Gene chip applications in environmental toxicology.

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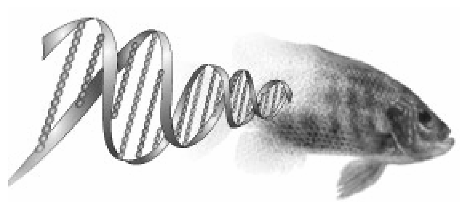

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Advantages of Gene chips

-Biomarkers of exposure

-Compound discrimination and quantification

-**Bioavailability**

The company- *what we do.*

EcoArray Inc. is a company that manufactures gene chips and provides support and services related to these products.

Our products and services are specifically tailored to the toxicology field.

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Existing technologies that can measure compounds

Technology	Limitations
• Water/sediment chemistry tests	• Fail to report on what is happening in an animal.
• *In vitro* assays (ie. YES assay).	• Can not report on metabolites of compounds.
• Whole animal bioassays.	• Insensitive endpoints.

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• *In vitro* assays (ie. YES assay).	• Can not report on metabolites of compounds.
• Whole animal bioassays.	• Insensitive endpoints.

… gene chips overcome these limitations.

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What are gene chips?

Small glass slides or membranes that contain genetic material.

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How gene chips work- *an overview*

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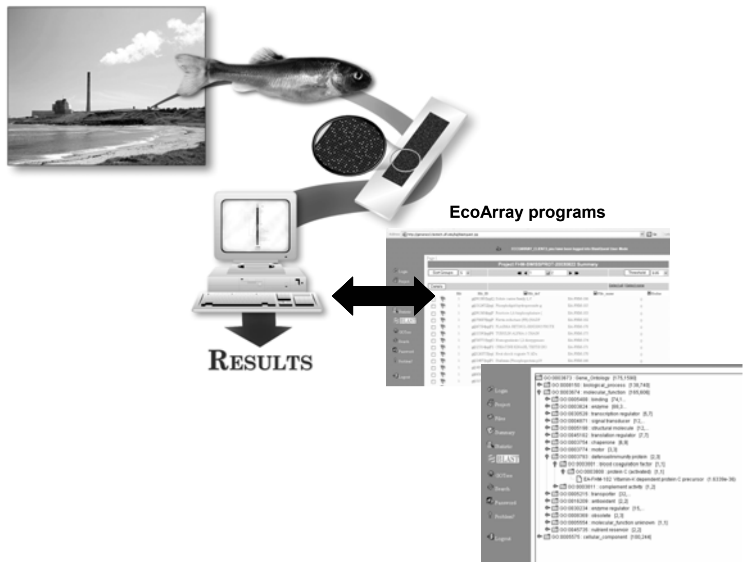
How gene chips work- *an overview*



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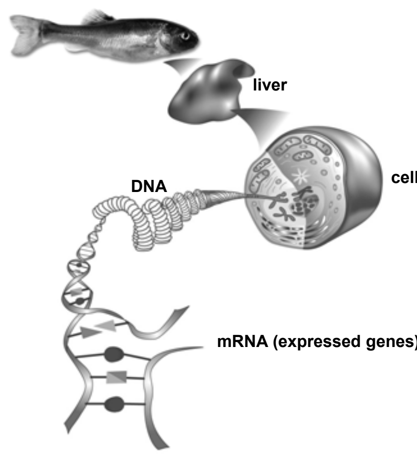
How gene chips work
-In more detail…



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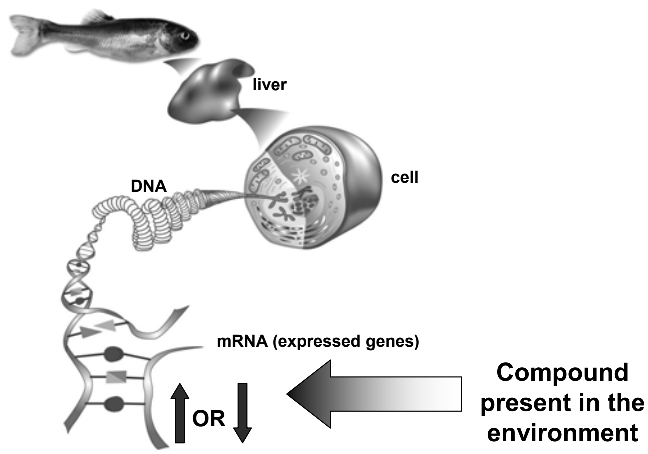
How gene chips work
-In more detail…



Very sensitive tests!!!

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Measure mRNA using gene chips

- Thousands of genes can be spotted onto chips
- mRNA changes can be quantitated
- Changes in mRNA can be used for the early detection of compounds BEFORE adverse effects are observed in animals.

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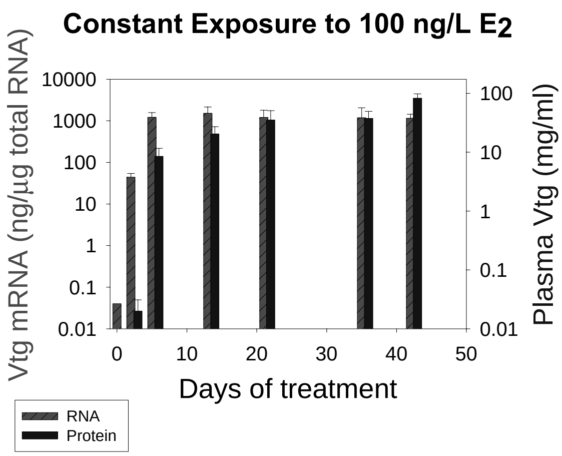
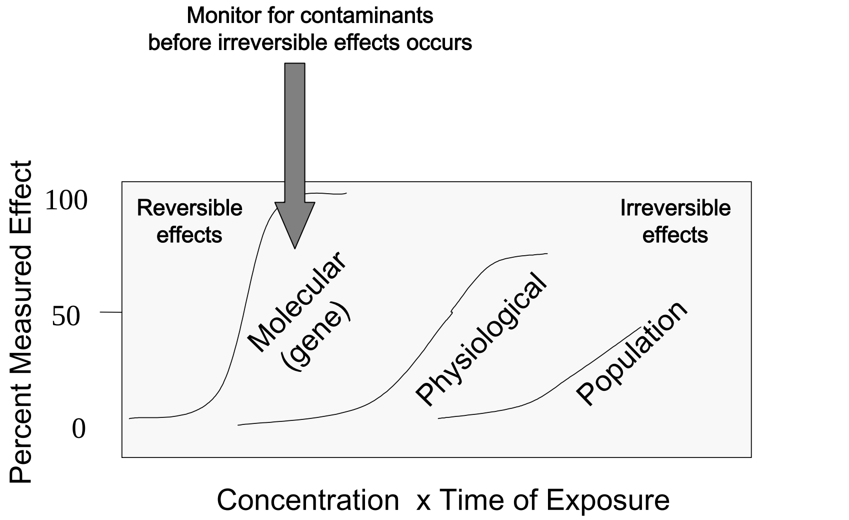
Early detection



Denslow et al.

Early detection



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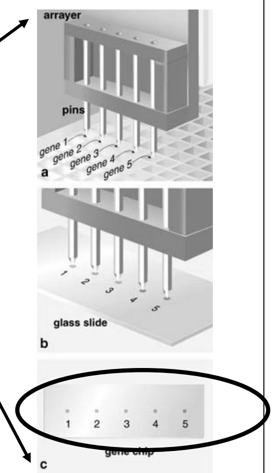
How gene chips are made

Robotics



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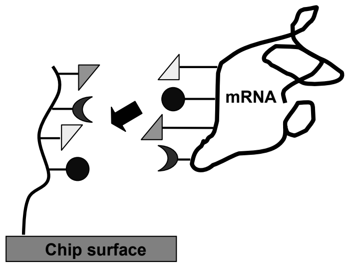
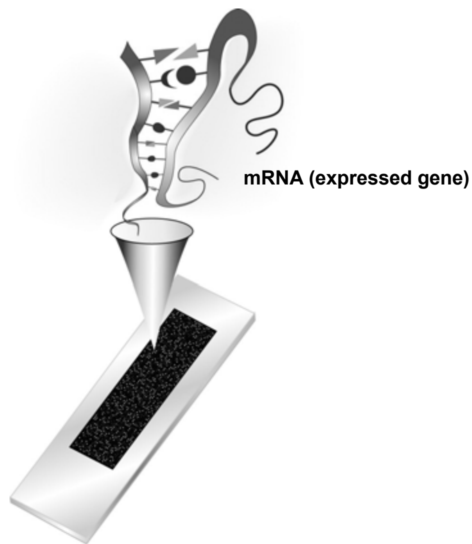
How gene chips work
-In more detail…





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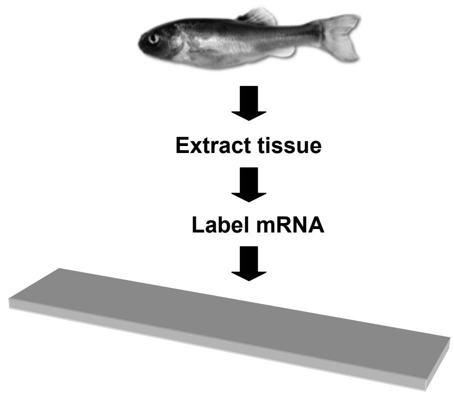
How gene chips are used



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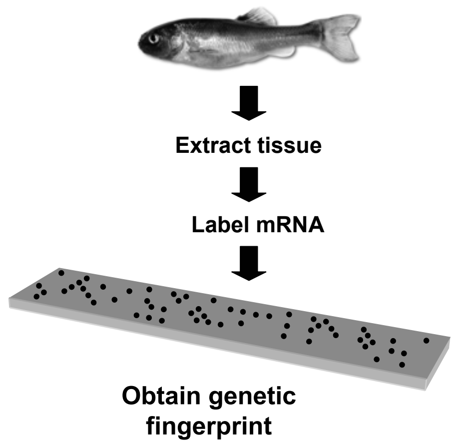
How gene chips are used



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Model species

Fathead minnow freshwater species that is commonly used for toxicology testing.

***Example*: Biomarkers for exposure**

Sheepshead minnow estuarine species that is commonly used for toxicology testing.

***Example*: Compound discrimination and quantification**

Largemouth bass important game fish found throughout much of the United States.

***Example*: Bioavailability**

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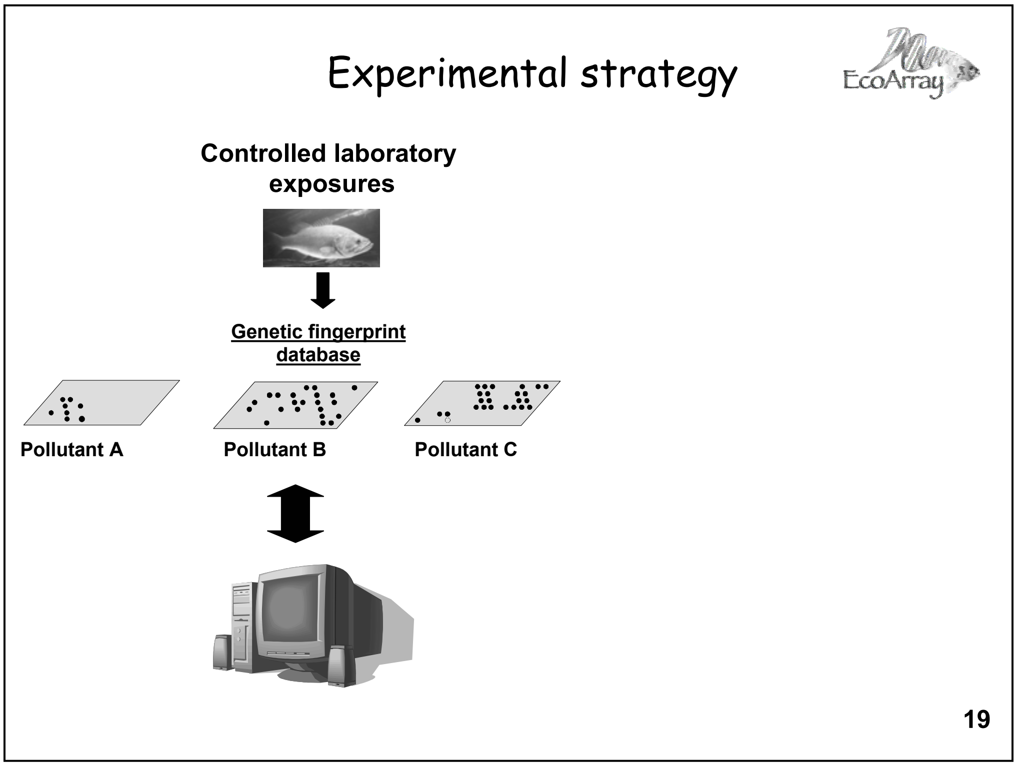
Experimental strategy
EcoArray
Controlled laboratory exposures
Genetic fingerprint database
Pollutant A
Pollutant B
Pollutant C
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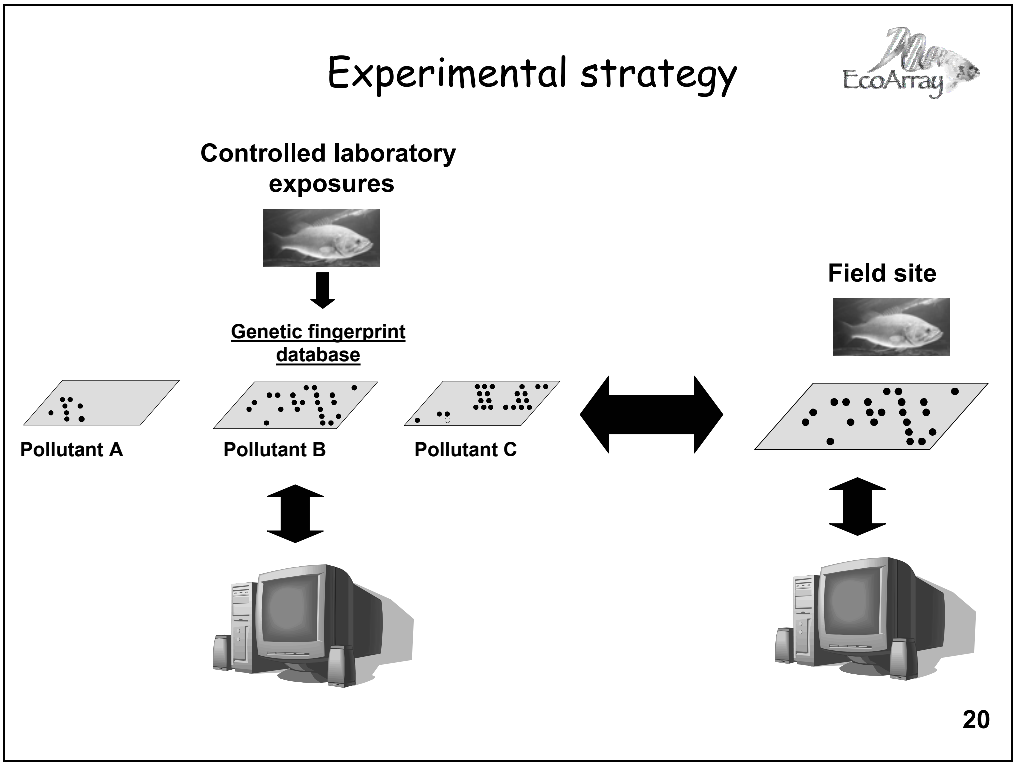
Experimental strategy
EcoArray
Controlled laboratory exposures
Genetic fingerprint database
Pollutant A
Pollutant B
Pollutant C
Field site
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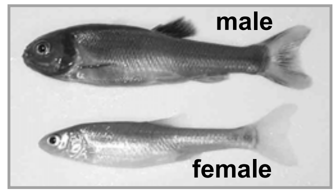
Fathead minnow



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FHMinnow chip

- 200 gene chip.
- Genes obtained from a variety of methods (cDNA libraries, directed cloning, and subtraction libraries).
- Genes are parts of multiple pathways.

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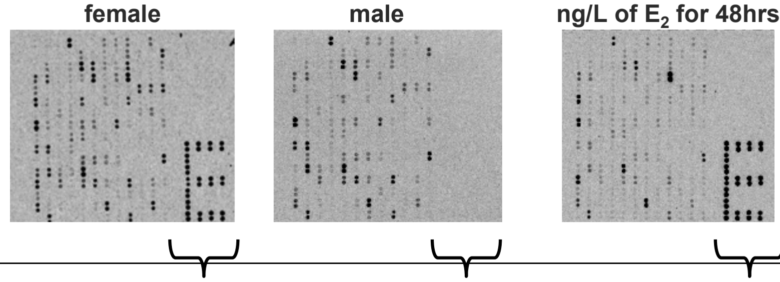
Fathead minnow experiments

EcoArray

FHMChips®

Un-exposed female	Un-exposed male	Male exposed to 400 ng/L of E_2 for 48hrs



Genetic biomarker for estrogens

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Fathead minnow experiments

We are developing a **2000+** oligonucleotide based gene chip in fathead minnows with the EPA.

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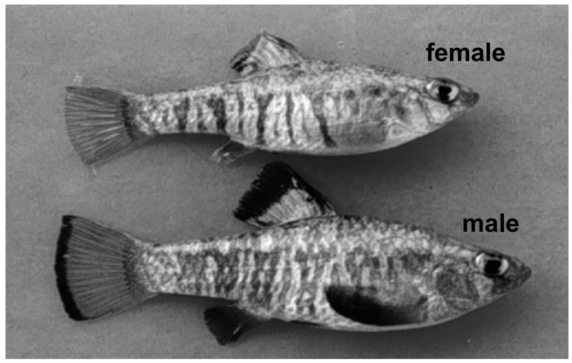
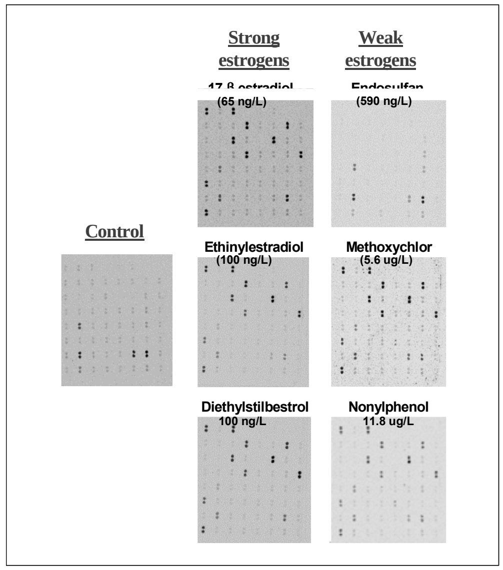
Sheepshead minnow



Compound discrimination

-SHMs exposed to several estrogenic compounds



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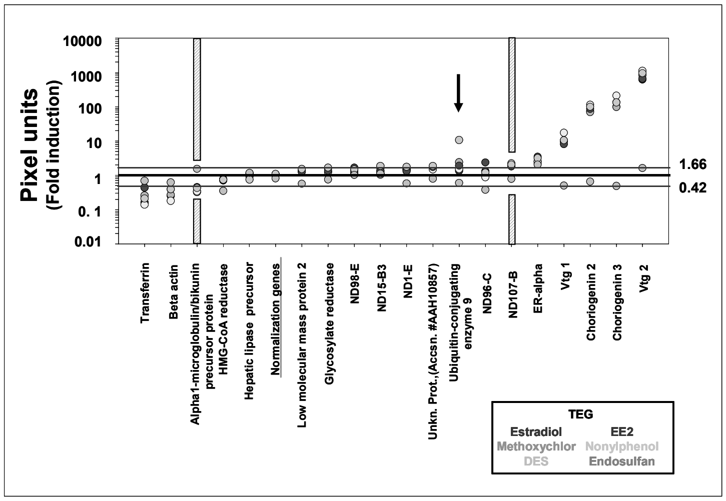
Compound discrimination

EcoArray



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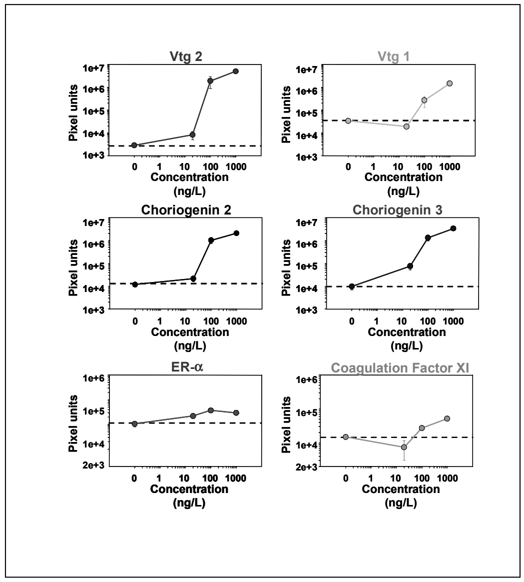
Larkin et al, EHP 2003

Quantification

-SHMs exposed to several different doses of EE2



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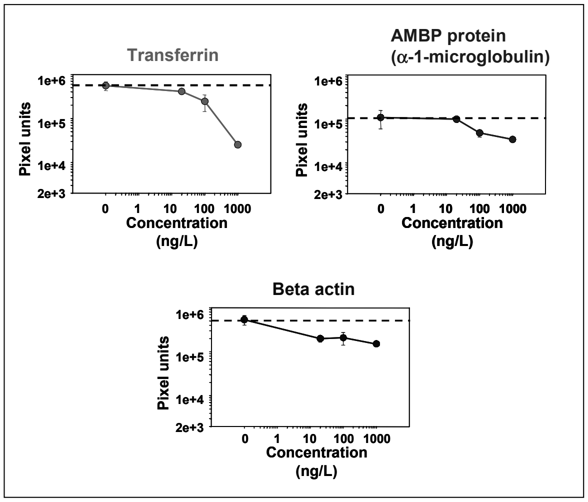
Larkin et al, EHP 2003

Quantification



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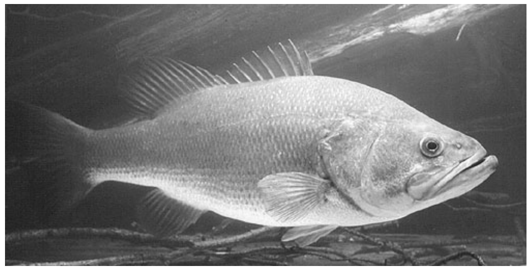
Larkin et al, EHP 2003

Largemouth bass model



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Area 7 field site

<u>OCPs levels in the muscles of fish :</u>

Chlordane
Dieldrin
p’p’-DDD
p’p’-DDE
p’p’-DDT
toxaphene

LMBass gene chip

- 500 gene chip.
- Genes obtained from a variety of methods (cDNA libraries, directed cloning, differential display, and subtraction libraries).
- Genes are parts of multiple pathways.

Largemouth bass model

Laboratory exposure

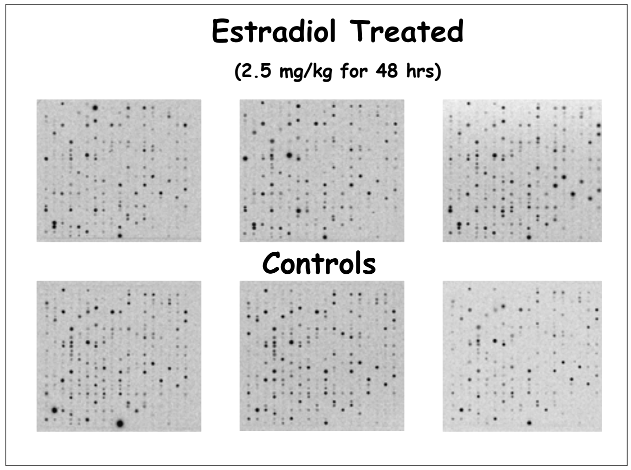
- **17-β estradiol exposure (2.5 mg/kg inject, 48 hrs).**
- **p'p-DDE (100 mg/kg inject, 48 hrs).**
- **11-ketotestosterone (2 mg/kg inject, 48 hrs).**
- **Characterize reproductive cycle**

Field analysis

- **Site of investigation**

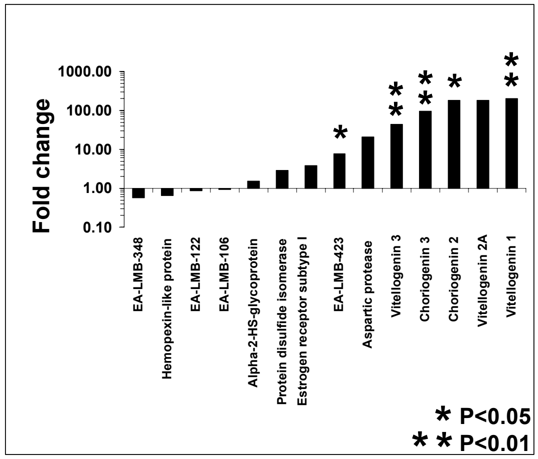
`

Estradiol laboratory exposures



Larkin et al, CBP 2002

Estradiol laboratory exposures



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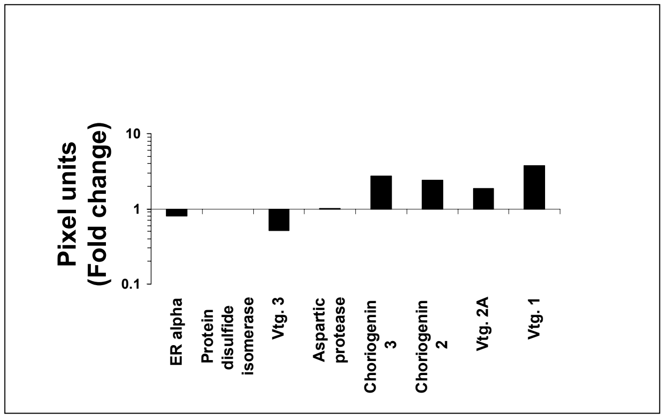
Larkin et al, CBP 2002

DDE laboratory exposures



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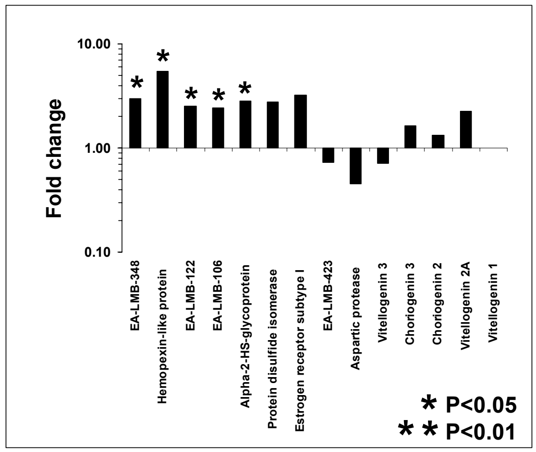
Larkin et al, CBP 2002

11KT laboratory exposures



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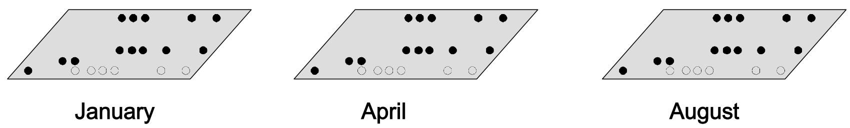
Bass reproductive cycle

Need to define “normal” fingerprint pattern in bass before one can identify atypical gene expression patterns in the field



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Bass reproductive cycle

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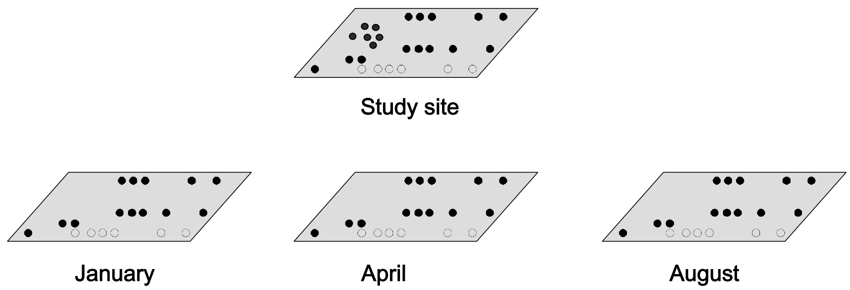
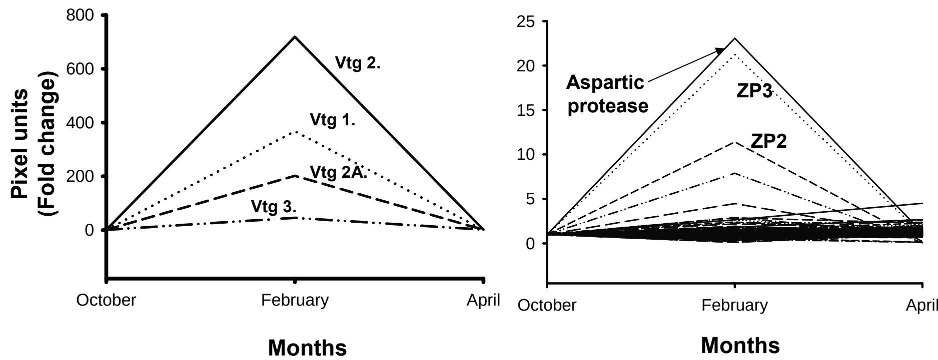


Bass reproductive cycle (females)



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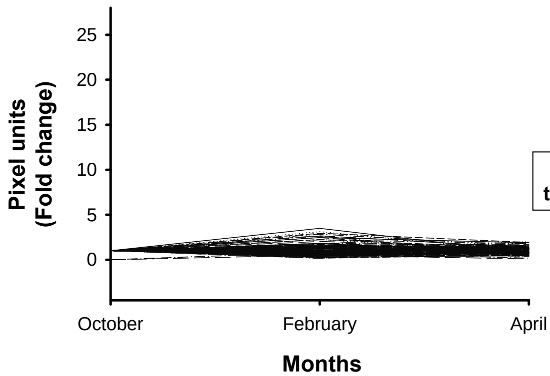
Bass reproductive cycle (males)



No significant change in the vtgs and choriogenins.

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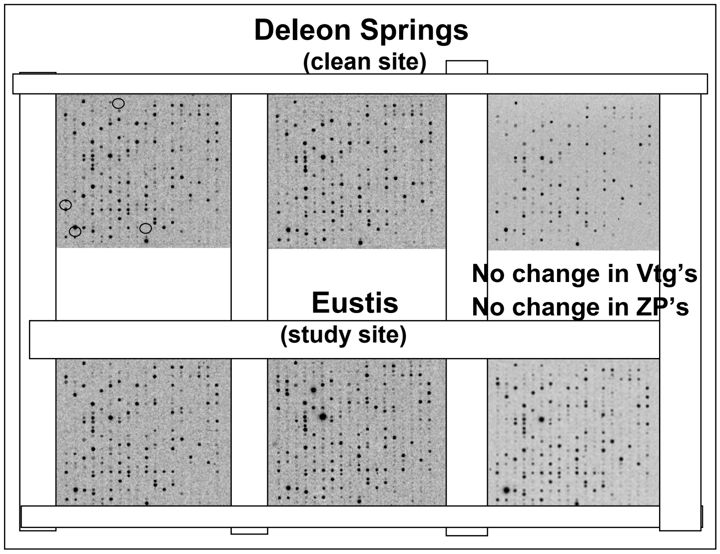
Field analysis



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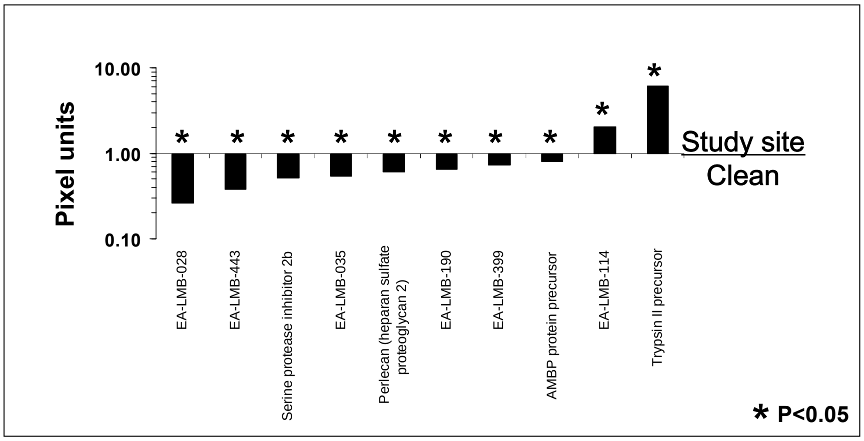
Field analysis



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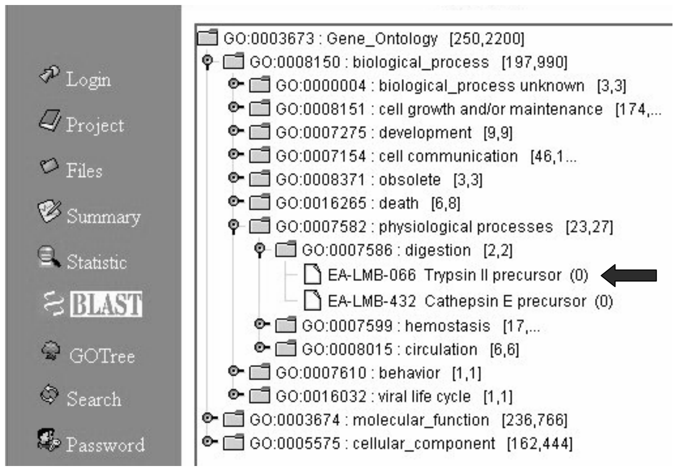
Gene ontology database



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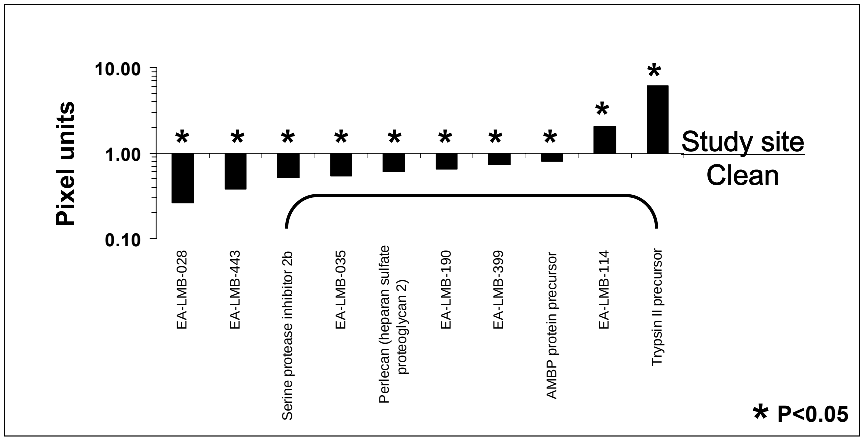
Field analysis



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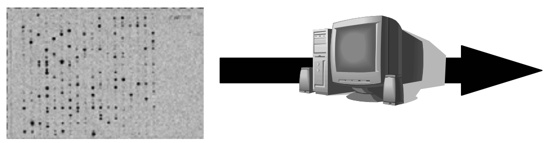
Field analysis



Digestive pathways may be affected in these animals

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Summary

-Gene chips can be used for biomarkers for exposure.

-Gene chips can be used to discriminate between compounds and can provide quantifiable data.

-Gene chips can provide information on bioavailability of compounds.

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Acknowledgements

EcoArray Inc
Barbara Carter

Fish and Wildlife Conservation Commision, Eustis FL
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Li Liu
Anuj Sahni

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SBRP (P42 ES 07375)

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Additional information

See www.ecoarray.com

Our website contains links to manuscripts using this technology and other information

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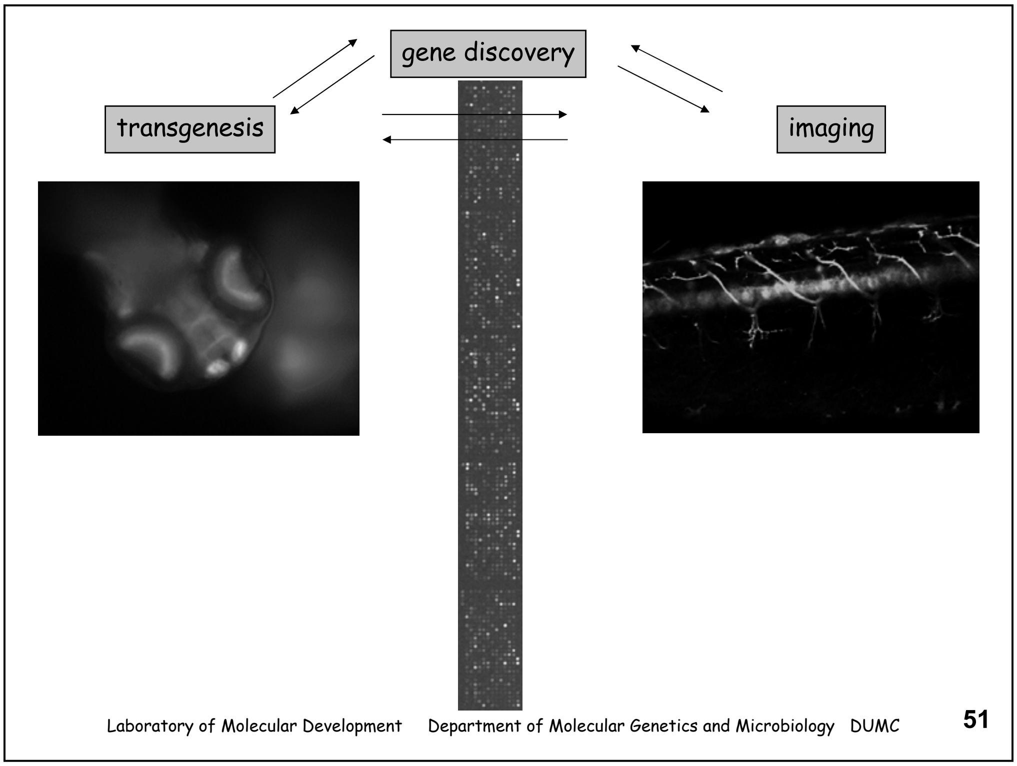
Biosensing with Zebrafish

Elwood Linney, Ph. D.
Molecular Genetics and Microbiology
Duke University Medical Center


gene discovery
transgenesis
imaging
Laboratory of Molecular Development Department of Molecular Genetics and Microbiology DUMC
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Assumptions we make:

1) toxicants are impacting upon normal, existing pathways

2) there can be a differential sensitivity to a toxicant depending upon whether the organism or target organ is developing or fully formed

3) there are common pathways in different organisms

4) differences between organisms should be represented by "differences" in their genomes

Our changing view of "biosensors"

1) transgenic indicator mice for snapshots of "retinoic acid activity"

2) transgenic, fluorescent zebrafish for live 4-D studies of activity

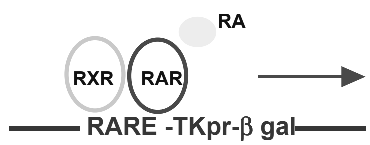
3) using zebrafish themselves as indicators or sensors for toxicant events

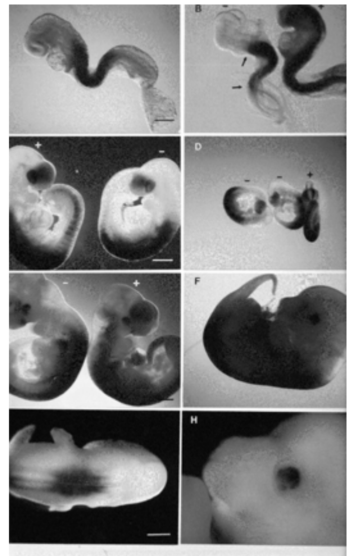
4) using discovery microarray analysis for identifying genes affected

→ Use results to design new biosensor transgenics

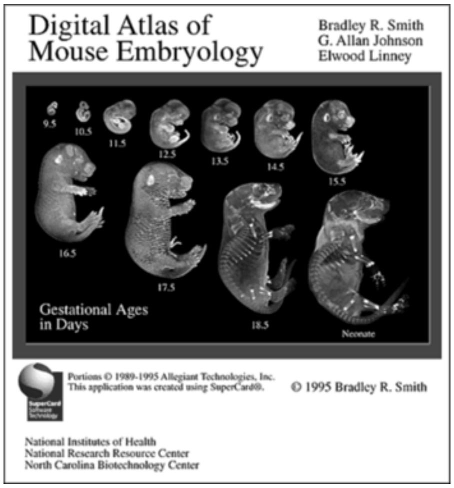
Reporter transgenic mice using constructed retinoic acid responsive promoter

Subset of retinoic acid receptor activity in reporter transgenic mouse:





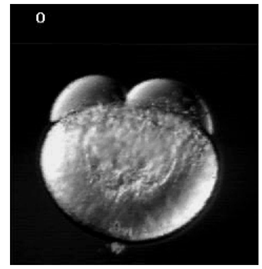
Size relationships, developmental time, changing in size with development:



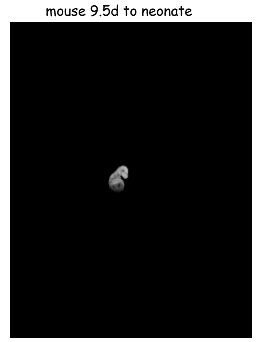
relative size
of embryos

Time-lapse 2 cell to 17 hours

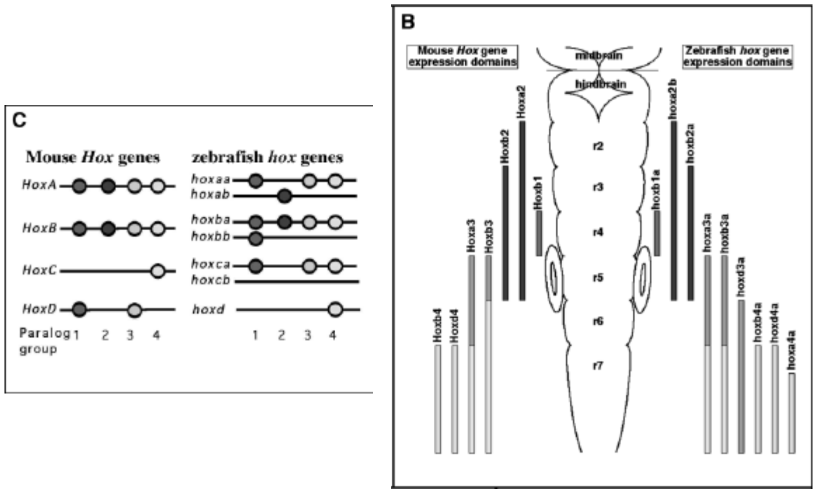
R.Kalstron and D. Kane



mouse 9.5d to neonate



Mouse and zebrafish homeobox genes:



Parallels in axial development between vertebrate species:

Development 121, 333-346 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Hox genes and the evolution of vertebrate axial morphology

Ann C. Burke, Craig E. Nelson, Bruce A. Morgan* and Cliff Tabin

Black bars denote
spinal nerves of
brachial plexus

level of curved line
represents the
level of limb or fin

shaded somites
represent level
of Hoxc-6 expression

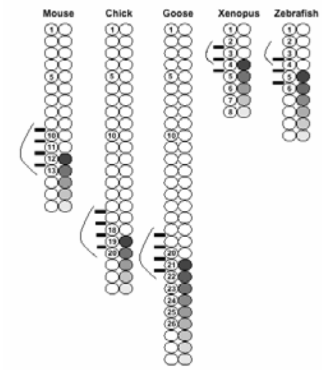


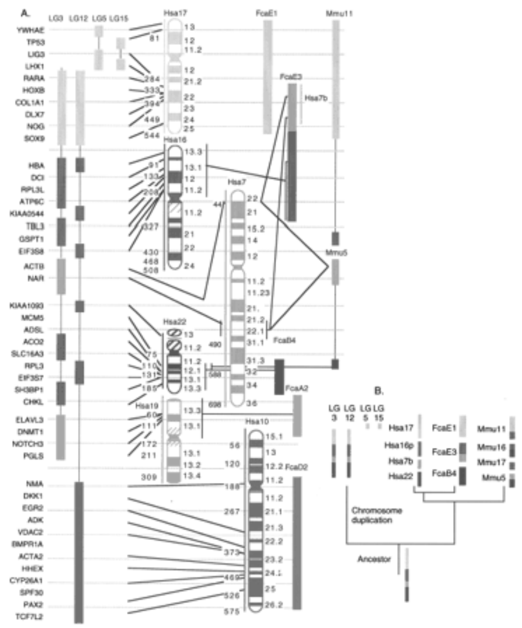
Fig. 11. Schematic representation of the somite levels bridging the cervical-thoracic transition in mouse, chick, goose, *Xenopus*, zebrafish. Black bars represent the spinal nerves of the brachial plexus, and the level of the limb or fin bud is indicated with a curved line. The shaded somite levels indicate the level of *Hoxc-6* expression as determined by whole-mount in situ hybridization, or immunohistochemistry. The level for the zebrafish is taken from Molven et al. (1990).

Syntenic relationships
between vertebrate genomes
--genes inherited as linked clusters during speciation

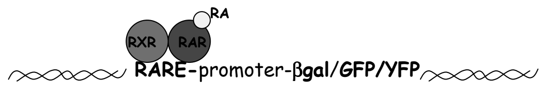
Vol. 10, Issue 12, 1890-1902, December 2000

Zebrafish Comparative Genomics and the Origins of Vertebrate Chromosomes

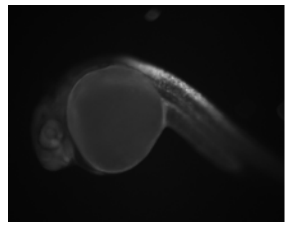
John H. Postlethwait,[1,3] Ian G. Woods,[2] Phuong Ngo-Hazelett,[1] Yi-Lin Yan,[1] Peter D. Kelly,[2] Felicia Chu,[2] Hui Huang,[2] Alicia Hill-Force,[1] and William S. Talbot[2]



Retinoic acid indicator embryos



sizes approximately to scale



24 hr live zebrafish embryo expressing
YFP from RARE zGT2 basal promoter sequence

retinoic acid responsive day 8.5
mouse embryo expressing lacZ
from RARE TKpr sequences

Vitamin A-Retinoid Relationships:

vitamin A(all-trans retinol)

↓ ↑ **retinol dehydrogenase**

retinaldehyde

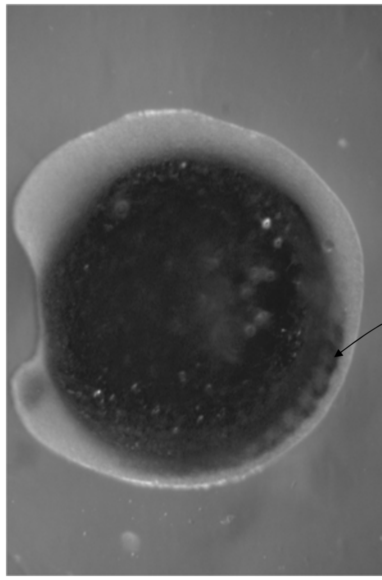
↓ **retinal dehydrogenase(RALDH)** → RA synthesis

retinoic acid [ligand for transcription factors]

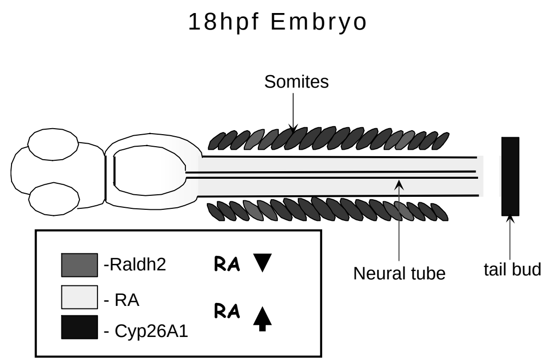
↓ **cytochrome p450RAI(cyp26)** → RA metabolism

4-oxoretinoic acid

Dual in situ hybridization for RALDH2(purple) and cyp26a1(red-orange):



Model of some of the retinoid events occurring in the trunk neural tube:



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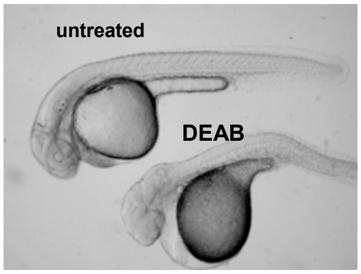
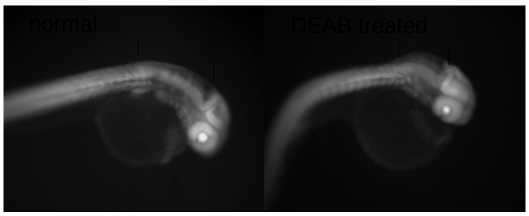
RALDH inhibitor
[our work]



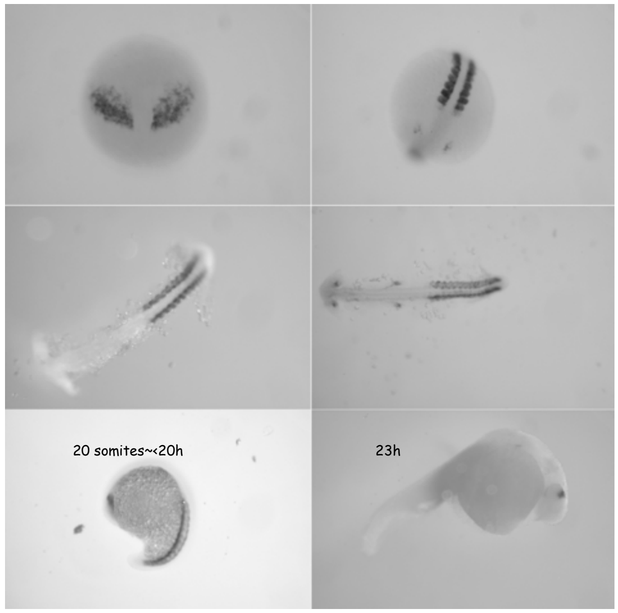


Neckless mutant in RALDH2

Begemann, Schilling, Rauch, Geisler and Ingham

Either chemical inhibition of Raldh's in zebrafish with DEAB or an isolated Raldh2 zebrafish mutant produced phenotypes paralleling the mouse Raldh2 knockout phenotypes:
- hindbrain changes
- forelimb or fin inhibition

In situs of zebrafish Raldh2 at different developmental stages[Kari Yacisin]:

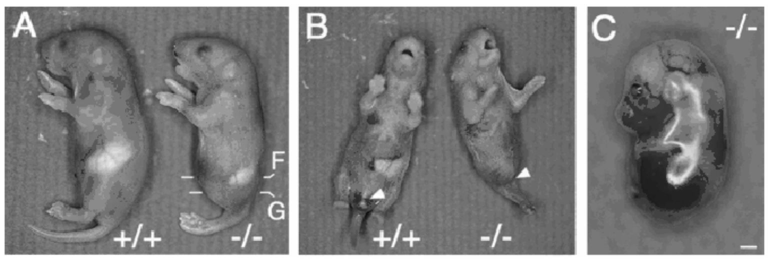


Point:
apparent turn-off of RALDH2 as neural tube ceases to grow

Two mouse KO studies of cyp26A1 revealed caudal truncations and occasional exencephaly

cyp26 knockout mice Abu-Abed, Dolle, Metzger Beckett, Chambon and Petkovich
[somewhat similar phenotypes from these authors]

cyp 26 knockout mice Sakai, Meno, Fujii, Nishino, Shiratori, Saijoh, Rossant, and Hamada



Summary:

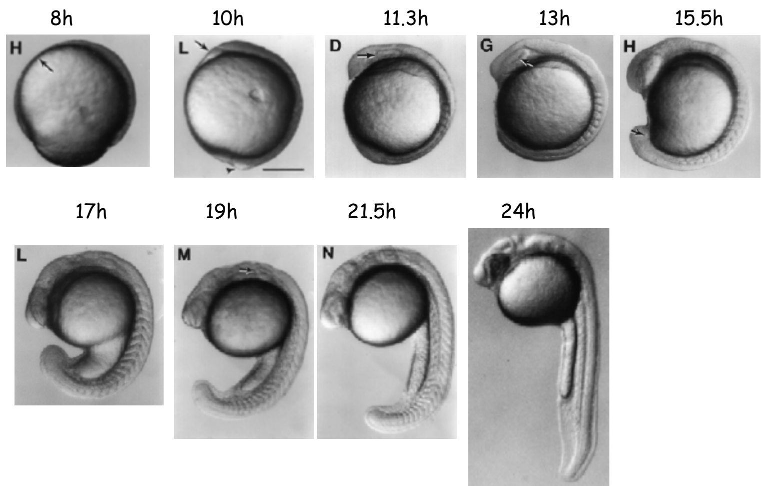
1)Raldh2 and cyp26a1(and cyp26b1) can be found adjacent to each other in the developing embryo creating functional "microgradients" of RA ligand for RAR activity

2)expression patterns and available mutants for these genes in mouse and zebrafish show consider homology

3)in zebrafish the Raldh2 promoter is directly repressed by RA and the cyp26a1 promoter is directly induced by RA

4)this system is being studied to determine whether there might be a genetic and/or environmental basis for neural tube defects

Developmental changes flanking and including neural tube growth which we are analysing with 8h, 10h, 12h, 14h, 16h, 18h, 20h, 22h, 24h microarray analysis:



Progression of microarray development:

1) oligos from 500 selected zebrafish genes

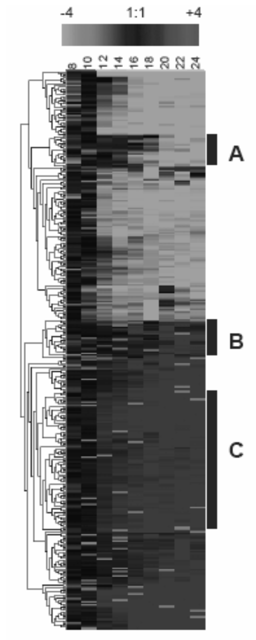
2)16k oligomer library from Compugen arrayed

3)now examining 22k zebrafish oligomer array produced by Agilent, bioinformatics through Paradigm

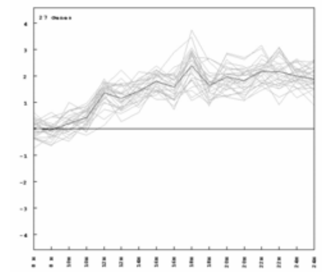
Affymetrix has produced zebrafish arrays but we have yet to use them

Genes regulated during segmentation

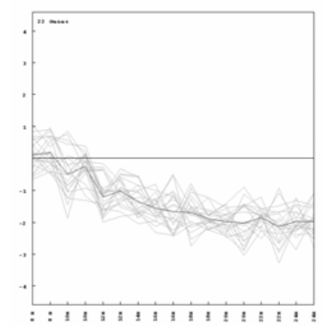
[work done in collaboration with R. Malek at TIGR]



expression
log base 2



upregulated
at 12 hpf



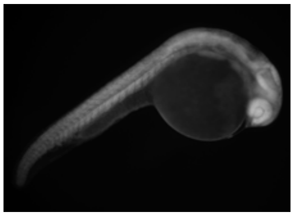
downregulated
at 12hpf

Some elements of our zebrafish toolbox:

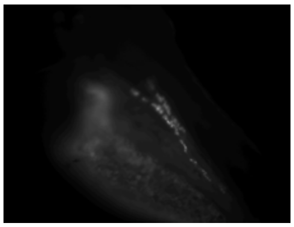
1) live, fluorescent, transgenic embryos

2) anti-sense morpholino knockdown of gene expression

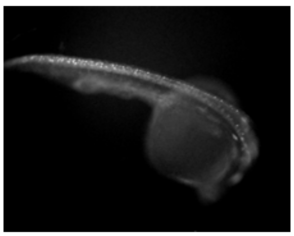
Some transgenic lines we made:



GFP off constitutive promoter



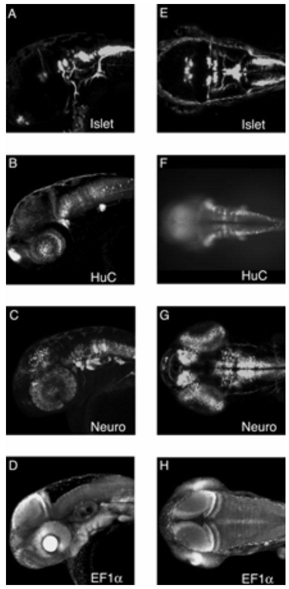
GFP off artificial construct--lights up cells migrating along pronephric ducts

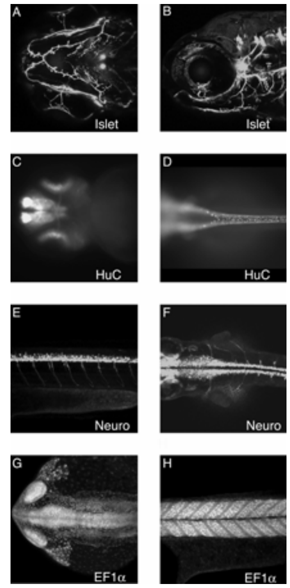


YFP of zHuC promoter that lights up developing nervous system

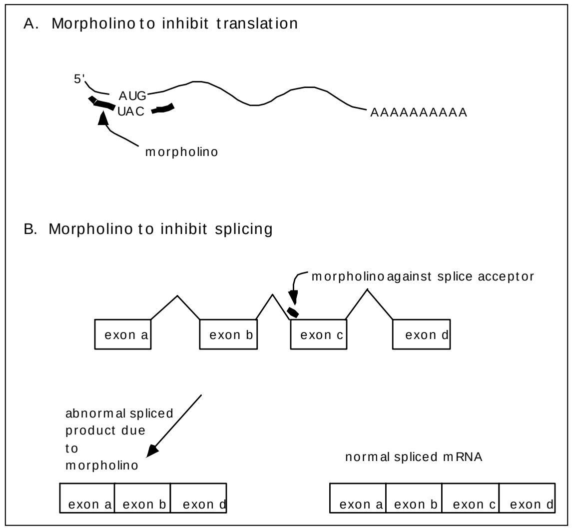
71

Four transgenic lines for examining nervous system:

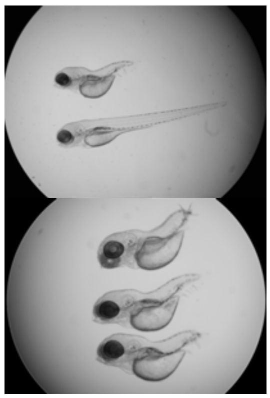


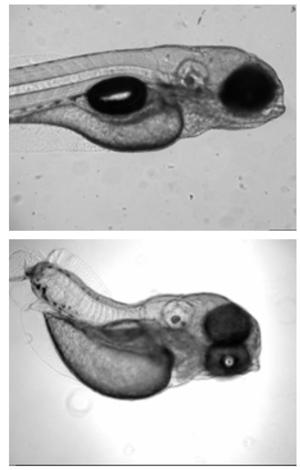


Antisense morpholino approaches use by zebrafish researchers to "knockdown" genes



no-tail (T-allele) morpholino we injected into a 1-cell zebrafish embryo--these are 4 day larvae after hatching--the phenotype is what is seen with real mutants in no-tail





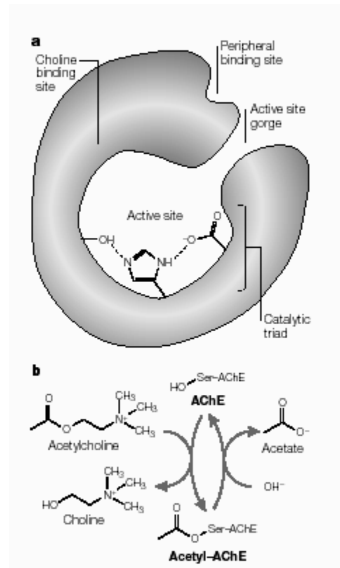
Chlorpyrifos studies

Acetycholinesterase

classical function of hydrolysing the neurotransmitter, acetylcholine

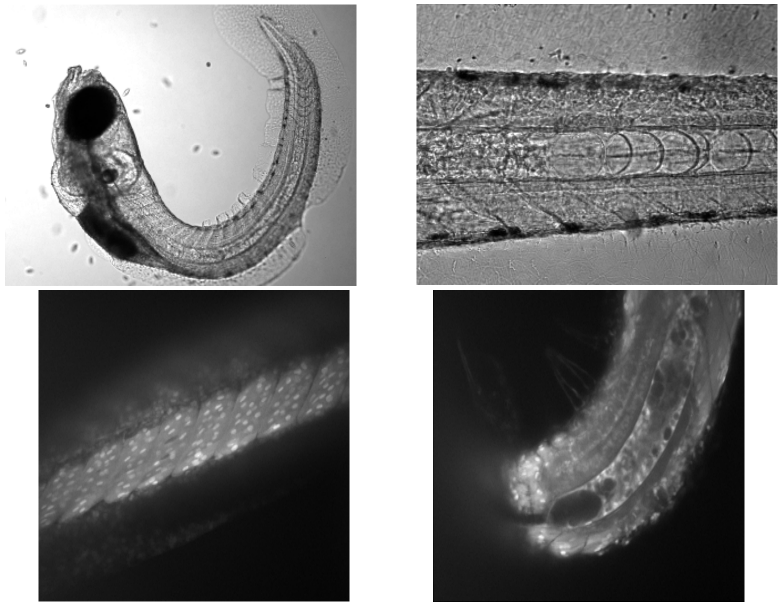
mice have AChE plus a butyrylcholinesterase so mouse KOs in AChE allow animals to at least be born and live ~21 days

zebrafish only has AChE, so AChE-/- embryos show defects in muscle fiber formation, innervation, and primary sensory neurons die prematurely--embryos are initially motile and then develop paralysis and die



from Soreq and Seidman, Nature Reviews Neuroscience(2001)

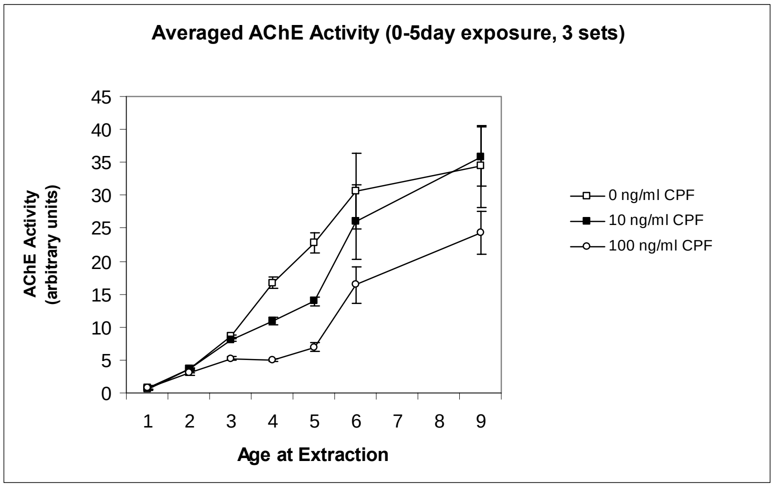
Chlorpyrifos treating zebrafish embryos--our work, high dose(500ng/ml):



Experimental Plan:

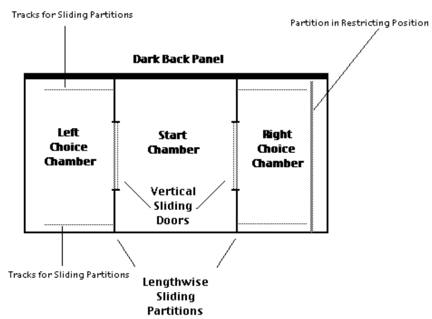
1) expose embryos for 5 days with chlorpyrifos
2) adult learning studies in E. Levin's lab
3) acetylcholine esterase assays during embryogenesis
4) AChE morpholino titration to CPF inhibition studies
5) adult learning studies and microarray analysis

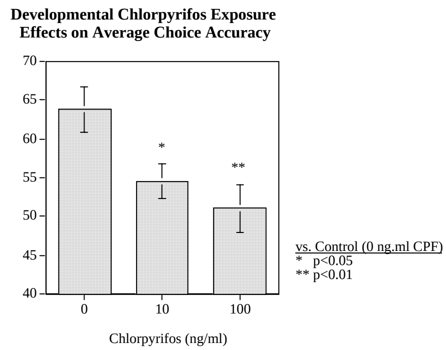
Acetylcholine esterase activity/chlorpyrifos exposure:



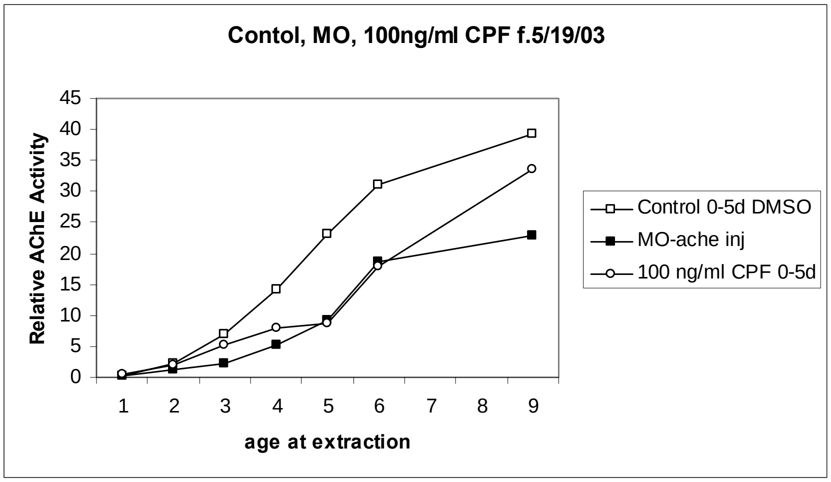
Collaborative work with E. Levin and E. Chrysanthis:

As part of our Superfund program we chlorpyrifos
treated embryos for 5 days, released them and grew them up and they tested
for learning in maze designed by E. Levin in our Psychiatry department:



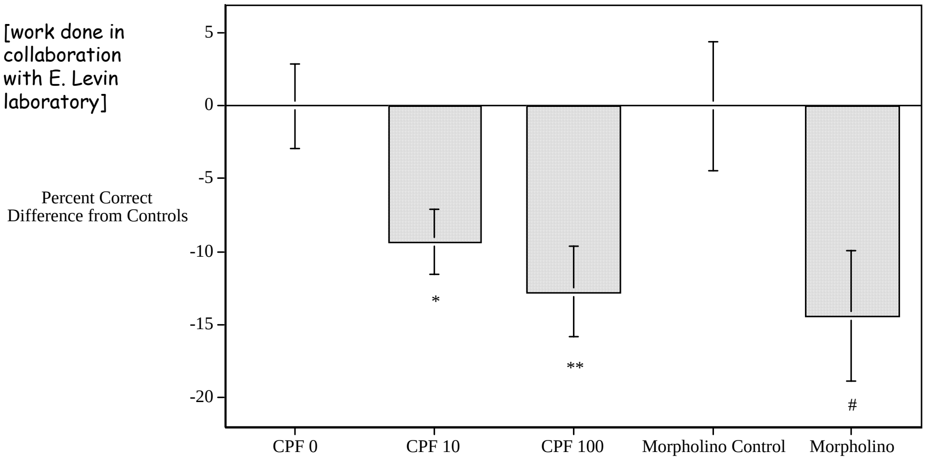


Targeting acetylcholine esterase with a morpholino:



Delayed Spatial Alternation Choice Accuracy with Developmental Chlorpyrifos or Morphoino Zebrafish

[work done in collaboration with E. Levin laboratory]



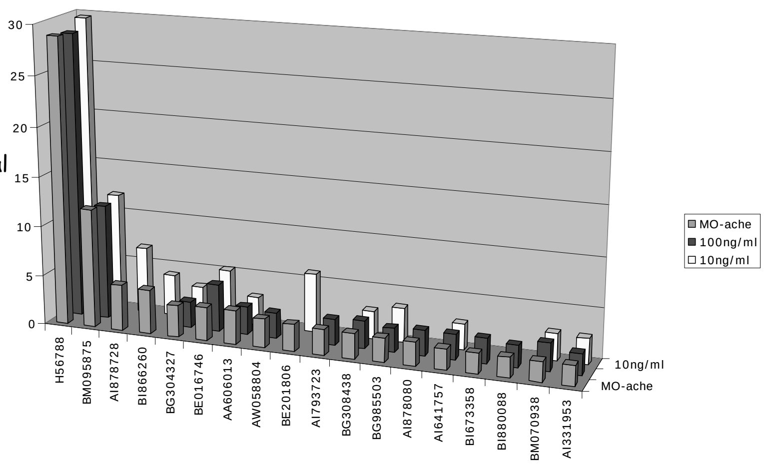
vs. CPF 0
* p<0.05
** p<0.01

vs. Morpholino Control
# p<0.025

Preliminary comparison of expression of MO-AChE, 100ng/ml CPF 10ng/ml CPF versus control using filter of 2x or above expression:

[3 day treated and untreated embryos]

expression of experimental divided by control



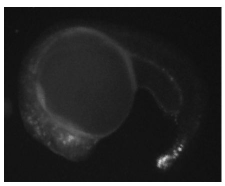
each bar individual gene (15 out of 37 genes overlap of MO vs. 100ng/ml CPF)

Future--Biosensors:

1) the generation of a series of responsive transgenics to small molecules (in progress, estrogen inducibility)

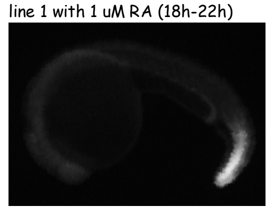
2) the use of the Sanger Centre zebrafish DNA assembly to identify clones for genes which show distinct responsiveness to environmental toxicants so that transgenics can be derived from their regulatory sequences

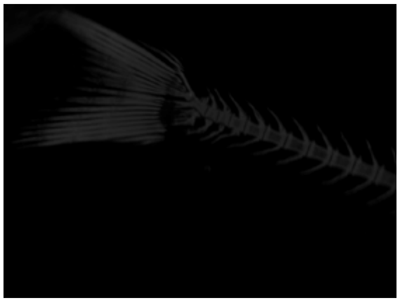
3) the analysis of the 22k array data to formulate potential pathways that toxicants are impacting upon



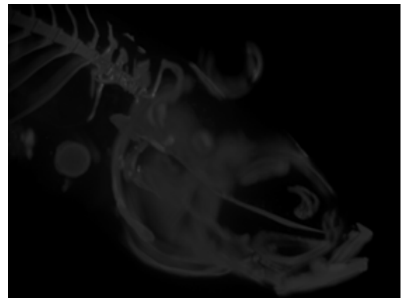
our zCyp26A1pr
transgenics with
RA inducible promoter

line 1 with 1 uM RA (18h-22h)





Collaborators:
Ed Levin
Elizabeth Chrysanthis
Renae Malek(TIGR)
Brad Smith(MRM)
G.A. Johnson(MRM)



Lab individuals involved in

our CPF work:	Neural tube work:
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Lucia Upchurch	Betsy Dobbs-McAuliffe
Stephen Huang	Margaret Lai

past members
Kari Yacisin
Keenan O'Leary
Qingshun Zhao

Research support from:
NIEHS Superfund
and Toxicogenomics
Consortium, NICHD

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