Ecotoxicology and Environmental Safety 164 (2018) 283–288

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

The impacts of chlorothalonil and diflubenzuron on *Apis mellifera* L. larvae reared *in vitro*

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ARTICLE INFO

Keywords:
Apis mellifera
Larvae
Toxicity
Chlorothalonil
Diflubenzuron

ABSTRACT

Chlorothalonil is a broad-spectrum fungicide and diflubenzuron is an insect growth regulator used to control many insect larvae feeding on agricultural, forest and ornamental plants. Honey bee larvae may be exposed to both *via* contaminated pollen, in the form of beebread, added to their diet by their adult nurse sisters. In this study, we determined how single (acute: 72 h mortality) and repeated (chronic: mortality until emergence as adults) exposure to chlorothalonil and diflubenzuron in their diet affected honey bee larvae reared *in vitro*. The tested doses of chlorothalonil (20, 100, or 200 mg/L) did not impact 72 h larval mortality acutely relative to that of the solvent control. The 72 h mortality of larvae exposed to 1.6 mg/L and higher doses of diflubenzuron acutely in their diet (47.2–63.9% mortality) was significantly higher than that of larvae fed the solvent control, with no predictable dose dependent pattern observed. In the chronic toxicity tests, consuming an artificial diet with 30 or 100 mg/L chlorothalonil and 0.8, 1.3 or 2 mg/L diflubenzuron significantly lowered the survival of honey bee larvae over that of larvae feeding on the solvent control diet. We calculated risk quotients (RQs) for both compounds using the data we generated in our experiments. Collectively, the RQs suggest that neither compound is likely to affect larval mortality directly at field relevant doses given that pollen composes only a fraction of the total larval diet. Nevertheless, our data do not preclude any sublethal effects that chronic exposure to either compound may cause.

1. Introduction

Western honey bee (*Apis mellifera* L.) colonies can be exposed to pesticides that are present in water, nectar, pollen, or propolis (Johnson et al., 2010; Johnson, 2015; Mullin et al., 2010; Badiou-Bénéteau et al., 2013; Rundlöf et al., 2015). In addition, pesticides and corresponding metabolites are brought back to the hive and stored in hive matrices to which adult and immature honey bees may be exposed (Johnson et al., 2010; Mullin et al., 2010; Sánchez-Bayo and Goka, 2014). Larval bees, specifically, may be exposed to pesticides *via* their diet (Human et al., 2014). To date, most of the honey bee-related toxicology studies conducted have focused on adult bees, while fewer have focused on immature ones. Those that have been conducted (Aupinel et al., 2007; Gregorc and Ellis, 2011; Wu et al., 2011; Gregorc et al., 2012; Charpentier et al., 2014; Thompson et al., 2014; Zhu et al., 2014; Dai et al., 2017; Staroň et al., 2017, and others) have focused on the more traditional neurotoxic insecticide chemistries rather than on pesticides such as fungicides or insect growth regulators (IGRs). This led to our decision to assess chlorothalonil (fungicide) and diflubenzuron (IGR) impacts on honey bee larvae.

Chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a broad spectrum agricultural and a contact nonsystemic fungicide (Caux et al., 1996; Cox, 1997) in the organochlorine class. It reduces fungal intracellular glutathione molecules to alternate forms that cannot participate in essential enzymatic reactions, ultimately leading to cell death (Tillman et al., 1973). Chlorothalonil is often applied to blooming crops when honey bees are present for pollination, thus providing a possible route of exposure. It has been shown to affect larval survival (Zhu et al., 2014), the structure and function of honey bee gut bacterial communities (Kakumanu et al., 2016), and honey bee susceptibility to *Nosema* infection (Pettis et al., 2012, 2013; Wu et al., 2012).

Diflubenzuron is an insect growth regulator (IGR) used as an

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https://doi.org/10.1016/j.ecoenv.2018.08.039
Received 18 April 2018; Received in revised form 4 July 2018; Accepted 10 August 2018

acaricide/insecticide to control immature forms feeding on agricultural, forest and ornamental plants (*e.g.* gypsy moths, mosquito larvae, rust mites) (EPA, 1997). Diflubenzuron, a benzoylphenylurea, interferes with chitin synthesis (Cohen, 1987), causing new cuticle to develop malformed (Hassan and Charnley, 1987).

Residues of chlorothalonil and diflubenzuron have been found in pollen collected by honey bees: chlorothalonil - from 1.1 to 98,900 µg/kg; diflubenzuron - from 15 to 128 µg/kg (Mullin et al., 2010; Sánchez-Bayo and Goka, 2014). Maximum residues of 15.8 µg/kg chlorothalonil have been found in nectar/honey (Sánchez-Bayo and Goka, 2014). Diflubenzuron residues have not been reported in nectar/honey. Low water solubility might account for diflubenzuron absence in nectar. Systemic insecticides stand out for their high prevalence among the residues in nectar/honey, but some insecticides with high lipophilicity, such as pyrethroids, such as bifenthrin, esfenvalerate and fenpropathrin are typically absent from nectar/honey, unless they are used as varroacides, such as tau-fluvalinate (Sánchez-Bayo and Goka, 2014). A fermented mixture of pollen and nectar, known as beebread, is added to the diet of larval bees by their adult nurse sisters (Haydak, 1970). This represents a clear route of exposure for honey bee larvae to these compounds. Consequently, we hypothesized that both compounds would impact the survival of honey bees exposed to the compounds while feeding as larvae. Accordingly, our objective was to determine how honey bee larvae reared *in vitro* are affected by acute (single) and chronic (repeated) exposure to chlorothalonil and diflubenzuron *via* their diet.

2. Materials and methods

2.1. Pesticides

The test pesticides chlorothalonil (Product no. N-11454-250MG, purity 98.0%, expiration 12/31/2019) and diflubenzuron (product number N-11722–250MG, purity 99.5%, expiration 8/30/2018) were purchased from Chem Service, Inc. (660 Tower Lane West Chester, PA 19380, United States). All compounds were handled and mixed into larval diets according to standard protocols to ensure that we achieved the target dose/concentration (Medrzycki et al., 2013). We weighed the pesticides using a precision balance (Mettler Toledo, Model #: AL204, Columbus, OH, USA). The balance and all pipettes (HandyStep®, Brand, BrandTech Scientific, Essex, CT, USA) undergo routine calibration. Furthermore, all pesticide/diet combinations were mixed thoroughly using a vortex mixer. All stock solutions were kept covered to avoid concentration changes due to solvent evaporation.

2.2. Honey bee larvae rearing conditions

Experiments were conducted at the Honey Bee Research and Extension Laboratory, Entomology and Nematology Department, University of Florida (Gainesville, FL, USA) during summer 2016. Honey bee larvae were reared *in vitro* according to Schmehl et al. (2016). Our discussion of the *in vitro* timeline corresponds to Schmehl 's et al. (2016) Table 3, column 3, where we discuss all timepoints from grafting as day D = 0 or D0 (grafted larvae are 87 ± 12 h old at this timepoint, and this includes the egg stage). Honey bee queens were caged on a wax comb (D-4) for 24 h to lay eggs. At D0, the larvae were transferred from the comb to 48-well tissue culture plates with 20 µL of diet A (royal jelly 44.25%, glucose 5.3%, fructose 5.3%, yeast extract 0.9% and water 44.25%) prepared in each well. On D2, each larva was fed 20 µL of diet B (royal jelly 42.95%, glucose 6.4%, fructose 6.4%, yeast extract 1.3% and water 42.95%). On D3, 4 and 5, each larva was fed 30 µL, 40 µL and 50 µL, respectively, of diet C (royal jelly 50%, glucose 9%, fructose 9%, yeast extract 2% and water 30%). Larvae were transferred from the larval culture plates to the prepared pupal culture plates when all available diet had been consumed (as early as D6). The larval culture plates were maintained at 94% R.H. and 35 °C, and pupal culture plates were maintained at 75% R.H. and 35 °C. Adult worker bees began to eclose as soon 18 days after grafting. Emerging adults were collected at least twice daily and were maintained in hoarding cages with *ad libitum* access to pollen and 50% sugar water solution (w/v) (Schmehl et al., 2016).

2.3. Single exposure

Pilot studies were conducted to determine a suitable concentration range to use for both compounds. Chlorothalonil and diflubenzuron were dissolved in acetone to prepare the stock solution. The stock solution concentrations were 1000, 5000 and 10,000 mg/L for chlorothalonil, and 39, 78, 156, 313, 625, 1250, 2500 and 5000 mg/L for diflubenzuron. We dissolved 30 µL stock solution into 1470 µL diet C respectively, and the solvent accounted for 2% of the volume in the final diets. The concentrations tested for both compounds were as follows: chlorothalonil: 20, 100 and 200 mg/L; diflubenzuron: 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 and 100 mg/L. A solvent control was included in the study. Three replicates were run for all concentrations of both compounds. Larvae from a different colony were used for each replicate. On D3, 36 larvae per treatment (12 larvae × 3 colonies = 36 larvae) were selected and each larva was fed 30 µL of diet C (Schmehl et al., 2016) containing the assigned treatments. Larval mortality was recorded on D4, D5 and D6 (project termination), at the same time each day. Mortality was determined by viewing larval movement and the activity of the spiracles under a dissecting microscope. Immobile larvae or larvae failing to respire (*i.e.* no spiracle movement) were considered dead.

2.4. Repeated exposure

We used information on the acute toxicity (our single exposure data) of chlorothalonil and diflubenzuron to bees and residue levels reported in pollen (Mullin et al., 2010) to develop our test ranges for both compounds in the repeated (chronic) exposure tests.

1) Chlorothalonil: 1 mg/L (1/3 mean residue in pollen, Mullin et al., 2010; 100× mean residue in nectar/honey, Sánchez-Bayo and Goka, 2014), 10 mg/L (3× mean residue in pollen, Mullin et al., 2010; 625× maximum residue in nectar/honey, Sánchez-Bayo and Goka, 2014), 30 mg/L (10× mean residue in pollen, Mullin et al., 2010; 2063× maximum residue in nectar/honey, Sánchez-Bayo and Goka, 2014) and 100 mg/L (maximum residue in pollen, Mullin et al., 2010; 6250× maximum residue in nectar/honey, Sánchez-Bayo and Goka, 2014)
2) Diflubenzuron: 0.13 mg/L (maximum residue in pollen, Mullin et al., 2010), 0.8 mg/L (10× mean residue in pollen, Mullin et al., 2010), 1.3 mg/L (10× maximum residue in pollen, Mullin et al., 2010), 2 mg/L (15× maximum residue in pollen, Mullin et al., 2010). Diflubenzuron residues have not been reported in nectar/honey.

The pesticides were dissolved in acetone to prepare stock solution, and the solvent accounted for 0.5% of the volume of the final diets.

The following treatments were conducted for each test solution: four concentrations of the test solution, negative control, solvent control, and 45 mg/L dimethoate as a positive control. Dimethoate is most commonly used as a positive control in toxicity tests due to its high toxicity to honey bees (OECD, 2016). Five replicates were used per treatment group with each replicate composed of larvae from a single, but different colony (N = 5 colonies). Additional plates of larvae reared *in vitro* were used at D2 to replace the larvae that died before the test diets were administered. On D2, 12 robust larvae from each of the five colonies were selected and fed 20 µL of diet B (Schmehl et al., 2016) containing one of the test treatments. From this point, diets in all future daily feedings (D3–D5, Schmehl et al., 2016) contained their respective