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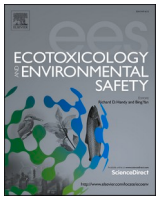

Integrative assessment of sulfoxaflor effects on gene expression, reproduction, and behavior in the bumblebee *Bombus impatiens*

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ABSTRACT

Social insect pollinators, such as bumblebees, face increasing threats from environmental agrochemicals; yet the sublethal effects of these compounds across different levels of biological organization remain poorly understood. This study uses an integrative approach to examine how chronic exposure to sublethal concentrations of the insecticide sulfoxaflor affects the common eastern bumblebee (*Bombus impatiens*). Microcolonies of *B. impatiens* worker bees were fed sulfoxaflor-treated sugar water for 21 days. We then assessed changes in molecular, physiological, and behavioral traits resulting from sulfoxaflor exposure. Transcriptomic analysis revealed extensive differential gene expression in ovaries, but not brains, of exposed bees. Bees exposed to sulfoxaflor showed upregulated cellular signaling pathways and downregulated genes associated with oogenesis and mitosis. Moreover, sulfoxaflor-exposed bees showed reduced tissue-biased gene expression, suggesting broader disruption in tissue-specific regulation. At the physiological level, exposed workers exhibited disrupted ovarian development and produced significantly fewer eggs. In addition, sulfoxaflor-exposed bees displayed significantly increased stinging behavior and decreased leg lifting behavior. Finally, exposed microcolonies exhibited reduced sugar water consumption and impaired nest building. Overall, these results indicate that reproductive tissues are more sensitive to sulfoxaflor exposure than neural tissues and that molecular disruptions manifest in impaired physiology and colony-level behaviors. This study highlights the value of assessing multiple levels of biological organization when investigating the nonlethal yet ecologically significant effects of agrochemicals on pollinator health.

1. Introduction

Global food supply relies on agricultural practices that require agrochemicals to manage pest insects. However, these chemicals often pose significant risks to beneficial non-target organisms, such as pollinating bees (Potts et al., 2016). The widespread use of neonicotinoid insecticides, in particular, has raised considerable concern due to their detrimental effects on pollinator populations. In fact, many neonicotinoid insecticides have been restricted or banned in various regions, including the European Union (Dirilgen et al., 2023). This has led to the development and use of alternative insecticides.

Sulfoxaflor (hereafter, SFX) was approved for use in the United States in 2013 (Watson et al., 2021). SFX has emerged as a prominent replacement insecticide for neonicotinoids. Marketed under names such as Isoclast, Transform, and Closer, SFX belongs to the sulfoximine class of insecticides. Like neonicotinoids, SFX targets insect nicotinic acetylcholine receptors (nAChRs) but is structurally distinct and may have different effects on insect physiology, behavior, and viability (Sparks et al., 2013). SFX has gained popularity due to its effectiveness against pest insects, including those resistant to neonicotinoids, and its lower toxicity to non-target pollinators compared to some traditional insecticides (Mundy-Heisz et al., 2022; Orr et al., 2025; Watson et al., 2021).

Despite the apparent advantages of SFX, emerging research has raised concerns about its ecological impact, particularly on pollinating bees. Studies have documented negative effects of SFX on survival,

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reproduction, and pollination efficiency in various bee species, including honeybees, stingless bees, and bumblebees (Boff et al., 2022; Capela et al., 2022; El-Din et al., 2022; Linguadoca et al., 2021; Siviter et al., 2018; Siviter and Muth, 2020). As such, the European Union banned SFX from outdoor use in 2022. However, SFX is still widely used in other parts of the world, including the United States. Further, recent work has demonstrated that bumblebees have a surprising preference for SFX-contaminated foods (Orr et al., 2025). Of particular concern is the observation that native bumblebees may be more sensitive to SFX than managed honeybees (Gradish et al., 2019; Mundy-Heisz et al., 2022). This differential sensitivity underscores the need for comprehensive research on the effects of SFX on diverse pollinator species.

The common eastern bumblebee, *Bombus impatiens* , represents an important organism for investigating the impacts of agrochemicals on social insect pollinators. *B. impatiens* is native to North America and also plays a vital role in the pollination of numerous plants, including those requiring buzz-pollination, a technique that honeybees cannot perform. This pollination method is crucial for over 20,000 plant species, such as tomatoes, eggplants, and blueberries (Pritchard and Vallejo-Marín, 2020).

The social structure of *B. impatiens*, characterized by a distinct caste system and division of labor within colonies, provides a complex framework for studying the potential cascading effects of insecticide exposure on pollinator communities (Goulson et al., 2008; Potts et al., 2016). New *B. impatiens* colonies are founded by a single queen in the spring. The queen produces workers that take over the tasks of colony maintenance and foraging. The colony grows to a size of several hundred workers through the summer and starts to produce new queens and males in late summer. These new queens and males will go on to mate, then queens hibernate, and found new colonies the following year.

The annual life cycle of *B. impatiens* colonies, led by a queen and supported by hundreds of mostly non-reproductive workers, offers opportunities to examine both individual and colony-level impacts of SFX exposure. Notably, workers will make a physiological shift in the absence of a queen and begin to lay haploid eggs that develop into male bees. Thus, microcolonies composed of workers can be used to investigate reproductive outcomes of insecticide exposure (Lehmann, 2022). Measuring reproductive traits in workers therefore provides a meaningful proxy for evaluating how chronic insecticide exposure may affect colony function. Overall, given the ecological importance of this bee species and the growing body of evidence suggesting potential harm from SFX exposure, there is an urgent need for targeted research investigating how insecticides affect *B. impatiens*.

In this study, we assessed the effects of chronic sublethal SFX exposure in worker microcolonies of *B. impatiens* at multiple levels of biological organization. This study had four aims. First, (1) we aimed to determine whether chronic SFX would cause differential patterns of gene expression in worker bees. We expected that chemical exposure would cause physiological changes that would lead to variation in gene expression, but such patterns would differ among tissues. Second, (2) we investigated how SFX affected the reproductive physiology of worker bees. We expected that SFX would impede ovary development and reduce egg-laying. Third, (3) we investigated how SFX affected behaviors of worker bees. We hypothesized that locomotory behaviors and disturbance responses would be impaired. Finally, (4) we investigated how SFX affected collective, nest-building behaviors. We specifically investigated nest construction activities of workers and predicted that chronic SFX exposure would impair nest construction.

Overall, this study takes a comprehensive approach to studying insecticide exposure in pollinators. We integrate behavioral, physiological, and molecular analyses of individuals and groups to determine how insecticides affect function at different levels of biological organization. By elucidating these impacts, we seek to contribute valuable insights to the ongoing discourse on insecticide regulation and pollinator conservation, ultimately informing more sustainable agricultural practices that balance pest management with the protection of essential pollinator species.

2. Materials and methods

2.1. Bumblebee husbandry

We acquired 8 queenright *Bombus impatiens* colonies commercially from Plant Products (Leamington, ON, Canada). Each colony was provided with unlimited 50% sugar syrup through a reservoir with a wick and pollen balls, which were replenished weekly. Colonies were kept in a dark, warm room at 25.4°C ± 0.01 (mean ± SEM) and Relative Humidity = 31.5% ± 0.2. We used a LogTag recorder (Aukland, New Zealand) to measure temperature and relative humidity at 30-minute intervals. All bee husbandry and bee manipulation were conducted under red light.

2.2. Insecticide chemistry

We created a stock solution of pure sulfoxaflor (ThermoFisher, Waltham, MA, USA) in deionized water. Spiked sugar syrup was made by performing a serial dilution of the stock solution into fresh 50% sugar water. We mailed all samples, including the control (0 mg/L), to AGQ Labs (Oxnard, CA, USA) for concentration verification. AGQ analyzed the samples using a validated multi-residue pesticide method (PE-674), which incorporates both LC-MS/MS and GC-MS/MS based on compound-specific properties. Quality assurance procedures included spike recover tests, calibration curve validation ($R^2 > 0.99$), blanks (method, matrix, and solvent), and periodic instrument checks (CCV, ICV) to ensure analytical accuracy and instrument stability. All quality control criteria were met. We used the measured concentrations (0, 0.16, and 0.31 mg/L SFX) in all subsequent analyses. The selected concentrations (0.16 and 0.31 mg/L) were chosen based on prior toxicity studies to represent sublethal exposures that are sufficiently high to reveal mechanistic effects while avoiding substantial mortality. Although these concentrations exceed most estimated field exposures, they enable detection of molecular, physiological, and behavioral responses across levels of biological organization and allow assessment of dose-dependent effects. This approach facilitates the identification of potential mechanisms of toxicity and sublethal effects, which can provide important insights for pollinator risk assessment.

2.3. SFX chronic exposures

We used replicated microcolony exposures to assess the chronic toxicity of SFX. We created 51 small experimental cages (15 ×15 x 5 cm) made from acrylic with ventilation holes. Each cage was prepared with clean sugar syrup in a 15 mL feeding tube and a fresh pollen ball. Twenty-seven cages were seeded with 3 callow (i.e., freshly emerged, <24 h old) workers from various origin colonies (Experiment A). Twenty-four cages were seeded with 10 sister workers of various ages from the same origin colony (Experiment B). We used two complementary experimental designs to balance experimental control with ecological relevance. Experiment A employed callow workers to minimize developmental and physiological variability for transcriptomic analyses. In contrast, Experiment B included mixed-age workers to generate microcolonies with sufficient social structure for disturbance assays. This design allowed us to examine molecular responses under controlled conditions while evaluating behavioral outcomes in a more natural social context. Overall, we sourced experimental bees from 8 queenright colonies and used a total of 297 worker bees.

Microcolonies were divided into three treatment groups of 17 colonies. Treatment groups were exposed to 0 (control), 0.16 (low), or 0.31 (high) mg/L SFX, to achieve a sublethal dose based on previous toxicity tests (Orr et al., 2025). Bees were exposed to SFX through spiked sugar syrup. In addition, bees were fed clean pollen *ad libitum*. The chronic exposure period lasted 21 days. Any mortalities were noted and

removed promptly but not replaced. Any microcolonies with no surviving workers were excluded from the analysis. Fresh pollen was replenished every 2–3 days. Sugar syrup was replaced twice weekly and weighed before and after to obtain information on consumption. After 21 days, bees from Experiment A were used in the crawling behavior assays and bees from Experiment B were used in the disturbance behavioral assays, as described below. Then, all individuals were flash frozen in liquid nitrogen for further analyses.

2.4. Tissue dissections, RNA extraction, and sequencing

We used all surviving bees from Experiment A for gene expression analyses. Brain and ovary tissues were dissected under an Olympus SZX16 microscope, preserved in sterile tubes on ice, and stored at −80°C. RNA was extracted using the Zymo Quick RNA Kit with DNAse treatment, and RNA purity was measured with NanoDrop One. We submitted RNA from 17 brain and 21 ovary samples for library preparation and RNA-sequencing on an Illumina NextSeq2000 at the Georgia Genomics and Bioinformatics Core (GGBC, UG Athens, GA RRID: SCR_010994). Samples were sequenced in two batches using P2–200 flow cells (9 samples in the first batch; 29 samples in the second batch).

2.5. RNA quality control and read mapping

Raw sequencing reads were evaluated using FastQC v0.12.1 (Andrews, 2010) to assess quality and identify adapter contamination. Reads were trimmed with fastp v0.23.4 (Chen et al., 2018) (parameters: -l 50 –trim_poly_g –trim_poly_x –low_complexity_filter), followed by re-evaluation with FastQC, and summary reporting using MultiQC v1.19 (Ewels et al., 2016) (Table S1). Trimmed reads were aligned to the *B. impatiens* genome (GCF_000188095.3_BIMP_2.2) using HiSat2 v2.2.1 (Kim et al., 2019) and RNA-Seq by Expectation-Maximization (RSEM) v1.3.3 (Li and Dewey, 2011). A reference index was prepared with the rsem-prepare-reference function and transcript abundance was quantitated using rsem-calculate-expression function. MultiQC was used to summarize mapping statistics (Table S1). Gene-level counts were combined into a count matrix (Table S2) using rsem-generate-data-matrix and analyzed with DESeq2 v1.30.1 (Love et al., 2014). The transcript per million (TPM) report (Table S3) was also checked for quality control purposes.

2.6. Tissue Specificity

The count matrix was transformed using DESeq2's variance stabilizing transformation (VST; Table S4). The VST matrix was used to gene tissue-specificity values, tau (τ), across control, low, and high treatment groups (Table S5). Tau was calculated as $\tau = \sum(1 - x_i)/n - 1$, where x_i is the expression level of a gene in tissue i divided by the maximum expression level across all tissues, and n is the number of tissues analyzed. Tau ranges from 0 (broadly expressed across tissues) to 1 (completely tissue-specific). Pairwise correlations were computed using the corr.test() function from the R package psych v2.4.12 (Revelle, 2024). Spearman's rank correlation was chosen to account for potential non-linear relationships between variables. The statistical significance of correlations was evaluated, and p-values were adjusted for multiple comparisons using the Bonferroni method to control the false discovery rate (FDR).

2.7. Differential Gene Expression Analysis

Genes with an adjusted p-value (FDR) < 0.05, as determined by DESeq2, were considered significantly differentially expressed. Principal components were also calculated (Table S6). DESeq2 results were cross validated with edgeR v3.20 (Robinson et al., 2010) (Table S7). Volcano plots generated with EnhancedVolcano v3.20 were used to visualize differentially expressed genes (DEGs) (Blighe, 2024).

2.8. Functional enrichment analysis

Gene Ontology (GO) terms from DEGs (Table S8) were analyzed across the three top-level GO categories: Biological Processes (BP), Molecular Functions (MF), and Cellular Components (CC). GO enrichment was assessed using Fisher's exact tests in topGO ((Alexa and Rahnenfuhrer, 2025) (Table S8) and visualized in Revigo v1.8.1 (Supek et al., 2011) (Table S8). ShinyGO v0.81 (Ge et al., 2020) was used to provide interactive visualizations of enriched GO terms.

2.9. Gene Co-expression network analysis

Gene co-expression networks were constructed using weighted gene co-expression network analysis (WGCNA) v1.70 (Langfelder and Horvath, 2008) including both brain and ovary samples. Tissue identity and treatment group were included as traits in the module–trait correlation analysis, allowing identification of modules associated with tissue differences as well as treatment effects within a unified analytical framework. The variance for each gene was calculated using the rowVars() function, and genes with variance below the 75th percentile were excluded using the quantile() function. A soft-thresholding power of 16 was chosen for downstream analysis based on the scale free topology model fit signed R^2 curve (SFT.R.sq; Table S9). Modules of co-expressed genes were identified using hierarchical clustering and dynamic tree cutting (networkType = "signed", pamRespectsDendro = F, deepSplit = 2, detectCutHeight = 0.75, minModuleSize = 30, maxBlockSize = 4000, reassignThreshold = 0, mergeCutHeight = 0.01), summarized by eigengenes, the first principal component of module expression. Module-trait correlations were analyzed to identify biologically relevant modules, and hub genes were identified based on connectivity (Table S9). Networks (Table S9) were visualized using Cytoscape v3.9.1 (Shannon et al., 2003).

2.10. Reproductive Physiology

To determine how chronic SFX exposure affects reproductive physiology, we counted the number of eggs produced by each microcolony at the end of the 21-day exposures from both Experiment A and Experiment B. All data was normalized by the number of surviving worker bees in each colony. We analyzed egg-laying data using a generalized linear mixed model (GLMM) with a negative binomial distribution to account for overdispersion and zero inflation. Treatment group was included as a fixed effect, and cage nested within maternal colony was included as a random effect to account for shared genetics and cage-level variation. Because all bees in the highest sulfoxaflor (SFX) treatment group (0.31 mg/L) laid zero eggs, this treatment level could not be estimated reliably in the model due to quasi-complete separation. Therefore, we combined the low and high SFX treatment groups into a single "SFX" category to assess the overall effect of pesticide exposure. Statistical analysis was performed in RStudio v.2025.05.1 and figures were created in GraphPad Prism v.10.1.

We further dissected out ovaries for phenotypic analysis from surviving bees from Experiment B using an Olympus SZX16 dissecting microscope and Teledyne Lumenera Infinity5 camera software. Ovaries were subsequently weighed using a Mettler Toledo ABI35-S scale. To analyze how SFX affected ovary mass we ran a generalized linear mixed model with SFX treatment as a fixed effect and microcolony as a random effect in JMP v.18. Figures were created in GraphPad Prism v. 10.1.

2.11. Individual behavioral assays

To assess how chronic SFX exposure impacted individual behavior, each cage of bees was subjected to one of two distinct behavioral assays at the end of the 21-day exposure. Bees from Experiment A were used in the crawling behavioral assay and bees from Experiment B were used in the disturbance behavioral assay.

First, we investigated bee crawling, which is how bees navigate and move around the nest. We recorded the bees undisturbed in their experimental cages ($n = 9$ per treatment) under red light with an iPhone 16 Camera for 15 min. We used this video footage to train deep-learning models in Social LEAP Estimates Animal Pose (SLEAP) v1.3.3 (Pereira et al., 2022) to track each bee's head, thorax, and abdomen movements. We calculated mean crawling velocity, distance traveled, time spent moving, and head movement frequencies for each bee. Statistical analysis was performed in JMP v.18. We ran a mixed-effects model with SFX treatment as a fixed effect and microcolony as a random effect to determine if SFX treatment affected crawling behavior.

Next, we used the microcolonies from Experiment B ($n = 8$ per treatment) to perform a disturbance behavioral assay and assess how SFX might affect a worker's ability to respond to an unknown threat. Each bee from an individual microcolony was placed in a small plastic cup and allowed to acclimate for approximately 1 h at least 1 m away from all other individuals. Then, observers, blinded to each bee's exposure history, inserted a small metal probe into each cup in front of each bee's head and recorded the following behaviors: leg lifts, bite, sting, or buzz. If a bee performed leg lifting, the observers noted how many legs were lifted (1 or 2). Biting was defined as the bee opening its mandibles. Stinging was defined as the bee violently attacking the probe and attempting to sting. Buzzing was recorded if the observer could feel vibrations through the cup. Each bee went through 3 identical trials by the same observers with approximately 15-minute breaks in between trials. At the end of the behavioral trials, bees were weighed and then euthanized by rapid cooling and stored in a −80°C freezer.

Statistical analysis for disturbance behaviors was performed in RStudio v2025.05.1. We analyzed disturbance behaviors using generalized linear mixed models (GLMMs) for binary outcomes (buzz and bite) and a cumulative link mixed model (CLMM) for ordinal DLR score (0–2). Treatment was included as a fixed effect, with random intercepts for bee identity nested within microcolony and maternal colony. To account for quasi-complete separation in the sting data (0 stings in the control group), we used a bias-reduced logistic regression (Firth correction).

2.12. Collective behavioral assays

We investigated two types of group behaviors in the *B. impatiens* microcolonies from both Experiment A and Experiment B. We first investigated honey pot construction by colonies. Honey pots are built collectively by workers from pollen and wax and used to store food for the colony. To test how chronic SFX exposure impacted honey pot building behavior, we counted the number of honey pot formations at the end of the 21-day exposures in each microcolony. We analyzed honey pot counts using a generalized linear mixed model (Poisson distribution) with treatment as a fixed effect and cage as a random effect in R v2025.05.1. Where applicable, microcolonies were nested within their maternal colony of origin. Microcolonies derived from mixed-maternal colonies were grouped under a single "mixed" category to allow model convergence.

We also calculated the sugar water consumed on a weekly basis. Sugar water was consumed by all individuals within microcolonies, and so sugar water consumption represented a group behavior. All data were normalized by the number of worker bees in each colony. To analyze how SFX affected sugar water consumption, we ran a mixed-effects model with a normal distribution in JMP v.18 with SFX treatment as a fixed effect and microcolony as a random effect. Where applicable, microcolonies were nested within their maternal colony of origin. Microcolonies derived from mixed-maternal colonies were grouped under a single "mixed" category to allow model convergence. All behavioral figures were created in GraphPad Prism v10.1.

3. Results

3.1. SFX induced strong differential gene expression in ovaries but not brains of bumblebees

We calculated the tissue-specificity metric tau (τ), which measures the extent to which a gene is preferentially expressed in one tissue compared to others. Tau values range from 0, indicating uniform expression across tissues, to 1, indicating completely tissue-specific expression. Spearman's rank correlations of tau between treatment groups ranged from 0.86 to 0.90, indicating a very strong relationship across the conditions (Figure S1). However, Wilcoxon rank sum tests found that tau values in the control group were significantly higher than in bees exposed to 0.16 mg/L SFX ($W = 321{,}312{,}046$, $p < 2.2e\text{-}16$) and 0.31 mg/L SFX ($W = 348{,}832{,}621$, $p < 2.2e\text{-}16$). Additionally, tau values in bees exposed to 0.16 mg/L were significantly higher than those in bees exposed to 0.31 mg/L ($W = 325{,}719{,}921$, $p < 2.2e\text{-}16$). Thus, genes showed substantial changes in their patterns of expression in the SFX treatments.

Differential expression analysis revealed that SFX exposure induced more transcriptional changes in ovaries compared to brains (Fig. 1). Differential expression in both tissues was most pronounced at the highest concentration of SFX tested (0.31 mg/L). However, transcriptional changes were also observed at the lower SFX concentration (0.16 mg/L).

Gene Ontology (GO) enrichment analysis revealed differentially expressed genes in ovary tissues displaying distinct biological processes related to SFX exposure (0.16 mg/L and 0.31 mg/L) in relation to the control conditions (Table S10). The highest impacts were observed at 0.31 mg/L, consistent with the dose-dependent nature of the exposure. There was no signal of GO term enrichment for biological processes in brain tissues under similar conditions.

3.2. SFX induced gene expression is driven by a few highly connected hub genes

Co-expression network analysis using WGCNA identified gene modules with strong associations to tissue type and experimental conditions (Fig. 2A). The module-trait relationship heatmap revealed that several modules, including the MEyellow, MEgreen, and MEred modules, were strongly associated with ovary tissue (Fig. 2B). Genes within these modules were enriched for reproductive and metabolic processes. Alternatively, we found that the turquoise module was correlated with brain gene expression and enriched in cellular messaging pathways.

SFX exposure also revealed unique associations of gene modules through co-expression network analysis, suggesting molecular pathways affected by SFX toxicity. In control bee tissues, expression of hub genes (those with the highest connectivity within a module) was primarily associated with G kinase-anchoring protein in module MEyellow. In contrast, tissues from SFX-exposed bees were associated with the expression of different hub genes, including calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase in module MEturquoise and DE-cadherin in module MEred (Fig. 2C). Thus, exposure to SFX changed the hub genes involved in regulating gene expression and altered the transcriptional networks operating in the bees.

3.3. Bumblebee reproduction and ovary development are impeded by SFX

After accounting for cage variability, results showed that chronic exposure to SFX significantly reduced the number of eggs laid per bee ($\beta = -1.97 \pm 0.65$ SE, $p = 0.0026$) (Fig. 3). Control microcolonies laid on average 2.43x more eggs than 0.16 mg/L microcolonies. Notably, none of the 0.31 mg/L microcolonies laid a single egg.

Further, we found that worker bees exposed to 0.31 mg/L SFX had significantly smaller ovaries by mass ($F = 4.4291$, $p = 0.0296$, $n = 81$) and appeared visibly underdeveloped (Fig. 3). On average, bees had an

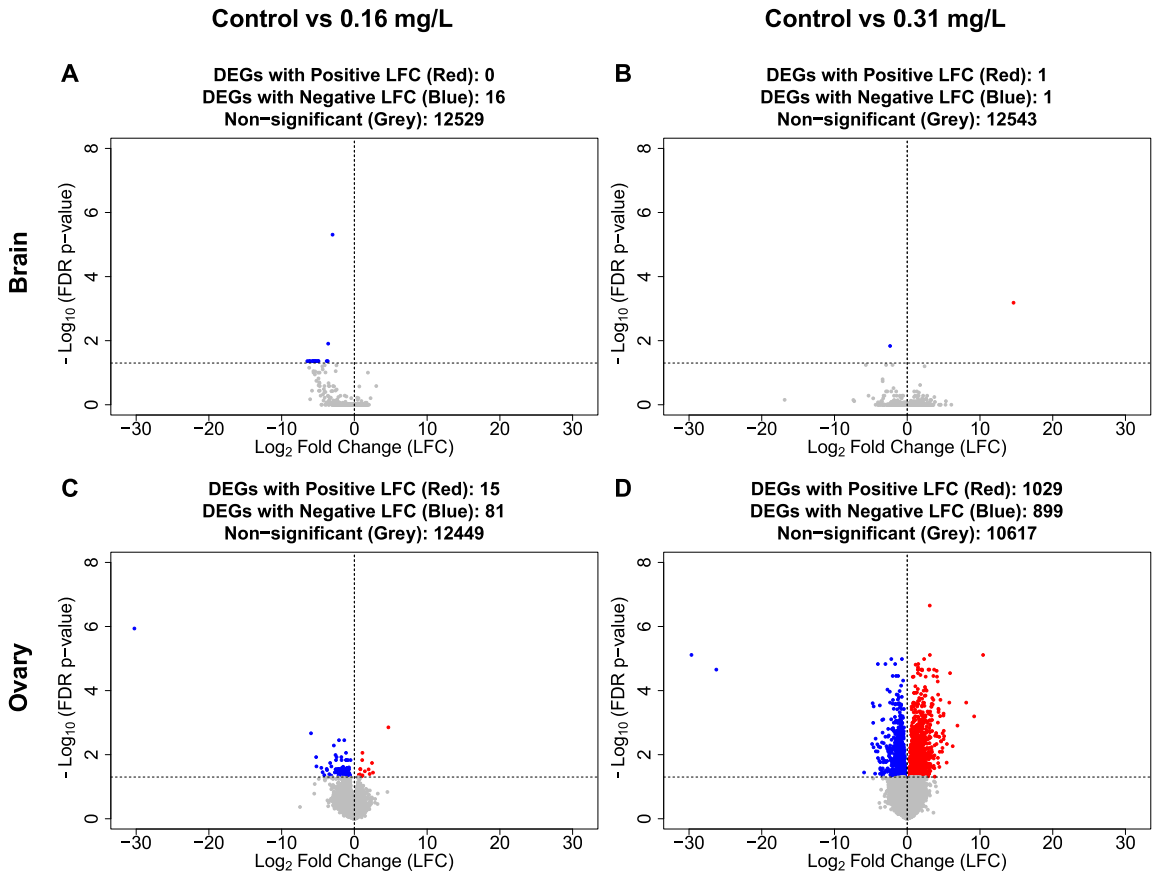


Fig. 1. Differential gene expression in brain and ovary across different sulfoxaflor (SFX) exposure levels. Differential expression between (A) control vs 0.16 mg/L SFX and (B) control vs 0.31 mg/L SFX in brain tissue. Differential expression between (C) control vs 0.16 mg/L SFX and (D) control vs 0.31 mg/L SFX in ovary tissue. SFX induced differential gene expression primarily in ovary tissue. Volcano plots highlight differentially expressed genes (DEG; false discovery rate (FDR) adjusted p-values < 0.05) with a positive $\log_2$ fold change (LFC; red), a negative LFC (blue), or non-significant (grey). Brain RNA-seq: $n = 17$ samples; ovary RNA-seq: $n = 21$ samples. Control RNA-seq: $n = 14$; 0.16 mg/L RNA-seq: $n = 13$; 0.31 mg/L RNA-seq: $n = 11$.

ovary mass of 13.81 ± 1.5, 14.20 ± 1.5, and 8.19 ± 1.2 (mean ± SEM) for microcolonies in the control group and those treated with 0.16, and 0.31 mg/L SFX, respectively.

Workers in microcolonies experienced some mortality in both the age-controlled, callow groups of 3 bees (Experiment A, 27%), and the non-age-controlled groups of 10 bees (Experiment B, 55%). Importantly, the mortality rates were not different among treatment groups, including the control group receiving no SFX, in either microcolonies of 10 ($F = 0.98$, $p = 0.2918$, $n = 8$) or microcolonies of 3 ($F = 0.82$, $p = 0.45$, $n = 9$). Thus, mortality in these experiments is likely explained by natural or age-related effects and not SFX treatment indicating that SFX exposure was sublethal, as expected. There were no significant differences between experimental setups (Experiment A vs Experiment B) on number of eggs produced per bee.

3.4. Individual bumblebee behavior is not strongly influenced by SFX

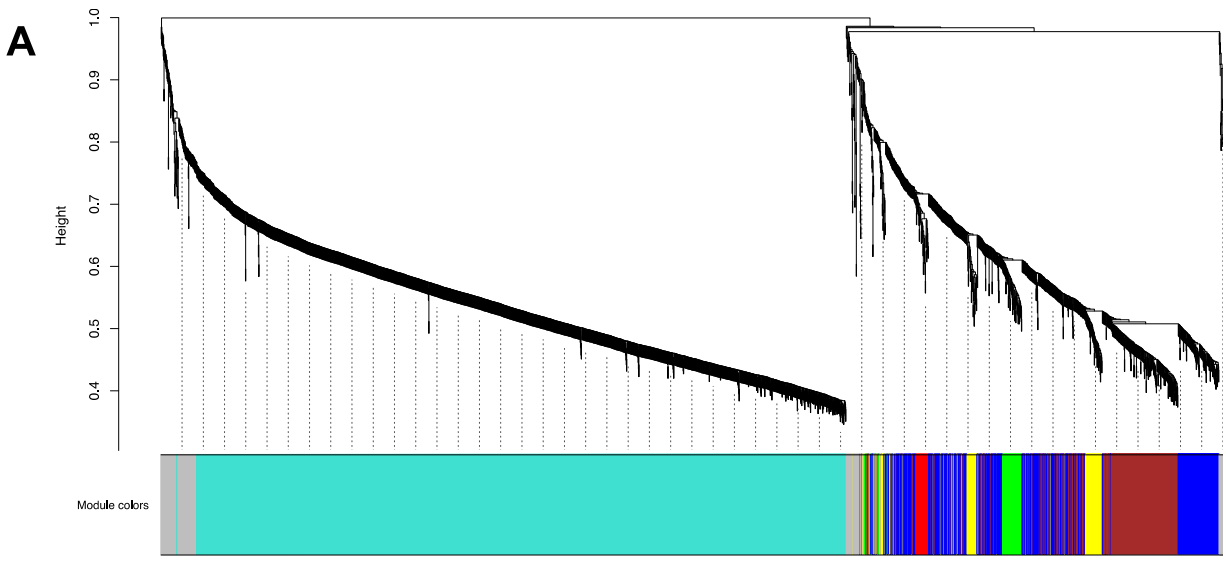
We found that chronic SFX exposure did not affect crawling velocity ($F = 0.4855$, $p = 0.6222$, $n = 54$), time spent moving ($F = 1.4408$, $p = 0.2620$, $n = 53$), distance traveled ($F = 0.4477$, $p = 0.6451$, $n = 54$), or head movement frequency ($F = 0.2010$, $p = 0.8195$, $n = 60$) at any concentration from Experiment A (Figure S2). Bees behaved similarly across treatment groups.

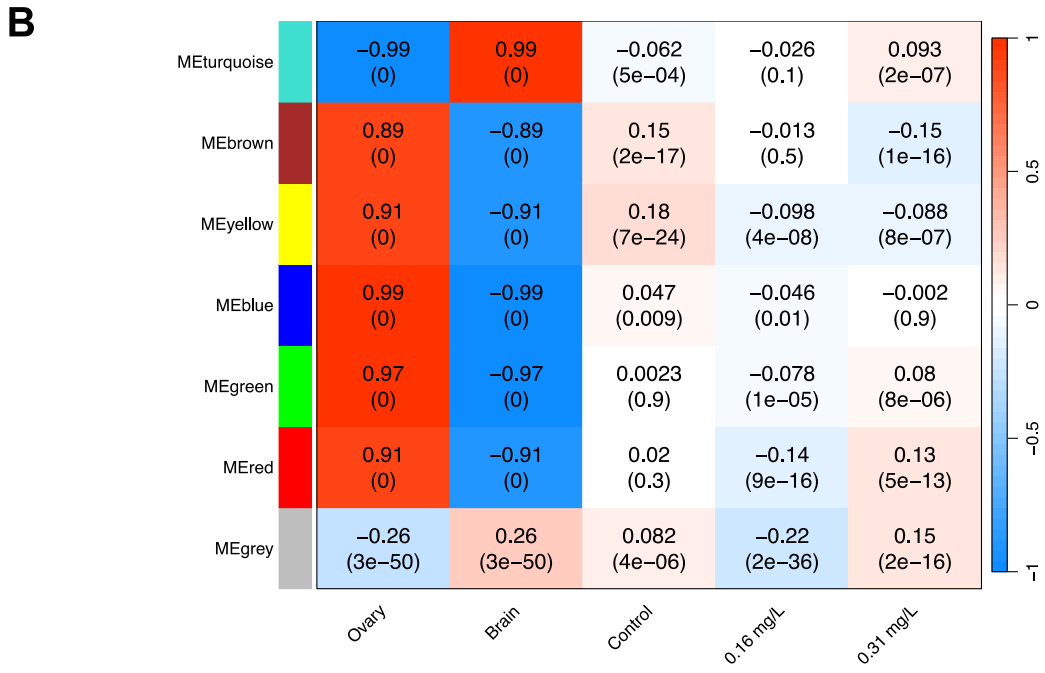
In contrast, we found that 0.16 mg/L SFX exposure significantly reduced leg-lifting (DLR) behavior ($\beta = -0.84 \pm 0.32$ SE, $p = 0.0098$), suggesting a dampened disturbance response (Fig. 4). No significant treatment effects were observed for buzzing ($z = -1.78$, $p = 0.076$) or biting ($z = 0.006$, $p = 0.995$) in response to the probe. However, bees exposed to 0.31 mg/L sulfoxaflor were significantly more likely to exhibit stinging behavior compared to controls ($\beta = 3.54 \pm 1.46$ SE, $p = 0.015$), corresponding to a ~34-fold increase in odds. Bees exposed to 0.16 mg/L SFX also showed an elevated sting response ($\beta = 2.45 \pm 1.48$ SE, $p = 0.097$), though this effect was marginal. These results suggest a dose-dependent increase in stinging behavior following developmental SFX exposure. Overall, this examination of the effects of SFX on behaviors showed that some behaviors, but not all, were affected by treatment.

3.5. Collective nest-building behavior and food consumption is disturbed by SFX

The data show that chronic exposure to SFX significantly reduced the number of honey pots built by microcolonies of workers ($F = 13.1084$, $p < 0.0001$, $n = 49$) (Fig. 5). On average, control microcolonies built 2.24x and 17.19x more honey pots per bee than 0.16 and 0.31 mg/L SFX colonies, respectively.

Further, we found that collective feeding behaviors were affected by SFX. Both concentrations of SFX significantly reduced the amount of sugar water consumed in microcolonies ($F = 13.2852$, $p < 0.0001$, $n = 49$) (Fig. 5). Control microcolonies consumed 1.61x and 2.44x more sugar water than 0.16 and 0.31 mg/L SFX colonies, respectively. Notably, there were no significant differences between experimental setups (Experiment A vs Experiment B) on honey pot production or percent sugar water consumed per bee.





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Module	SeqName	Description
MEturquoise	LOC100743073	calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1
MEbrown	LOC100741465	uncharacterized protein
MEyellow	LOC100743297	G kinase-anchoring protein 1
MEblue	LOC100740735	uncharacterized protein
MEgreen	LOC100745242	probable G-protein coupled receptor Mth-like 1
MEred	LOC100741934	DE-cadherin

Fig. 2. Co-expression network analysis across tissues and sulfoxaflor (SFX) exposure levels. (A) Cluster dendrogram showing gene modules identified through hierarchical clustering, with modules color-coded by similarity. (B) Module-trait heatmap displaying correlations between gene modules and tissue type (ovary or brain) and exposure levels (control, 0.16 mg/L, or 0.31 mg/L), with correlation values and p-values indicated in each cell. (C) List of hub genes, which are the most highly connected genes within each module and are expected to drive the expression of other genes in the network. Co-expression analyses were conducted using the RNA-seq dataset (brain: $n = 17$; ovary: $n = 21$; control: $n = 14$; 0.16 mg/L: $n = 13$; 0.31 mg/L: $n = 11$).

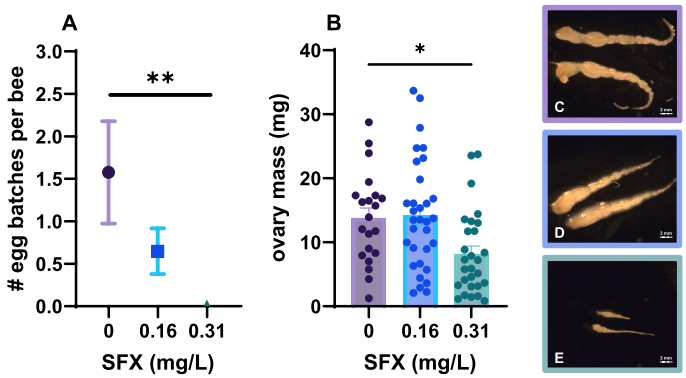

Fig. 3. Sulfoxaflor (SFX) reduced (A) egg-laying in microcolonies; each point represents the mean of colony egg production, normalized by number of surviving bees, and error bars show the standard error of the mean ($n = 16$–17 microcolonies per treatment; total $n = 49$). SFX also reduced (B) ovary development in microcolonies; each point represents an individual bee, the bar represents mean of all individuals within each treatment group and error bars show the standard error of the mean ($n = 81$ bees). Photos show representative ovarian tissue from bees exposed to (C) 0, (D) 0.16, and (E) 0.31 mg/L SFX for 3-weeks. Scale bars represent 3 mm. *, $p < 0.05$; **, $p < 0.01$.

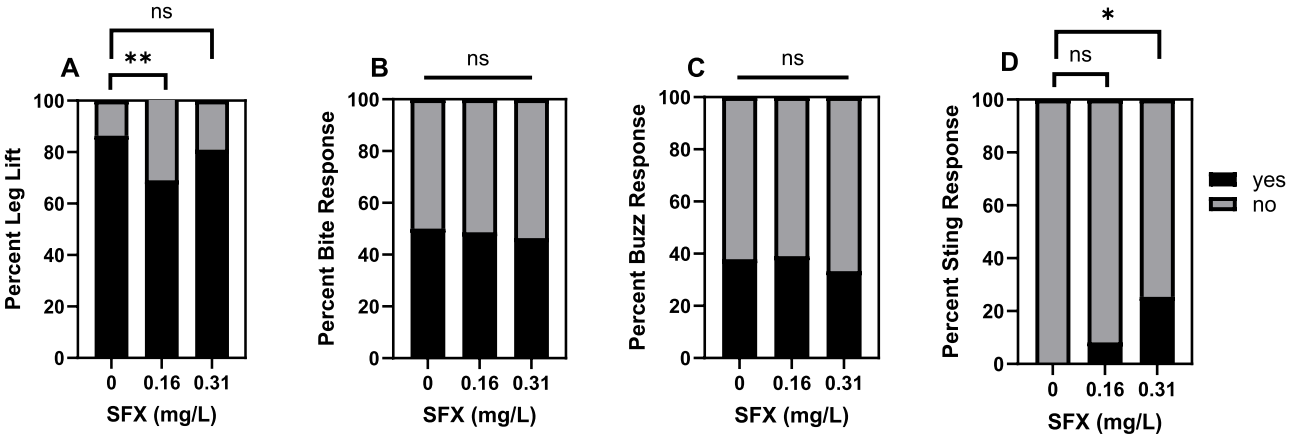

Fig. 4. Sulfoxaflor (SFX) exposure significantly decreased (A) leg lift response but did not affect (B) bite response or (C) buzz response. SFX-exposed bees had an elevated (D) sting response. Data represents the percentage of assayed bees that performed each behavior (yes). Disturbance responses were scored per individual bee (control $n = 22$, 0.16 mg/L $n = 35$, 0.31 mg/L $n = 28$; 3 trials per bee) from $n = 7$ microcolonies per treatment. Statistical notations are as follows: ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

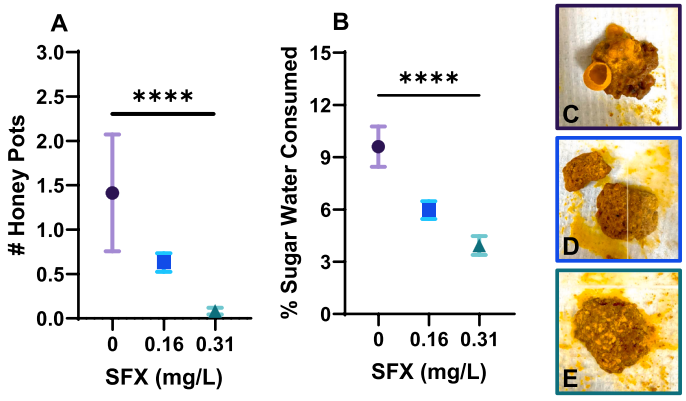

Fig. 5. Chronic sulfoxaflor (SFX) exposure alters group behaviors including honey pot building (A) and sugar water consumption (B) in microcolonies. Each point represents the mean per colony; error bars show the standard error of the mean. All data is normalized by the number of surviving bees per microcolony. Representative nest images for microcolonies exposed to (C) 0, (D) 0.16, and (E) 0.31 mg/L SFX for 3-weeks. ****, $p < 0.0001$. Group-level measures were assessed at the microcolony level ($n = 16$–17 microcolonies per treatment; total $n = 49$).

4. Discussion

Using an integrative framework spanning gene expression, physiology, and behavior, we showed that chronic exposure to the pesticide sulfoxaflor (SFX) primarily disrupted reproductive physiology in *Bombus impatiens*. Ovarian tissues exhibited strong transcriptional

responses and impaired development, while neural transcriptional responses and most individual behaviors remained comparatively stable. These findings suggest that SFX toxicity in bumblebees may operate primarily through reproductive and physiological pathways rather than widespread neural disruption. Integrating molecular, physiological, and behavioral endpoints provides a clearer picture of how sublethal pesticides disrupt pollinator function.

4.1. SFX induced strong differential gene expression in ovaries but not brains

We found that ovary tissues from workers subjected to different levels of SFX exposure displayed more differentially expressed genes compared to brain tissues (Fig. 1). This pronounced ovarian response suggests that reproductive tissues are a primary target of SFX toxicity. Further, many *B. impatiens* genes exhibited tissue-specific expression related to ovary and brain tissues (Figure S1). These patterns were generally consistent across experimental conditions, as genes showing tissue-specific expression in one treatment group tended to retain similar specificity in others. However, despite these strong correlations, tissue-specific expression estimates, measured by tau (τ), decreased with increasing SFX exposure. This reduction suggests that SFX disrupts normal regulatory mechanisms that maintain tissue-specific gene expression, leading to a broader expression of genes across tissues. The stronger reduction at higher SFX concentrations further suggests that SFX impairs the transcriptional regulation required to maintain tissue-specific functions.

The relatively small transcriptional response observed in brain tissue may reflect several non-exclusive mechanisms. First, the concentrations tested may primarily target reproductive physiology, resulting in stronger molecular effects in ovary tissue than in brains. Second, chronic exposure may allow partial physiological compensation or acclimation, reducing the magnitude of transcriptional changes detectable at the time of sampling (21 days in this study). Third, neural responses to toxicants may be subtle, transient, or mediated through post-transcriptional processes that are not captured by differential expression analyses. Thus, limited brain differential expression does not necessarily imply an absence of functional neural effects.

We found that genes that were upregulated in ovary tissues in response to SFX exposure, relative to the control group, were associated with key biological processes including G protein-coupled receptor signaling pathways, homophilic cell adhesion, calcium ion transport, and glucosylceramide catabolic processes (Table S10). The upregulation of genes in these pathways is indicative of cellular stress responses triggered by SFX exposure. The predominance of upregulated genes in *B. impatiens* ovary tissue (control vs 0.31 mg/L) mirror similar trends reported in *A. mellifera* head tissues following SFX exposure (Wang et al., 2024).

In contrast, downregulated genes in ovary tissues in response to SFX exposure were predominantly involved in reproductive and metabolic processes, such as calcineurin-mediated signaling, neuron fate specification, and triglyceride biosynthetic processes (Table S10). Genes involved in chromatin assembly and chromosome segregation were also strongly downregulated in ovary tissue in SFX-exposed bees compared to the control group. These findings align with the physiological findings in this study as well with previous findings that SFX is driving fecundity reduction in bees (Linguadoca et al., 2021; Siviter et al., 2018; Siviter and Muth, 2020). RNA-seq studies in honeybees have also revealed important genes in worker ovary activation including yellow proteins and odorant-binding proteins (Niu et al., 2014), suggesting potential parallels in SFX's impact on reproductive gene regulation across bee species.

Together, these patterns suggest that SFX exposure activates stress- and signaling-related pathways while suppressing processes required for ovary activation and reproductive function. These findings are consistent with prior work showing caste- and tissue-specific transcriptional differences in bee ovaries(Kang et al., 2021) and tissue-specific responses to insecticide exposure in *A. mellifera* (Witwicka et al., 2023). Thus, reproductive tissues are especially sensitive to SFX, with likely consequences for fertility and colony function.

4.2. SFX induced gene expression is driven by a few highly connected hub genes

Co-expression network analysis highlighted the tissue-specific and dose-dependent effects of SFX exposure, particularly in ovary tissue (Fig. 2). Ovary differential expression appeared to be organized around several hub genes including G kinase-anchoring protein, G-protein coupled receptor (GPCR), and DE-cadherin. GPCRs play a crucial role in various reproductive processes in insect ovarian tissues, including uptake of vitellogenin (Bai and Palli, 2016; Wang et al., 2012). Cadherins also play a role in gametogenesis and gonad development in ovarioles (Piprek et al., 2020). Together, this suggests that SFX may disrupt conserved molecular networks involved in maintaining ovarian function.

Brain differential expression in response to SFX was generally low. Nevertheless, patterns of differential gene expression were driven primarily by calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase, an enzyme that regulates cyclic nucleotide second messengers at the cellular level. The expression of this gene has previously been shown to be altered by imidacloprid exposure in honeybee brains (Christen et al., 2018). The identification of a phosphodiesterase hub gene suggests that even subtle modulation of central signaling pathways could influence neural function. Because phosphodiesterases regulate cyclic nucleotide signaling, small changes in their activity can alter neuronal excitability or synaptic plasticity, potentially producing behavioral effects that are not readily detected by the assays used here. Thus, these results highlight the possibility that molecular perturbations in the brain may precede or underlie more subtle changes in behavior. Together, these network analyses suggest that SFX-induced responses are organized around a small number of regulatory hubs, particularly in ovary tissue, making these genes strong candidates for future mechanistic studies.

4.3. Bumblebee reproduction and ovary development are impeded by SFX

SFX dramatically reduced bumblebee reproductive success (Fig. 3). In fact, no microcolonies exposed to 0.31 mg/L laid a single batch of eggs over the 3-week experimental window. Similar reductions in reproduction have been reported in *B. terrestris* at even lower concentrations of SFX (5 ppb ~ 0.005 mg/L) (Siviter et al., 2018; Siviter and Muth, 2020), which may be exacerbated by nutritional stress (Linguadoca et al., 2021). Egg-laying reduction has also been demonstrated in other social insects exposed to SFX including two ant species, *Lasius niger* and *Myrmica rubra* (Svoboda et al., 2023).

In addition to these reproductive effects, this study is the first to show a significant reduction in ovary development in response to SFX. We found that chronic exposure to 0.31 mg/L SFX contaminated sugar water strongly impaired ovary development in worker bees (Fig. 3). Control bees developed ovary tissues that were on average 1.68x larger than bees treated with high SFX. This result suggests that SFX is physiologically impeding ovary development, which may be exacerbated by consuming less food (Linguadoca et al., 2021).

Although ovarian effects have not been directly documented in bees exposed to lower concentrations of SFX before (Siviter and Muth, 2020), other groups have reported impaired ovary development in other *Bombus sp.* workers exposed to high concentrations of imidacloprid (Laycock et al., 2012) and thiamethoxam (Baron et al., 2017). Further, a solitary bee, *Osmia bicornis*, has been shown to experience delayed ovary development in response to field-realistic mixtures of clothianidin and propiconazole (Sgolastra et al., 2018). Collectively, these studies point to substantial reproductive costs of agrochemical exposure in

pollinators, raising concern for their persistence in agroecosystems and wild habitats.

4.4. Individual bumblebee behavior is not strongly influenced by SFX

Chronic SFX exposure did not affect crawling behavior (Figure S2). Previous studies have similarly reported mixed and often limited impacts of SFX on behavior endpoints. For example, SFX does not appear to affect bee foraging or pollination efficiency (Straw et al., 2023; Tamburini et al., 2021). Similarly, olfactory learning and memory are not impacted by SFX exposure in bumblebees (Siviter et al., 2019; Vaughan et al., 2022). However, other investigations have observed that SFX decreases foraging (Boff et al., 2022; El-Din et al., 2022) and the ability of bees to return to their hives (Capela et al., 2022). These mixed findings suggest that behavioral effects of SFX may depend strongly on context, endpoint, and experimental design.

SFX altered some disturbance responses, such as leg-lifting and stinging, but not others, such as biting and buzzing. Leg-lifting behavior decreased at 0.16 mg/L SFX (Fig. 4), which may reflect impaired threat perception or altered neuromotor output due to sulfoxaflor's action on nicotinic acetylcholine receptors (Watson et al., 2021). By contrast, bees exposed to SFX had a significantly elevated 'sting' response (Fig. 4). Although increased stinging may indicate heightened defensiveness, it could also reflect stress reactivity or altered sensory processing rather than increased aggression per se. Because sulfoxaflor targets nicotinic acetylcholine receptors, subtle disruption of cholinergic signaling may shift response thresholds or threat perception.

In contrast, biting and buzzing were unaffected by SFX treatments (Fig. 4). The absence of parallel changes in biting or buzzing supports the interpretation that SFX affects specific behavioral pathways rather than broadly elevating aggression, though direct measures of neural or stress responses are needed to clarify mechanism. Consistent with this interpretation, most behaviors, including crawling performance, were unaffected, while leg-lifting and stinging were altered. This pattern suggests selective modulation of internal state or motivation rather than generalized impairment of motor or neural function.

Only a few studies have investigated aggressive behaviors in response to insecticide exposure in bees. Interestingly, one study found that highly aggressive honeybees were more tolerant to neonicotinoid insecticides, suggesting a relationship between behavior and toxicant susceptibility (Rittschof et al., 2015). Another group showed that exposure to the herbicide bentazone increased aggression in honeybees (Belsky and Joshi, 2020). Neonicotinoid exposure has also been shown to enhance aggression in other social insects, such as ants (Barbieri et al., 2013). Taken together, these mixed findings suggest that the behavioral effects of SFX are context-dependent and may be more apparent in ecologically complex settings than under controlled laboratory conditions.

4.5. Collective nest-building behavior and food consumption is disturbed by SFX

We found a highly significant decrease in sugar water consumption in *B. impatiens* microcolonies exposed to SFX (Fig. 5). Previous research has highlighted a reduction in food intake by bees when exposed to sugar syrup contaminated with SFX (Albacete et al., 2023; Kenna et al., 2023; Nebauer et al., 2024; Orr et al., 2025; Parkinson et al., 2023). One study found that *B. terrestris* workers reduced their intake of SFX-contaminated syrup even when it was the only available food source, indicating an active form of feeding deterrence (Catania et al., 2024). This suggests that bees may be capable of detecting SFX post-ingestively or associating its consumption with negative physiological consequences, leading to learned avoidance or conditioned taste aversion. Alternatively, reduced consumption may reflect a generalized physiological stress response leading to a suppression of appetite. Distinguishing between these possibilities will require targeted assays of feeding motivation and metabolic status. Notably, the decrease in food consumption could help explain a variety of other observed phenotypes including decreases in ovary development and egg-laying.

An important question is whether reduced food consumption alone can explain the complete absence of egg production at the highest SFX concentration, or whether direct toxic effects on ovarian physiology are the primary driver, with nutritional changes acting secondarily. The transcriptomic analyses revealed substantial dysregulation of genes involved in oogenesis, cell signaling, and mitotic processes in ovary tissue, suggesting that SFX directly disrupts reproductive pathways. At the same time, microcolonies exposed to SFX consumed less sugar water, raising the possibility that reduced nutrient intake could exacerbate reproductive impairment. One plausible scenario is that SFX first interferes with ovarian function, reducing reproductive demand and thereby decreasing food consumption and nest-building activity. Alternatively, feeding deterrence or physiological malaise could reduce intake, leading to energetic limitation that constrains ovary activation. Although this data cannot fully disentangle these pathways, the strong molecular signature in reproductive tissues alongside complete reproductive failure at high dose suggests that direct ovarian toxicity likely plays a primary role, with reduced consumption acting as a reinforcing mechanism.

We also discovered that nest building activity, as measured by honey pot production, was significantly lower when *B. impatiens* microcolonies were exposed to SFX (Fig. 5). Many studies have shown a decrease in overall colony performance in response to insecticide exposure (Gill et al., 2012). However, to our knowledge, this study is the first to demonstrate a reduction in collective nest building behavior in response to SFX exposure. We were able to document these differences because bumblebee microcolonies provide an excellent framework to observe collective nest building behavior (Klinger et al., 2019). Similarly, a previous investigation found a reduction in honey pot production in bumblebee microcolonies exposed to another chemical, azadirachtin (Barbosa et al., 2015). These results are consistent with this finding and suggest a general effect of insecticides on group function and, importantly, on colony fitness, with potential consequences for pollination services.

4.6. Integrative toxicological response to SFX exposure

Across levels of biological organization, these results show that chronic SFX exposure primarily disrupts reproduction, highlighting the need for ecologically realistic insecticide assessments. Transcriptional disruption in ovary tissue aligns closely with the observed reductions in egg-laying and ovary development. The observed reductions in sugar water consumption and honey pot construction may also be explained by these reproductive impairments. Honey pot construction and collective food storage is driven by the demand of the colony and thus, we speculate that the lack of reproduction in SFX colonies drove reduced nest building behavior. Likewise, the limited transcriptional response in brain tissue is also consistent with the relatively modest and selective behavioral effects observed at the individual level.

The divergence between minimal brain differential expression and pronounced changes in reproduction and collective behavior raises the possibility that SFX effects are mediated primarily through endocrine or physiological pathways rather than direct disruption of complex neural circuits. In social insects, reproductive status strongly shapes behavior through hormonal signaling and metabolic state, suggesting that ovarian dysfunction could cascade to influence nest construction and defensive responses even in the absence of widespread transcriptional changes in the brain.

An important limitation of this study is that we did not directly compare ovary development in experimental microcolonies with bees remaining in untreated source colonies. As a result, we cannot exclude the possibility that higher SFX doses disrupted the establishment of typical microcolony social organization, potentially altering

reproductive cues that drive ovary activation, honeypot construction, and aggression. Thus, some observed differences may reflect treatment effects on social context in addition to direct physiological toxicity. In addition, workers in Experiment B were not age-controlled, which introduces additional biological variation but better reflects natural colony demographics. Age differences can influence reproductive physiology and behavior in social insects; however, incorporating mixed-age cohorts allows assessment of treatment effects under socially realistic conditions. Because treatment effects were consistent across endpoints, we do not expect age variation to have obscured the major patterns observed, though it may contribute to increased variance.

Overall, these results show that sublethal pesticide exposure can impair pollinator reproduction even when neural transcriptional responses and many individual behaviors appear largely unaffected. By integrating gene expression, ovary physiology, and individual and collective behavior, we show that sulfoxaflor primarily disrupts reproductive function rather than broadly altering neural activity or behavior. These findings underscore the need for risk assessments to move beyond acute mortality and incorporate chronic, mechanistic, and ecologically relevant endpoints. Developing testing frameworks that reflect natural exposure conditions and account for effects on behavior, physiology, and gene regulation will improve predictions of population-level consequences for pollinators.

CRediT authorship contribution statement

Michael A. Catto: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Jixiang Xu**: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Kayla A. Murray**: Writing – review & editing, Methodology, Investigation, Data curation. **Emma Leigh M. Bossard**: Writing – review & editing, Methodology, Investigation, Data curation. **Michael A. D. Goodisman**: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition. **Sarah E. Orr**: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.120101.

Data availability

The sequence data generated in this study are available in the NCBI BioProject database under accession number PRJNA1212201. Other data from this study are available via DRYAD at DOI: 10.5061/dryad.xsj3tx9rj.

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