Determination of Acute Oral Toxicity of Flumethrin in Honey Bees

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ABSTRACT Flumethrin is one of many pesticides used for the control and treatment of varroatosis in honey bees and for the control of mosquitoes and ticks in the environment. For the control of varroatosis, flumethrin is applied to hives formulated as a plastic strip for several weeks. During this time, honey bees are treated topically with flumethrin, and hive products may accumulate the pesticide. Honey bees may indirectly ingest flumethrin through hygienic behaviors during the application period and receive low doses of flumethrin through comb wax remodeling after the application period. The goal of our study was to determine the acute oral toxicity of flumethrin and observe the acute effects on motor coordination in honey bees (*Apis mellifera anatoliaca*). Six doses (between 0.125 and 4.000 μg per bee) in a geometric series were studied. The acute oral LD_{50} of flumethrin was determined to be 0.527 and 0.178 μg per bee (n = 210, 95% CI) for 24 and 48 h, respectively. Orally administered flumethrin is highly toxic to honey bees. Oral flumethrin disrupted the motor coordination of honey bees. Honey bees that ingested flumethrin exhibited convulsions in the antennae, legs, and wings at low doses. At higher doses, partial and total paralysis in the antennae, legs, wings, proboscises, bodies, and twitches in the antennae and legs were observed.

KEY WORDS *Apis mellifera anatoliaca*, flumethrin, oral, acute toxicity

The honey bee, *Apis mellifera*, is an economically important insect for humans, producing honey, pollen, royal jelly, propolis, and wax. Honey bees also play a major role in agricultural production because bees pollinate crops for the production of high quality, commercial seeds and fruits (Iwasa et al. 2004). Because honey bees encounter numerous agrochemicals in apiculture as they forage in agricultural areas, bees may encounter a wide variety of pesticides, drugs, or other chemical agents. Pesticides and drugs, despite their ability to control a wide variety of agricultural pests and honey bee diseases, are toxins that may also have harmful effects on honey bees. The effects of chronic low exposure (topical or oral) of acaricides through accumulation in hive materials are not well understood. Although acaricide toxicities are carefully studied in honey bees during product development, further studies are needed to better understand acaricide effects on the >20 subspecies of honey bees that may differ in sensitivity to acaricides under diverse conditions of apiculture in different regions of the world. Such studies can be used to adapt local honey bee management practices for regional conditions.

One group of acaricides, the pyrethrins, is composed of naturally occurring compounds with insecticidal properties that are found in pyrethrum extract from certain chrysanthemum flowers (Leahey 1985). The pyrethrins are often used in household insecticides and products to control insects on pets or livestock. Pyrethroids are manufactured chemicals that are very similar in structure to the pyrethrins, but are often more toxic to insects as well as to mammals and pyrethroids last longer in the environment than pyrethrins. Pyrethrins and pyrethroids affect the insect nervous system by causing multiple action potentials in the nerve cells by delaying the closing of ion channels. Increased excitability of neuronal tissue causes fatal nervous system failure and muscle spasms (Klaassen et al. 1996, Costa 1997).

Flumethrin is a synthetic pyrethroid ectoparasiticide commonly used in veterinary medicine that is applied topically to sheep, cattle, and goats as a 1% wt:vol pour-on or plunge dip solution for the control of ticks, lice, and mites. In beekeeping, plastic strips impregnated with 3.6 mg flumethrin are fastened between combs in beehives so that bees receive topical treatment for varroatosis by contact with the strip (The European Agency for the Evaluation of Medicinal Products [EMEA] 1998). Flumethrin may also be used to control mosquitoes and ticks in the environment, and it could be poisoning honey bees (Unal et

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al. 2010). The acute toxicity of flumethrin is variable and dependent on the solvent vehicle. Liquid paraffin, 2-octyldodecanol and Solvesso 150, Solvesso 200, Solvesso 150, and emulsifiers, Miglyol and Cremophor are vehicles used in different commercial preparations of flumethrin (EMEA 1998). Toxicological bioassays can track the pesticide susceptibility of honey bees by determining the median lethal doses or concentrations (LD_{50} or LC_{50}). Insecticides are classified as highly toxic (acute LD_{50}, <2 μg per bee), moderately toxic (acute LD_{50}, 2–10.99 μg per bee), slightly toxic (acute LD_{50}, 11–100 μg per bee), and essentially nontoxic (acute LD_{50}, >100 μg per bee) to adult bees (Washington State Department of Agriculture [WSDA] 2010). Santiago et al. (2000) studied the contact toxicity of flumethrin (3% emulsifying concentrate) in honey bees and reported the LD_{50} for flumethrin to be 0.05 μg per bee (95% CI).

Honey bees can be exposed to pesticides such as flumethrin by contact. However, honey bees might also be exposed to pesticides through the oral ingestion of contaminated nectar and pollen (French Food Safety Agency [AFSSA] 2009). Beeswax and honey can also contain flumethrin (Johnson et al. 2010, Serra–Bonvehi and Orantes–Bermejo 2010). Therefore, oral toxicity testing of flumethrin is important. To our knowledge, there have been no primary literature reports on the oral toxicity of flumethrin in honey bees. The goal of this study was to determine the acute oral 24- and 48-h toxicity of flumethrin (Akarvil) for the Anatolian honey bee (*Apis mellifera anatoliaca*) and to observe the toxic effects (postadministration motor coordination and LD_{50} values) of flumethrin in honey bees.

Materials and Methods

In the current study, the LD_{50} study was performed as described in the European and Mediterranean Plant Protection Organization (European and Mediterranean Plant Protection Organization [EPPO] 1998) and The Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals, Honey bees, Acute Oral Toxicity Test (OECD 213 1998) but was modified as follows. Young, foraging, adult worker honey bees (*Apis mellifera anatoliaca*) were collected from a water source located 20–30 m from the hives and next to the Uludag University Beekeeping Development-Application and Research Center on 23 July, 2010. Honey bee workers begin foraging at the age of 15–20 d. As they age, foraging bees become worn out, and their hair darkens. The bees used in this experiment were ≈20–30 d old (Winston 1987). The hives (n = 80 hives) from which the bees were collected showed no obvious signs of disease during routine colony maintenance and bee collections. The honey bees were collected into plastic containers between 07:00 and 08:00 hours in the morning, and individual bees were placed singly in paper cups (upper diameter: 8 cm, bottom diameter: 6 cm, and height: 10 cm) covered with a nylon mesh (0.2-cm holes) in the laboratory. The collected bees were randomly assigned to flumethrin (n = 180) or control (n = 30) treatment groups. The bees were starved for up to 30–45 min before the initiation of the test. Moribund bees were rejected and replaced by randomly selected healthy bees, which were collected as substitutes for this situation before starting the test.

Flumethrin was tested in pilot studies as a formulated product (Akarvil, 7.5%, Vilsan, Istanbul) at a broad range of doses (0.05–15 μg per bee) to establish the range of concentrations toxic to bees. With Cremophor EL as the vehicle of flumethrin, the commercial formulation dissolved easily in water. Therefore, this commercial formulation of flumethrin was chosen, and flumethrin is currently in use against varroa (EMEA 1998, Loucif–Ayad et al. 2008, Giriskin and Aydin 2010). Akarvil was dissolved in deionized water to obtain the desired concentrations of flumethrin, six doses in a geometric (factor 2.0) series. Sucrose was then added to the test solutions at final concentrations of 50% sucrose (wt:vol). The concentrations of Cremophor EL in the test solutions containing flumethrin are harmonious (<1%) with the OECD procedure. The bees in the control treatment groups were fed with 10 μl of 50% (wt:vol) sucrose in deionized water by using an automatic pipette. All bees used in this study were fed with a 10 μl test solution for each bee. Three replicate treatments (n = 10 bees per treatment) were dosed with each test concentration. Administration doses of flumethrin were between 0.00 and 4.00 μg per bee with six doses as 0.00, 0.125, 0.25, 0.50, 1.00, 2.00, and 4.00 μg per bee. The solutions were vortexed vigorously after preparation and before use. The bees were held in the dark at room temperature (25 ± 1°C), but all experimental applications to bees were performed in the light. The relative humidity during the experiment was between 45 and 65%. After consuming oral doses of flumethrin, the bees were fed with sucrose (50% wt:vol) solution ad libitum at 1 h postingestion and every 4 h during the day. Mortality was recorded at 4 h after the beginning of the test and thereafter at 6, 9, 20, 24, 36, and 48 h. All abnormal behavioral effects (clearly affected motor coordination of the proboscis, antennae, wings, and legs) observed during the testing period were recorded. After 48 h, the mortality was between 15 and 20% in the control treatment group. Therefore, the study was ended, and the results were evaluated for the 24 and 48 h time points, according to the protocol.

Statistical analyses. The median lethal dosage (LD_{50}) based on cumulative mortality per treatment was estimated by the maximum likelihood of Probit Analysis (Finney 1971) using the Minitab (Minitab Inc. 2001) statistical program. Logarithmic transformation (Log_{10}) was applied to the flumethrin doses before analysis. The data distribution and the model were fitted adequately according to the goodness-of-fit tests (P values = 0.449, 0.296). We calculated the LD_{50} of flumethrin and 95% CIs individually for three replicate treatments (for 10 bees) and as a whole for pooled treatments (for 30 bees). The frequency of mortality at each observation period (4–48 h) was

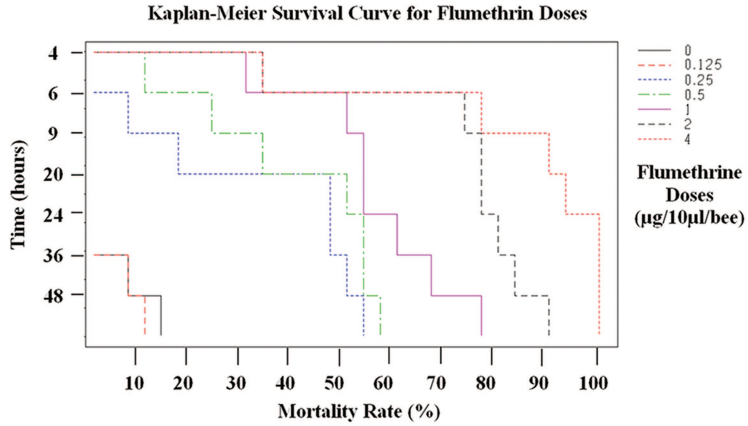

Fig. 1. Kaplan–Meier survival curve for flumethrin doses.

tested among treatments by contingency χ^2 analysis in JMP 9.0 (SAS Institute 2010).

Results

Honey bees fed with serial dilutions of flumethrin displayed a broad range of effects on motor coordination and mortalities. Oral doses of flumethrin clearly affected motor coordination of the proboscis, antennae, wing, and legs. No abnormal behavior was observed at the lowest dose (0.125 μg per bee). However, convulsions and paralysis were observed at higher doses of flumethrin (0.25–4.0 μg per bee) between 3 and 20 min after treatment in affected honey bees. Honey bees that experienced convulsions or paralysis lacked the motor skills to feed or move around in containers. Some honey bees were not much affected and able to recover, especially those treated with 0.25–0.50 μg flumethrin.

We observed a sigmoidal flumethrin dose-mortality curve, with the lowest dose (0.125 μg per bee) showing no mortality at 24 h, and all other doses causing a higher mortality than control treatments. The cumulative mortality of flumethrin was presented in Fig. 1 by Kaplan–Meier Survival Curve. The acute oral LD_{50} of flumethrin was determined to be 0.527 and 0.178 μg per bee ($n = 210$; 95% CI) for 24 and 48 h, respectively. The cumulative frequency of mortality generally increased during the 24-h postingestion period, but the frequencies of mortality at the 4–24 h observation times in the 0.25-μg treatment were different from the frequencies at the same times in the 0.5–4.0-μg treatments. The bees fed with 0.25 μg flumethrin displayed mortality later than the bees in the 0.5–4.0-μg treatments ($\chi^2 = 48.166$; df = 16; $P < 0.001$). In the 0.25-μg flumethrin treatments, the earliest mortality was observed at 6 h postingestion, whereas bees fed with 0.50 μg per bee or higher displayed their earliest mortality at 4 h postingestion. The treatments in which bees were fed 0.5–4.0 μg flumethrin per bee were similar in their onset of mortality during the 4–24 h observation periods ($\chi^2 = 20.923$; df = 12; $P = 0.052$).

Discussion

Our study revealed that orally administered flumethrin was highly toxic to bees, with oral LD_{50} dose of 0.527 and 0.178 μg per bee ($n = 210$; 95% CI) for 24 and 48 h, respectively. The LD_{50} at 48 h was ≈3 times less than LD_{50} at 24 h. We used the insecticide classification of WSDA (2010), in which pesticides having an LD_{50} <2 μg per bee are considered highly toxic to honey bees. Although no published data are available for direct comparison of acute oral toxicity, the contact (topical, thorax, 24 h) LD_{50} of flumethrin in Bayticol (3%, emulsifying concentrate, Bayer) of 0.05 μg per bee (95% CI) is consistent with our conclusion that this acaricide is highly toxic to honey bees (Santiago et al. 2000). In this study, oral LD_{50} values at 24 and 48 h are 10.5 and 3.5 times higher, respectively, than the contact (topical, thorax) LD_{50} of flumethrin. Other pyrethroids such as cypermethrin (U.S. Environmental Protection Agency [EPA] 2008), deltamethrin (Tomlin 2006, EPA 2010), permethrin (EPA 2006a), and resmethrin (EPA 2006b) are also classified as highly toxic to bees and act as contact poisons that disrupt the insect nervous system (Klaassen et al. 1996) similar to flumethrin. The oral LD_{50} of deltamethrin was found to be 0.051 μg per bee (Tomlin 2006), similar to the topical LD_{50} of flumethrin reported in Santiago et al. (2000), but lower than the oral LD_{50} doses of flumethrin in the current study. The differences in reported LD_{50} values may be caused by several factors: dose application route, time, carrier molecule in the commercial formulation, or test colony characteristics. The topical and oral dose toxicities may differ by an order of magnitude or more between species (Lagadic et al. 1993). The sensitivity of adult bees to toxins varies with gender (Charnetski 1988), species (Del Lama and Peruqueitti 2006), and such colony characteristics as larval brood temperature (Medrzycki et al. 2010) and physiological conditions (Wahl and Ulm 1983). The detoxification of pyrethroids by P450 activity (Johnson et al. 2006), similar to the immune response (Evans and Pettis 2005), may

differ among colonies because protein metabolism and energetic or past selection pressures in the colony affect the trait.

The OECD (1998) recommends that acute oral toxicity tests for honey bees use test cages containing ten bees. Each treatment of 10 bees should be fed with 100–200 μl of 50% sucrose solution in water containing the test substance at the appropriate concentrations in these cages. In the current study, we chose to place bees singly in paper cups to ensure that they consumed the full dose of the flumethrin. Housing bees individually provided the added benefit of being able to characterize the responses of individual bees at time periods during the experiment, better revealing the frequencies of honey bee mortality and loss of motor coordination at the different observation times. The study was ended when the mortality reached between 15 and 20% in the control group according to the OECD procedure (1998). There has not been a report regarding mortality rates in control groups for honey bees related to 48 h flumethrin toxicity studies. Santiago et al. (2000) studied the contact toxicity of flumethrin for 24 h.

At the lowest dose of flumethrin (0.125 μg per bee), motor coordination was no different than that of control bees. However, loss of motor coordination was observed at doses of 0.25–4.0 μg per bee. At these higher doses, loss of motor coordination manifested as convulsions in antennae, legs, and wings, and, in more severe cases, partial and total paralysis of antennae, legs, wings, proboscis, and bodies. Based on these observations, honey bees that orally consume doses above 0.125 μg flumethrin in hives or surrounding environments may not possess the motor coordination to return to their colony from the environment. Plastic strip applications of flumethrin slowly release low doses of flumethrin that are simultaneously effective against mites and are barely detectable in beeswax after a single application (Szerletics–Turi 1999, Floris et al. 2001). However, with levels of flumethrin at 158 μg/kg in Spanish beeswax and 31.2–34.8 mg/kg in recycled acaricide-treated beeswax (Bogdanov et al. 1998, Serra–Bonvehi and Orantes–Bermejo 2010), honey bees would only have to work 3.3 g of Spanish beeswax or 15.1–16.9 mg of recycled beeswax to contact a dose at the LD_{50} reported in our study. To better understand the threat of contaminated beeswax, we need to know the rate at which flumethrin is absorbed from beeswax by honey bees.

Flumethrin is highly toxic to honey bees, similar to other pyrethroids. The careless use of flumethrin in beekeeping or in environments where bees forage may lead to colony losses. This is especially important for pyrethroids because they are among the most lipophilic acaricides, capable of accumulating in recycled beeswax and propolis (Bogdanov et al. 1998). Honey bee exposure to pesticides is among the most important factors affecting colony health, but contradictions abound (Pimentel et al. 1980, Johnson et al. 2010, vanEngelsdorp et al. 2010). For example, acaricides promote colony health by controlling mite infestations (vanEngelsdorp et al. 2010) but also may diminish colony health through a possible reduction in brood growth metabolism (Nielsen et al. 2000), through known effects on reproductive castes and through possible sublethal effects of multiple accumulated acaricides in hive materials (reviewed in Johnson et al. 2010). Under field conditions, flumethrin could interact with many factors, particularly the application conditions (e.g., rate, time, route of exposure, and colony characteristics) to affect the motor coordination and mortality of honey bees. Honey bees are directly exposed to flumethrin during the control and treatment of varroatosis, indirectly from the control of mosquito and ticks in the environment, and through nectar and pollen contamination with flumethrin. Therefore, monitoring programs for acaricide contamination in beeswax may help avoid one of the most severe potential threats to honey bees as described by Johnson et al. (2010). Further studies are needed to integrate acute and chronic effects of flumethrin with behavioral and ecological performance of honey bees.

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