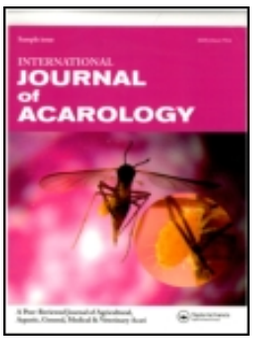

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ROLE OF DETOXIFICATION IN *VARROA DESTRUCTOR* (ACARI: VARROIDAE) TOLERANCE OF THE MITICIDE TAU-FLUVALINATE

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ABSTRACT – The varroa mite (*Varroa destructor* Anderson and Trueman) is a devastating pest of honey bees (*Apis mellifera* L.). Beekeepers have relied on the pyrethroid pesticide tau-fluvalinate as a principal agent of varroa mite control. While this miticide was quite effective at controlling varroa mites through the 1990s, its efficacy has waned as resistance to tau-fluvalinate has appeared in many populations of mites. Resistance in some populations of varroa mites has been associated with elevated detoxification of tau-fluvalinate. Honey bees tolerate miticidal tau-fluvalinate applications principally through rapid detoxification mediated by cytochrome-P450 mono-oxygenases, with the other detoxification enzyme families, the carboxylesterases and glutathione-S-transferases, playing much smaller roles in miticide tolerance. The goal of this study was to test the capability of the glutathione-S-transferase enzyme inhibitors diethyl maleate and curcumin, which should interfere minimally with honey bee detoxification, to elevate the toxicity of tau-fluvalinate to a population of varroa mites. Additionally, to test the role of cytochrome P450s and esterases in any detoxification-mediated resistance, varroa mites were also treated with the enzyme inhibitors piperonyl butoxide and S,S,S-tributyl phosphorotrithioate. None of the tested enzyme inhibitors increased the toxicity of tau-fluvalinate, suggesting that detoxification plays a minimal role in the tolerance of tau-fluvalinate in the population of varroa mites in this study.
Key words – Glutathione-S-transferase, varroa, *Apis mellifera*, pesticide resistance, pyrethroid, cytochrome P450 mono-oxygenase, curcumin.

INTRODUCTION

The Western honey bee (*Apis mellifera* L.), is among the most economically important insect species in North America; beyond its contributions to the US economy in the form of goods such as honey, wax, propolis, and pollen, the value of its service as a pollinator of plants used as food for humans and livestock exceeds $15 billion annually (Morse and Calderone, 2000). In recent years, the economic contribution of honey bee colonies has been threatened by colony collapse disorder (CCD) (Oldroyd, 2007). Although the cause of CCD has yet to be determined, the general consensus is that bee deaths from CCD are likely to be the result of a combination of diseases, parasites, and exposure to environmental toxicants (Watanabe, 2008). One ectoparasite of honey bees, the varroa mite (*Varroa destructor* Anderson and Trueman), has been killing colonies in the US since its introduction in the 1980s (Watanabe, 1994). As mite populations build up in a colony over several years, the bees, if untreated, will ultimately succumb to "parasitic mite syndrome" and the entire colony may die. Individual bees are directly harmed by varroa inasmuch as the mites suck

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hemolymph and parasitized bees are at increased risk of infection from the viruses vectored by mites and from the immunosuppression caused by varroa parasitism (Yang and Cox-Foster, 2005; Boecking and Genersch, 2008). Varroa mites may well be a contributing factor to CCD through their ability to transmit bee viruses (Yang and Cox-Foster, 2005), including Israeli acute paralysis virus, which may be associated with CCD (Chen and Evans, 2007; Cox-Foster *et al.*, 2007).

Beekeepers often use in-hive miticides to treat infested colonies and prophylactically to keep varroa populations low. In 1992, the first synthetic miticide, Apistan®, was registered for use in the USA. Apistan® treatment consists of suspending plastic strips impregnated with the pyrethroid pesticide tau-fluvalinate between frames within the hive for 6–8 weeks, during which time the pesticide is slowly released onto the bees that contact the strip. Tau-fluvalinate is a subset of isomers of fluvalinate; as a pyrethroid it inhibits deactivation of voltage-gated sodium channels (Davies *et al.*, 2007) and can kill mites at low concentrations and bees at substantially higher concentrations (Atkins, 1992).

Key to the success of tau-fluvalinate as an in-hive miticide is its relatively low toxicity to honey bees (Santiago *et al.*, 2000). As a class, the pyrethroids include some of the most toxic of all pesticides to honey bees (Atkins, 1992). Cytochrome P450 monooxygenases (P450s) and carboxylesterases (COEs) play a role in pyrethroid metabolism in bees (Pilling *et al.*, 1995). A bioassay study using inhibitors of P450s, COEs, and glutathione-S-transferases (GSTs) with tau-fluvalinate and other pyrethroids demonstrated that P450s are of primary importance in detoxifying these pesticides, with COEs and GSTs playing a lesser role (Johnson *et al.*, 2006).

Although tau-fluvalinate was initially quite effective at controlling mite infestations and exhibited low toxicity to bees, ostensibly because of rapid P450-mediated detoxification (Atkins, 1992; Johnson *et al.*, 2006), varroa mite populations rapidly developed resistance wherever tau-fluvalinate was used (Lodesani *et al.*, 1995; Milani, 1995; Elzen *et al.*, 1998; Mozes-Koch *et al.*, 2000; Goodwin *et al.*, 2005). Acquisition of resistance by mites was probably aided by the long-term low-dose exposure associated with Apistan® treatment, although resistance has also appeared in varroa populations with no history of miticide exposure (Goodwin *et al.*, 2005; Sammataro *et al.*, 2005).

The mechanism of tau-fluvalinate resistance in varroa doubtless follows the mechanisms of pyrethroid resistance that have developed in other pest arthropods: altered behavioral response, decreased penetration through the cuticle, decreased target site sensitivity, and elevated detoxification (Scott and Georghiou, 1986; Casida and Quistad, 1998). In varroa, mutations in the sodium channel target site have been identified that may contribute to resistance (Wang *et al.*, 2002). In terms of the contribution of detoxification enzymes to resistance, the fact that mites possess cytochrome P450 mono-oxygenases, GSTs, and COEs has been confirmed, but the role of these enzymes in resistance is not clear.

Esterase activity has been confirmed in mites (Gerson *et al.*, 1991). Whereas no difference in enzyme activity was observed between resistant and susceptible strains in studies using the model substrate naphthyl acetate (Wu *et al.*, 2003; Sammataro *et al.*, 2005), a 2.5-fold increase in esterase activity was documented in resistant mites using *p*-nitrophenyl acetate as a substrate (Mozes-Koch *et al.*, 2000). No P450 activity was observed in varroa when methoxyresorufin (Wu *et al.*, 2003), benzo(*a*)pyrene, or benzphetamine was used as a model substrate (Mozes-Koch *et al.*, 2000); *p*-nitrophenol, however, was metabolized and assays using resistant mites demonstrated a 20-fold increase in *p*-nitroanisole activity (Mozes-Koch *et al.*, 2000). Aryl hydrocarbon hydroxylase activity, based on another model P450 substrate, has also been reported in mites (Baars and Driessen, 1984). Bioassays using piperonyl butoxide (PBO) as an inhibitor of P450-mediated detoxification of tau-fluvalinate have produced similarly mixed results, with one study demonstrating a substantial increase in the toxicity of tau-fluvalinate in resistant populations of mites when treated with PBO, but only a minor increase in toxicity in susceptible populations (Hillesheim *et al.*, 1996). Other studies found no synergism between PBO and tau-fluvalinate in either susceptible or resistant mites (Bell *et al.*, 1999; Wu *et al.*, 2003). Early work demonstrated GST activity in varroa through the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB), a model substrate (Baars and Driessen, 1984; Gerson *et al.*, 1991). No difference in conjugation of CDNB was observed between resistant and susceptible strains of mites (Wu *et al.*, 2003). Bioassays using *N*-ethylmaleimide and diethylmaleate (DEM) found no synergistic increase in the toxicity of tau-fluvalinate to resistant mites (Bell *et al.*, 1999). However, DEM treatment increased the toxicity of pyrethroids to other mite species, suggesting that GST-mediated detoxification plays a central role in pyrethroid tolerance in mites (Yang *et al.*, 2001).

As both honey bees and mites rely on a diversity of biochemical and physiological mechanisms for detoxification of pesticides, identifying taxon-specific resistance mechanisms can provide important tools for management of resistance and continued use of chemically based management approaches. To clarify

the role detoxification plays in Apistan® tolerance, we used enzyme inhibitors specific to each of the three major detoxification enzyme families. Cytochrome P450s are inhibited with PBO, esterases are inhibited with S,S,S-tributyl phosphorotrithioate (DEF), and GSTs are inhibited with DEM.

Enzyme inhibitors have been commonly used as synergists to increase the toxicity of pyrethroids to pest insects (Ishaaya, 1993). Using enzyme inhibitors in the hive may partially restore the efficacy of tau-fluvalinate against resistant populations of varroa mites. While traditional enzyme inhibitors would be unsuitable for use in the hive as they are highly toxic and would be unacceptable in honey, some naturally occurring phytochemicals are effective inhibitors of detoxification enzymes and are classified as "generally recognized as safe" (GRAS). Curcumin is an inhibitor of GSTs and is already a common compound in many human diets as a constituent of the spice turmeric (Oetari *et al.*, 1996). The utility of curcumin as a synergist for tau-fluvalinate was also explored.

MATERIALS AND METHODS

Mites – Varroa mites (*Varroa destructor*) were collected from four colonies at the Michigan State University apiary in East Lansing, MI, between 15 September and 12 October 2006. No chemical control was used to limit the mite populations in 2006, although the hive boxes and frames may have come into contact with Apistan® in previous years. Mites were collected using the "sugar shake" method of Macedo and Ellis (2002). Approximately 350 bees were brushed from brood frames into a plastic basin and then poured into 473-ml wide-mouth Mason jars fitted with screen lids (3 × 3-mm mesh). Approximately 8 g powdered sugar was sifted through the screen and the jar was shaken for 10 seconds. The mites then fell through the screen onto a piece of paper. Mites were cleaned of sugar dust using a moistened paintbrush, placed in groups of 20 inside treated 20-ml glass scintillation vials, and kept at 27°C in a dark incubator. Two layers of KimWipe (Kimberly-Clark, Roswell, GA) sheets were placed underneath the vial cap to control moisture build-up.

LC_{50} determination – Values of the 50% lethal concentration (LC_{50}) were determined by placing varroa mites in 20-ml glass scintillation vials following the methods outlined in Elzen *et al.* (1998). The enzyme inhibitors DEM, DEF (Chem Services, West Chester, PA), and PBO (TCI America, Portland, OR) were dissolved in acetone and curcumin (Chem Services) was dissolved in ethanol. Maximum sublethal concentrations of each inhibitor were determined (1 µg/vial PBO and DEF; 100 µg/vial DEM and curcumin) and applied to vials. Solvent was allowed to evaporate as the vials were constantly rolled using a hot dog roller (HDG-598, The Helman Group, Oxnard, CA). Tau-fluvalinate (Chem Services) in acetone (12–6000 ng/vial) was then added to the vials and the solvent was again allowed to dry.

Twenty mites were placed inside each prepared vial and provided with a worker pupa for food 6 h later. Mortality was scored after 24 h, with mites that showed no response when contacted with a metal probe scored as dead. A log-probit line was fitted for each treatment from which the LC_{50} values and 95% confidence intervals were calculated using the *R* statistical package (R Development Core Team, 2008) with correction for heterogeneity when needed (Finney, 1971). Treatments with non-overlapping 95% confidence intervals are considered to demonstrate significant differences in toxicity.

RESULTS AND DISCUSSION

Inhibitor toxicity – Curcumin did not cause mite mortality at any tested concentration, even at the highest concentration, 1000 µg/vial. Diethyl maleate displayed intermittent toxicity at very high concentrations, with a calculated LC_{50} of 300.3 µg/vial; however, this quantity of liquid synergist probably killed some mites by physical submersion. Calculated LC_{50} values for DEF and PBO were 33.3 µg/vial and 73.5 µg/vial, respectively.

Measuring resistance – The population of mites evaluated in this study appears to be mildly resistant to tau-fluvalinate, with an LC_{50} of 384 ng/vial. Using the same bioassay technique, Wu *et al.* (2003) established an LC_{50} of 127 ng/vial for susceptible mites from Nebraska and 777–2957 ng/vial for mites from a resistant population from Cable, Wisconsin. Elzen *et al.* (1998) estimated the LC_{90} for susceptible mites from Texas using this assay at 2.4 µg/vial. The LC_{90} for the population of mites used in this study is 8.99 µg/vial (3.71–41.25 µg/vial), suggesting that this population of varroa mites is somewhat resistant to tau-fluvalinate. It is difficult to compare tau-fluvalinate toxicity statistics with others reported, as much previous work was performed using a different method in which tau-fluvalinate was incorporated into paraffin wax (Milani, 1995). The relatively shallow slope of the fitted probit line, 0.91 ± 0.06, suggests heterogeneity in the resistance of individual mites. Hillesheim *et al.* (1996) found the slope of the probit line was much steeper, 4.5 ± 0.8 and 6.7 ± 1.1, for two susceptible strains of mites, but similarly shallow, 1.6 ± 0.3 and 2.8 ± 0.5, for resistant strains. Clearly, resistance to tau-fluvalinate is widespread, relative to the susceptibility exhibited by mites historically, and

Table 1. Lethal concentration of tau-fluvalinate for 50% mortality (LC_{50}) and 95% confidence intervals (CI) to varroa mites when treated simultaneously with curcumin or DEM (100 μg/vial), both glutathione-S-transferase inhibitors, DEF (1 μg/vial), a carboxylesterase inhibitor, or PBO, a cytochrome P450 inhibitor. None of the inhibitor treatments significantly influenced the LC_{50} of tau-fluvalinate.

Inhibitor treatment	Total no. of mites	Tau-fluvalinate LC_{50} (ng/vial) 95% CI	Slope ± SE	Chi-squared	df
None	935	384 (219–579)	0.91 ± 0.06	24.7	8
Curcumin	700	651 (384–1280)	0.93 ± 0.07	24.2	8
DEM	680	448 (296–726)	1.04 ± 0.08	18.3	8
DEF	660	413 (298–601)	0.96 ± 0.08	8.1	7
PBO	680	432 (267–723)	0.88 ± 0.07	17.6	8

DEF, S,S,S-tributyl phosphorotrithioate; DEM, diethyl maleate; PBO, piperonyl butoxide.

appears to be present to some degree in the apiary used in this study despite the fact that Apistan® treatment had not been used for several years.

Synergism – No significant differences were observed in the toxicity of tau-fluvalinate to mites exposed to any of the inhibitor treatments (Table 1). This result indicates that detoxification contributes little to the tolerance of tau-fluvalinate in this population. Lack of synergism between PBO and tau-fluvalinate supports the findings of some previous bioassays using both susceptible and resistant mites (Bell *et al.*, 1999; Wu *et al.*, 2003), but it conflicts with the substantial synergism reported by others (Hillesheim *et al.*, 1996; Mozes-Koch *et al.*, 2000). This may be evidence that different resistant populations of varroa mites carry different mechanisms of resistance. Alternatively, differences in PBO synergism could be the result of differences in methods of PBO administration. Hillesheim *et al.* (1996) found synergism when PBO was applied topically to the dorsum of mites, but studies in which PBO has been administered indirectly to mites on the surface of a vial, including the present study, have failed to find PBO-synergism. To be practical, any in-hive synergist must be applied at the same time as the tau-fluvalinate treatment. It is possible that P450s could play a role in tau-fluvalinate resistance, but high concentrations of PBO or other P450 inhibitors would be required to restore the toxicity of tau-fluvalinate. Such concentrations, based on the results of past studies, would also inhibit honey bee metabolism of tau-fluvalinate and greatly increase the toxicity of this miticide to bees (Johnson *et al.*, 2006).

Although COE enzyme activity has been found in varroa mites, at best only a weak synergism has been found between DEF and tau-fluvalinate in bioassays (Hillesheim *et al.*, 1996). Lack of synergism could also be the result, as with PBO, of differences in inhibitor administration, but it seems unlikely that COEs actually make any substantial contribution to tau-fluvalinate resistance.

Both DEM and curcumin failed to synergize tau-fluvalinate at the concentrations tested. Both compounds appear to be relatively non-toxic to mites in the absence of tau-fluvalinate, suggesting that GSTs play a minor role in both the detoxification of tau-fluvalinate and mite survival in general. Curcumin, as the only solid inhibitor, was probably poorly absorbed through the cuticle of mites. It even appeared to slightly decrease the toxicity of tau-fluvalinate, probably because as a powder it formed a solid crust protecting mites from the tau-fluvalinate on the vial.

Inhibition of detoxifying enzymes failed to increase the toxicity of tau-fluvalinate to this population of varroa mites. This finding suggests that insensitivity of the voltage-gated sodium channel to tau-fluvalinate (Wang *et al.*, 2002; Liu *et al.*, 2006) is the basis for the elevated tolerance of tau-fluvalinate. That other populations of mites appeared to achieve resistance through detoxification indicates that varroa mites may be capable of multiple forms of resistance to tau-fluvalinate.

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