

# DOSE-RESPONSE MODELING OF MICROARRAY DATA TO IDENTIFY A BIOCHEMICAL MARKER OF EFFECT FOR PYRETHROID INSECTICIDES AFTER ACUTE EXPOSURE *IN VIVO*: SPECIFIC UPREGULATION OF CAMK1 $\gamma$ 1

J.A. Harrill<sup>1</sup>, Z. Li<sup>2</sup>, F.M. Wright<sup>2</sup>, K.M. Crofton<sup>3</sup> • <sup>1</sup>Curriculum in Toxicology, UNC-CH, Chapel Hill, NC, <sup>2</sup>Department of Biostatistics and Carolina Environmental Bioinformatics Research Center, UNC-CH, Chapel Hill, NC, <sup>3</sup>Neurotoxicology Division, NHEERL, ORD, USEPA, RTP, NC.

TOXICOGENOMICS

## Abstract

Pyrethroid insecticides produce neurotoxicity in mammals by interacting with a number membrane bound ion channels in excitable nerve membranes. Pyrethroid use has increased as the use of other pesticide compounds has declined. There is a dearth of information concerning the intracellular response of neurons following the interaction of pyrethroids with these molecular targets. Nor is a sensitive, specific, dose-responsive biomarker of effect available that correlates with pyrethroid-induced disruption of nervous system function. This study used a combination of statistical regression methods to identify dose-responsive transcripts from a microarray data set. The goal is to identify and characterize potential biochemical markers of pyrethroid effects. Long-Evans rats ( $n = 8-12$ /group) were acutely exposed via oral gavage to corn-oil vehicle, permethrin (1, 10, 100 mg/kg), or deltamethrin (0.3, 1.0, 3.0 mg/kg) and cerebrocortical tissue was collected at 6 hr post-exposure. Affymetrix Rat 230 2.0 GeneChip<sup>®</sup> microarrays were used to obtain transcriptional profiles. Two methods for identifying dose-responsive transcripts were compared: a quantitative regression (Significance Analysis of Microarrays software), and an isotonic regression model that assumes a monotonic dose-response relationship, but otherwise makes no assumptions about the precise form of the dose-response curve (M-score, Hu et al. 2005). Permutation-based calculations of false discovery rates were used in both cases to provide multiple comparison error control. Quantitative regression provided a superior estimate of false discovery rates compared to isotonic regression. These analyses revealed several candidate transcripts that respond in a dose-related fashion for both compounds. qRT-PCR confirmed a dose-responsive increase in the expression of Camk1 $\gamma$ , and decreases in the expression of prominin 1 and dopa decarboxylase, for both deltamethrin and permethrin. Use of Camk1 splice variant specific probes demonstrated an effect restricted to Camk1 $\gamma$ 1. A qRT-PCR time course study of Camk1 $\gamma$  and Ddc expression (1, 3, 6 & 9 hr) confirmed the previous findings and demonstrated peak times of effect between 3 and 6 hr post-exposure. This work has identified several potential biomarkers of effect for pyrethroids in the mammalian central nervous system. *This abstract does not necessarily reflect the policy of the US EPA.*

## Background

**Primary Site of Pyrethroid Action** Nerve membranes

**Molecular Target(s)** Primary (✓): Voltage sensitive sodium channels (Narahashi, 1996)  
Secondary (°): Voltage sensitive calcium channels (Symington & Clark, 2005)  
Chloride permeable anion channels (Barr & Ray, 2004)  
GABAergic receptors (Lawrence et al., 1985)  
calmodulin / calcineurin (Enom & Matsumura, 1992)

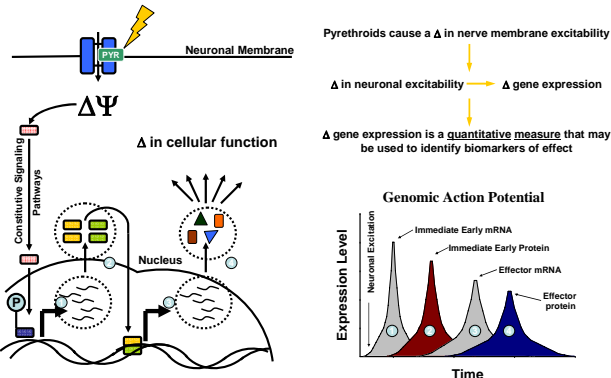
**Physiological Action**  $\Delta$  neuronal excitability  $\rightarrow$  neurotoxicity

**Unknowns**

- 1) Which molecular targets are critical mediators of pyrethroid toxicity *in vivo*.
- 2) The integrated effect on neuronal function that results from simultaneous pyrethroid action at multiple target sites in the same cell type *in vivo*.
- 3) The consequence of pyrethroid-induced nerve membrane disruption on downstream signaling / gene expression which, in turn, **MAX** result in previously unidentified adverse outcomes.

**Goal**  
Identify dose-responsive biomarker(s) of effect for pyrethroid action in the central nervous system *in vivo*.

**Hypothesis**  
Compounds in the pyrethroid insecticide class will induce alterations in the expression of genes that are regulated by neuronal excitability.



## Model

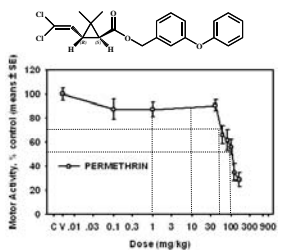
**Model:** Long-Evans Rat  
**Route:** Acute, oral  
**Age:** PND59 – PND68  
**Tissue:** Cerebral cortex

**Time:** Dose-Response - 6 hours  
**Time Course:** 1, 3, 6, 9 hours

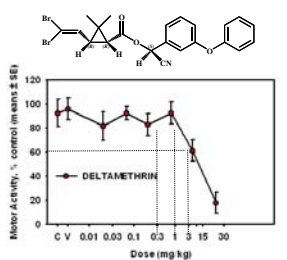
Doses based on behavioral data from Wolansky et al., (2006)

## Test Compounds

### Model Type I: Permethrin



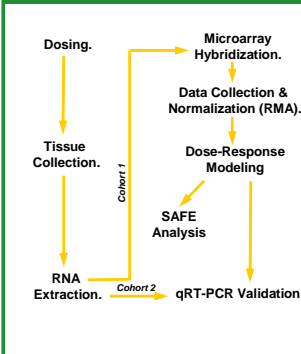
### Model Type II: Deltamethrin



	sub-NOAEL	Threshold	ED <sub>10</sub>	ED <sub>50</sub>
Permethrin	1 <sup>a</sup>	10 <sup>a</sup>	40 <sup>b</sup>	100 <sup>b</sup>
Deltamethrin	0.3 <sup>a</sup>	1 <sup>a</sup>	3 <sup>b</sup>	

<sup>a</sup> = Dose levels included in microarray study  
<sup>b</sup> = Dose levels included in qRT-PCR study only

## Experimental Flowchart



## Regression Models

### Quantitative Regression (SAM)

Tusher et al., (2001), Ghu et al., (2004)

**Regression Model:**  $x_{ij} = \beta_0 + \beta_1 y_i + \epsilon_{ij}$

**Test Statistic:**  $d_j$

§ Standard linear function  
§ Tests if response ( $x_j$ ) is linear

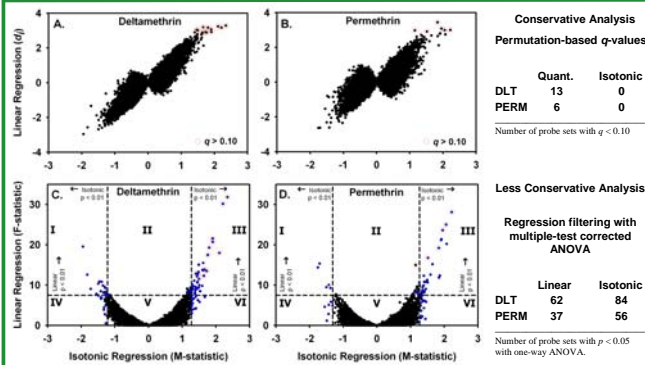
### Isotonic Regression

Wu et al., (2005), Storey & Tibshirani (2003)

**Regression Model:**  $x_{ij} = \hat{f}(x_j) + \epsilon_{ij}$

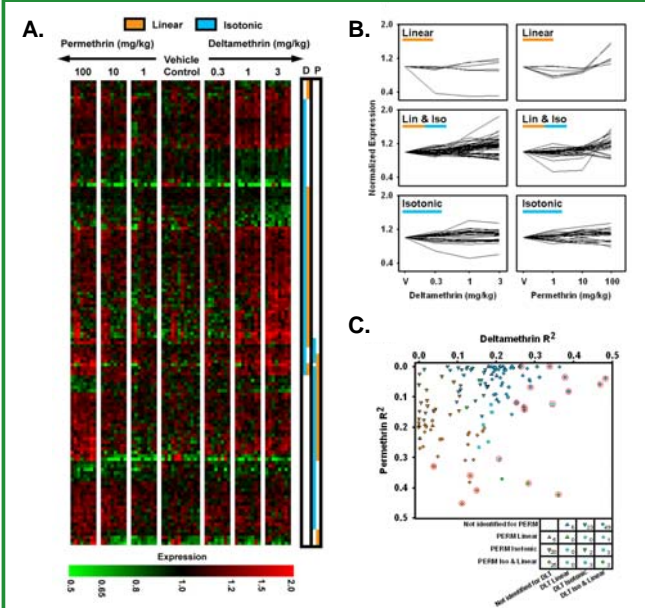
**Test Statistic:**  $M_j$

§  $\hat{f}(x_j)$  is an unknown monotonic function  
§ Tests if response ( $x_j$ ) increases or decreases monotonically, regardless of shape.



**Comparison of regression methods.** The top panels are volcano plots of test statistics from penalized isotonic regression (x-axis) versus those from SAM-based penalized quantitative regression (y-axis) of microarray data for DLT (A) and PERM (B). The lower panels are volcano plots of the test statistics from a standard linear regression (y-axis) versus penalized isotonic regression in panels (C) and (D) for DLT (C) and PERM (D) respectively. Probe sets with a false discovery rate  $q$ -value  $< 0.10$  for SAM-based quantitative regression are circled in red in all panels. Horizontal and vertical dotted lines in panels C & D represent a filtering threshold of  $p < 0.01$  for an empirical  $p$ -values from the standard linear and penalized isotonic regressions, respectively. Points in blue from panels C & D had a Benjamini-Hochberg corrected  $p < 0.05$  for a one-way ANOVA for main effect of dose.

## Comparison Across Compounds

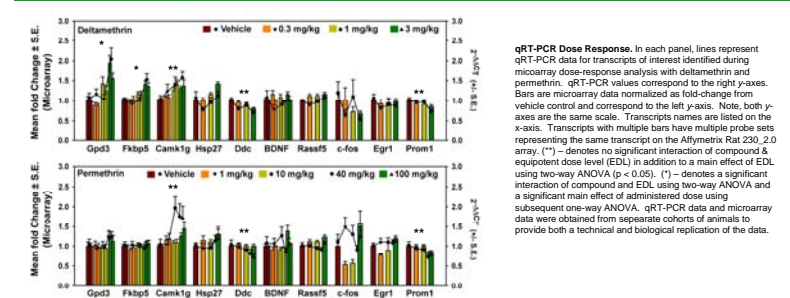


**Figure 3. Comparison of microarray data across compounds.** (A) Gene expression heatmap including all probe sets of interest discovered using regression-based microarray analysis scheme. The colors are normalized to the mean expression value of the control group for each probe set: green: upregulated, red: downregulated, black: no change. The strip of color to right denotes which regression method detected the dose-related change with either deltamethrin (D) or permethrin (P); isotonic (blue), linear (gold). Colorbar for fold-change expression is at the bottom. (B) Dose-response patterns detected using isotonic and linear regression methods for both compounds. (C) Plot of linear regression correlation coefficients ( $R^2$ ) values for deltamethrin (x-axis) and permethrin (y-axis). Symbol is located at bottom right corner of panel. Points circled in red had permutation based  $q$ -values  $< 0.10$  using SAM quantitative regression method.

## SAFE Analysis

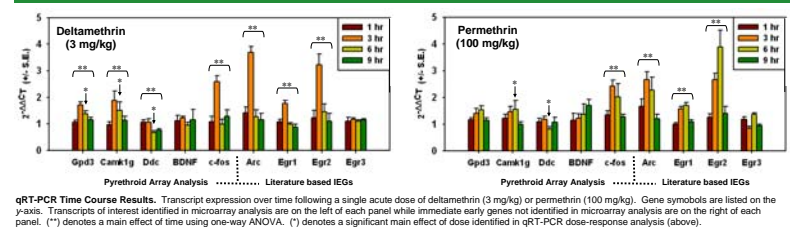
Category I.D. and name	size	Adjusted p-value	Category I.D. and name	size	DLT p-value	PERM p-value
<b>DELTAMETHRIN</b>			<b>GO Biological Process</b>			
KEGG Pathway			GO:0041875, 'branching morphogenesis of a tube'	66	0.0171	2.00e-4
KEGG:00564, 'Phenylethanolamine metabolism'	73	0.0404	GO:0001763, 'morphogenesis of a branching structure'	67	0.0172	2.00e-4
KEGG:00400, 'Phenylalanine, tyrosine and tryptophan metabolism'	12	0.0928	GO:0001569, 'patterning of blood vessels'	31	0.0661	3.00e-4
			GO:0007162, 'negative regulation of cell adhesion'	27	0.0175	0.0025
			GO:0009880, 'embryonic pattern specification'	40	0.1259	5.00e-4
			GO:0015718, 'monocarboxylic acid transport'	39	0.0051	0.0125
			GO:0007498, 'mesoderm development'	57	0.0105	0.0067
<b>PERMETHRIN</b>			<b>GO Cellular Component</b>			
GO:0041875, 'branching morphogenesis of a tube'	66	0.0349	GO:0005954, 'Ca <sup>2+</sup> /2Ca <sup>2+</sup> -dependent protein kinase complex'	25	0.0053	0.0146
GO:0001763, 'morphogenesis of a branching structure'	67	0.0349				
GO:0001569, 'patterning of blood vessels'	31	0.0405				
GO:0009880, 'embryonic pattern specification'	49	0.0554				
GO:0045655, 'regulation of monocyte differentiation'	32	0.0932				
			<b>GO Molecular Function</b>			
			GO:0046915, 'transition metal ion transport activity'	44	0.0026	0.0348

## qRT-PCR: Dose Response



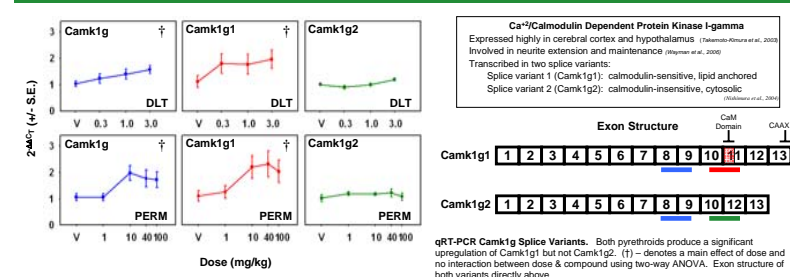
**qRT-PCR Dose Response.** In each panel, lines represent qRT-PCR data for transcripts of interest identified during microarray dose-response analysis with deltamethrin and permethrin. qRT-PCR values correspond to the right y-axis. Bars are microarray data normalized as fold-change from vehicle control and correspond to the left y-axis. Note, both y-axes are the same scale. Transcripts names are listed on the x-axis. Transcripts with multiple bars have multiple probe sets representing the same transcript on the Affymetrix Rat 230\_2.0 array. (\*) – denotes no significant interaction of compound & equipment dose level (EDL) in addition to a main effect of EDL using two-way ANOVA ( $p < 0.05$ ). (†) – denotes a significant interaction of compound and EDL using two-way ANOVA and a significant main effect of administered dose using subsequent one-way ANOVA. qRT-PCR data and microarray data were obtained from separate cohorts of animals to provide both a technical and biological replication of the data.

## qRT-PCR: Time Course



**qRT-PCR Time Course Results.** Transcript expression over time following a single acute dose of deltamethrin (3 mg/kg) or permethrin (100 mg/kg). Gene symbols are listed on the x-axis. Transcripts of interest identified in microarray analysis are on the left of each panel while immediate early genes not identified in microarray analysis are on the right of each panel. (\*) denotes a main effect of time using one-way ANOVA. (†) denotes a significant main effect of dose identified in qRT-PCR dose-response analysis (above).

## Ca<sup>2+</sup>/calmodulin dependent protein kinase 1-gamma



**Ca<sup>2+</sup>/Calmodulin Dependent Protein Kinase I-gamma**  
Expressed highly in cerebral cortex and hypothalamus (Thambisetty et al., 2003)  
Involved in neurite extension and maintenance (Hirayama et al., 2000)  
Transcribed in two splice variants:  
Splice variant 1 (Camk1g1): calmodulin-sensitive, lipid anchored  
Splice variant 2 (Camk1g2): calmodulin-insensitive, cytosolic (Hirayama et al., 2000)

**qRT-PCR Camk1g Splice Variants.** Both pyrethroids produce a significant upregulation of Camk1g1 but not Camk1g2. (†) – denotes a main effect of dose and no interaction between dose & compound using two-way ANOVA. Exon structure of both variants directly above.

## Conclusions

- » For this data quantitative regression with SAM identified a greater number of potential biomarkers-of-pyrethroid effect than isotonic regression.
- » Several transcripts (Camk1g, Gpd3, Ddc, & Prom1) reliably change as a function of dose following acute exposure to both compounds.
- » Distinct temporal patterns of gene expression are observed in CNS following acute exposure to pyrethroids.
- » Both compounds tested produce specific upregulation of Camk1g1 and not Camk1g2