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The Utility of a Bumble Bee (*Bombus spp.* [Hymenoptera: Apidae]) Brood Test for Evaluating the Effects of Pesticides

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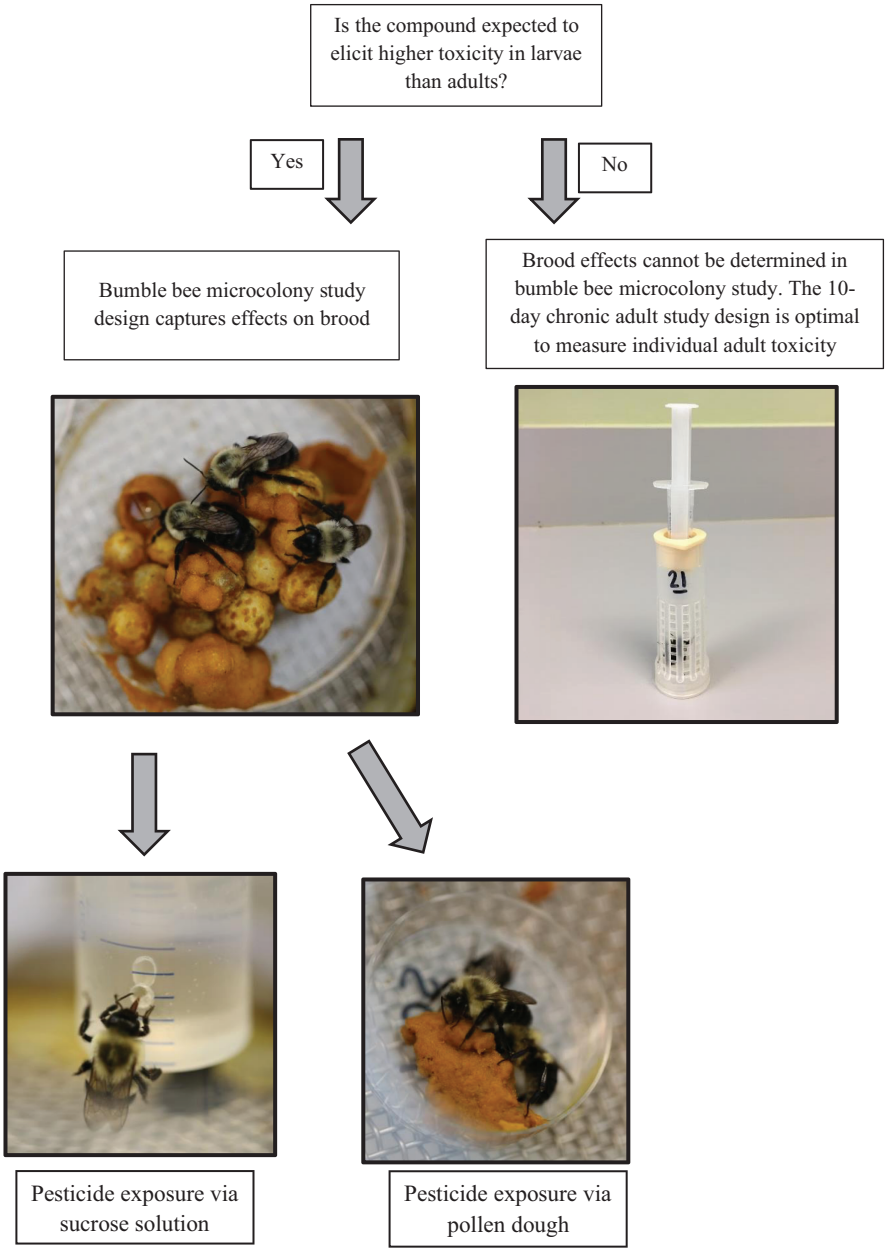
Abstract

Risk assessment for chemicals in the United States relies upon the honey bee (*Apis meliffera* L. [Hymenoptera: Apidae]) as a surrogate for other bee species. There is uncertainty in extrapolating honey bee toxicity data to bumble bees due to differences in life history strategies, food consumption, and nest structure. Here we evaluated the design of a queenless bumble bee microcolony test that could be considered for generating larval toxicity data. Three microcolony studies were conducted with *Bombus impatiens* to evaluate the effects of exposure to 1) diflubenzuron in pollen, 2) dimethoate in pollen, and 3) dimethoate in sucrose. Immature drone bee emergence, worker survival, pollen, and sucrose utilization were measured throughout the study duration. For dimethoate, a 10-d chronic adult bumble bee study was also conducted to compare microcolony endpoints to toxicity endpoints on individual adults. Microcolonies exposed to 10 mg diflubenzuron/kg pollen produced fewer adult drones despite no effects on worker survival. Microcolonies treated with dimethoate at ≥3 mg a.i./kg pollen and ≥0.1 mg a.i./kg sucrose solution produced fewer drones. Exposure to dimethoate in the 10-d chronic adult study resulted in direct mortality to the adult workers at ≥0.1 mg a.i./kg diet. Results from the 10-d study suggest direct effects of dimethoate on workers in the microcolony will alter provisioning of diet to the brood, resulting in lower drone production in the microcolony. Our data suggest that the microcolony study is only appropriate to assess brood effects to bumble bees for substances with low toxicity to adults, as demonstrated with diflubenzuron.

Graphical Abstract



The microcolony study design is optimal for assessing the effects of substances to larvae when direct effects to adults are not predicted. A test item can be delivered via both the pollen dough and sucrose solution.

Key words: bumble bee, pesticide, hazard assessment, microcolony

Insect pollinators are vital for the pollination of many fruits and vegetables grown across the world. Both pesticides and pollinator services are key contributors to crop yield and quality (Gallai et al. 2009, Savary et al. 2019). The total value of global pollination services to crop production is estimated at €153 billion (Gallai et al. 2009). Additionally, significant crop yield losses of up to 41% (Savary et al. 2019) can be reduced by using pesticides and other management practices to control harmful weeds, fungi, and insects. Pesticide manufacturers follow guidance from regulatory authorities to ensure that pesticide use patterns recommended on the labels do not cause unreasonable adverse effects on pollination services, pollinator populations, or hive products (US EPA 2014, Spurgeon et al. 2016). Standardized toxicity test guidelines are established for honey bees (*Apis mellifera* L. [Hymenoptera: Apidae]) to characterize the toxicity profile of a substance for acute contact exposure of adults (US EPA 1996a, OECD 1998a), acute oral exposure of adults (OECD 1998b), chronic oral exposure of adults (OECD 2017a), the toxicity of residues on foliage (US EPA 2012), and acute exposure of larvae (OECD 2013). Additional work by the Organization of Economic Co-operation and Development (OECD)

has focused on finalizing methods to evaluate toxicity to honey bee larvae following repeated exposures (OECD 2016). Until recently, the regulatory authorities across regions have relied on the honey bee as a surrogate for other non-honey bee (non-*Apis)* species of bees and other insect pollinators (Alix et al. 2014, US EPA 2014). However, as attention mounts for the decline in wild bee populations (Grixti et al. 2009, Cameron et al. 2011, Scheper et al. 2014) there are doubts that the current pesticide risk assessment framework for pollinators is protective for all species of bees due to the differences in body size, life history traits, food consumption, among other factors (Scott-Dupree et al. 2009).

With more than 20,000 identified bee species across the world (Michener 2000) it is not feasible to test every species. The honey bee is relied upon as a surrogate species for risk assessment of pesticides (US EPA 2014) since it is widely used for pollination services, has an expansive geographical distribution, has a longer active season compared to other bees, well-established husbandry methods, and lives in large colonies which can provide individuals to be used as test organisms. A meta-analysis of historical toxicity data indicates the sensitivity of honey bees is fairly representative of the other bee species (Barmaz et al. 2010, Arena and Sgolastra 2014). However, empirical toxicity data on non-*Apis* bee species can expand our understanding on how the honey bee-based surrogacy risk models relate to the broader pollinator community and identify areas where the current risk assessment framework may require modifications. Bee species with life histories different from the honey bee are being considered as additional surrogates for pesticide testing under a standardized approach (European Food Safety Authority 2013, Cabrera et al. 2016, Uhl et al. 2018). Species that are used for pollination services, including bumble bee species such as *Bombus impatiens* C. (Hymenoptera: Apidae) and *B. terrestris* L. (Hymenoptera: Apidae) and some solitary bee species including *Osmia spp*. (Hymenoptera: Megachilidae) and *Megachile spp*. (Hymenoptera: Megachilidae), are commercially-available and therefore easily obtained for toxicity testing (European Food Safety Authority 2013, Uhl et al. 2018). Of these, two bumble bee species have emerged as potential surrogates for pesticide testing, *B. impatiens,* commercially reared and utilized for crop pollination in North America (Cabrera et al. 2016), and *B. terrestris*, commercially reared and utilized for crop pollination in Europe (Velthuis and Doorn 2006).

The goal of a standardized laboratory assay is to generate toxicity endpoints that can be compared to exposure estimates to determine the risk potential for pesticides as a screening-level Tier I evaluation (US EPA 2014). Standardized test methods allow for the collection of robust data (e.g., consistent test performance across different laboratories) that can be utilized for regulatory decision-making. The intention of developing standardized non-*Apis* test guidelines is to validate the current honey bee-based risk assessment framework as protective of other bee species or identify additional data needs when assessing risks to other bee species. Multi-stakeholder working groups, such as the International Commission for Plant-Pollinator Relationships (ICPPR), are developing standardized laboratory tests for non-*Apis* species of bees. Test guidelines are now available for assessing the acute contact and oral toxicity of a test substance on bumble bee adult workers (OECD 2017b, 2017c). There is an ongoing effort to evaluate methods at an international scale for assessing chronic oral toxicity to bumble bee adult workers (Exeler 2018). Currently, there is not a standardized laboratory method established for evaluating pesticide effects to female worker bumble bee larvae.

Here, we evaluated the utility of a bumble bee microcolony assay for assessing chronic effects of a substance on bumble bee brood. While microcolonies are defined differently in the literature and can include both queen-led and worker-led (pseudo-queen) small colonies (Klinger et al. 2019), we define a microcolony as a pseudo-queen led colony that is initially established with five newly eclosed (emergence of the adult insect from the cocoon), unmated female workers. Within the first 5–7 d, one of the five workers establishes its dominance over the other four workers, develops its ovaries, and begins functioning as a pseudo-queen by laying unfertilized eggs that ultimately develop into adult male (drone) bumble bees. Within the microcolony study design, adult workers care for the developing drone brood by transferring the provisioned diet of pollen and sucrose solution to the drones during the larval stage of development (Klinger et al. 2019). While the unfertilized eggs develop into drones, the timeline for development of drones and workers is almost identical (Cnaani et al. 2002), and drone, worker, and queen brood are all fed the same diet (Pereboom 2000) although each caste develops differently. The microcolony study design has historically been used as an attempt to overcome some of the challenges associated with working with individual queens or whole colonies (Génissel et al. 2002, Mommaerts et al. 2006, Gradish et al. 2013) and may provide insight into chemicals that are toxic to larvae through potential exposure via pollen and nectar. Furthermore, studies suggest that haploid individuals may be more susceptible to the deleterious effects of insecticides (Mobley and Gegear 2018, Friedli et al. 2020). Thus, the microcolony study design should provide robust toxicity endpoints that are protective of the most sensitive larva.

The majority of the bumble bee microcolony studies has been conducted with the European species *B. terrestris* (Tasei et al. 2000, Mommaerts et al. 2006, Laycock et al. 2014, Livesey et al. 2019) and it is uncertain how this species can be compared to the North American *B. impatiens*. Here we conducted a series of experiments with the goal of determining whether *B. impatiens* microcolonies can be successfully used to assess the toxicity of a substance to developing bumble bee brood.

We selected two pesticides; diflubenzuron, a substance with low-predicted adult toxicity and high-predicted brood toxicity, and dimethoate, a substance with high-predicted adult toxicity and low predicted brood toxicity, to evaluate the utility of the microcolony study design. Diflubenzuron, an insect growth regulator toxic to larvae, was chosen to evaluate the microcolony test design for substances that are preferentially toxic to the larval life-stage (i.e., in the absence of worker impairment). Dimethoate was chosen to characterize the impact on brood production if there was direct impairment to adults and is the preferred reference substance in OECD honey bee test guidelines (OECD 2017a). We conducted three microcolony experiments: 1) dimethoate and 2) diflubenzuron provisioned to adult workers through pollen and transferred to developing brood, and 3) dimethoate provisioned to adult workers through a sucrose feeding solution.

We hypothesized that direct effects to adults may be prevented in the microcolony study design if a substance is dosed through pollen, given that exposure of a substance to the brood is only facilitated by the adult workers (Klinger et al. 2019) and that adult consumption of the pollen is minimal (Tasei 2001, Heinrich 2004). Additionally, we provisioned dimethoate through the sucrose solution matrix to characterize the impact on brood production if there was direct impairment to adults. We further characterized the direct effects observed on adults exposed to dimethoate by directly dosing individual bumble bee adult workers in a 10-d chronic adult exposure study design.

Materials and Methods

Three *B. impatiens* microcolony brood toxicity studies were conducted from February 2017–August 2018 to assess the effects of two toxic reference substances on immature bee (drone) emergence and worker survival. Bumble bee microcolonies were dosed with either diflubenzuron through a pollen diet, dimethoate through a pollen diet, or dimethoate through a sucrose diet (50% w/w, sugar/water solution, and designated as sucrose from here on). Worker survival, daily drone emergence, pollen utilization, and sucrose utilization were recorded as endpoints that provide useful information for assessing potential effects of a pesticide (US EPA 2014). For dimethoate, a 10-d chronic adult bumble bee study was also conducted to compare microcolony dimethoate endpoints with adult dimethoate toxicity endpoints. Analytical verification for the dosing and homogeneity of the substances in the provided diet was conducted in conjunction with the dimethoate and diflubenzuron pollen microcolony studies and the 10-d chronic adult study.

Test Substances

Dimethoate (Item # 45449, Lot # BCBS9338V) and diflubenzuron (Item # 45446, LOT#SZBF111XV) were sourced from Sigma–Aldrich. Dimethoate, is an organophosphate, is water soluble, and did not require the use of a solvent at the concentrations included in the study (Sanderson and Edson 1964). Dimethoate stock solutions were prepared in 0.01 M acetic acid and sodium acetate buffer at pH 4 for the microcolony studies to increase the half-life to 156 d (FAO Specifications 1991). The dimethoate stock solution was stored at 4°C throughout 42-d study duration. No buffer was used for the dimethoate stock solutions in the 10-d chronic adult study.

A solvent was necessary to prepare diflubenzuron in a pollen- and water-based diet at the study concentrations. Acetone, a standard solvent in toxicity testing, was selected as the carrier for diflubenzuron. A diflubenzuron stock solution was prepared at the onset of provisioning the treated matrix to the bumble bees (day 10) and stored at 4°C throughout 42-d study duration.

Microcolony Study Design

Each custom-made microcolony chamber consisted of a 15 cm metal soil sieve (Newark Wire Cloth Company, Clifton, NJ, US STD 6in SS No. 10) fit with a custom-made acrylic base and lid (Fig. 1A). The lid contained several ventilation holes, one hole that fits a 20 ml syringe (Thermo Scientific, SUN-SRi Luer-Slip Syringes), and one hole with a plug that was used as an access port while maintaining the microcolonies. The 20 ml syringes were used to feed the bees sucrose and were modified by drilling a 2.8 mm hole into the side of the syringe at approximately the 2 ml mark. The syringe eccentric tip was capped with a rubber stopper (BD Syringe Luer Tip Cap 308341) and placed upright into each chamber. The syringes were replaced at each maintenance interval every 2–3 d. A paper towel was placed on the acrylic base to collect nest debris and defecation that fell through the sieve and was replaced weekly. A 55 mm Petri dish base was placed in each chamber to serve as a designated brood nest dish and was not removed from the chamber during the study. A 35 mm Petri dish (top or base) was used to provision the pollen after day 10 once the brood nest was well-established on the 55 mm Petri dish base. The 35 mm petri dish was removed and replaced at each diet provisioning in order to weigh the utilized pollen.

For each microcolony study, 125 callow workers were ordered from Biobest and shipped overnight in groups of 25 for a total of five boxes. Upon arrival, one apparently healthy worker was selected from each of the five shipment boxes and transferred to each microcolony chamber, so that five workers were newly introduced to one another on day 0 and any effects of shipment box were accounted for. Any workers that died within the first 24 h after placement were replaced with workers from the original shipment.

Pollen diet for the microcolonies was prepared by grinding Indiana- and North Carolina-sourced honey bee wildflower pollen (stored at –20°C for a maximum duration of 3 yr) with a small blender (Magic Bullet) and mixing with 1:1 sucrose (as described above) at a 3:1 or 2:1 (w/w) pollen:sucrose ratio based upon the consistency of the diet. The variation in diet consistency was likely due to differences in the water content of the honey-bee collected pollen, but this assumption was not verified. Sucrose diet was prepared by mixing table-grade sugar with distilled water at a 1:1 (w/w) ratio and stored for a maximum duration of seven days at 4°C.

To ensure a homogenous distribution of the test item, 10 g of pollen was first mixed with 10 g of sucrose solution (1:1, preparation as described above) using a hand-mixer (Mainstays). A 250 µl aliquot of the prepared stock solutions (as described above) was added dropwise using a 250 µl Microman positive-displacement pipette (Gilson) onto the 1:1 pollen:sucrose diet. For the diflubenzuron pollen diet, the carrier solvent acetone was evaporated from the surface by directing a stream of nitrogen on each of the droplet

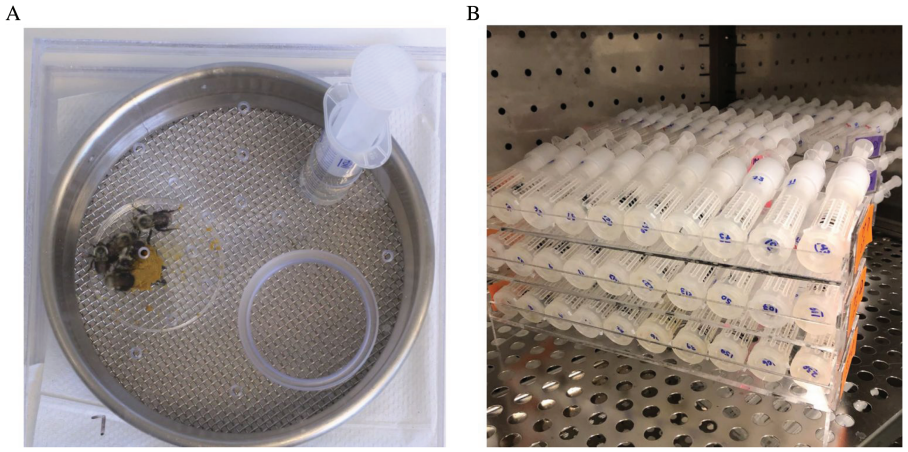


Fig. 1. (A) The microcolony test chamber was constructed from a metal sieve. Each sieve was fitted with a custom plastic lid with small holes to allow for ventilation. A 1.25 cm hole held the sucrose feeding syringe, and another 7.6 cm opening allowed for access to the colony for feeding events and removal of dead bees. (B) The workers in the 10-d chronic adult study were housed in individual modified honey bee queen hair roller cages.

locations for up to 70 s until it visibly disappeared. Pollen diets were mixed for at least 1 min with the hand-mixer to incorporate the test item before the remaining 20 g of pollen was added. The pollen diets were prepared weekly and were stored for a maximum duration of seven days at 4°C.

Each microcolony was provided sucrose solution ad libitum through a modified syringe feeder replaced at each maintenance interval. The filled feeder syringes were weighed in on an analytical balance (OHaus Pioneer PA64), and then weighed out at the subsequent maintenance interval. On day 0 and day 3, each microcolony was provided ~1.5 g of untreated pollen diet directly into the designated brood dish. On day 6 and day 8, each microcolony was provided ~1 g of untreated pollen into the same designated brood dish. After day 10, each microcolony was provided pollen diet *ad libitum* in a separate feeding dish that was replaced at each maintenance interval. After day 10 the weight of the utilized diet was measured and recorded at each maintenance interval.

Additional test chambers were set up and provisioned without bees to account for potential evaporative loss in the pollen and sucrose diets. Pollen utilization and sucrose utilization measurements were adjusted for evaporative loss before analysis. To control for potential variation within the incubator, an evaporative control was placed on each of the three shelves and the evaporative loss was considered the average across the shelves.

Treated diets were administered to the microcolonies on day 10. The dosing schedule was based on the assumption that a pseudo-queen requires five days to develop ovaries and lay its first clutch of eggs (Röseler 1977, Bloch et al. 1996), and five additional days are required for the eggs to hatch (Cnaani et al. 2002). Therefore, day 10 is the expected date for the first bumble bee larvae to be present. The test substance for each of the microcolony studies were, 1) diflubenzuron delivered through pollen at three concentrations (1.0, 3.0, and 10.0 mg a.i./kg treated matrix) with four replicates per treatment group, 2) dimethoate delivered through pollen at five concentrations (0.1, 0.3, 1.0, 3.0, and 10 mg a.i./kg treated matrix) with three replicates per treatment group, and 3) dimethoate delivered through sucrose solution at five concentrations (0.03, 0.1, 0.3, 1.0, and 3.0 mg a.i./kg treated matrix) with three replicates per treatment group. An untreated control diet (0 mg a.i./kg treated matrix, 3 replicates per study) was included for each study and a solvent-control group (Acetone, 3 replicates) was included for the diflubenzuron microcolony study that required the use of a solvent to integrate the test substance into the pollen matrix.

Each of the three microcolony studies were housed in an incubator (Percival I-30) at 25 ± 2°C, 60 ± 10 % RH (OECD 2017c) with constant darkness, Microcolonies were maintained twice a week for the first week and three times a week after establishment. Maintenance of the microcolonies included measuring and replacing sucrose and pollen, recording worker survival and drone emergence, and the removal of dead workers and emerged drones. The paper towels underneath each chamber were replaced once a week to remove waste and nest debris.

All microcolonies were frozen at test termination (42 d after test initiation). Nest weight, number of eggs, viable larvae, dead larvae, pupae, and drones were counted and recorded (Supp Table 1 [online only]), but not included in our statistical analysis.

Ten-Day Chronic Adult Study Design

A 10-d chronic adult toxicity test was conducted to evaluate the toxicity of dimethoate to individual bumble bee workers. The test was initiated by randomly selecting 320 worker bees of unknown age from nine *B. impatiens* queen-right commercial colonies (Koppert Biological Systems, Howell, MI) within one week of receipt. Individual adult worker bumble bees were placed into modified honey bee queen hair roller cages (QC-117, Mann Lake LTD.) and modified yellow cell cup holders (QC-127, Mann Lake LTD) to accommodate a 3 ml syringe (Sigma Aldrich, Z116858) (Fig. 1B). *B. impatiens* workers were individually weighed before the initiation of the study. The worker bees in the lowest and highest 8% of weights were removed, and a total of 30 individual worker replicates were randomly assigned to each of the nine treatment groups (n = 270).

Feeders were created for the study by trimming the tip of each 3-ml syringe to a length of about 2 mm. This simultaneously allowed for the bumble bees to reach the sucrose solution with their proboscis while preventing the sucrose solution from leaking. Each of the cages was placed into a cell in a custom-made lattice to distribute the cages within the incubator. The individual test chambers were housed in an incubator (Percival I-30) at 25 ± 2°C, 60 ± 10 % RH (OECD 2017c) with constant darkness for the three microcolony studies and the 10-d chronic adult study.

Sucrose diet was prepared by mixing table-grade sugar with distilled water at a 1:1 (w/w) ratio and stored for a maximum duration of seven days at 4°C. The dimethoate stock solution was used to prepare fresh diets on days 0, 3, and 6 of the test and stored at 4°C for a maximum of 4 d. The adult workers were acclimated for one day before 10 days of continuous exposure to the test substance. Dimethoate concentrations evaluated in the 10-d chronic adult study were 0, 0 (5% acetone), 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg a.i./kg diet.

Analytical Verification

Analytical verification was performed for two microcolony studies: diflubenzuron exposure via pollen and dimethoate exposure via pollen. Three ~1 g aliquots of each newly prepared test diet were taken from different locations within the prepared diet and stored at –20°C for up to 5 wk. Aliquots were weighed directly into Omni Bead Ruptor tubes (#19–6358 Hard Tissue Homogenizing Mix 30 ml Tubes with 2.8 mm ceramic beads). Samples were removed from the freezer, and 0. 5 ml deuterated internal standard of dimethoate (O,O-dimethyl-d_6, 1000 ng/ml, Sigma Aldrich, Lot # AM2B21G062) or diflubenzuron (4-chlorophenyl-d_4, 1000 ng/ml, CDN D-7169, Lot # P-191) were added to the sample with 9. 5 ml of ACN:Water (1:1). Samples were vigorously mixed using the Bead Ruptor (Omni Bead Ruptor 24, Kennesaw, GA) to separate the test substance from the pollen diet. Approximately 2 g of $MgSO_4$ and ~0.5 g of NaCl were added to each sample and mixed again on the Omni Bead Ruptor (S = 4.00, T = 1.00, C = 01, D = 01:00) before centrifuging at 1800 g for 7 min. An aliquot of the top layer from each sample extract was transferred to a 1. 8 ml HPLC autosampler vial and analyzed by LC/MS/MS (Thermo TSQ Quantum AM, Phenomenex Luna C18 (2)-HST 55 mm × 2.0 mm, 2.5 um particle size). The homogeneity of diflubenzuron in the pollen was evaluated by taking five random samples from different parts of freshly prepared batches for each of the three test levels.

Concentrations of dimethoate in sucrose were also analytically verified for the 10-d adult chronic study. Samples were collected from freshly prepared treated diet on day 6 and again from the same batch of diet at day 9 after three days of storage in the refrigerator. Samples were then stored in the freezer at –20°C and sent for analysis (Bayer CropScience, Monheim am Rhein, Germany). Aliquots of 0.1–1.0 g of stored diets were weighed out and spiked with 0. 5 ml of internal

standard deuterated dimethoate (100 µg/ml, Sigma Aldrich, Lot # AM2B21G062), and 9. 5 ml of ACN:Water (1:1). A 100 µl aliquot of the internal standard spiked diet was transferred to a 1. 8 ml HPLC autosampler vial with 900 µl diluted internal standard and analyzed by LC/MS/MS, (Phenomenex Luna C18 (2)-HST 50 mm × 2.0 mm, 2.5 um particle size). Monitored mass transitions 230 →199 for dimethoate and 236 →205 for dimethoate internal standard.

Statistical Analysis

Statistical analyses for all studies were conducted in R 3.6.1 (Team 2019), utilizing the 'rstatix' (Kassambara 2020), 'stats' (Team 2019), 'DescTools' (Signorell et al. 2020), and 'PMCMRplus' (Pohlert 2019) packages. The analytical methods align with OECD Guidance for the Statistical Analysis of Ecotoxicity Data (OECD 2006).

Data were first tested for normality and heterogeneity of variance using the Shapiro–Wilk test and Levene's test, respectively. Data that showed evidence of divergence from normality (i.e., Shapiro–Wilk $P < 0.05$) or heterogeneity of variances (i.e., Levene's $P < 0.05$) were analyzed using the non-parametric Kruskal–Wallis ANOVA and Dunn's many-to-one Rank Comparison test. Data that followed a normal distribution and had homogeneous variance were analyzed using one-way ANOVA and Dunnett's post-hoc tests. Differences in drone emergence, worker survival, sucrose utilization, and pollen utilization were analyzed to determine the No Observed Effect Concentration (NOEC) for each endpoint. Additionally, bumble bee mortality in 10-d chronic adult study was analyzed using Fisher's Exact Test for trend, and diet consumption was analyzed using a Kruskal–Wallis Test. The non-solvent and solvent control results (when applicable) were combined for ad-hoc statistical comparisons to the treatment groups if no significant differences were observed between the control treatment groups. The statistical tests used to analyze each endpoint and the statistical outputs are detailed in Supp Table 2 (online only) (microcolony studies) and Supp Table 3 (online only) (10-d chronic adult study).

Results

Acetone was used as a carrier solvent in the diflubenzuron microcolony study. A solvent control was included in the study, but combined with the non-solvent control for all statistical analyses since there were no statistical differences in drone emergence (t.test; t = 0, $P = 1$; df = 4), worker mortality (Wilcoxon; w = 6, $P = 0.6193$), sucrose utilization (t.test; t = –0.11, $P = 0.92$; df = 4), and pollen utilization (Wilcoxon; w = 0, $P = 0.08$).

Microcolony Drone Emergence

Drone emergence was measured across the three microcolony studies as an indicator of potential effects to developing bumble bee brood (Fig. 2). Diflubenzuron delivered via pollen (Fig. 2A) resulted in a significant reduction in drone emergence (ANOVA;

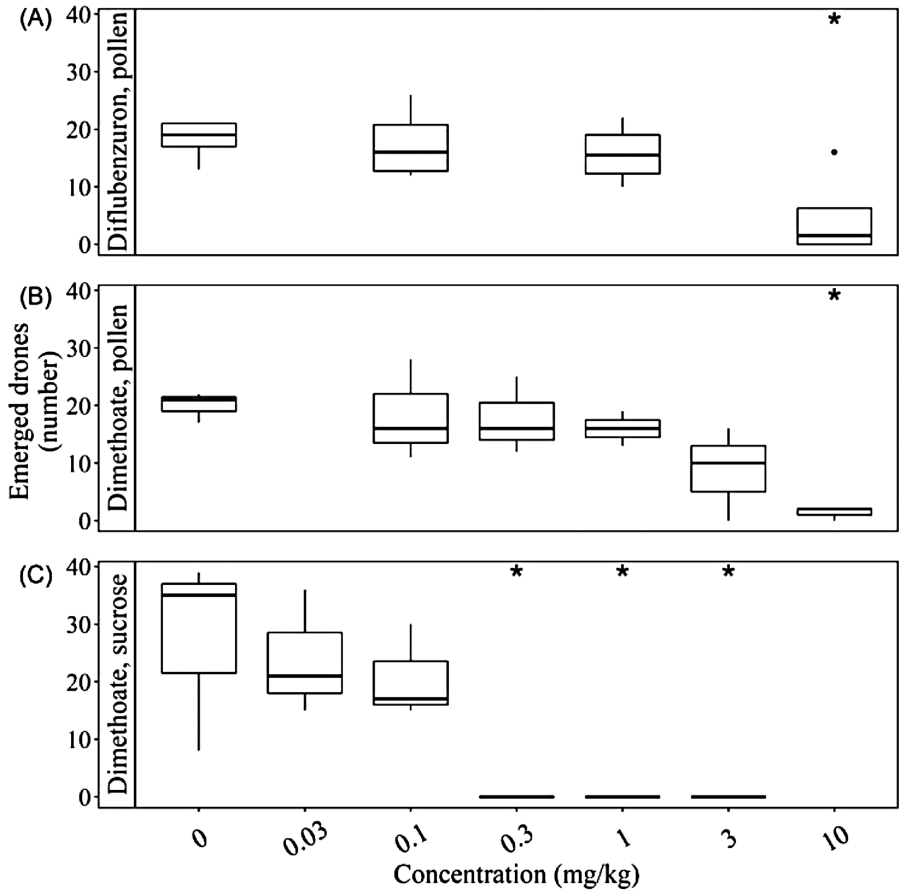


Fig. 2. Comparative box plots of drone emergence per treatment for three microcolony studies. There were significant decreases observed in drone emergence from (A) diflubenzuron exposure via pollen (ANOVA; $P = 0.0108$, $F = 5.44$; df = 3), (B) dimethoate exposure via pollen (ANOVA; $P = 0.014$, $F = 4.611$; df = 5), and (C) dimethoate exposure via sucrose (Kruskal–Wallis; $P = 0.0115$, $\chi^2 = 14.76$; df = 5). Concentrations presented represent milligrams (mg) of active ingredient per kilogram (kg) of treated matrix, adjusted for evaporation. Significant differences from the control group ($P < 0.05$) are indicated with '*'.

$P = 0.0108$, $F = 5.44$; df = 3). A Dunnett's post-hoc test showed significant decrease in drone emergence at 10 mg a.i./kg treated matrix ($P = 0.0058$), resulting in a No Observed Effect Concentration (NOEC) of 1 mg a.i./kg treated matrix and a Lowest Observed Effect Concentration (LOEC) of 10 mg a.i./kg treated matrix. Dimethoate delivered via pollen (Fig. 2B) resulted in a significant reduction in drone emergence (ANOVA; $P = 0.014$, $F = 4.611$; df = 5). A Dunnett's post-hoc test showed significant decrease in drone emergence at 10 mg a.i./kg treated matrix ($P = 0.0082$), resulting in a NOEC of 3 mg a.i./kg treated matrix and LOEC of 10 mg a.i./kg treated matrix. Dimethoate delivered via sucrose (Fig. 2C) resulted in a significant reduction in drone emergence (Kruskal–Wallis; $P = 0.0115$, $\chi^2 = 14.76$; df = 5). A Dunn's post-hoc analysis revealed significant difference from the control in drone emergence at concentrations greater than and equal to 0.3 mg a.i./kg treated matrix ($P < 0.05$), resulting in a NOEC of 0.1 mg a.i./kg treated matrix and a LOEC of 0.3 mg a.i./kg treated matrix. All detailed statistics for post-hoc tests are reported in Supp Table 2 (online only).

Microcolony Worker Mortality

Worker mortality was measured across the three microcolony studies to determine whether the test substance caused direct mortality to the adults (Fig. 3). Diflubenzuron delivered via pollen (Fig. 3A) did not result in any significant difference in worker mortality across any treatments when compared to the control (Kruskal–Wallis; $P = 0.157$, $\chi^2 = 5.23$; df = 3), resulting in a NOEC of ≥10 mg a.i./kg treated matrix. Dimethoate delivered via pollen (Fig. 3B) resulted in significant worker mortality (Kruskal–Wallis; $P = 0.048$, $\chi^2 = 11.17$; df = 5). A Dunn's post-hoc test showed significant increases in worker mortality at concentrations greater than and equal to 3 mg a.i./kg treated matrix ($P < 0.05$), resulting in a NOEC of 1 mg a.i./kg treated matrix and a LOEC of 3 mg a.i./kg treated matrix. Dimethoate delivered via sucrose (Fig. 3C) resulted in significant worker mortality (Kruskal–Wallis; $P = 0.0126$, $\chi^2 = 14.52$; df = 5). A Dunn's post-hoc analysis revealed significant decrease in worker survival greater than or equal to 0.3 mg a.i./kg treated matrix ($P < 0.05$), resulting in a NOEC of 0.1 mg a.i./kg treated matrix and a LOEC of 3 mg a.i./kg treated matrix. All detailed statistics for post-hoc tests are reported in Supp Table 2 (online only).

Microcolony Sucrose Utilization

Sucrose utilization (*i.e.*, diet consumed or utilized for building nest matrix) was measured across the three microcolony studies (Fig. 4). Diflubenzuron delivered via pollen (Fig. 4A) did not result in any significant difference in sucrose consumption rates across any treatments when compared to the control (ANOVA; $P = 0.0157$, $F = 2.021$; df = 3), resulting in a NOEC of ≥10 mg a.i./kg treated matrix. Dimethoate delivered via pollen (Fig. 4B) resulted in a significant difference in sucrose utilization (ANOVA; $P = 0.0005$,

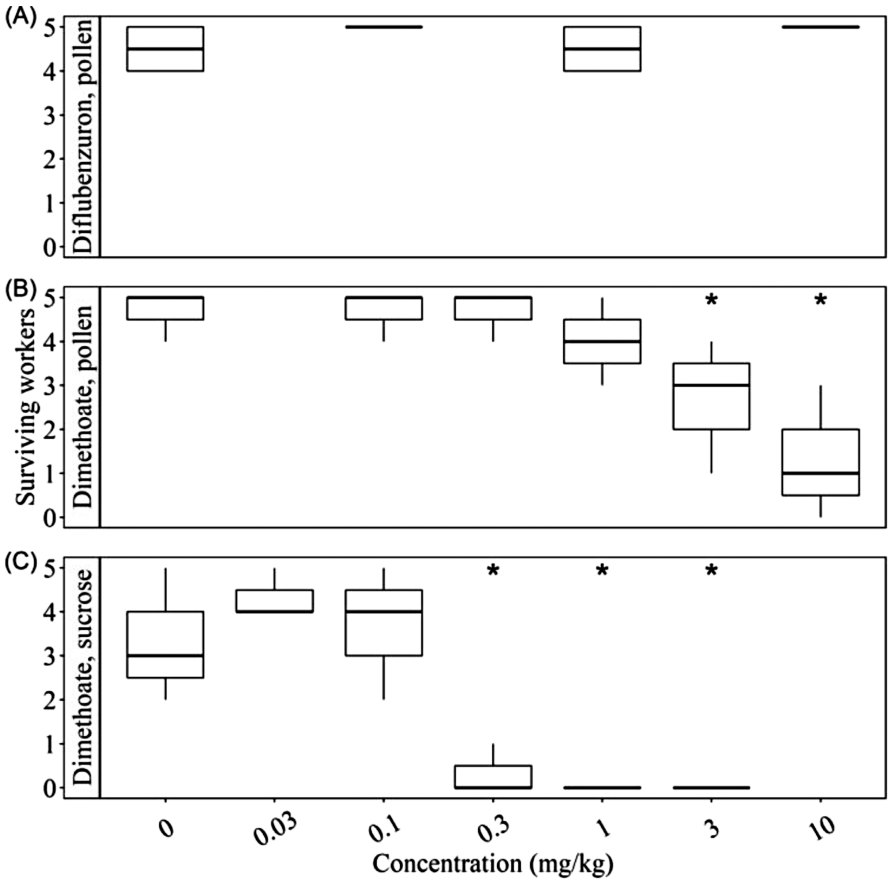


Fig. 3. Comparative box plots of worker survival per treatment for three microcolony studies. Diflubenzuron exposure via pollen (A) resulted in no significant differences in worker survival up to 10 mg a.i./kg treated matrix (Kruskal–Wallis; $P = 0.157$, $\chi^2 = 5.23$; df = 3). In contrast, significant decreases were observed for worker survival relative to the controls from (B) dimethoate exposure via pollen (Kruskal–Wallis; $P = 0.048$, $\chi^2 = 11.17$; df = 5) and (C) dimethoate exposure via sucrose (Kruskal–Wallis; $P = 0.0126$, $\chi^2 = 14.52$; df = 5). Concentrations presented represent milligrams (mg) of active ingredient per kilogram (kg) of treated matrix, adjusted for evaporation. Significant differences from the control group ($P < 0.05$) are indicated with '*'.

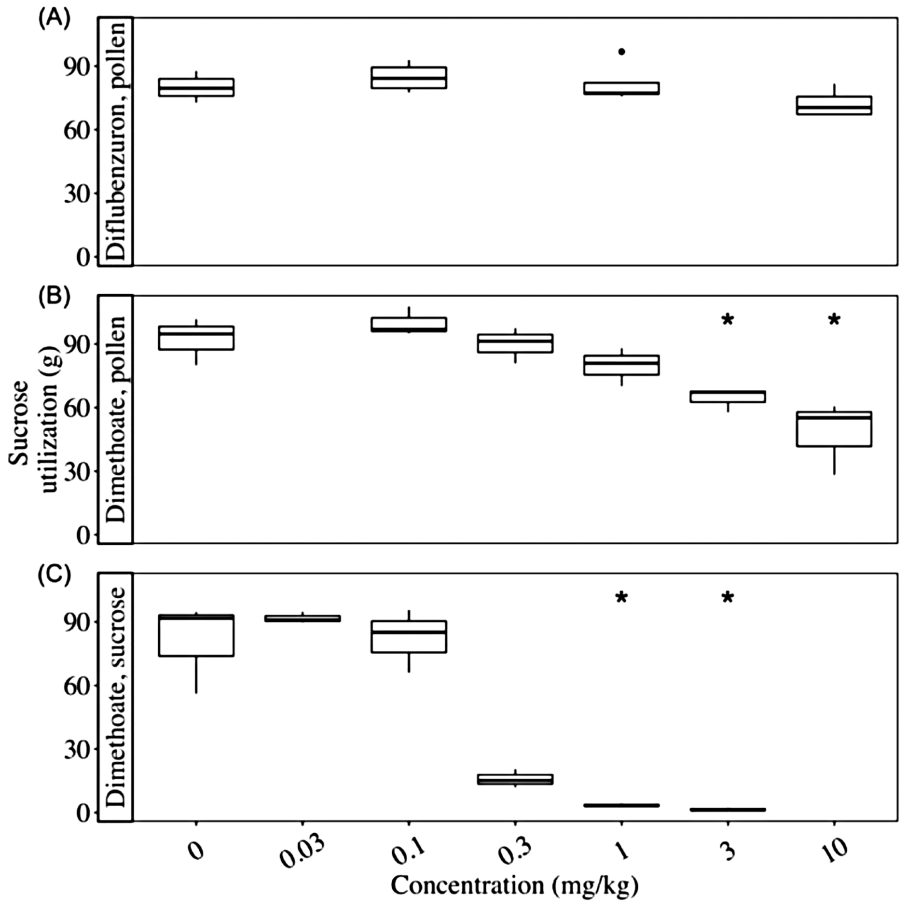


Fig. 4. Comparative box plots of sucrose utilization per treatment for three microcolony studies. Diflubenzuron exposure via pollen (A) resulted in no significant differences in sucrose utilization up to 10 mg a.i./kg treated matrix (ANOVA; $P = 0.0157$, $F = 2.021$; df = 3). In contrast, significant decreases were observed for sucrose utilization relative to the controls from (B) dimethoate exposure via pollen (ANOVA; $P = 0.0005$, $F = 10.48$; df = 5) and (C) dimethoate exposure via sucrose (Kruskal–Wallis; $P = 0.0115$, df = 5). Concentrations presented represent milligrams (mg) of active ingredient per kilogram (kg) of treated matrix, adjusted for evaporation. Significant differences from the control group ($P < 0.05$) are indicated with '*'.

F = 10.48; df = 5). A Dunnett's post-hoc test showed significant decrease in sucrose utilization at concentrations greater than and equal to 3 mg a.i./kg treated matrix ($P < 0.05$), resulting in a NOEC of 1 mg a.i./kg treated matrix and a LOEC of 3 mg a.i./kg treated matrix. Dimethoate delivered via sucrose (Fig. 4C) resulted in significant difference in sucrose utilization (Kruskal–Wallis; $P = 0.0115$, $\chi^2 = 14.75$, df = 5). A Dunn's post-hoc analysis revealed significant decrease in sucrose utilization at concentrations greater than and equal to 1 mg a.i./kg treated matrix ($P < 0.05$), resulting in a NOEC of 0.3 mg a.i./kg treated matrix and a LOEC of 1 mg a.i./kg treated matrix. All detailed statistics for post-hoc tests are reported in Supp Table 2 (online only).

Microcolony Pollen Utilization

Pollen utilization (*i.e.*, diet consumed or utilized for building nest matrix) was measured across the three microcolony studies (Fig. 5). Diflubenzuron delivered via pollen (Fig. 5A) did not result in any significant difference in pollen consumption rates across any treatments when compared to the control (ANOVA; $P = 0.8870$, $F = 0.212$; df = 3). Dimethoate delivered via pollen (Fig. 5B) resulted in a significant difference in pollen utilization (ANOVA; $P = 0.0002$, $F = 13.19$; df = 5). A Dunnett's post-hoc test showed significant decrease in pollen utilization at greater than and equal to 3 mg a.i./kg treated matrix ($P < 0.05$), corresponding to a NOEC of 1 mg a.i./kg treated matrix. Dimethoate delivered via sucrose (Fig. 5C) resulted in significant difference in pollen utilization (Kruskal–Wallis; $P = 0.0120$, $\chi^2 = 14.64$; df = 5). A Dunn's post-hoc analysis revealed significant decrease in pollen utilization at concentrations equal to and greater than 1 mg a.i./kg treated matrix ($P < 0.05$), corresponding to a NOEC of 0.3 mg a.i./kg treated matrix and a LOEC of 1 mg a.i./kg treated matrix. All detailed statistics for post-hoc tests are reported in Supp Table 2 (online only).

Ten-Day Chronic Adult Exposure to Dimethoate

A 10-d chronic adult study was conducted with dimethoate to correlate direct effects on individual adults to what was observed within the dimethoate microcolony studies. Mortality did not differ significantly between the control and the solvent control group (Wilcoxon; w = 0, $P = 0.3337$). Thus, the two control groups were combined in the worker mortality analysis. A Fisher's Exact test for trend was used to analyze worker mortality in *B. impatiens* adult workers. Dimethoate delivered through sucrose directly to individual adult workers (Table 1) resulted in a significant decrease in worker survival at test concentrations greater than and equal to 0.2 mg a.i./kg diet ($P = 1.94E-07$), which correlates with a NOEC of 0.1 mg a.i./kg diet and a LOEC of 0.2 mg a.i./kg diet.

Cumulative sucrose consumption for each treatment group was measured and recorded for each individual adult worker. There

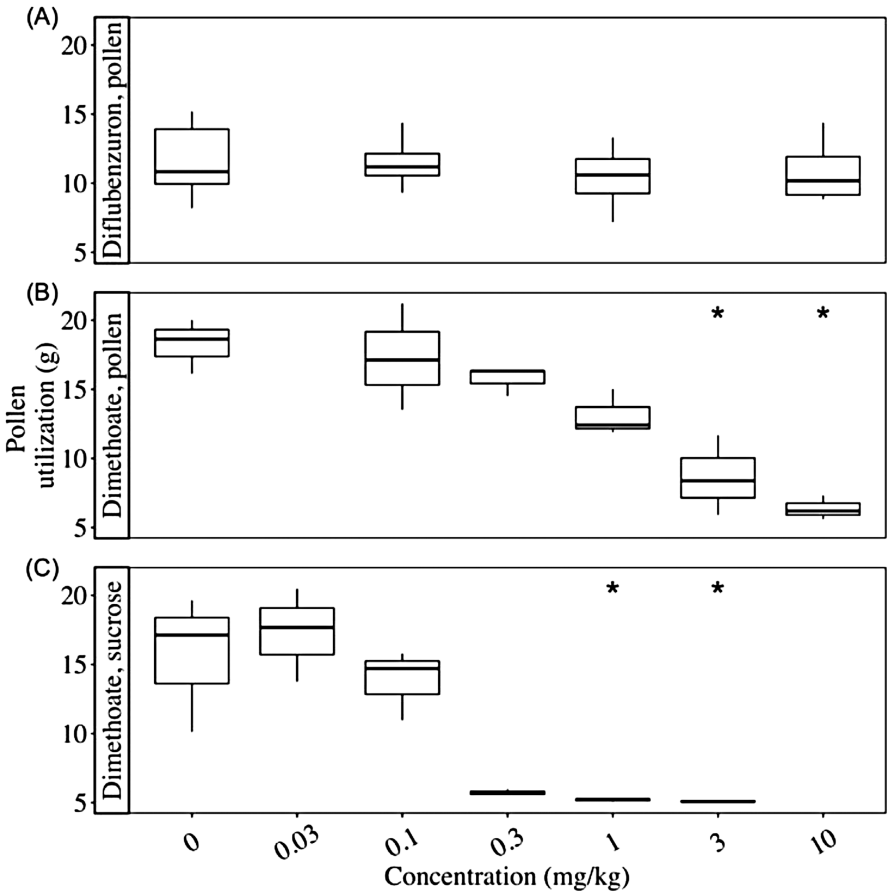


Fig. 5. Comparative box plots of pollen utilization per treatment for three microcolony studies. Diflubenzuron exposure via pollen (A) resulted in no significant differences in pollen utilization up to 10 mg a.i./kg treated matrix (ANOVA; $P = 0.8870$, $F = 0.212$; df = 3). In contrast, significant decreases were observed for pollen utilization relative to the controls for (B) dimethoate exposure via pollen (ANOVA; $P = 0.0002$, $F = 13.19$; df = 5) and (C) dimethoate exposure via sucrose (Kruskal–Wallis; $P = 0.0120$, $\chi^2 = 14.64$; df = 5). Concentrations presented represent milligrams (mg) of active ingredient per kilogram (kg) of treated matrix, adjusted for evaporation. Significant differences from the control group ($P < 0.05$) are indicated with '*'.

Table 1. 10-d chronic adult bumble bee mortality and mean consumption when exposed to differing concentrations of dimethoate through a sucrose diet

Dimethoate Concentration (mg/kg)	Exposed (n)	Total Mortality (n)	Proportion Dead	Total sucrose consumption (g)
Acetone	30	0	0	2.92
0	30	1	0.03	3.33
0.025	30	1	0.03	3.09
0.05	30	1	0.03	2.7
0.1	30	2	0.07	2.22*
0.2	30	14	0.47*	1.65*
0.4	30	29	0.97*	0.96*
0.8	30	30	1*	0.46*
1.6	30	30	1*	0.24*

A Fisher exact test indicated a significant reduction in survival at 0.2 mg a.i./kg diet ($P = 0.0204$). Similarly, there were significant differences in sucrose consumption at 0.1 mg a.i./kg diet (Kruskal–Wallis; $P < 2.2E\text{-}16$, df = 8). Significant differences form the control group ($P < 0.05$) are indicated with an asterisk.

was a statistically significant difference in sucrose consumption between the control and the solvent control (t.test; $t = 2.13$, $P = 0.037$; df = 58) and therefore were not combined for the sucrose consumption analysis. Cumulative sucrose utilization at day 10, adjusted for evaporation, was significantly different across the 9 treatment groups (Kruskal–Wallis; $P = 2.2E\text{-}16$, df = 8). A Dunn's post-hoc analysis revealed a significant decrease in sucrose consumption at concentrations greater than and equal to 0.1 mg a.i./kg treated matrix ($P < 0.05$) and results in a NOEC of 0.05 mg a.i./kg diet and a LOEC of 0.1 mg a.i./kg treated matrix. All detailed statistics for post-hoc tests are reported in Supp Table 3 [online only].

Analytical Verification of Test Concentrations

Recovery rates of diflubenzuron (Supp Table 4 [online only]) were on average 81, 85, and 82 % for test levels 0.1, 1.0 and 10 mg a.i. /kg

treated matrix respectively, which is within the EPA-recognized acceptable recovery range of 70–120% (US EPA 1996b). Furthermore, the coefficients of variation were 8.2, 5.4, and 7.1 % for the test levels 0.1, 1.0 and 10 mg/kg, respectively. The later values show a narrow distribution around the mean, indicative of a relatively uniform distribution of the test material in the pollen diet.

Analytical verification of the test levels was also conducted for diflubenzuron and dimethoate delivered via the pollen on a weekly basis. Recovery rates assessed each week for the duration of the 5-wk exposure period were within our target range of 70–120% for diflubenzuron pollen diets (Supp Table 5 [online only]) and dimethoate pollen diets (Supp Table 6 [online only]). Concentrations were also verified for 3 of the 6 test levels for the dimethoate treated sucrose solutions from the 10-d chronic adult study with recoveries of 86–99%.

Discussion

Our results demonstrate that bumble bee microcolonies can provide useful insight into how bumble bee larvae are exposed to a test substance within a colony and may be a useful tool for addressing immature bumble bee toxicity in the absence of a validated in vitro rearing method for individual bees. The test design simulates a more realistic exposure pathway to the larvae and provides insight into the barrier of exposure between workers and larvae.

Adult consumption of pollen is minimal, yet the microcolony pollen route of exposure did not prevent exposure to the adult worker bees based upon the mortality observed from dimethoate delivered via pollen. The worker survival NOEC for dimethoate was 1 mg a.i./kg treated matrix and the drone emergence NOEC for dimethoate was 3 mg a.i./kg treated matrix from a pollen route of exposure. Exposure through sucrose resulted in a lower endpoint for both worker survival (NOEC = 0.1 mg a.i./kg treated matrix) and drone emergence (NOEC = 0.1 mg a.i./kg treated matrix). The relationship between worker survival and drone emergence endpoints for exposure through pollen and through sucrose is not unexpected. The drone emergence endpoints are directly related to the worker survival endpoint, regardless of the route of delivery when a substance, like dimethoate, yields higher toxicity to the adult life stage than to the larval life stage. In contrast, exposure to diflubenzuron, a substance preferentially toxic to the larval life stage, did not impact adult bumble bee worker survival but still resulted in a significant decrease in drone emergence that is indicative of a greater sensitivity to diflubenzuron in the immature life stage. A recently published microcolony study exposed *B. impatiens* to diflubenzuron through sucrose and demonstrated a significant reduction in drone production and pollen consumption at 0.1 mg a.i./kg treated matrix, 100-fold lower than the effect threshold we found with exposure through pollen (Camp et al. 2020). The reduced sensitivity observed through the treated pollen matrix is a result of lower pollen consumption relative to sucrose consumption (Tasei et al. 2000, Řehoř et al. 2014, Richardson et al. 2015, Camp et al. 2020). Reduced pollen consumption yields a higher concentration-based endpoint as observed in our study. The total pollen utilized in our microcolony studies ranged from 6.9 to 9.1-fold less by weight than the amount of sucrose utilized. A limitation within the microcolony study design is that the provisioned sucrose and pollen are both consumed and used for constructing the nest matrix. Similarly, this is true at the full-colony level where a dietary dose to the individual or colony cannot be determined, but rather provides a concentration-based toxicity endpoint.

One limitation of the microcolony study design is that a specific dietary dose of a test item to individual bees or the colony cannot be determined. Predicted adult and larval toxicity should be considered when determining the appropriate route of exposure and may be based upon previously-collected honey bee laboratory toxicity data. However, other physiochemical properties of the substance of interest may also need to be taken into consideration. While oral exposure through sucrose has been the most frequent exposure route mostly for ease of mixing and quantification (Mommaerts et al. 2006, Gradish et al. 2013, Klinger et al. 2019), dosing through pollen can alleviate issues with stability and solubility and can allow for the use and removal of a solvent when integrating the test item into the diet. With the mixing strategy outlined in the present study, we found that a test item could be successfully integrated and quantified in the pollen diet (Supp Tables 4–6 [online only]). The exact mechanism of exposure to brood when dosed through pollen is unknown and is an aspect of bumble bee biology that needs further research. Workers themselves consume pollen and use it for nest construction in addition to provisioning developing larvae (Pereboom 2000, Dornhaus and Chittka 2005). From the amount of treated pollen or sucrose provisioned, it is difficult to assess the quantity consumed by both the larvae and the workers, as well as the amount used for nest construction. Despite this uncertainty, the lack of significant worker mortality in combination with significant differences in drone emergence from the diflubenzuron microcolony study suggests larvae are exposed to the test substance when delivered through pollen.

Exposure to the test item in the present study was initiated 10 d after workers were placed in microcolony chambers which allowed initial nest construction to be completed with untreated pollen and sucrose. Exposure initiation was based on the established timeline of when the laying female develops her ovaries and eggs are first laid, as documented in other studies (Röseler 1977, Cnaani et al. 2002) since our objective for the current study was to assess effects on bumble bee brood. We consider the chronic exposure in our study of 32 d as representative of a worse-case scenario and included multiple egg-laying events despite the 10-d acclimation and nest establishment period.

The microcolony study design is less adapted for assessing direct effects of substances that may yield a greater toxicity to adult bees than immature life stages. The drone emergence NOEC for dimethoate in sucrose in the microcolony study design was nearly equivalent to the worker survival NOEC in the 10-d chronic adult dimethoate study design. Our data confirm that direct brood effects cannot be observed within the microcolony test design when provisioned with a substance that elicits a direct effect on the adults, due to the reliance on adult workers to provision the diet to the brood. Our data suggests that the impairment of the workers by exposure to the test item will affect brood care, even if brood is not directly impacted by the test substance.

Adult honey bees and bumble bees consume considerably more nectar than pollen (Gradish et al. 2019) so exposure to the test substance through nectar has been adopted as a worst-case exposure both for relevance and ease of diet preparation (Dance et al. 2017). For worker bumble bees, daily sucrose consumption is estimated at 73–149 mg sucrose/bee/day compared to the pollen estimate, at 26.6–30.3 mg pollen/bee/day (Spurgeon et al. 2016). However, in our 10-d chronic adult study, we documented even higher sucrose consumption at 256 mg/bee/d. The difference in consumption rates between sucrose and pollen directly correlates with the oral dose. For example, honey bee colonies exposed to clothianidin through sucrose or through pollen resulted in a

toxicity endpoint nearly 20 times lower when dosed through sucrose (Olmstead 2018, US EPA 2020). Additionally, our microcolony study results support the use of sucrose treated matrix as a surrogate for nectar and as a more optimal matrix than pollen in the microcolony study as long as the test substance is soluble and/or homogeneous within the sucrose matrix. The onset of exposure to the developing brood is more immediate when the test substance is provisioned through sucrose, rather than through the pollen. The initial 5 g of pollen provisioned to the microcolony for nest establishment prevents the immediate consumption of treated pollen when it is first provisioned, however treated sucrose is readily consumed by the bees when first provisioned on day 10 of the study design.

Pollen may be considered for an alternative dosing mechanism to sucrose in the context of the microcolony study design when working with substances of low solubility and stability. The pollen delivery may also prevent the potentially-detrimental effects of a solvent if evaporated from the test diet as demonstrated in our study. This approach to working with volatile and potentially toxic solvents has been used in the field of aquatic toxicology and is recommended in honey bee guidelines as well (Cornement et al. 2017). The advantage of eliminating solvent effects with a pollen route of exposure should be considered when deciding between routes of exposure.

There are several limitations for the use of a microcolony study as a toxicity bioassay for use in a pollinator risk assessment. There is no direct equivalent of the microcolony assay for honey bees, making species sensitivity comparisons more difficult. As a test for developmental effects on immature bees, larvae are exposed through worker-provisioned food at varying amounts, therefore study endpoints are limited to concentration and entirely dependent upon the diet provisioning by the adult bees. Increased consumption through individual larval development as well as competition for food among clustered larvae also makes calculating individual doses not possible. Our primary goal was to determine whether exposure to adults could be prevented by dosing through the pollen diet. Our data clearly rejects our initial hypothesis and illustrate that worker exposure cannot be eliminated from the test system as evidenced by worker mortality in both sucrose and pollen routes of exposure with dimethoate (Fig. 3). If there is any repellency or feeding deterrence, both worker survival and drone emergence will be impacted over the duration of the study. Rather, the microcolony study design is optimal for assessing effects to bumble bee brood when no adverse effects are predicted to occur to the adult life stage, as in the case of diflubenzuron. It is difficult to predict toxicity to the adult stage in the absence of data, however data requirements within the current risk assessment framework generally rely upon the honey bee as a surrogate species in the pollinator risk assessment (US EPA 2014) and can provide the foundational data needed designing a study with a different bee species.

Another foundational question in our study was to understand the relative toxicity of our two test substances between honey bees and bumble bees to further inform the need for non-*Apis* testing. Our 10-d chronic adult bumble bee study allows for a direct comparison to honey bee endpoints. The 10-d chronic adult bumble bee survival endpoint resulted in a NOEC of 0.1 mg a.i./kg diet (Table 1). Our NOEC corresponds with a No Observed Effect Daily Dose (NOEDD) of 0.019 µg/bee/d based upon our mean measured daily sucrose utilization of 189.3 mg for bumble bees assigned to the test level 0.1 mg a.i./kg diet. A recently published international ring test across 17 laboratories determined a honey bee NOEC for dimethoate 0.28 mg a.i./kg diet, respectively (Kling and Schmitzer 2015). This honey bee NOEC corresponds with a NOEDD of 0.011 µg ai/bee/d. The NOEC is lower in our study relative to the honey bee endpoints, however, there is minimal difference in sensitivity between the species when accounting for dose due to the differences in food consumption. The bumble bees in the control group in our study consumed a mean daily amount of 256.3 mg sucrose solution, approximately 6-fold what honey bees consume in a day under laboratory conditions. Together, these results support the current EPA risk assessment framework that relies upon the honey bee as protective of other species of bees (US EPA 2014). A direct comparison on individual larval bee toxicity endpoints for dimethoate (Pollinator Research Task Force 2017) was not possible due to the limitations of the worker-mediated transfer of treated diet in the microcolony test design and the direct impacts we observed on adults.

Our diflubenzuron microcolony study resulted in a concentration-based endpoint that can be related to published honey bee larval endpoints. Since there was no significant worker mortality, drone emergence numbers are more representative of brood effects than in the dimethoate studies where there was significant worker mortality (Fig. 2). Dai et al. (2018) determined a LOEC of 45 mg a.i./liter det (equivalent to ~40 mg a.i./kg diet) and a NOEC of 6 mg/liter (equivalent to ~5.4 mg a.i./kg diet) based upon immature bee survival through adulthood after chronic exposure to diflubenzuron in the larval diet (Dai et al. 2018). In our diflubenzuron pollen microcolony study we determined a LOEC of 10 mg a.i./kg treated matrix and a NOEC of 1 mg a.i./kg treated matrix. As previously mentioned, we are unable to directly calculate a dose within the microcolony study design, however our results suggest that immature bumble bees may be more sensitive on a concentration-basis. Since the NOEC is dependent upon the spacing factor, additional studies are needed to further refine whether immature bumble bees demonstrate a greater sensitivity than immature honey bees.

Our study examined the utility of a microcolony study design for use in assessing the effects of a substance to immature bees. There is uncertainty around whether these worker-led microcolonies could be a suitable surrogate for a whole colony (Klinger et al. 2019). EFSA guidance suggests using microcolonies as higher tier testing (Spurgeon et al. 2016) however there is much debate about whether effect levels identified at the microcolony level would be protective of those for a queenright colony, with some data suggesting colonies are more sensitive than tested individuals (Whitehorn et al. 2012). Our data suggest that microcolonies provisioned with dimethoate through sucrose resulted in a similar endpoint as individually-housed workers in a 10-d chronic adult test. For substances with a greater sensitivity to adults than to immature bees, we would expect effects on workers would be the main driver of colony-level impacts. This is supported by Mommaerts et al. 2009 who found foraging pseudo-queen led microcolonies were predictive of the NOEC for foraging colonies. While these findings suggest that the microcolony may be an appropriate measure of worker-mediated colony failure, more research is needed to evaluate the relationship of a laboratory-based endpoints from the microcolony or individual adult worker to a colony level response of a substance.

The microcolony test design can provide valuable insight into the effects of a pesticide on brood within a bumble bee colony. This work is a foundation for a standardized approach to relating immature honey bee brood effects to other bee taxa, such as *B. impatiens*. Furthermore, our data support the surrogacy of honey bee toxicity data within the current pollinator risk assessment framework.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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Data Accessibility

Data pertaining to this manuscript are deposited in figshare at https://figshare.com/s/d645bff1038cd881db43 (DOI 10.6084/m9.figshare.12442607 has been reserved and will be made public pending acceptance of the manuscript).

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