

Synergistic Interactions Between In-Hive Miticides in *Apis mellifera*

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ABSTRACT The varroa mite, *Varroa destructor* Anderson & Trueman, is a devastating pest of honey bees, *Apis mellifera* L., that has been primarily controlled over the last 15 yr with two in-hive miticides: the organophosphate coumaphos (Checkmite+), and the pyrethroid tau-fluvalinate (Apistan). Both coumaphos and tau-fluvalinate are lipophilic compounds that are absorbed by the wax component of the hive, where they are stable and have the potential to build up over repeated treatments such that bees could be exposed to both compounds simultaneously. Although these compounds were chosen as in-hive miticides due to their low toxicity to honey bees, that low toxicity depends, at least in part, on rapid detoxification mediated by cytochrome P450 monooxygenase enzymes (P450s). In this laboratory study, we observed a large increase in the toxicity of tau-fluvalinate to 3-d-old bees that had been treated previously with coumaphos, and a moderate increase in the toxicity of coumaphos in bees treated previously with tau-fluvalinate. The observed synergism may result from competition between miticides for access to detoxicative P450s. These results suggest that honey bee mortality may occur with the application of otherwise sublethal doses of miticide when tau-fluvalinate and coumaphos are simultaneously present in the hive.

KEY WORDS cytochrome P450 monooxygenase, tau-fluvalinate, coumaphos, colony collapse disorder, *Varroa destructor*

The honey bee, *Apis mellifera* L., plays a significant role in the U.S. economy, contributing \$15 billion annually, primarily through pollination services (Morse and Calderone 2000). However, honey production and agricultural pollination have been threatened in recent years by colony collapse disorder (Oldroyd 2007). Although the cause of colony collapse disorder has yet to be determined, it seems likely that a combination of stresses, potentially including viruses (Cox-Foster et al. 2007) or pesticides (Frazier et al. 2008), may be contributing to bee decline.

A major source of stress for honey bees is the varroa mite, *Varroa destructor* Anderson & Trueman. Varroa mites weaken colonies directly by sucking the hemolymph of adults and brood and indirectly by serving as a vector of bee viruses (Chen et al. 2004). Colony survival in the presence of varroa mites depends on intervention by beekeepers, because an unmanaged colony is likely to succumb to "parasitic mite syndrome" after sustaining an untreated infestation for several years (Boecking and Genersch 2008). In-hive miticide applications, such as Apistan and Checkmite+, have been among the principal tools available to beekeepers for varroa management; however, their presence in the hive can be an additional stress on bees (Van Buren et al. 1992, Sokol 1996, Pettis et al. 2004).

Apistan was the first synthetic miticide registered for use in the United States in 1990. Apistan treatment

consists of suspending plastic strips impregnated with the pyrethroid pesticide tau-fluvalinate between brood frames inside the hive, where they slowly release tau-fluvalinate over the course of 6 to 8 wk. Tau-fluvalinate is a subset of isomers of fluvalinate; its mode of action is as an agonist of the voltage-gated sodium channel. Although tau-fluvalinate was initially quite effective at controlling mite infestations and exhibited low toxicity to bees (Atkins 1992), the efficacy of this agent has been compromised because resistance has become widespread in mite populations worldwide (Lodesani et al. 1995, Elzen et al. 1998). Resistance in mites has been gained through mutations in the voltage-gated sodium channel, thereby reducing the binding of tau-fluvalinate (Wang et al. 2002).

In 1998, in response to the failure of tau-fluvalinate to control varroa, the organophosphate pesticide coumaphos was approved in the United States both as a miticide and as a treatment for the small hive beetle, *Aethina tumida* Muray (Federal Register 2000). Coumaphos, marketed under the name Checkmite+, also is administered in pesticide-impregnated plastic strips hung between brood frames in the hive for 42–45 d. Coumaphos or its oxon metabolite inactivate acetylcholinesterase, thereby interfering with nerve signaling and function, leading to the death of the insect. Thus, as an acetylcholinesterase inhibitor, coumaphos acts via a different mode of action than that of tau-fluvalinate and it initially proved very effective at

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killing varroa resistant to tau-fluvalinate (Elzen et al. 2000). However, resistance to coumaphos developed rapidly, with U.S. mites demonstrating significant resistance to coumaphos as early as 2001 (Elzen and Westervelt 2002).

Among the challenges in developing new miticides for use in varroa management is that pesticides used within the hive must be minimally harmful to the bees, while maintaining toxicity to mites. Honey bees, however, are notoriously sensitive to a wide range of pesticides (Atkins 1992). The recent sequencing of the honey bee genome provided a possible explanation for this sensitivity; relative to other insect genomes, the honey bee genome is markedly deficient in the number of genes encoding detoxification enzymes, including cytochrome P450 monooxygenases (P450s), glutathione transferases, and carboxylesterases (Claudianos et al. 2006). Honey bee tolerance of miticidal concentrations of tau-fluvalinate is attributable in large part to rapid cytochrome P450-mediated detoxification (Johnson et al. 2006).

Despite its widespread in-hive use, the mechanism of tolerance to coumaphos in honey bees has yet to be determined. As a phosphorothionate, the organophosphate pesticide coumaphos is bioactivated to the more toxic coumaphos oxon through a P450-mediated reaction. However, P450s are also known to participate in the detoxification of organophosphates, which could be the basis for the tolerance of coumaphos in honey bees. Heterologously expressed CYP6A1, which is overexpressed in populations of house flies, *Musca domestica* L., resistant to the organophosphate diazinon, catalyzes the detoxificative ester cleavage of diazinon to 2-isopropyl-4-methyl-6-hydroxypyrimidine at a greater rate than it catalyzes the bioactivating desulfuration reaction producing diazoxon (Sabourault et al. 2001). As well, P450-mediated detoxification of phosphorothionates is mediated by house fly CYP12A1 (Guzov et al. 1998) and *Drosophila melanogaster* (Meigen) CYP6A2 (Dunkov et al. 1997).

If in fact *A. mellifera* relies on P450-mediated metabolism to tolerate coumaphos, the potential exists for synergistic or antagonistic interactions with tau-fluvalinate, which is metabolized by P450s. In the realm of human medicine, the potential for synergistic interactions among drugs that interact with P450s is well understood, as certain drug combinations are known to be deadly, but synergism among therapeutic agents in insects has never been considered. The potential for simultaneous exposure to both coumaphos and tau-fluvalinate exists because both compounds are lipophilic and can reside in the wax of colonies for years (Martel et al. 2007, Frazier et al. 2008). In this study, we used bioassays with selective enzyme inhibitors to determine the extent to which detoxificative metabolism contributes to honey bee tolerance of coumaphos. Additionally, we examined the synergistic effects of exposure to sublethal doses of one miticide, either tau-fluvalinate or coumaphos, on the toxicity of the other miticide.

Materials and Methods

Chemicals. Technical grade tau-fluvalinate (95%) and coumaphos (99%) were purchased from Chem Services (West Chester, PA). Three enzyme inhibitors were used to assess the extent to which different detoxification enzymes contribute to tolerance (Johnson et al. 2006). The glutathione transferase inhibitor diethyl maleate (DEM; 97%) was obtained from Sigma-Aldrich (St. Louis, MO); the esterase inhibitor S,S,S-tributylphosphorotrithioate (DEF; 98%) was purchased from Chem Services; and the cytochrome P450 inhibitor piperonyl butoxide (PBO; 90%) was obtained from TCI America (Portland, OR).

Insects. Late-stage capped brood frames were collected from healthy colonies at the University of Illinois Bee Research Facility on the Urbana-Champaign campus and at the University of Illinois Phillips Tract Research Area, near Urbana (Champaign County), IL, in June and July 2008. Colonies were maintained using standard preventative treatment for bee pests and diseases: American foulbrood (*Paenibacillus larvae*) was prevented with terramycin, *Nosema* spp. infection was prevented with Fumidil B, and varroa mite (*V. destructor*) infestation was controlled with powdered sugar treatment and Apilife Var. Neither Apistan nor Checkmite+ had been used in any of the equipment for at least 5 yr. Collected frames of late-stage brood were placed in a dark, humid incubator at 32–34°C. Newly eclosed adults were brushed from frames daily and placed in screen-topped wooden boxes (330 cm³) in groups of 100–200 and provisioned with bee candy (equal parts powdered sugar and heavy sucrose syrup) before their use in bioassays.

LD₅₀ Bioassays. Three- to 4-d-old worker bees were used for all bioassays. All compounds were dissolved in 1 μ l of acetone and applied to the thorax of worker bees with a microliter syringe mounted on a PB-600 repeating dispenser (Hamilton, Reno, NV). In treatments with enzyme inhibitors, the inhibitor was administered 1 h before the insecticide application, at maximum sublethal dose: 100, 10, or 10 μ g per bee for DEM, DEF, and PBO, respectively (Johnson et al. 2006). In tests of pesticide interactions, bees were treated with doses of coumaphos (0.1, 0.3, 1, 3, or 10 μ g) or tau-fluvalinate (0.1, 0.3, 1, or 3 μ g) 1 h before application of the other pesticide. No mortality was observed in any control bees that received only the pretreatment dose, except for bees receiving 3 μ g of tau-fluvalinate as pretreatment. In this case, Abbott's method (Abbott 1925) was used to correct for control mortality.

All bioassays included a solvent control and doses eliciting 0 and 100% mortality 24 h after treatment, as well as at least four doses causing >0 and <100% mortality. All bees were treated in groups of 20 and were placed in wax-coated paper cups (177 cm³; Sweetheart, Owings Mills, MD) covered with cotton cheesecloth secured by two rubber bands. Bees were provisioned with 1:1 sugar water in a punctured 1.5-ml plastic tube and maintained in an incubator. Mortality was assessed 24 h after treatment; bees incapable of

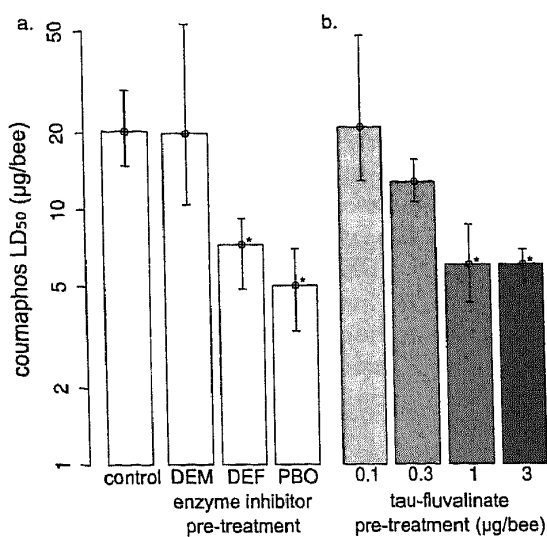


Fig. 1. (a) Coumaphos, 24 h. LD₅₀ (micrograms per bee), with 95% confidence intervals for bees pretreated with an acetone control (LD₅₀ = 20.29 [14.88–29.44] µg per bee; *n* = 480; slope = 2.67 ± 0.24; χ^2 = 14.8; df = 4); DEM, a glutathione transferase inhibitor (LD₅₀ = 19.92 [10.48–53.45] µg per bee; *n* = 460; slope = 1.95 ± 0.19; χ^2 = 49.2; df = 6); DEF, a carboxylesterase inhibitor (LD₅₀ = 7.29 [4.88–9.22] µg per bee; *n* = 300; slope = 4.77 ± 0.52; χ^2 = 8.37; df = 3); or PBO, a cytochrome P450 inhibitor (LD₅₀ = 5.04 [3.34–7.01] µg per bee; *n* = 480; slope = 3.85 ± 0.32; χ^2 = 23.75; df = 4). (b) Coumaphos LD₅₀ after pretreatment with a range of sublethal tau-fluvalinate doses: 0.1 µg per bee (LD₅₀ = 20.97 [12.9–47.86] µg per bee; *n* = 280; slope = 2.39 ± 0.28; χ^2 = 23.53; df = 5), 0.3 µg per bee (LD₅₀ = 12.77 [10.63–15.59] µg per bee; *n* = 400; slope = 3.34 ± 0.29; χ^2 = 9.54; df = 5), 1 µg per bee (LD₅₀ = 6.05 [4.29–8.68] µg per bee; *n* = 540; slope = 1.5 ± 0.14; χ^2 = 12.96; df = 6) or 3 µg/bee (LD₅₀ = 6.06 [5.15–6.94] µg per bee; *n* = 380; slope = 2.38 ± 0.27; χ^2 = 3.12; df = 4). Treatments with nonoverlapping confidence intervals are considered significantly different. Treatments different from control are indicated with an asterisk (*).

righting themselves were scored as dead. Each bioassay was replicated a minimum of three times, with bees taken from different colonies for each replicate.

Statistical Analysis. Log-probit analyses were performed using the R statistical package (R Development Core Team 2008) with MASS libraries (Venables 2002). LD₅₀ values with 95% confidence intervals were calculated using Fieller's method, with correction for heterogeneity where appropriate (Finney 1971). Treatments with nonoverlapping 95% confidence intervals were considered significantly different.

Results

The LD₅₀ toxicity of coumaphos in the absence of enzyme inhibitors was 20.3 µg per bee, consistent with previously published values (Van Buren et al. 1992; Klochko et al. 1994; Fig. 1a). Pretreatment with the glutathione transferase inhibitor DEM did not significantly alter the toxicity of coumaphos. Both DEF and PBO exhibited a synergistic interaction with coumaphos, enhancing toxicity 2.8-fold and 4.0-fold, respectively. The toxicity of coumaphos increased with higher doses of tau-fluvalinate pretreatment: up to

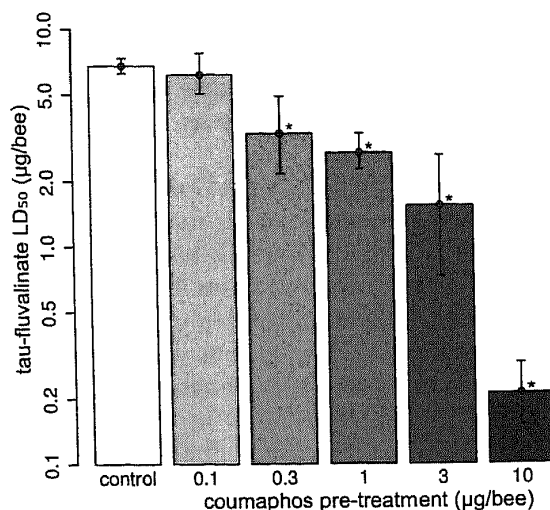


Fig. 2. Tau-fluvalinate, 24 h. LD₅₀ (micrograms per bee), with 95% confidence intervals for bees pretreated with an acetone control (LD₅₀ = 6.75 [6.24–7.33] µg per bee; *n* = 1,260; slope = 2.32 ± 0.13; χ^2 = 4.78; df = 5) or a range of sublethal coumaphos doses: 0.1 µg per bee (LD₅₀ = 6.14 [5.03–7.71] µg per bee; *n* = 780; slope = 1.86 ± 0.14; χ^2 = 10.68; df = 6), 0.3 µg per bee (LD₅₀ = 3.29 [2.15–4.87] µg/bee; *n* = 700; slope = 1.42 ± 0.12; χ^2 = 25.34; df = 7), 1 µg per bee (LD₅₀ = 2.68 [2.25–3.28] µg/bee; *n* = 760; slope = 1.89 ± 0.13; χ^2 = 10.25; df = 7), 3 µg per bee (LD₅₀ = 1.53 [0.72–2.6] µg per bee; *n* = 840; slope = 1.56 ± 0.11; χ^2 = 64.92; df = 7), or 10 µg per bee (LD₅₀ = 0.21 [0.15–0.29] µg per bee; *n* = 640; slope = 1.61 ± 0.12; χ^2 = 13.81; df = 7). Treatments with nonoverlapping confidence intervals are considered significantly different. Treatments different from control are indicated with an asterisk (*).

3.4-fold synergism was effected by pretreatment with either 1 or 3 µg of tau-fluvalinate (Fig. 1b).

Synergism of tau-fluvalinate also was observed when coumaphos was used as a pretreatment, with its toxicity exhibiting 2.1-fold synergism in the presence of as little as 0.3 µg of coumaphos (Fig. 2). The toxicity of tau-fluvalinate increased as the amount of coumaphos used as a pretreatment increased; pretreatment with 1, 3, and 10 µg of coumaphos resulted in 2.5-, 4.4-, and 32.1-fold synergism, respectively.

Discussion

Both *in vitro* metabolism assays and bioassays with detoxicative enzyme inhibitors have demonstrated that pyrethroid pesticides are metabolized principally by cytochrome P450 monooxygenases and to a lesser extent carboxylesterases (Pilling et al. 1995; Johnson et al. 2006). Although phosphorothionate organophosphates are known to be bioactivated by P450s in some species, the synergism of coumaphos toxicity by PBO, a P450 inhibitor, provides evidence that P450s serve to detoxify coumaphos in honey bees. However, even with P450 inhibition, coumaphos is still much less toxic to honey bees than most other organophosphates (Atkins 1992), suggesting that tolerance of this pesticide in honey bees involves more than simply rapid detoxification.

Given the reduced inventory of P450 genes in the honey bee genome, with less than half the number of

genes found in most other insect genomes (Claudianos et al. 2006), as well as the multiplicity of functions served by P450s in insects other than detoxification, including hormone and pheromone biosynthesis (Feyereisen 2005), the possibility exists that synthetic organic insecticides may be metabolized in honey bees by a very small number of detoxicative P450 enzymes. The synergistic interactions observed between coumaphos and tau-fluvalinate may even result from competition between these compounds for access to the same P450 enzyme.

Pesticide levels leading to synergism in this study are within the range that can be encountered in managed hives. Two Checkmite+ strips, the recommended dosage for coumaphos treatment, contain 2.8 g of coumaphos, and two Apistan strips contain 1.4 g of tau-fluvalinate. Assuming that 10% of the active ingredient diffuses out of the strips (Bogdanov et al. 1998, Tremolada et al. 2004) and a colony of 20,000 individuals is treated for the recommended duration of treatment (6 wk for Checkmite+ and 8 wk for Apistan), the daily dose for each individual can be approximated at 0.33 μg per bee per day for coumaphos and 0.125 μg per bee per day for tau-fluvalinate. Bioassays in this study showed that coumaphos treatment at this level effectively doubles susceptibility to tau-fluvalinate. There is evidence that some bees may be subject to much higher exposure as miticides do not diffuse evenly over the population of the hive; some bees come into contact with the plastic strips more frequently and receive higher doses. Concentrations of coumaphos as high as 3.2 μg per bee have been reported in colonies treated with Checkmite+ (Haarmann et al. 2002).

Tremolada et al. (2004) modeled the disposition of miticides inside the hive environment. When the treatment is first applied, bees experience high doses of miticide, which is either rapidly metabolized by the bees or deposited in the hive in newly secreted wax. Coumaphos and tau-fluvalinate are both lipophilic compounds, and a majority of the unmetabolized miticide is sequestered in beeswax, where both are quite stable and can persist for years, even accumulating with repeated miticide treatments (Sokol 1996, Bogdanov et al. 1998, Wallner 1999). Wax in brood frames adjacent to the strips, however, take in more of the miticide and form hot spots of contamination (Lodesani et al. 1992, Haarmann et al. 2002). Concentrations as high as 66 μg of coumaphos per gram of wax have been reported, with an average wax concentration over several studies of ≈ 1.1 $\mu\text{g}/\text{g}$ (reviewed in Tremolada et al. 2004). Tau-fluvalinate concentrations as high as 7 $\mu\text{g}/\text{g}$ wax have been reported, with an average concentration of 1.1 $\mu\text{g}/\text{g}$ (Lodesani et al. 1992, Bogdanov et al. 1998). Both also can occur in wax of colonies that have never been treated with either miticide. Beekeepers often move potentially miticide-contaminated frames within and between hives. Additionally, both coumaphos and tau-fluvalinate survive and are concentrated by the wax recycling process used to make new foundation (Bogdanov et al. 1998, Martel et al. 2007). In a recent survey of in-hive chem-

ical residues conducted in the wake of colony collapse disorder, both compounds were found in 100% of wax samples from both healthy and collapsed colonies (Frazier et al. 2008).

It is unclear what proportion of the tau-fluvalinate and coumaphos sequestered in the wax of hives can find its way into the bees, in which it could cause synergistic effects either with newly applied miticide treatments or with another miticide also stored in wax. Wax contaminated with coumaphos (10 $\mu\text{g}/\text{g}$) or fluvalinate (100 $\mu\text{g}/\text{g}$) leaches enough miticide to affect varroa mite survival (Fries et al. 1998). Bees from colonies treated the previous year with Apistan strips contained as much as 0.1 μg of tau-fluvalinate per bee (Haarmann et al. 2002). Miticides can move from the wax into honey, where they are available for ingestion by bees. Approximately 0.2% of either miticide present in wax will move into the honey (Tremolada et al. 2004) where both compounds are stable (Korta et al. 2001). The half-life of coumaphos in a colony environment has been estimated at 69 d in honey and 115–346 d in comb wax (Martel et al. 2007). For both miticides, it has been estimated that 5 yr is required for complete disappearance (Bogdanov 2004).

Bioassays performed with adult worker bees in this study clearly demonstrate interactions between miticides that enhance toxicity, but, given that the sensitivity to many pesticidal compounds varies developmentally, synergistic impacts may be more or less acute in bees of different castes or ages. Two-week-old worker bees, for example, are twice as susceptible to coumaphos, when ingested in sugar water, than are 3-d-old bees (Van Buren et al. 1992). Drones from Apistan-treated colonies were less likely to survive after emergence as adults and weighed less than drones from untreated colonies (Rinderer et al. 1999). Queen larvae exposed to coumaphos either failed to develop or showed morphological and behavioral abnormalities, with more severe effects observed in colonies with wax containing tau-fluvalinate residue from prior Apistan treatment (Haarmann et al. 2002). Queens that survived coumaphos exposure weighed less than queens that had not been exposed to coumaphos (Pettis et al. 2004). Although not addressed in this study, honey bee brood may be at greater risk for suffering from synergistic interactions as brood can be up to an order of magnitude more susceptible to pesticides than adult bees (Atkins 1992). Larvae are also more likely to experience continuous exposure to tau-fluvalinate and coumaphos through contact with contaminated beeswax, in which they are effectively encased.

To manage varroa resistance to both tau-fluvalinate and coumaphos, beekeepers have been encouraged to adopt a rotation program alternating between Apistan and Checkmite+ (Elzen et al. 2001). In light of the potential for synergistic interactions between tau-fluvalinate and coumaphos, other miticides with no known potential for P450 interactions, such as the organic acids (Underwood and Currie 2005), should be considered for management of varroa. Further research is needed to determine the potential for the

observed synergism between coumaphos and tau-fluvaliante to harm whole bee colonies and to elucidate the risk to bees posed by wax contaminated with years of accumulated miticide treatments. The flagging effectiveness of the miticides tau-fluvalinate and coumaphos, combined with their propensity to accumulate in wax and synergize each other, is compelling evidence of an urgent need to develop alternative approaches to varroa management in commercial apiculture.

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