The Joint Toxicity of Type I, II, and Nonester Pyrethroid Insecticides

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ABSTRACT Evidence suggests that there are separate binding domains for type I and II pyrethroid insecticides on the voltage gated sodium channel of the nerve cell axon, but there are no studies that have examined the mixture toxicity of nonester pyrethroids and type I and II pyrethroids. Therefore, we examined the effect of nonester pyrethroid (etofenprox), type I (permethrin), and type II (cypermethrin) pyrethroid insecticides alone and in all combinations to *Drosophila melanogaster* Meigen. The combination of permethrin + etofenprox and permethrin + cypermethrin demonstrated antagonistic toxicity, while the combination of cypermethrin + etofenprox demonstrated synergistic toxicity. The mixture of permethrin + cypermethrin + etofenprox demonstrated additive toxicity. The toxicity of permethrin + cypermethrin was significantly lower than the toxicity of cypermethrin alone, but the combination was not significantly different from permethrin alone. The toxicity of permethrin + cypermethrin + etofenprox was significantly greater than the toxicity of both permethrin and etofenprox alone, but it was significantly lower than cypermethrin alone. The mixture of permethrin and etofenprox was significantly less toxic than permethrin. The explanation for the decreased toxicity observed is most likely because of the competitive binding at the voltage-gated sodium channel, which is supported by physiological and biochemical studies of pyrethroids. Our results demonstrate that the assumption that the mixture toxicity of pyrethroids would be additive is not adequate for modeling the mixture toxicity of pyrethroids to insects.

KEY WORDS ecotoxicology, antagonism, pesticide, mixture toxicity, cumulative exposure

Use of pyrethroid insecticides has increased substantially throughout the world as organophosphate, carbamate, and organochlorine insecticides are being phased out of use (USDHHS 2007, Spurlock and Lee 2008, USEPA 2010b). Pyrethroids represent ≈23% of the global insecticide market, with >3,500 registered formulations, and are widely used in agriculture, residential areas, public health, and food preparation (Casida and Quistad 1998, USEPA 2010a). Permethrin ((3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2dimethylcyclopropane carboxylate) and cypermethrin ((*RS*)-α-cyano-3-phenoxybenzyl (1*RS*,3*RS*;1*RS*, 3*SR*)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane1-carboxylate) are the most widely used pyrethroids in the United States (USEPA 2008, 2009). Nonester pyrethroids (also known as pseudopyrethroids) have not been widely used in the United States, but etofenprox (2-(4-ethoxyphenyl)-2-methylpropyl 3-phenoxybenzyl ether) recently was registered for the management of adult mosquitoes, with crop uses currently being evaluated (USEPA 2007).

There are three types of pyrethroids categorized based on their structure and toxicology, including those lacking the α-cyano group on the phenoxybenzyl moiety (type I), those with a α-cyano group on the phenoxybenzyl moiety (type II), and the nonester pyrethroids (nonester pyrethroids; Fig. 1) (Schleier and Peterson 2011). Pyrethroids act very quickly to produce symptoms of lost coordination and paralysis that are known as the knockdown effect and are often accompanied by spasms and tremors that induce intense repetitive activation in sense organs and in myelinated nerve fibers (Soderlund and Bloomquist 1989, Soderlund 1995, Breckenridge et al. 2009).

As a class, pyrethroids do not act in a similar fashion on the voltage-gated sodium channels (VGSC) of nerve cell axons and the classifications of toxicology for the pyrethroid types is not absolute with respect to type for either invertebrates or vertebrates (Wang et al. 2006, Breckenridge et al. 2009, Schleier and Peterson 2011). Type I pyrethroids modify the sodium channels in the closed state, whereas type II pyrethroids modify the open but not inactivated sodium channels (Soderlund 2010).

Type I pyrethroids modify the sodium channels such that there is a slight prolongation of the open time of the VGSC, which results in multiple long action potentials (Wright et al. 1988, Breckenridge et al. 2009). Type II pyrethroids significantly prolong the open time of the VGSC resulting in increased resting membrane potential and often inducing depolariza-

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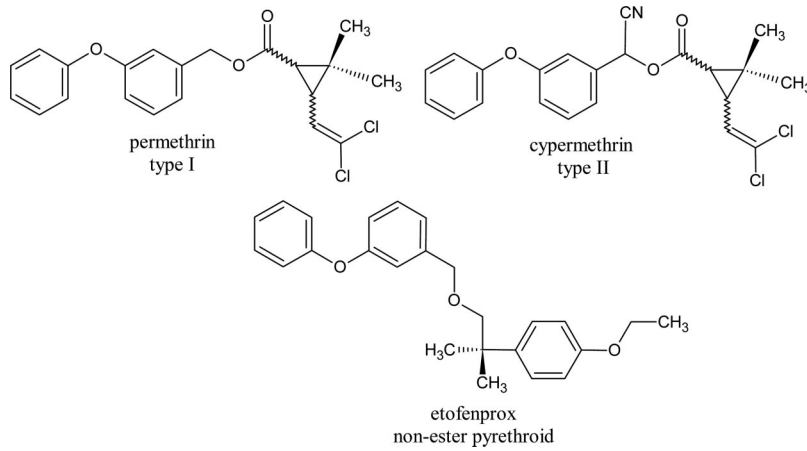

Fig. 1. Chemical structure of type I, type II, and nonester pyrethroids.

tion dependent block of action potentials (Wright et al. 1988, Schleier and Peterson 2011). Nonester pyrethroids induce repetitive discharges prolonging the opening of the VGSC similar to type I pyrethroids (Nishimura et al. 1996).

There is evidence that there are separate binding domains on the VGSC for type I and II pyrethroids that could explain why competitive binding may reduce the toxicity of pyrethroid mixtures (Vais et al. 2003, Brander et al. 2009, Hu et al. 2011, Schleier and Peterson 2011). Type I and II pyrethroids have been shown to segregate into separate types suggesting separate binding domains on the VGSC based on electrophysiological patch clamp experiments (Vijverberg et al. 1982, Breckenridge et al. 2009). In addition, there is evidence from resistance studies demonstrating that populations resistant to type I pyrethroids are susceptible to type II pyrethroids and nonester pyrethroids (Karunaratne et al. 2007, Perera et al. 2008). However, when mosquitoes were resistant to type II pyrethroids they were also resistant to nonester pyrethroids, suggesting that the binding sites for type II and nonester pyrethroids may be similar (Karunaratne et al. 2007, Perera et al. 2008). Brander et al. (2009) demonstrated that the type I pyrethroid permethrin and the type II pyrethroid cyfluthrin can be antagonistic to one another, lowering the overall toxicity of the mixture to *Daphnia magna* Straus.

To our knowledge, there are no studies examining the effect of nonester pyrethroids on the toxicity of type I and II pyrethroids. Because of the lack of studies examining the mixture toxicity of pyrethroid types, we examined the effect of the type I (permethrin), II (cypermethrin), and nonester pyrethroid (etofenprox) alone and in all combinations to *Drosophila melanogaster* Meigen. Our objective was to determine if the three pyrethroid types follow the assumption of dose-addition that chemicals with the same mode of action with the same binding domains have an additive toxicity when combined.

Materials and Methods

Eggs of wild-type Oregon strain *D. melanogaster* were obtained from Carolina Biological Supply Company (Burlington, NC). *D. melanogaster* was used as a surrogate for small nontarget insects and is a model organism for toxicity testing in a wide range of biological experiments (Kasbekar and Hall 1988, Daborn et al. 2002). *D. melanogaster* were reared on Formula 4–24 instant Drosophila media (Carolina Biological Supply Company) with yeast on laboratory bench (22.38 ± 0.04°C, photoperiod of 16:8 [L:D] h). We used female *D. melanogaster* that were allowed to mature for 2 d for all experiments.

Technical grade permethrin (98% purity, 50:50 mixture of the toxicology active *cis* and *trans* isomers), cypermethrin (98% purity, 50:50 mixture of the toxicology active *cis* and *trans* isomers), and etofenprox (98% purity) were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions and dilutions were prepared in high pressure liquid chromatography acetone (99.7% purity; EMD Chemicals, Gibbstown, NJ). The dose–response curves were determined under laboratory conditions using methods similar to those of Schleier and Peterson (2010). Serial dilutions were prepared in acetone for each active ingredient listed and all paired mixtures were composed of 1:1 ratios and the combination of the three insecticides was a 1:1:1 ratio of each insecticide. A 0.5-ml aliquot of test solution was dispensed into 20-ml glass scintillation vials with a total inside surface area of 40.26 cm^2 (Thermo Fisher Inc., Waltham, MA). Acetone was used as the control. Vials were placed on hot dog rollers (model HDR-565, The Helman Group, Ltd., Oxnard, CA) and rotated mechanically so that the acetone dried and the insecticide was uniformly coated in the vial. One female *D. melanogaster* was placed in each vial and covered with a cap. Treated vials were placed on large plastic trays and left on the laboratory bench with the same temperature, humidity, and photoperiod used for rearing. Mortality was assessed at 24 h, and female *D. melanogaster* that did

Table 1. LC_{50} values, slope coefficient estimates for the logistic regression model, 95% confidence interval (CI) for the coefficient estimates, *P* value for the slope coefficients for the logistic quasi-binomial model, and the mixture toxicity

Chemical mixture	LC_{50} ($\mu g/cm^2$)	Coefficient estimate	95% CI coefficient estimate	*P* value	Mixture toxicity
Permethrin	0.075	25.29	19.1–37.5	<0.0001	—
Etofenprox	0.1075	11.41	7.4–19.3	0.0066	—
Cypermethrin	0.0185	106.26	82.3–153.2	<0.0001	—
Permethrin + etofenprox	0.221	8.88	6.6–13.3	0.0002	Antagonistic
Permethrin + cypermethrin	0.081	24.38	16.4–40.1	0.0035	Antagonistic
Cypermethrin + etofenprox	0.019	102.37	77.6–150.9	0.0001	Synergistic
Permethrin + cypermethrin + etofenprox	0.0345	56.95	44.3–81.8	<0.0001	Additive

not move when stimulated by shaking the vial were considered dead.

To establish the concentration-mortality relationships, treatments were performed over time. The experimental design for each experiment was a randomized complete block with eight vials (individuals) per concentration, and nine concentrations (blocks). The treatments were permethrin, etofenprox, cypermethrin, permethrin + etofenprox, permethrin + cypermethrin, etofenprox + cypermethrin, and permethrin + etofenprox + cypermethrin. Each concentration was replicated seven times for a total of 56 individuals at each concentration for all treatments. If control mortality was greater than two individuals, the experiment was discarded and performed again.

Data were analyzed using R Statistical Package version 2.12.2 (The R Foundation for Statistical Computing, Vienna, Austria) and dose-mortality regressions were estimated by logistic regression analysis. A full model was fit to the data where all combinations were included in the model. Overdispersion was identified so a quasi-binomial model with an estimated overdispersion parameter of 13.25 was used to correct the standard errors (Agresti 2007). The quasi-binomial model method allows for departures from the usual assumption for binomial data, such as overdispersion caused by correlated observations or an unobserved explanatory variable (Agresti 2007). Abbott's formula was used to correct for control mortality (Abbott 1925, Perry et al. 1998). Significant differences between slopes were determined by using the 95% confidence interval overlap test.

Mixture toxicity was modeled by the concentration-addition equation:

$$C_{mix} = \sum_{i}^{n} \frac{c_i}{LC50_i} \quad [1]$$

where C_{mix} is the summed ratios of the insecticides in the mixture, c_i is the concentration ($\mu g/cm^2$) of chemical *i* in the mixture, and the LC_{50i} ($\mu g/cm^2$) is the lethal concentration that kills 50% of a population for chemical *i* in the mixture (Altenburger et al. 2000). Concentration-addition models are used for chemicals that exert effects on the same mode of action (Altenburger et al. 2000, Vijver et al. 2011).

Determination of additive, antagonist, and synergist interactions was modeled using the deviation from addition model:

$$DA = \frac{C_x}{C_{mix}} \times 100 \quad [2]$$

where DA is the deviation from addition, C_x is the observed LC_{50} from the mixture toxicity experiments, and C_{mix} is the value obtained from equation 1. Values of 100% represent an additive response, values exceeding 100% indicate synergism, and values <100% indicate antagonism (Vijver et al. 2011).

Results

The full model used to estimate the seven dose–response curves had significant coefficients for all mixtures, demonstrating a good fit of the model (Table 1). The order of toxicity was cypermethrin > cypermethrin + etofenprox > permethrin + cypermethrin + etofenprox > permethrin > permethrin + cypermethrin > etofenprox > permethrin + etofenprox (Table 1). The deviation from additive model estimates for permethrin + etofenprox, permethrin + cypermethrin, cypermethrin + etofenprox, and + cypermethrin + etofenprox was 41, 58, 332, and 97%, respectively. The combination of permethrin + etofenprox and permethrin + cypermethrin demonstrated antagonistic activity, while the combination of cypermethrin + etofenprox was synergistic (Table 1). The mixture of permethrin + cypermethrin + etofenprox demonstrated additive toxicity (Table 1).

The toxicity of permethrin + cypermethrin was significantly less than the toxicity of cypermethrin alone, but the combination was not significantly different from permethrin alone (Table 1; Fig. 1 and 2). The toxicity of permethrin + cypermethrin + etofenprox was significantly greater than the toxicity of both permethrin and etofenprox alone, but it was significantly less than cypermethrin alone (Table 1; Figs. 2–4). The mixture of permethrin and etofenprox was significantly less toxic than permethrin (Table 1; Fig. 2 and 4). The slope coefficient estimates for all mixtures containing cypermethrin were similar to cypermethrin alone suggesting cypermethrin may be the dominant toxicant overwhelming the toxicity of permethrin and etofenprox (Table 1).

Discussion

The order of toxicity for the three pyrethroids alone was similar to previous estimates (Siegfried 1993).

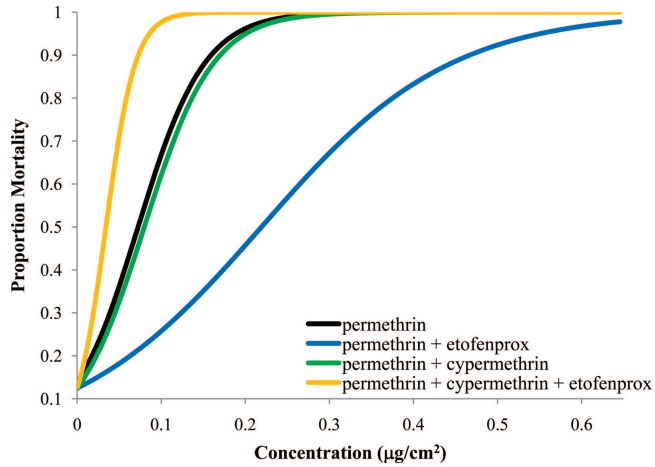

Fig. 2. Fitted concentration-response functions for permethrin, permethrin + cypermethrin, permethrin + etofenprox, and permethrin + cypermethrin + etofenprox using *D. melanogaster*. (Online figure in color.)

Because the mode-of-action for permethrin, cypermethrin, and etofenprox is the same, with different binding domains, according to the mixture toxicity hypothesis additive toxicity would be expected (Berenbaum 1985, Gardner et al. 1998, Altenburger et al. 2000). However, we found that the permethrin + etofenprox and permethrin + cypermethrin mixtures were 48 and 52% less toxic than would be expected if the toxicity was additive, respectively. The toxicity of permethrin + cypermethrin + etofenprox was similar to what would be expected if the toxicity was additive. The toxicity of permethrin + cypermethrin + etofenprox was significantly greater than the toxicity of both permethrin and etofenprox alone, but it was significantly less than cypermethrin alone, which is most likely because of the greater toxicity of cypermethrin compared with permethrin and etofenprox. The additive toxicity of the permethrin + cypermethrin + etofenprox may be the result of counteracting synergistic and antagonistic toxicity.

The mixture of permethrin and etofenprox was significantly less toxic than permethrin. The lower toxicity of permethrin + cypermethrin + etofenprox when compared with cypermethrin alone is most likely because of the competitive binding at the VGSC of permethrin with etofenprox and cypermethrin, which is known as competitive antagonism (Cassee et al. 1998). Type II pyrethroids are more potent in depolarizing the nerves than both type I pyrethroids and nonester pyrethroids (Salgado et al. 1983, Wright et al. 1988, Breckenridge et al. 2009). In addition, type II pyrethroids are metabolized more slowly than type I pyrethroids which may account for the differences in the toxicity observed in the current study (Salgado et al. 1983, Wright et al. 1988, Breckenridge et al. 2009).

The toxicity of cypermethrin + etofenprox was synergistic but the toxicity was similar to cypermethrin alone. Because the mixture was a 1:1 ratio of etofenprox and cypermethrin, this suggests that the presence of cypermethrin may have increased the toxicity of

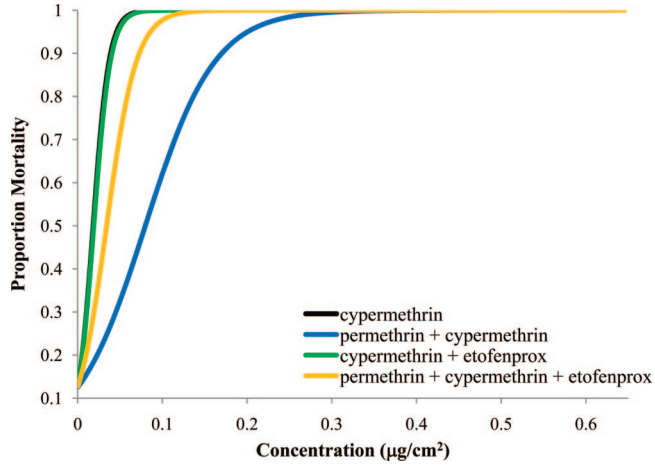

Fig. 3. Fitted concentration-response functions for cypermethrin, permethrin + cypermethrin, cypermethrin + etofenprox, and permethrin + cypermethrin + etofenprox using *D. melanogaster*. (Online figure in color.)

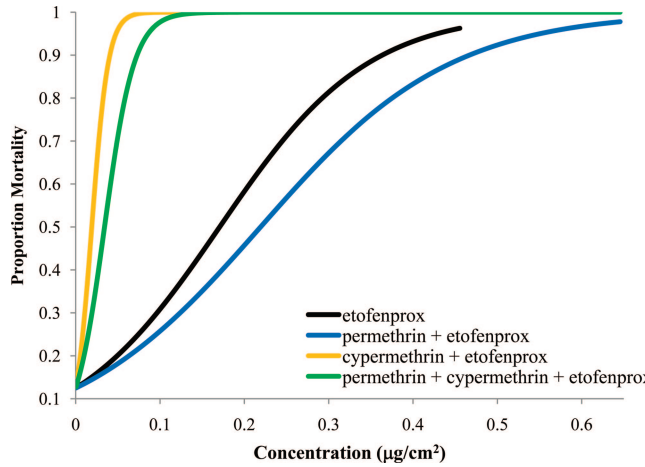

Fig. 4. Fitted concentration-response functions for etofenprox, permethrin + etofenprox, cypermethrin + etofenprox, and permethrin + cypermethrin + etofenprox using *D. melanogaster*. (Online figure in color.)

etofenprox. A similar binding site for nonester pyrethroids and type II pyrethroids is plausible because fenvalerate ((*RS*)-α-cyano-3-phenoxybenzyl (*RS*)-2-(4-chlorophenyl)-3-methylbutyrate; type II pyrethroid) provided the base molecule for synthesizing nonester pyrethroids (Katsuda 1999, Schleier and Peterson 2011).

The most likely explanation for decreased toxicity of the most toxic pyrethroid is because of the competitive binding at the VGSC, which is supported by physiological, biochemical, and organismal studies (Chang and Plapp 1983, Scott and Wen 2001, Vais et al. 2003, Brander et al. 2009, Schleier and Peterson 2011). Vais et al. (2003) examined the effects of mutations of the VGSC on the susceptibility of type I and II pyrethroids and found that, depending on the mutation, the sensitivity to one pyrethroid type but not the other type was decreased. Differing sensitivity to type I and II pyrethroids has been shown to result from a mutation on the VGSC which did not reduced the toxicity of type II pyrethroids, but decreased the toxicity to type I pyrethroids (Du et al. 2009a, b; Hu et al. 2011).

Resistance studies of multiple mosquito species have demonstrated that populations that are resistant to type I pyrethroids are susceptible to both type II pyrethroids and nonester pyrethroids (Perera et al. 2008). When mosquitoes are resistant to type II pyrethroids, they have been shown to be resistant to nonester pyrethroids (Karunaratne et al. 2007). Our results and results from other studies suggest that type II pyrethroids and nonester pyrethroids may act at the same binding domain on the VGSC, but biochemical studies will need to be performed with respect to the binding site of nonester pyrethroids. Our results indicating no reduction in the toxicity of cypermethrin when mixed with etofenprox support the current weight-of-evidence that nonester pyrethroids and type II pyrethroids bind to a similar site on the VGSC. The reduction in toxicity of permethrin and etofenprox when mixed provides evidence to support the hypothesis that type I pyrethroids competitively bind with type II pyrethroids and nonester pyrethroids. Thus, there seem to be pyrethroid type-specific binding domains on the VGSC.

Mixture toxicity of pyrethroids has been assumed to be additive because of a common binding site. Weston et al. (2005) found mixtures of both type I and II pyrethroids in sediment using additive toxicity models would result in toxic effects to nontarget benthic organisms. However, our study and Brander et al. (2009) show that this assumption may not be correct for mixtures of pyrethroids in aquatic environments. In aquatic environments, mixtures >1:1 ratio, like those studied in the current study, will most likely not be encountered because of differing pyrethroid use across areas (Weston et al. 2005, Amweg et al. 2006, Weston and Lydy 2010). Therefore, determination of toxicity based on additive models may overestimate the toxicity to organisms in aquatic environments. If the concentration of type II pyrethroid is greater than the other types it may be the dominant toxicant because of the greater toxicity, but in sediment permethrin and bifenthrin are generally present in the highest concentrations (Amweg et al. 2005, 2006), thus reducing the overall toxicity when mixed with type II or nonester pyrethroids.

The competitive binding of pyrethroid types potentially could affect malaria control using both indoor residual sprays and bed nets. Currently, type II pyrethroids and etofenprox are used for indoor residual sprays and the type I pyrethroid, permethrin, is used to treat bed nets (Alaii et al. 2003; Sreehari et al. 2007, 2009; Raghavendra et al. 2011). Because adult mosquitoes rest on the inside walls of buildings and seek a blood meal by landing on bed nets, mosquitoes could encounter a mixture of a type I and either nonester or type II pyrethroids reducing the toxicity. Therefore, pyrethroid types should be carefully considered when indoor residual sprays and bed nets are used together.

Our results are the first to demonstrate the effect of nonester pyrethroids on the toxicity of type I and II

pyrethroids. The mixture toxicity experiments support the findings of previous physiological and resistance studies that mixtures of pyrethroid types can inhibit the toxicity of the most toxic constituent. The toxicity of cypermethrin is not inhibited by etofenprox most likely because of a common binding site for type II and nonester pyrethroids that could account for the increased the toxicity of etofenprox when combined with cypermethrin. Conversely, permethrin displayed competitive binding with cypermethrin and etofenprox. The results of our study and those of Brander et al. (2009) demonstrate that the additive toxicity hypothesis is not accurate for modeling pyrethroid mixtures.

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