Eminent Toxicologist Lecture Series

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Society of Toxicology

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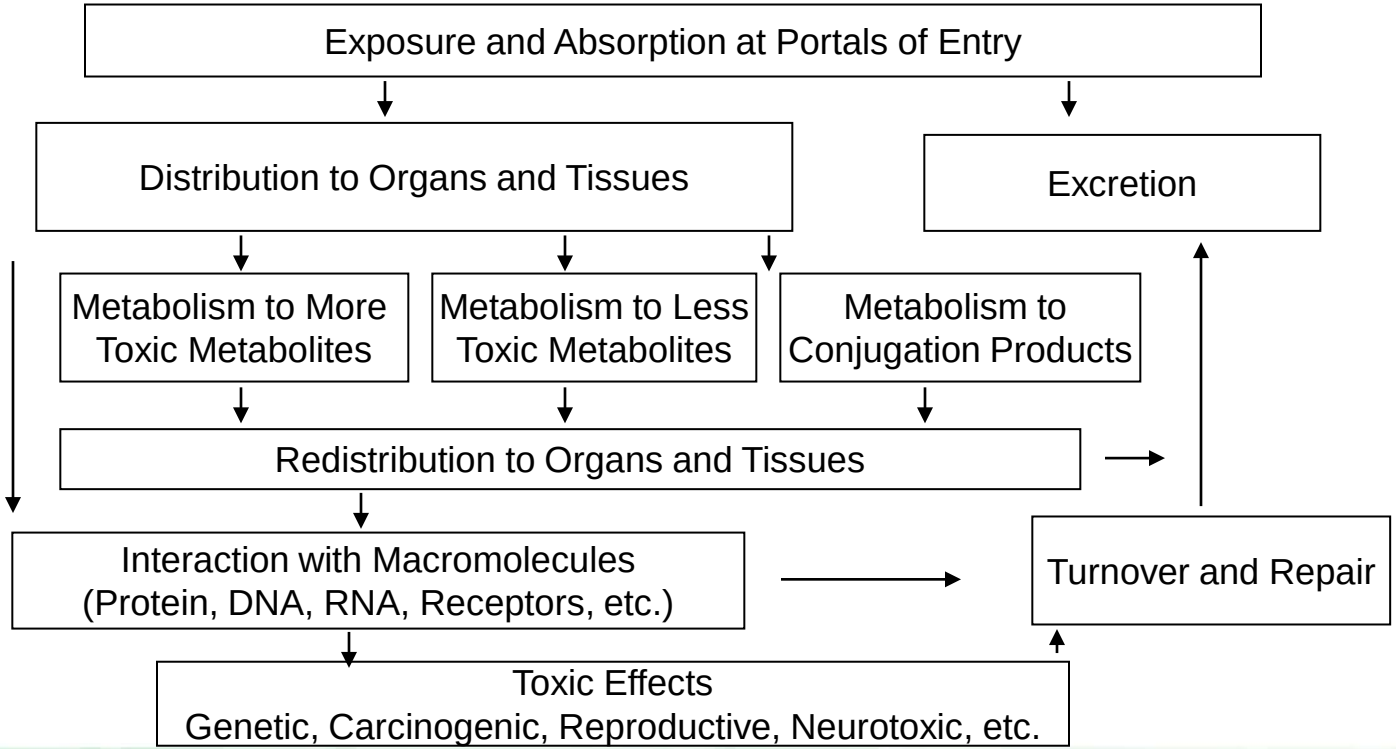
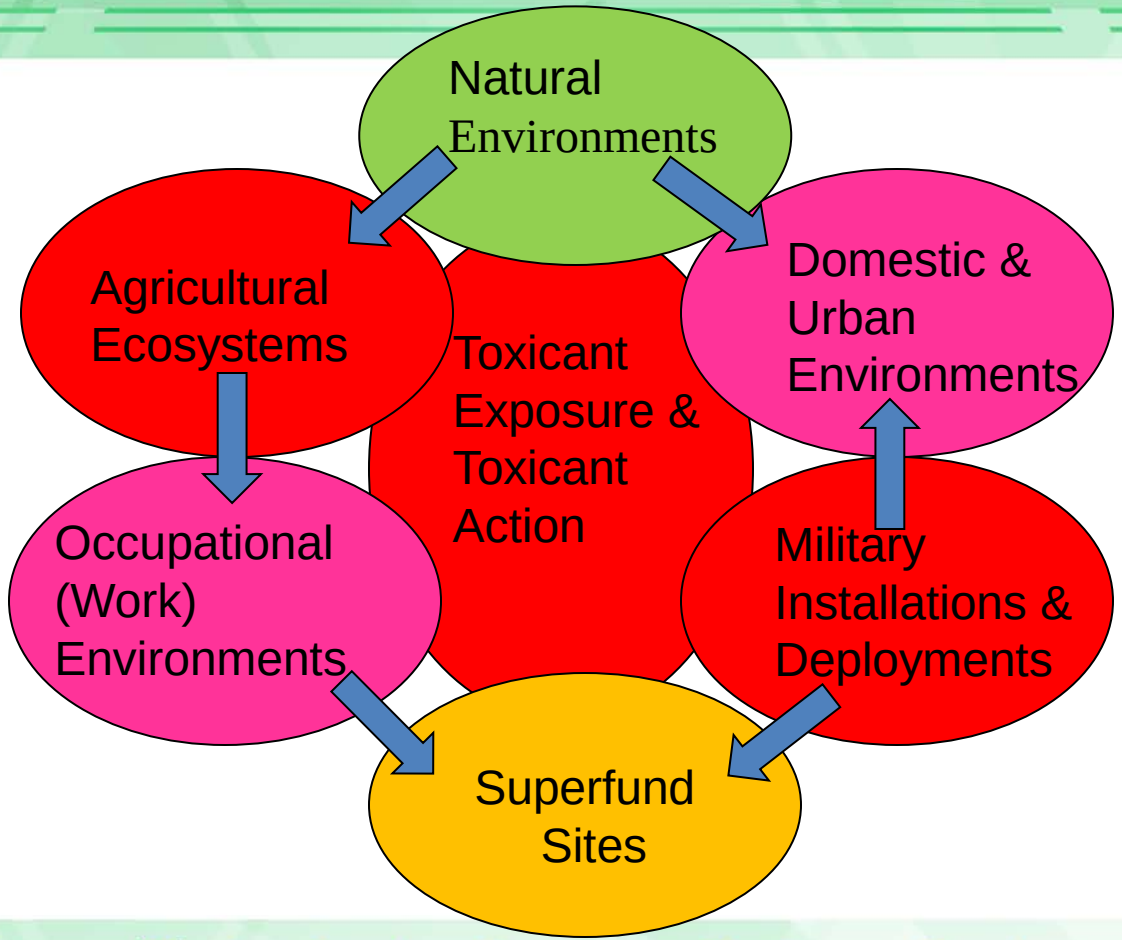
We Are Not Rodents: Environmental Toxicants and the Role of Human Studies

Society of Toxicology

Metabolism Affects Disposition and Distribution of Environmental Chemicals




Natural Environments
Agricultural Ecosystems
Domestic & Urban Environments
Toxicant Exposure & Toxicant Action
Occupational (Work) Environments
Military Installations & Deployments
Superfund Sites

The Exposome

The almost infinite variety of exposure scenarios presents a challenge for molecular epidemiology if molecular biomarkers of exposure and/or effect are to be linked to actual exposure. This has led to the exposome concept; an attempt to characterize all lifetime exposures that utilizes data from exposure sciences, epidemiology and other sciences. First advanced by Wild (2005) this concept has received recent support from the National Academy of Science (2010).

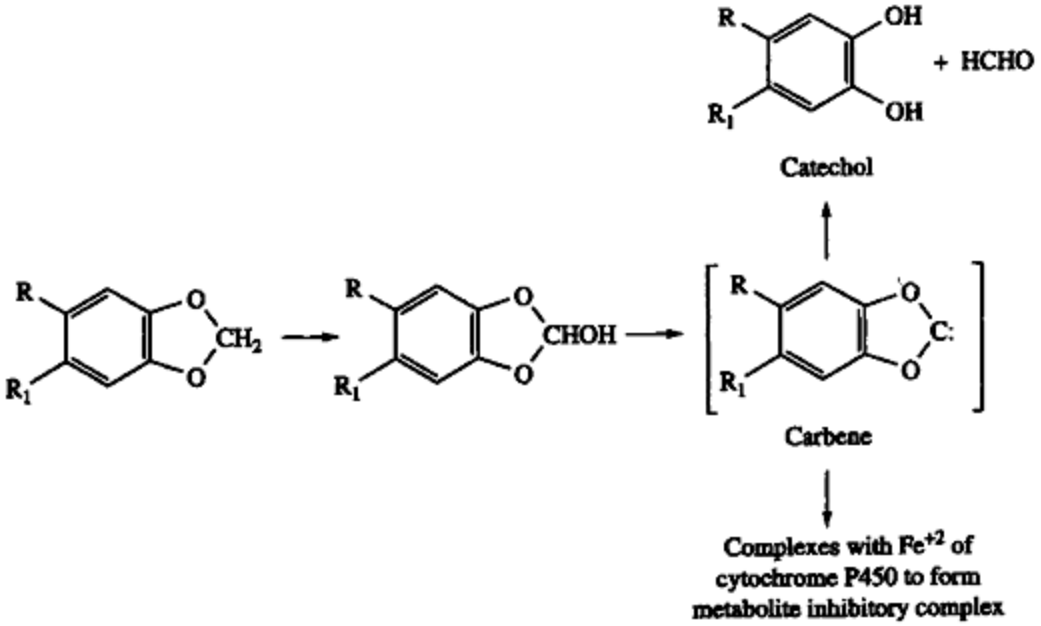
The Path to Human Studies

- Pesticide metabolism in non-human mammalian species.
- Characterization of insect cytochrome P450.
- Cytochrome P450 and insecticide resistance.
- Molecular biology of insect cytochrome P450.
- Interactions based on inhibition in non-human mammalian species.
- Interactions based on induction in non-human mammalian species.
- Inhibition and induction by methylenedioxyphenyl compounds.

Methylenedioxyphenyl Chemicals

- Biologically active secondary plant chemicals (e.g., safrole, isosafrole) and widely used commercial synergists (e.g., piperonyl butoxide).
- It had previously been shown that they affected P450-dependent oxidations in both insects and mammals.
- Two aspects were of interest to us and others and both became controversial; mechanism of inhibitory action and induction in mammals.

Monooxygenation of methylenedioxyphenyl compounds



Induction by MDP Chemicals

Following a long series of studies (and publications) involving Nancy Adams, Y-C Chui, Jon Cook, Pat Levi, Margaret Lewandowski, Doug-Young Ryu and myself, we concluded that MDP chemicals induced CYP1A1 via the AhR but induced CYP1A2 by a non-AhR dependent mechanism. This was largely ignored or disparaged until Frank Gonzalez produced the first Ah knockout mouse. To our relief (and gratification) piperonyl butoxide and acenaphthylene did not induce CYP1A1 in knockout mice but did, *mirabilis dictu*, induce both CYP1A2 and 1B1.

Ryu, D-Y, Levi, P. E., Fernandez-Salguero, P., Gonzalez, F. J. and Hodgson, E. (1996) Piperonyl butoxide and acenaphthylene induce P4501A2 and 1B1 mRNA in aromatic hydrocarbon-responsive receptor knock-out mouse liver. Mol. Pharmacol. 50:443-446.

Human Studies Contribute to Human Health Risk Assessment and PBPK Modeling

Past and Current. Exposure and Epidemiology

Current. Defining human variation

Showing the potential for human-specific interactions.

Defining populations or individuals at increased risk.

Facilitating new molecular approaches to human health risk assessment.

Showing the potential for human-specific interactions.

Providing insight into uncertainty factors.

In Vitro Human Toxicant Metabolic Interactions

- Enzymes involved in toxicant metabolism
- Isoforms and polymorphic variants
- Variation between individuals
- Xenobiotic-xenobiotic interactions
- Xenobiotic-endogenous metabolite interactions
- Enzyme induction in human hepatocytes
- Cytotoxicity

Human Metabolism: Some Substrates Investigated

Agrochemicals

Chloroacetamide herbicides

Chlorpyrifos	Fipronil
Carbaryl	Endosulfan
Carbofuran	Permethrin

Repellents

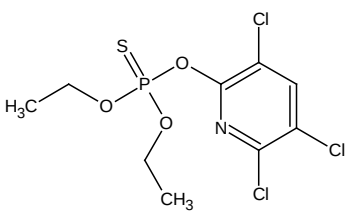
DEET

Diesel Fuel and Diesel Fuel Components

Nonane	Naphthalene

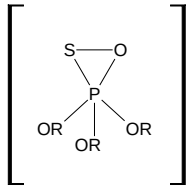
Investigations may include human variation, isoform identification, effect of SNPs and metabolic interactions.

Chlorpyrifos Metabolism

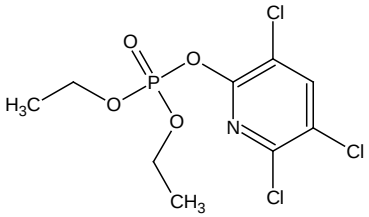


Chlorpyrifos

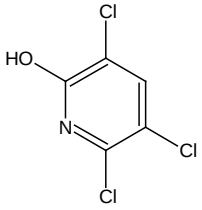
CYP



S +

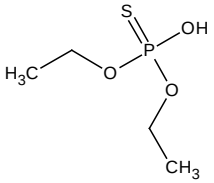


Chlorpyrifos-oxon



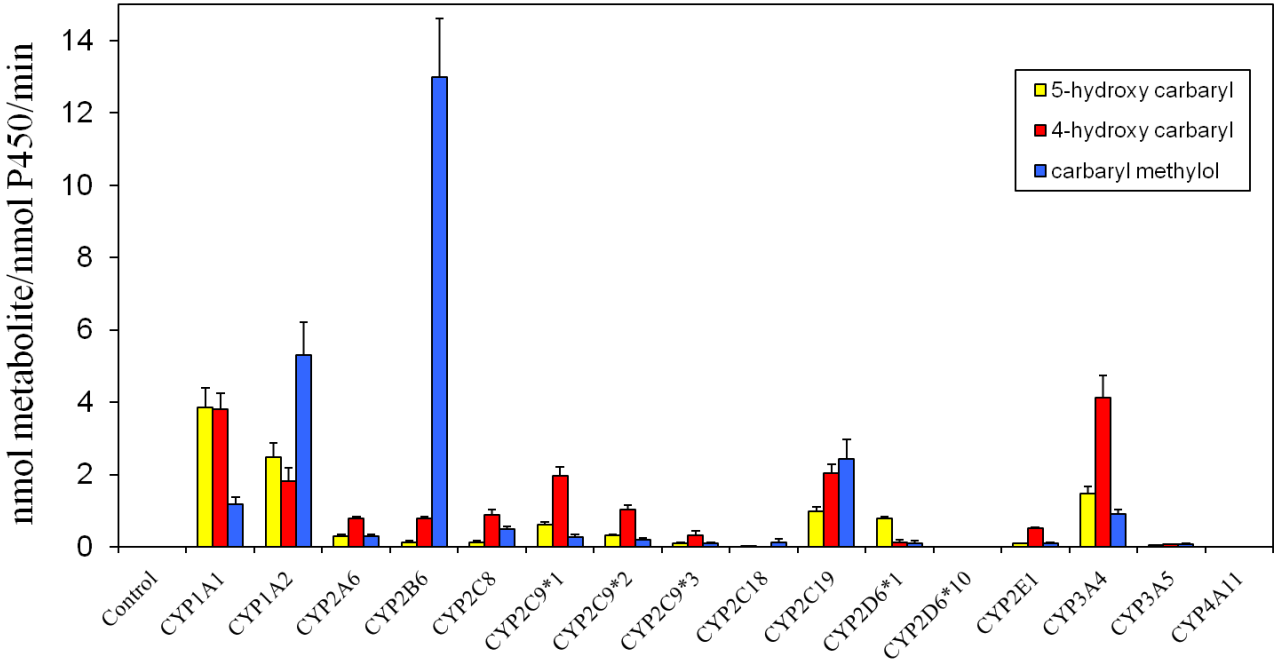
3,5,6-trichloro-2-pyridinol

+

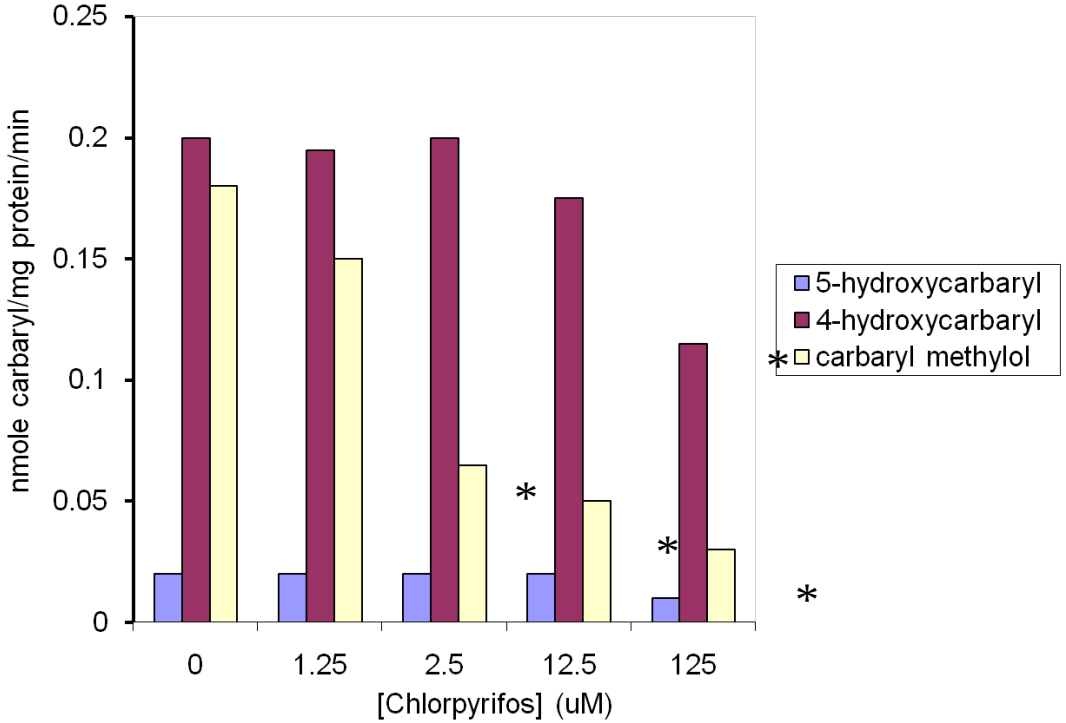


Diethyl phosphorodithioate

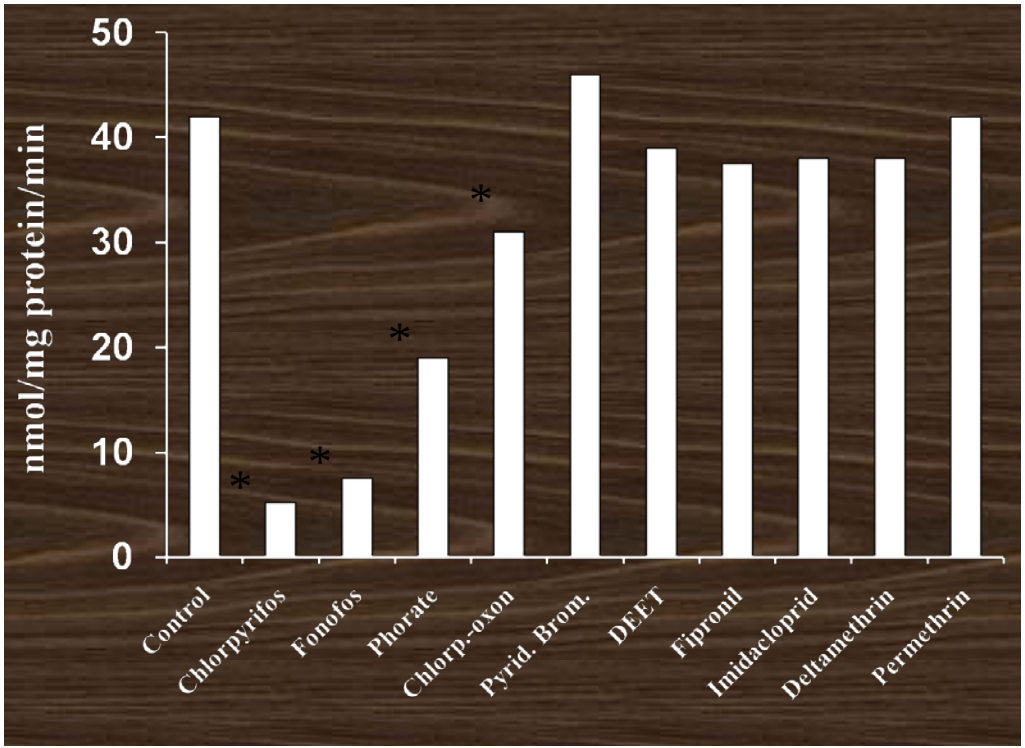
Isoform Specificity for Carbaryl Metabolism



Chlorpyrifos Inhibition of Carbaryl Metabolism *In Vitro*



Inhibition of Testosterone Metabolism by Pesticides



Effects on *In Vitro* Testosterone Metabolism

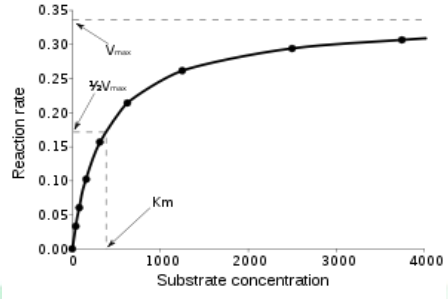
- 6β-hydroxytestosterone accounts for approximately 86% of all testosterone metabolites produced by human liver microsomes.
- CYPs 3A4 and 3A5 account for most of the 6β-hydroxytestosterone formed.
- Preincubation of CYP3A4 with chlorpyrifos (2 μM) resulted in 98% inhibition of major testosterone metabolite production (100 μM testosterone substrate concentration).

Examples of Metabolic Interactions in Humans Based on Inhibition

Enzyme	Substrate	Inhibitor
CYP2B6	Carbaryl	Chlorpyrifos
CYP3A4	Fipronil	Chlorpyrifos
CYP2B6	Nonane	Chlorpyrifos
CYP3A4	Testosterone	Chlorpyrifos, Fonofos, Phorate
CYP3A4	Estradiol	Chlorpyrifos, Fonofos, Permethrin, Deltamethrin
CYP1A2	Estradiol	Chlorpyrifos, Fonofos, Carbaryl, Naphthalene

Importance of CYP2B6

- Although there is normally more CYP3A4 than CYP2B6 in human liver, CYP2B6 has the lowest Km and is more effective
- Foxenberg et al.,(2008) reported Km constants for Chlorpyrifos oxon production.
  - CYP3A4 27µM
  - CYP2B6 0.81µM



CYP2B6 and CPS Toxicity

- CYP2B6 activity predicted Chlorpyrifos oxon production.
- CYP2B6 inhibition reduced Chlorpyrifos oxon production.
- CYP2B6*6/*6 samples could produce Chlorpyrifos oxon in large amounts.
- Individuals with high CYP2B6 could be at greater risk of chlorpyrifos poisoning.

Eminent Toxicologist Lecture Series

Induction by Environmental Chemicals

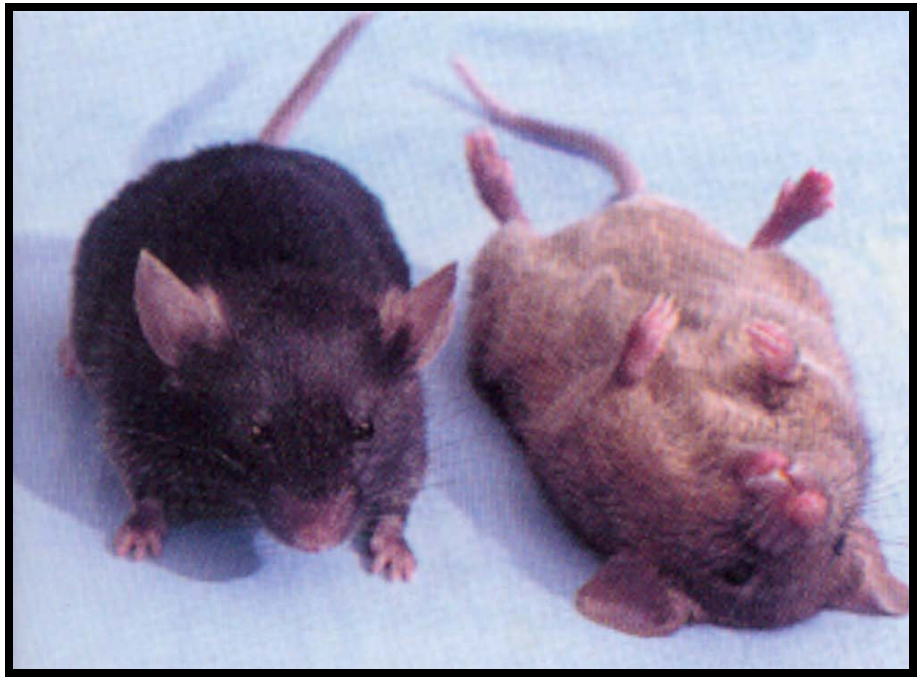
The induction of xenobiotic-metabolizing enzymes has been studied extensively in surrogate animals for several decades, since the landmark review of Alan Conney and it is now clear that most induction involves one or more nuclear receptors.

However, since an intact cell with functional sub-cellular organelles is required for experimental work, studies of induction in humans are more recent, and studies involving environmental chemicals even more so. Examples of chemicals investigated for induction and cytotoxicity in human hepatocytes include: Fipronil, Chlorpyrifos, Endosulfan.

Endosulfan–*In Vivo* Effects

1. Reduces sleep time in wild type mice.
2. Does not reduce sleep time in PXR-null mice.
3. Reduces sleep time in humanized PXR mice.

Increased Metabolism and Reduction in Sleep Time



Z Z Z Z Z

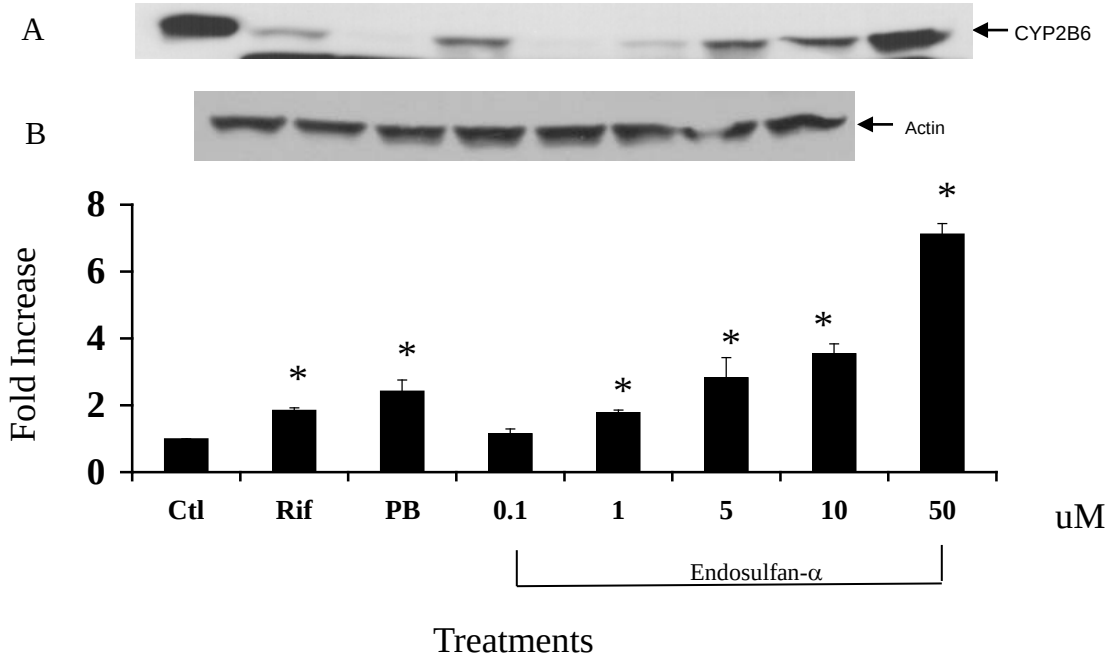
+ Compound **Control**

Adapted from
JBC 276: 37739-42.

Effects of Endosulfan on Human Hepatocytes

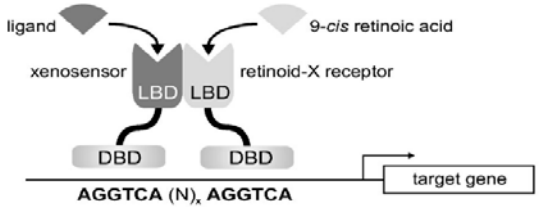
1. Is cytotoxic.
2. Induces CYP2B6 and CYP3A4.
3. Induction is PXR-dependent.

Endosulfan-a Increases CYP2B6 Levels in Human Hepatocytes



Pregnane X Receptor (PXR)

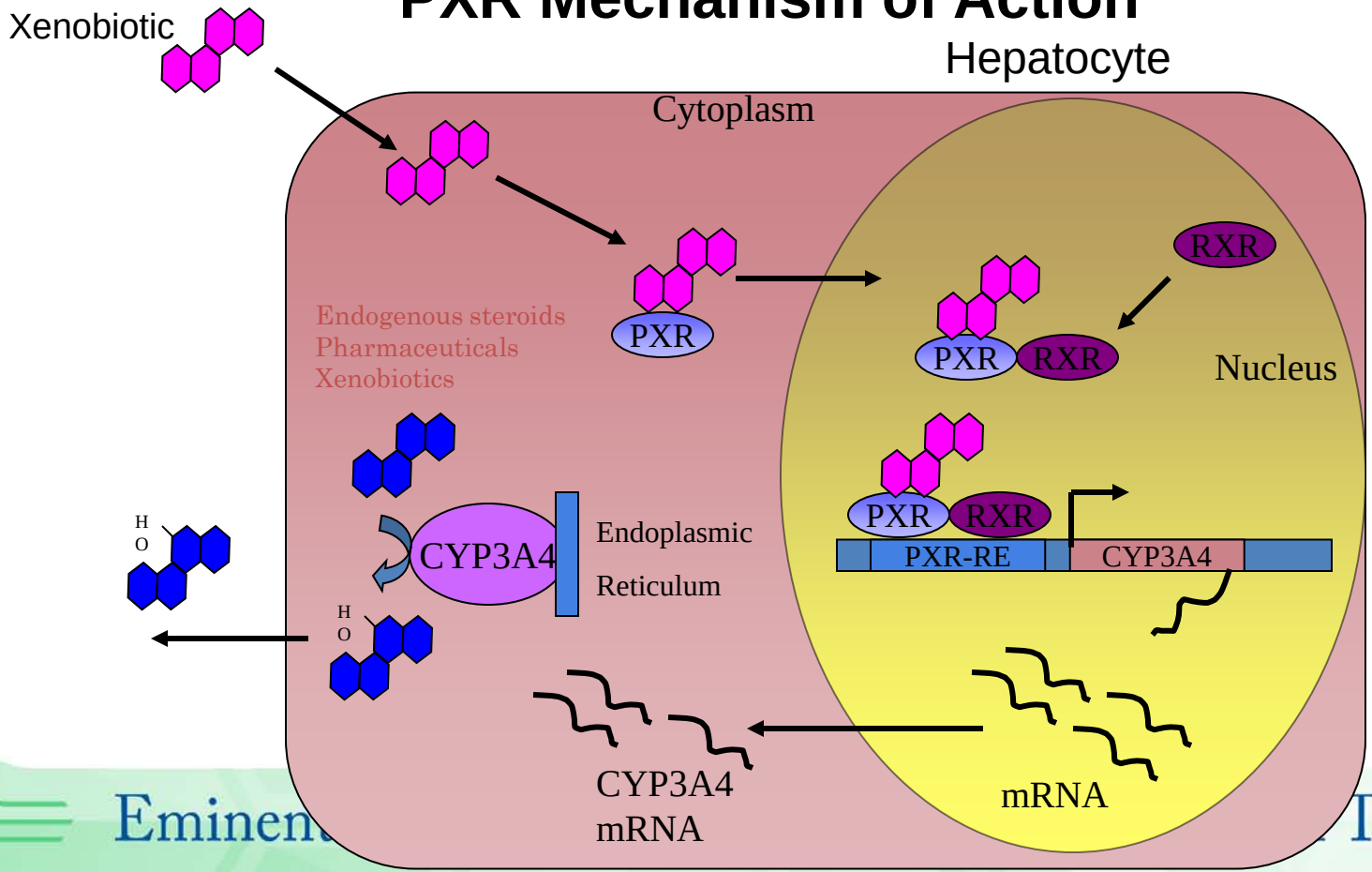
- **Orphan member of the nuclear receptor family**
- **Xenosensor of toxic substances; binds xenobiotics and endogenous compounds and induces xenobiotic metabolizing enzyme gene expression**
- **Highly expressed in liver and intestine**



DNA binding of nuclear receptors

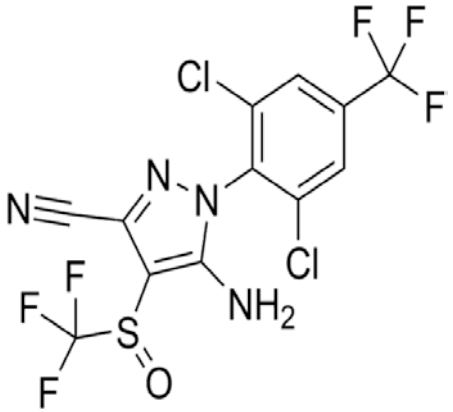
Handschin (2003)

PXR Mechanism of Action



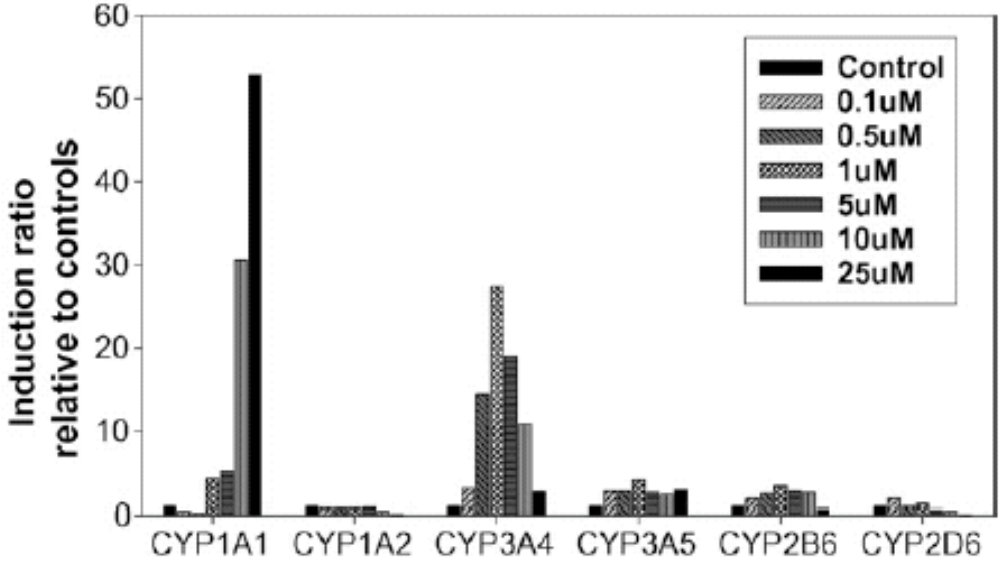
Fipronil

- **Fipronil is a member of the phenylpyrazole class of insecticides.**
- **Non-competitive GABA receptor Cl channel blocker.**

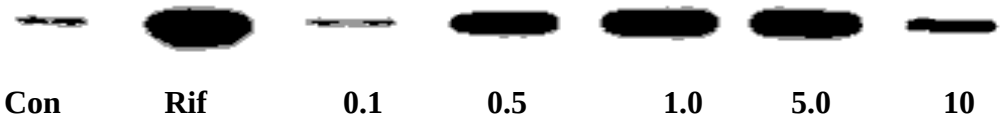


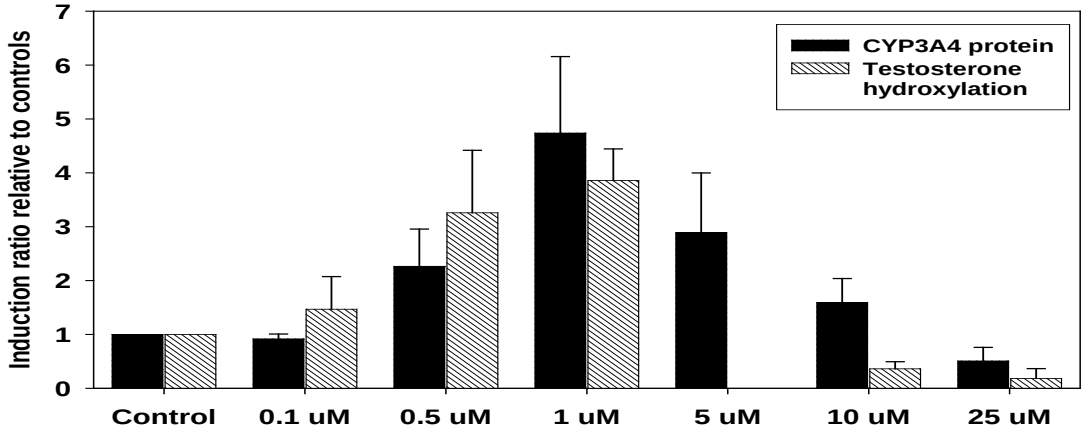
Fipronil

Effect of Fipronil on CYP mRNA levels in Human Hepatocytes

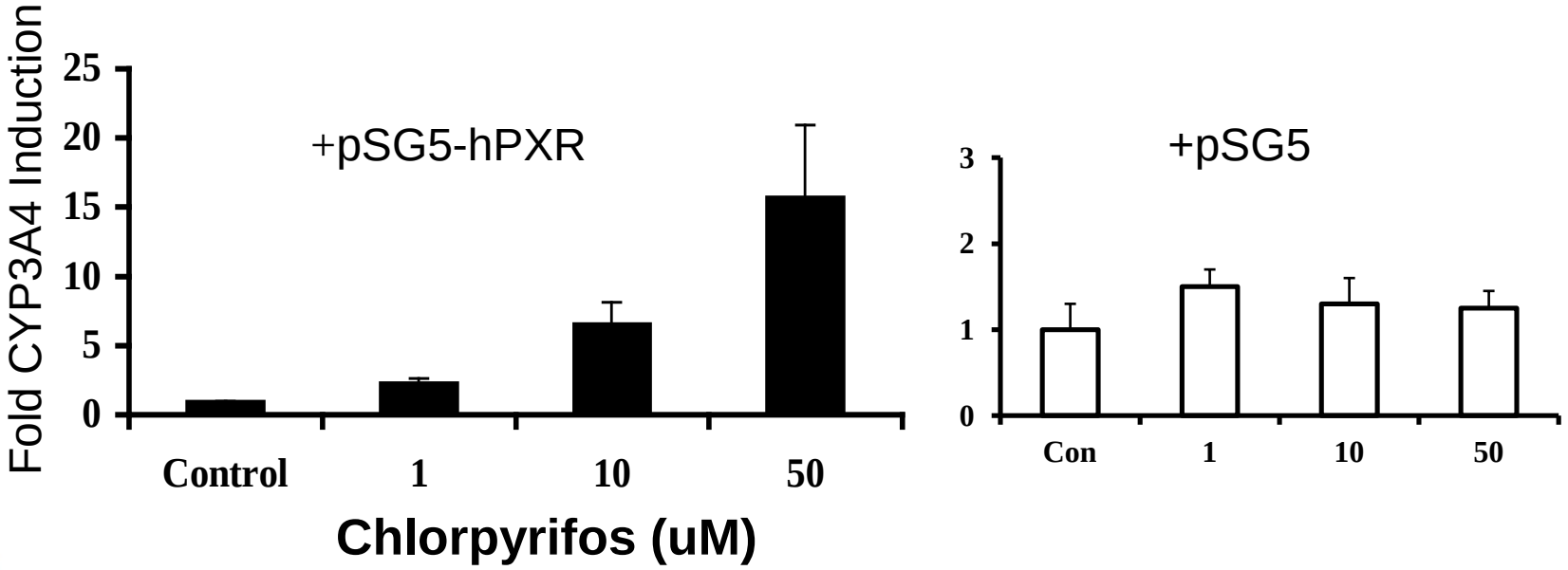


Effect of Fipronil on CYP3A4 Protein and Activity in Human Hepatocytes





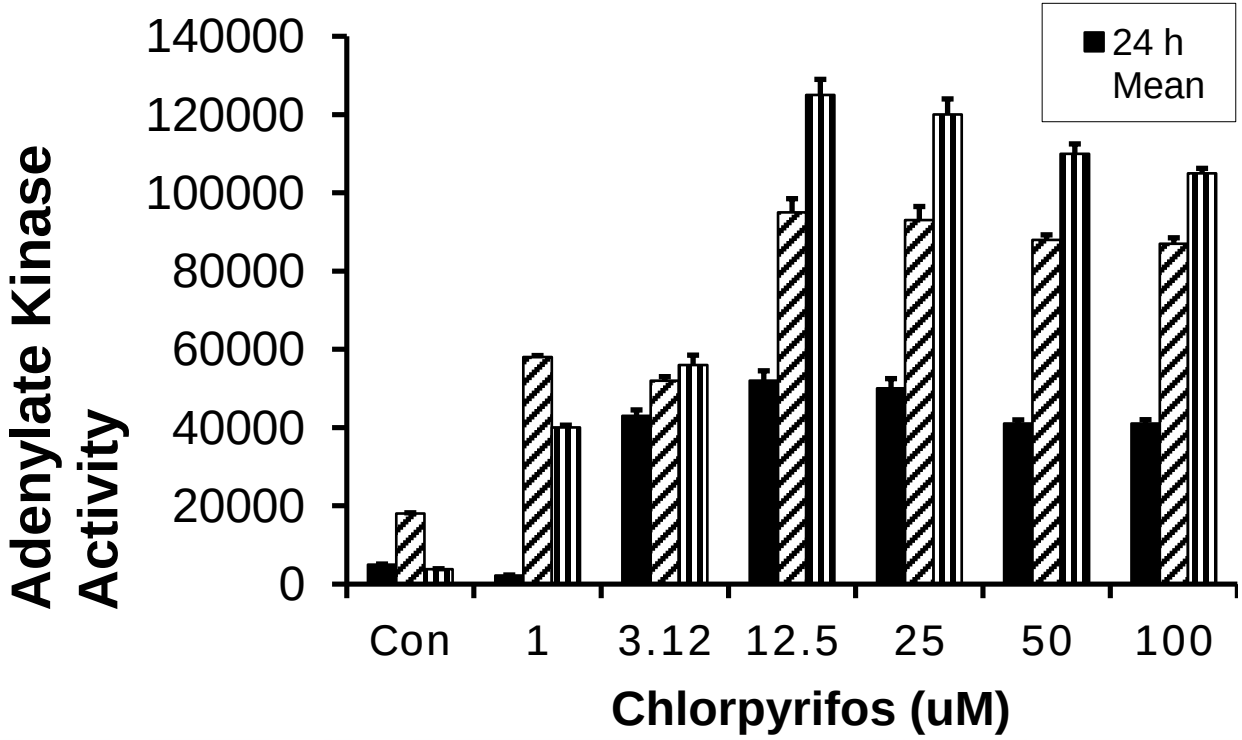
CYP3A4-Luc Induction in HepG2 Cells after Chlorpyrifos Exposure



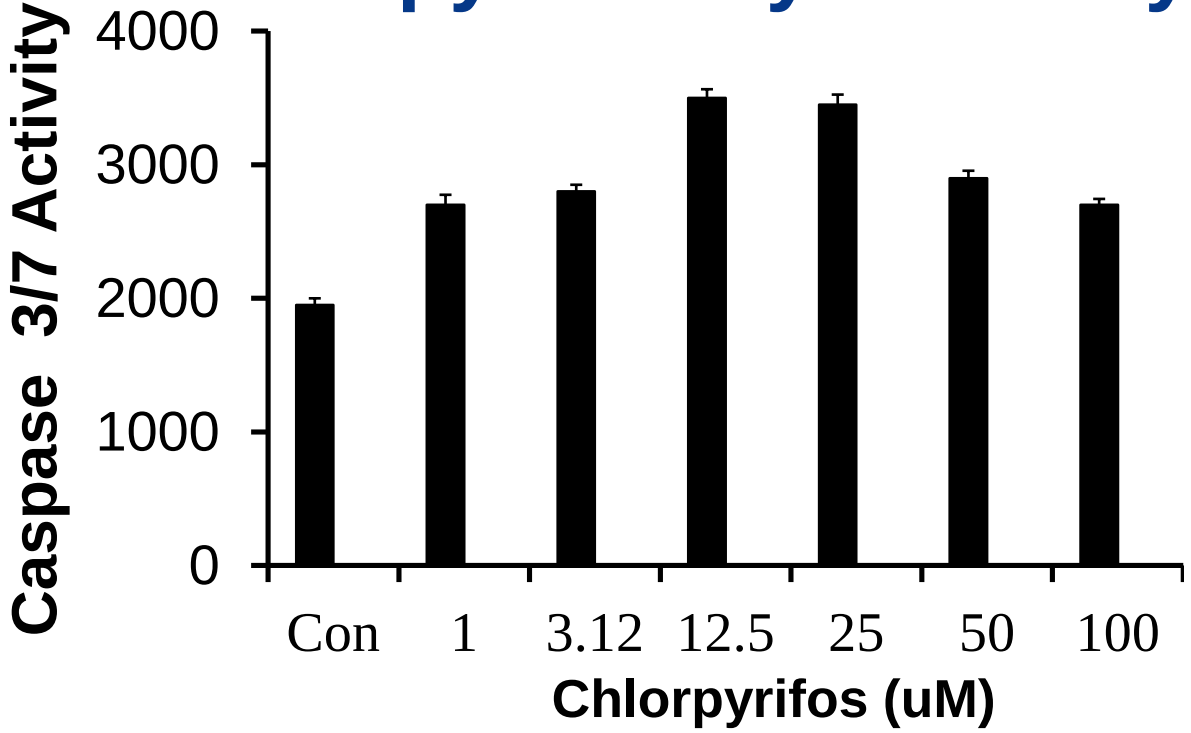
Fipronil Cytotoxicity: Human Hepatocytes and HepG2 Cells

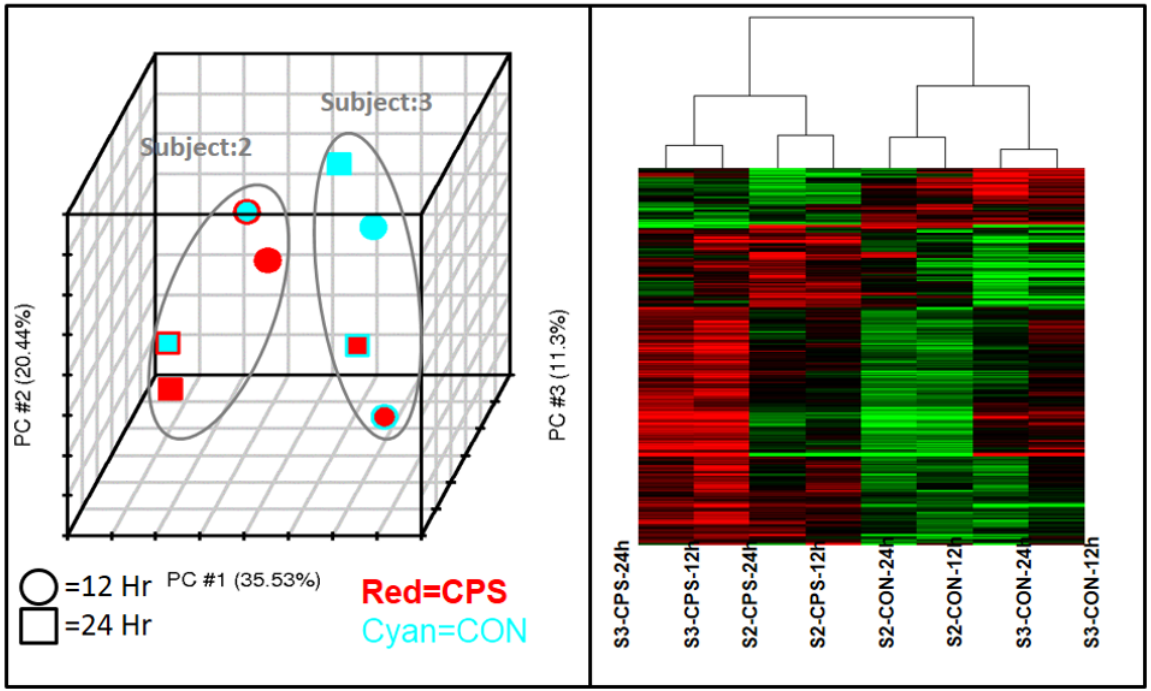
Cell Type	Threshold dose
Adenylate kinase release	
HepG2	<0.5µM
Hepatocytes	<25µM
Caspase 3/7 activity	
HepG2	<0.1µM
Hepatocytes	<25µM

Chlorpyrifos Cytotoxicity



Chlorpyrifos Cytotoxicity





Enabling Science via Analytical Informatics

Identification of Chlorpyrifos Regulated Genes in Human Hepatocytes by Microarray

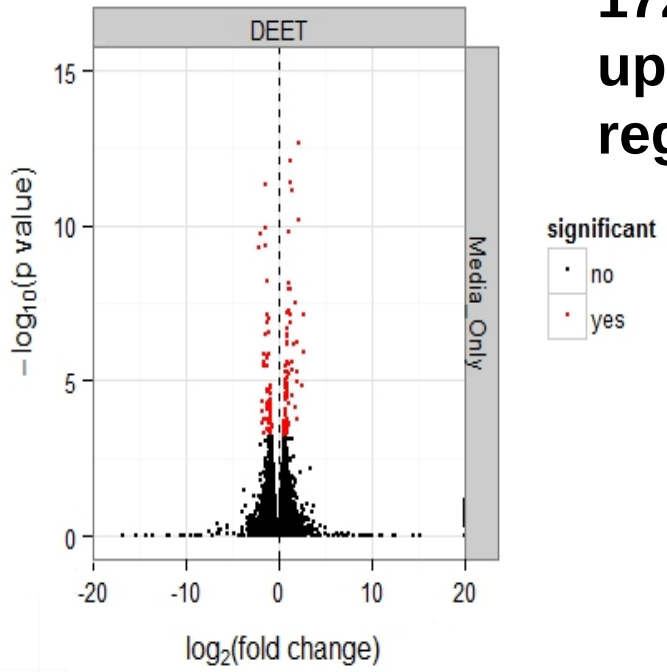
Gene Name	Fold Change
alcohol dehydrogenase 1B	3.59
glycine-N-acyltransferase	2.30
transferrin	2.25
Cytochrome P450, family 1, subfamily A, polypeptide 2	-2.46
Insulin-like growth factor binding protein 1	-2.71
Uridine phopsphorylase 1	2.48
Heat shock protein70 kDa family member 13	-2.00

Identification of Biological Pathways Regulated after Chlorpyrifos Exposure

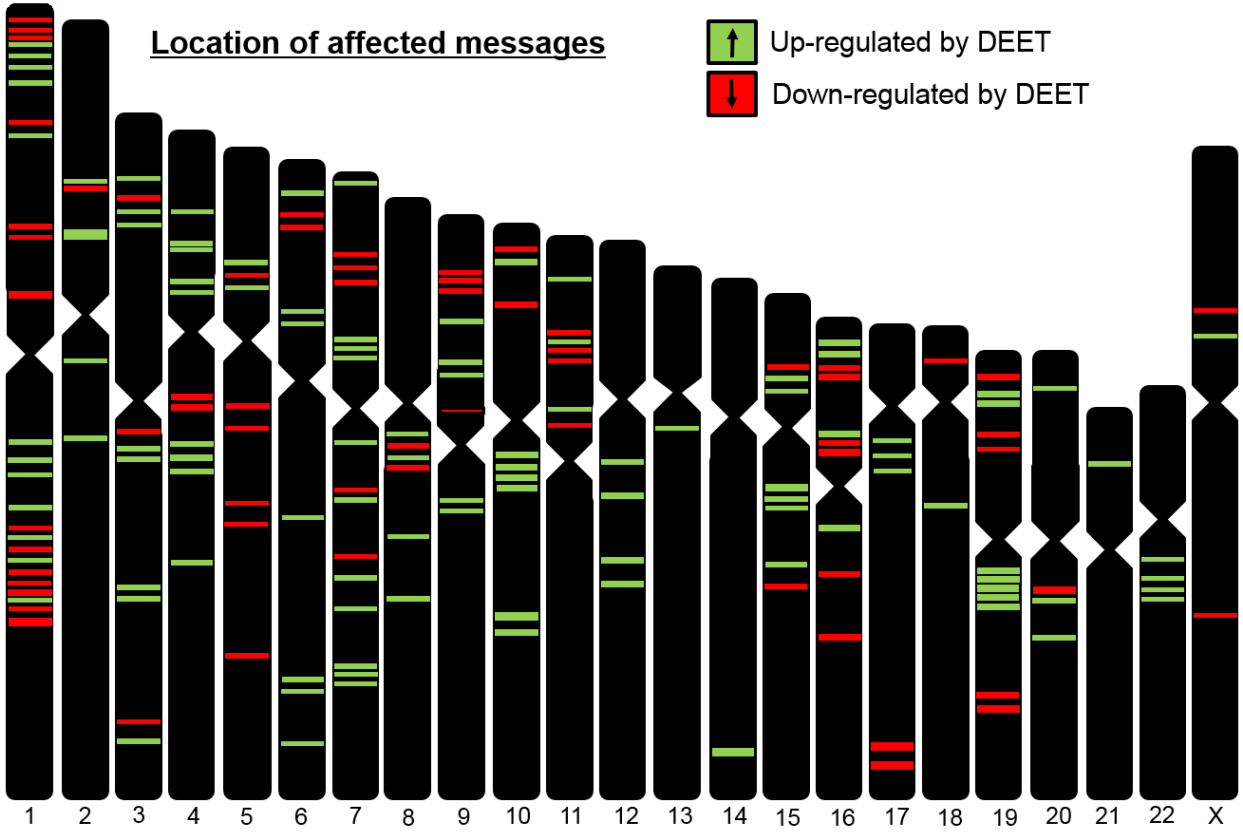
Kyoto Encyclopedia of Genes and Genomes (KEGG)	Number of Regulated Genes in Pathway	Unadjusted P-value
Retinol metabolism	7	6.24E-05
Metabolism of xenobiotics by CYPs	7	0.0002
Porphyrin and chlorophyll metabolism	4	0.0037
Long-term potentiation	5	0.0128
MAPK signaling pathway	11	0.0134
Drug metabolism enzymes	5	0.0087
Regulation of actin cytoskeleton	9	0.0182

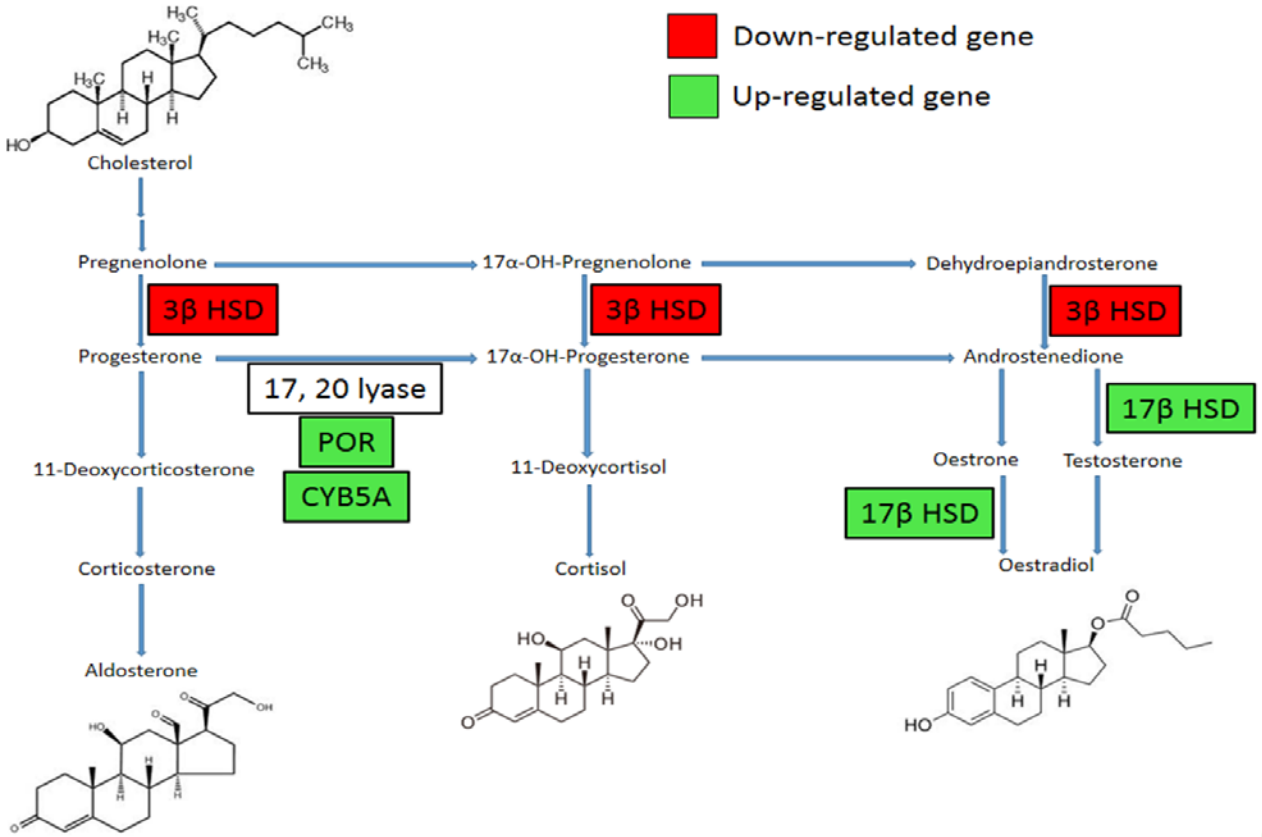
What is the impact of DEET on global gene expression in primary human hepatocytes after a 72 h exposure?

Volcano Plot of Comparisons between DEET and Media Only Data



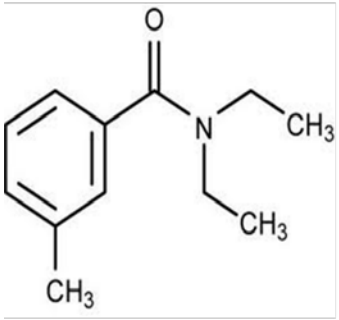
172 messages up or down regulated


Location of affected messages
Up-regulated by DEET
Down-regulated by DEET
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X

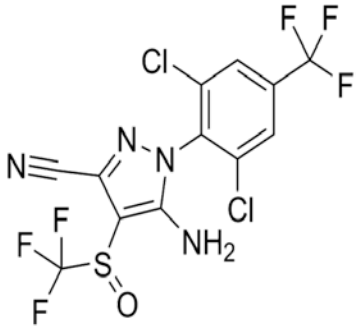

Down-regulated gene
Up-regulated gene
Cholesterol
Pregnenolone
17α-OH-Pregnenolone
Dehydroepiandrosterone
3β HSD
3β HSD
3β HSD
Progesterone
17α-OH-Progesterone
Androstenedione
17, 20 lyase
POR
CYB5A
17β HSD
11-Deoxycorticosterone
11-Deoxycortisol
Oestrone
Testosterone
17β HSD
Corticosterone
Cortisol
Oestradiol
Aldosterone

Sequence Name	Sequnece Description	Seq. Length	#Hits	min. eValue	mean Similarity	#GOs	expression with DEET	Chromosome
gi|28476829|ref|NR_001278.1|	ncRNA_cytochrome p450 2b6-like (CYP2B7P1)	3000	20	0	94.30%	10	up	chr19:41430169-41456565
gi|153791676|ref|NR_003610.1|	ncRNA_pyridoxal-dependent decarboxylase domain-containing containing 2, pseudogene (PDXDC2P)	4298	20	0	97.65%	7	down	chr16:70010201-70099851
gi|141801873|ref|NR_002934.2|	ncRNA_scavenger receptor protein family member (LOC619207)	4918	10	0	88.50%	2	down	chr10:135267431-135281953
gi|223555922|ref|NR_026816.1|	ncRNA_psoriasis susceptibility 1 candidate gene 3 (PSOR1C3)	600	20	1.19E-47	70.95%	6	down	chr6:31141511-31145676
gi|284813500|ref|NR_033266.1|	ncRNA_was protein family homolog 2-like; WAS protein family 5 homolog pseudogene (WASH5P)	1137	20	4.20E-24	93.95%	9	down	chr19:60950-70966
gi|223972624|ref|NR_026936.1|	ncRNA_isoform cra_a; chromosome 5 open reading frame 27 (C5orf27)	1931	20	2.61E-57	82.00%	14	down	chr5:95187935-95195836
gi|226053461|ref|NR_027440.1|	ncRNA_unnamed portein product; uncharacterized LOC100272217 (LOC100272217)	2159	20	4.77E-25	66.55%	4	down	chr9:133452736-133454881
gi|213385260|ref|NR_024456.1|	ncRNA_histone demethylase uty-like; uncharacterized LOC100190986 (LOC100190986)	2453	20	5.62E-32	81.10%	16	down	chr16:21443344-21445776
gi|327412331|ref|NR_038080.1|	ncRNA_chromosome 17 open reading frame 55; long intergenic non-protein coding RNA 482 (LINC00482)	2970	20	6.02E-147	77.00%	9	down	chr17:79276623-79283048
gi|219555684|ref|NR_002817.2|	ncRNA_aquaporin adipose; aquaporin 7 pseudogene 1 (AQP7P1)	3180	20	2.34E-51	92.25%	15	down	chr9:67270214-67289492
gi|302699227|ref|NR_036530.1|	ncRNA_unnamed protein product; uncharacterized LOC100289230 (LOC100289230)	1876	19	5.31E-25	73.68%	0	down	chr5:98264837-98266713
gi|345842504|ref|NR_027455.3|	ncRNA_flj44451 fis; uncharacterized LOC100131434 (LOC100131434)	2348	13	5.26E-96	91.69%	0	down	chrX:148609131-148621312
gi|341932552|ref|NR_040662.1|	ncRNA_hla complex isoform cra_a; HLA complex P5 (non-protein coding)(HCP5)	2547	11	1.14E-61	70.73%	1	up	chr6:31430956-31433586
gi|255306270|ref|NR_028272.1|	ncRNA_nuclear paraspeckle assembly transcript 1 (non-protein coding)(NEAT1)	3756	5	9.82E-43	97.80%	0	down	chr11:65190268-65194003
gi|378548194|ref|NR_046377.1|	ncRNA_isoform cra_a; hCG1813624 (LOC728040)	763	3	6.49E-39	83.67%	2	down	chr4:74374519-74394250
gi|338797704|ref|NR_040023.1|	ncRNA_endogenous retrovirus group K13, member 1 (ERVK13-1)	8336	n/a	n/a	n/a	n/a	down	chr16:2708389-2723440
gi|207113128|ref|NR_002819.2|	ncRNA_metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)(MALAT1)	8708	n/a	n/a	n/a	n/a	down	chr11:65265232-65273939
gi|342307077|ref|NR_039981.2|	ncRNA_uncharacterized LOC100216546 (LOC100216546)	9419	n/a	n/a	n/a	n/a	down	chr7:104622193-104631612
gi|211938434|ref|NR_024368.1|	ncRNA_uncharacterized LOC402483 (FLI45340)	10263	n/a	n/a	n/a	n/a	down	chr7:128281294-128301052

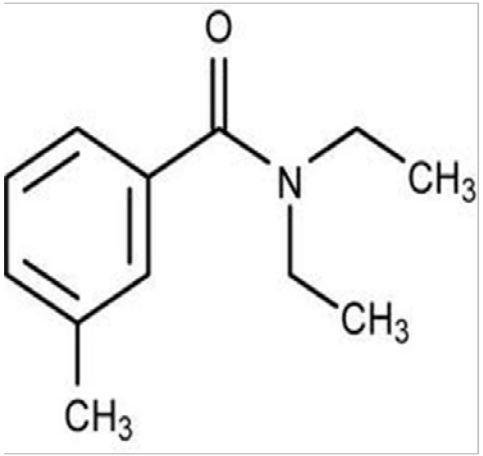
What is the impact of fipronil mixtures with DEET on primary human hepatocytes?



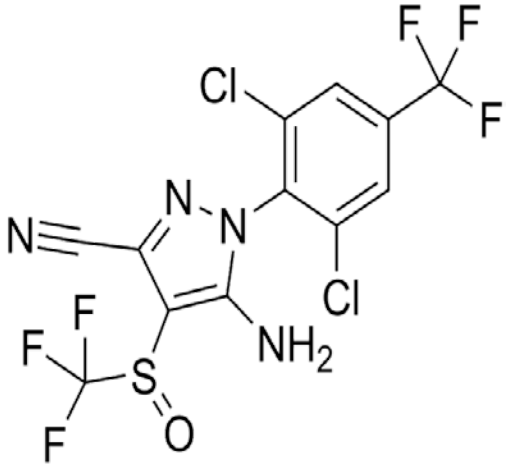
DEET



Fipronil



DEET

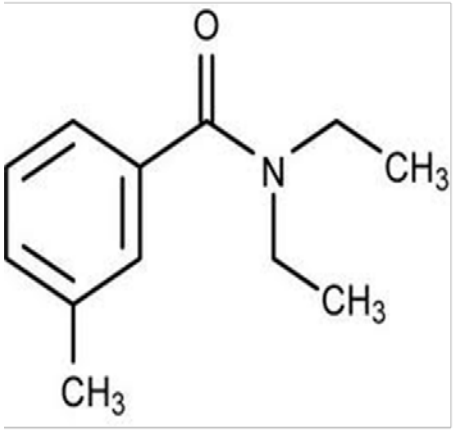


Fipronil

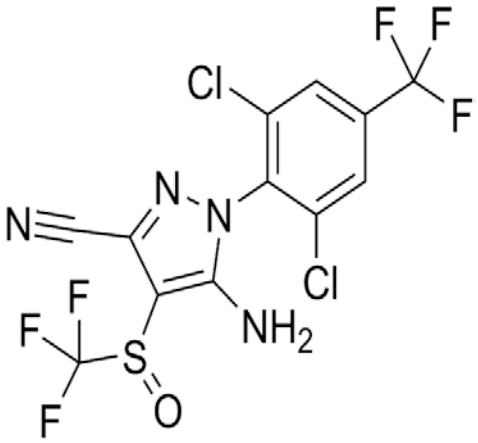
172 genes differentially expressed (p-value of 0.05)

3703 genes differentially expressed (p-value of 0.05)

Mixture: Greater than Additive Effect



DEET



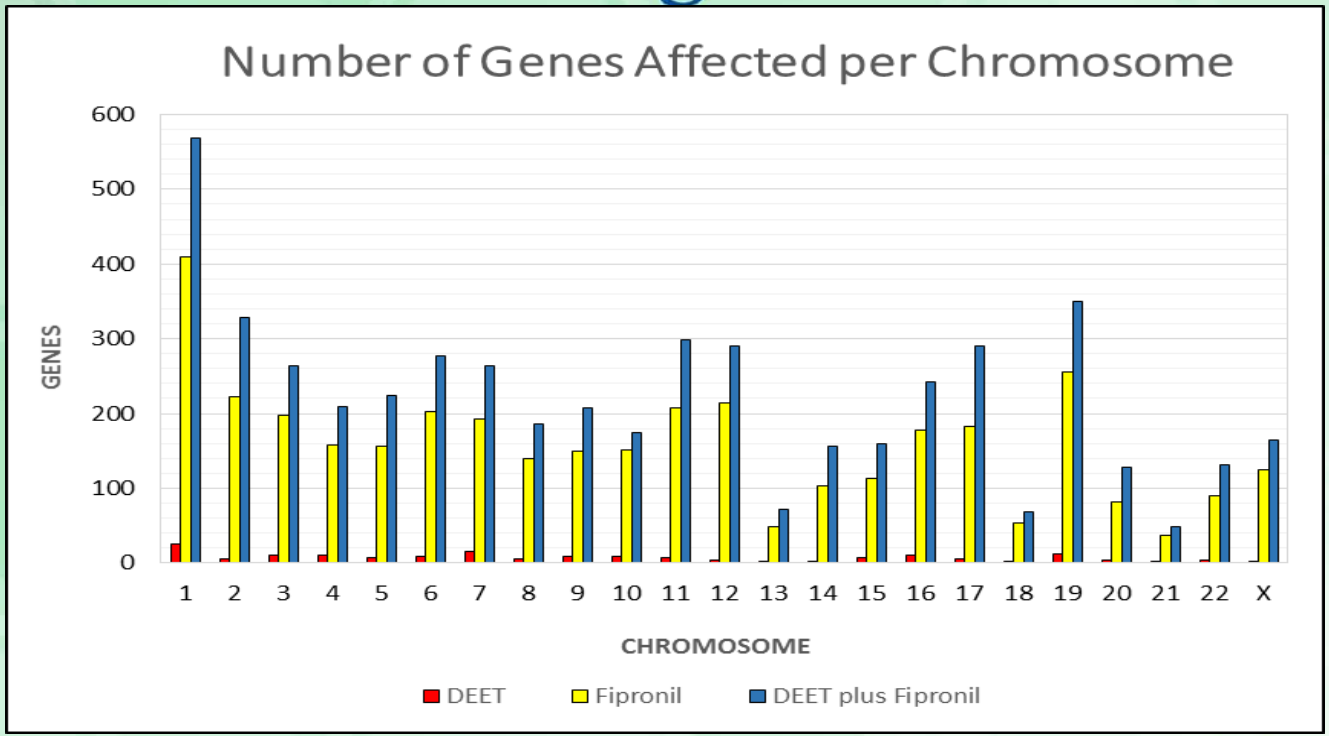
Fipronil

Additive Total 3,875

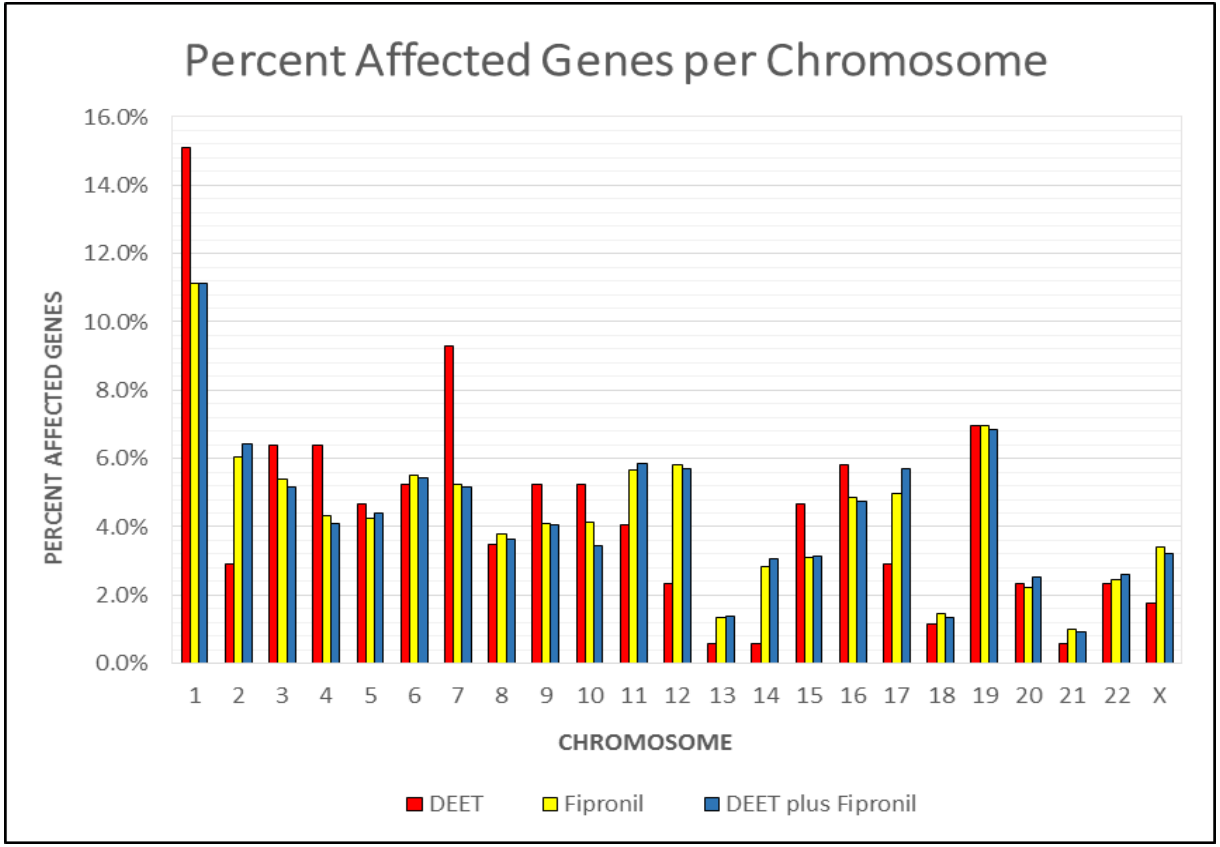
DEET + Fipronil vs Media

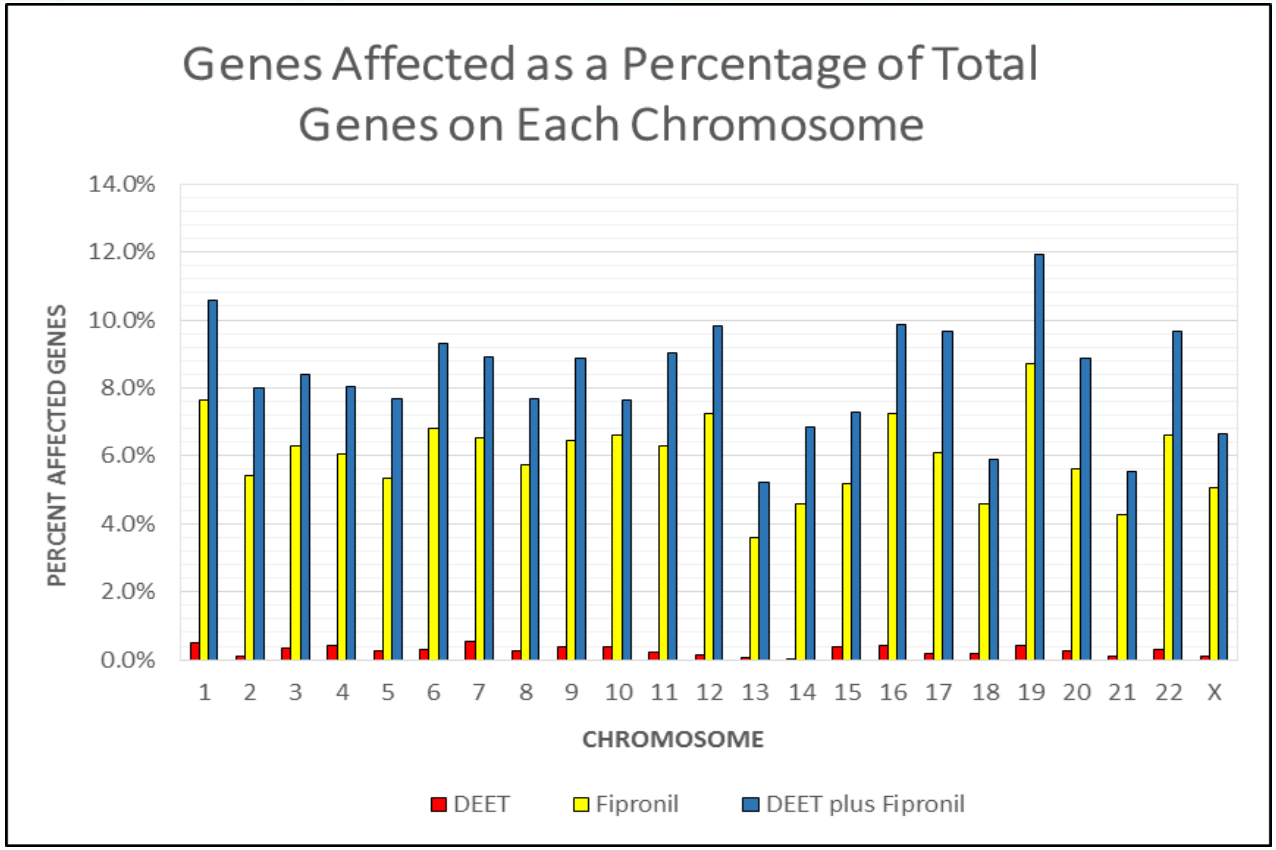
5146 genes differentially expressed (p-value of 0.05)

Eminent Toxicologist Lecture Series

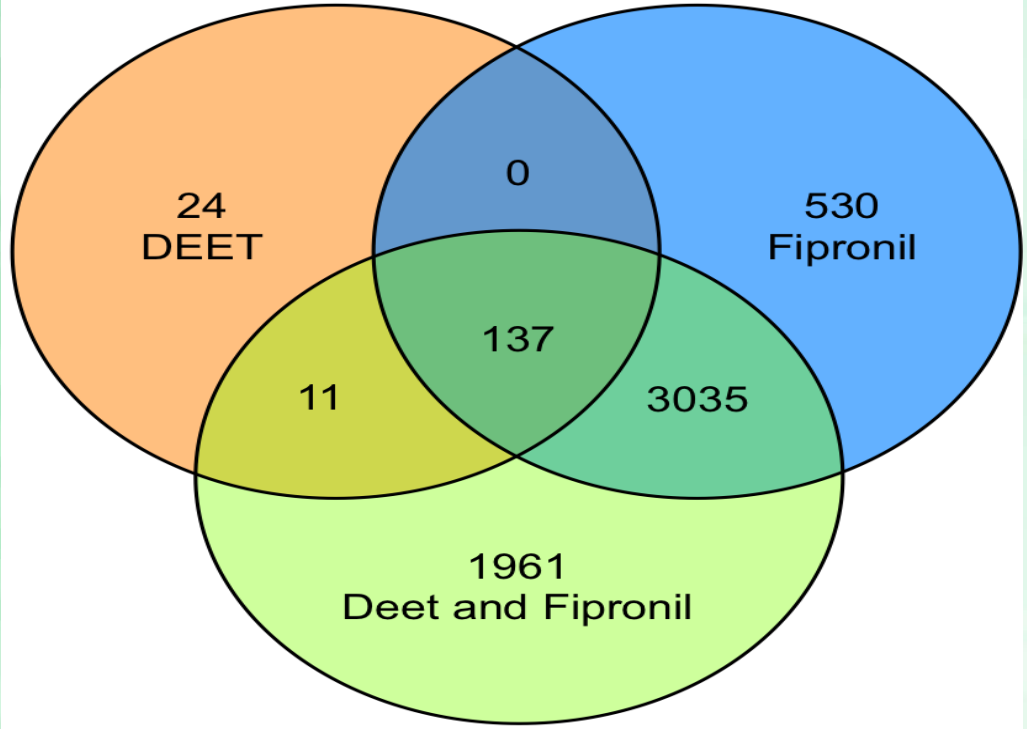


Society of Toxicology

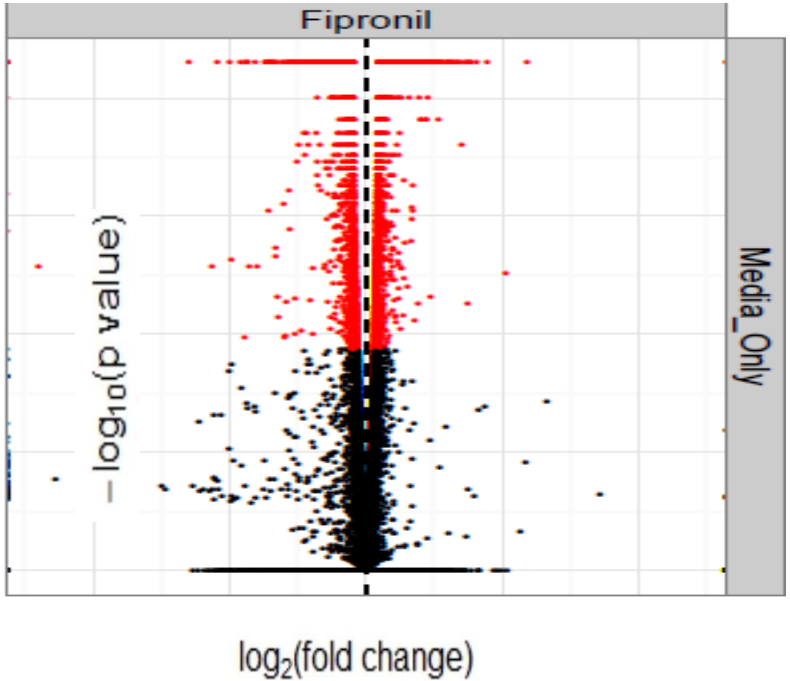

Percent Affected Genes per Chromosome
16.0%
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
PERCENT AFFECTED GENES
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X
CHROMOSOME
DEET
Fipronil
DEET plus Fipronil

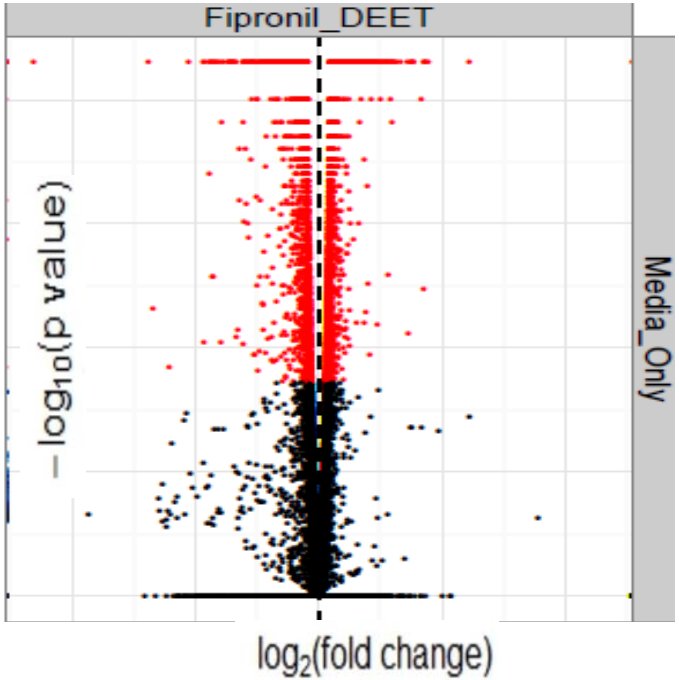

Genes Affected as a Percentage of Total Genes on Each Chromosome
PERCENT AFFECTED GENES
14.0%
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X
CHROMOSOME
DEET
Fipronil
DEET plus Fipronil

Eminent Toxicologist Lecture Series



Society of Toxicology


Fipronil
Media_Only
$-\log_{10}$(p value)
$\log_2$(fold change)


Fipronil_DEET
Media_Only
$-\log_{10}$(p value)
$\log_2$(fold change)