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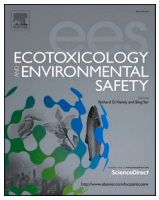

Flupyradifurone reduces nectar consumption and foraging but does not alter honey bee recruitment dancing

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ABSTRACT

Foraging is essential for honey bee colony fitness and is enhanced by the waggle dance, a recruitment behavior in which bees can communicate food location and quality. We tested if the consumption of nectar (sucrose solution) with a field-realistic concentration of 4 ppm flupyradifurone (FPF) could alter foraging behavior and recruitment dancing in *Apis mellifera*. Foragers were repelled by FPF. They visited the FPF feeder less often and spent less time imbibing sucrose solution (2.5 M, 65% w/w) with FPF. As a result, bees feeding on the FPF treatment consumed 16% less nectar. However, FPF did not affect dancing: there were no effects on unloading wait time, the number of dance bouts per nest visit, or the number of dance circuits performed per dance bout. FPF could therefore deter bees from foraging on contaminated nectar. However, the willingness of bees to recruit nestmates for nectar with FPF is concerning. Recruitment can rapidly amplify the number of foragers and could overcome the decrease in consumption of FPF-contaminated nectar, resulting in a net inflow of pesticide to the colony. FPF also significantly altered the expression of 116 genes, some of which may be relevant for the olfactory learning deficits induced by FPF and the toxicity of FPF.

1. Introduction

Flupyradifurone (FPF) is a relatively new systemic butanolide insecticide that was developed by Bayer CropScience and first commercially registered in 2014 as the active component of SIVANTO® (Nauen et al., 2014). FPF is modified from a natural product, stemofoline, to increase its toxicity (Nauen et al., 2014). Like the neonicotinoids, FPF interacts with insect nicotinic acetylcholine receptors (nAChR) but has a lower binding affinity to insect nAChRs than neonicotinoids (Nauen et al., 2014). FPF is therefore used at higher application levels than neonicotinoids, and is effective against a wide range of pest insects, including many that are resistant to the neonicotinoids (Glaberman and White, 2014; Chen et al., 2017). It is also used on multiple crops such as vegetables, fruits, grapes, dates, coffee, and cocoa through diverse application methods (foliar spray, soil drench, or seed treatment) throughout the world (Nauen et al., 2014; Chen et al., 2017).

FPF is reported to be relatively safe for the European honey bees, *Apis mellifera*, (Campbell et al., 2016; Glaberman and White, 2014), but there is growing evidence of detrimental effects for beneficial pollinators such as honey bees, which are widely used to pollinate agricultural crops treated with FPF. This pesticide can reduce bee survival and is associated with altered expression of bee immune and detoxification genes (Naggar and Baer, 2019). FPF also has multiple behavioral effects. It can impair bee flight ability (Tong et al., 2019), increase abnormal behaviors (Tosi and Nieh, 2019), and create motor disabilities and disturbed motor behavior (Hesselbach and Scheiner, 2019). In line with research showing that sick or poisoned bees can exhibit altruistic suicide by remaining longer outside the nest (Rueppell et al., 2010), chronic exposure to FPF can lead to premature bee foraging (Hesselbach et al., 2020). FPF can also harm gustatory responsiveness and olfactory learning at high concentrations in *A. mellifera* (Hesselbach and Scheiner, 2018) and impair learning in *Apis cerana* workers exposed as larvae or adults (Tan et al., 2017). However, at lower doses (0.44–0.52 μg FPF/bee/day) sucrose responsiveness was not altered (Bell et al., 2019), highlighting the importance of doses and protocol in determining FPF effects.

It is unclear if FPF attracts foragers. Two commonly used neonicotinoids are attractive to foragers at field-realistic concentrations: Kessler et al. (2015) found that honey bees and buff-tailed bumble bees preferred sucrose solutions containing imidacloprid or thiamethoxam at

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levels found in nectar and pollen over pure sucrose solutions. Foragers consumed less food overall within 24 h, an effect of pesticide consumption, but the preferences exhibited are concerning for pollinator health (2015). Interestingly, this preference may be related to activation of nAChR because honey bees also prefer to collect sucrose solutions containing low concentrations of nicotine, which (like neonicotinoids) activates these receptors (Singaravelan et al., 2005). Wood et al. (2020) reported that syrup consumption by worker bees was not affected by the thiamethoxam at field-realistic doses in a 30-day chronic exposure assay. These consumption preferences may thus manifest over shorter exposure durations (Kessler et al., 2015).

One of the most complex and sophisticated honey bee behaviors is the waggle dance, which communicates the quality and location of good resources to nestmates (Frisch, 1967). Waggle dancing can be important for colony fitness (Sherman and Visscher, 2002), depending upon the distribution of food sources in the landscape (Dornhaus and Chittka, 2004). Previously, Eiri and Nieh (2012) reported that acute exposure (ingestion) of the neonicotinoid pesticide, imidacloprid, could impair waggle dancing by reducing the number of dance circuits per recruiting forager, even if the dancer was recruiting for pure sucrose solution without any pesticide. Zhang et al. (2020) reported that imidacloprid contributed error to the waggle dance, increasing variance in the divergence angle of the waggle phase and in the waggle dance return phases. Similarly, the pyrethroid pesticide, deltamethrin, reduced the precision of honey bee waggle dancing and thereby decreased its information content (Zhang et al., 2019). Interestingly, although these dances were more variable, bees produced more dance circuits per 15 s, usually a measure of excitation about a resource (Zhang et al., 2019).

Much remains to be learned about how pesticide-induced changes in gene expression harm bees. However, growing data on the honey bee transcriptome profile after exposure to pesticide could aid in understanding the effects of pesticides on honey bee health (Lewis et al., 2016; Shi et al., 2017; Wang et al., 2018; Wu et al., 2017a). These studies find interesting trends in differentially expressed genes (DEGs) that are likely involved in multiple functions related to behavior (Shi et al., 2017; Wang et al., 2018; Wu et al., 2017b). For example, sublethal exposure to imidacloprid and thiamethoxam down-regulated odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), potentially reduce honey bee chemosensory abilities (Shi et al., 2017; Wu et al., 2017a). Prior studies also found evidence of upregulated detoxification via cytochrome P450s in bees exposed to carbendazim or thiamethoxam (Shi et al., 2017; Wang et al., 2018). Researchers found that imidacloprid down-regulated muscle-related genes and this may be one source of honey bee climbing impairment (Wu et al., 2017a). Further study of how pesticides affect honey bee gene expression may therefore contribute to our understanding of the complex interactions between pesticides and honey bees.

Our goals were therefore to determine if honey bee foragers exhibited preferences for, were neutral to, or avoided sucrose solution contaminated with a field realistic concentration (4 ppm) of FPF and if such exposure altered dance recruitment, specifically, the number of dance circuits performed. We also used RNA-seq and analyzed the transcriptome profile to detect global changes in gene expression following FPF exposure.

2. Methods

2.1. Testing for FPF effects on forager behavior

2.1.1. Colonies

We studied three honey bee observation colonies maintained at an apiary at UCSD (32°53′13.1″N; 117°13′48.6″W). Each colony consisted of three combs housed inside a wooden case (65 cm high x 45 cm wide x 5 cm deep) and contained approximately 4500 bees, as determined by photographic estimation (Park and Nieh, 2017). Colonies were maintained at 33 °C ± 2 °C inside a trailer whose wall was pierced with a 3 cm diameter tube per colony to allow bees to enter and exit. All colonies were healthy as determined by standard beekeeping inspection practices (Dietemann et al., 2013).

2.1.2. Foraging cage

At the entrance of the colony under use, the focal colony, we placed a mesh cage on a table (74 cm high) to prevent otherother colonies from feeding on the sugar solutions offered (Fig. S1). The framework consisted of 1.27 cm diameter polyvinyl chloride pipes creating a cube (60 cm × 60 cm x 60 cm). Black polyester mesh (1.5 mm × 1.0 mm mesh size) screened the cage, which also contained a mesh panel that could be opened to change the feeding solutions. The nest entrance tube led into this cage, whose mesh was sealed against the tube with permanent magnets. On the upper left side of the cage, we cut a 22 cm × 15 cm opening that allowed foragers to leave the cage to collect nectar and pollen from natural food sources.

When this cage was placed in front of the colony, bees rapidly adapted within one day, and foragers learned to exit the cage to collect natural food sources. However, bees trained to the feeder placed inside the cage simply flew back through the cage entrance without venturing outside the cage. By marking all bees that visited the feeder with different colors of acrylic paints on their thoraces, we confirmed that foragers were from the focal colony. In the rare cases in which bees came from a different colony, the experimenter aspirated the bees and subsequently froze them to avoid recruitment from other colonies and the potential contamination of thesethe colonies with pesticide. Inside the cage, we placed a GoPro Hero 3+ Silver camera to video-record individual foraging trips.

Feeders consisted of plastic circular cell culture dishes (5.7 cm diameter x 1 cm high) covered with aluminum metal mesh (1.75 mm × 1.5 mm mesh size) that was glued to the side of the dish. This design prevented bees from falling into the sugar solution in the dish and allowed up to 20 bees to feed simultaneously, without crowding, from 10 ml of solution. We used 2.5 M reagent-grade sucrose solution (65% w/w sucrose prepared in double distilled water) to provide a high sugar concentration that elicited recruitment and dancing.

For our pesticide treatment, we prepared a 400 ppm stock FPF solution in double distilled water and stored this in a glass bottle covered with aluminum foil to exclude light degradation at 4 °C. At this concentration, FPF dissolves readily in water, and we observed no particulate matter at any time inside the bottle. From this stock, we made dilutions with 2.5 M sucrose solution to achieve 4.0 ppm FPF. The control sucrose solution was identical but contained no pesticide. New sucrose solution and fresh pesticide dilutions were made each week and stored in the dark, inside glass vials covered with aluminum foil. All solutions were stored at 4 °C. Prior to feeding the bees, we took the requisite quantities of solution for a one-day trial and allowed it come to room temperature for 90 min so that bees would not be repelled by a cold solution.

We tested a 4 ppm FPF concentration because, although the field-realistic concentration of FPF covers a wide range, FPF was found at 4.3 ppm and 4.1 ppm in the nectar brought back by bees foraging on oilseed rape 1 and 3 days after FPF spraying, respectively (Glaberman and White, 2014). Bees can consume FPF at higher concentrations in pollen (21 ppm in oilseed rape) and in nectar (22 ppm in cotton, 39 ppm in apple, and 68 ppm in blueberry) (Glaberman and White, 2014). Recently, Tong et al. (2019) showed that a 4 ppm dose, in combination with nutritional stress and season, can reduce bee survival, flight success, thermoregulation, and food consumption (a decrease of 14%).

We trained bees to the feeder by first placing a pure 2.5 M sucrose solution feeder (no pesticide) directly in front of the nest entrance and allowing bees to visit. Once approximately five bees had visited and were individually paint marked, we moved the feeder to the center of the cage floor, 30 cm from the colony entrance and began our experiment.

2.1.3. Measuring consumption

We estimated the amount of solution that each bee imbibed based upon its video-recorded imbibing time. To determine the volume imbibed, we used a separate group of bees from the three test colonies, trained them to the feeder, and allowed them to imbibe while recording them. When they left the feeder to return to the nest, we captured them gently with an aspirator, placed each bee in a plastic vial, chilled it on ice for 4 min to reduce its motion, and then gently squeezed its ventral and dorsal sides to cause it to regurgitate the contents of its honey stomach into a 50 μl microcapillary tube. We calibrated these tubes by using a micropipette to dispense 10, 20, 30, and 40 μl of sucrose solution that was then taken up by the tube. The length that each volume occupied in the tube was then measuredme.

We separately measured the correlation between imbibing time and the volume imbibed for bees consuming FPF in 2.5 M sucrose solution or pure 2.5 M sucrose solution but found no difference and therefore pooled these data, calculated the linear regression of imbibing time and volume, and used the regression slope to estimate the volume imbibed per second of imbibing time.

2.1.4. Experimental phases

We only conducted one trial per day, beginning at approximately 9 a.m. on clear, sunny days. Each trial consisted of three phases: training, before treatment, and during treatment. Only one feeder was placed in the cage in each phase. We tracked the same set of bees in all three phases and video recorded all visits to the feeder. Per bee in the *before* and *during* treatment phases, we measured total foraging time, total number of feeder visits, imbibing time, time spent unloading food to nestmates inside the nest (unloading time, including all unloadings that were >1 s), number of dance bouts per nest visit, and the total number of dance circuits per nest visit. Given the close distance of the food source to the nest, bees performed round dances, which are part of a continuum of dance behavior that is generally described as waggle dancing (Frisch, 1967; Gardner et al., 2008). In the case of round dancing, a single dance circuit consists of a circular movement that end with a sharp reversal, often of 180° in dancer direction (Frisch, 1967). A dance bout can consist of multiple dance circuits. Bees would often interrupt their dancing to either unload collected food to nestmates, move to a different section of the comb, or both. We defined one dance bout as a continuous dance performance. A dancing pause >1 s would be scored as a different dance bout. A bee that did not dance at all during a nest visit was scored as performing 0 dance bouts and 0 dance circuits.

In the *training phase* (40 min), we trained five individually marked bees, removing excess bees with an aspirator. We used this phase to identify bees that reliably and repeatedly visited the feeder and would dance to recruit for it. In the *before phase* (60 min), we typically focused on two foragers that we identified from the training phase and monitored all returns of these foragers inside the nest. Behavior inside the nest was manually recorded by the observer with the help of digital timers. During the before phase, all bees fed only on pure 2.5 M sucrose solution from a clean feeder replaced at the beginning of this phase to capture their behavior before pesticide exposure.

We then began the *treatment phase* (60 min) and tracked the same foragers. In this phase, we again replaced the feeder with a clean one and fed bees either pure 2.5 M sucrose solution (control) or FPF sucrose solution (pesticide treatment). We ran control and FPF trials on alternating days with identical feeders. Thus, in any given trial, all bees were exposed to the same treatment, but we randomly selected which trials would have pesticide treatments. The experimenter was blind to the treatment being used. Only bees that continued to visit the feeder in all three phases were used for our analyses.

2.2. Testing for FPF effects on forager transcriptome

2.2.1. Treating bees

In a separate experiment, we exposed bees from three full-sized colonies to 4 ppm FPF and tested for transcriptome effects. Three combs with capped cells, each from a different colony was maintained in an incubator at 30 °C and 70% relative humidity in darkness. Newly emerging bees (<12 h old) were collected from these combs so that each colony accounted for one third of all the bees used. We placed 50 bees in each cage and used eight cages, feeding the incubated bees with 50% sucrose (w/v) solution and pollen for 48 h *ad libitum*. Half of the cages were then fed with pure 50% sucrose (control) and the other half were fed 4 ppm FPF in 50% sucrose solution (FPF treatment). After 12 h of exposure to these treatment solutions, bees were knocked from their cages into liquid nitrogen. Frozen bee heads were severed and collected in centrifuge tubes (one tube per treatment type) filled with liquid nitrogen. Heads were stored at −80 °C for 24 h before being sent for analysis (Biomarker Biotechnology Co., Ltd) in dry ice. In total, we analyzed 99 bee heads from the control group and 99 heads from the pesticide group (33 heads per treatment per colony), performing RNA-seq with three replicates from the FPF treatment and three replicates from the control treatment (33 heads per replicate per treatment type, total of six samples and three replicates for RNA extraction).

2.2.2. RNA-seq

RNA concentration was measured with a NanoDrop 2000 (Thermo-Fisher Scientific) and RNA quality was assessed with an RNA Nano 6000 Assay Kit (Agilent Bioanalyzer 2100 system, Agilent Technologies, CA, USA). We used 1 μg RNA per sample. Sequencing libraries were generated with the NEBNext UltraTM RNA Library Prep Kit (Illumina, NEB, USA) following manufacturer's recommendations and index codes were added to each sequence. mRNA was purified from total RNA with poly-T oligo-attached magnetic beads. Fragmentation was conducted with divalent cations in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized with random hexamer primers and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed with DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends with exonuclease and polymerase. After adenylating the 3′ ends of DNA fragments, NEBNext Adaptors with hairpin loop structures were ligated to prepare for hybridization (Manjon et al., 2018). To select cDNA fragments approximately 240 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was incubated with size-selected, adaptor--ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer (PCR conditions: 98 °C for 30s, followed by 40 cycles of 98 °C for 10s, 65 °C for 30s, 72 °C for 30s, and then 72 °C for 5min). Finally, PCR products were purified (AMPure XP system) and library quality assessed (Agilent Bioanalyzer 2100 system).

The clustering of the index-coded samples was performed with a cBot Cluster Generation System using the TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina platform and paired-end reads were generated. For quality control, raw reads in FASTQ format were processed with in-house Perl scripts. We then removed low quality reads and reads containing adapters or ploy-N. Subsequently, Q20, Q30, and GC-content and the sequence duplication level were calculated. All downstream analyses were based on clean data of high quality. Clean reads were mapped to the reference *A. mellifera* genome sequence (version Amel-HAv3.1 downloaded from NCBI) with Hisat2 software. Reads with ≤1 mismatch were further analyzed and annotated based upon the reference genome.

To validate our RNAseq data, we performed qRT-PCR with 99 heads from the control treatment and 99 heads from FPF treatment (see above), usingthree biological replicates. We randomly selected five up-regulated and five down-regulated genes (Wu et al., 2017b). Primers used are listed in Table S3. Thermal cycling was set at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative expression of genes was normalized by comparison with the expression

of β-actin using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

2.3. Statistics

For the number of visits to the feeder or the nest, we used Repeated Measures General Linear Models (GLM) with colony as a fixed effect and bee identity nested within treatment. We first tested for overdispersion, but then eliminated this if no overdispersion was found. To further analysis differences in the number of feeder visits, we applied a Dunn-Sidak correction ($k = 2$) and denote significant tests with ^{DS}.

We log-transformed the number of round dance bouts per nest visit and the number of dance circuits per nest visit and used Repeated Measures Linear Mixed Models (REML algorithm) with colony as a random effect and bee identity nested within treatments. Phase (before vs. during treatment phases) was a fixed effect. To analyze time data, we used Repeated-Measures Linear Mixed Models (REML algorithm) with colony as a random effect and bee identity nested within treatment. We log-transformed unloading time. Residuals analysis confirmed that our models met parametric assumptions. Full models with all interactions were initially run, and then the interactions eliminated if they were not significant. We used limited contrast tests, based upon visual inspection of the data. To determine the volume imbibed per unit time, we used a Linear Mixed Model (REML algorithm) with colony as a random effect.

Differential gene expression analysis was performed with the DESeq R package (1.10.1), using a negative binomial distribution. The resulting *P* values were adjusted with the Benjamini and Hochberg correction to control for false discoveries. We accepted as significant only expression differences >1.2 fold and for which $P < 0.05$ after Benjamini Hochberg (B–H) correction. We used Gene ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) with GOseq R packages and a Wallenius non-central hyper-geometric distribution (Young et al., 2010) that can adjust for gene length bias in DEGs.

3. Results

3.1. Behavioral effects of FPF

In total, from three colonies, we tracked the feeder visit behavior of 100 bees of which 78 danced during the treatment phase.

3.1.1. Bees visited the FPF sucrose solution feeder significantly less than control bees

For *visits to the nest*, there were no significant effects of treatment (L-R chi-square = 0.20, 1 df, $P = 0.66$), phase (L-R chi-square = 3.34, 1 df, $P = 0.07$), or the interaction of treatment x phase (L-R chi-square = 1.70, 1 df, $P = 0.19$, GLM, Poisson distribution, reciprocal link, Fig. 1).

For foraging *visits to the feeder*, there were no significant effects of treatment (L-R chi-square = 0.94, 1 df, $P = 0.33$) or phase (L-R chi-square = 1.41, 1 df, $P = 0.24$), but a significant interaction of treatment x phase (L-R chi-square = 14.96, 1 df, $P < 0.0001$, GLM, Poisson distribution, log link). For control bees, the number of visits increased during the treatment phase as compared to the control phase (L-R chi-square = 13.73, 1 df, $P = 0.0002^{DS}$). Before pesticide treatment (before treatment phase), the number of visits was not significantly different between control bees and bees randomly designated to receive pesticide (L-R chi-square = 3.47, 1 df, $P = 0.06$). However, in the treatment phase, bees treated with FPF visited the feeder significantly less (−31% less) than control bees (L-R chi-square = 11.65, 1 df, $P = 0.0006$ ^{DS}, Fig. 1).

3.1.2. No effects of FPF on time spent foraging or unloading food

The total time spent foraging was not significantly affected by treatment ($F_{1,32} = 0.93$, $P = 0.34$), phase ($F_{1,33} = 1.23$, $P = 0.27$), or the interaction treatment x phase ($F_{1,32} = 2.76$, $P = 0.11$, model $R^2 = 0.12$). Similarly, there was no effect of FPF on the *time that bees spent unloading* their collected food to nestmates in the colony ($R^2 = 0.59$). There were no significant effects of treatment ($F_{1,34} = 0.19$, $P = 0.66$), phase ($F_{1,26} = 0.004$, $P = 0.95$), or the interaction treatment x phase ($F_{1,28} = 0.006$, $P = 0.94$).

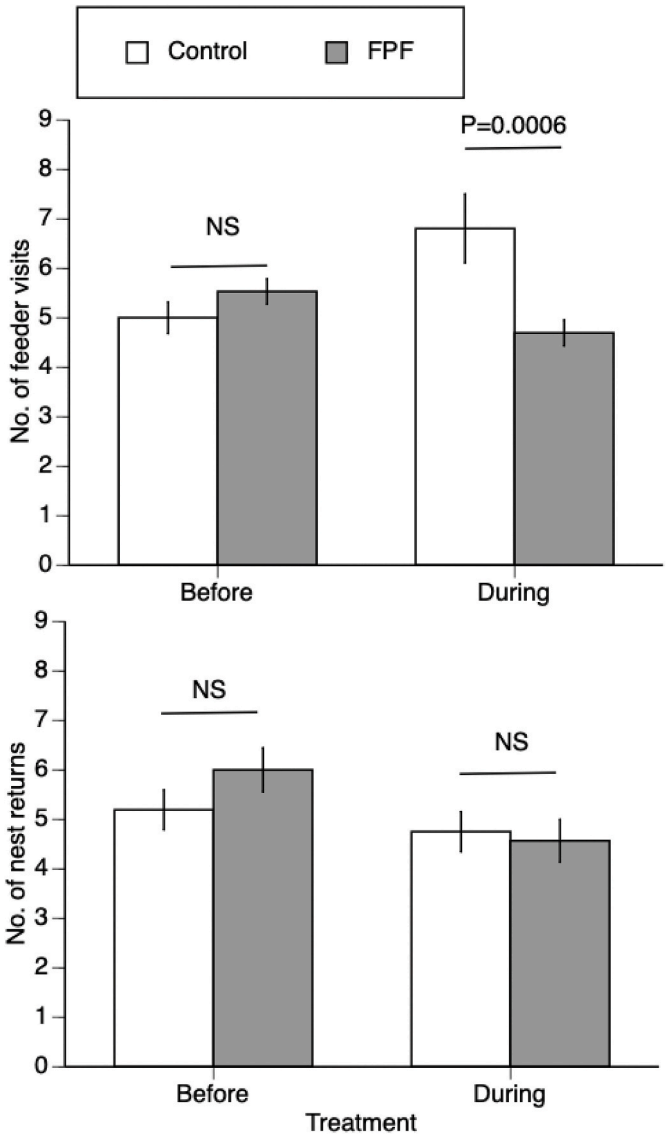

Fig. 1. Effect of FPF on feeder visits and nest returns. The mean ± 1 standard error is shown (NS: $P \geq 0.05$). There were no significant differences between the number of feeder visits and nest returns for control bees before and during treatments phases and for FPF bees before and during treatment phases (see Results). However, control bees visited the control feeder significantly more during the treatment phase than FPF bees (L-R chi-square = 11.65, 1 df, $P = 0.0006$ ^{DS}).

3.1.3. No effects of FPF on dance bouts or dance circuits

FPF did not significantly alter the number of *dance bouts* per *nest visit*: ($F_{1,33} = 0.12$, $P = 0.74$), phase, ($F_{1,63} = 2.42$, $P = 0.13$), or the interaction treatment x phase ($F_{1,65} = 1.87$, $P = 0.18$, whole model $R^2 = 0.38$). FPF also did not change the *number of dance circuits* per *nest visit* ($F_{1,48} = 1.37$, $P = 0.25$), phase ($F_{1,57} = 0.04$, $P = 0.84$), or the interaction treatment x phase ($F_{1,58} = 0.60$, $P = 0.44$, whole model $R^2 = 0.36$, Fig. 2).

3.1.4. Bees made fewer trips to imbibe less FPF sucrose solution than pure sucrose solution

In our imbibing quantification calibration experiment, there was a significant positive relationship ($R^2 = 0.57$) between the volume consumed and the drinking time ($F_{1,30} = 33.45$, $P < 0.0001$), yielding a

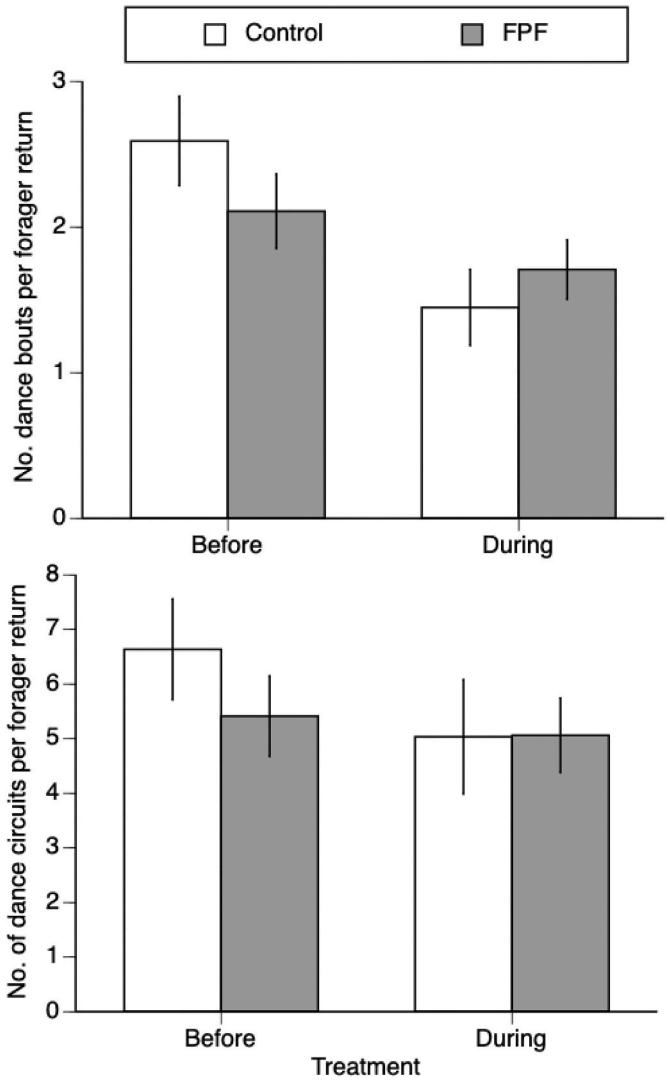

Fig. 2. Effect of FPF on the number of dance bouts and dance circuits per forager return to the nest. A dance bout is defined as a continuous, uninterrupted performance of dance circuits. A pause (bee largely stationary on the comb) of >1 marked the end of one dance bout. In contrast, the number of dance circuits is the total number of dance circuits performed during a forager return to the nest. A forager return began when the forager returned to the nest from the feeder and ended when she left the nest for the feeder again. There were no significant effects of treatment, phase, or their interaction. The mean ± 1 standard error is shown.

regression equation of y = 0.29x+1.98. Bees therefore consumed 0.29 µl of sugar solution per second (Fig. 3). The amounts of sucrose solution and FPF that bees were estimated to imbibe, based upon this equation, are shown in Table 1.

For the total amount consumed per phase, before treatment, all bees consumed amounts that were not significantly different between treatment controls or the control (contrast test, $F_{1,179} = 0.33$, $P = 0.56$), as expected given that no bees received FPF before treatment. There was a significant interaction of treatment x phase ($F_{1,97} = 6.01$, $P = 0.016$) because FPF reduced imbibing during the treatment phase. There were no significant effects of treatment ($F_{1,97} = 1.27$, $P = 0.26$) and phase ($F_{1,97} = 0.12$, $P = 0.73$, model $R^2 = 0.53$). During the treatment phase, *bees the received FPF imbibed significantly less (16%) than control bees* (contrast test, $F_{1,177} = 5.75$, $P = 0.017$, Fig. 3).

There were no effects of treatment ($F_{1,97} = 0.28$, $P = 0.60$), phase ($F_{1,99} = 0.52$, $P = 0.47$), or the interaction treatment x phase ($F_{1,97} = 0.03$, $P = 0.87$) on the average imbibing time *per visit* (model $R^2 = 0.51$).

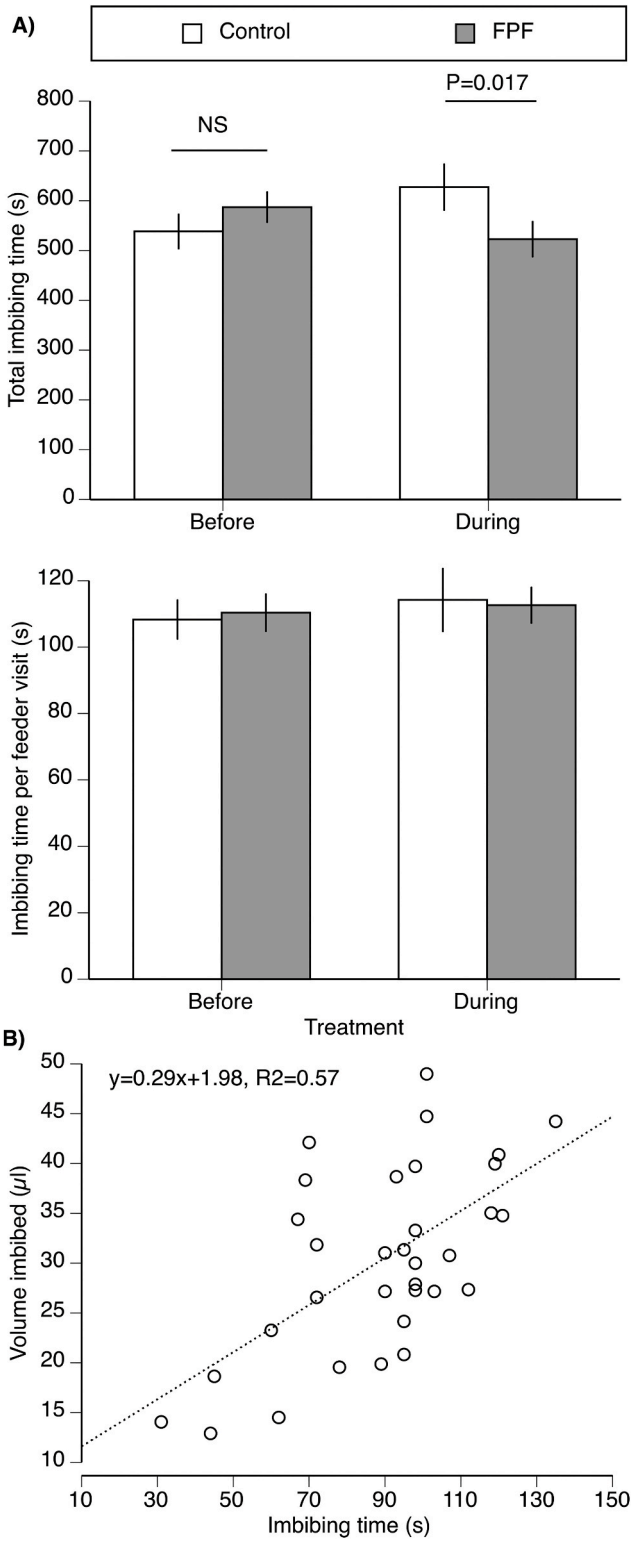

Fig. 3. Effect of FPF on nectar consumption (imbibing time). A) Total imbibing time per bee during the entire treatment phase and per feeder visit (mean ± 1 standard error). Time is shown because the volume imbibed and the amount of FPF consumed (Table 1) is a linear function of imbibing time. There were no significant effects of treatment or phase, but a significant interaction of treatment x phase that is shown in the significant contrast test ($F_{1,177} = 5.75$, $P = 0.017$). B) Results of a separate experiment measuring the volume of pure sucrose solution (no FPF) consumed per second of imbibing time. The linear regression equation (including colony as a random factor) is shown (dashed line).

Table 1
Quantity of sucrose solution and FPF imbibed overall and per visit to the feeder. No FPF was fed to any bees during the before phase.

Treatment	Phase	N bees	Volume imbibed per bee		FPF consumed per bee	
			Total (μl)	Per visit (μl)	Total (ng)	Per visit (ng)
Control	Before	66	159 ± 10	34 ± 2	0 ± 0	0 ± 0
Control	During	66	185 ± 13	35 ± 3	0 ± 0	0 ± 0
FPF	Before	59	170 ± 9	34 ± 2	0 ± 0	0 ± 0
FPF	During	59	155 ± 10	35 ± 2	620 ± 41	450 ± 21

Thus, the reduction in total imbibing time was driven by the reduction in visits to the FPF feeder, not in reduced consumption per trip.

3.1.5. *FPF treatment changed the bee transcriptome*

We obtained approximately 36 million clean reads that mapped to the *A. mellifera* reference genome from each of the six libraries after filtering, and we identified 10,637 honey bee genes (Table S1). There were 116 significantly differentially expressed genes (DEGs) between control and FPF treated groups (B–H corrected<0.05 and value fold changes >1.2, see Supplemental Table S2) of which 58 genes were up-regulated and 58 were down-regulated.

Ten randomly selected genes (5 up-regulated and 5 down-regulated genes) showed the same expression profiles in the qRT-PCR assays and the RNA-seq data (Fig. 4). This agreement indicates that the abundance of the Illumina reads closely mirrored the actual expression levels of the DEGs.

Gene ontology (GO) term enrichment analysis ($P < 0.05$) was performed to further understand the function of these DEGs, which were clustered in nine categories, of which the three largest categories contained genes involved in (1) biological processes, (2) cellular components, and (3) molecular functions. Genes in the biological processes category are involved in responses to stimuli, localization, signaling, cellular component organization or biogenesis, and detoxification. In the cellular components category, genes related to cells, cell parts, and cell membranes were highly represented. Finally, in the molecular functions category, genes related to catalytic activity, binding, and transporter activity were differentially expressed (Fig. 4).

The potentially most relevant genes are in the biological processes category and are related to responses to stimuli: eight genes of LOC412774, LOC410765, Hsp 90, LOC409994, LOC100577252, LOC551930, LOC410415, LOC412949 (see Table S2 and Table S3). The last four DEGs (LOC100577252, LOC551930, LOC410415, LOC412949) were also enriched and related to signaling. In addition, DEGs related to transporter activity within the molecular function category were enriched: LOC412949, LOC725165, LOC412217, LOC410780, and LOC100577451. Among those, LOC412217 is predicted to be a multi-drug resistance-associated protein 1. Given that we were examining foraging behavior, we also checked for effects of FPF on the foraging genes Amfor, Amoctα-R1 and AmlnR-2 (George et al., 2019) but found no significant effects ($P < 0.05$).

4. Discussion

Bees were significantly repelled by 4 ppm FPF in sucrose solution. They visited the FPF feeder less often in the treatment phase, as compared to control bees (Fig. 1), and spent less time imbibing sucrose solution than control bees (Fig. 3). In addition, bees feeding on the FPF treatment consumed 16% less nectar (Table 1), which is similar to the 14% decrease in food consumption in caged bees fed 4 ppm FPF in

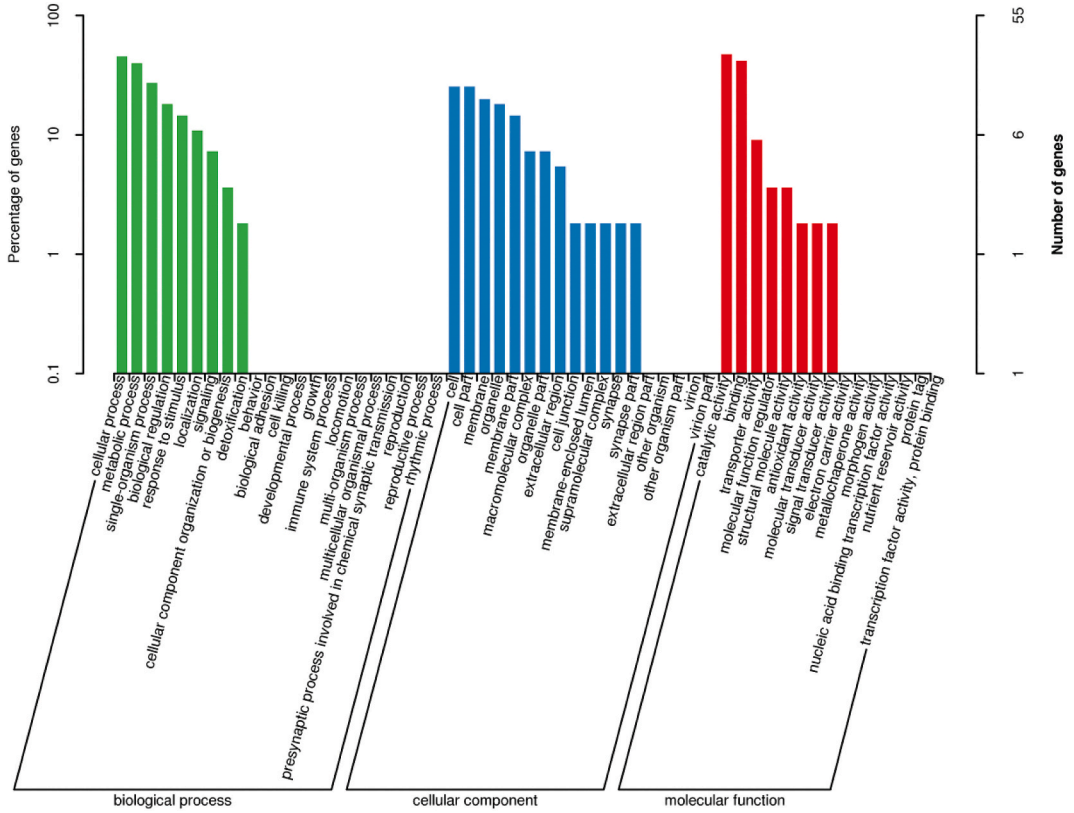

Fig. 4. Significantly enriched gene ontology (GO) terms ($P < 0.05$) in all DEGs in bees fed 4 ppm FPF in 2.5 M sucrose solution as compared to control bees fed only pure 2.5 M sucrose solution. The genes belong to three major categories: biological processes (GOBP), molecular functions (GOMF), and cellular components (GOCC) and are respectively shown in green, red, and blue. (For a color version of this figure, the reader is referred to the Web version of this article.)

sucrose solution in an incubator (Tong et al., 2019). However, there were no effects of FPF on the average unloading wait time, the number of dance bouts per next visit, or the average number of dance circuits performed per dance bout. We also found that FPF significantly altered the expression of 116 genes, some of which are relevant to olfaction, olfactory learning, and detoxification pathways. These gene changes may not be linked to the behavioral changes observed in this study, but could be relevant to the olfactory learning deficits induced by FPF and the relatively low toxicity of FPF to honey bees as compared withwith the neonicotinoids (Lewis et al., 2016).

The number of control feeder visits, but not FPF feeder visits, increased during the treatment phase. This increase in the number of control visits may be a function of time of day or increased familiarity that bees had with the food source. The significant decrease in feeder visits to the FPF treatment was likely due to comparatively increased visitation to the control feeder in the during treatment phase. The design of our experiment required us to consistently provide FPF second, since providing it first could result in FPF-altered behavior in bees fed pure sucrose solution in a subsequent "FPF-free" phase. Thus, there was an unavoidable sequence (or time) effect. However, even given such an effect, there was a significant elevation of visits to the control solution in the treatment phase, an increase not seen in visits to the FPF feeder.

During the treatment phase, foragers brought back solution with FPF back into the nest, and this was subsequently distributed to multiple nestmates via trophallaxis. Some of these nectar receivers could have subsequently become control group foragers, thereby influencing our results. Previously, we used a design in which we fed foragers with pesticide, isolated them, and then reintroduced them to the nest to avoid this potential issue (Eiri and Nieh, 2012). However, a criticism of this approach is that bees foraging on pesticide-contaminated nectar would continue to do so over multiple visits and be exposed during each visit. We therefore used a design in which bees could multiply visit FPF sucrose solution. On any given day, we only ran one type of trial (control or FPF). The experiments were conducted over several months and examination of the first control day before any colonies were exposed to FPF (or after at least a one month pause in which colonies were not exposed to FPF) showed no evident differences between unexposed control bees and potentially FPF-exposed control bees. Recent data suggest that honey bees are efficient at detoxifying FPF because long term chronic feeding does not result in an expected additively increasing mortality (Tosi et al. in review). Our data also show that FPF exposure can result in upregulation of LOC412217, a gene potentially associated with detoxification. Nonetheless, the use of a separate set of control colonies that were never exposed to FPF would have improved our experiments.

Another limitation of our study was the use of a feeder placed only 30 cm away from the nest, which is not a typical foraging distance. We used this very short distance because we needed a group of bees that would reliably visit the same feeder location very frequently to encompass the three different phases (training, before, and during) that we used each experimental day and to provide them ample opportunity to dance during each phase. We also wanted to make the feeder as valuable as possible (close by and 2.5 M sucrose solution) to increase the number of dance circuits and thereby have more power to discriminate a statistically significant effect on dancing. Future studies examining more realistic training distances of several hundred meters would be valuable, but would likely only be possible in an environment with an extreme dearth of food sources to encourage reliable feeder visitation and good dancing.

Because we focused on round dancing, which does not typically have a waggle phase, particularly at distances very close to the nest (Frisch, 1967), we did not examine variance in the waggle phase, which has been reported to increase upon exposure to different pesticides (Zhang et al., 2020, 2019). Such detailed examination of the effects of FPF on the waggle dance would be desirable for future studies. However, in our study, we found no effects of FPF on other related behaviors: time spent foraging, time spent unloading food, number of dance bouts per nest visit, or number of dance circuits per dance bout. In stingless bees (*Melipona quadrifasciata*), neonicotinoid pesticides can reduce social behaviors such as antennation and trophallaxis (Boff et al., 2018). However, we found no evidence of impaired food exchange as measured in terms of unloading wait times for foragers bringing back 4 ppm FPF, which is not a neonicotinoid, in nectar.

Measurements of nectar loads show that foragers can carry about 32–40% of their body mass (approximately 20 μl of a 40% sucrose solution w/w, correcting sucrose density) (Feuerbacher, 2003). On average, our bees consumed 34–35 μl of sucrose solution, which is within the range for proventricular nectar loads reported for *A. mellifera* returning to the nest after foraging on sucrose solution feeders (34–59 μl) (Roubik and Buchmann, 1984). The reduction in imbibing FPF contaminated sucrose solution is interesting, but the mechanism for this change in consumption is unclear, as it is for the increased consumption patterns exhibited by bees for nectar with certain concentrations of caffeine (Kessler et al., 2015).

The number of differentially expressed genes (116 DEGs) altered by FPF at a field-realistic level, seems to broadly correlate with its toxicity: thiamethoxam (609 DEGs), imidacloprid (578 DEGs) and carbendazim (247 DEGs) are all more toxic per dose (Lewis et al., 2016). Within these DEGs, there are broad categories of affected genes, including those relevant to defense, immunity, odor recognition, chemical communication, learning, memory, and detoxification (Shi et al., 2017; Wang et al., 2018; Wu et al., 2017a).

We used unscented sucrose solution, but olfaction plays an important role in multiple aspects of bee communication and foraging and reduced or numbed olfaction could influence consumption. Our transcriptome analyses showed that LOC412949 (glutamate receptor ionotropic, kainate 5) was up-regulated by FPF. This gene is integral to antennal sensory systems that are involved in thermosensation, hygrosensation, and olfaction (Abuin et al., 2011; Enjin et al., 2016; Knecht et al., 2016). Certain pesticides could alter bee olfactory perception. Like LOC412949, odorant-binding protein genes are involved in odor recognition. Shi et al. (2017) fed four-day-old bees with sublethal concentration of thiamethoxam for ten days and reported that the odorant-binding protein genes Obp3, Obp17, Obp21, and CSP3 all showed significantly decreased expression.

The gene expression impairments that we observed are likely more relevant to the FPF-induced reduction in olfactory learning shown in *A. mellifera* (Hesselbach and Scheiner, 2018) and in *Apis cerana* (Tan et al., 2017). Ca^{2+} signals play a key role in learning and memory. The processing of odorant information from detection by the sensilla of the antenna to integration, learning, and memorization uses Ca^{2+} signals at all steps. We found that FPF exposure significantly up-regulated the gene LOC408430 (voltage-dependent L-type calcium channel subunit beta-2-like). The beta subunit of voltage-dependent calcium channels contributes to their function by increasing peak calcium current, shifting the voltage dependencies of activation and inactivation, modulating G protein inhibition and controlling alpha-1 subunit membrane targeting. Thus, FPF may alter olfactory perception and the pathways involved in olfactory learning.

A key aspect of FPF is its relatively lower toxicity to honey bees in comparison with neonicotinoids (Glaberman and White, 2014; Nauen et al., 2014). In addition to the role of cytochrome P450s in detoxification (Manjon et al., 2018), we found that gene LOC412217 (multidrug resistance-associated protein 1) was enriched in “transporter activity” and up-regulated by FPF. This protein mediates the export of organic anions and certain drugs from the cytoplasm (Conseil et al., 2006; Leier et al., 1994; Sjölinder et al., 1999). Our transcriptome results highlight the need for additional studies on FPF, particularly given its growing and widespread use on crops pollinated and visited by honey bees.

Author credit statement

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Conceptualization, Formal analysis, Data curation, Funding acquisition. Patrick Pasberg, Methodology, Investigation, writing, Conceptualization. Qing-Yun Diao, Methodology, Investigation, writing. James C. Nieh, Methodology, Investigation, writing, Conceptualization, Formal analysis, Data curation, Funding acquisition, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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