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COMPLEMENTARY SESSION PAPER

Ethomics for Ecotoxicology: Automated Tracking Reveals Diverse Effects of Insecticides on Bumble Bee Foraging and In-Nest Behavior

Anupreksha Jain [*,1], Acacia Tsz So Tang [*,†] and James Crall [*]

[*]Department of Entomology, University of Wisconsin-Madison, Madison, WI 53706, USA; [†]Department of Integrative Biology, University of Wisconsin-Madison, Madison, WI 53706, USA.

[1]E-mail: anuprekshajain98@gmail.com

Synopsis The majority of flowering plants depend on insect pollination for reproduction and declining pollinator populations pose a threat to biodiversity as well as critical crop pollination services globally. Widespread insecticide use negatively impacts pollinator physiology and behavior even at environmentally realistic concentrations below lethal toxicity, leading to reduced fitness and long-term population declines. However, significant gaps remain in our understanding of how insecticides affect diverse aspects of behavior and ultimately influence pollinator populations and pollination services. These gaps partly stem from the challenge of quantifying sublethal effects of pesticides on the complex behavioral repertoires of insects. Current methods often focus on a narrow set of behaviors at a time, limiting our ability to capture the comprehensive range of impacts within management-relevant timescales. The emergence of low-cost techniques for high-throughput behavioral quantification, or "ethomics," holds enormous potential to address this knowledge gap. Here, we used automated, computer vision-based tracking implemented on open-source hardware (Raspberry Pis) to investigate the sublethal effects of an emerging "bee-safe" butenolide insecticide (flupyradifurone), as well as a neonicotinoid insecticide (imidacloprid), on bumble bee (*Bombus impatiens*) behavior. We simultaneously quantified the behavior of uniquely tagged individual workers both within the nest, and during foraging in a semi-field environment, to assess the holistic effects of insecticides under naturalistic conditions. Both insecticides increased mortality risk and altered behavior, but in distinct ways across behavioral contexts. Imidacloprid modified nest behavior by decreasing activity, while flupyradifurone altered spatial behavior within the nest (shifting bees toward the brood). Imidacloprid—but not flupyradifurone—reduced overall foraging activity, while both affected floral preference. Overall, our results highlight the complex potential mechanistic links between sublethal insecticide exposure, behavior, and pollinator health. This work emphasizes the need—and possibility—for rapid and holistic pesticide risk assessment under realistic environmental conditions using high-throughput ethomics, and could inform the development of sustainable agricultural practices and conservation strategies.

Introduction

Bees and other insect pollinators are critical for ecosystem functioning as well as human well-being. Most flowering plants (both wild and domesticated) depend on animal pollination, including ~75% of crop species that account for one-third of global food production (Klein et al. 2007; Ollerton et al. 2011). Pollinator-dependent crops are especially important for human health because of their high nutritional value (Chaplin-Kramer et al. 2014; Smith et al. 2022). Thus, recent declines of pollinator populations have sparked significant concern over potential implications for biodiversity conservation and food security (Potts et al. 2016).

While the underlying causes of pollinator decline are diverse and complex, mounting evidence has demonstrated that widespread pesticide use is a significant contributor (Goulson et al. 2015; Dicks et al. 2021; Janousek et al. 2023). Neonicotinoid insecticides are an important class of pesticides widely used in agri-

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cultural, horticultural, and veterinary applications, and often contaminate natural areas due to drift, leaching, and uptake by non-target plants (Bonmatin et al. 2015; Mitchell et al. 2017). Neonicotinoids are neuroactive compounds that bind to the insect nicotinic acetylcholine receptor (nAChR), disrupting nervous system function, causing paralysis and death at high concentrations (Simon-Delso et al. 2015). However, even at low, environmentally realistic concentrations, neonicotinoids can disrupt many aspects of insect biology, ranging from metabolism and physiology to cognition and behavior, in both pests and non-target beneficial species (Guedes et al. 2016; Teder and Knapp 2019; Crall and Raine 2023). In insect pollinators, such "sub-lethal effects" of neonicotinoids can contribute to population declines (Whitehorn et al. 2012; Woodcock et al. 2016; Deynze et al. 2024), as well as impair plant reproductive fitness and crop pollination (e.g., *via* shifts in floral preference and constancy: Gill and Raine 2014; Stanley et al. 2015; Stanley and Raine 2016).

Given concerns over the negative impacts of neonicotinoids on beneficial organisms, restrictions have been imposed in many parts of the world, such as a ban on outdoor agricultural application in the European Union and expanding regulation by state and local governments in the United States. In response to both emerging restrictions and increasing insecticide resistance in many pest species (Bass et al. 2015), there is a rising demand for novel insecticides to serve as substitutes for neonicotinoids. One emerging substitute is flupyradifurone, commercially available as Sivanto Prime ("FPF" hereafter). FPF is a butenolide insecticide with a distinct chemical structure but shared molecular target (nAChRs) and mode of action as neonicotinoids (Nauen et al. 2015). In light of its lower acute toxicity, FPF is marketed as "bee-safe" and—unlike neonicotinoids—approved for foliar applications during flowering, increasing exposure risk via both contact and ingestion pathways (Jeschke et al. 2015; Mundy-Heisz et al. 2022). However, recent work has demonstrated significant adverse effects on the behavior and fitness of both solitary and social bees (Boff and Ayasse 2024; Chen et al. 2024; Richardson et al. 2024; Siviter et al. 2024).

Increasing evidence for the negative impacts of neonicotinoids and emerging substitutes highlights the fundamental challenges of assessing and predicting the complex effects of novel pesticides on pollinators under real-world conditions. First, they can affect many different aspects of organismal performance. The receptor targets of butenolide and neonicotinoid insecticides (nAChRs) are ubiquitous within the insect central nervous system, and the sublethal impacts of these compounds are correspondingly varied, ranging from deficits in learning and navigation to olfactory processing and social interactions (Siviter and Muth 2020; Crall and Raine 2023). Beyond neuroreceptor interference, insecticide exposure can also impact gene expression (e.g., linked to metabolism and immunity) and other biological processes in diverse and complex manners (Witwicka et al. 2025). Second, the impacts of pesticides vary strongly depending on environmental conditions. For example, resource availability and ambient temperature can both modulate the effects of neonicotinoids (Stuligross and Williams 2020; Kenna et al. 2023; Fischer et al. 2024). As a result, lab-based controlled-environment studies may underestimate the risk posed by these compounds under dynamic, naturalistic conditions. Given these complexities, developing a comprehensive understanding of pesticide impacts on organismal performance (e.g., behavior and physiology) and their links to long-term colony and population health might take years, or even decades, especially because traditional approaches are time-, labor-, and cost-intensive. Thus, there is a critical need for approaches that can rapidly characterize the holistic effects of pesticides on pollinators in naturalistic environmental conditions to improve our understanding of these complex effects and ultimately promote pollinator health.

Recent technological advances for automated phenotyping and tracking hold enormous potential to address this challenge. For example, the rapid emergence of techniques for high-throughput, quantitative analysis of animal behavior (i.e., "ethomics") has significantly advanced our understanding of how animals interact with each other and their environment (Branson et al. 2009; Brown and de Bivort 2018). Hardware and software tools for quantifying behavior are increasingly low-cost, accessible, and deployable in field conditions, opening new opportunities for their application in understanding sublethal impacts of stressors on pollinator behavior and potential applications in environmental risk assessments. Radio-frequency identification technology, for example, has shed light on pesticides' impacts on foraging and homing behavior (Gill et al. 2012; Feltham et al. 2014; Henry et al. 2014; Stanley et al. 2016), while advances in computer vision and automated behavioral tracking of individuals have demonstrated effects of pesticide exposure on social behaviors within bee nests, such as nursing and thermoregulation (Crall et al. 2018; Smith et al. 2022; Easton-Calabria et al. 2023). However, no studies to date (to our knowledge) have assessed impacts of pesticides across these behavioral contexts (within-nest behavior and foraging outside the nest) simultaneously.

Here, we employ a combination of computer vision and machine learning powered imaging tools and in-

dividual tag tracking to investigate the sublethal effects of FPF as well as imidacloprid ("IMD," a common neonicotinoid pesticide), on social behavior within nests and the foraging behavior of bumble bees (Hymenoptera: Apidae: *Bombus impatiens* Cresson) in a semi-field environment. IMD is the most widely used and researched neonicotinoid pesticide, and has been shown to disrupt locomotion, thermoregulation, foraging, homing ability, and more, in important pollinator species (Anderson and Harmon-Threatt 2021; Crall and Raine 2023), providing an important comparison for understanding the sublethal effects of FPF. Using an integrated approach, we address two questions: (1) what are the diverse behavioral impacts of sublethal insecticide exposure, and (2) do these impacts vary across compounds? Specifically, we quantify the effects of insecticide exposure on within-nest space use and movement, foraging activity, and flower visitation, hypothesizing that both compounds will disrupt nursing and foraging behavior of bumble bee workers similarly given their shared mode of action.

Materials and methods

Study system: bumble bees

The common eastern bumble bee (*B. impatiens*) is native to eastern North America and an important wild as well as managed pollinator throughout its range. It is a primitively eusocial insect species, in which workers engage in foraging (i.e., nectar and pollen collection) and nest care tasks (e.g., nursing, incubation, and larval feeding) important for colony development. For this experiment, we sourced 10 *B. impatiens* colonies from Koppert Biological Systems (Howell MI, USA) between July and September of 2023. Each experimental round consisted of creating three microcolonies from a single source colony. Microcolonies are groups of worker bees isolated in queenless environments that display analogous social and foraging behaviors to queen-right colonies, and are an established method for studying bumble bee colonies while allowing for standardization (e.g., of colony sizes) and high replication (Klinger et al. 2019; though see Van Oystaeyen et al. 2021).

We created microcolonies by CO_2-anesthetizing and transferring 20 workers and approximately 10 brood cells (late-stage larvae or pupae) to custom behavioral monitoring chambers made of 3-D printed and laser-cut plastic parts (modified from the BumbleBox; Easton-Calabria and Crall 2025). The BumbleBox is a Raspberry Pi-based system for continuous, automated behavioral monitoring of individually tagged workers within bumble bee nests. To quantify foraging activity during the semi-field trials (in addition to tracking behavior within the nest), we modified one wall of the BumbleBox nest chamber to include a foraging tunnel to allow the bees to enter and exit the nest freely (Fig. 1A and Fig. S1; see "Foraging Activity" below).

While still anesthetized, each worker was tagged with a unique ArUco marker printed on water-resistant paper using ethyl cyanoacrylate super glue (tag width = 2.5 mm; Garrido-Jurado et al. 2014). This tagging allows automated identification of individual workers and their movements within the nest and while foraging during the trial. The microcolonies were placed in a dark room with *ad libitum* access to an artificial nectar substitute supplied through a cotton wick (50% sugar solution with a feeding stimulant and essential amino acids [Honey-B-Healthy, Cumberland MD, USA], and sorbic acid for preservation; hereafter nectar), and a ~10 g pollen ball made using honey bee collected pollen (Koppert Biological Systems) and nectar. Fresh pollen balls were added every 2 days. After anesthetization, microcolonies acclimated for 1–3 days before the onset of insecticide exposure.

Insecticide exposure and experimental design

Each of the three microcolonies in an experimental round was randomly assigned to one of three treatments provided through the nectar reservoir: control (nectar only), IMD (6 ppb), or FPF (500 ppb). Both the concentrations chosen are sub-lethal (well below LD_{50} values reported for *Bombus* spp.: Reid et al. 2020; Mundy-Heisz et al. 2022) but within field-realistic ranges of exposure in floral nectar (Krischik et al. 2007; Sanchez-Bayo and Goka 2014; Campbell et al. 2016; Siviter et al. 2024).

Technical grade IMD and FPF (Sigma-Aldrich, St. Louis MO, USA) were used to make stock solutions of 0.1 mg/ml and 1 mg/ml concentrations, respectively, and stored in a refrigerator in glass conical flasks covered with foil to minimize photo- and thermal degradation. Nectar was made fresh for each round, and the insecticide treatments were prepared by dissolving 12 µl of the IMD stock solution into 200 ml nectar for a final concentration of 6 µg/L, or 100 µl of the FPF stock solution into 200 ml nectar for a final concentration of 500 µg/L.

The onset of exposure was staggered by 1 day for each treatment within a round, and the order randomized for each round, so that the microcolony acclimation time varied but the insecticide exposure time was the same for each treatment. After 1 day of exposure in lab, the microcolonies were deployed in a hoophouse one at a time, and in-nest and foraging behaviors were recorded. This procedure was repeated for 10 experimental rounds (or $n = 30$ microcolonies, $n = 600$ individual workers total).

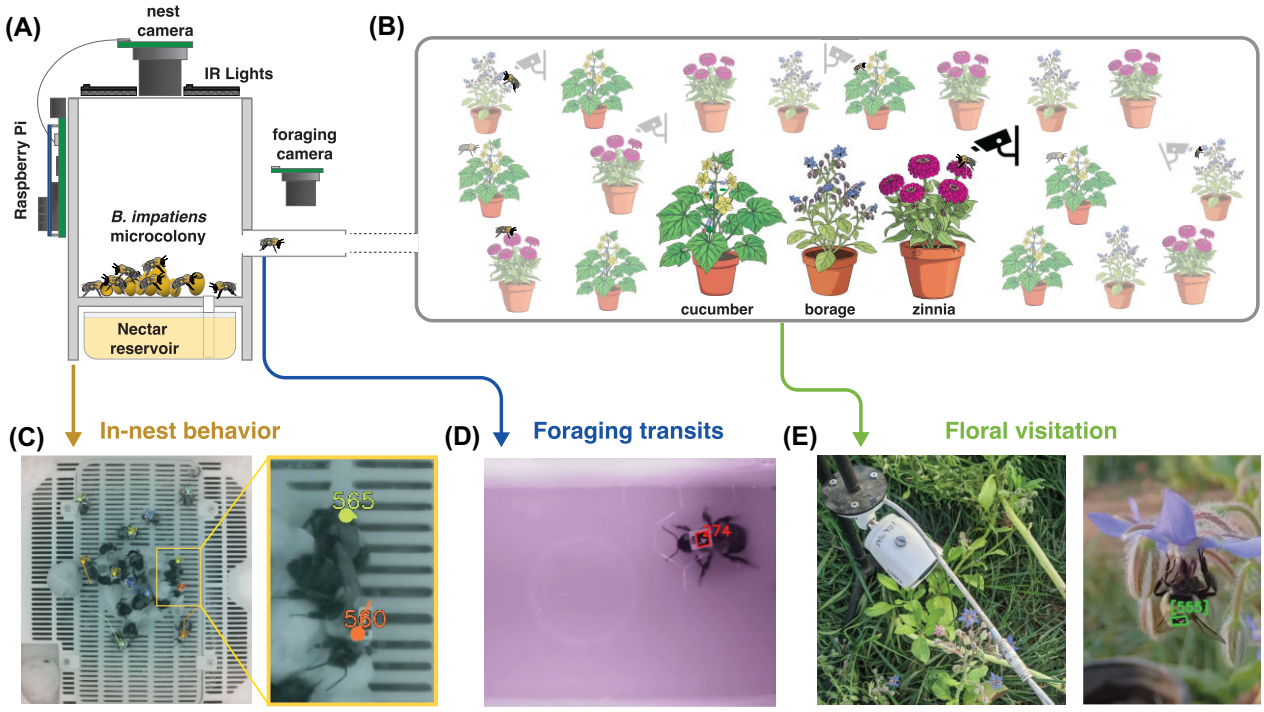

Fig. 1 Simultaneous automated tracking of within-nest and foraging behavior of *B. impatiens* microcolonies in a semi-field environment. Schematic representations of (A) BumbleBox chamber for within-nest behavioral monitoring, modified to incorporate a foraging tunnel (adapted from Fig. 6a in Easton-Calabria et al. 2023), and (B) hoophouse with potted flowering plants and cameras to monitor free-foraging behavior. Sample tracked images of (C) within-nest behavior, (D) foraging activity, and (E) flower visitation.

Hoophouse setup

The semi-field trials were conducted at Arlington Agricultural Research Station in Columbia County, Wisconsin in irrigated, plastic-enclosed hoophouses (29.26 m × 6.1 m × 3.66 m). Plants of four species were grown from seed in 1-gallon pots containing field soil: cucumber (Cucurbitaceae: *Cucumis sativus* L. var. A & C pickling; Seed Savers Exchange, Decorah IA, USA), zinnia (Asteraceae: *Zinnia violacea* Cav. var. Lilliput; American Meadows, Shelburne VT, USA), borage (Boraginaceae; *Borago officinalis* L.; American Meadows), and sunflower (Asteraceae: *Helianthus annuus* L. var. Domino; American Meadows). All of these species are known to be visited by bumble bees, and were chosen for this study because their floral morphology is compatible with our automated imaging system (see "Flower Visitation" below), specifically that larger flowers and longer handling times facilitate more consistent tag reading. We seeded successively over 6 weeks in May and June of 2023 to ensure flowering throughout the course of the experiment.

Experimental trials occurred in a second adjacent hoophouse with identical dimensions, containing an array of 45 potted plants, 15 each of cucumber, borage, and zinnia (Fig. 1B and Fig. S2). Sunflower was used instead of zinnia in Round 4 only and this round was excluded from floral visitation analyses. Plants were replaced for each experimental round and the plant species positions were randomized. Bumble bee microcolonies were ground transported to the field site and placed next to the hoophouse inside a waterproof plastic container (41.2 cm × 40 cm × 54.6 cm) with ventilation holes, and additionally shaded with a canopy tent (Fig. S3). The BumbleBox foraging tunnel was connected to the hoophouse *via* plastic PVC tubing (~90 cm length and 1.6 cm inner diameter). At the hoophouse end of this tubing, we affixed a yellow landing pad to facilitate returning foragers in locating the nest entrance. Each microcolony was deployed at approximately 17:00 and allowed to forage freely in the hoophouse for approximately 24 h before being removed (also at approximately 17:00). Nest behavior, foraging activity, and flower visitation were automatically recorded during these 24 h (see below). Any bees still foraging in the hoophouse at the end of the experimental trial were collected with an Insect Vac (Bioquip, Rancho Dominguez CA, USA).

Nest behavior

We quantified behavior of uniquely marked *B. impatiens* workers within the nest using the BumbleBox sys-

tem. A Raspberry Pi 4 model B microcomputer connected to a Raspberry Pi High-Quality camera (12 MP, 4056 × 3040 resolution) recorded a 20-s video (at 4 frames per second) of the microcolony every 5 min and stored these videos to a local external USB storage device (Fig. 1C and Fig. S1). The nest was illuminated by infrared lighting (850 nm) from above.

From these videos, the locations of individual ArUco tags were tracked for each frame offline using the ArUco module implemented in the OpenCV library in Python (Fig. 1C; Garrido-Jurado et al. 2014). To estimate the background of each nest (i.e., digitally remove bees), we subsampled one frame from each video, and generated aggregate nest images for each microcolony by calculating median pixel values across these frames (Fig. S4). The spatial locations of nest features (i.e., developing larvae and pupae [brood], waxpots, etc.) were manually mapped using LabelMe (Wada 2016). For each worker, we then calculated five focal spatial metrics and two movement-based metrics across all tracked frames in each video. The spatial metrics were: mean distance from social center (defined as the mean centroid of all tracked locations), mean distance from other bees, median distance to closest bee, mean distance to brood, and median distance to closest brood cell. The movement metrics were: proportion of time spent moving (activity), and mean speed when moving. These metrics have been shown in previous work to be linked to within-nest task allocation and response to stress (Crall et al. 2018; Easton-Calabria et al. 2023). We classified the time of day for each video as "night" if recorded between sunset on deployment day and sunrise the next day, and as "day" otherwise.

To ensure data quality, we excluded cases where individual bees were tracked for fewer than 20 frames in an 80-frame video, or had mean speed greater than 1000 pixels/frame, and videos that had fewer than three total bees tracked. From manually reviewing the nest images and the number of videos from each trial, we determined that eight microcolonies had missing data or poor video quality due to intermittent hardware issues, and excluded them from nest behavior analysis, resulting in 22 ($n = 6$, $n = 7$ IMD, and $n = 9$ FPF) microcolonies (though excluded microcolonies were included in other analyses where appropriate). We also excluded the first hour of each trial to account for hoophouse acclimation.

Worker mortality

Several bees died during the lab acclimation period before hoophouse deployment. We checked for mortality by looking for bees that were immobile for the duration of the 24 h observation period (or for the full duration of available video data) by manually screening the same still images used for background estimation (Fig. S4). Bees that did change locations but only due to physical contact with other live bees were also recorded as dead; mortality in these bees was further confirmed by reviewing two random videos from daytime hours and looking for lack of antennal movement. Even for videos with poor video quality and insufficient tracking, we could nonetheless count the number of dead bees. Dead workers with readable tags were excluded from within-nest behavior analyses.

Foraging activity

The foraging tunnel was imaged from above using a 1.3 megapixel USB camera (1280 × 960; ELP, Shenzhen Ailipu Technology Co., China) connected to the same Raspberry Pi microcomputer recording nest behavior, and illuminated with infrared lighting. We wrote a custom Python script using the OpenCV library that recorded images to local USB storage whenever there was movement in the camera's field of view. Recorded images were processed offline to obtain the identity and position of individual bees based on their ArUco tags (Fig. 1D). Multiple images (∼250 ms delay between image captures) were recorded for each transit through the tunnel. To separate these into unique foraging transits, we conducted hierarchical clustering of timestamps from frame-level detections. To be considered unique transits, clusters had to be separated by at least 2 min. We excluded any foraging transits that occurred during the first hour of the trial, as well as any that occurred during the night, defined as the time period between sunset on deployment day and sunrise the next day, as these detections were unlikely to represent realistic foraging behavior. Based on manual review of tracked timelapses and the number of triggered images from each trial, we excluded 12 microcolonies that had missing data or poor lighting leading to inconsistent tag tracking, yielding 18 total microcolonies ($n = 7$ control, $n = 6$ FPF, and $n = 5$ IMD). Finally, since the exact deployment duration varied between trials (21–25 h), we standardized the number of foraging transits per bee for 24 h.

Flower visitation

We monitored flower visitation using a network of security cameras ($n = 30$; Lorex, Markham, Ontario, Canada) focused on a subset of 30 flowers in the hoophouse, randomly chosen from the grid of 45 total plants to include 10 each of cucumber, borage, and zinnia (Fig. 1B). Round 4 used sunflower instead of zinnia because of insufficient availability of flowering zinnias, and was not included in this analysis. The cameras were connected to Raspberry Pi 4 Model B microcomputers (15

cameras to a single Raspberry Pi) through an ethernet hub. Insect visits to flowers were detected using a lightweight deep learning classification network (MobileNetV2) custom-trained on an open-source iNaturalist dataset (450,000 images, 8,000 insect species). Images were sequentially taken from each camera on the network and analyzed with this classification model using an Edge TPU coprocessor (Google Coral Accelerator) to enable high-speed inference. When insects were detected (with an intentionally "permissive" threshold setting to minimize false negatives [undetected bee visits] while allowing false positives [empty videos]) from this model, brief (10 s) videos were recorded to a local hard drive for subsequent, offline processing. The system would then delay for a minimum of 90 s before recording another video from the same camera (data capture from other cameras would proceed in parallel). This inference occurred at ∼1 Hz per camera.

Recorded videos were then analyzed offline for the presence of tagged bees using ArUco tag processing (Fig. 1E; similar to nest and forage tunnel tracking). Round 7 was excluded due to inconsistent data recording. Similarly to the foraging tunnel transits, subsequent detections of the same bee at the same flower were clustered to minimize multiple countings (with a threshold cutoff of 3 min). We determined the number of visits to each flower species per bee, as well as the total number of flower visits, after standardizing for a 24 h deployment.

Statistical analysis

We used linear mixed models (LMMs) and generalized linear mixed models (GLMMs) in R v. 4.3.0 (R Core Team 2025) to test the lethal and sublethal effects of IMD and FPF exposure on bumble bee behavior, constructed using the packages *lme4* (Bates et al. 2015) and *glmmTMB* (Brooks et al. 2017) respectively. We used the *DHARMa* package to evaluate model fits, and the *lmerTest* package to calculate *P*-values (Kuznetsova et al. 2017; Hartig 2024).

We evaluated 24 h mortality from microcolonies with any amount of behavioral recordings within the nest (control $n = 10$, IMD $n = 9$, FPF $n = 9$). The number of dead bees per microcolony was analyzed using a zero-inflated GLMM with a negative binomial distribution, treatment as a fixed effect, and round as a random effect.

To analyze spatial metrics of nest behavior, we first conducted dimensionality reduction via principal component analysis (PCA) on $\log_{10}$-transformed data (after adding 0.001 to all metrics to avoid values of negative infinity) from the five metrics calculated for each bee in each video. The first principal component (PC1, hereafter "spatial centrality") explained 67.4% of the variation, and was negatively correlated with the five measurements of distance from nest (Table S1). We then calculated the means of spatial centrality (PC1) and the two movement metrics (activity and speed) for each individual bee across videos, separated by daytime and nighttime behavior. We also calculated the interquartile range (IQR) of activity, as we wanted to capture the effects on intra-individual variability. Bees with either consistently high activity or consistently low activity would have low activity IQR, while those that vary between high and low activity would show high activity IQR. Increased variability compared to control could indicate erratic behavior, while decreased variability could indicate lower behavioral flexibility or disruption of a cyclical pattern (Stamps et al. 2012).

We assessed the effects of insecticide and time of day on these four metrics of nest behavior using treatment and time of day as interacting fixed effects, and unique bee nested within microcolony, in turn nested within round, as random effects: mean spatial centrality, mean activity, mean speed, and activity IQR. We analyzed mean spatial centrality using an LMM, mean activity using a GLMM with beta family, and mean speed using a log-linear model. Activity IQR was only analyzed for bees observed in at least 25 videos (to ensure there was enough variability to be captured), using a GLMM with beta family. The effects of treatment were assessed by *post hoc* comparisons made using estimated marginal means with the *emmeans* package, separated by time of day, and the *P*-values were adjusted with the "Tukey" method (Lenth 2025).

We tested the effects of insecticide treatment on the number of foraging transits undertaken by each bee in a microcolony (including "zeros" for non-foraging bees that were detected as alive within the nest) using a zero-inflated GLMM with a negative binomial distribution, treatment as fixed effect, and microcolony nested within round as random effects.

Finally, to assess the effects of insecticides on flower visitation, we employed log-linear models with treatment as a fixed effect and microcolony nested within round as random effects. We first tested the effects on the total number of flower visits per bee. Then we tested the effects on flower visits per bee for each plant species by adding species as an interacting fixed effect, and conducted *post hoc* comparisons using the *emmeans* package, adjusting the *P*-values with the "Tukey" method (Lenth 2025).

We report our results in the language of evidence framework rather than statistical significance testing (Muff et al. 2022). Alongside test statistics and *P*-

values, we state if the data indicates little or no evidence ($P > 0.1$), weak evidence ($0.1 > P > 0.05$), moderate evidence ($0.05 > P > 0.01$), strong evidence ($0.01 > P > 0.001$), or very strong evidence ($P < 0.001$).

Results

Mortality

We found strong evidence for FPF and moderate evidence for IMD that insecticide treatment increased bee mortality risk compared to control after 1 day of insecticide exposure in lab conditions (FPF: $z = 2.77$, $P = 0.0056$; IMD: $z = 2.505$, $P = 0.0123$; Fig. S5, Table S2).

Nest behavior

Overall, we tracked 3.32 million tag locations from $n = 369$ unique workers (109 bees from 6 control microcolonies, 150 bees from 9 FPF microcolonies, and 110 bees from 7 IMD microcolonies) after data filtering and cleaning. We found moderate evidence that spatial centrality increased under FPF treatment, i.e., the bees were closer to the nest center, and no evidence for an IMD effect (FPF: $t = 2.254$, $P = 0.045$; IMD: $t = -0.675$, $P = 0.513$; Table S3). *Post hoc* comparisons (Fig. 2A; Table S4) revealed moderate evidence that spatial centrality was higher for FPF bees than IMD bees during day time (t ratio $= 3.194$, $P = 0.019$), while at night time, there was weak evidence that FPF bees were more spatially centered than control (t ratio $= -2.372$, $P = 0.08$) and strong evidence that they were more spatially centered than IMD (t ratio $= 3.938$, $P = 0.0049$).

For movement metrics, first there was very strong evidence that mean activity was lower for IMD bees than control bees or FPF bees both at day time and night time, and strong evidence that the difference was starker at night time (IMD effect: $z = -6.064$, $P < 0.0001$; time of day effect: $z = -7.216$, $P < 0.0001$; IMD $*$ night interaction effect: $z = -3.147$, $P = 0.0016$; Fig. 2B,Tables S5 and S6). Next, we found strong evidence that FPF decreased activity IQR throughout the trial, and very strong evidence that IMD decreased it only at night time (FPF effect: $z = -2.857$, $P = 0.0043$; time of day effect: $z = 1.927$, $P = 0.054$; IMD $*$ night interaction effect: $z = -8.65$, $P < 0.0001$; Fig. 2C, Tables S7 and S8). Finally, we found no evidence that when active, FPF or IMD bees overall had higher mean speeds than control bees, but there was very strong evidence for an effect of time of day and its interaction with IMD treatment (time of day effect: $t = -5.428$, $P < 0.0001$; IMD $*$ night interaction effect: $t = -3.773$, $P = 0.0002$; Table S9). *Post hoc* comparisons indicate moderate evidence that speed was higher for FPF bees than IMD bees during night time (t ratio $= 3.175$, $P = 0.016$; Fig. 2D, Table S10).

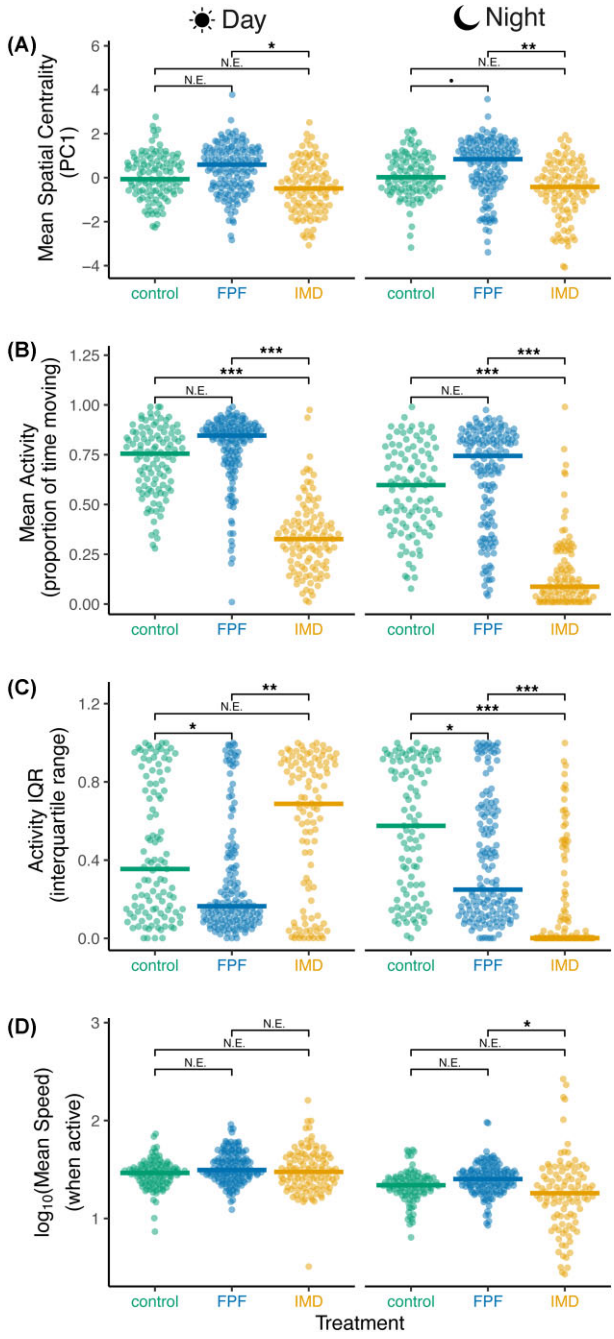

Fig. 2 Day and night nest behavior metrics for bees under different insecticide treatments: (A) mean spatial centrality (PC1; Tables S3 and S4), (B) mean activity (Tables S5 and S6), (C) activity IQR (Tables S7 and S8), and (D) mean speed (Tables S9 and S10). Horizontal lines represent medians, while circles represent individual bees. Asterisks denote level of evidence for difference between the treatments (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, • $P < 0.1$, N.E. [no evidence] $= P > 0.1$).

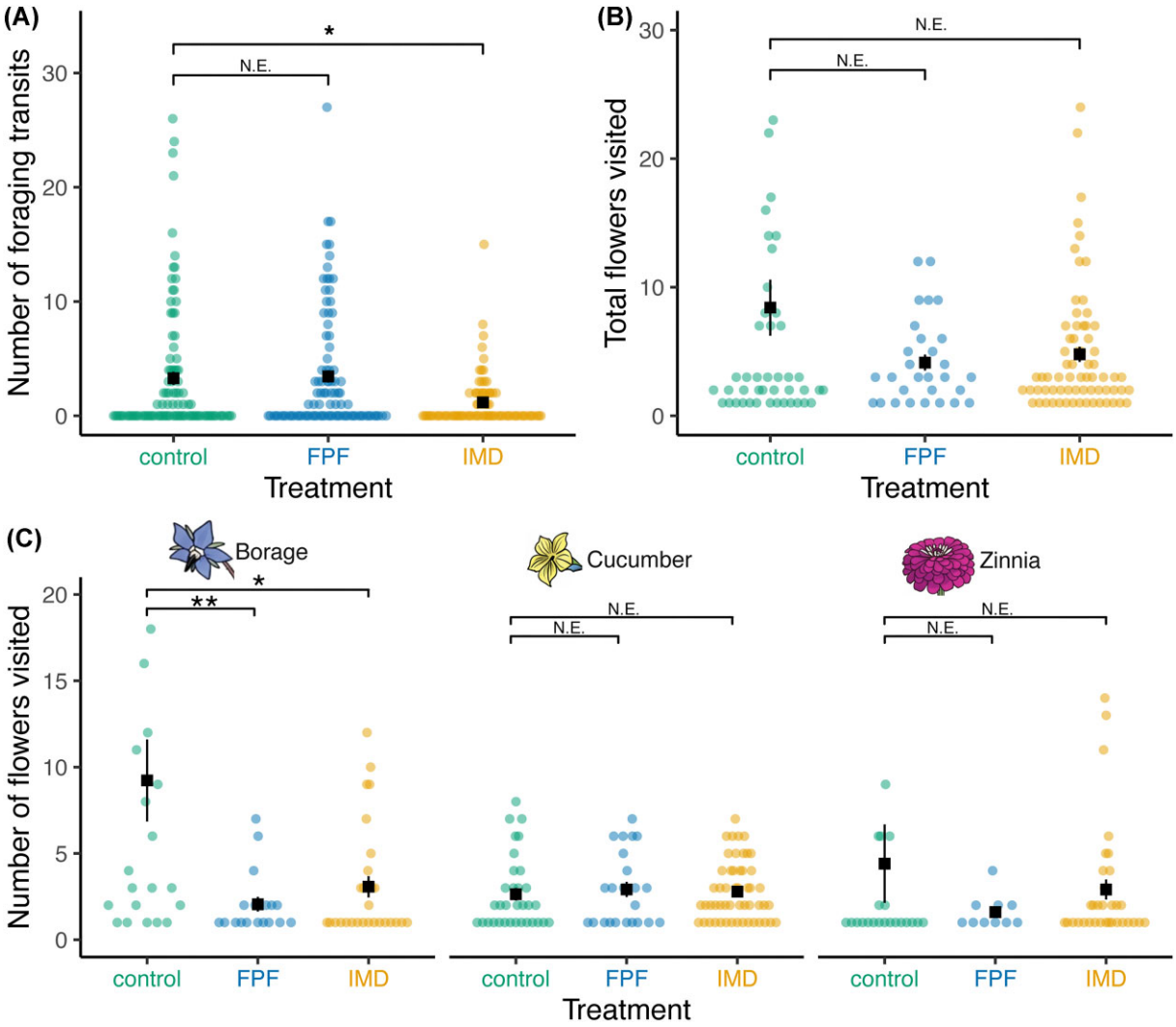

Fig. 3 Foraging behavior metrics for bees under different insecticide treatments: (A) daily foraging transits (Table S11), (B) total flower visitation (Table S12), and (C) flower visitation to different plant species (Tables S13 and S14). Black squares and bars represent means and standard errors, while colored circles represent individual bees. Asterisks denote level of evidence for difference between the treatments (** $P < 0.01$, * $P < 0.05$, N.E. [no evidence] = $P > 0.1$).

Foraging behavior

We analyzed the number of foraging transits undertaken by $n = 275$ bees in 24 h from 17 microcolonies: 100 bees from 7 control microcolonies, 96 bees from 7 FPF microcolonies, and 79 bees from 7 IMD microcolonies. We found moderate evidence that bees under IMD exposure undertook fewer transits through the forage tunnel, and no evidence for an FPF effect on this metric (FPF: $z = 0.614$, $P = 0.5395$; IMD: $z = -1.99$, $P = 0.0462$; Fig. 3A and Table S11).

144 bees from 24 microcolonies were detected visiting flowers (eight microcolonies from each treatment). There was no evidence that bees from different insecticide treatments differed in the total number of flowers visited (FPF: $z = -0.509$, $P = 0.611$; IMD: $z = -2.265$, $P = 0.791$; Fig. 3B and Table S12). However, looking at plant species-specific visitation, we found complex effects (Table S13). *Post hoc* comparisons revealed moderate and strong evidence, respectively for IMD and FPF that treated bees had fewer detections on borage flowers compared to control (FPF: t ratio = 3.55, $P = 0.0014$; IMD: t ratio = 2.933, $P = 0.0104$), but no evidence for any change in detections on cucumber or zinnia flowers (Fig. 3C, Table S14).

Discussion

In a semi-field experiment investigating the effects of two insecticides (the neonicotinoid imidacloprid, and the butenolide flupyradifurone) on multiple aspects of bumble bee behavior and health, we found that

both FPF and IMD had widespread effects on behavior within the nest as well as during foraging, however these sublethal effects differed strongly between pesticide treatments. IMD suppressed locomotor activity within the nest and foraging, whereas FPF impacted spatial centrality within the nest and variability in locomotor activity. Both compounds altered floral preference, specifically reducing visitation to borage. In addition, we found that both IMD and FPF increased mortality risk after only 1 day of exposure when administered at field-realistic concentrations (Fig. S5).

Both insecticide treatments had significant effects on multiple behavioral metrics within nests, particularly spatial distribution and activity, and these effects were more pronounced at night. Critical within-nest behaviors include thermoregulation, nest structure building and maintenance, feeding, incubating the brood, etc., and previous work has linked disruptions in nest behavior (including spatial centrality and activity) to impaired colony development (Easton-Calabria et al. 2023). We hypothesized that the effects of FPF would be qualitatively similar to IMD, given that the two insecticides target the same neuroreceptors. Instead, we found divergent impacts between the two compounds, consistent with previous findings that insecticides may differ in their effects on behavior (Siviter and Muth 2020). IMD markedly reduced the proportion of time bumble bees spent moving. In contrast, FPF increased spatial centrality and reduced variability in activity, suggesting a possible disruption of the appropriate regulation of locomotor activity cycles. This could contribute to impaired brood development observed in the literature (Fischer et al. 2023; Richardson et al. 2024), but potentially *via* a different behavioral mechanism than IMD. However, the same compounds can also have differential effects on behaviors at different concentrations (i.e., "hormesis": Cutler et al. 2022); future work exploring the impacts of different compounds across a range of concentrations will be key to disentangling the effects of concentration vs. compound on sublethal behavioral effects.

The number of foraging transits undertaken by each bee was significantly reduced under IMD but not under FPF. These effects of IMD are generally consistent with findings that neonicotinoids can impair foraging and pollination services (Gill and Raine 2014; Stanley et al. 2015). Reduced foraging performance could result in nutritional stress and negatively impact colony health (Feltham et al. 2014; Carnell et al. 2020). The lack of effect of FPF on foraging activity is consistent with a lack of effect on in-nest activity levels and suggests that FPF may not substantially impact locomotion at this concentration (500 ppb). An important consideration is that the provision of *ad libitum* nectar and pollen to microcolonies within the nest, and the relatively low number of plants available for foraging in the hoophouse, may have contributed to low baseline foraging motivation and number of foraging transits, and thus masked some effects, in addition to the relatively brief (24 h) foraging period.

In a multi-species flowering plant assemblage, neither IMD nor FPF affected the total number of flowers visited by bees, but both shifted floral preference, specifically reducing visitation to borage. This pattern may be attributable to the morphological complexity of borage flowers, as like many pendant flowers, they require bees to adopt an inverted posture while hanging from the flower to access nectar (Fig. 1E). A shift in floral preference has been observed in other studies of neonicotinoid effects on bumble bee behavior (Gill and Raine 2014; Stanley and Raine 2016). This dietary change could lead to suboptimal nutrition and imperiled colony health (Vaudo et al. 2015), as well as adverse outcomes for plant reproduction. Floral species and crops with higher morphological complexity could see stronger reductions in visitation, pollen transfer, and seed set when pollinators are exposed to insecticides (e.g., buzz-pollinated species with poricidal anthers: Switzer and Combes 2016; Whitehorn et al. 2017).

By advancing our knowledge of the sublethal impacts of two insecticides on bumble bee behavior, this work could directly inform the development of sustainable agricultural practices and conservation policies (e.g., for the critically endangered species *Bombus affinis:*Hatfield et al. 2015). Our findings also underscore the importance (and potential) of quantifying behavior across multiple contexts (e.g., foraging and within-nest behavior), given that disruption of any of these critical behaviors could lead to impaired colony growth and fitness. Furthermore, conducting these experiments in complex, naturalistic settings, such as multi-species plant assemblages, is essential for capturing nuances that can reveal species-specific impacts (e.g., reduction in borage visitation).

Despite extensive evidence from several pesticide formulations linking sublethal physiological and behavioral impacts to long-term colony and population declines, pesticide risk assessment and regulation practices typically only require testing for short-term lethal toxicity, leading to an unfortunate cycle of novel "sustainable" pesticides later determined to be hazardous (Fisher et al. 2023). Moreover, evaluations for new approvals currently only involve a limited number of species (e.g., *Apis mellifera*) even though bee species vary in pesticide sensitivity (Arena and Sgolastra 2014; Hayward et al. 2019; Kline et al. 2025). Numerous calls have emerged for holistic risk assessment that in-

corporates both sublethal impacts and non-*Apis* bees (Thompson 2003; Franklin and Raine 2019; Sgolastra et al. 2020), which are increasingly being integrated into regulatory guidelines and frameworks (EFSA et al. 2023). However, existing methods for systematically profiling behavioral effects remain limited (Franke et al. 2021; Klein et al. 2022). Our study highlights the potential of using an ethomics approach to address this knowledge and regulatory gap.

In our experiment, computer vision tools enabled the detection of insecticide effects on the variability of locomotor activity, and not just a central tendency. Indeed, both intra- and inter-individual variability are important but understudied metrics that could elucidate vulnerabilities as well as potential for resilience in response to stress (Cabirol et al. 2023). Further, we observed within-nest behavioral effects varying substantially by time of day, which confirms results from prior studies that also used automated behavioral quantification to assess circadian patterns (Crall et al. 2018; Tasman et al. 2020). Such automated approaches could be an asset for higher tier (semi-field and field) studies in risk assessment frameworks. The BumbleBox system used here, for example, can also be used for larger (e.g., queenright) colonies with complex nest structures, and field-deployed for extended time periods (Easton-Calabria and Crall 2025). A similar ethomics framework could be further applied for studying an even larger suite of behaviors not captured in our study. For example, computer vision can facilitate learning and memory assays, and radiotelemetry can elucidate pollinator movement in open landscapes (Hagen et al. 2011; Paus-Knudsen et al. 2023). Finally, data from such studies could be used to inform models predicting colony and population health outcomes (Becher et al. 2018; EFSA et al. 2025).

Overall, our study underscores the complex behavioral responses of bumble bees to pesticide exposure in realistic environments. Moreover, it offers a robust framework for high-throughput, multifaceted analysis of pollinator behavior under various stressors, which is particularly valuable for ecotoxicological assessments, and can further inform management recommendations and conservation policy.

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Supplementary data

Supplementary Data available at *ICB* online.

Conflict of interest

The authors have no conflicts of interest to declare.

Data availability

The data and code used in this work are publicly available at the GitHub repository https://github.com/Crall-Lab/ICB2025_ethotoxicology. Code includes files for recording, tracking, and analyzing behavior. The MobileNetV2 flower visit detection model is available upon request.

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