51} = 2.327$ ,  $\text{MSE} = 0.236$ ,  $P = 0.046$ ) and trial ( $F_{5,285} = 54.95$ ,  $\text{MSE} = 0.117$ ,  $P < 0.001$ ) having significant effects of PER, whereas neither the condition effect ( $F_{1,51} = 0.018$ ,  $\text{MSE} = 0.236$ ,  $P > 0.05$ ) nor the trial  $\times$  condition interaction ( $F_{5,285} = 0.476$ ,  $\text{MSE} = 0.117$ ,  $P > 0.05$ ) were significant (Figure 2c).

### 3.4 Experiment 4: acute LD<sub>50</sub> (48 h) FPF

Following feeding in their harnesses, there was significantly less mortality in the control group than in the FPF group (0% versus 8.4%, Fisher exact test  $P < 0.001$ ). The proportion of bees that were excluded for inappropriate responses (more than two PER responses to water, or no PER responses at all) was also significantly lower in the controls (46.3% versus 60.3%; Fisher exact tests  $P = 0.019$ ).

There was a significant effect of condition on SRT, with the FPF group responding to significantly lower concentrations of sucrose than controls ( $F_{1,124} = 5.521$ ,  $P = 0.02$ ) (Figure 1d). No significant effect of colony on SRT was found ( $F_{14,124} = 1.31$ ,  $\text{MSE} = 0.872$ ,  $P > 0.05$ ).

With respect to the pattern of responses across the ascending sucrose concentrations, when all trials were considered, including water trials, a significant effect of condition was detected, with controls exhibiting fewer responses to sucrose overall ( $F_{1,124} = 6.017$ ,  $\text{MSE} = 0.202$ ,  $P = 0.016$ ). There was no main effect of colony ( $F_{14,124} = 0.912$ ,  $\text{MSE} = 0.202$ ,  $P > 0.05$ ). As expected,

there was a significant effect of trial ( $F_{11,1518} = 112.3$ ,  $MSE = 0.094$ ,  $P < 0.001$ ); however, the trial  $\times$  condition interaction was not significant ( $F_{11,1518} = 1.60$ ,  $MSE = 0.094$ ,  $P > 0.05$ ), indicating that the pattern of responses across the conditions did not differ.

When only trials with the varying concentrations of sucrose were considered, there was a main effect of condition ( $F_{1,124} = 7.33$ ,  $MSE = 0.301$ ,  $P = 0.008$ ), with the FPF bees producing significantly more PERs across the trials than controls. The colony effect was not significant ( $F_{14,124} = 0.994$ ,  $MSE = 0.301$ ,  $P > 0.05$ ). There was a significant effect of trial ( $F_{5,690} = 77.45$ ,  $MSE = 0.147$ ,  $P < 0.001$ ); however, the trial  $\times$  condition effect was not significant ( $F_{5,690} = 0.707$ ,  $MSE = 0.147$ ,  $P > 0.05$ ) (Figure 2d).

## 4 DISCUSSION AND CONCLUSION

Neither acute nor chronic 2- or 4-day field-realistic exposure to FPF altered either SRT or the overall pattern of responsiveness to varying concentrations of sucrose. At the  $LD_{50}$  (48 h) dose, we observed a significant reduction in SRT: FPF bees exhibited PER to significantly lower concentrations of sucrose than controls. There was no interaction between trial and condition, indicating that, although FPF bees were more responsive to sucrose, the overall shape of the curve of responses across the concentrations did not differ.

That we failed to find any effect of FPF at field-realistic doses on PER or consumption, but did detect effects at  $LD_{50}$  is important. First, the lack of an effect at lower doses adds to the literature concerning the safety of FPF for pollinators. At the same time, the  $LD_{50}$  experiment serves as an important positive control – showing not only that FPF was biologically active, but also that FPF has demonstrable behavioral effects, as one would predict given its known activation of nAChR receptors. Tosi *et al.*<sup>32</sup> recently reported that FPF, in conjunction with a fungicide, was associated with increased hyperactivity in foragers. In our case, we found that a higher proportion of bees treated with FPF at  $LD_{50}$  responded to sucrose. It is possible, but unclear, whether this elevated responsiveness is similar to the hyperactivity observed by Tosi *et al.*<sup>32</sup> We saw both a lower average response rate, and a higher SRT across all of our experiments compared with Eiri *et al.*<sup>18</sup> The likely reason is three-fold. First, Eiri *et al.*<sup>18</sup> did not have water trials among their sucrose trials, and second, we excluded bees that exhibited two or more water responses. Given that individuals in our experiments displaying such behavior tended to respond to almost all or all stimuli, had they been left in the analysis, the response rate would have been higher and the SRT would also have been lower. Thirdly, Eiri *et al.*<sup>18</sup> studied the effects of a different compound, imidacloprid.

In summary, we failed to detect an effect of FPF on SRT and abnormal response rate over three separate experiments with different field-realistic exposure regimes. Although we did find an effect on mortality at 48 h for experiment 2, this was not replicated for experiment 3. The three experiments were conducted at different times (winter and early spring), using bees from different colonies, and by different researchers. For the two chronic exposure experiments, we also failed to detect an effect on consumption of sucrose containing FPF relative to controls. Pesticide effects might be found under a different set of experimental conditions. However, we were able to detect significant differences, using the same measures with bees exposed to  $LD_{50}$  (48 h) acute doses. This suggests that, at least for foragers in winter and early spring, our negative results may reflect a true lack of effect rather than Type II error.

Moving forward, more thorough assessments of FPF residues found in honey bee products, such as wax and honey, should be

conducted, and what constitutes field-realistic exposure for different bee castes and during different seasons should be revisited. Synergistic effects with other pesticides, stressors, bee caste, and season may occur and should continue to be explored, not only based on mortality, but also by examining the multiple behaviors that key to honey bee colony fitness and survival.

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