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Safety of methionine, a novel biopesticide, to adult and larval honey bees (*Apis mellifera* L.)

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ABSTRACT

Methionine is an essential/indispensible amino acid nutrient required by adult and larval honey bees (*Apis mellifera* L. [Hymenoptera: Apidae]). Bees are unable to rear broods on pollen deficient in methionine, and reportedly behaviorally avoid collecting pollen or nectar from florets deficient in methioinine. In contrast, it has been demonstrated that methionine is toxic to certain pest insects; thus it has been proposed as an effective biopesticide. As an ecofriendly integrated pest management agent, methionine boasts a novel mode of action differentiating it from conventional pesticides, while providing non-target safety. Pesticides that minimize collateral effects on bees are desirable, given the economic and ecological concerns about honey bee health. The aim of the present study was to assess the potential impact of the biopesticide methionine on non-target adult and larval honey bees. Acute contact adult toxicology bioassays, oral adult assessments and chronic larval toxicity assessments were performed as per U.S. Environmental Protection Agency (EPA) requirements. Our results demonstrated that methionine fits the U.S. EPA category of practically nontoxic (*i.e.* lethal dose to 50% mortality or LD_{50} > 11 μg/bee) to adult honey bees. The contact LD_{50} was > 25 μg/bee and the oral LD_{50} was > 100 μg/bee. Mortality was observed in larval bees that ingested DL-methionine (effective concentration to 50% mortality or EC_{50} 560 μg/bee). Therefore, we conclude that methionine poses little threat to the health of the honey bee, due to unlikely exposure at concentrations shown to elicit toxic effects.

1. Introduction

Mounting resistance in pest insect species to currently-registered synthetic compounds has led to a pressing need for insecticide resistance management (IRAC, 2014), including new effective control options. However, the development of new tools is increasingly difficult due to few novel modes of action to target pests, compounded by demanding registration requirements. This reduced ability to protect our food, our animals and ourselves from pests has underscored the need for integrated pest management (IPM). IPM is the integrated use of several tools or tactics for sustainable management of a pest (Ehler, 2006). Effective biopesticides are necessary additions to the IPM arsenal as alternatives to traditional synthetic pesticides. Biopesticides are agents that contain a natural product, such as a living microorganism, organic, or inorganic material, as the active ingredient (Chandler et al., 2011). Examples of biochemicals commonly used for IPM include the pyrethrins from *Chrysanthemum cinerariaefolium* (Trevis.) Vis. (Asteraceae), and the spinosyn derivatives from *Saccharopolyspora* spp. (Pseudonocardiaceae). Examples of microorganism-based biopesticides include *Bacillus thuringiensis* (Berliner; Bacillaceae), *Beauveria bassiana* (Bals.–Criv.) Vuill. (Cordycipitaceae), and *Metarhizium anisopliae* (Metschn.) Sorokin (Clavicipitaceae).

The non-essential non-protein amino acid canavanine has been studied for potential use in pest control (Rosenthal, 2001). Canavanine is produced by leguminous plants as a natural toxin for protection against phytophagous insects and other herbivores. It was found to be effective against the tobacco horn worm *Manduca sexta* L. (Lepidoptera: Sphingidae), causing reduced body weight, prolonged developmental times, and reduced fecundity and fertility (Dahlman and Rosenthal, 1975; Dahlman, 1977). However, the detoxification pathways of some

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pest insects led to substantial resistance to canavanine (Berge et al., 1986; Rosenthal, 2001).

Unlike canavanine, methionine is an essential/indispensible amino acid, meaning that it cannot be synthesized *de novo*. Methionine has been demonstrated to be active against certain pest insects at concentrations somewhat above their low baseline levels. As an indispensible nutrient, methionine is unlikely to cause the development of resistance in pest targets, because it is required in miniscule amounts for their survival (House, 1965; Nation, 2015).

Investigations into using methionine as a biopesticide began following the initial discovery in *M. sexta* (Feldman et al., 2000). Methionine disrupts the function of the cation-amino acid transporter/channel, CAATCH1, an insect gut epithelium amino acid transporter exhibiting cation channel properties (Feldman et al., 2000; Quick and Stevens, 2001; Stevens et al., 2002; Liu et al., 2003). Exposure of *Manduca* CAATCH1 to L-methionine enhanced net K^+ current conductance while simultaneously blocking Na^+ currents (Quick and Stevens, 2001). On the other hand, the amino acid, proline, unlike methionine that binds and disrupts this transporter, binds CAATCH1 to promote desirable conductances of both cation currents (Feldman et al., 2000; Quick and Stevens, 2001). For this reason, proline is often used as a negative control for studying methionine effects on target organisms. These *in vitro* studies, completed with *M. sexta*, led to a series of *in vivo* experiments demonstrating pesticidal effectiveness of methionine in different pest organisms including the insects *M. sexta*, the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), and the giant swallowtail *Heraclides (Papilio) cresphontes* Cramer (Lepidoptera: Papilionidae), as well as the pest nematodes *Belonolaimus longicaudatus* Rau (Belonolaimidae) and *Mesocriconema ornata* Raski (Criconematidae) (Long et al., 2003; Long, 2004; Crow et al., 2009; Zhang and Crow, 2010; Zhang et al., 2010; Lewis et al., 2011). In each case, the control amino acid proline had no significant effect on survival of these pests in contrast to the observed lethal effects of methionine.

Crystalline DL-methionine is utilized worldwide as an aquaculture feed supplementation for farmed fish and shrimp (Nunes et al., 2014), and routinely on a massive scale in livestock feedlots for bovine, poultry, and porcine nutritional supplementation (Zhang et al., 2015). The racemic DL-form of methionine is used, rather than the isomers, because it is considerably less expensive than the L-methionine stereoisomer, and it is nutritionally as effective as L-methionine in animals raised for meat (Business Research Store, 2017). Relating to pesticidal activity, our studies have shown that DL-methionine has the same effects as L-methionine against *Aedes aegypti* L. (Diptera: Culicidae) larvae (Long, 2004), with mortalities due to either the L- or D-methionine stereoisomer being similar. In the bioassays for *H. cresphontes*, L- and DL-methionine were both effective (Lewis et al., 2011). Therefore, we utilized DL-methionine in the present study.

Regarding non-target beneficial insects, studies show minimal effects of methionine on the mottled water hyacinth weevil (*Neochetina eichhorniae* Warner [Coleoptera: Brachyceridae]) and the spotted lady beetle (*Coleomegilla maculata* De Geer [Coleoptera: Coccinellidae]), which were topically exposed to and feeding on leaves treated with 1% methionine, an exceedingly high concentration (Long, 2004). Additionally, there was no effect on a common aphid parasitoid *Lysiphlebus testaceipes* Cresson (Hymenoptera: Braconidae) when measuring aphid (*Aphis gossypii* Glover [Hemiptera: Aphididae]) parasitism on cotton plants (Long, 2004).

While these studies are promising in demonstrating the selectivity of methionine as a biopesticide to certain pest insects, the tested species are not common surrogates used to evaluate non-target effects by the U.S. Environmental Protection Agency (EPA) and other global regulatory authorities. The most common surrogate species for non-target insects is the western honey bee (*Apis mellifera* L. [Hymenoptera: Apidae]). The U.S. EPA requires substantial data on the honey bee for any pesticide applied where bees and other pollinators may be present, including data on potential toxicity to the adult and immature developmental stages (EPA, 2012, 2014, 2017).

In the present study, we hypothesized that DL-methionine would be safe (without adverse effects) to both adult and larval *A. mellifera*. We sought to define a specific potentially toxic dose of methionine to *A. mellifera* in both larval and adult life stages in conditions of both acute and chronic exposures. For these conditions, high dose limit tests were conducted at 25 μg/bee for topical application and 100 μg/bee for acute oral exposure. In the chronic oral exposure to adult and larval bees treated food was supplied with a concentration of up to 1% DL-methionine. In addition to testing the diluent (specific to each bioassay), negative control treatments employed proline instead of methionine, and the positive control treatment was dimethioate.

We anticipated that the experimental results would contribute to the comprehensive risk assessment conducted prior to the deployment of methionine as a pesticide, anticipating that it would exert minimal or non-existent collateral damage to bees. Experimental confirmation of the hypothesis would be useful in supporting future registration for methionine as a biopesticide.

2. Materials and methods

2.1. Honey bees

The honey bees were collected from hives maintained at the University of Florida (UF) Bee Unit located in Gainesville, FL. The adult worker bees originated from healthy, queen-right colonies (*i.e.* colonies with queens) that were not treated with any miticides for at least one month prior to the initiation of the study. Adult bees were obtained by removing combs of late-stage capped brood (< 1 day until eclosion) from the hives and transporting the combs within a ventilated box to a laboratory incubator at the UF Institute of Food and Agricultural Sciences (IFAS) Entomology and Nematology Department, Gainesville, FL. The combs were held at 34.5 °C for up to 24 h. The following day, < 1 day-old adult bees were indiscriminately collected from the box and treated before being placed into the bioassay cages used in the experiments (cages described in next section). Larvae were obtained according to the methods of Schmehl et al. (2016). A colony's queen was confined on a single frame on an open section of comb using a cage. After ~ 24 h, the queen was released from the cage. The comb with ~ 1st instar larvae was transported into the laboratory after an additional 87 ± 12 h and the larvae were grafted into *in vitro* rearing plates within 3 h.

2.2. Adult bee assays

Experimental adult bees were maintained in cages constructed from 0.59 L plastic cups (Fig. 1), secured upside down with a rubber band to a 9 cm Petri-dish. A sheet of beeswax (~ 5 × 7 cm) was placed on the interior side of the cup and held in place with five brass fasteners. A hole covered with plastic screen on the opposite side of the cup provided ventilation. Unless otherwise specified, adult bees were provided *ad libitum* water and sugar solution (1:1 sucrose/water; w/v). The liquids were provided separately in 1.5 mL microcentrifuge tubes with small holes in their tips to permit feeding. The microcentrifuge tubes were inserted through holes in the cage, the sugar in the top and the water on the side of the cage.

2.3. Adult acute contact exposures

The U.S. EPA guideline for honey bee acute toxicity testing was followed (EPA, 2012). As discussed above, our *a priori* assumption was that DL-methionine would have low toxicity to bees, particularly through contact exposure. Therefore, a limit test, to be conducted when a test substance (TS) is expected to have relatively low toxicity (EPA, 2012), was conducted at one dose, namely 25 μg TS/bee. Bees were restrained by grasping their wings with the fingertips and an U.S. EPA