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*Communication*

# Toxicity of Formulated Systemic Insecticides Used in Apple Orchard Pest Management Programs to the Honey Bee (*Apis mellifera* (L.))

**Sarah Heller [1,2], Julia Fine [3], Ngoc T. Phan [4,5], Edwin G. Rajotte [2], David J. Biddinger [1,2] and Neelendra K. Joshi [4,*]**

1 Penn State Fruit Research and Extension Center, Biglerville, PA 17307, USA; saraheshugrue@gmail.com (S.H.); djb134@psu.edu (D.J.B.)
2 Department of Entomology, The Pennsylvania State University, University Park, PA 17307, USA; uvu@psu.edu
3 Invasive Species and Pollinator Health Research Unit, US Department of Agriculture-Agricultural Research Service, 3026 Bee Biology Rd., Davis, CA 95616, USA; julia.fine@usda.gov
4 Department of Entomology and Plant Pathology, University of Arkansas, Fayetteville, AR 72701, USA; pearlp@uark.edu
5 Research Center for Tropical Bees and Beekeeping, Gia Lam, Hanoi 100000, Vietnam
* Correspondence: nkjoshi@uark.edu or neeljoshi1005@gmail.com

**Abstract:** Honey bees (*Apis mellifera*) are one of the most important pollinating species of flowering plants. Recently, populations of honey bees have been declining due to a combination of factors, including the widespread use of agricultural pesticides. Laboratory studies were conducted to determine the acute oral toxicity of different formulated pesticides to honey bee adults. In particular, we assessed the acute oral toxicity of two neonicotinoids (acetamiprid, Assail 30SG and thiamethoxam, Actara 25WDG) and two other systemic insecticide products (sulfoxaflor, Closer 2SC and flupyradifurone, Sivanto 200SL), all of which are generally used in pest management programs in commercial apple orchards in the Eastern United States. Honey bees were fed a range of doses of each pesticide in order to create a response curve, and $LC_{50}$, $LC_{90}$, and $LD_{50}$ values were determined. The pesticide formulation containing flupyradifurone as the active ingredient was found to be the least toxic to honey bees followed by the formulations containing sulfoxaflor and acetamiprid. The toxicity values obtained in this study differ from other studies testing only technical active ingredient compounds, suggesting the need to evaluate formulated products while conducting ecotoxicological risk assessment.

**Keywords:** neonicotinoids; bees; environmental toxicology; insecticides; pollinator health; pesticide formulations



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## 1. Introduction

Pollinators provide essential ecosystem services, supporting approximately 85% of the world's flowering plants [1] and 35% of the global crop production [2]. Pollinator health has become a pressing issue as populations of both managed and native pollinators are declining worldwide [3]. Honey bees (*Apis mellifera* L.) are the most studied pollinators and are considered the most valuable [4]. Honey bee populations, however, have been affected by a combination of environmental factors in recent years [5]. Studies suggest that bee declines, generally, are caused by the interaction of many stress factors, including pathogens, parasitic mites, pesticides, lack of forage and nesting habitat due to intensive monocultures, and stress from poor nutrition and the transport of hives for commercial pollination [6–9]. Pesticide exposure has received a disproportionate amount of media attention due to the presence of agricultural chemicals detected in honey bee wax and pollen [10–12].

One class of pesticides, known as neonicotinoids, has been implicated in increased mortality of honey bee colonies [13–15] and has become the subject of much debate [16] and regulatory restrictions on its use [17]. Neonicotinoids are systemic pesticides that, once applied by foliar sprays, trunk injection, or root drenching, are transported throughout the plant vascular tissue and protect against sap-feeding insects. Neonicotinoids act as nicotinic acetylcholine receptors, blocking the transmission of nerve impulses in the central nervous system of insects [18,19]. Bees can encounter systemic insecticides, such as neonicotinoids, in many ways, including direct contact with foliar sprays or particles released during treated seed planting, contacting residues on treated plants or nesting substrate, or consuming contaminated pollen, nectar, and water [20–22]. Neonicotinoids may also have indirect effects on honey bees by altering the community structure of the bee gut microbiome [23]. However, because of their lower mammalian toxicity, neonicotinoids are sometimes considered lower risk relative to other pesticides [24]. Different pesticides, including neonicotinoids and other reduced-risk insecticides, are used in insect pest management in apple orchards [25–30]. In the Eastern US, apple orchards, insecticides are generally not applied during the 7 to 10-day bloom period. Therefore, oral exposure through contaminated pollen and nectar from the systemic movement in the plant of pre-bloom applications is the primary route of exposure in orchards [31], and such exposure could be toxic to non-target species as the $DT_{50}$ values of systemic insecticides used in orchard pest management are usually longer than the bloom period.

Although much research has been undertaken on the effects of some neonicotinoids on bee health [32–36], few studies have been performed on the newer neonicotinoids and systemic active ingredient compounds that are applied as foliar sprays in tree fruit crops such as apples [37]. Federal safety regulations for pesticide registration require assessing pollinator risk by performing toxicity laboratory studies on honey bees, using the active ingredient of a substance rather than the formulated product [38]. However, formulated pesticides usually contain inert ingredients in higher amounts than the active ingredient, and recent studies have shown that consumption of these inert ingredients can adversely affect target and non-target insects [39,40].

The main objective of this study was to determine the risk to honey bees associated with the use of formulated neonicotinoids and neonicotinoid-related products as categorized by the Insecticide Resistance Action Committee (IRAC). In particular, we assessed the acute oral toxicities of two formulated neonicotinoids (IRAC group 4A) and two other systemic insecticide products (IRAC group 4C and 4D) commonly used in apple production in the Eastern United States.

## 2. Materials and Methods

### *2.1. Insects and Experimental Design*

Adult worker bees were sourced from three different hives in the Penn State apiary at University Park, PA, located within about 60 m of each other. House bees or middle-aged bees were selected by shaking frames of honey [41]. These hives were maintained according to best management practices [42], and the bees were in good health. After bees were collected from the hives, they were starved for four hours and placed into group feeding cages. Five bees from each hive were placed into each cage to eliminate hive effects for 15 bees per cage in total. Cages were constructed as described in Biddinger et al. [43]. A Petri dish (100 mm × 20 mm) encased the open ends of a 100 mm-long wire mesh cylinder. Each Petri dish had a hole made with a heated cork-borer, one side with a larger hole to put bees in and remove dead bees, and the other side with a smaller hole where the feeder hung. The feeder was a 1.7-milliliter centrifuge tube with two pin-sized holes in the bottom to allow *ad libitum* feeding.

### *2.2. Pesticide Treatments*

The commercial formulations (AI%; manufacturer) of the treatments were the neonicotinoids (IRAC Code 4A) Assail 30SG (acetamiprid 30%; United Phosphorous Inc., King of

Prussia, PA, USA) [44] and Actara 25WP (25.0% thiamethoxam, Syngenta, Wilmington, DE, USA) [45], and the neonicotinoid-related compounds (IRAC Code 4D and 4C, respectively) Sivanto 200SL (flupyradifurone 17.09%, Bayer CropScience, Research Triangle Park, NC, USA) [46] and Closer 240SC (sulfoxaflor 21.8%, Dow AgroSciences LLC, Indianapolis, IN, USA) [47]. One dose of Cygon 400 (dimethoate 43.5%; Drexel Chemical Company, Memphis, TN, USA) [48] was included as a positive control [49], and negative control bees had access to 50% sucrose solution.

The feeding protocol was adapted from the Organization for Co-operation and Development Test No. 213: Honeybees, Acute Oral Toxicity Test [50]. Treatment doses were chosen based on previously published median lethal doses for the active ingredient of each pesticide (acetamiprid [51], thiamethoxam [52], flupyradifurone [53], and sulfoxaflor: average derived from multiple reports [54,55]). The median lethal dose, or $LD_{50}$, is the dose required to kill half of a test population. For each pesticide formulation used in this study, a range of five dose treatment concentrations (10, 20, 40, 80, and 160 ppm) were extrapolated from the published data, aiming to produce mortalities between 10 and 90%, as suggested in Robertson et al. [56]. These values were converted into a concentration per bee, with 50% sucrose solution as the solvent. After the starvation period, bees were given a feeder with 400 microliters of the assigned treatment. Bees were watched until approximately 200 microliters of the treatment was consumed volumetrically, at which point the feeder was replaced with a feeder containing 50% sucrose solution only. All bees consumed the 200 microliters within 4 h. Three replicate cages of bees were tested with each treatment, for a total of 45 bees per dose and 225 bees per chemical. Cages were placed inside large plastic containers, which also contained a jar of saturated NaCl solution to maintain ~75% relative humidity. All plastic containers were kept in a growth chamber at 34.5 °C in darkness.

### *2.3. Data Collection and Analysis*

The amount of treatment solution consumed by weight was recorded and converted into an average dose per bee assuming equal consumption by individuals in a treatment cage. Mortality and behavioral observations were recorded at zero, 12, 24, and 48 h post-exposure. Bees were considered dead when they remained absolutely still during the 30 s observation period (similar to Laurino et al. [57]). Quantal response regressions were estimated assuming the normal distribution (i.e., probit model) with the computer program POLOPlus 2.0 (LeOra Software [58]) as described by Robertson et al. [56]. The median lethal concentration ($LC_{50}$ and $LC_{90}$) values with 95% confidence intervals were also calculated using POLOPlus 2.0, and the median lethal dose ($LD_{50}$) values were calculated based on the procedure described in Phan et al. [59].

## 3. Results and Discussion

In this study, the negative control mortality (n = 45) was 0%, and positive control mortality (n = 45) was 4.4%. Response curves (Figure 1) and $LC_{50}$ and $LC_{90}$ values (Table 1) were generated for pesticide formulations containing acetamiprid, flupyradifurone, and sulfoxaflor as individual active ingredients. A valid toxicity response curve could not be generated by POLOPlus 2.0 for pesticide formulation containing thiamethoxam because the data points were concentrated at very low dose mortality, producing too much variance for a correct calculation of toxicity values. However, we expected to be able to create response curves for all four chemicals, because the doses were based on published data and we used the full range necessary for POLOPlus to generate valid curves [56,60]. The sources of variation could be the differences in age [61] or genetic lineage [62] of the bees used in our study.

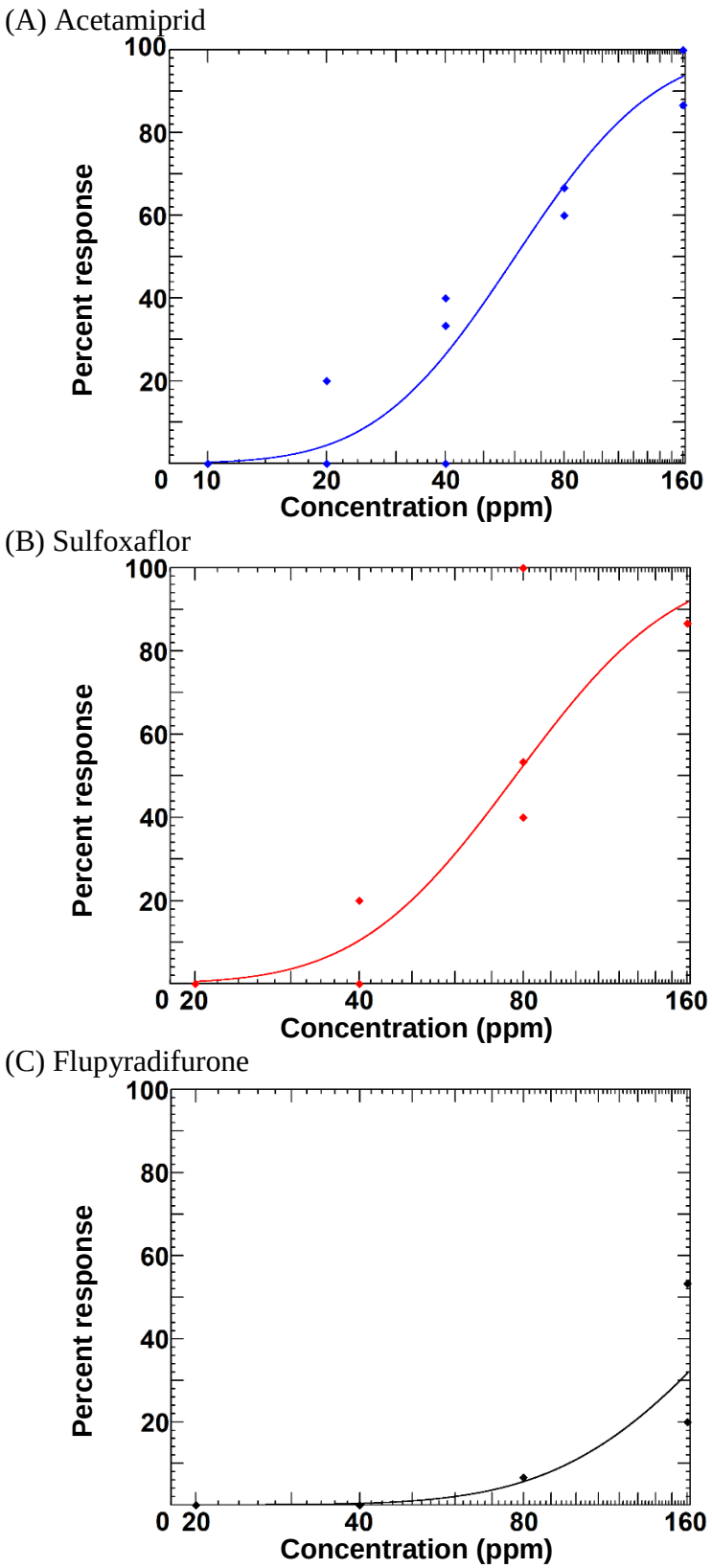


**Figure 1.** Toxicity responses of *Apis mellifera* to different systemic pesticides: acetamiprid (**A**), sulfoxaflor (**B**), and flupyradifurone (**C**) through ingestion bioassays (at 48 h after treatment). The points represent percent response; when two replications had the same percent response, the points overlapped.

**Table 1.** Response curve slopes, $LC_{50}$, and $LC_{90}$ values for different pesticides using mortality observations at 48 h post-feeding.

| Product (Active Ingredient) | n ‡ | Slope ± SE | $LC_{50}$ (ppm) (95% CL) | $LC_{90}$ (ppm) (95% CL) |
|---|---|---|---|---|
| Assail (acetamiprid) | 225 | 3.58 ± 0.41 | 59.93 (48.51–74.92) | 136.77 (103.57–218.05) |
| * Actara (thiamethoxam) | 135 | - | - | - |
| Closer (sulfoxaflor) | 180 | 4.39 ± 0.55 | 77.32 (60.85–98.32) | 151.37 (115.21–259.39) |
| Sivanto (flupyradifurone) | 180 | 3.69 ± 0.97 | 214.96 (167.77–406.45) | 477.85 (294.85–2025.36) |

* Values were not calculated as the data points were concentrated in the very low mortality zone. ‡ n is the number of individuals included in the analysis.

Conversely, the range of mortalities observed was great enough to calculate valid $LC_{50}$ and $LC_{90}$ values for acetamiprid, flupyradifurone, and sulfoxaflor-based product formulations. The number of individuals included in the analysis (n), the slope of the response curve, and 95% confidence intervals for the $LC_{50}$ and $LC_{90}$ values are also reported (Table 1). These values are not usually reported in conventional toxicity testing, but they are essential for making comparisons between different chemicals, different bee species, different populations of the same species, and for extrapolating the toxicity of a known dose [56,60]. A similar trend in the toxicity was also observed in the $LD_{50}$ values (µg/bee) of these formulated products in this study. The flupyradifurone active ingredient-based formulation was the least toxic ($LD_{50}$ = 2.87 µg/bee, 95% CL = 2.24–5.42), followed by sulfoxaflor ($LD_{50}$ = 1.03 µg/bee, (95% CL = 0.81–1.31), and acetamiprid ($LD_{50}$ = 0.80 µg/bee, 95% CL = 0.65–1.00). These toxicity values differ from other studies conducted using technical compounds [51,54,55,63–66]. For instance, the $LD_{50}$ value (2.87 µg/bee) for the formulation containing flupyradifurone was over two times higher than the $LD_{50}$ value reported by the US EPA [66] and Nauen et al. (1.20 µg/bee) [67]. However, the $LD_{50}$ value for flupyradifurone in our study was slightly lower than the $LD_{50}$ value (2.995 µg/bee) reported by Tosi and Nieh [66] in a recent study. Toxicities of these formulated products may also vary among products containing the same active ingredient. For instance, in a laboratory study, the $LD_{50}$ value for a different acetamiprid-based formulated product was 1.69 µg/bee [68]. Such differences could be likely due to the differences in the composition of inactive ingredients.

Most toxicity bioassays are generally conducted using technical active ingredient compounds. However, pesticide formulations contain several other inactive ingredients, and toxicity differences in the current study could be likely due to the use of formulated products compared to previous studies where only technical active ingredients (i.e., active substances) were evaluated [51,54,55,63–66]. In general, the inactive ingredients in pesticide formulations are assumed to be inert and are not included in the required pesticide registration toxicity tests for pollinators [39]. There is much debate over the oral toxicity of the inactive ingredients of formulated pesticides, but consumption of these "inert" ingredients has been shown to cause adverse effects, including learning impairments in honey bees [39,40]. The impact of pesticide formulations on pollinator health must be further investigated, and using formulated products is necessary for pesticide toxicity testing, and in addition, differences in the toxicity of technical active ingredients and associated formulated products highlight the need for future studies in this direction.

Much of the mortality occurred within 12 h of feeding. However, all bees were sustained on sucrose solution long after the experiment was over, and only the untreated control bees survived beyond 7 days, indicating delayed mortality in treated bees. The poisoning symptoms observed mirrored those already reported in similar studies [59,69]. Sublethal effects such as staggering, partial paralysis, abdomen tucking, and twitching were observed to some degree in all treated bees during the feeding period, but most

often in those treated with thiamethoxam and sulfoxaflor. These observations could be an indication of sublethal and chronic effects of pesticide consumption. These effects are not observed in the 48 h acute pesticide testing and can impact longevity, reproduction, and colony health [70]. Therefore, it is important to conduct both short- and long-term toxicity studies while conducting pesticide risk assessment.

To further advance pesticide registration testing, moving beyond using *A. mellifera* as a surrogate species is necessary. Previous research has found that there are differences in the contact toxicity of systemic pesticides to *A. mellifera* and another apple pollinator, *Osmia cornifrons* (Radoszkowski, 1887) [43]. Although it is impossible to test all bee species, including a few other surrogates, such as an *Osmia* (mason bee) or *Bombus* (bumble bee) species, in pesticide registration and extension of testing would greatly improve our knowledge of the threat to pollinators.

Additional research is necessary on the toxicity of the inert ingredients in systemic pesticide formulations and their ability to move to the pollen and nectar, as consumption is a primary route of exposure for pollinators. Additionally, the data reported on neonicotinoid toxicity to pollinators varies greatly due to inconsistent testing methods [71]. The development of more field-realistic toxicity testing is vital as honey bees are exposed to insecticides through various routes [20,72]. This would include using pesticide formulations, as well as toxicity testing on all life stages, long-term studies to observe sublethal effects, choice tests, field-scale assays, and varying bee species [73]. This range of exposure testing is not currently considered during the registration or regulation of insecticides by the United States Environmental Protection Agency [74]. Moving forward, we believe field-scale studies where pesticide application is highly controlled and bee behavior and reproduction can be observed would be best. A uniform toxicity testing technique that emphasizes field-realistic conditions with formulated pesticides could reduce the variability in reported data and produce results that better represent the effects seen in the field. Additionally, legally requiring reporting of response curve slopes and confidence intervals around $LD_{50}$ values would allow more detailed conclusions to be drawn, such as which chemical is more toxic and at what dose. With more reliable and consistent toxicity data, integrated pest management programs can be adjusted to protect pollinators while controlling pests [75].

## 4. Conclusions

In this study, the toxicities of different systemic insecticide formulations to honey bees were established. In terms of the $LC_{50}$ and $LD_{50}$ values, the formulation based on the active ingredient flupyradifurone was found to be the least toxic, followed by sulfoxaflor and acetamiprid-based formulations. The toxicity of formulated insecticide products to honey bees may differ from the toxicity of technical active ingredients. The toxicity values determined in this study are based on the formulated insecticide products that growers apply on their farms, which is more realistic than testing active ingredients alone as honey bees are exposed to formulated pesticides and not just the technical active ingredients. There is increasing evidence of differences in the toxicity of technical active ingredients and their associated formulated products. The current pesticide risk assessment framework could be refined further to incorporate formulated pesticides while conducting ecotoxicological risk assessments since many 'inert ingredients' can cause adverse effects in honey bees and other non-target species.

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