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# Toxicity Screening using Zebrafish Embryos: Form and Function

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**Office of Research and Development**
National Health and Environmental Effects Research Laboratory, Integrated Systems Toxicology Division

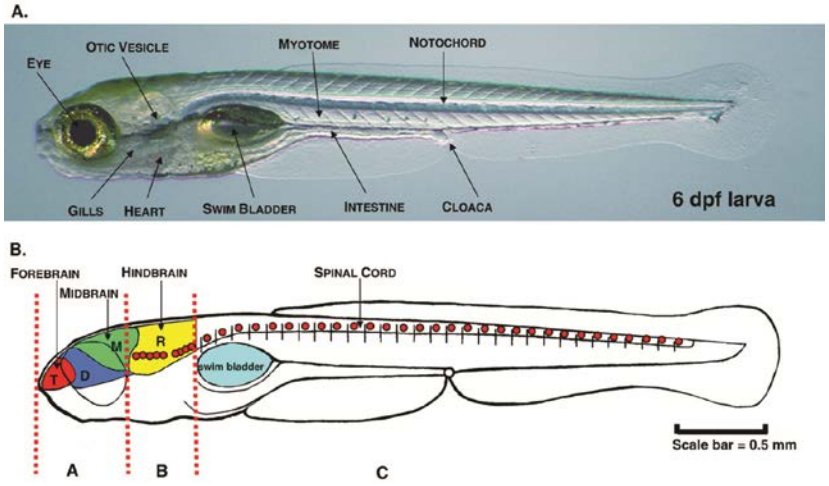
# Zebrafish Screening

- Integrated, highly conserved model of development
- Applicable to both human and eco toxicology
- “Form”: basic developmental toxicity screening
  - Padilla *et al, Reprod. Toxicol*., 2011
- “Function”: more subtle, functional endpoints

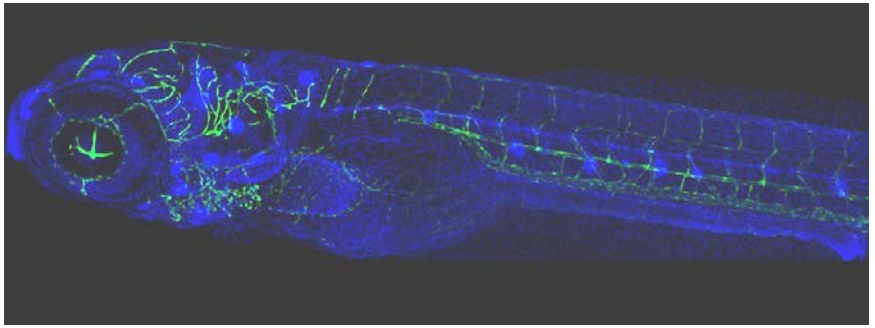
# Zebrafish Development

## practical considerations

- Rapid development
- Transparent embryo
- Developmental homology
- Easy to manipulate genome
- Inexpensive maintenance
- Hundreds of embryos
- Duration of experiment: 6 days




From Airhart *et al* (2007)



Courtesy of J. Olin, A. Tennant, and K. Jensen

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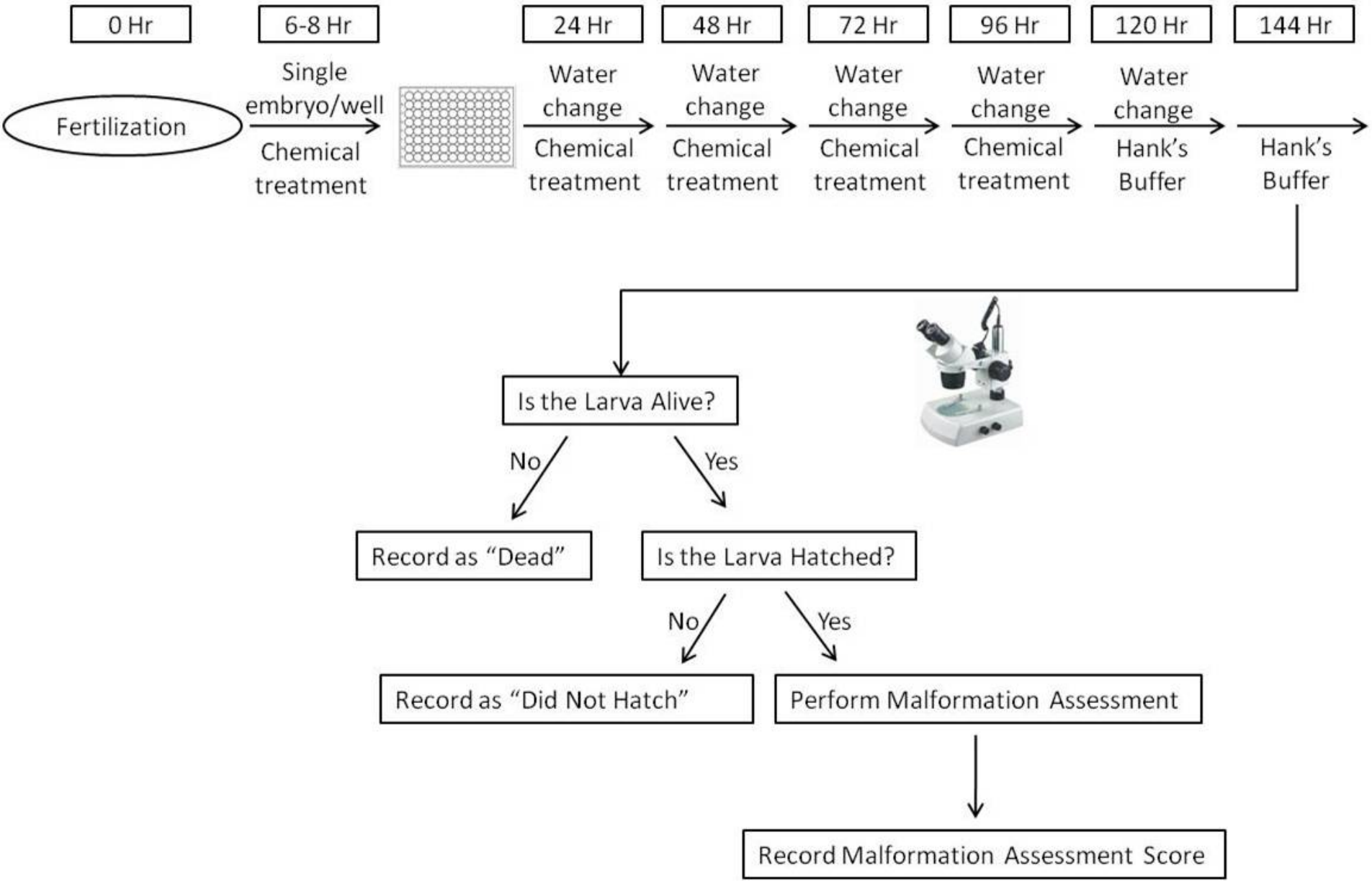
# ToxCast_320 Chemicals

- 309 chemicals: primarily pesticides and pesticide metabolites
- Intra- and inter-plate duplicates and triplicates
- Many of the chemicals have gone through testing in mammals, including guideline tests for developmental toxicity in rat and/or rabbit.

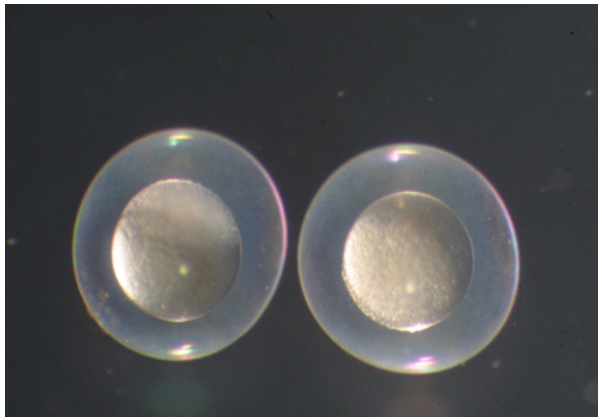
  (ToxRef Database;**http://www.epa.gov/ncct/toxrefdb**)

# Zebrafish Developmental Assay



# Malformation Assessment

| | | |
|---|---|---|
| Irregular Fins | | Enlarged Organs |
| Missing Fin | | Granular Organs |
| Stunted Fin(s) | | Hemorrhage |
| Acardia | | Lumps/Tumors |
| Bradycardia | | Jaundice |
| Tachycardia | | Kink in Tail |
| Cardiac enlargement | | Lordosis |
| Tube Heart | | Scoliosis |
| Brachycephalic | | Eel-Like Body |
| Dolichocephalic/Beak Face | | Emaciated |
| Microcephalic | | Stunted Growth |
| Microphthalmia | | Rolling to Side |
| Ocular Edema | | Floating |
| Under-Developed Jaw | | Gasping |
| Enlarged Otoliths | | Lying on Side |
| Distended Thoracic Region | | Not Moving/Can't Swim |

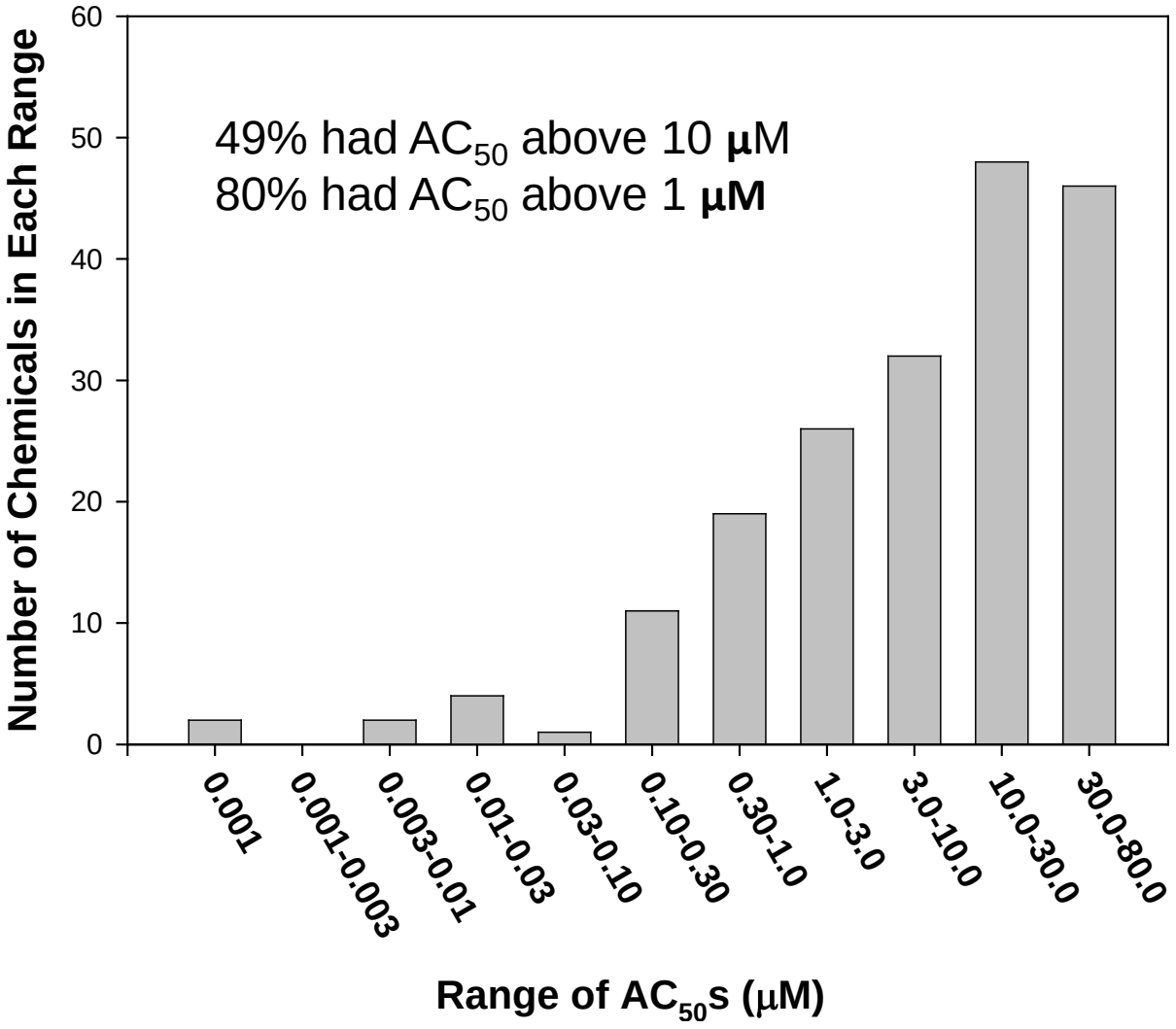


6-8 hr post fertilization

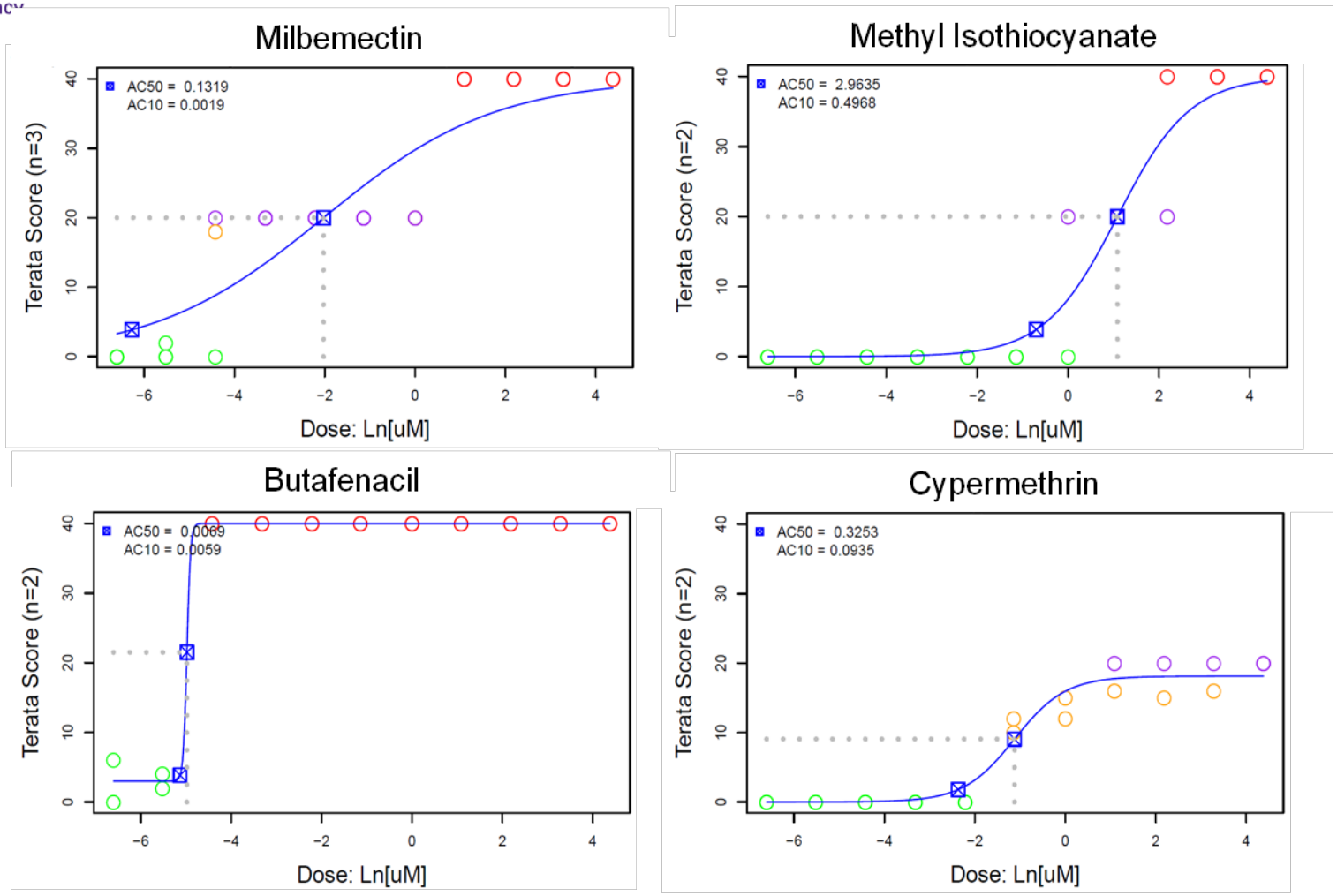


6 days post fertilization

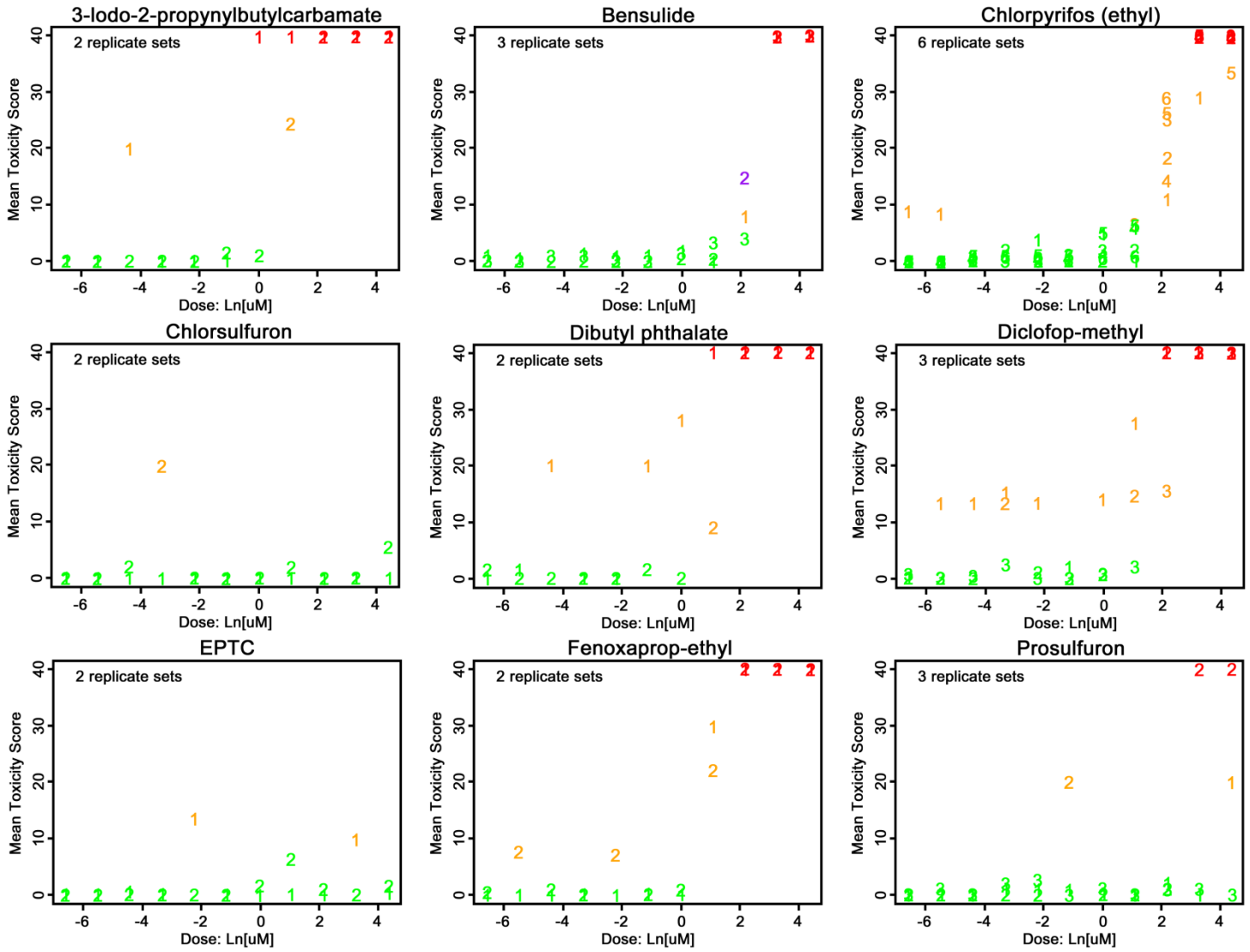
# Distribution of Potencies



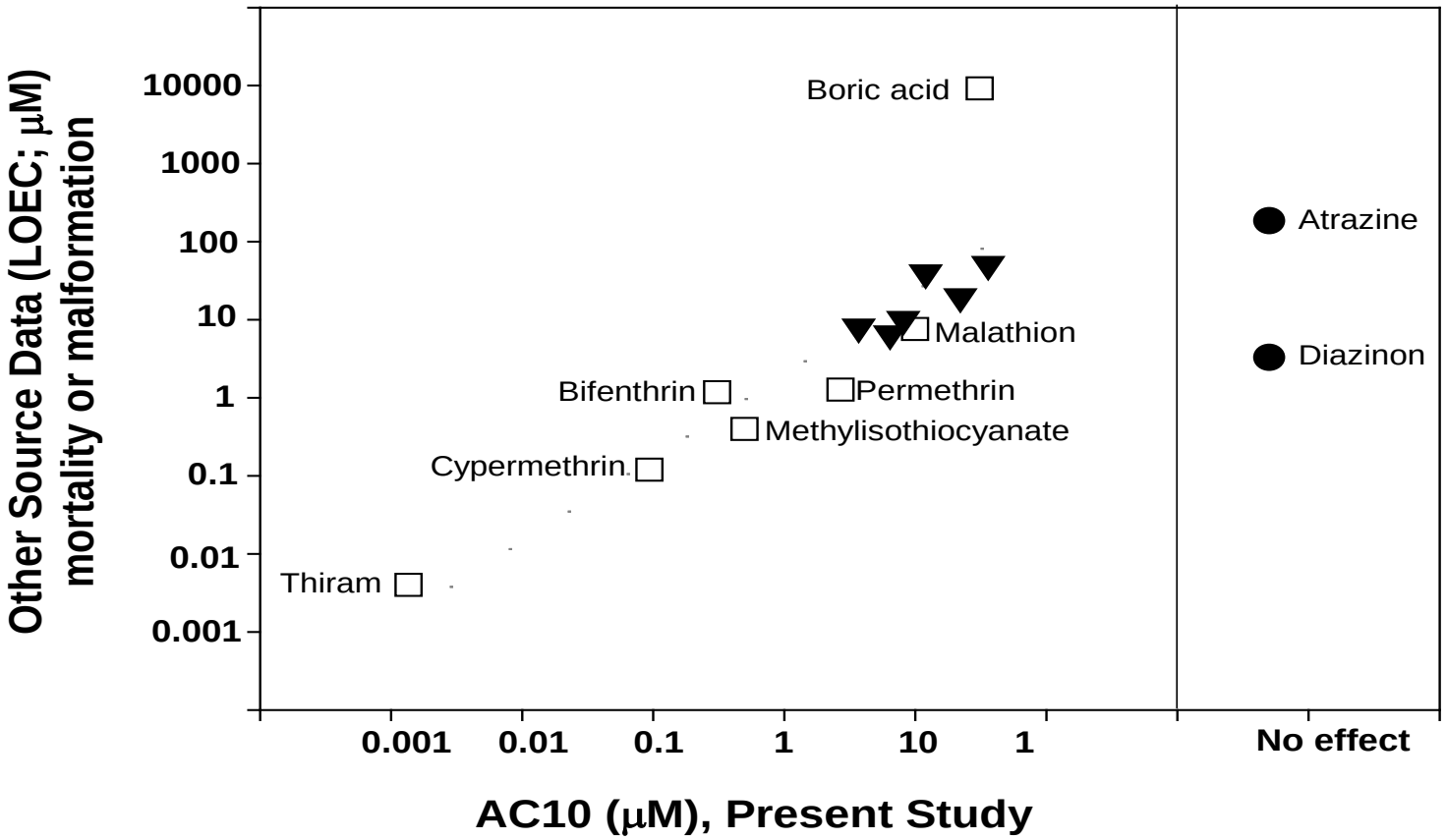
# Example Dose-Response Curves



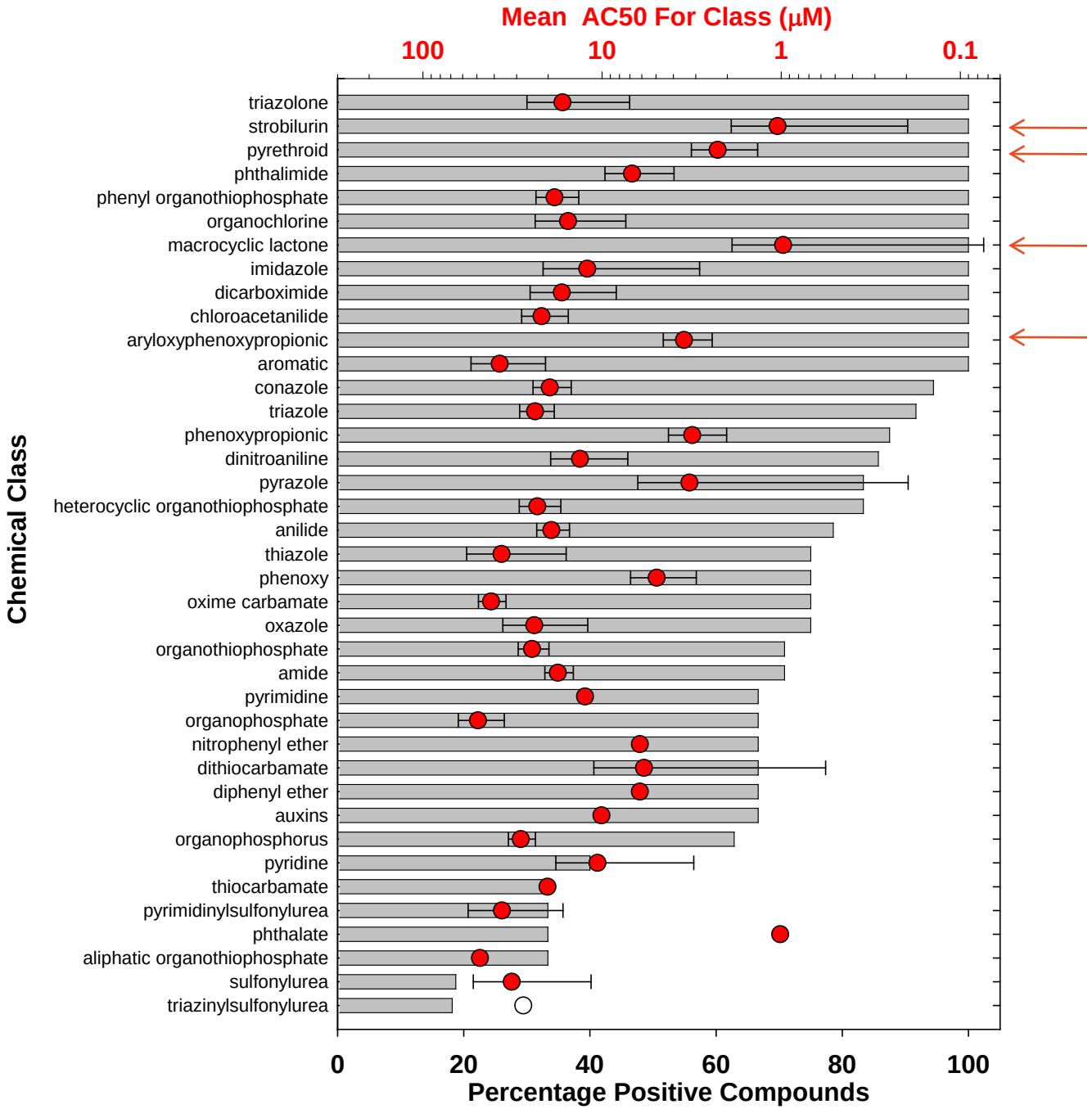
# Concordance Among Replicates

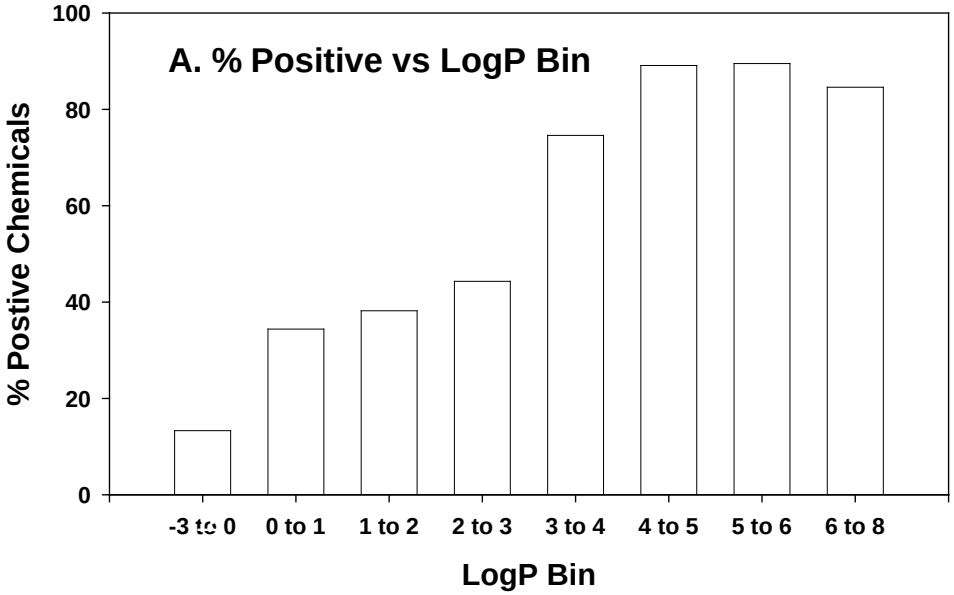


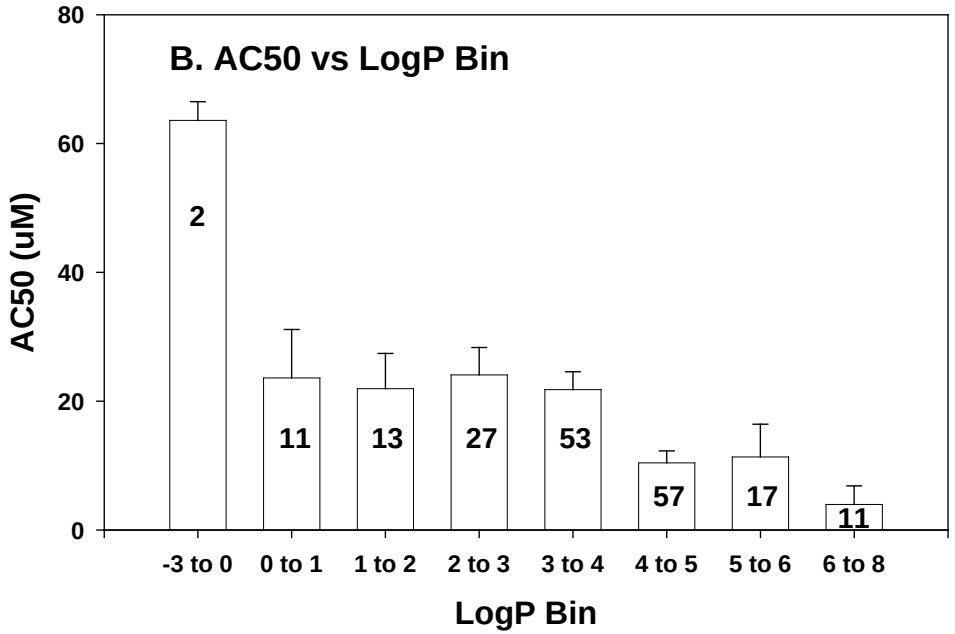
# Concordance with Previous Data

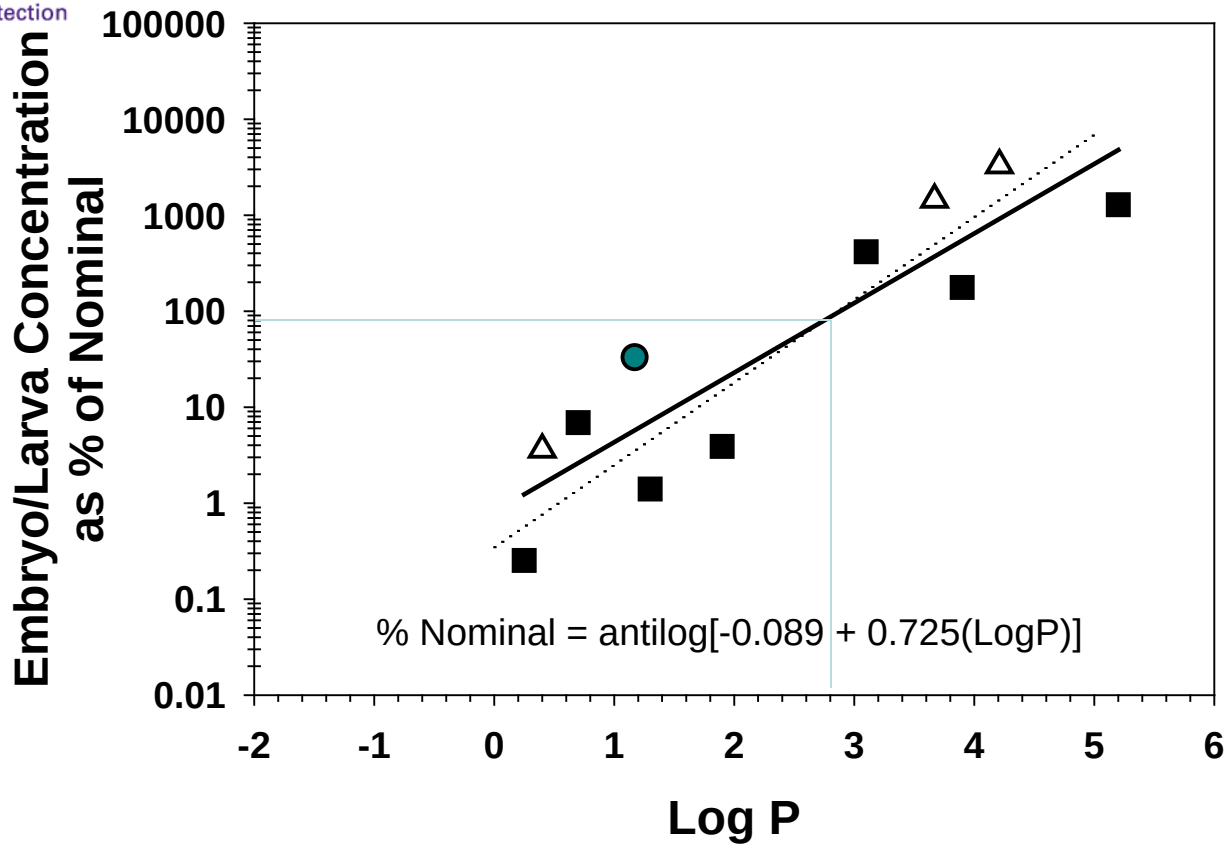



Comparison of the present data with the zebrafish embryo toxicity data in the ECOTOX database as well as recently published paperson the toxicity of triazole derivatives Hermsen, SA et al, 2011) □ = chemicals that were positive in the ECOTOX Database and in the present study; ● = chemicals that were positive in the ECOTOX Database, but negative in the present assay; ▼= triazole derivatives tested in the above publications. The correlation line (dashed line) fit to the positive chemicals resulted in a slope 1.07 and $R^2$=0.79.


Mean AC50 For Class (μM)
100
10
1
0.1
triazolone
strobilurin
pyrethroid
phthalimide
phenyl organothiophosphate
organochlorine
macrocyclic lactone
imidazole
dicarboximide
chloroacetanilide
aryloxyphenoxypropionic
aromatic
conazole
triazole
phenoxypropionic
dinitroaniline
pyrazole
heterocyclic organothiophosphate
anilide
thiazole
phenoxy
oxime carbamate
oxazole
organothiophosphate
amide
pyrimidine
organophosphate
nitrophenyl ether
dithiocarbamate
diphenyl ether
auxins
organophosphorus
pyridine
thiocarbamate
pyrimidinylsulfonylurea
phthalate
aliphatic organothiophosphate
sulfonylurea
triazinylsulfonylurea
Chemical Class
0
20
40
60
80
100
Percentage Positive Compounds


A. % Positive vs LogP Bin
% Postive Chemicals
100
80
60
40
20
0
-3 to 0
0 to 1
1 to 2
2 to 3
3 to 4
4 to 5
5 to 6
6 to 8
LogP Bin


B. AC50 vs LogP Bin
AC50 (uM)
80
60
40
20
0
2
11
13
27
53
57
17
11
-3 to 0
0 to 1
1 to 2
2 to 3
3 to 4
4 to 5
5 to 6
6 to 8
LogP Bin

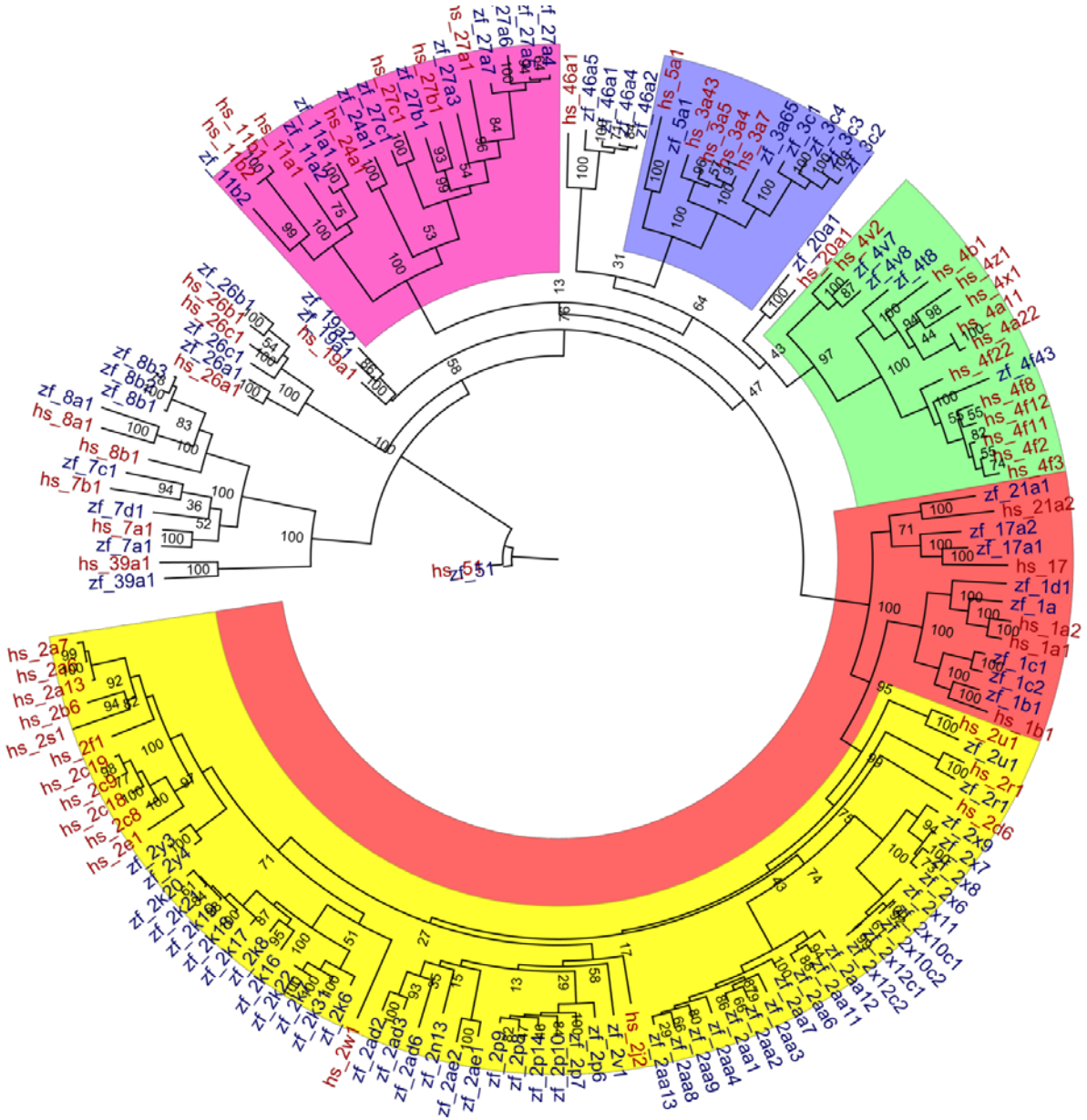



Relationship between LogP and body burden of chemical in zebrafish embryos/larvae. These data are taken from Berghmans et al, 2008 (■ = mean of 3 dpf and 7 dpf measures); Gustafson et al, 2012 (△); and Thomas et al, 2009 (grey circle). Linear regression (—) of these combined data gives an equation relating the concentration in the embryo to the LogP of the chemical: % nominal concentration in the embryo/larva = antilog[-0.089 + 0.725(LogP)]. The $r^2$ of this linear regression is 0.81. The dashed line is the relationship between LogP and BioConcentration Factor (BCF) calculated by Petersen and Kristensen, 1998 where Log BCF= -0.46 + 0.86 (LogP) for a group of lipophilic compounds tested in zebrafish embryos and larvae.



Padilla, 2012

# Maximum likelihood phylogenetic tree of all zebrafish and human CYPs



Goldstone et al, 2010

## Table 3 Synteny comparison between zebrafish and human CYPs

| Zebrafish | Human | Zebrafish | Human | Zebrafish | Human |
|---|---|---|---|---|---|
| CYP1A | CYP1A1/1A2 | CYP3A65 | CYP3A-se1,-se2[a] | CYP19A1,2 | CYP19A1 |
| CYP1B1 | CYP1B1 | CYP3C1-4 | CYP3A3,4,7 | CYP20A1 | CYP20A1 |
| CYP1C1,2 | - | CYP4F43 | CYP4F | CYP21A1 | CYP21A2 |
| CYP1D1 | CYP1D1P | CYP4V7,8 | CYP4V2 | CYP24A1 | CYP24A1 |
| CYP2Ks | CYP2W1 | CYP4T8 | - | CYP26A1 | CYP26A1/C1 |
| CYP2N13 | CYP2J2 | CYP5A1 | CYP5A1 | CYP26B1 | CYP26B1 |
| CYP2Ps | CYP2J2 | CYP7A1 | CYP7A1 | CYP26C1 | - |
| CYP2R1 | CYP2R1 | CYP7B1 | CYP7B1 | CYP27A3-7 | CYP27A1 |
| CYP2U1 | CYP2U1 | CYP7C1 | - | CYP27B1 | - |
| CYP2V1 | CYP2J2 | CYP8A1 | CYP8A1 | CYP27C1 | - |
| CYP2X1-10 | - | CYP8B1-3 | CYP8B1 | CYP39A1 | CYP39A1 |
| CYP2Y3,4 | CYP2A/B/F/S | CYP11A1,2 | CYP11A1 | CYP46A1 | CYP46A1 |
| CYP2AA1-12 | - | CYP11C1 | - | CYP46A2,4,5 | - |
| CYP2AD2,3,6 | CYP2J2 | CYP17A1,2 | CYP17A1 | CYP51A1 | CYP51A1 |
| CYP2AE1,2 | - | | | | |

[a] pseudogene (single remnant exon)

Goldstone et al, 2010

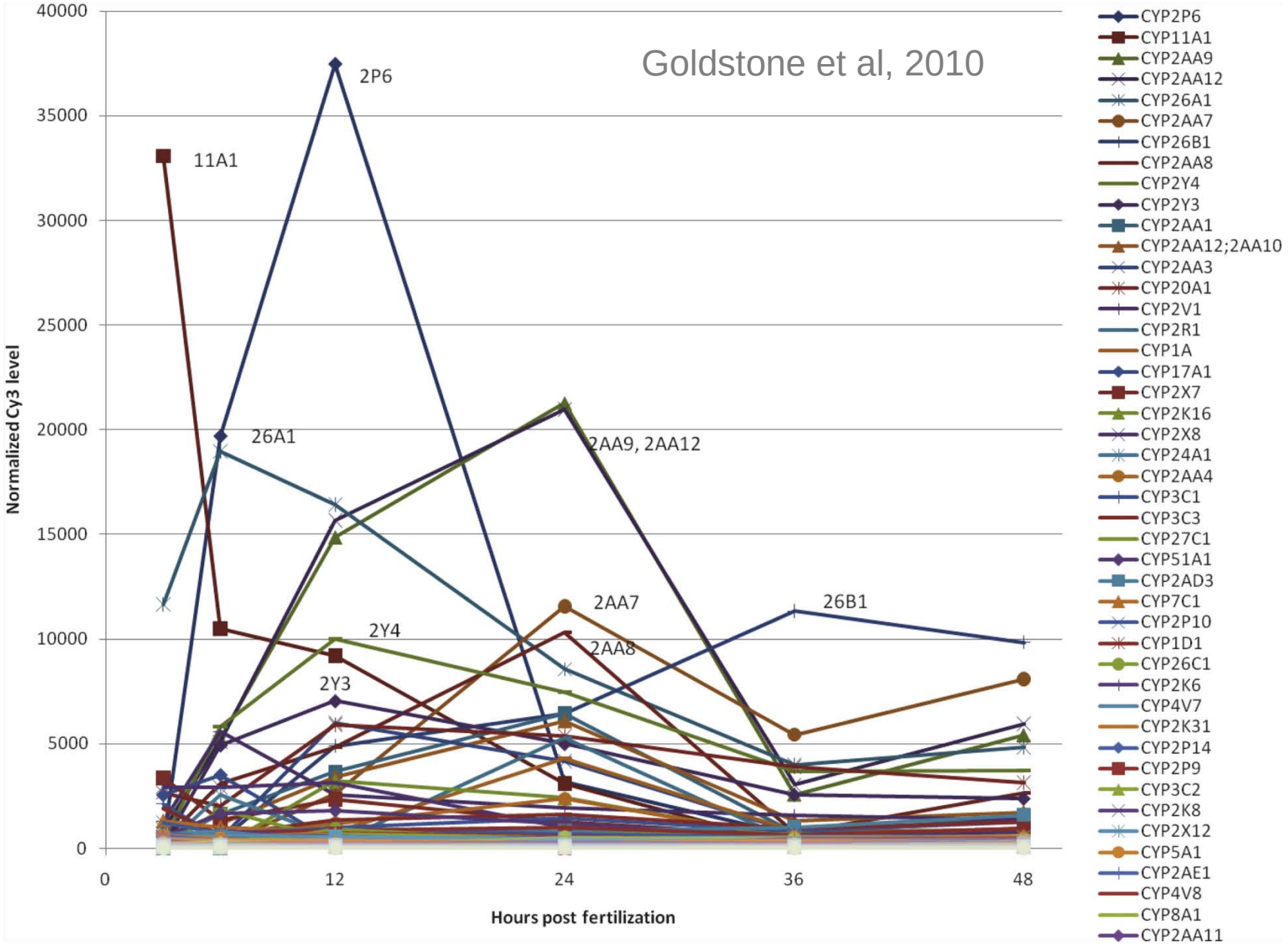



**Figure 4 Expression of 88 CYP genes in zebrafish during the first 48 hours of development**. Single color microarray analyses of CYP gene expression throughout early development (3-48 hours post fertilization, hpf) shows that while some CYP genes are expressed in the whole embryo at high levels, most CYP genes are expressed at levels significantly above background (~5 fluorescent units; see Additional File 2, Table S3). Strong temporal signals are apparent.

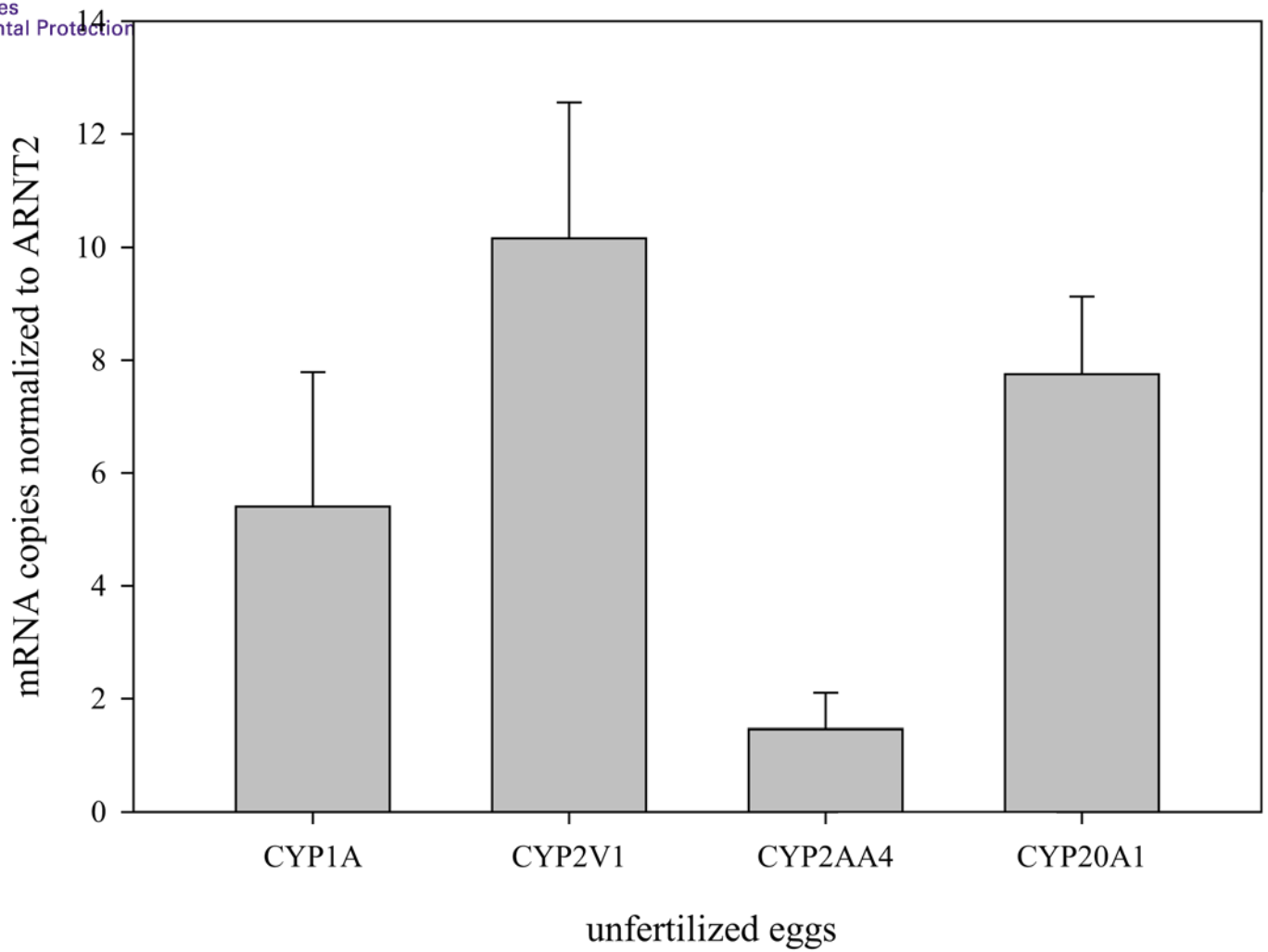



**Figure 7 Expression of four CYP genes in unfertilized oocytes**. The maternal contribution to transcript abundance of selected CYPs was determined using qPCR on unfertilized oocytes. Eggs were expressed from gravid female zebrafish (n = 3) by gentle squeezing of anesthetized fish. Data was normalized using ARNT2.

| Metabolic Pairs | | Outcome | |
|---|---|---|---|
| Parent | Metabolite | Parent | Metabolite |
| Dimethyl Phthalate | Methyl Hydrogen Phthalate | — | — |
| Atrazine | 6-Deisopropylatrazine | — | — |
| Methoxychlor | 2,2-Bis(4-hydroxyphenyl)-1,1,1-Trichloroethane (HPTE) | + 2.63 | + 24.68 |
| Metam-Sodium | Methyl Isothiocyanate | + 21.63 | + 2.96 |
| Diethylhexyl Phthalate | Monoethylhexyl Phthalate | — | +0.5665 |
| Metiram-Zinc | Ethylenethiourea | + 1.44 | — |
| Maneb | Ethylenethiourea | _ | — |
| Mancozeb | Ethylenethiourea | — | — |
| Dibutyl Phthalate | Monobutyl Phthalate | + 1.46 | — |
| Malathion | Malaoxon | + 23.5 | — |
| Diazinon | Diazoxon | — | + 28.99 |
| Chlorpyrifos (Ethyl) | Chlorpyrifos-oxon (Ethyl) | + 8.5 | + 0.4 |

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# Main Conclusions

- The majority (62%) of ToxCast Phase 1 chemicals were toxic to the developing zebrafish.
- Both toxicity incidence and potency were correlated with chemical class and hydrophobicity (logP)
- Need to understand dose, which is related to logP
- Inter-and intra-plate replicates showed good agreement.
- Hepatic metabolism is present.

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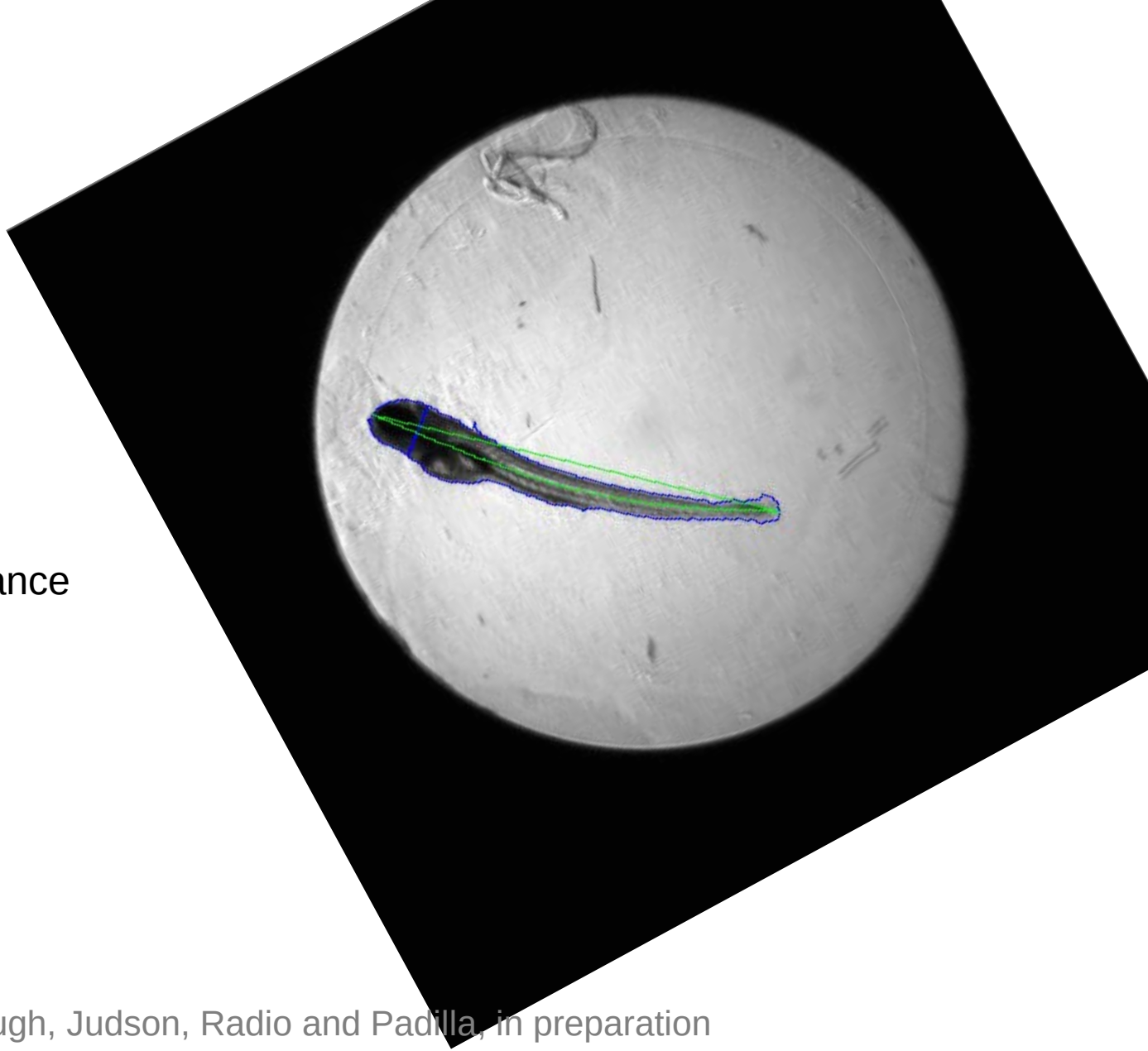
## Measures

- Area
- P2A
- LWR
- Head Tail Distance
- Spine length
- Width
- Straightness
- Convexity
- Curvature



Frady, Houck, Wambaugh, Judson, Radio and Padilla, in preparation

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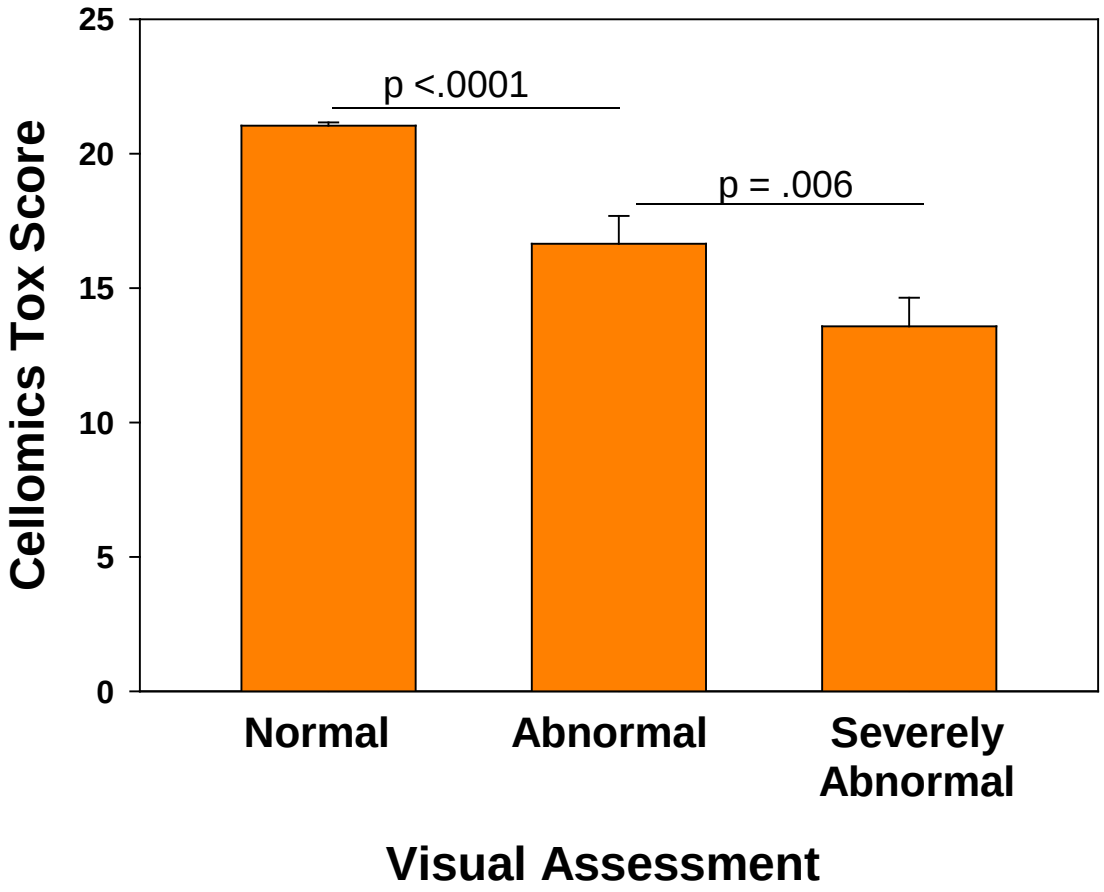
# Comparison of Human Visual Assessment with Cellomics Automated Assessment of Larvae



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# Lessons Learned and Future Directions

- Larval zebrafish assay has excellent reproducibility even with n=2-3.
- Good correlation between single high dose study and dose response study.
- Larval zebrafish have metabolic capability.
- Larval zebrafish assay may correlate with mammalian assays, but it won't be simple. (Sipes *et al*, 2011)
- Future Directions
  - Automated assessment of dysmorphology
  - ToxCast Phase II

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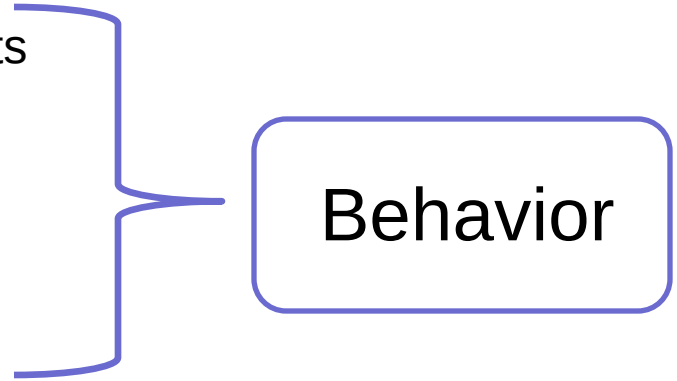
# Function

- Integration of Development
  - Spatial and temporal aspects of nervous system development
- Functional Assessments
- Sensory Assessments
  - Threshold
- Learning and Memory




Take advantage of the whole animal approach

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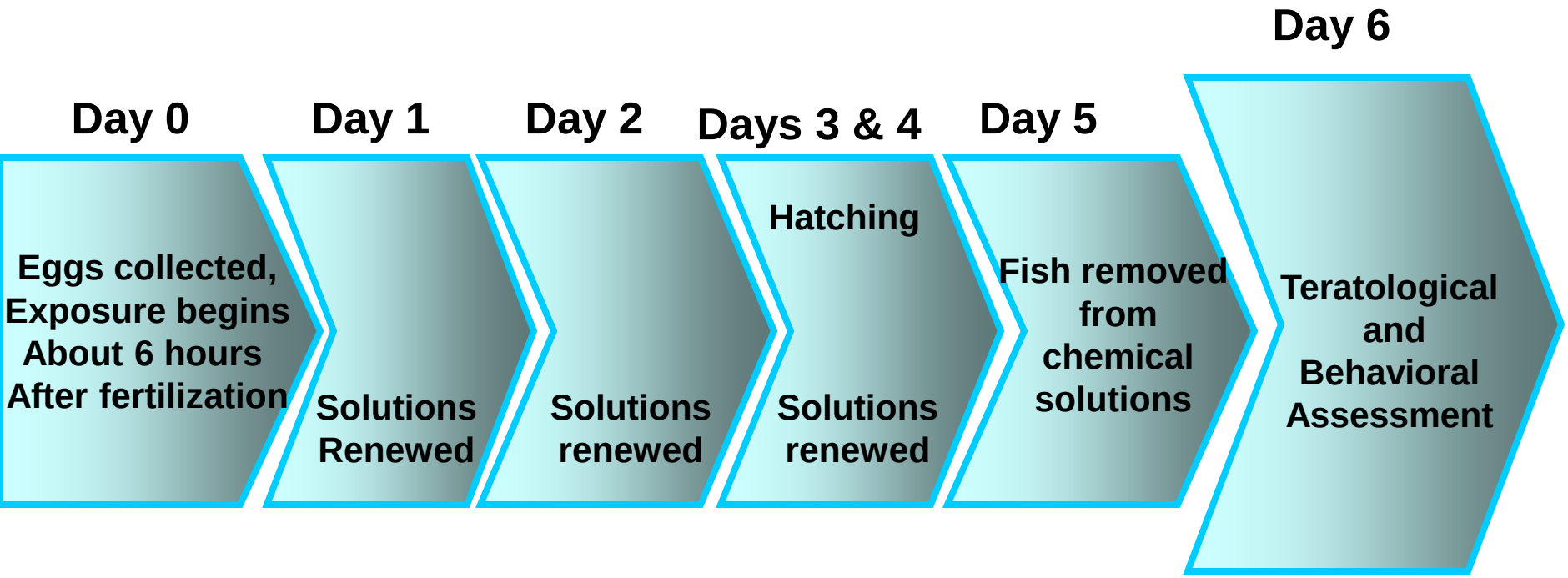
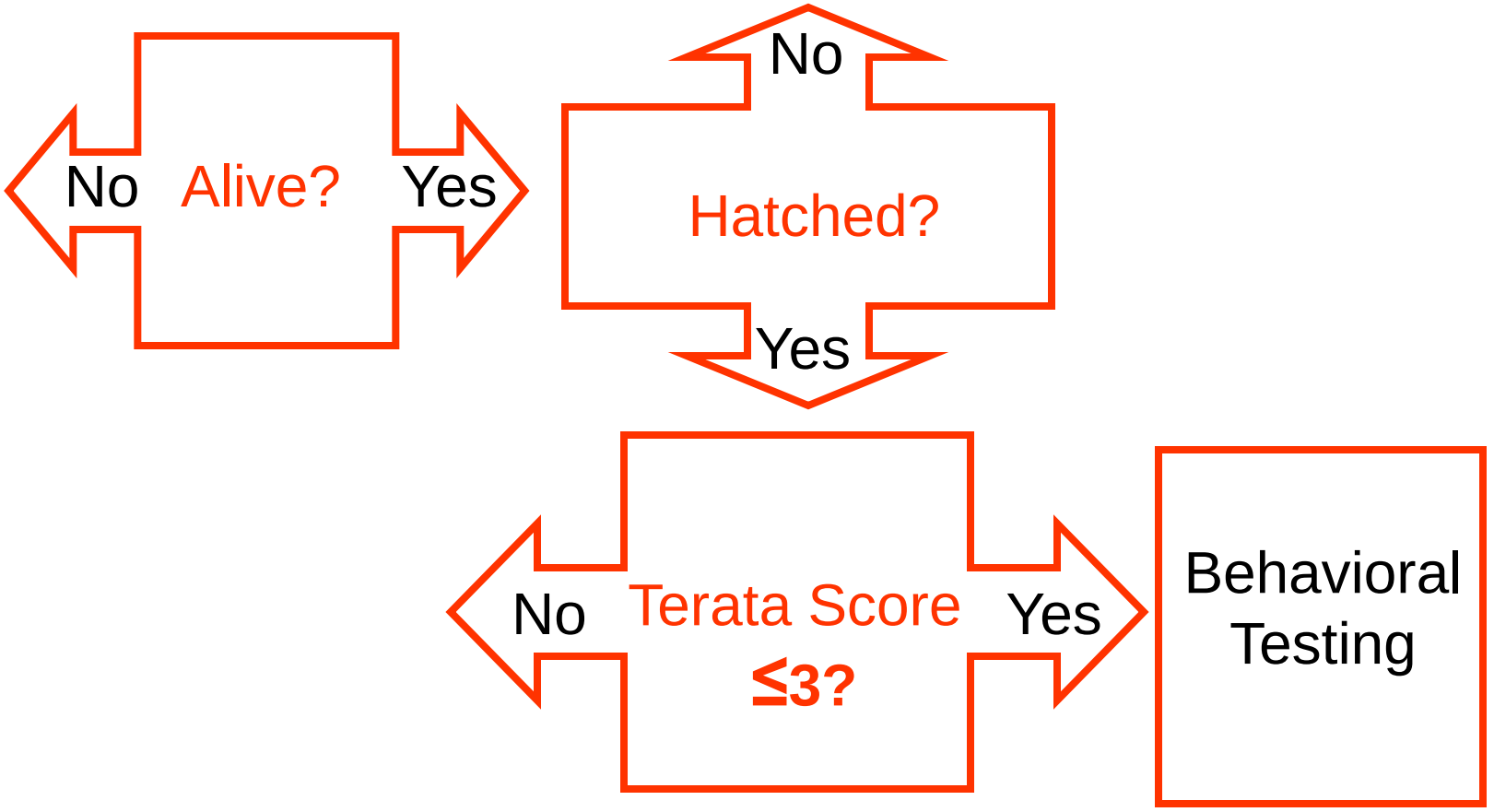
# General Experimental Approach

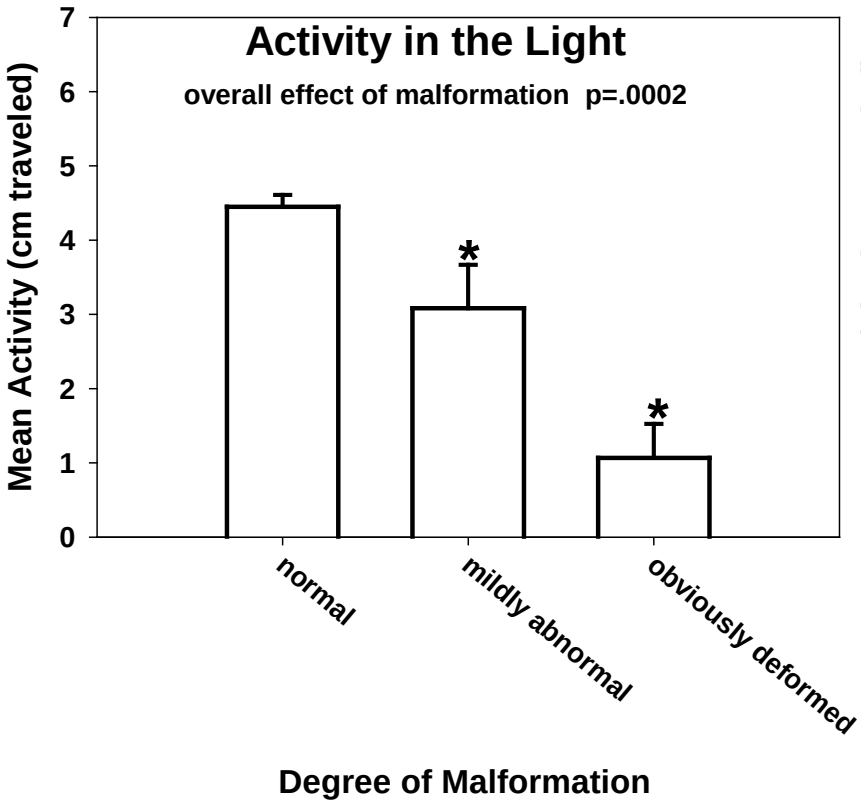


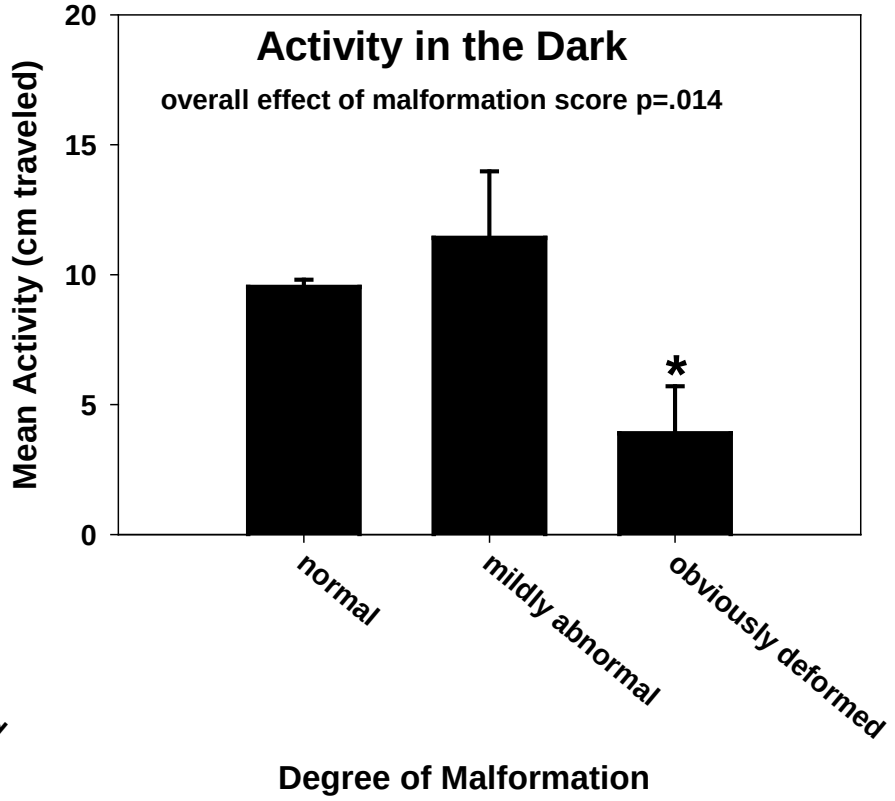

**All eggs/larvae kept at 26°C with 14:10 hr light:dark cycle.**

# Decision Tree for Day 6 Larvae




Note: No testing reported on abnormal larvae


Activity in the Light
overall effect of malformation p=.0002
Mean Activity (cm traveled)
0
1
2
3
4
5
6
7
*
*
normal
mildly abnormal
obviously deformed
Degree of Malformation


Activity in the Dark
overall effect of malformation score p=.014
Mean Activity (cm traveled)
0
5
10
15
20
*
normal
mildly abnormal
obviously deformed
Degree of Malformation

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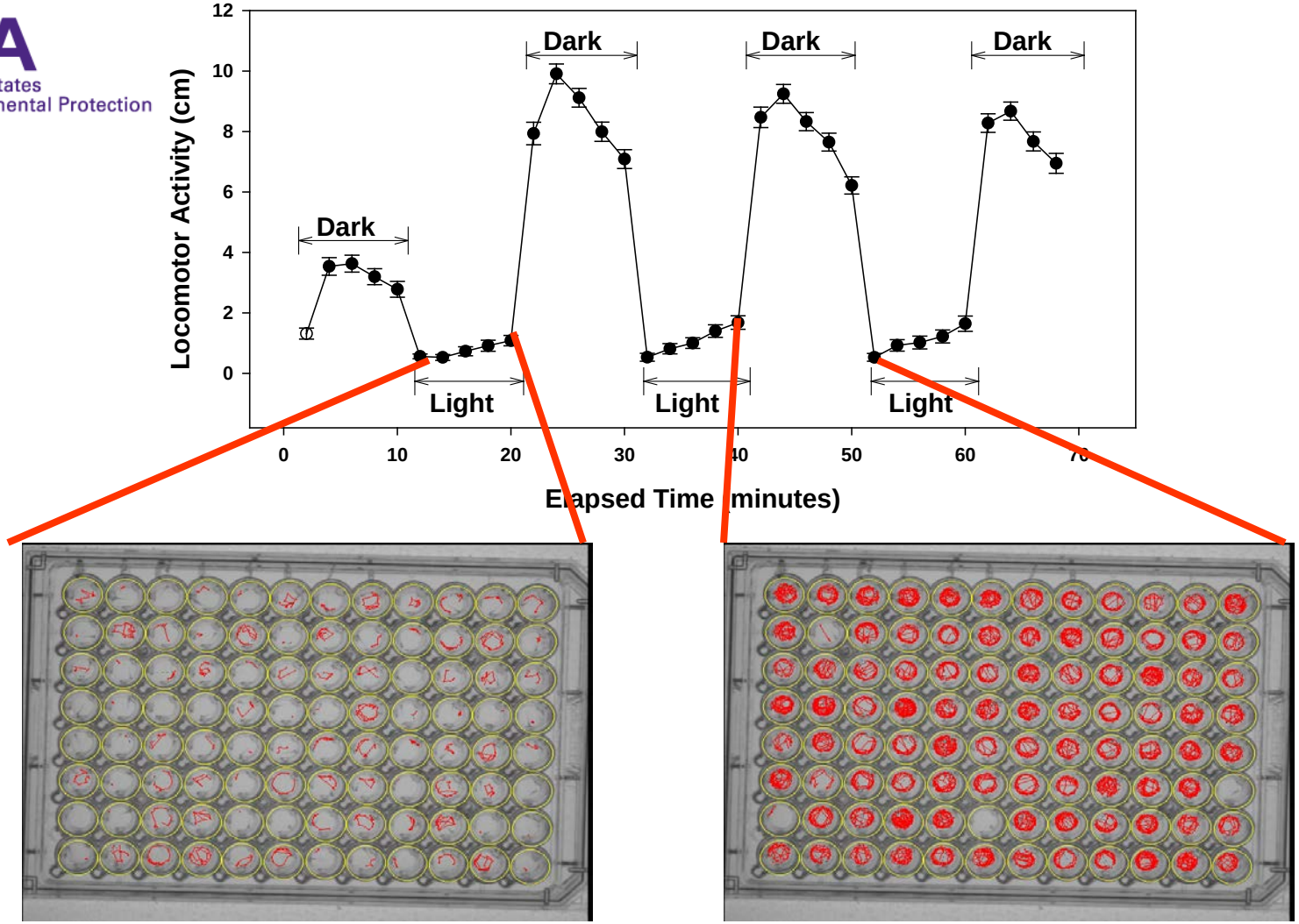


## Noldus video tracking system with Ethovision Software

From: R.C. MacPhail, J. Brooks, D.L. Hunter, B. Padnos, T.D. Irons, and S. Padilla. Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol, *NeuroToxicology*, 2009.

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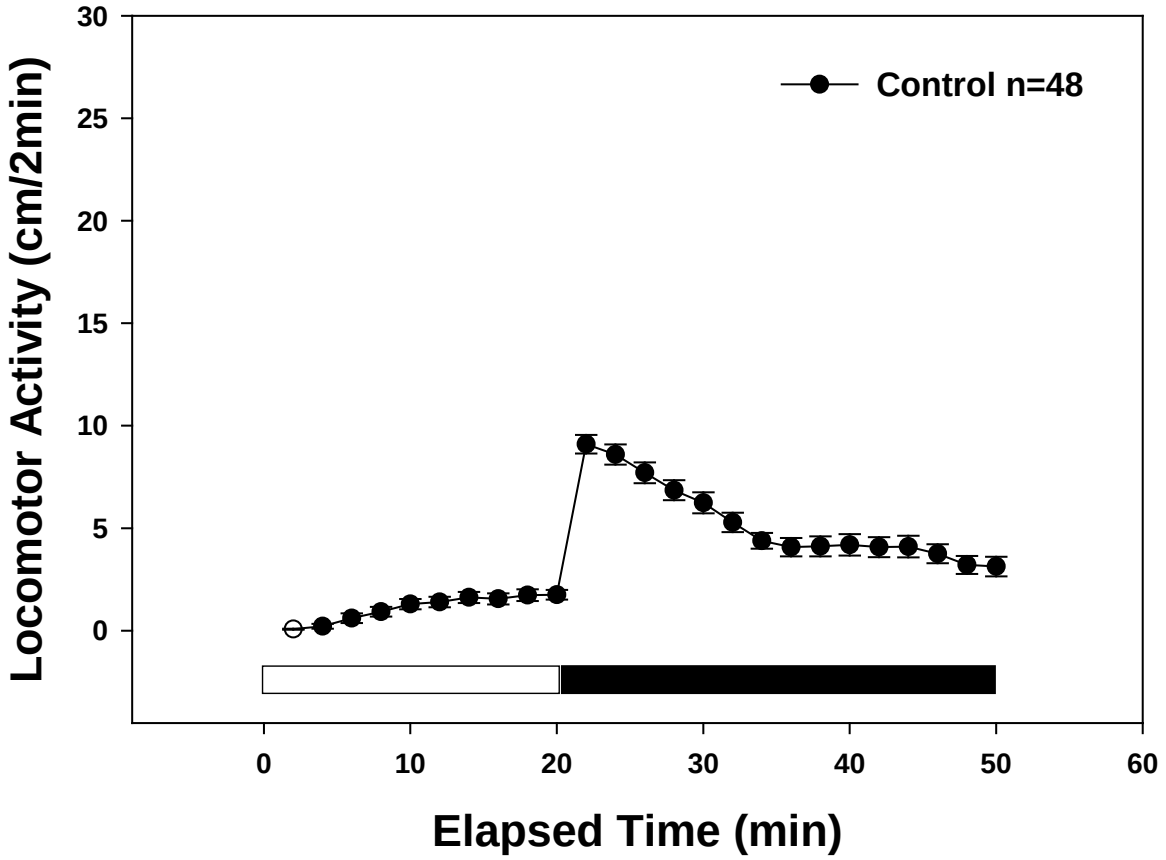
# Developmental Heptachlor



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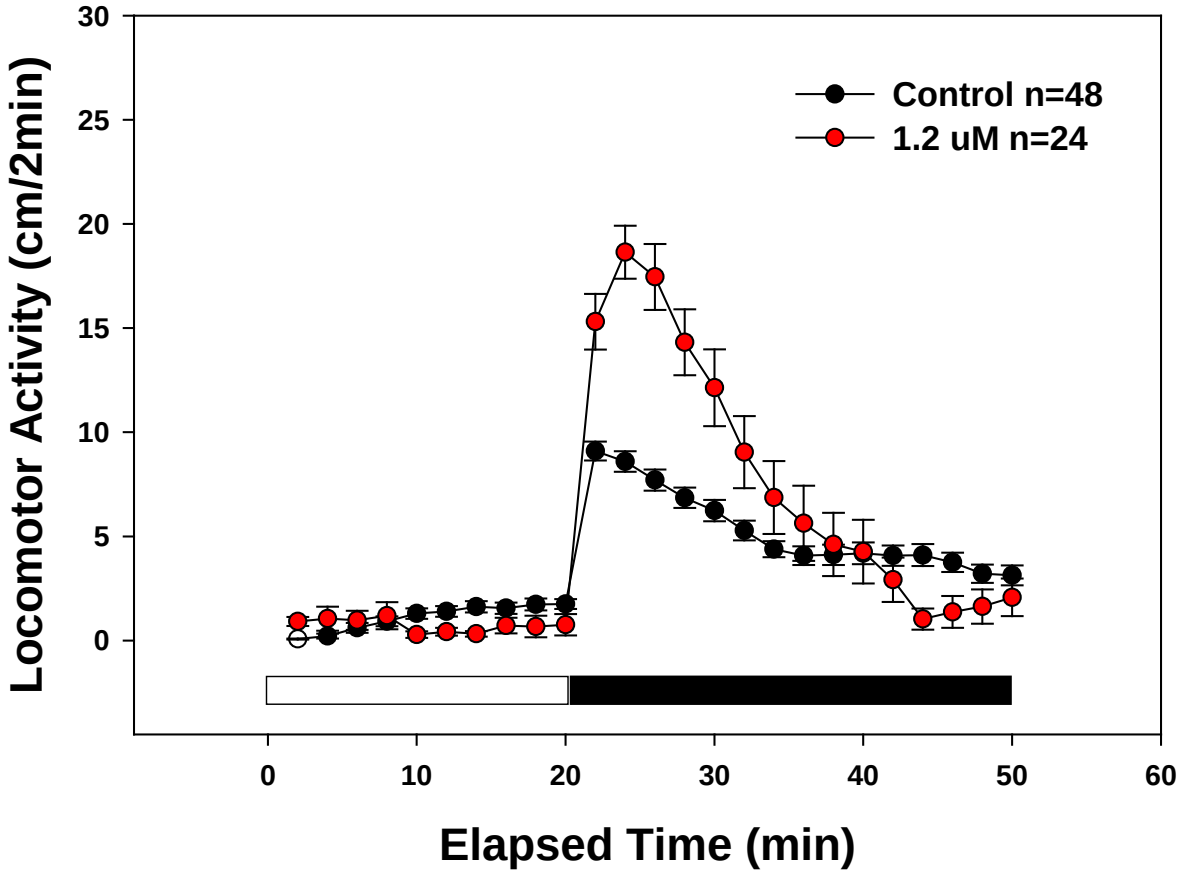
# Developmental Heptachlor



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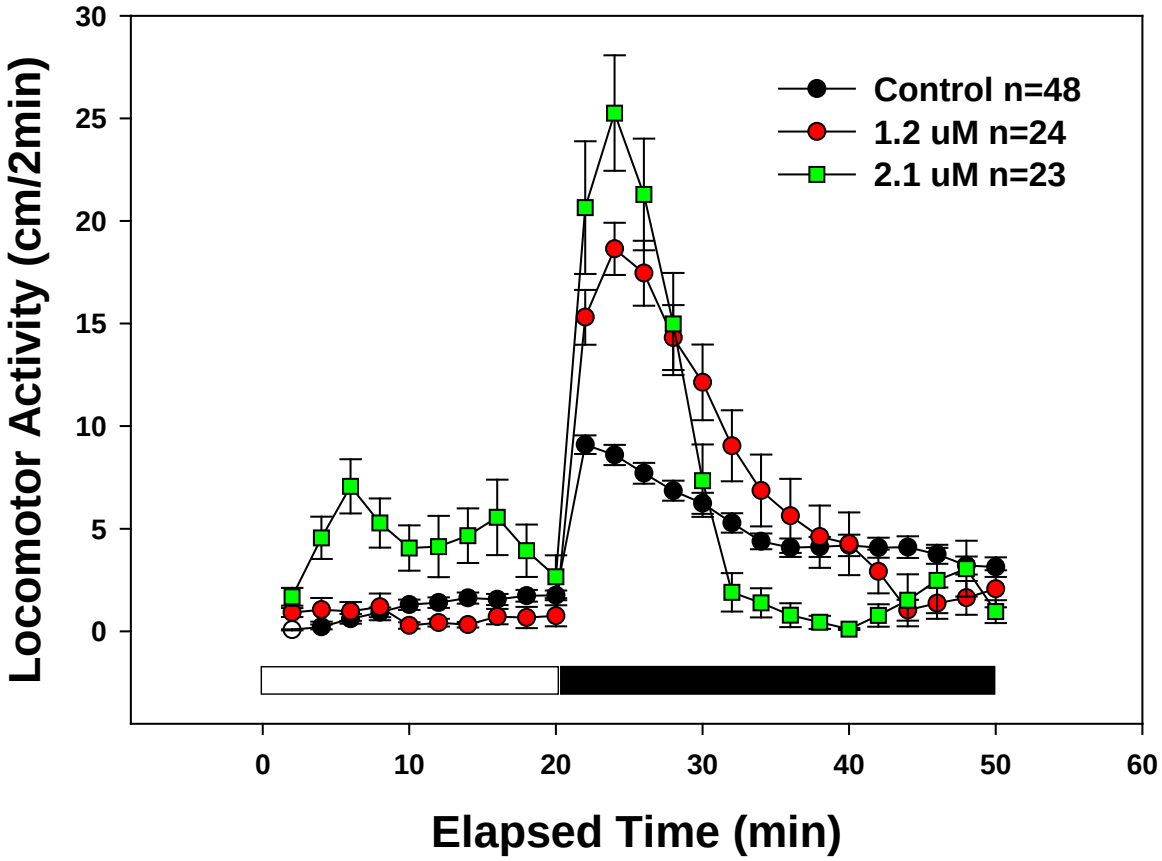
# Developmental Heptachlor



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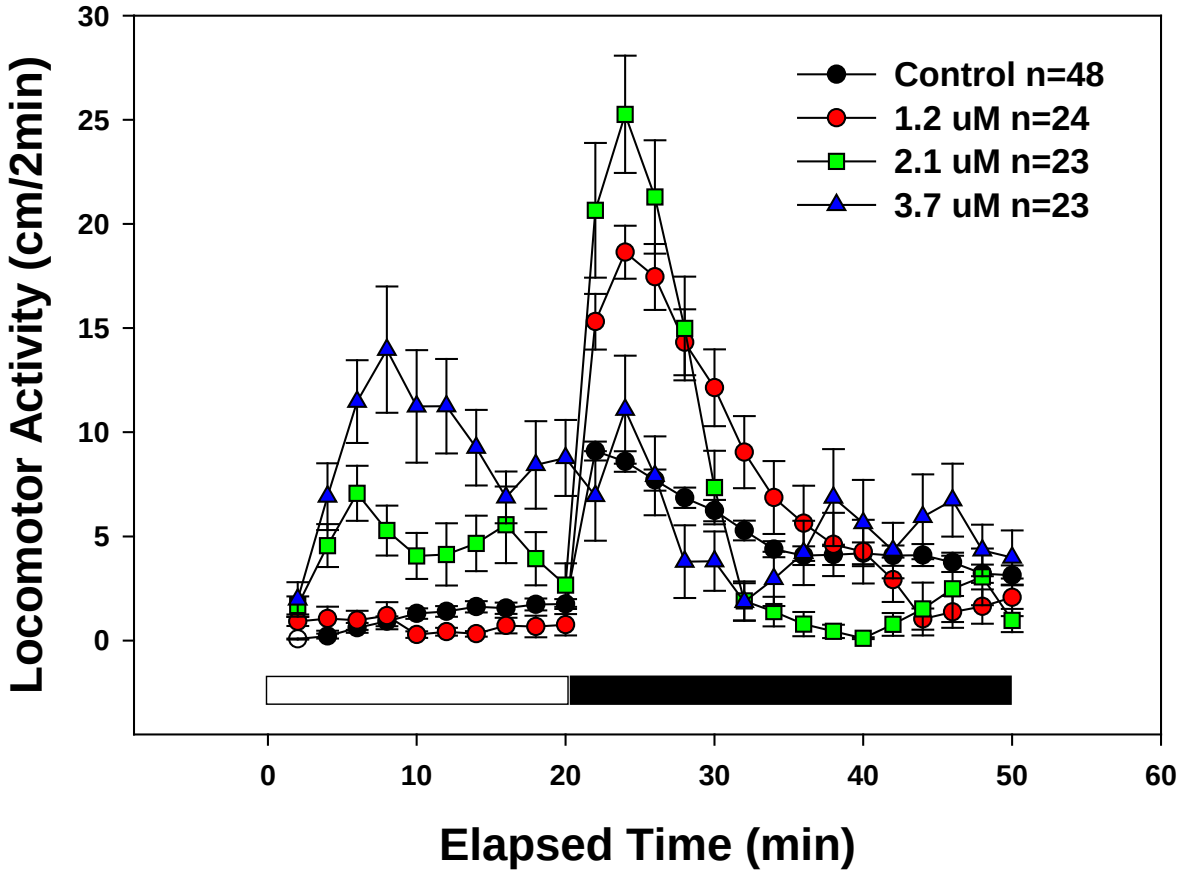
# Developmental Heptachlor



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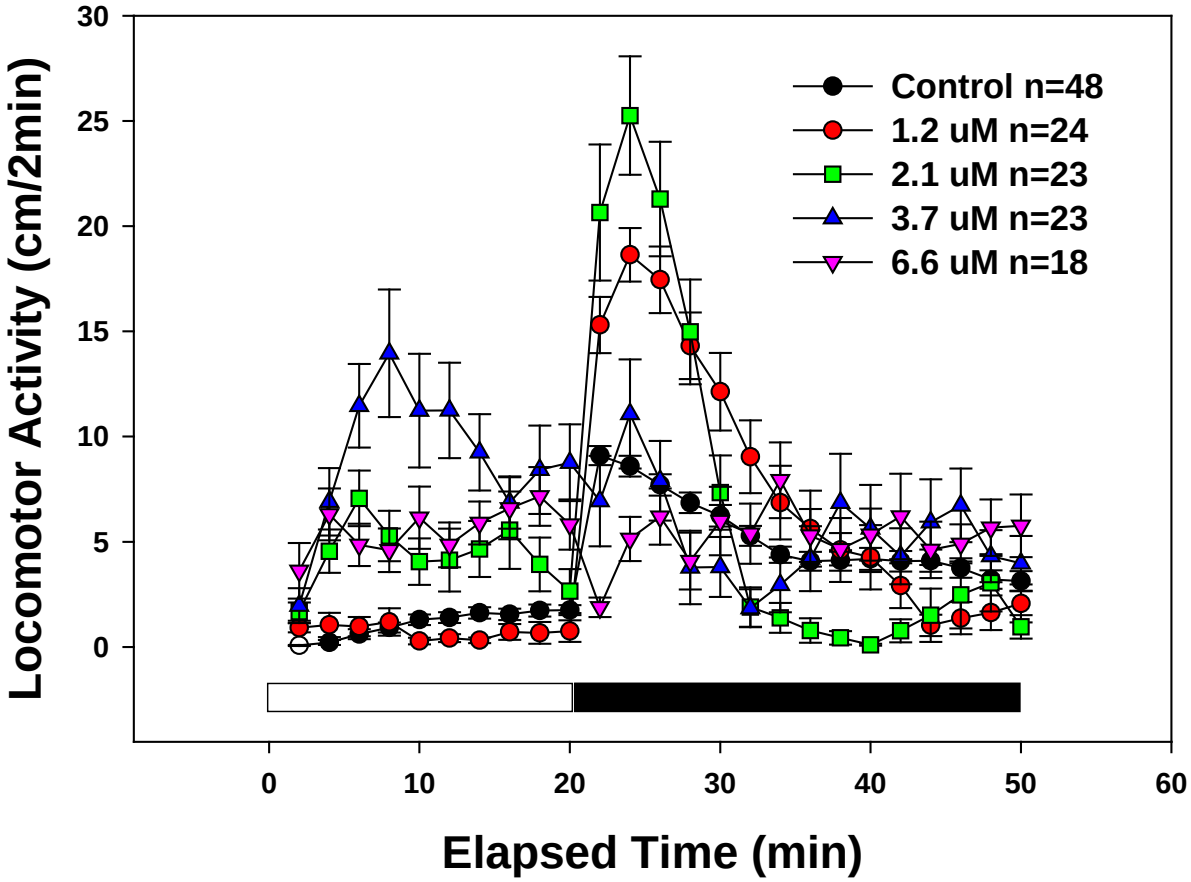
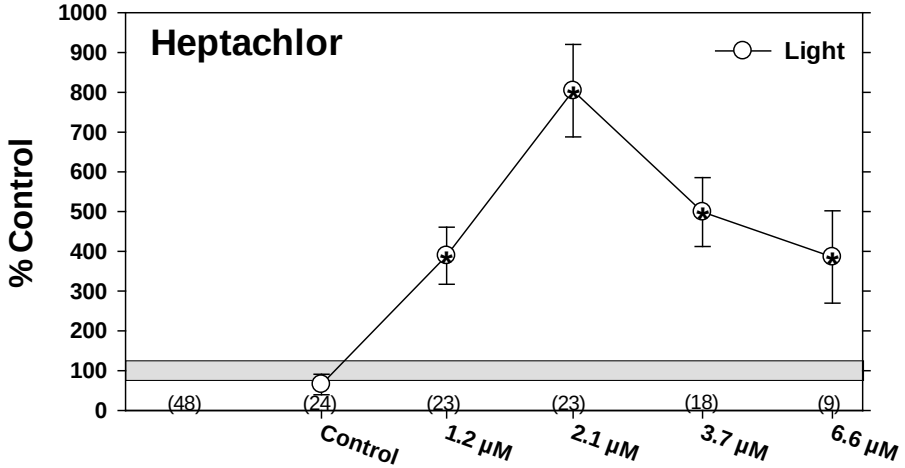
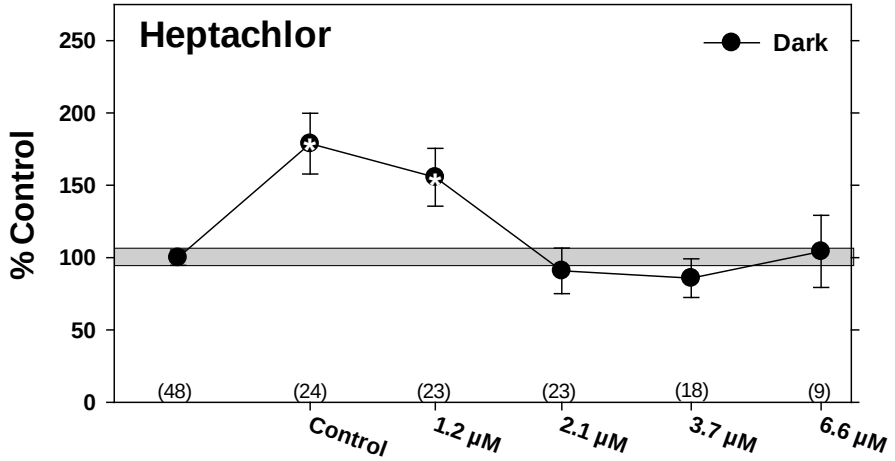
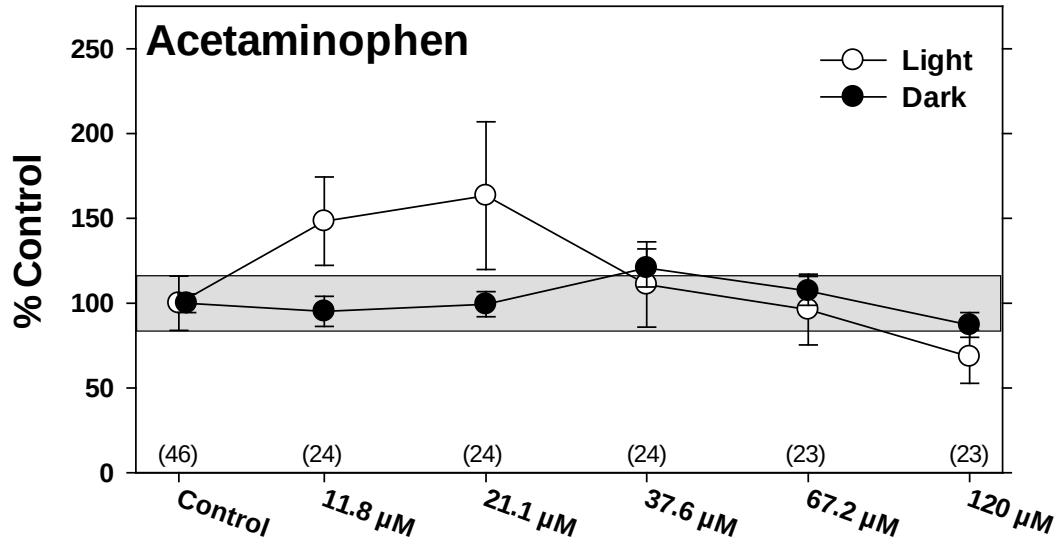
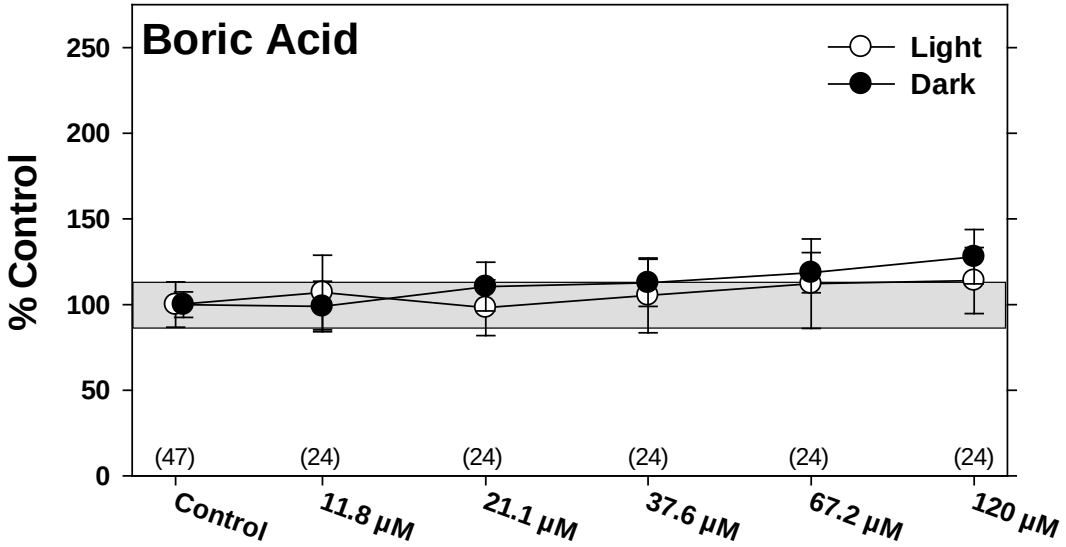
# Developmental Heptachlor




Heptachlor
Light
% Control
1000
900
800
700
600
500
400
300
200
100
0
(48)
(24)
(23)
(23)
(18)
(9)
Control
1.2 µM
2.1 µM
3.7 µM
6.6 µM


Heptachlor
Dark
% Control
250
200
150
100
50
0
(48)
(24)
(23)
(23)
(18)
(9)
Control
1.2 µM
2.1 µM
3.7 µM
6.6 µM


Acetaminophen
Light
Dark
% Control
250
200
150
100
50
0
(46)
(24)
(24)
(24)
(23)
(23)
Control
11.8 µM
21.1 µM
37.6 µM
67.2 µM
120 µM


Boric Acid
Light
Dark
% Control
250
200
150
100
50
0
(47)
(24)
(24)
(24)
(24)
(24)
Control
11.8 µM
21.1 µM
37.6 µM
67.2 µM
120 µM

United States Environmental Protection Agency

# Specific Accomplishments

1. We have developed a convenient method for assessing behavior in larval zebrafish in 96-well plates.
   - **Zebrafish larvae respond differently to light and dark conditions.**
     - **Toxic chemicals may have different effects on each**
   - **An optimal test is at least 50 minutes under both light and dark conditions.**
   - **Need to take into consideration**
     - **Dysmorphology**

2. Thus far, the behavioral test is capable of identifying developmental neurotoxicants, and non-neurotoxic chemicals.

   **With 24 chemicals tested so far we have a Sensitivity of 82% and a Specificity of 80%.**