Environmentally Relevant Mixing Ratios in Cumulative Assessments: A Study of the Kinetics of Pyrethroids and Their Ester Cleavage Metabolites in Blood and Brain; and the Effect of a Pyrethroid Mixture on the Motor Activity of Rats.

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Abstract
National surveys of United States households and child care centers have demonstrated that pyrethroids are widely distributed in indoor habited dwellings and this suggests that co-exposure to multiple pyrethroids occurs in nonoccupational settings. The purpose of this research was to use an environmentally relevant mixture of pyrethroids to assess their cumulative effect on motor activity and develop kinetic profiles for these pyrethroids and their hydrolytic metabolites in brain and blood of rats. Rats were dosed orally at one of two levels (1.5x or 5.0x the calculated dose that decreases rat motor activity by 30%) with a mixture of cypermethrin, deltamethrin, esfenvalerate, *cis*-/*trans*-permethrin, and β-cyfluthrin in corn oil. At 1, 2, 4, 8, or 24 hours after dosing, the motor activity of each animal was assessed and the animals sacrificed. Concentrations of pyrethroids in brain and blood, and the following metabolites: *cis*-/*trans*-dichlorovinyl-dimethylcyclopropane-carboxylic acid, 3-phenoxybenzoic acid, 3-phenoxybenzyl alcohol, 4-fluoro-3-phenoxybenzoic acid, and *cis*-dibromovinyl-dimethylcyclopropane-carboxylic acid were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). Using this pyrethroid mixture in rats, the results suggest there is greater metabolism of *trans*-permethrin prior to entering the systemic circulatory system. All pyrethroids had tissue half-lives ($t_{1/2}$) of less than five hours, excepting esfenvalerate in brain. At early time points, relative pyrethroid brain concentrations approximated their dose mixture proportions and a sigmoidal E_{max} model described the relationship between motor activity decrease and total pyrethroid brain concentration. In blood, the $t_{1/2}$'s of the cyclopropane metabolites were longer than the phenoxybenzoic metabolites. However, relative to their respective precursors, concentrations of the phenoxybenzoic acids were much higher than concentrations of the cyclopropane metabolites. Brain concentrations of all metabolites were low relative to blood concentrations. This implies limited metabolite penetration of the blood-brain barrier and little metabolite formation within the brain. In conclusion: toxicokinetic differences between the pyrethroids did not appear to be important determinants of their relative potency and their effect on motor activity was consistent with a pyrethroid dose additive model.

Keywords: Pyrethroids, Pyrethroid Metabolites, Toxicokinetics, Half-Life

1 Introduction

Pyrethroids are a class of synthetic, neurotoxic, insecticides that are structurally based on purified extracts (pyrethrins) of *Chrysanthemum sp*. flowers. Characteristically, pyrethroids are described as phenoxybenzoic and cyclopropane moieties joined by an ester bond. Most pyrethroids have chiral centers at carbons 1 and 3 of the cyclopropane component, resulting in *cis*-/*trans*- configurations and pyrethroids are frequently grouped according to the absence (Type I) or presence (Type II) of a cyano group at the α-carbon of the phenoxybenzoic constituent. Both Type I and Type II pyrethroids lengthen the duration of voltage gated sodium channel opening (Narahashi et al.,1998; Soderlund et al., 2002), although Type I/II high dose differences in acute neurobehavioral effects have been noted (Verschoyle and Aldridge, 1980).

Sold for both commercial and retail purposes, pyrethroids have approved applications in many sectors including public health, agricultural, and residential pest control. Demonstrating their ubiquitous distribution in habited spaces, surveys of households (Stout II et al., 2009) and child care facilities in the U.S. (Morgan et al., 2004; Tulve et al., 2006) found at least one pyrethroid (permethrin) in nearly 3/4 of samples taken from indoor surfaces. Further, re-analysis (Tornero-Velez et al., 2012) of the Tulve et al. (2006) data showed that multiple pyrethroids are frequently co-located which suggests that non-occupational simultaneous exposure to multiple pyrethroids is common. Characterizing the combined effects resulting from simultaneous exposure to low levels of co-occurring pyrethroids is important because pyrethroid mixtures produce dose additive effects in rats (Wolansky et al., 2009) and the U.S. Environmental Protection Agency (U.S. EPA) has proposed using cumulative exposure (U.S. EPA, 2009) when estimating pyrethroid risk under the Food Quality Protection Act (FQPA, 1996).

Due to the likelihood of multiple pyrethroid exposure, it is important to understand the kinetics and metabolism of pyrethroid mixtures. However, most *in vitro* (Godin et al., 2006; Scollon et al., 2009) and *in vivo* (Anadón et al., 1991; Anadón et al., 1996; Anadón et al., 2006; Cole et al., 1982; Ding et al., 2004; Gaughan et al., 1977; Ohkawa et al., 1979) kinetic studies evaluated individual pyrethroids and did not include measurements of metabolic products. A recent toxicokinetic study of a pyrethroid mixture in rats (Starr et al., 2012) linked total pyrethroid brain concentration and motor activity, but did not measure tissue levels of the metabolites.

All published intentional human dosing reports are single pyrethroid studies. In these studies, the ester cleavage metabolites in urine were measured after dosing with β-cyfluthrin or cypermethrin (Eadsforth and Baldwin, 1983; Eadsforth et al., 1988; Leng et al., 1997a; Woollen et al., 1992). These studies provide insight into pyrethroid metabolism and elimination rates via certain metabolic pathways and the use of metabolites produced by ester cleavage has persisted in subsequent human occupational (Hardt and Angerer, 2003; Leng et al., 1996; Leng et al., 1997b; Leng et al., 2003; Shan et al., 2004; Smith et al., 2002), and non-occupational (Heudorf and Angerer 2001) biomonitoring research. However, considering that most pyrethroid biomarkers are not specific to individual pyrethroids, the interpretation of metabolite data can be problematic, particularly in non-occupational studies where both exposure level and pyrethroid identity are unknown.

Compared to additive approaches from single analyte studies, *in vivo* pyrethroid mixture studies can offer a more realistic basis for understanding the toxicokinetics and metabolism of the pyrethroid combinations predicted to occur in habited environments. The toxicokinetic data can

inform cumulative risk assessments more precisely, while simultaneously generated multi-pyrethroid metabolite data can reduce uncertainty in the dose mixture-excretion models used in human biomonitoring studies.

In this study, rats were dosed (oral gavage) with a mixture of pyrethroids most frequently detected in a national survey of child care centers (Tulve et al., 2006). Kinetic profiles were developed for cypermethrin, deltamethrin, esfenvalerate, *cis*-/*trans*-permethrin, and β-cyfluthrin and their ester cleaved metabolites; *cis*-/*trans*-dichlorovinyl-dimethylcyclopropane-carboxylic acid (*cis*-/*trans*-DCCA), 3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), and *cis*-dibromovinyl-dimethylcyclopropane-carboxylic acid (DBCA). 3-phenoxybenzyl alcohol (3-PBAlc), a precursor of 3-PBA was also measured. Finally, the motor activity of each rat was assessed to further inform the relationship between pyrethroid brain concentration and gross physical function.

2 Materials and Methods

2.1 Selection of Pyrethroids and Pyrethroid Metabolites

Figure 1 shows the structures and relationships of all study analytes. The pyrethroid mixture consisted of cypermethrin, deltamethrin, esfenvalerate, *cis*-/*trans*-permethrin, and β-cyfluthrin. The process by which the study pyrethroids were selected has been described previously (Starr et al., 2012; Tornero-Velez et al., 2012). Briefly, data from a national survey of child care centers in the United States (Tulve et al., 2006) was used to identify the predominate pyrethroids and calculate their relative proportions in the solutions used to dose the animals.

The metabolites 3-PBA, 4-F-3-PBA, *cis-/trans*-DCCA, and DBCA were selected because of the widespread use in occupational (Leng et al., 1996; Leng et al., 1997b; Leng et al., 2003; Shan et al., 2004; Smith et al., 2002) and non-occupational biomonitoring studies. 3-phenoxybenzyl alcohol (3-PBAlc) was included because it is an intermediate in the metabolism of Type I pyrethroids to 3-PBA.

2.2 Chemicals and Standards

Pesticide grade cyclopentane and acetonitrile (Honeywell Burdick & Jackson (Muskegon, MI), as well as methanol, ethyl acetate, hexanes, acetone (Fisher Scientific, Pittsburgh, PA) were used for sample processing. Water (18.2 MΩ resistance) was purified onsite. Standards used to prepare calibration curves were as follows: cypermethrin, deltamethrin, esfenvalerate, c*is*-permethrin, *trans*-permethrin, and β-cyfluthrin from Absolute Standards (Hamden, CT); 3-PBA, 4-F-3-PBA, and *cis-*/*trans*-DCCA from Cambridge Isotope Laboratories (Andover, MA); DBCA and 3-PBAlc from EQ Laboratories (Atlanta, GA). Excepting *trans*-permethrin (94%) and 4-F-3-PBA (95%), the purity of all standards, was ≥ 98%.

Analytes used to prepare internal and surrogate standards were obtained from these sources: $^{13}C_6$ *cis*-permethrin, $^{13}C_6$ *trans*-permethrin, $^{13}C_6$ cypermethrin, $^{13}C_6$ 3-PBA, $^{13}C_6$ 4-F-3-PBA and $^{13}C_6$ *trans*-DCCA were all purchased from Cambridge Isotope Laboratories (Andover, MA); biphenyl acid was provided at no cost by FMC Corporation (Philadelphia, PA). $^{13}C_6$ *trans*-permethrin and biphenyl acid were used as surrogates for the pyrethroids and metabolites respectively, and all other labeled compounds functioned as internal standards. The purity of all internal and surrogate standards was ≥ 97%.

All calibration standards were prepared by addition of pesticides or metabolites to processed and reconstituted blood and brain samples taken from unexposed rats. Three ranges of calibration curves (0.25 - 2.5 $ng*mL^{-1}$, 1.0 - 100 $ng*mL^{-1}$, and 25 - 1500 $ng*mL^{-1}$) were used for sample analysis. Samples were first analyzed with the 1.0 - 100 $ng*mL^{-1}$ curve and samples with concentrations outside this range were reanalyzed using one of the other two curves, as appropriate.

The physical/chemical properties of the pyrethroids used in the dosing solutions were reported by Wolansky et al. (2006). Permethrin and cypermethrin were provided by FMC Corporation (Philadelphia, PA), deltamethrin and ß-cyfluthrin were contributed by Bayer CropScience (Research Triangle Park, NC), and esfenvalerate was supplied by Dupont Crop Protection, (Wilmington, DE). All pyrethroids used for dosing were supplied as solids (technical grade). Corn oil (0.9 g/mL @ 25 ^{o}C) was purchased from Sigma Chemical.

2.3 Animals

Male, two month-old Long Evans rats were supplied by Charles River Laboratories (Raleigh, NC). Prior to the study, all rats were acclimated for at least 4 days in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. Pairs of rats were housed in 45 cm × 24 cm × 20 cm cages lined with heat-treated pine shavings bedding. Temperature (21 ± 2^{o}C), humidity (50 ± 10%), and light:dark periods (12L:12D) were controlled. Access to feed (Purina Rodent Chow 5001, Barnes Supply Co., Durham, NC) and tap water was not restricted.

2.4 Study Design

There were two pyrethroid mixture groups (high and low dose), and one vehicle (corn oil) control group in this study. The total pyrethroid dose in each of the two mixture groups was based on 1) a study by Wolansky et al. (2006) showing comparative decreases in motor activity of rats (relative potency) following oral administration of individual pyrethroids, and 2) research demonstrating the additive effect of multi-pyrethroid mixtures (Wolansky et al., 2009). Those studies determined the relative potency of each pyrethroid as well as the Effective Dose30 (ED_{30}- the pyrethroid dose expected to result in a 30% loss of motor activity relative to control animals) for pyrethroids individually or in combination. For this study, the total pyrethroid concentrations were 1.5× (low dose) and 5.0× (high dose) of the ED_{30} and were predicted by the pyrethroid dose additive model (Wolansky et al., 2009) to produce decreases in motor activity of 40 ± 20% and 60 ± 45% respectively. The concentrations of each pyrethroid and its *cis*-:*trans*- composition used in the dosing solutions are provided in Table 1.

All dosing was via oral gavage, using a corn oil vehicle of 1 $mL_{*}kg^{-1}$ body mass. Control rats received 1 $mL_{*}kg^{-1}$ corn oil only. Before dosing, appropriate concentrations of stock pyrethroid mixtures were made by combining the appropriate mass of each pyrethroid (technical grade). The mixture was then dissolved in corn oil by stirring at 40 - 45^{o} C for at least 15 minutes.

At 1, 2, 4, 8, or 24 hours after dosing, each animal's motor activity was assessed for one hour. One half hour after the end of motor activity assessment, the animal was anesthetized using CO_2 and exsanguinated by cardiac puncture. Whole blood samples were collected in 2 mL aliquots

and frozen in a methanol/dry ice bath. Brain samples were frozen using liquid nitrogen. Four animals were used for each dose and the control group at each of the five time points.

2.5 Motor Activity Assessment

The motor activity of each rat was assessed over a one hour period using photo-transistor/photodiode pairs to detect motion in a figure-eight maze. Both horizontal and vertical motions were counted and total counts were used to determine motor activity. The assessment methodology has been described in detail by Wolansky et al. (2006).

2.6 Tissue Processing

Two replicate blood and brain samples were used for each animal and both the pyrethroids and their metabolites were extracted from the same sample. Brain tissue was pulverized to a homogeneous powder while still frozen, then divided into samples weighing 350-400 mg. Blood was processed using the 2 mL aliquots. Both brain and blood were placed in borosilicate culture tubes and spiked with $^{13}C_6$-*trans*-permethrin and biphenyl acid (surrogate standards).

Pyrethroids were extracted by adding 5 mL acetone:hexane (2:8, V:V) to each sample. The samples were then vortexed for 10 minutes, followed by centrifugation at 3000 rpm for 10 minutes. The organic layer was transferred to another glass culture tube and extraction/clarification was repeated two additional times using 3 mL acetone:hexane (2:8, V:V) each time. The combined sample extracts were dried under N_2 and reconstituted in 1 mL hexane. Pyrethroid extracts were cleaned using 500 mg silica Solid Phase Extraction (SPE) columns (Waters, Inc., Milford, MA), with 5 mL of 6% ethyl acetate in hexane used as the eluting

solvent. After elution of the pyrethroids, the SPE tubes were rinsed with methanol and the rinsates were set aside to recover any metabolites that had been co-extracted with the pyrethroids. The cleaned pyrethroid extracts were then dried under nitrogen, and internal standards were added. The samples were reconstituted in 1 mL methanol:water (9:1, V:V) and transferred to autosampler vials.

Following the pyrethroid extraction, the brain and blood samples were acidified to increase metabolite partitioning into the organic solvent. To do this with brain, 1 mL water (pH 3) and 2 mL water (pH 1) were added to each sample. The samples were vortexed and adjusted to pH ≤ 3 by dropwise addition of HCl as needed. Blood samples were prepared in the same manner except 1 mL of water (pH 1) and 1 mL of water (pH 3) were added in the initial step. The pH of all samples was monitored throughout acidification using pH indicator strips (J.T. Baker, Phillipsburg PA). After the pH of a sample was ≤ 3, 100 mg NaCl was added to each sample to facilitate precipitation of macromolecules.

Metabolites were extracted by partitioning the acidified samples against acetone:hexane (2:8, V:V). After addition of the organic solvent (3 mL brain, 4 mL blood), the samples were vortexed and centrifuged with the same settings that were used for the pyrethroids. The organic layer was collected and the samples were each partitioned two additional times.

Brain extracts (metabolites) were combined with the rinsates from the pyrethroid extraction (used to recover any metabolites that had been co-extracted with the pyrethroids). Samples were loaded onto 500 mg C18 SPE cartridges in 5 mL water:methanol (9:1,V:V), eluted with 7 mL

methanol:water (75:25,V:V), then dried to 0.4 mL under N_2. Internal standards and 0.5 mL methanol were added and the samples transferred to autosampler vials.

Extracts of metabolite in blood were combined with the appropriate rinsates (collected during clean-up of the parent pyrethroids) and dried completely under N_2. Samples were reconstituted in 2 mL pH 3 water and partitioned (3x) against hexane (1:1, v:v). The hexane fractions were combined and dried to completeness under N_2; internal standards were added and the samples were reconstituted in 1 mL pH 3 water:methanol (4:6), and transferred to autosampler vials.

2.7 Sample Analysis

Chromatography of all samples was done using an Agilent (Palo Alto, CA) model 1100 high performance liquid chromatograph (LC). An AB SCIEX (Framingham, MA) model API 4000-Tandem Mass Spectrometer (MS/MS) system configured with a turbo ion spray served as the detector for all analytes except 3-PBAlc which was measured using a fluorescence detector. The specifics of the instrument settings are located in Supplementary Tables 1-3 in the Supplementary Online Data.

2.8 Method Validation, Limits of Detection/Quantitation (LOD/LOQ) and Quality Control/Quality Assurance (QA/QC)

The procedures used to validate the recoveries and estimate the LOD/LOQ for the analytes have been described previously (Starr et al., 2012). Briefly, percent recoveries and LOD/LOQ were determined by repetitive spiking of blood (2 mL) and brain (350-400 mg) samples taken from unexposed animals, then using the finalized methods to calculate concentrations. The concentrations of most analytes used for validation were 25 and 50 ng/sample and that of most

analytes used to determine LOD/LOQ were 1, 5, 10, and 15 ng/sample. The exceptions to this were *cis*- and *trans*-DCCA which were purchased as a 3:7 mixture (total DCCA equaled the listed concentration). The concentrations of 3-PBAlc used to estimate LOD/LOQ were 5, 25, 50, and 75 ng/sample.

The QA/QC processes used in this study have also been detailed previously (Starr et al., 2012) in a study that included only the parent pyrethroids. In summary, calibration curves were run before and after each batch of samples and during each run, a blank and midlevel standard were injected after every six samples. In contrast to the previous report (Starr et al., 2012), the acceptable surrogate recovery range was expanded to 70-130% (due to low metabolite recovery) and all sample concentrations were surrogate corrected.

2.9 Data Analysis

All parent pyrethroid and metabolite tissue concentration data were processed and analyzed using SAS/STAT software, version 9.2 (SAS Institute, Cary, NC) as described previously by Starr et al., (2012). Briefly, only tissue concentrations at or above the LOQ were used, with statistical significance assigned at a probability of ≤ 0.05 for all tests performed. Prior to comparing selected sample hours and dose level concentrations, distributions using pairwise Student's t-tests, conditions of data set normality were evaluated using a Shapiro-Wilk statistic. The assumption of normality was appropriate for more than 88% of all possible concentration distributions when stratified by substance of interest, tissue, sample hour, and dose level. Therefore, all statistical analyses were done assuming a normal distribution of the data.

When estimating analyte half-lives ($t_{1/2}$), first order elimination rates were assumed and all estimates and homogeneity of regression analyses were done using a general linear model (PROC GLM). The $t_{1/2}$ of each analyte, in each tissue, was first compared statistically across dose, then pooled $t_{1/2}$ estimates were generated using dose normalized tissue concentrations. The pyrethroid concentrations were normalized by dividing the tissue concentrations by the dose concentration. The metabolites were normalized using the average factor difference in the high and low dose levels of pyrethroids, a value of 3.33.

The relative percent concentration of each pyrethroid in blood and brain was calculated for the first tissue sampling period (2.5 hours after dosing) by dividing each individual pyrethroid concentration by the summed concentration of all pyrethroids. These values were compared with an expected percent contribution as calculated from administered dose (Table 1).

Metabolite concentrations in the blood and brain of each animal were divided by the respective concentration of that animal's 3-PBA. In addition, (excluding 3-PBAlc), individual metabolite concentrations in both blood and brain were compared at each time point to their respective precursor pyrethroid concentrations. Specifically, concentrations of 3-PBA (and 3-PBAlc) were compared to the sum of all pyrethroids, excluding cyfluthrin. 4-F-3-PBA was compared to cyfluthrin and DBCA with deltamethrin. *Cis*-DCCA and *trans*-DCCA were normalized by summing permethrin, cypermethrin and cyfluthrin and using the *cis*- to *trans*- isomer ratios of the pyrethroids listed in Table 1.

A four parameter logistic, or sigmoidal E_{max} model (Dmitrienko et al., 2007; Starr et al., 2012) was used to relate variability in motor activity (the response, *Y*) to total pyrethroid brain concentration (*X*) :

$$Y = E_{max} + \frac{(E_{min} - E_{max})}{1 + \left({}^{X}/_{EC_{50}}\right)^{h}}$$

Using a control-normalized response, upper and lower bounds of the response are approximated (E_{max} and E_{min}), along with the sigmoidal curve inflection (EC_{50}), the concentration at which the median response is attained, and *h*, the absolute maximum curve slope determined at the median response.

3 Results

3.1 Method validation

The method limits of detection, quantitation and percent recovery of spiked analytes are listed in Table 2. With the exception of 3-PBAlc, which was analyzed using a fluorescence detector, all limits of detection were below 7.0 pmol/g. Generally, sensitivities and recoveries were better for the pesticides than for the metabolites and the estimated detection limits for all analytes were lower in blood than in brain. The recovery of spiked metabolites from the 25 ng matrix spikes was less than that of the 75 ng spikes for all analytes except 3-PBalc. This may have resulted from a small amount of analyte binding to the matrix or glassware, which was more evident at the lower concentration.

3.2 Pyrethroids in blood and brain

The mean concentrations of the study pyrethroids in both dose groups, at each time point, are presented in Table 3. Statistical comparisons of the low dose group tissue concentrations at 2.5

and 3.5 hours indicated no significant difference between the two time points for any pyrethroid in brain tissue. In blood samples from the low dose group, cypermethrin ($p = 0.04$), deltamethrin ($p = 0.04$), esfenvalerate ($p = 0.03$), and β-cyfluthrin ($p = 0.03$) were significantly lower at 3.5 hours than at 2.5 hours. Comparison of high dose group concentrations at these same time points showed no statistical differences ($p \leq 0.05$) in concentration between hours 2.5 and 3.5 in blood or brain. After adjusting for dose (tissue concentration divided by administered dose), there were no significant differences between high and low dose group concentrations at 2.5 hours for any pyrethroid in blood, while this same comparison in brain found significant high and low dose differences for cypermethrin, esfenvalerate, and *cis*-permethrin. At 3.5 hours there were no significant differences between dose levels for any pyrethroid in either tissue. Comparison of brain / blood ratios at 2.5 hours by dose level showed a significant difference for esfenvalerate only ($p = 0.02$). This same comparison at 3.5 hours found no between dose level differences for any pyrethroid.

Table 4 lists the estimated $t_{1/2}$'s of each pyrethroid, for both dose groups, in blood and brain. Between dose homogeneity of regression tests on the elimination rates in blood indicated that only esfenvalerate had a significant dose related difference. The same comparisons in brain showed no significant dose related differences for any pyrethroid. However, neither the high and low dose esfenvalerate slopes in brain were significantly different from zero.

Table 4 also contains dose-pooled (after adjusting for dose) $t_{1/2}$ estimates for each pyrethroid in both tissues. After pooling the doses, the $t_{1/2}$'s of all pyrethroids in blood were between 3 and 4 hours and homogeneity of regression tests showed there were no significant differences between

them. In brain, there was a wider range of estimated half-lives, bounded by *trans*-permethrin (2.7 hours) and esfenvalerate (7.1 hours). Homogeneity of regression tests showed that the $t_{1/2}$ of *trans*-permethrin was statistically different than esfenvalerate and the $t_{1/2}$'s of all other pyrethroids were significantly different from *trans*-permethrin and/or esfenvalerate.

Comparisons of each pyrethroid / total pyrethroid blood concentration at 2.5 hours with its relative percent in the dosing solution (Table 1) showed that the relative percent concentration (mean ± se) of cypermethrin (60% ± 1.0), and deltamethrin (7.9% ± 0.2) in blood were higher than that predicted by relative administered dose. *Trans*-permethrin (2.0% ± 0.3) was lower than expected while *cis*-permethrin (17% ± 0.8), esfenvalerate (3.6% ± 0.1), and β-cyfluthrin (9.7% ± 0.4) were generally consistent with their respective dose proportions. To a large extent, the discrepancies in blood concentration (relative to dose) were offset by differences in partitioning from the blood to the brain at the 2.5 hours sample period as both *cis*- and *trans*-permethrin had brain / blood ratios greater than one, while the ratios of all other pyrethroids were less than one. Thus, the relative concentrations of cypermethrin (23% ± 0.4), deltamethrin (3.4% ± 1.2), esfenvalerate (1.7% ± 0.1) and β-cyfluthrin (7.9% ± 0.3) in brain are similar to their relative percent predicted by administered dose. Although *cis*-permethrin (59% ± 1.3) and *trans*-permethrin (5.7% ± 0.5) were respectively, much higher and lower than predicted by dose, total permethrin was only about 15 percentage points higher than predicted by its relative dose proportion.

3.3 Motor activity and pyrethroid brain concentration

The relationship between motor activity and total pyrethroid brain concentration fit a sigmoidal E_{max} model ($p = 0.0003$) with maximum and minimum response thresholds approximately 81% ($p < 0.0001$) and 24% ($p = 0.04$) of control motor activity, respectively. The pyrethroid concentration that resulted in a 50% reduction of motor activity (EC_{50}) was 1,044 pmol/g (421 ng/g) brain. The mean percent decrease in motor activity at the time of peak effect (1-2 hours post dosing low dose group; 2-3 post dosing high dose group) was 27 ± 16% for the low dose group and 61 ± 28% for the high dose group.

3.4 Metabolites in blood and brain

Mean tissue concentrations of all metabolites, in both dose groups, are provided in Table 3. The predominant metabolite in blood and brain was 3-PBA, which is consistent with it being a hydrolytic product of all of the pyrethroids administered to the rats with the exception of β-cyfluthrin. Excepting DBCA and the intermediate 3-PBAlc, most samples contained measurable metabolite concentrations. In brain, DBCA was present at concentrations above the detection limit in only 18 (out of 40) samples while 3-PBAlc was not detected in any sample. In blood, there were measurable concentrations of DBCA and 3-PBAlc in 39 and 22 (out of 40) samples, respectively. All other metabolites were detected in all blood samples. No significant differences ($p \leq 0.05$) in metabolite levels were observed between 2.5 hour and 3.5 hour time points in blood or brain, excepting 4-F-3-PBA which was significantly higher at the earlier time ($p = 0.028$) in low-dose animals blood samples.

The metabolite $t_{1/2}$ estimates are presented in Table 4. Statistical comparison of the half-lives in blood and brain showed the only significant dose-related differences to be 3-PBA and 4-F-3-PBA

in blood. Several metabolite $t_{1/2}$ estimates were not significantly different than zero including: DBCA (brain, low and high dose), *cis*-DCCA (brain, low dose), *trans*-DCCA (blood, low dose), 3-PBAlc (blood high dose). Considering the cyclopropane metabolites, the inability to estimate a $t_{1/2}$ for *cis*-/*trans*-DCCA was probably due to the relatively short duration of the experiment, whereas for DBCA, the low detection frequency especially at later time points, may have also contributed. The $t_{1/2}$ of 3-PBAlc in the low dose blood group was likely short enough to have been estimated within the experimental timeframe, but the sensitivity of the detector was inadequate to measure the low dose tissue concentrations. All estimated dose pooled half-lives for both tissues were significantly different than zero and, in both blood and brain, the phenoxybenzoic acid metabolites were consistently eliminated at a faster rate than the cyclopropane carboxylic acid metabolites.

The mean $t_{1/2}$ of the phenoxybenzoic metabolites in both blood and brain were comparable, or slightly longer, than those of their precursors. Half-lives of the phenoxybenzoic metabolites in the brain appeared slightly longer than in the blood, and were also longer than most of the parent pyrethroids in brain. In contrast, with $t_{1/2}$'s $\geq$ 9.5 hours, the cyclopropane metabolites in both blood and brain were much more stable than their parent compounds.

Based only on the relative concentration of each pyrethroid in the dosing solution (Table 1), the metabolite with the highest initial tissue concentrations was expected to be either 3-PBA or 3-PBAlc. And, assuming equal hydrolysis, no isomerization, and 37 : 63 *cis*-:*trans*-β-cyfluthrin isomer percentages, the expected initial relative concentrations of 3-PBA : 4-F-3-PBA : DBCA : *cis*-DCCA : *trans*-DCCA would be 1.00 : 0.15 : 0.04 : 0.49 : 0.62 (not estimated for the

intermediate 3-PBAlc). However, as seen in Table 5, when tissue metabolite concentrations were normalized to 3-PBA, only 4-F-3-PBA in both blood and brain was present at the predicted relative concentrations. DBCA, not found at measurable concentrations in brain tissue, was about 100-fold lower in blood than predicted at 2.5 hours. *Cis*-DCCA was about 20 times lower than expected in both blood and brain while *trans*-DCCA concentrations were approximately 10-fold (brain) and 20-fold (blood) less than predicted at 2.5 hours. In brain, the mean (± se) *trans*-/*cis*-DCCA ratio (dose groups pooled) ranged from 3.2 ± 0.5 at 2.5 hours to 4.1 ± 2.8 at 25.5 hours. In blood, this ratio was 1.6 ± 0.5 at 2.5 hours and 2.9 ± 0.4 at 25.5 hours. The mean *trans*-/*cis*-DCCA ratio during the entire study period was 3.4 ± 0.3 and 2.1 ± 0.1 for blood and brain respectively. The cyclopropane/phenoxybenzoic ratio ((DBCA + *cis-DCCA* + *trans*-DCCA) / (3-PBA + 4-F-3-PBA + 3-PBAlc)) in brain was 0.1 ± 0.02 at 2.5 hours and 0.76 ± 0.1 at 25.5 hours. This ratio in blood was 0.08 ± 0.1 at 2.5 hours and 1.1 ± 0.3 at 25.5 hours.

Since most of the study metabolites have more than one parent pyrethroid, the relative concentrations (metabolite concentration/sum of precursor pyrethroid concentrations) in blood and brain at each time point were calculated and are presented in Table 6. The data in this table incorporates the relative tissue concentrations of individual pyrethroids in the mixture for comparison with the appropriate metabolite. This table shows that the ratios for 3-PBA and 4-F-3-PBA in both blood and brain were generally (excepting *trans*-DCCA at 25.5 hours) greater than that of the other metabolites. For 3-PBA and 4-F-3-PBA the 2.5 hour metabolite/pyrethroid ratios in the brain were respectively 13- and 12-fold lower than in the blood. For *cis*-DCCA and *trans*-DCCA these initial ratio differences between blood and brain were 11- and 4-fold, respectively.

4 Discussion

In this study, an environmentally relevant pyrethroid ratio was used to study the toxicokinetics, metabolism, and the neurobehavioral effect of this mixture in rats. This approach may prove to be a practical way to study chemicals sharing a common mode of action when generating data for use in cumulative risk assessment. The inclusion of the hydrolytic metabolites further informed interpretation of the relative pyrethroid tissue concentrations in rats and provided data for use in human pyrethroid dose-excretion models.

4.1 Pyrethroids in blood and brain

The estimated $t_{1/2}$'s of the pyrethroids in blood were an average of 2.1 ± 0.3 hours longer than observed in a previous study (Starr et al., 2012) where the same combination of pyrethroids was used. Since one of the dosing levels (1.5 x ED_{30}) in that study was the same as the low dose level used in this study, the difference can be attributed to the inclusion of data from the 25.5 hour time point in the current analysis. In the earlier study, the 25.5 hour time point was not used due to the high frequency of non-detects, and data were limited to the first 9.5 hours after dosing. Inclusion of the 25.5 hour time point provided additional data for all pyrethroids except esfenvalerate, which was only found in blood samples from hours 2.5 - 9.5. And, as seen in Table 5, the $t_{1/2}$ of esfenvalerate in blood (low dose) was shorter than that of the other pyrethroids. The impact of using the 25.5 hour time point was verified by a re-analysis of the other pyrethroids in the current data set using only the 2.5 - 9.5 hour time points. This change resulted in shorter estimated $t_{1/2}$'s that were consistent with those of the previous study and eliminated the esfenvalerate dose dependent difference observed here. This shift in the estimated

$t_{1/2}$ is similar to that seen in an *in vivo* study by Anadón et al. (1996) of deltamethrin in plasma where the elimination curve was bi-phasic with relatively rapid clearance from hours 2- 12, followed by a markedly slower elimination rate thereafter.

The low blood concentration of *trans*-permethrin was especially notable because it was the pyrethroid administered at the highest dose and its $t_{1/2}$ in blood was not different than the other pyrethroids. Starr et al. (2012) showed that these low blood levels were not a result of greater *trans*-permethrin fat sequestration and suggested that differences in protein binding were also insufficient to explain the difference. It is possible that the low *trans*-permethrin levels may have resulted from intestinal metabolism prior to its entering the systemic circulatory system. Crow et al. (2007) used rat tissues to demonstrate *in vitro* hydrolysis of *trans*-permethrin by intestinal microsomes and cytosol, but found that these same preparations were not active against deltamethrin. Intestinal microbial metabolism of pyrethroids might also explain the differential degradation of permethrin isomers. Maloney et al. (1998) reported on the higher hydrolysis of *trans*-permethrin than *cis*-permethrin in microbial cultures isolated from soils. In addition, Gaughan et al. (1977) showed that rat fecal excretion of metabolized and un-metabolized *trans*-permethrin was less than fecal excretion of metabolized and un-metabolized *cis*-permethrin. Since age related changes in intestinal flora occur, it would be useful to see if the relatively low *trans*-permethrin blood levels are replicated in rats of differing age groups.

The consistency of all pyrethroids $t_{1/2}$ in blood is noteworthy considering their different concentrations in the dosing solutions and the use of two different dosing levels. The similarities contrast with differences in metabolic rates reported in the single pyrethroid *in vitro* and *in vivo*

studies cited earlier, and suggest a simplified rat mixture pharmacokinetic model since detoxification rates in blood appear independent of dose and pyrethroid identity.

There was an obvious difference in the *cis*- and *trans*-permethrin (Type I pyrethroids) blood/brain ratios compared to Type II pyrethroids. Apparently, the relatively low permethrin blood concentrations (especially *trans*-permethrin) were partially offset by greater *cis*-/*trans*-permethrin penetration of the blood brain barrier. Comparison of HPLC retention times indicated that permethrin is more hydrophobic than the other study pyrethroids. However, the octanol-water partition coefficient of permethrin is similar to that of esfenvalerate, β-cyfluthrin, and cypermethrin so solubility differences may not entirely explain the higher concentrations of permethrin in brain.

The dose proportionate brain concentrations seen in this study have been reported previously (Starr et al., 2012). This suggests that differences in the relative potencies of the study pyrethroids reported by Wolansky et al. (Wolansky et al., 2006) are not explained by toxicokinetic differences that result in higher brain concentrations of the more potent pyrethroids. Similarly, in general, the duration of the pyrethroids $t_{1/2}$ does not reflect their relative potency. However, it is interesting that esfenvalerate, with the longest $t_{1/2}$, also has the highest relative potency.

4.2 Motor activity and pyrethroid brain concentration

The two doses used in this study were 1.5x and 5.0x the ED_{30} and motor activity decrease was predicted by the sigmoidal E_{max} model with high and low response thresholds. The mean percent

461 decrease in motor activity at the time of peak effect were consistent with the pyrethroid dose
462 additive model proposed by Wolansky et al. (2009) which predicts a peak effect decrease of
463 approximately 40 ± 20% at a dose equal to 1.5x ED_{30}, and 60 ± 45% decrease at 3.7x ED_{30} or
464 greater. The results were also consistent with those obtained by Starr et al. (2012), although the
465 estimated EC_{50} in the cited study was lower, there was not a significant difference between the
466 two studies in estimates of the upper and lower response bounds.
467

468 **4.3 Metabolites in blood and brain**

469 As shown in Figure 1, this study focused on those pyrethroid metabolites that are formed via
470 ester bond cleavage. And these are the metabolites commonly used in pyrethroid biomonitoring
471 studies (Eadsforth and Baldwin, 1983; Eadsforth et al., 1988; Hardt and Angerer, 2003; Leng et
472 al., 1996; Leng et al., 1997b; Leng, 2003; Shan et al., 2004; Smith et al., 2002; Woollen et al.,
473 1992). Esterase mediated hydrolysis of pyrethroids appears to be a primary mechanism by
474 which ester cleavage occurs (Kaneko and Miyamoto, 2001). However, oxidase catalyzed
475 hydroxylation at the α carbon has been proposed as an additional mechanism for ester cleavage
476 of both Type I and Type II pyrethroids with the *cis*- configuration (Casida and Ruzo, 1980).
477 Pyrethroid hydroxylation also occurs at other sites, and exhaustive schematics of pyrethroid
478 metabolism (Kaneko and Miyamoto, 2001) show that, in rats, these sites include the gem-
479 dimethyl group of the cyclopropane moiety and the 2' and especially the 4' -position of the
480 phenoxy group. However, these hydroxylates are not generally used in human biomonitoring
481 and the metabolites which are used constitute a metabolic subset where the ester bond of the
482 parent pyrethroid has been broken and the functional group of the phenoxybenzoic moiety has
483 been further oxidized, but the cyclopropane has not.

The ratio of 4-F-3-PBA to 3-PBA (Table 5) throughout the experiment, in both blood and brain, was consistent with their relative proportions in the dosing solution. Since 4-F-3-PBA was only produced from a Type II pyrethroid whereas 3-PBA precursors were a Type I and II mixture, the *in vitro* esterase / oxidase, Type I vs. Type II differences seen in metabolic studies using liver microsomal preparations (Scollon et al., 2009; Soderlund and Casida, 1977), may be minimized in whole animal studies.

Relative to the cyclopropane metabolites, the shorter blood $t_{1/2}$ of the phenoxybenzoic moieties implies that the intermediate reactions needed to oxidize 3-PBAlc to 3-PBA and 4-F-3-PBA occur rapidly and were not rate limiting. In blood, the concentrations of 3-PBA and 4-F-3-PBA were much higher than each of the cyclopropane metabolites and this difference was greater than predicted based on the relative number of precursor molecules (Table 6). Considering this, and the longer $t_{1/2}$ of the cyclopropane metabolites, it is possible that there was more pre-hydrolysis hydroxylation of the cyclopropane moiety, while the phenoxybenzoic acids were oxidized largely after hydrolysis. However, this could not be determined our study since hydroxylated species were not measured and rat excretion studies of permethrin (Gaughan et al., 1977) and decamethrin (Ruzo et al., 1978) suggest that there is relatively little hydroxylation of the gem-dimethyl group. In addition, Anadon et al. (1996) found significant concentrations of 4'-OH-deltamethrin in the brains of rats dosed with deltamethrin. It is also possible that the phenoxybenzoic acids were conjugated more rapidly than the cyclopropane metabolites, but again, the relevant species were not assessed in this study.

The pyrethroid metabolites are not generally considered to be neurotoxicants, and therefore, metabolite formation is regarded as a detoxification step. The high metabolite / precursor ratios for 3-PBA, 4-F-3-PBA, and *cis*-/*trans*-DCCA in blood relative to brain (Table 6), as well as the absence of DBCA and 3-PBAlc in brain, suggests that little pyrethroid hydrolysis occurred in the brain and the majority of the metabolites present may have either diffused or been transported across the blood / brain barrier. Ghiasuddin and Soderlund (1984) found low levels of pyrethroid hydrolysis by soluble esterase isolated from mice brains. Of the measured pyrethroids in that study, fenvalerate was the most rapidly hydrolyzed by the brain esterases whereas in this study it had the longest brain $t_{1/2}$. This suggests that brain esterase activity may be species specific and additional research to isolate any isoforms responsible for hydrolysis in the brain of rats would be informative.

At the 2.5 and 3.5 hour time points, the mean ratio of *trans*- : *cis*-DCCA in blood, were 1.6:1 and 2:1 respectively. These ratios are similar to the 1.3:1 *trans*- : *cis*-DCCA ratio predicted by the relative proportions of their pyrethroid precursors in the dosing solution. Considering this, and the similarity in $t_{1/2}$ of *cis*- with *trans*-DCCA, and permethrin with the other pyrethroids in blood, suggests that more extensive metabolism of *trans*-permethrin prior to entering the systemic circulation. Since *trans*-permethrin in blood was only 2% the pyrethroid total at 2.5 hours, it appears that most of the *trans*-DCCA formed during this hydrolysis did not enter the circulatory system. However, Gaughan et al. (1977) found that neither *trans*-permethrin nor any *trans*-cyclopropane metabolites were present at appreciably higher concentrations than *cis*-permethrin or *cis*-DCCA cyclopropane metabolites in the feces of rats dosed with *cis*- or *trans*-permethrin.

530 It is also possible that the high *trans*-DCCA concentrations resulted from differences in
531 metabolic pathway (i.e. *trans*- hydrolysis > *cis*- hydrolysis and *cis*- oxidation > *trans*- oxidation)
532 of the various pyrethroids. Ross et al. (2006) found that Type I pyrethroids are hydrolyzed more
533 easily than Type II and *trans*-isomers more rapidly than *cis*- by liver microsomes, and in this
534 study, the ratios in blood of both DBCA and *cis*-DCCA (dose pooled, all time points) to their *cis*-
535 pyrethroid precursors, were significantly lower than that of *trans*-DCCA to its *trans*-pyrethroid
536 precursors (Table 6). This supports the prevailing hypothesis that the hydrolytic metabolic
537 pathway is not as important for the *cis*-pyrethroids. But, given the similarities of the pyrethroids
538 $t_{1/2}$ in blood, this does not explain the low blood concentration of *trans*-permethrin, relative to the
539 other pyrethroids.
540
541 The one hour motor activity test precluded collection of urine samples but the mean ratio of free
542 *trans*-:*cis*-DCCA in blood was 2:1 during the 25.5 hours of this study. Interestingly, the *trans*-
543 *cis*-DCCA ratio (free and conjugated) found in 24 hour urine samples of rats dosed orally with
544 permethrin was 3:1 (Gaughan et al., 1977). In contrast, the phenoxybenzoic to the cyclopropane
545 metabolite ratios which ranged from 14:1 at 2.5 hours to 1.5:1 at 25.5 hours, in our study were
546 different than the urinary phenoxybenzoic:cyclopropane ratio of 2:1 (free and conjugated) found
547 in that study (Gaughan et al.,1977). This discrepancy may be due to differences in the
548 composition of the pyrethroid mixture compared to that of permethrin, used alone in the cited
549 study. Alternatively, there could be differential binding of some metabolites to un-excreted
550 macromolecules. Another explanation is that the phenoxybenzoic metabolites are further
551 oxidized prior to conjugation and/or excretion. In rats, following permethrin administration,
552 hydroxylated phenoxybenzoic acids are excreted in concentrations ≥ 3-PBA (Elliott et al., 1976;

553 Gaughan et al., 1977). While it is not clear that this species is formed from hydroxylation of 3-
554 PBA or the parent pyrethroid prior to hydrolysis, Huckle et al. (1981) demonstrated significant
555 hydroxylation of 3-PBA to 4' HO3-PBA in rat liver. Interestingly, the relative proportions of the
556 phenoxybenzoic and cyclopropane metabolites in the cited studies were similar to those found in
557 the urine samples from single Type II pyrethroid human oral dosing (Eadsforth et al.,1988;
558 Eadsforth and Baldwin 1983; Woollen et al., 1992), inhalation (Leng et al., 1997a), and
559 occupational exposure studies (Leng et al., 1997b) where the *trans*-:*cis*- ratios of the
560 cyclopropane metabolites were approximately 2:1 and the cyclopropane:phenoxybenzoic ratios
561 ranged from 0.6:1 (Leng et al., 1997a) to 2:1 (Leng et al., 1997b). Clearly, a mixture study in
562 which blood, feces, and urine samples were collected would increase understanding of the
563 relevance of a pyrethroid mixture rat model to human biomonitoring studies.
564

565 **5 Conclusions**

566 In this study, we examined the toxicokinetics of a simultaneously administered mulit-pyrethroid
567 mixture and their hydrolytic metabolites in rats. The pyrethroid pesticides in this mixture study
568 elicited a sigmoidal decrease in motor activity and the magnitude of the decrease was consistent
569 with a dose additive model. Relative concentrations of the pyrethroids in the brain at the early
570 time points approximated their proportions in the dose mixture, indicating toxicokinetic
571 differences are not the source of relative potency differences observed in single pyrethroid motor
572 activity studies. Absorption of metabolites into the brain appeared limited, and, in the brain,
573 there was little evidence of significant pyrethroid hydrolysis. In blood, the relative proportions
574 of the metabolites were consistent with urinary metabolite ratios found in single pyrethroid

575 human studies suggesting this dose mixing paradigm may prove useful in building human dose
576 excretion models of pyrethroid mixtures.

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Conflict of Interest Statement
The authors declare there is no conflict of interest.

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Pyrethroid	Administered Dose (µmol/kg)		Composition		Relative Potency[b]
	Low	High	% of Total	*cis*-: *trans*-[a]	
cypermethrin	7.7	25.7	30	48.7 : 51.3	0.235
deltamethrin	0.9	3.0	3	100:0	1.000
esfenvalerate	0.7	2.4	3	NA[c]	2.092
permethrin	14.0	46.4	50	40 : 60	0.059[d]
β-cyfluthrin	3.4	11.5	13	32-42 : 58-68	1.136
Total Dose	26	89			

47.

48. [a] *Cis*- to *trans*- isomer ratios (Wolansky et al., 2006).

49. [b] Potency based on pyrethroid motor activity ED_{30} relative to deltamethrin (Wolansky et al., 2006).

50. [c] Not applicable.

51. [d] Relative potencies of *cis*-/*trans*-permethrin isomers not determined independently.

	Quantitation / Detection Limits[a] (pmol/g)				Percent Recovery from Spiked Tissue[b] (mean ± SE)			
	LOD		LOQ		25 ng		75 ng	
Analyte	**Brain**	**Blood**	**Brain**	**Blood**	**Brain**	**Blood**	**Brain**	**Blood**
cyfluthrin	0.24	0.02	0.79	0.04	99 ± 3	97 ± 1	95 ± 2	100 ± 3
cypermethrin	0.38	0.08	1.26	0.26	105 ± 2	95 ± 2	99 ± 4	95 ± 4
deltamethrin	0.12	0.09	0.41	0.28	104 ± 2	97 ± 1	97 ± 6	95 ± 4
esfenvalerate	1.15	0.25	3.85	0.84	106 ± 3	99 ± 4	98 ± 6	91 ± 4
trans-permethrin	0.80	0.05	2.65	0.15	102 ± 5	111 ± 7	99 ± 4	99 ± 4
cis-permethrin	0.38	0.23	1.26	0.75	102 ± 5	99 ± 2	97 ± 4	95 ± 4
3-PBA	6.54	0.89	21.82	2.99	66 ± 4	37 ± 4	94 ± 12	75 ± 5
4-F-3-PBA	5.34	0.69	17.84	2.28	69 ± 5	55 ± 3	91 ± 12	80 ± 7
DBCA	3.09	0.60	10.23	2.08	63 ± 6	65 ± 3	94 ± 7	78 ± 7
cis-DCCA	3.54	1.39	11.72	4.59	42 ± 5	61 ± 1	93 ± 10	76 ± 7
trans-DCCA	6.99	2.20	23.25	7.32	68 ± 7	54 ± 4	77 ± 5	82 ± 7
3-PBAlc	35.05	5.68	116.8	18.95	82 ± 9	67 ± 9	85 ± 5	69 ± 4

52.

53. [a] Four tissue samples at each of four concentrations, with each injected four times.

54. [b] Four tissue samples at each of the two spiked concentrations.

Tissue /Dose[a]	Hour	Pyrethroid Concentration (Mean ± SE, pmol/g)						Metabolite Concentration (Mean ± SE, pmol/g)					
		cypermethrin	deltamethrin	esfenvalerate	*cis*-permethrin	*trans*-permethrin	cyfluthrin	3-PBA	4-F-3-PBA	DBCA	*cis*-DCCA	*trans*-DCCA	3-PBAlc
Brain low dose	2.5	105 ± 11[a]	22 ± 15	9 ± 0.7	277 ± 19	25 ± 4	35 ± 3	583 ± 30	72 ± 7	ND[b]	18 ± 3	52 ± 16	ND
	3.5	82 ± 12	9 ± 1	8 ± 1.2	270 ± 31	14 ± 2	24 ± 5	499 ± 80	54 ± 8	ND	30 ± 4	70 ± 10	ND
	5.5	51 ± 11	5 ± 0.7	7 ± 0.5	243 ± 43	9 ± 2	14 ± 1	385 ± 59	42 ± 9	ND	23 ± 2	74 ± 24	ND
	9.5	15 ± 3	3 ± 0.5	ND[b]	125 ± 17	3 ± 0.3	5 ± 0.8	244 ± 40	30 ± 5	ND	19	54 ± 6	ND
	25.5	1[c]	ND	ND	7 ± 1	ND	0.7	ND	ND	ND	ND	49	ND
Brain high dose	2.5	219 ± 22	24 ± 1	14 ± 2	556 ± 46	60 ± 6	83 ± 7	1,362 ± 165	159 ± 34	ND	44 ± 14	139 ± 53	ND
	3.5	247 ± 60	35 ± 7	24 ± 5	674 ± 140	58 ± 18	91 ± 25	1,412 ± 303	157 ± 33	ND	45 ± 2	141 ± 18	ND
	6.0	159 ± 34	26 ± 7	21 ± 5	646 ± 124	33 ± 9	55 ± 14	1,078 ± 144	115 ± 20	ND	38 ± 13	121 ± 41	ND
	9.5	66 ± 21	13 ± 4	12 ± 3	359 ± 67	13 ± 4	17 ± 8	963 ± 319	104 ± 36	ND	62 ± 19	229 ± 65	ND
	25.5	3 ± 1	0.6 ± 0.2	ND	25 ± 7	ND	1 ± 0.2	123 ± 7	ND	ND	22 ± 8	69 ± 17	ND
Blood low dose	2.5	503 ± 99	66 ± 12	28 ± 5	141 ± 37	17 ± 5	76 ± 15	13,804 ± 907	1,919 ± 108	91 ± 21	419 ± 64	704 ± 89	78 ± 18
	3.5	218 ± 50	30 ± 7	11 ± 4	76 ± 14	10 ± 4	29 ± 8	10,166 ± 1,526	1,256 ± 203	66 ± 13	288 ± 59	705 ± 217	51 ± 9
	6.0	83 ± 39	10 ± 4	5 ± 2	32 ± 15	4 ± 2	10 ± 5	8,866 ± 1,490	1,051 ± 174	141 ± 19	580 ± 69	1,006 ± 100	ND
	9.5	11 ± 2	1 ± 0.2	1 ± 0.3	3 ± 0.4	0.3 ± 0.1	1 ± 0.2	6,058 ± 1,359	630 ± 162	56 ± 10	289 ± 45	731 ± 169	ND
	25.5	2 ± 0.5	0.8 ± 0.4	ND	0.9	0.2 ± 0	0.3 ± 0.1	297 ± 113	16 ± 5	20 ± 7	122 ± 52	277 ± 95	ND
Blood high dose	2.5	1,299 ± 160	170 ± 16	82 ± 10	378 ± 35	45 ± 20	226 ± 28	25,711 ± 1,658	3,542 ± 581	181 ± 30	971 ± 306	1,486 ± 464	303 ± 49
	3.5	1,089 ± 394	168 ± 61	66 ± 23	335 ± 113	38 ± 20	220 ± 85	25,703 ± 5,486	3,477 ± 886	215 ± 48	936 ± 186	1,533 ± 294	287 ± 102
	6.0	338 ± 111	40 ± 14	20 ± 7	86 ± 34	12 ± 8	43 ± 15	21,961 ± 4,860	2,710 ± 709	124 ± 40	772 ± 328	1,390 ± 481	70
	9.5	189 ± 127	25 ± 16	13 ± 9	58 ± 41	4 ± 2	27 ± 18	18,744 ± 4,338	2,402 ± 889	216 ± 19	1,310 ± 122	2,166 ± 235	78 ± 35
	25.5	7 ± 1	1 ± 0.1	1 ± 0.4	2 ± 1	0.3 ± 0.1	1 ± 0.2	1,530 ± 423	99 ± 25	46 ± 19	231 ± 94	626 ± 209	ND

55. [a] n= 4 for each tissue and dose group.

56. [b] Analyte either not detected or below the limit of quantitation.

57. [c] Single data point, standard error not estimated.

	Mean $t_{1/2}$ (hrs) (95% upper and lower confidence bounds)					
	Brain			**Blood**		
Analyte	**Low Dose**	**High Dose**	**Pooled Dose**	**Low Dose**	**High Dose**	**Pooled Dose**
cypermethrin	3.4 (2.8 - 4.2)	3.6 (3.1 - 4.3)	3.5[e] (3.2 - 3.9)	3.3 (2.7 - 4.5)	3.3 (2.7 - 4.4)	3.3 (2.8 – 4.0)
deltamethrin	3.3 (2.1 - 7.3)	3.9 (3.3 - 4.7)	3.8[d,e] (3.3 - 4.5)	3.8 (2.8 – 6.0)	3.5 (2.7 - 4.8)	3.7 (3.0 - 4.6)
esfenvalerate	9.4[a]	10[a]	7.1[d] (4.1 - 26)	1.5 (1.1 - 2.4)[b]	3.9 (2.7 - 6.9)	3.5 (2.7 - 5.3)
cis-permethrin	4.2 (3.8 - 4.7)	4.6 (3.9 - 5.6)	4.4[d,f] (4.0 - 4.9)	2.7 (1.9 - 4.5)	3.3 (2.6 - 4.7)	3.2 (2.6 - 4.1)
trans-permethrin	2.5 (2.0 - 3.3)	2.9 (1.9 - 6.8)	2.7 (2.1 - 3.6)	3.6 (2.4 - 7.1)	3.8 (2.6 - 7.1)	3.7 (2.8 - 5.4)
cyfluthrin	3.9 (3.2 - 5.1)	3.9 (3.3 - 4.8)	3.9[d,e] (3.4 - 4.4)	3.2 (2.5 - 4.5)	3.2 (2.5 - 4.5)	3.2 (2.7 – 4.0)
3-PBA	5.5 (3.8 - 9.3)	6.6 (5.3 - 8.7)	6.2 (5.3 - 7.5)	4.0[b] (3.5 - 4.6)	5.2 (4.3 - 6.8)	4.5 (4.0 - 5.2)
4-F-3-PBA	5.9 (3.5 - 17)	8.9[a]	6.9 (4.3 - 17)	3.3[b] (2.9 - 3.7)	4.2 (3.5 - 5.4)	3.7[g] (3.3 - 4.2)
DBCA	ND[c]	ND	ND	9.7 (6.7 - 17)	10 (6.6 - 21)	9.6[g,h] (7.2 - 14)
cis-DCCA	41[a]	25[a]	18[g] (9.7 - 155)	10 (7.1 - 20)	10 (6.4 - 25)	10[g,h] (7.5 - 16)
trans-DCCA	82 [a]	22[a]	23[g] (12 - 126)	13 (8.6 - 28)	16 (8.9 - 89)	14[g,h] (9.7 - 27)
3- PBAlc	ND	ND	ND	1.9[b]	3.3 (1.7 - 92)	3.4[i] (2.0 - 12)

58.

59. [a] Estimated decay rate not statistically different than zero.

60. [b] Estimated decay rate statistically different by dose (within tissue).

61. [c] Analyte either not detected or below limit of quantitation.

62. [d] Estimated decay rate statistically different than *trans*-permethrin

63. [e] Estimated decay rate statistically different than esfenvalerate

64. [f] Estimated decay rate statistically different than cypermethrin

65. [g] Estimated decay rate statistically different than 3-PBA

66. [h] Estimated decay rate statistically different than 4-F-3-PBA

67. [i] Estimated decay rate statistically different than *trans*-DCCA

Tissue	Time	[Metabolite] / [3-PBA] (mean ± SE) 3-PBA	4-F-3-PBA	DBCA	*cis*-DCCA	*trans*-DCCA
Brain	2.5	1[a]	0.12 ± 0.009	ND[b]	0.03 ± 0.004	0.09 ± 0.017
	3.5	1	0.11 ± 0.008	ND	0.05 ± 0.009	0.13 ± 0.011
	5.5	1	0.11 ± 0.008	ND	0.05 ± 0.008	0.15 ± 0.038
	9.5	1	0.11 ± 0.012	ND	0.07 ± 0.011	0.24 ± 0.021
	25.5	1	ND	ND	0.19 ± 0.07	0.64 ± 0.148
Blood	2.5	1	0.14 ± 0.006	0.01 ± 0.001	0.03 ± 0.005	0.05 ± 0.008
	3.5	1	0.13 ± 0.005	0.01 ± 0.001	0.03 ± 0.003	0.07 ± 0.009
	5.5	1	0.12 ± 0.004	0.01 ± 0.003	0.05 ± 0.01	0.09 ± 0.016
	9.5	1	0.11 ± 0.008	0.01 ± 0.001	0.06 ± 0.008	0.12 ± 0.009
	25.5	1	0.06 ± 0.004	0.06 ± 0.018	0.34 ± 0.135	0.73 ± 0.164

68. [a] n=4
69. [b] Analyte(s) either not detected or below the limit of quantitation.

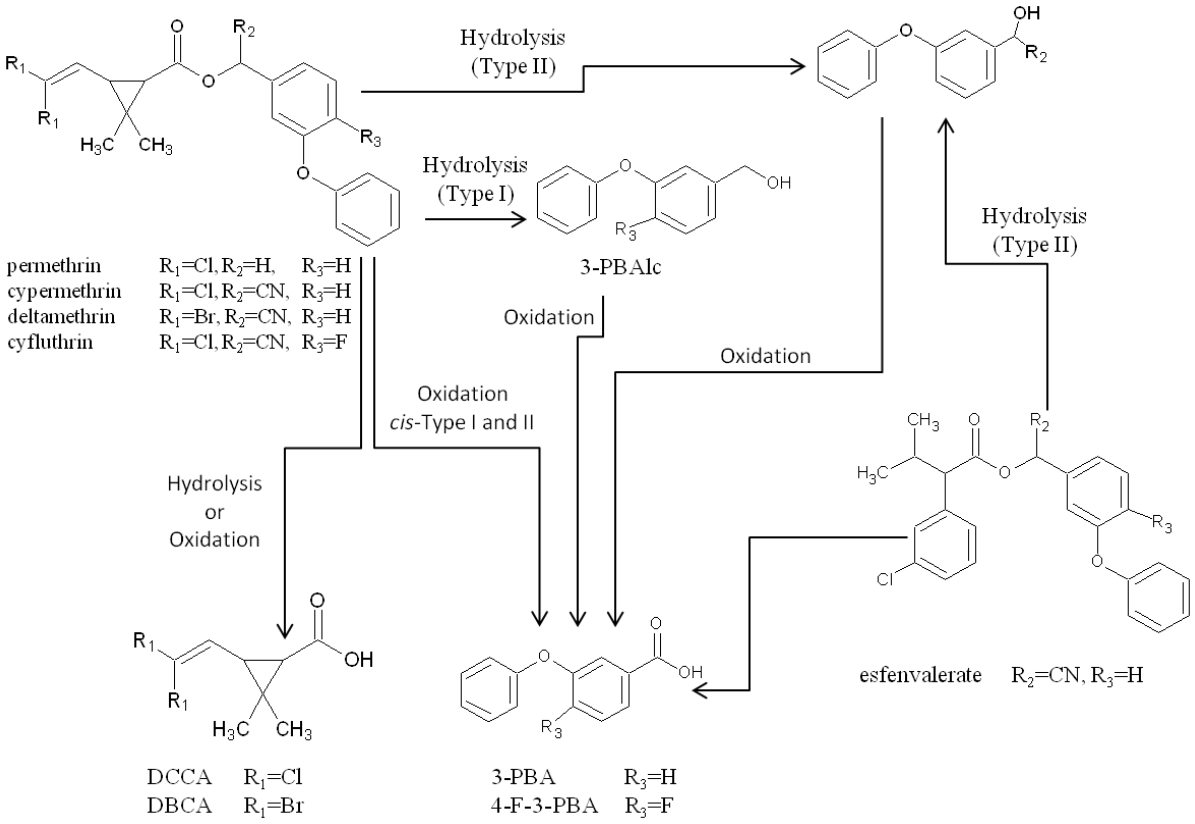
		[Metabolites[a]] / [Precursor Pyrethroids]				
Tissue	**Time**	**3-PBA**	**4-F-3-PBA**	**DBCA**	***cis*-DCCA[b]**	***trans*-DCCA[b]**
Brain	2.5	1.4 ± 0.1	2 ± 0.2	ND[c]	0.1 ± <0.1	0.6 ± 0.1
	3.5	1.3 ± 0.1	2.1 ± 0.2	ND	0.1 ± <0.1	0.9 ± 0.1
	5.5	1.3 ± 0.1	2.6 ± 0.3	ND	0.1 ± <0.1	1.3 ± 0.4
	9.5	1.8 ± 0.1	6.0 ± 0.6	ND	0.1 ± <0.1	4.1 ± 0.2
	25.5	4.0 ± 1.0	ND	ND	0.7 ± <0.1	ND
Blood	2.5	18 ± 2.7	23 ± 4.8	1.5 ± 0.5	1.1 ± 0.3	2.3 ± 0.7
	3.5	29 ± 6.0	39 ± 8.6	2.4 ± 0.6	1.7 ± 0.5	4.9 ± 1.3
	5.5	75 ± 14	121 ± 30	12 ± 3.7	7.9 ± 2.6	19 ± 6.3
	9.5	252 ± 51	325 ± 74	36 ± 7.1	28 ± 5.3	71 ± 14
	25.5	110 ± 23	71 ± 14	40 ± 12	44 ± 14	152 ± 38

70.

71. [a] Relative metabolite concentration = metabolite concentration (nM) / sum of precursor pyrethroid concentrations (nM)

72. [b] Assumes no *cis*-/*trans*- isomerization occurred and that the proportion of *cis*-:*trans*- cyfluthrin in the dosing solution was 37:63

73. [c] Analyte either not detected or below the limit of quantitation.


Hydrolysis
(Type II)
Hydrolysis
(Type I)
3-PBAlc
Oxidation
Oxidation
Oxidation
cis-Type I and II
Hydrolysis
(Type II)
Hydrolysis
or
Oxidation
permethrin R1=Cl, R2=H, R3=H
cypermethrin R1=Cl, R2=CN, R3=H
deltamethrin R1=Br, R2=CN, R3=H
cyfluthrin R1=Cl, R2=CN, R3=F
esfenvalerate R2=CN, R3=H
DCCA R1=Cl
DBCA R1=Br
3-PBA R3=H
4-F-3-PBA R3=F