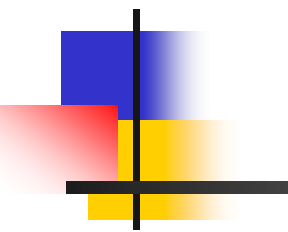
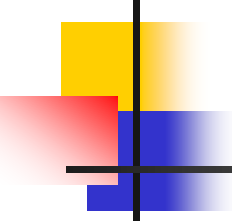


Bioanalytical methods to detect contaminants of emerging concern



Nancy Denslow
Center for Environmental and Human
Toxicology
University of Florida

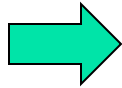


Outline

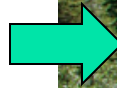
- Introduction
- High throughput assays
- In vivo models → systems biology
- Fathead minnow story
- Mosquitofish story
- Conclusions

Sources of CECs

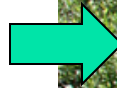
Septic Tanks



Groundwater



Runoff
(Urban, Agriculture)



Industrial
Discharge

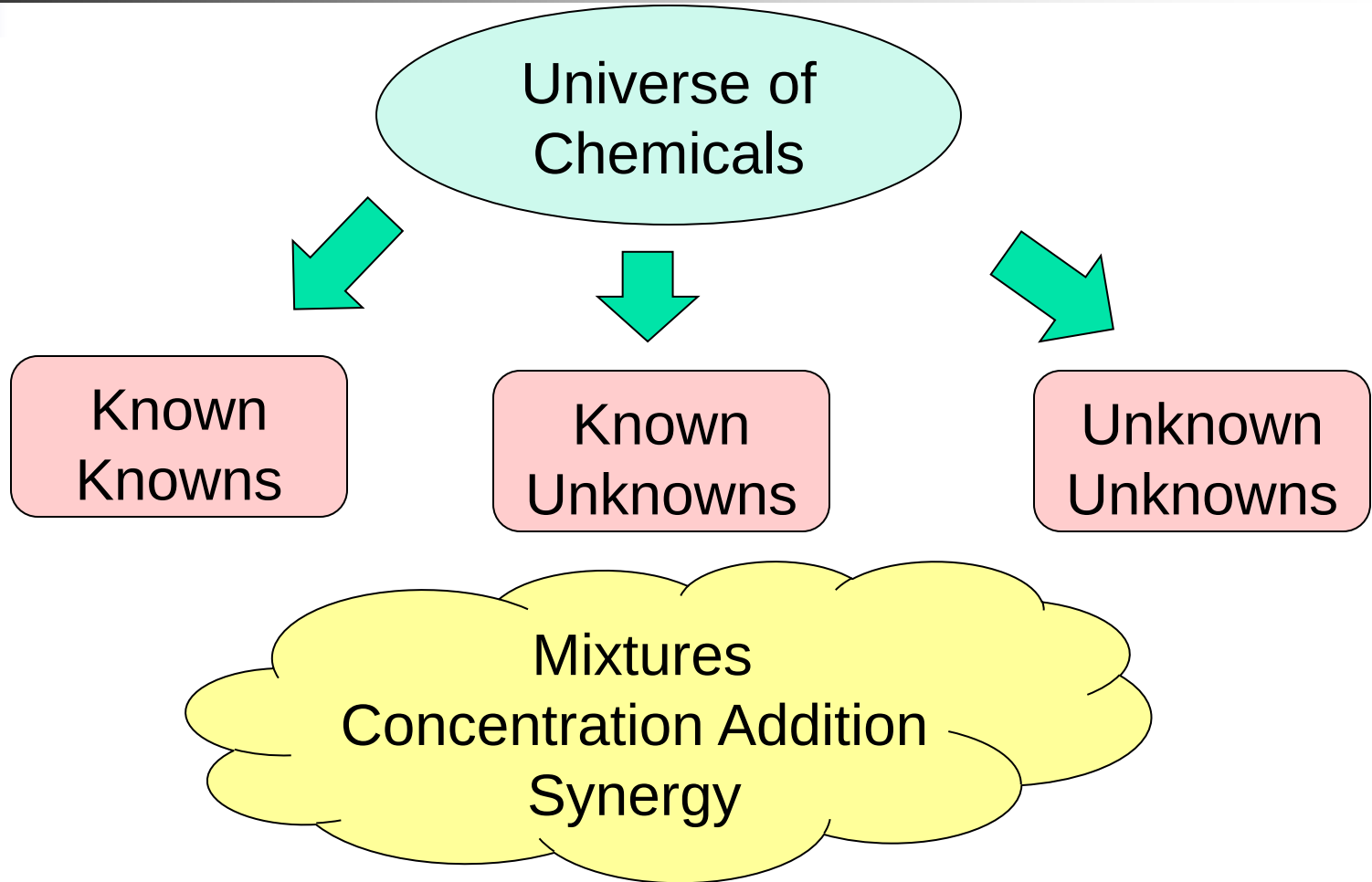
Sewage treatment
Plants



Atmospheric Disposition



Contaminants of emerging concern

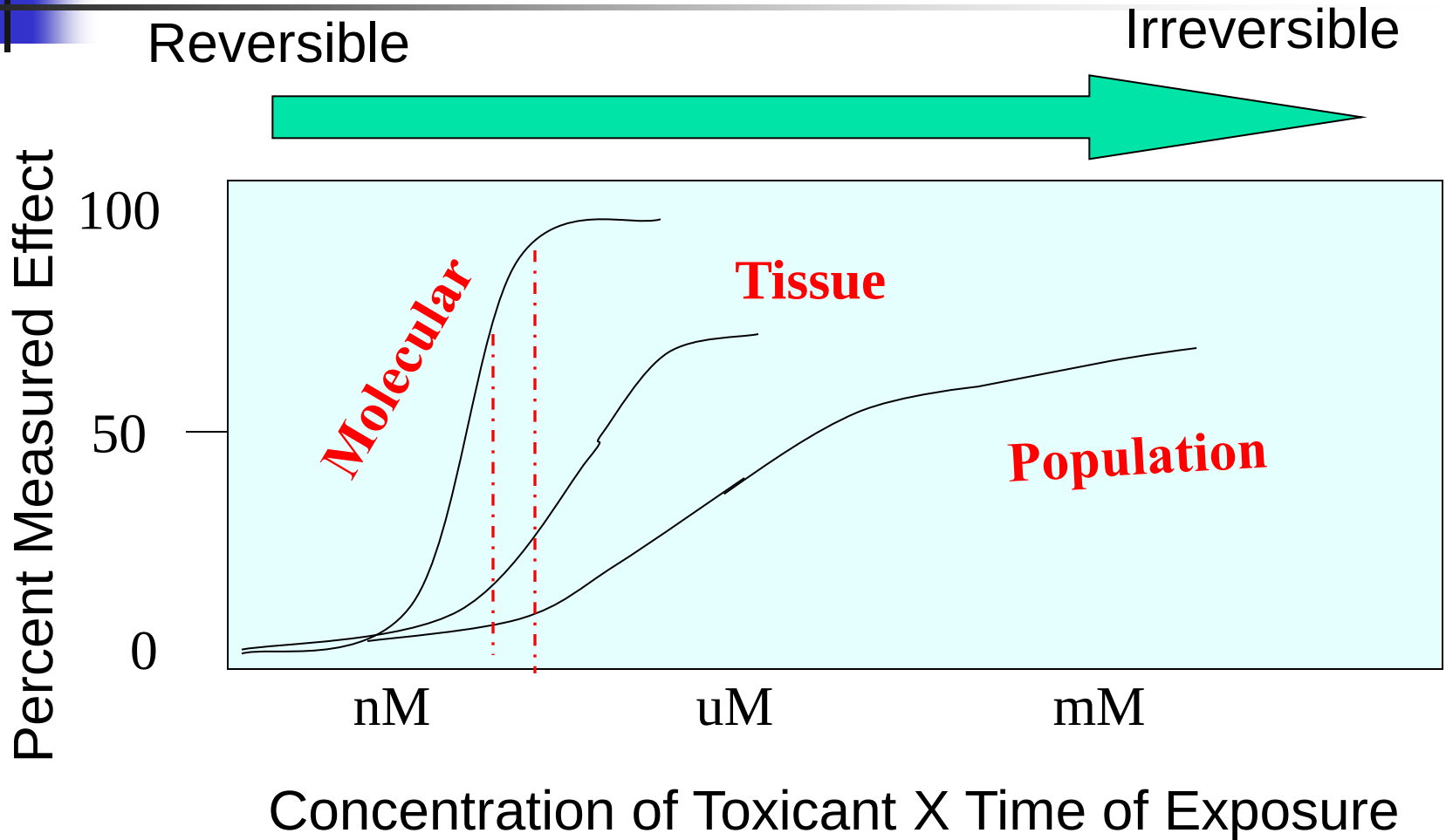


Chemicals Recommended for Monitoring in CA Receiving Waters

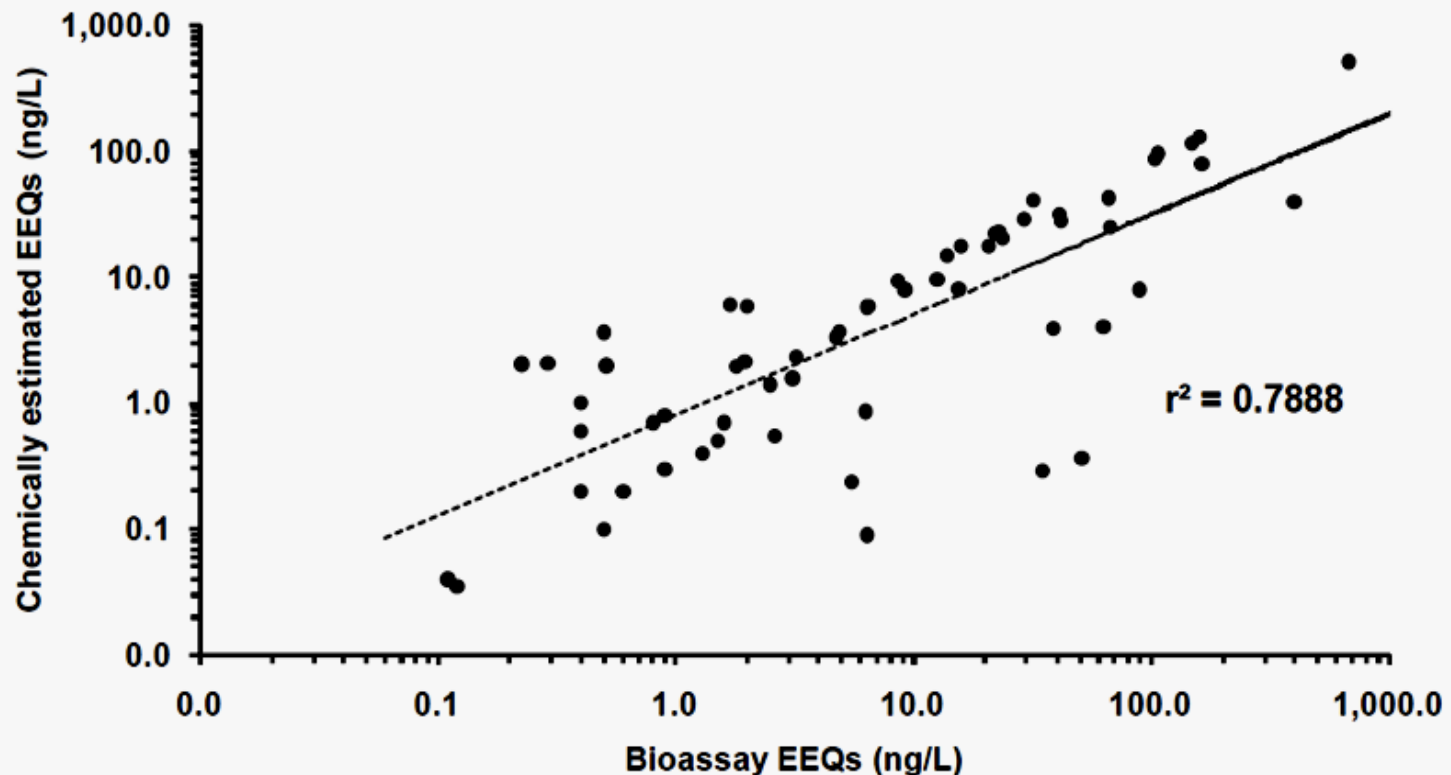
CEC Science Advisory Panel

Compound	Scenario 1 Inland Waters Aqueous	Scenario 2 Embayment Aqueous	WWTP Effluent	FW Stream - Storm- water (Aqueous and Sediment)	Scenario 2 Embayment Sediment	Scenario 3 Marine Sediment	All Scenarios Tissue
Bis(2-ethylhexyl) phthalate	NA	NA	M-O	NA	NA	M	NA
Bisphenol A	M	M	M-E/F	M	NA	NA	NA
Bifenthrin	M	M	M-E/F	M	M	NA	NA
Butylbenzyl phthalate	NA	NA	M-O	NA	NA	M	NA
Permethrin	M	M	M-E/F	M	M	NA	NA
Chlorpyrifos	M	M	M-E/F	M	NA	NA	NA
Estrone	M	M	M-E/F	M	NA	NA	NA
Ibuprofen	M	NA	M-F	M	NA	NA	NA
17-beta estradiol	M	M	M-E/F	M	NA	NA	NA
Galaxolide (HHCB)	M	M	M-E/F	M	NA	NA	NA
Diclofenac	M	NA	M-F	M	NA	NA	NA
p-Nonylphenol	NA	NA	M-O	NA	NA	M	NA
PBDE -47 and 99	NA	NA	M- E/F/O	M	M	M	M
PFOS	NA	NA	M- E/F/O	M	M	M	M
Triclosan	M	NA	M-F	M	NA	NA	NA

Biomarker sensitivity



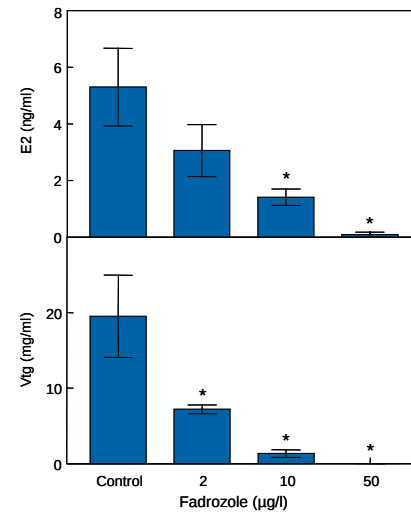
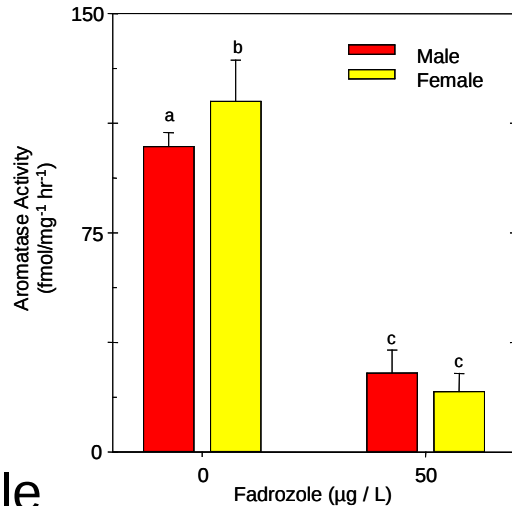
Estrogen equivalency



Correlation between chemically estimated EEQs and bioassay EEQs (Bulloch *et al.*, 2010).

Linkage of biomarker to population level effects

Testosterone → estrogen

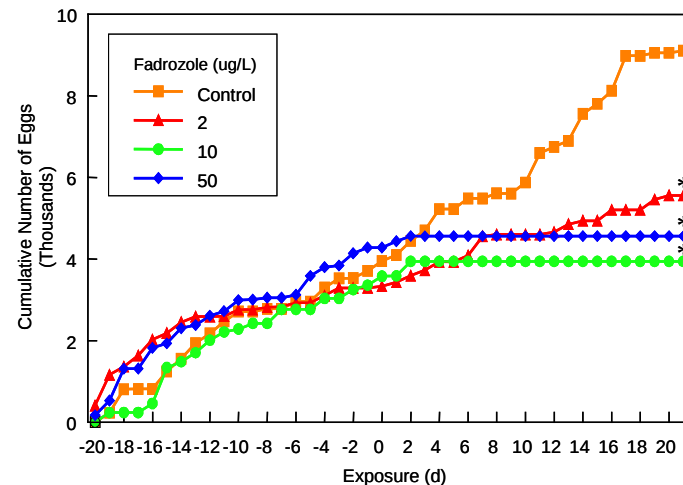
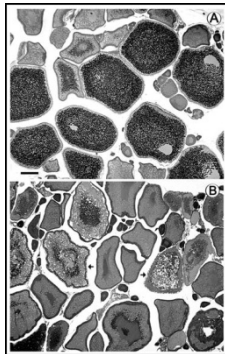


Plasma E2

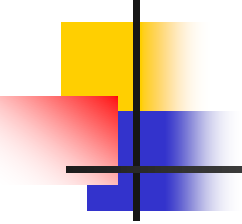
Plasma Vtg

Fadrozole
aromatase

Histology



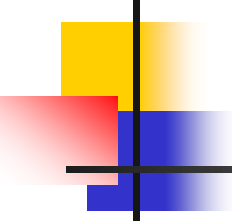
Number
of eggs



Vitellogenin assay: protein

Protein assay

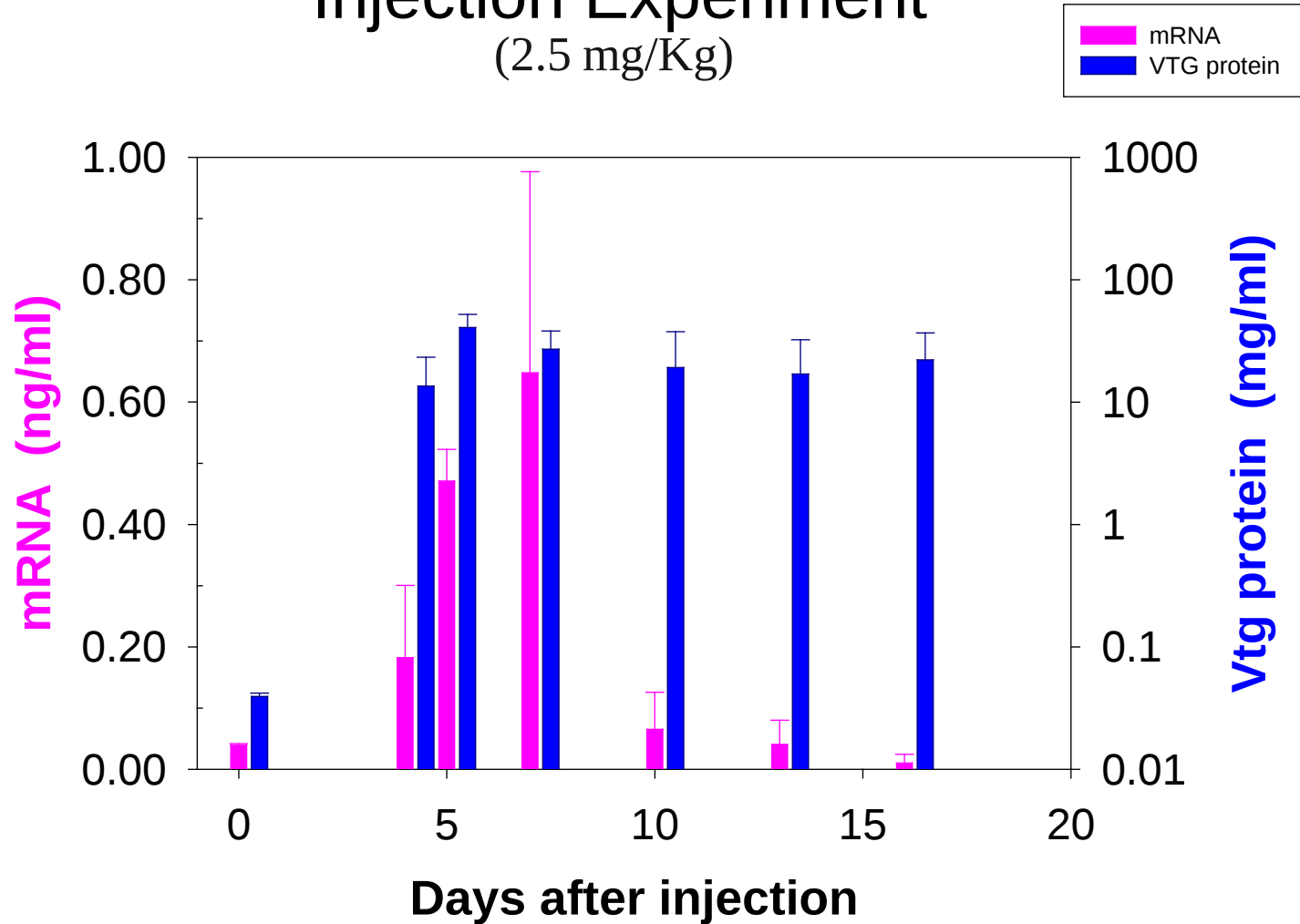
- Monoclonal antibodies that cross-react with most fish species
- Developed against a number of fish Vtg's
- Check crossreactivity by western blot
- Make standard curve based on purified Vtg of fish species of interest
- Always add standards to same concentration of “clean” male plasma at dilution used for assay
- Look for linearity of assay



Vitellogenin mRNA assay

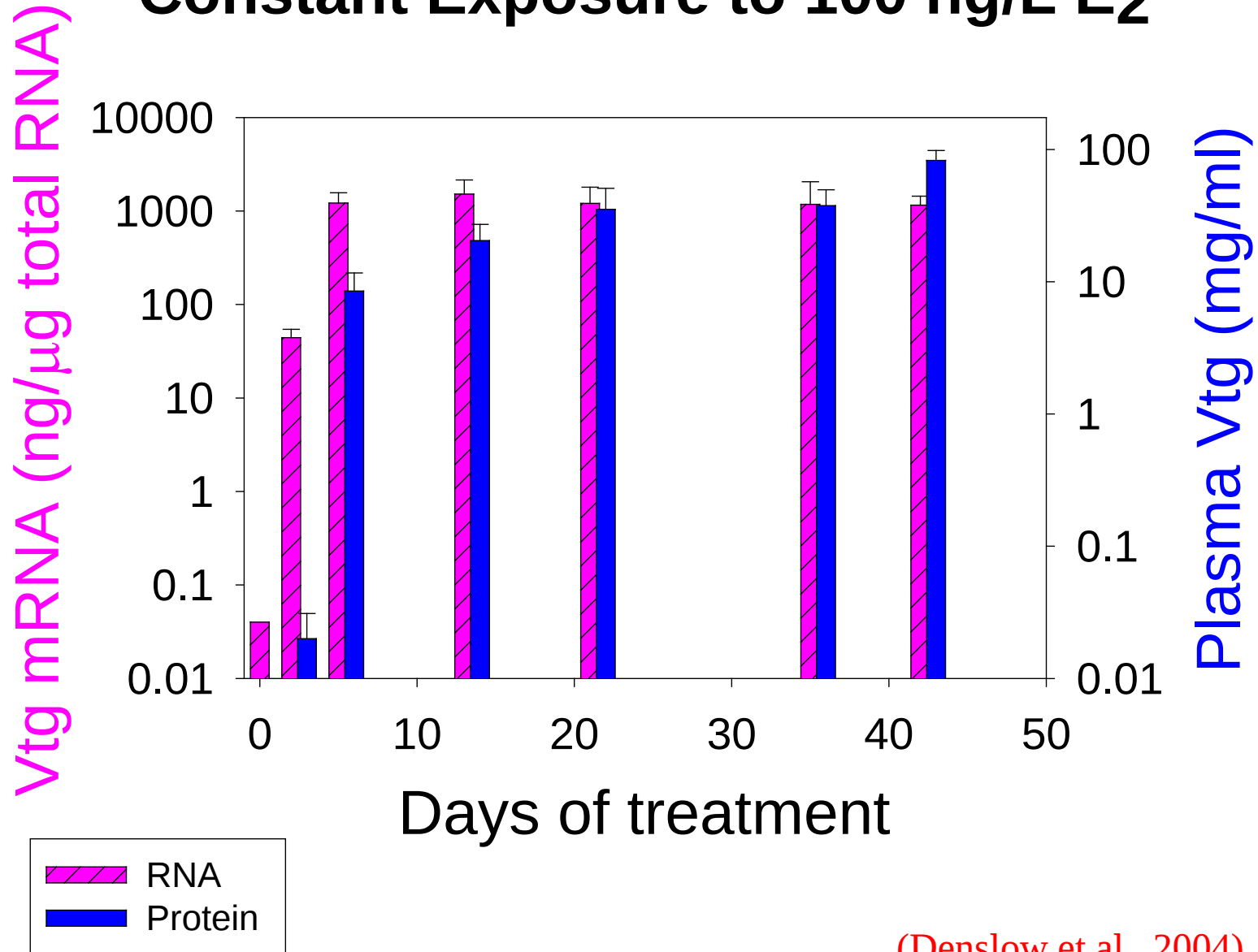
- Get liver samples from fish
- Extract total RNA
- Develop Vtg specific primers for species of interest
- Validate primers for linearity and efficiency of amplification
- Perform quantitative PCR assay

Injection Experiment (2.5 mg/Kg)

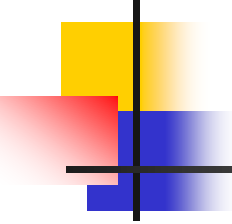


(Denslow et al., 2004)

Constant Exposure to 100 ng/L E₂



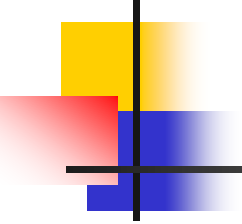
(Denslow et al., 2004)



Evaluation of plasma hormone levels

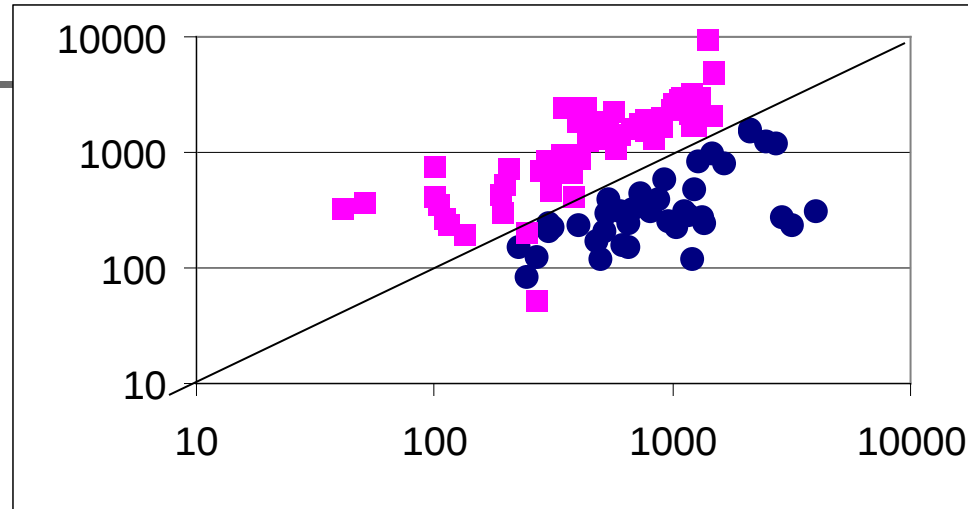
- Add tracer levels of [^3H] E2 or [^3H]T
- Extract hormones from plasma using liquid-liquid technique using 1-chlorobutane
- Perform assay using ELISA for E2, T or 11-KT
- Correct results based on extraction of added tracer
- Quantify plasma E2, T, 11-KT/ml

Steroid Hormones – Control Site



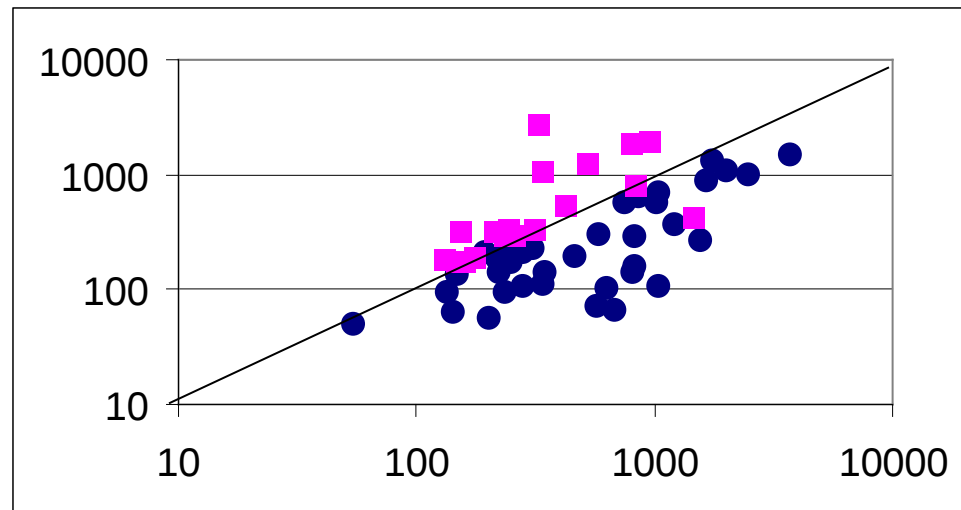
Spring

E_2



Summer

E_2

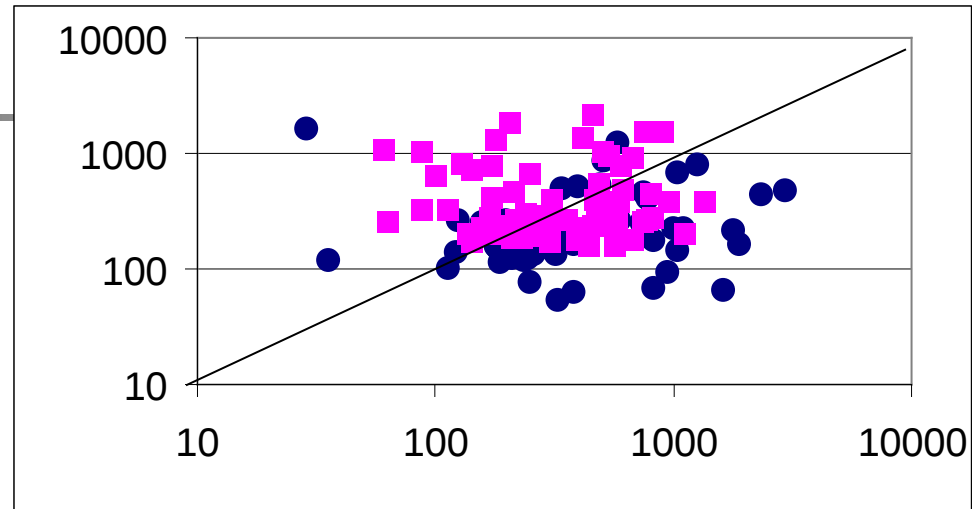


T

Steroid Hormones – Impacted Site

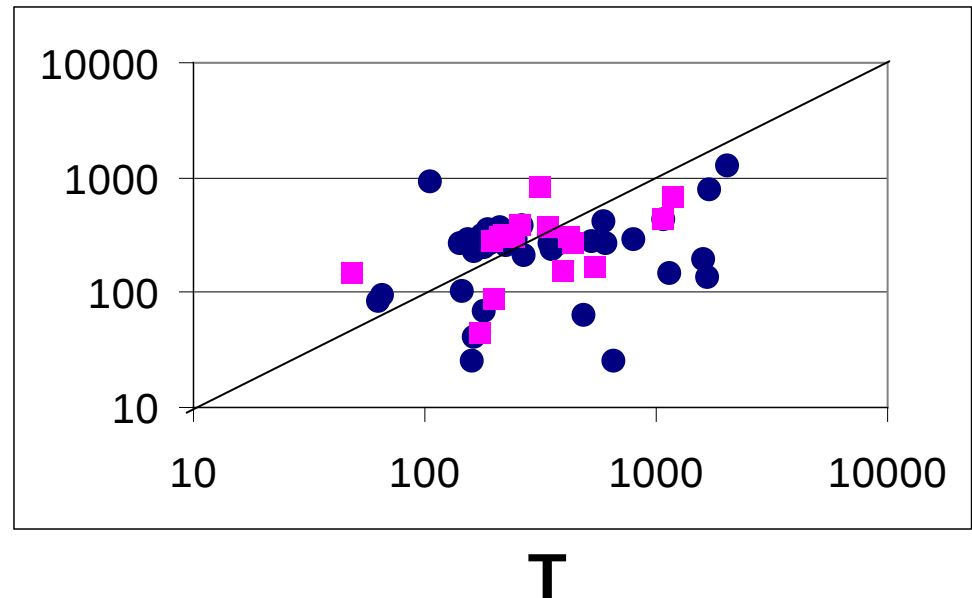
Spring

E_2

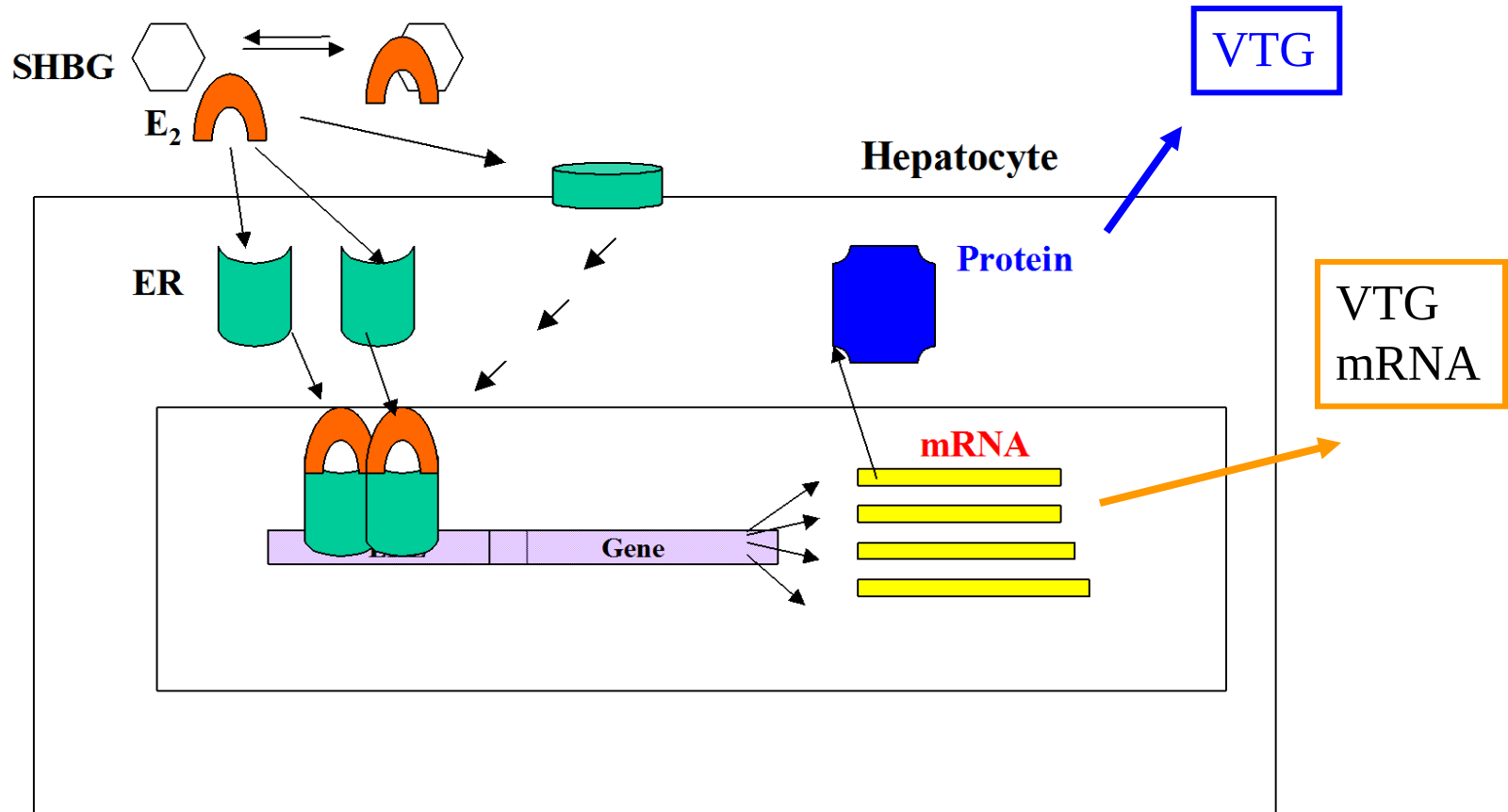


Summer

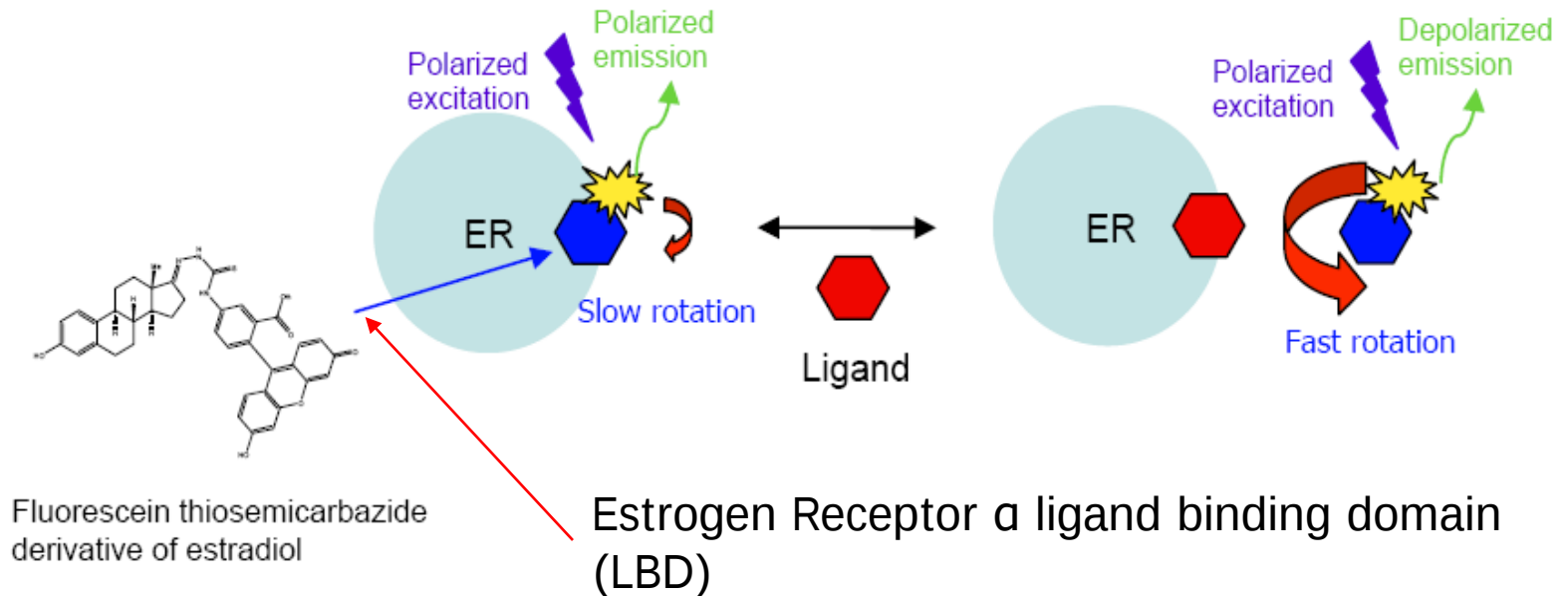
E_2



Gene Regulation through the estrogen receptor



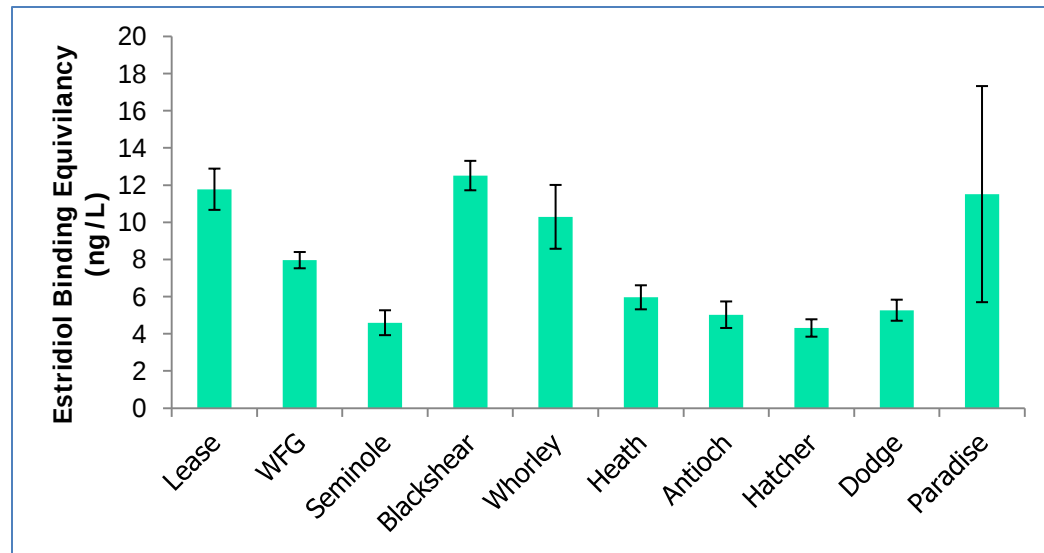
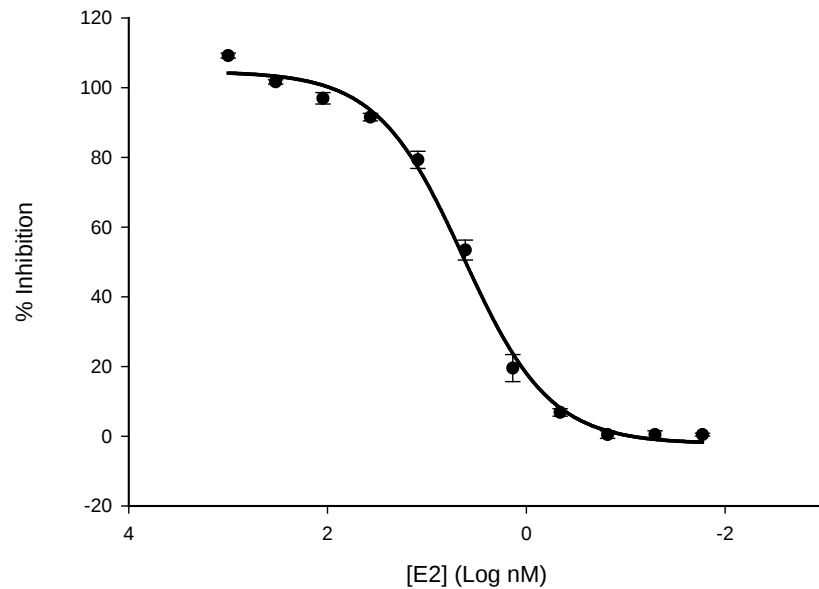
Fluorescence polarization: how it works



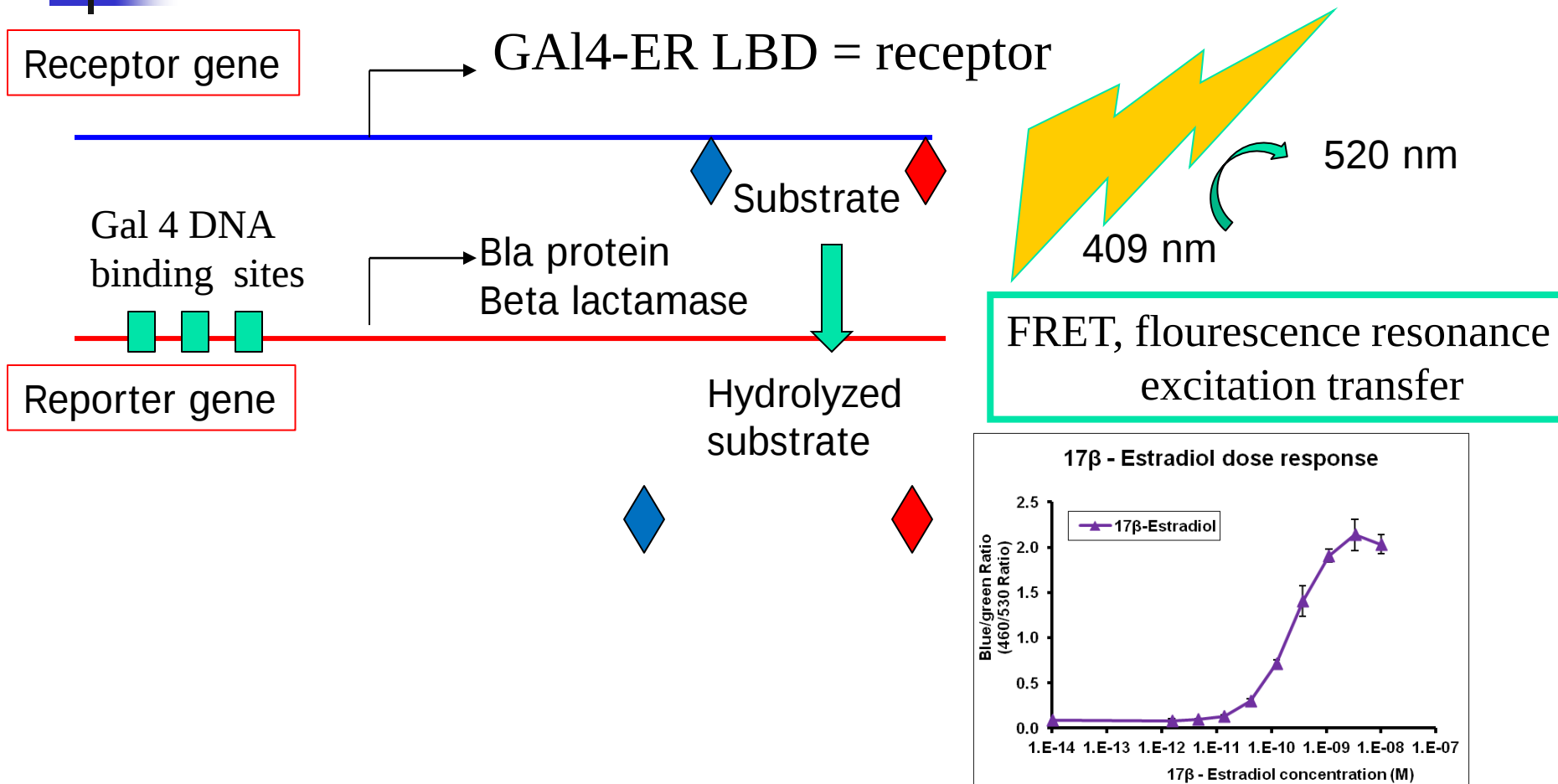
Advantages:

- Homogeneous, sensitive assay
- Non-radioactive
- Amenable to view binding events at equilibrium
- High-throughput format (96-well plates)

Fluorescence polarization: Example

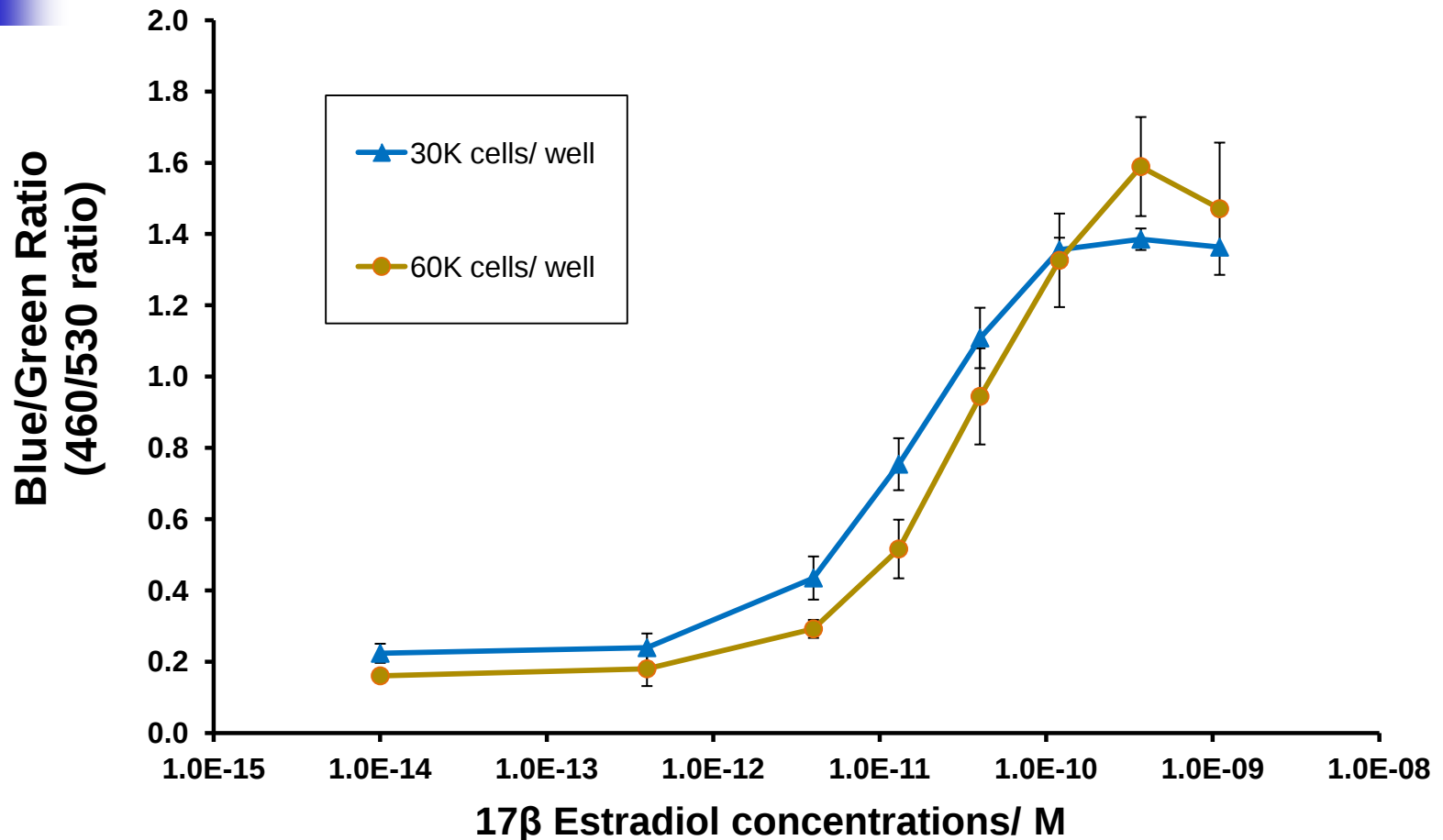


High throughput assays –ER & AR InVitrogen



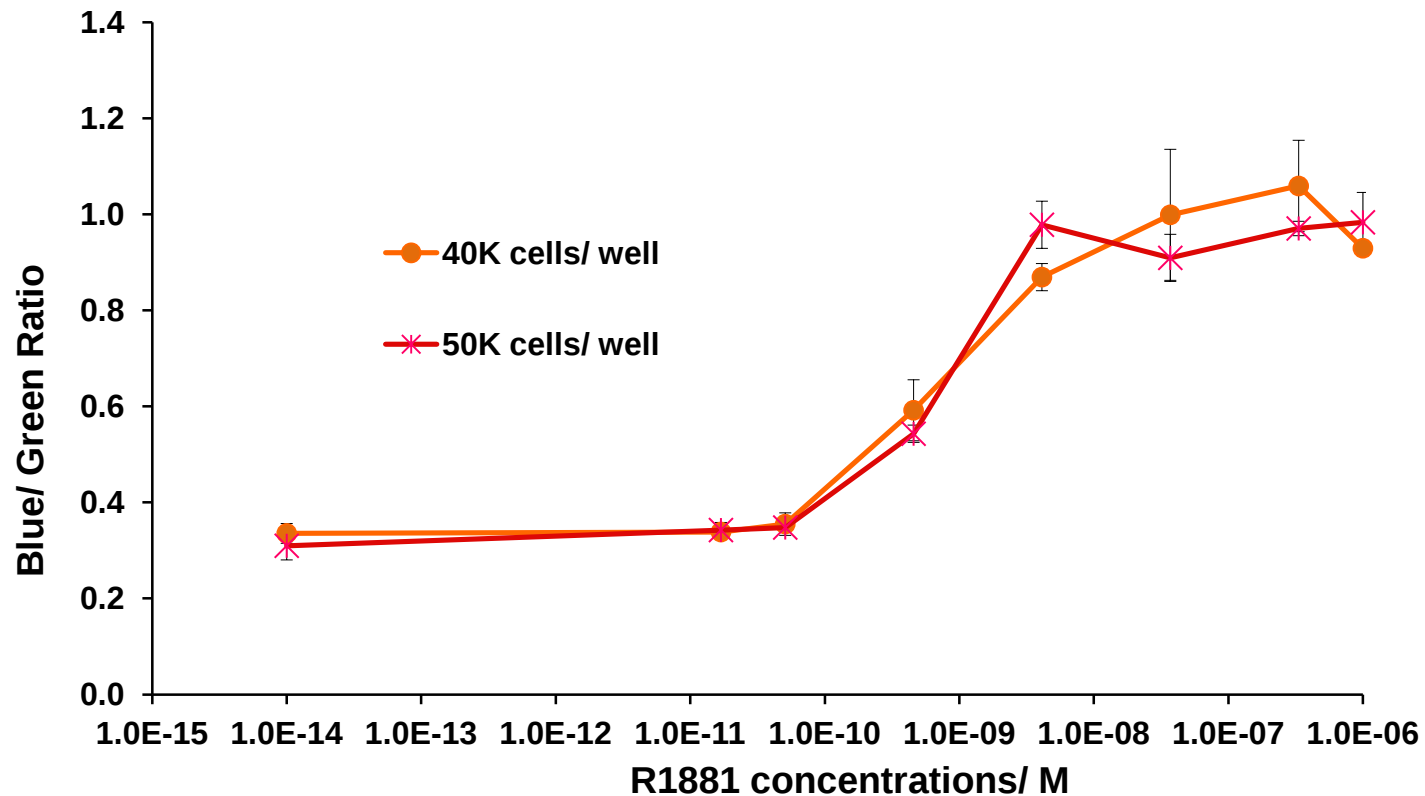
GeneBLAzer ER α Assay

E2 dose response with 30K and 60K cells per well



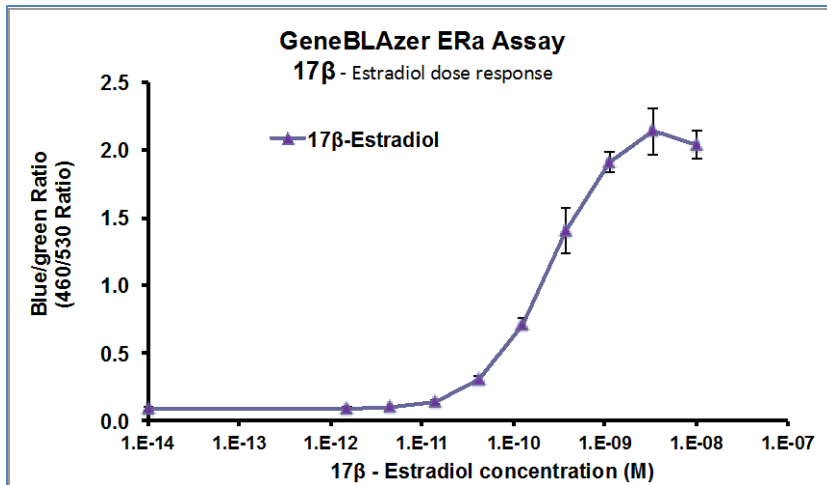
GeneBLAzer AR Assay

Dose response with 40K and 50K cells per well

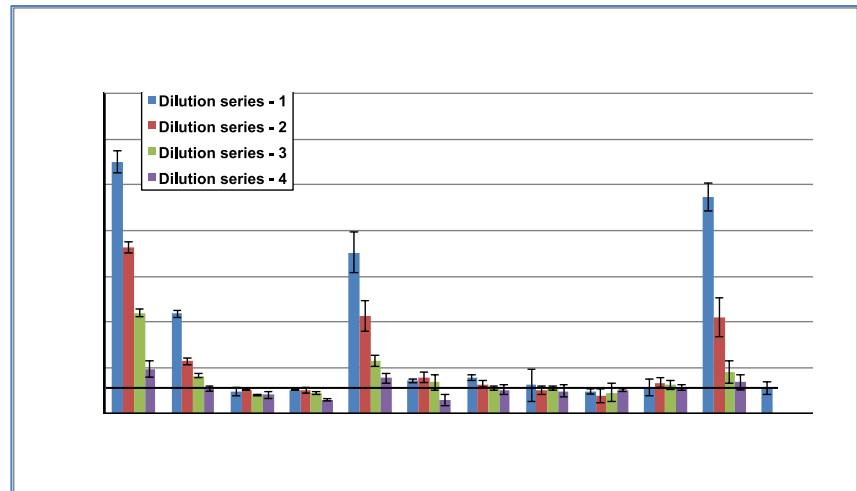


ER assay and evaluation of water

Standard curve



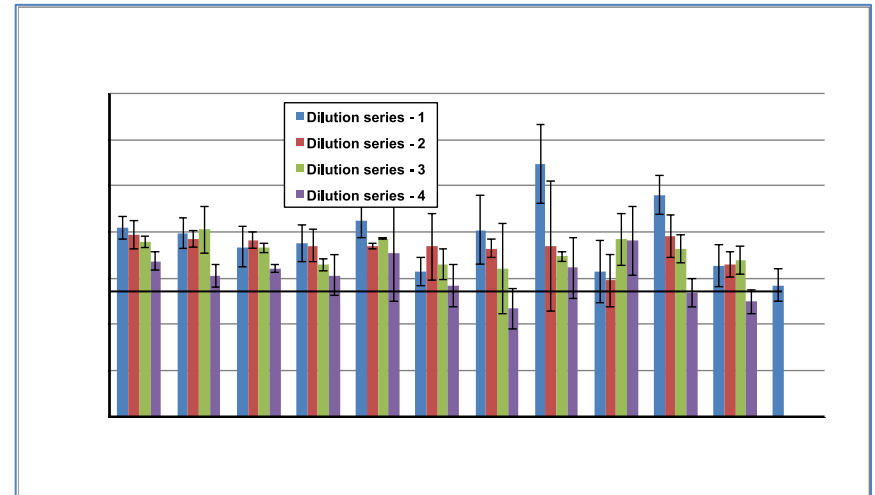
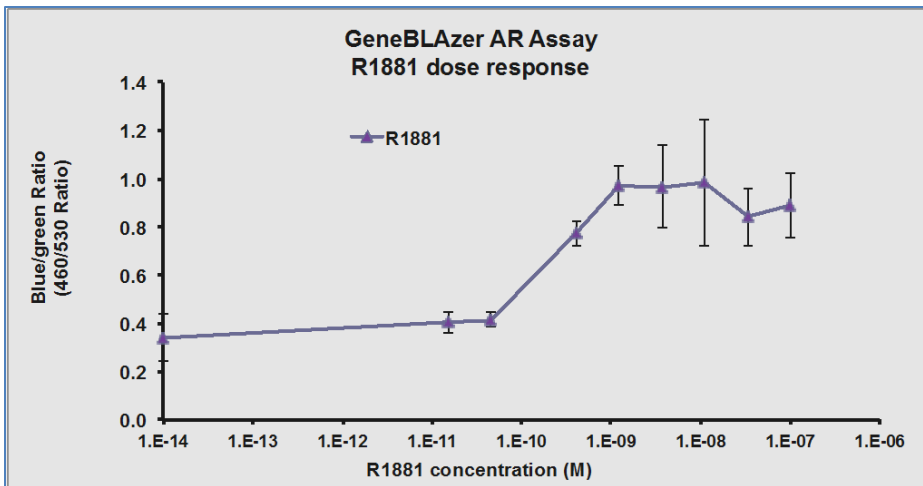
Water samples



Samples A, E, CA-A: Secondary treated effluent

Others: Secondary effluent receiving advanced treatment (e.g. membrane filtration; RO; ozonation & biologically activated carbon filtration; drinking water inlet/outlet stormwater (sample J); blank

AR assay and water samples



Samples A, E, CA-A: Secondary treated effluent

Others: Secondary effluent receiving advanced treatment (e.g. membrane filtration; RO; ozonation & biologically activated carbon filtration; drinking water inlet/outlet stormwater (sample J); blank

Relating molecular to organism level responses

■ Higher order endpoints

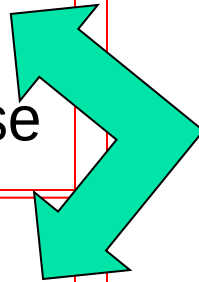
- Growth
- Survival
- Reproduction
- Susceptibility to disease

■ In vitro bioassay

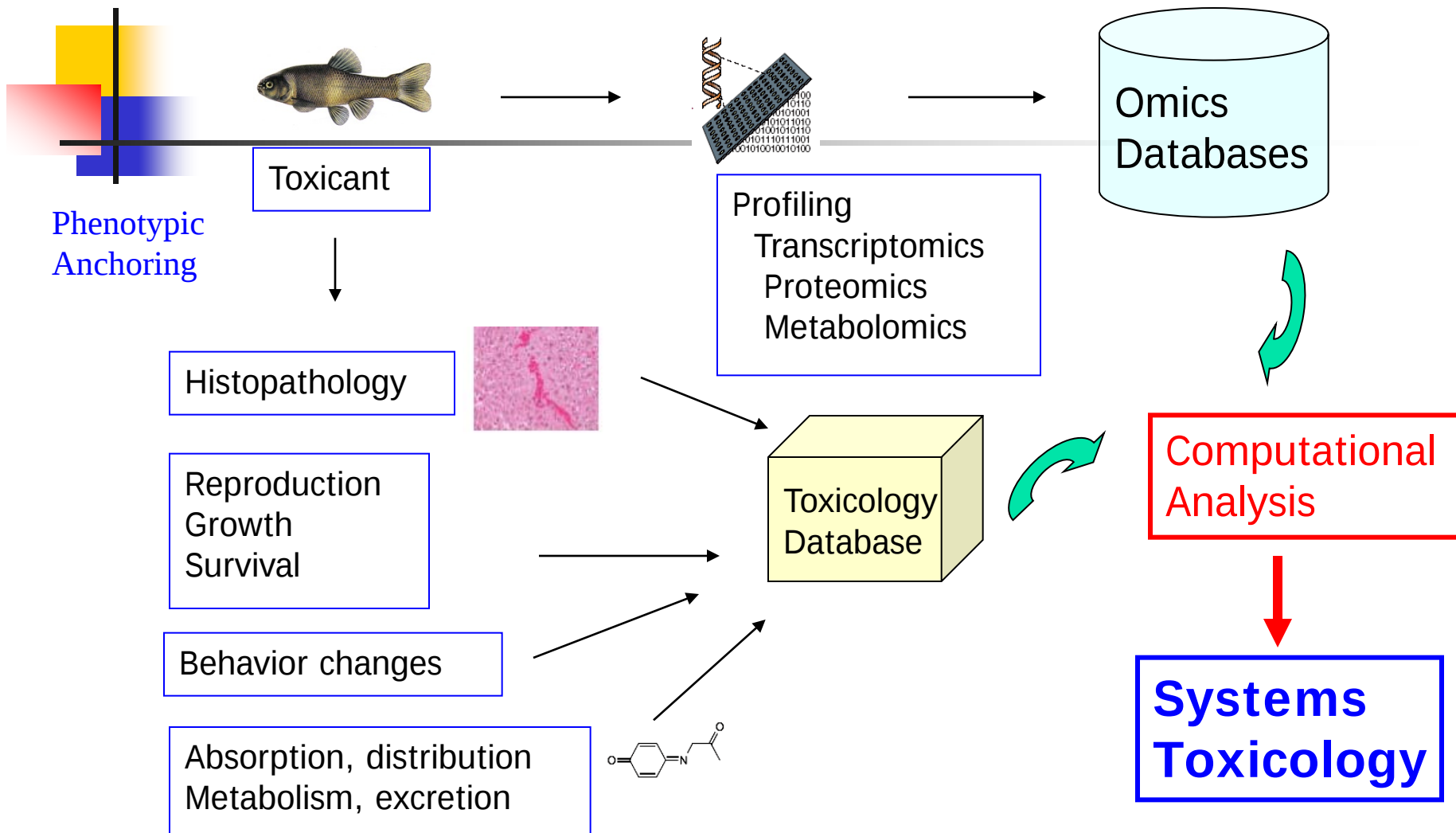
- EEQs via ER test results

■ Molecular biomarkers

- Plasma Vtg, hormones
- IGF-1, GH, brain aromatase, amh, dmrt1
- Vtg, ER α , ER β , StAR, AR

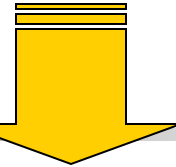
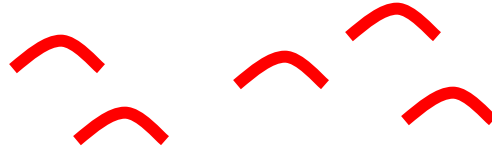


Omics and Systems Toxicology

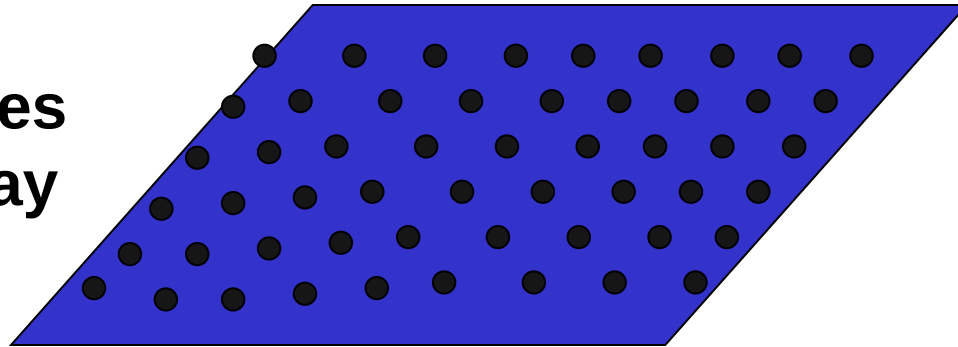


How microarrays work

Total RNA
from individual
fish is
labeled
(probes)

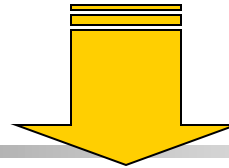
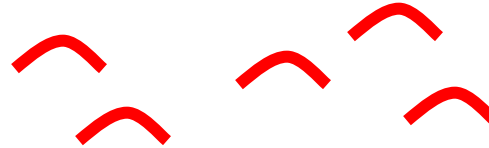


Oligonucleotides
spotted on array
(targets)

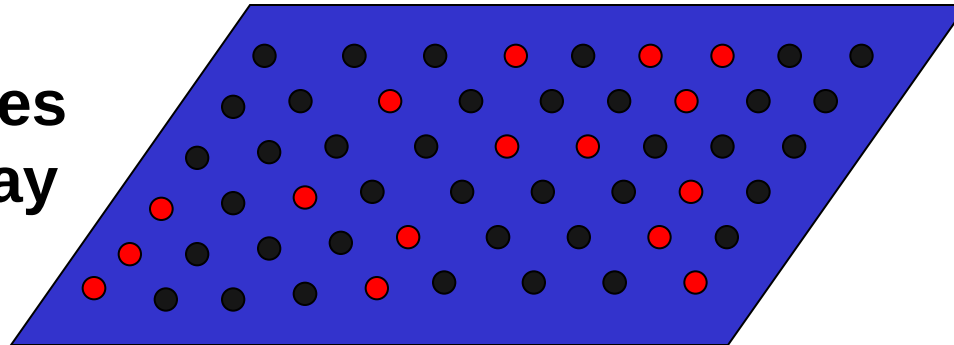


How microarrays work

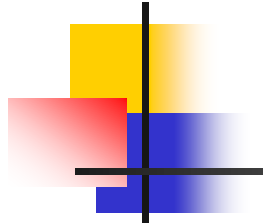
Total RNA
from individual
fish is
labeled
(probes)



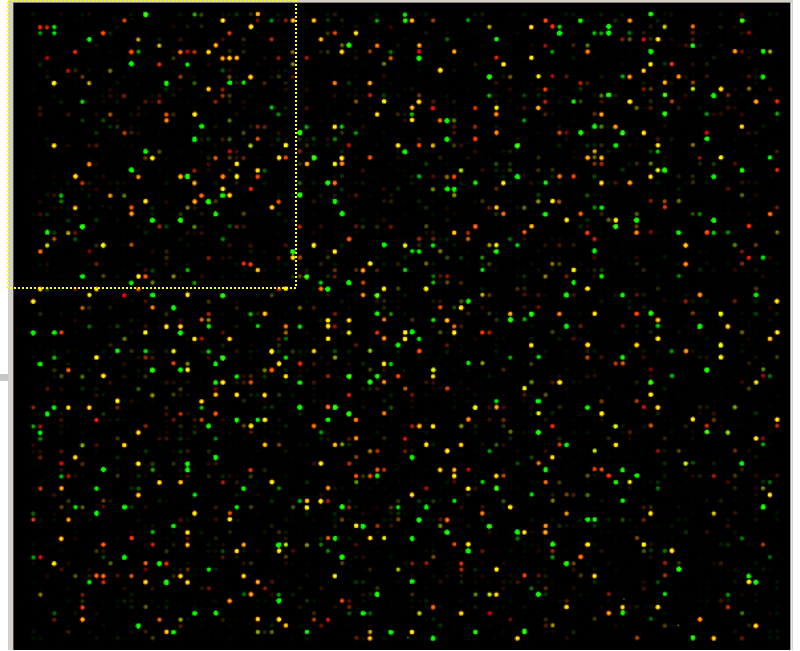
Oligonucleotides
spotted on array
(targets)



Analysis by microarray



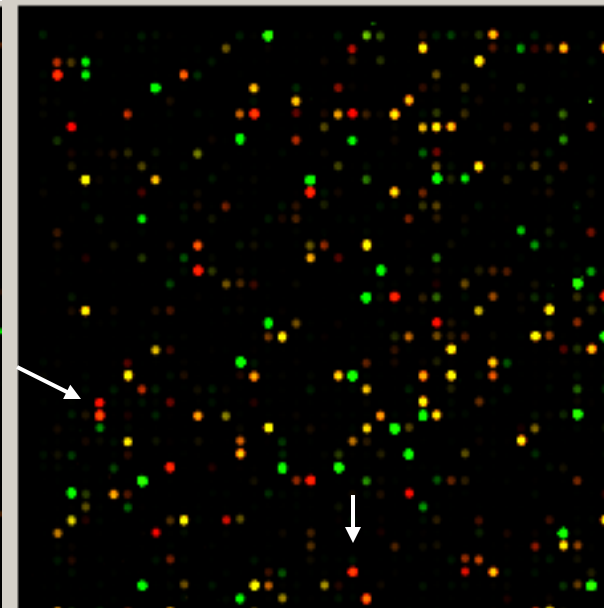
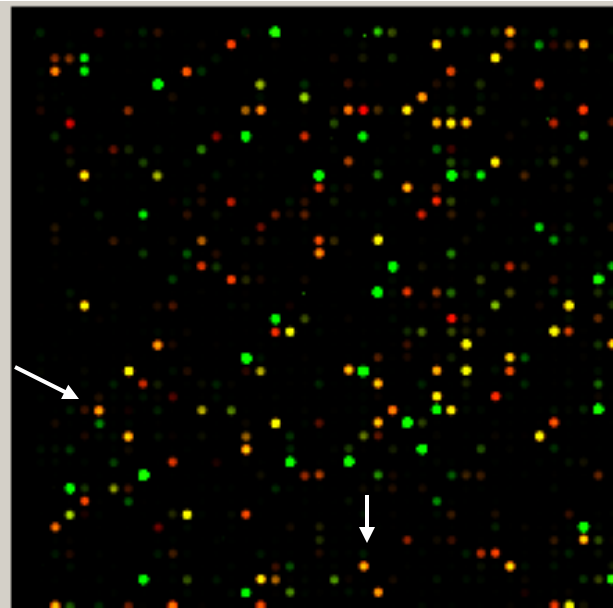
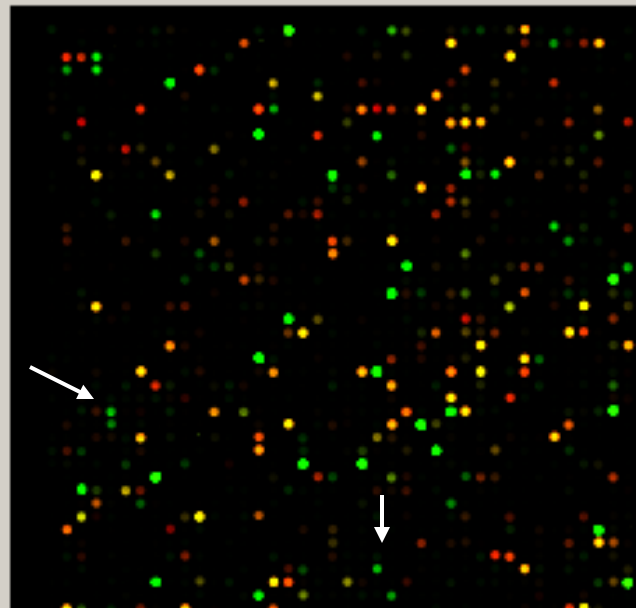
Fish $\pm$ treatment $\rightarrow$ organ $\rightarrow$ total RNA $\rightarrow$ cRNA $\rightarrow$ array



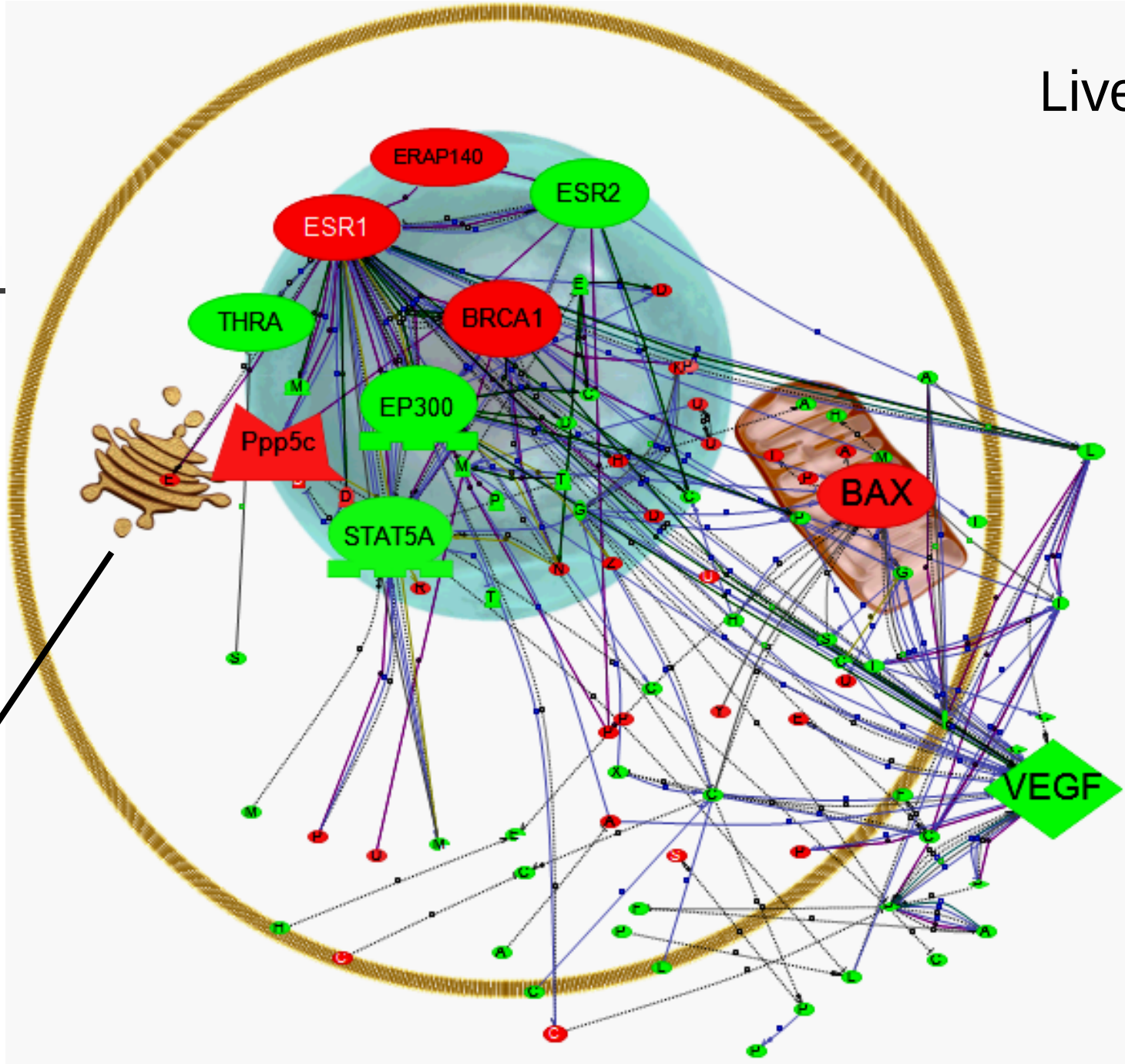
Control

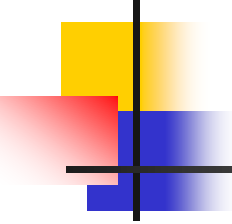
20 ng/L E2

100 ng/L E2



Liver





Endocrine Disruption

- Hormone receptor activity
- Steroidogenesis
- Metabolism
- Immune system dysfunction
- Neuroendocrine control
- Apoptosis → oocyte atresia
- Cardiovascular function

Pharmaceuticals and Personal Care Products -- PPCPs

■ Pharmaceuticals

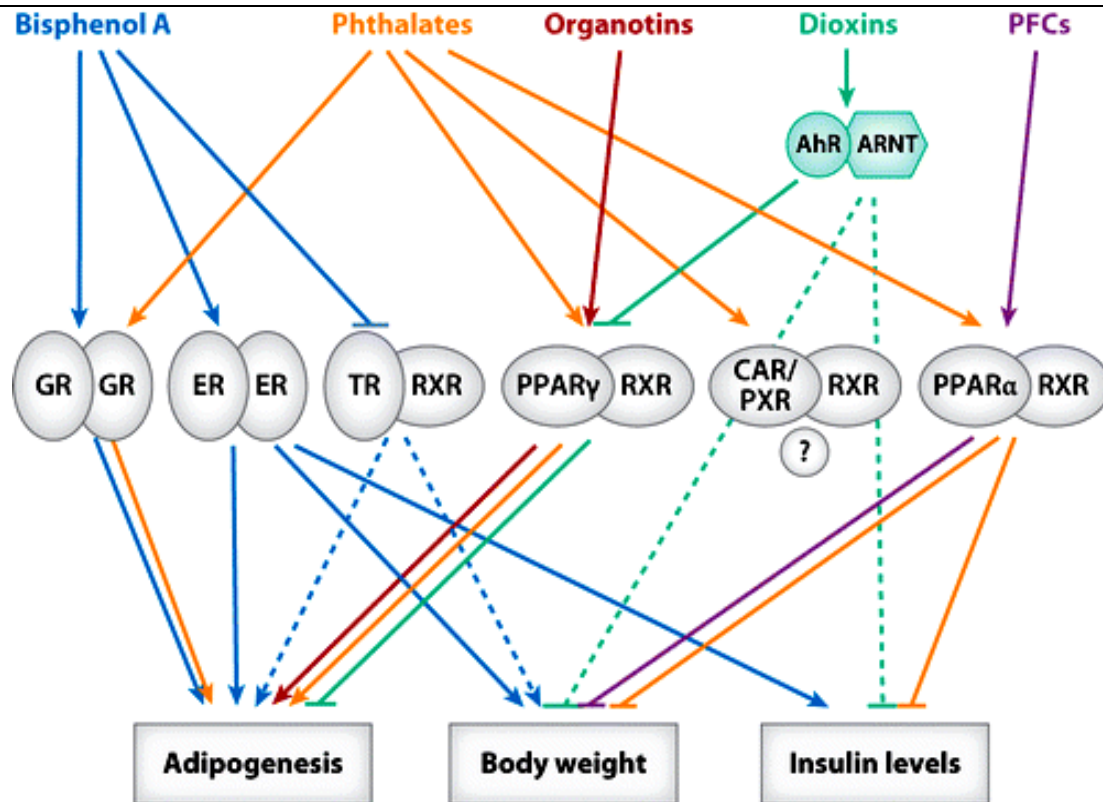
- Hormones/ Mimics
- Antibiotics
- Blood lipid regulators
- Anti-inflammatory drugs – NSAIDs
- Heart drugs – β -blockers
- Anti-depressants – serotonin uptake inhibitors

■ Personal Care Products

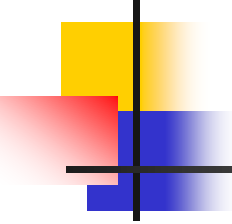
- Fragrances
- Preservatives
- Disinfectants
- Sunscreen agents



Expression analysis



Casals-Casas C, Desvergne B. 2011.
Annu. Rev. Physiol. 73:135–62



Phenotypic changes in fish

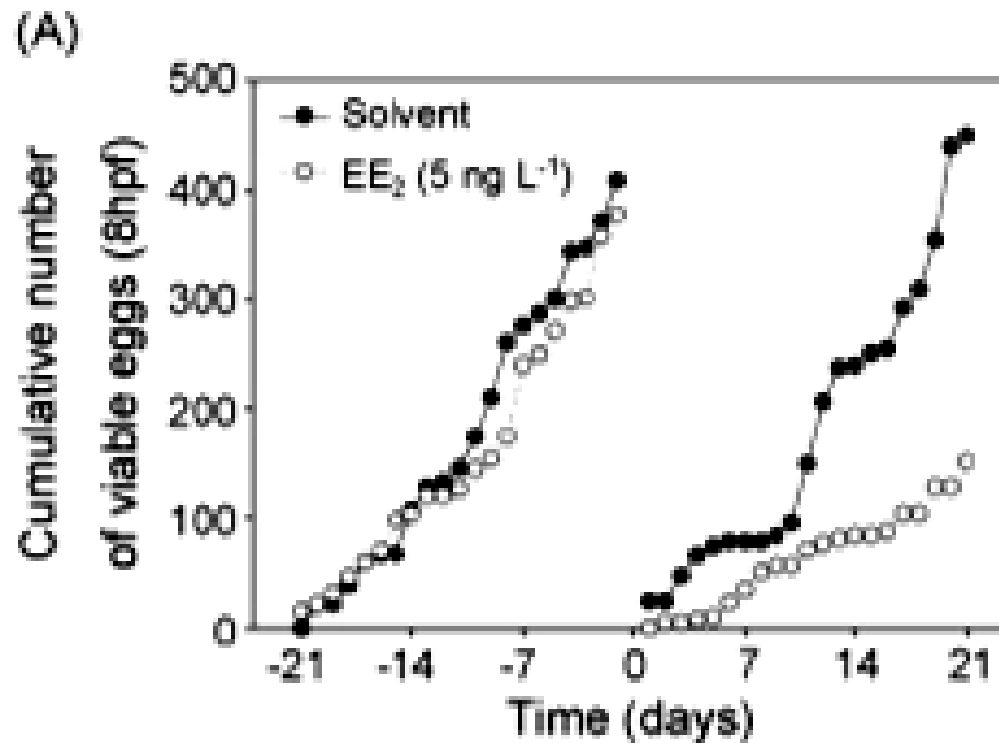
Estrogen exposures

- Male fish synthesize the egg-yolk protein; become feminized in secondary sex characteristics and behavior
- Female fish diminish egg production – linked to population level effects.

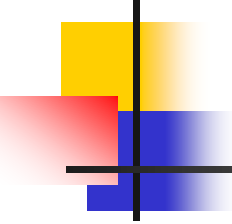
Androgen exposures

- Male fish become more aggressive
- Female fish develop male secondary sex characteristics
- Female fish diminish egg production → Pop. effects

Effect of 5 ng/L EE2 on egg production



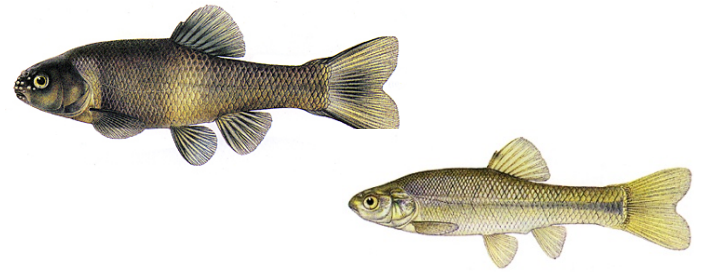
(Santos et al., 2007)



Fresh water species

- Fathead minnow

Pimephales promelas



- Largemouth bass

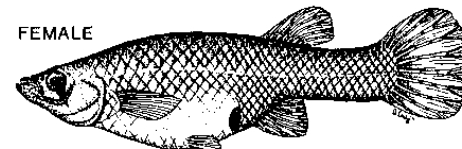
Micropterus salmoides



MOSQUITOFISH
Gambusia spp.

- Mosquitofish

Gambusia holbrooki

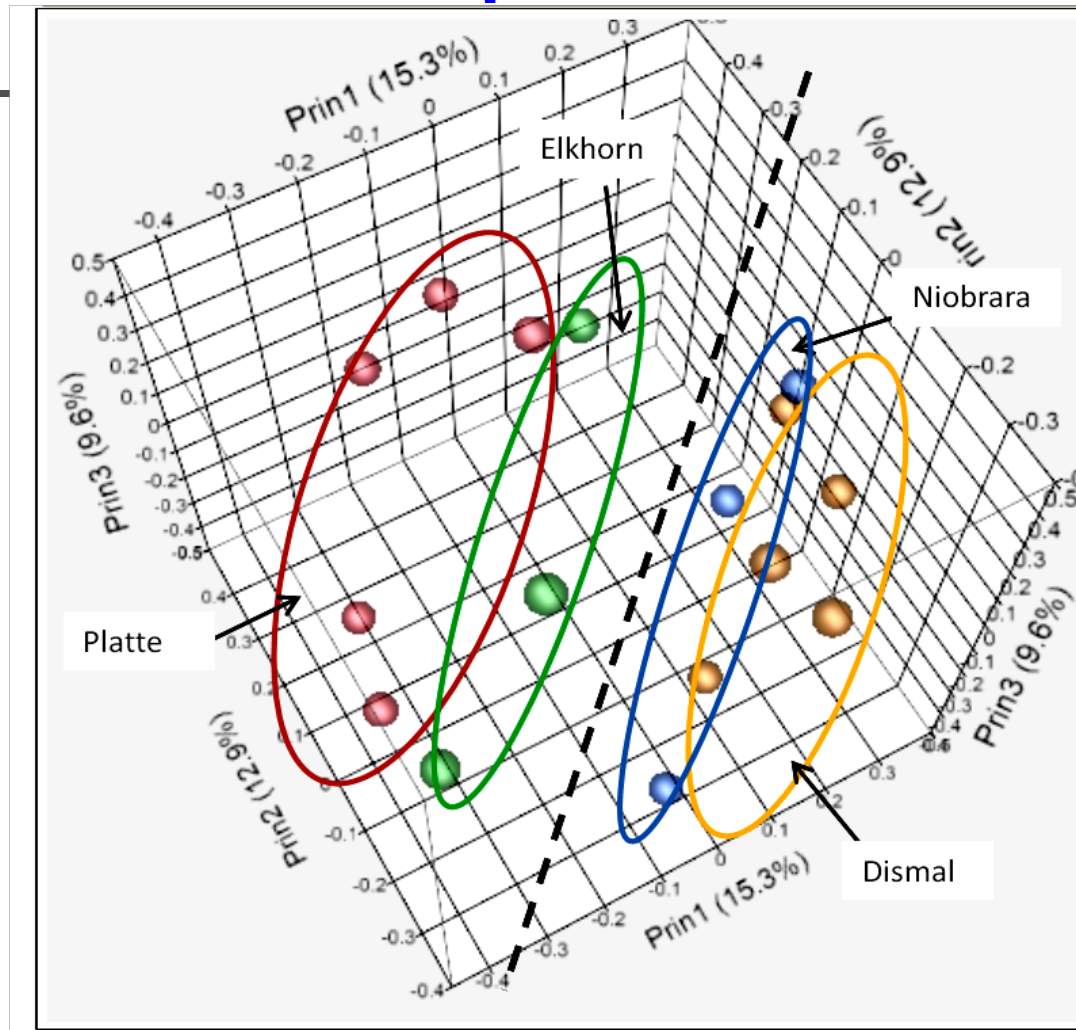


Case study- Nebraska watersheds

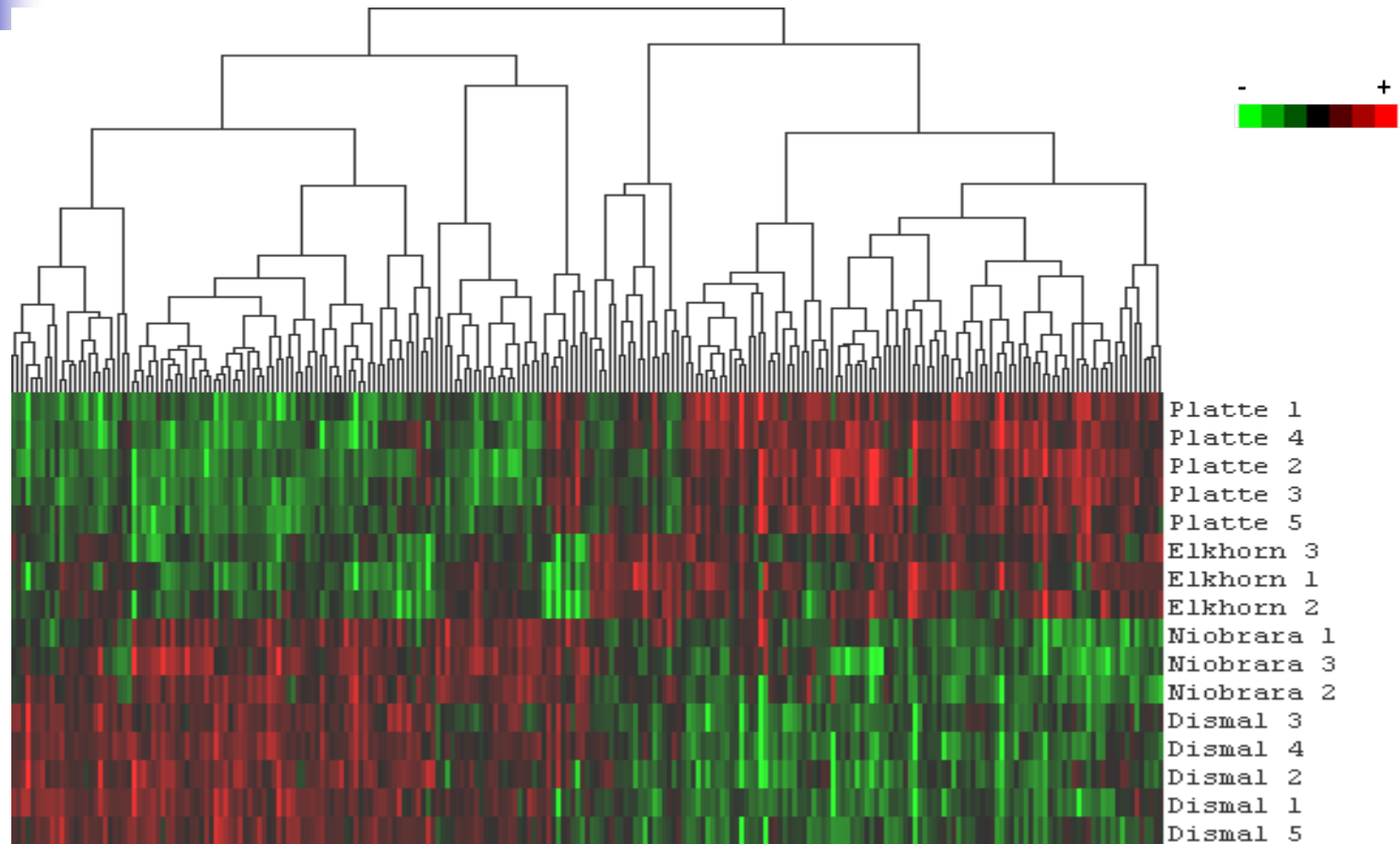


- Four watersheds characterized by different land use
 - Elkhorn, Platte, Dismal and Niobrara sites
- At each site 3 cages of 12 fathead minnows were deployed for 7 days (no lab control)
- POCIS deployed alongside for water chemistry analysis
- Male livers (n= 5 per site) used in microarray study
 - Cy3 labeling and hybridization onto a 15K microarray (EcoArray)*
- ANOVA (pairwise analysis) and GO analysis

FHM transcriptomes



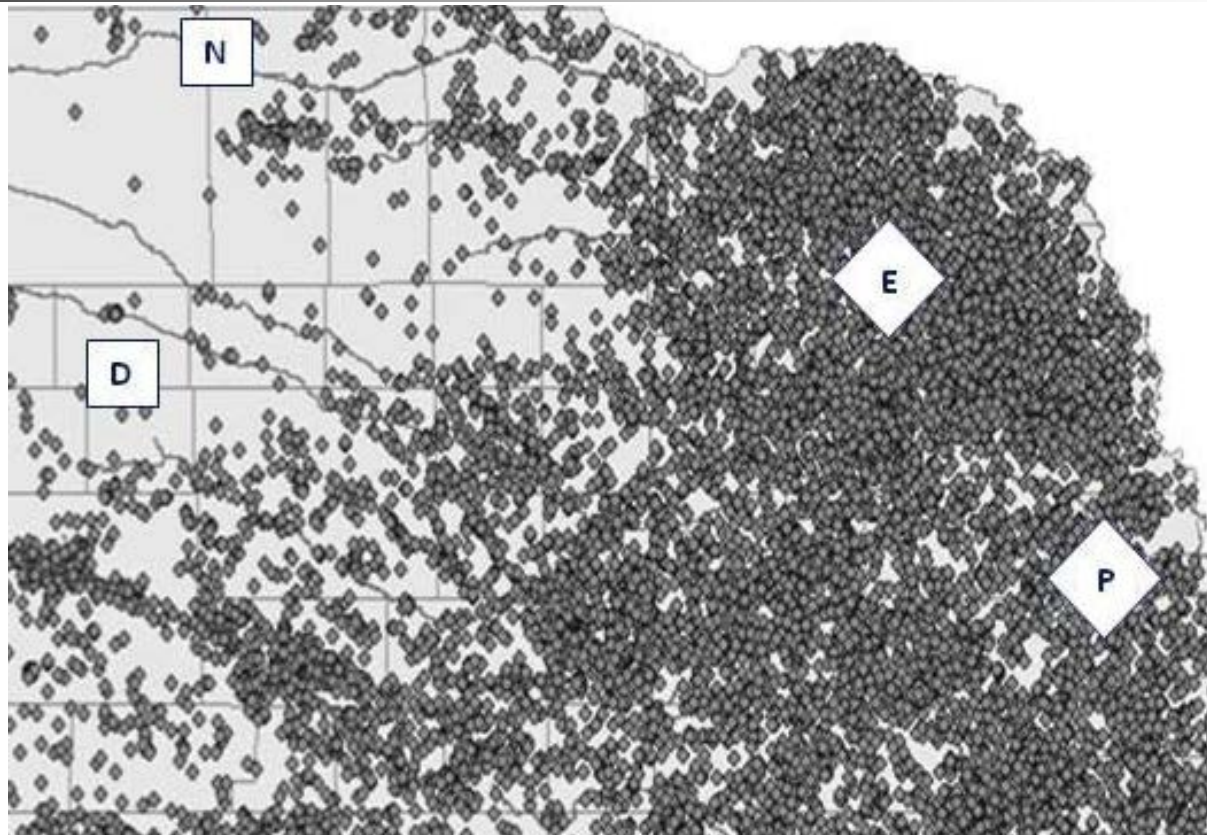
Clustering analysis



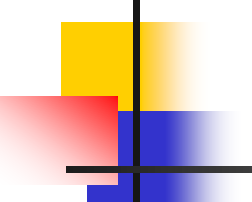
Functional analysis

	Elkhorn vs Dismal	Platte vs Dismal	Elkhorn vs Niobrara	Platte vs Niobrara
DNA-dependent DNA replication		x		x
DNA repair		x		x
Response to DNA damage stimulus		x		x
Chromatin assembly and disassembly		x		x
Microtubule-based movement		x	x	x
Cholesterol biosynthesis	x		x	
Lipid catabolism	x		x	
Steroid biosynthesis	x		x	

Map of the watersheds



Small grey circles indicate beef feedlots with over 500 head of cattle.

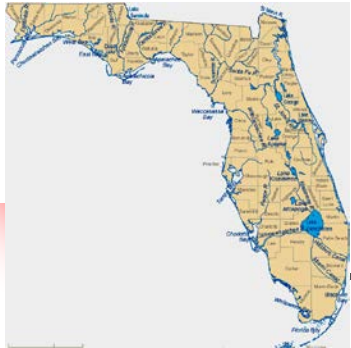


Water chemistry (POCIS)

	Dismal	Niobrara	Elkhorn	Platte
Stream flow	6.40	24.4	64.4	379
(m ³ /s)	18.2	20.5	21.8	24.7
Temp (° C)				
Acetochlor (ng)	n.d	n.d	98	194
Alachlor (ng)	n.d	n.d	n.d	12
Atrazine (ng)	11	13	967	1983
Propazine (ng)	n.d	n.d	19	33
Metolachlor (ng)	n.d	n.d	379	775
Simazine (ng)	n.d	n.d	n.d	11

Case study: Papermill effluents

Androgens



Elevenmile creek
Fenholloway

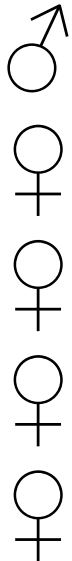
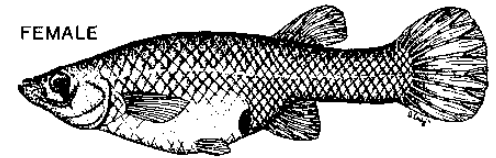


MOSQUITOFISH
Gambusia spp.

MALE



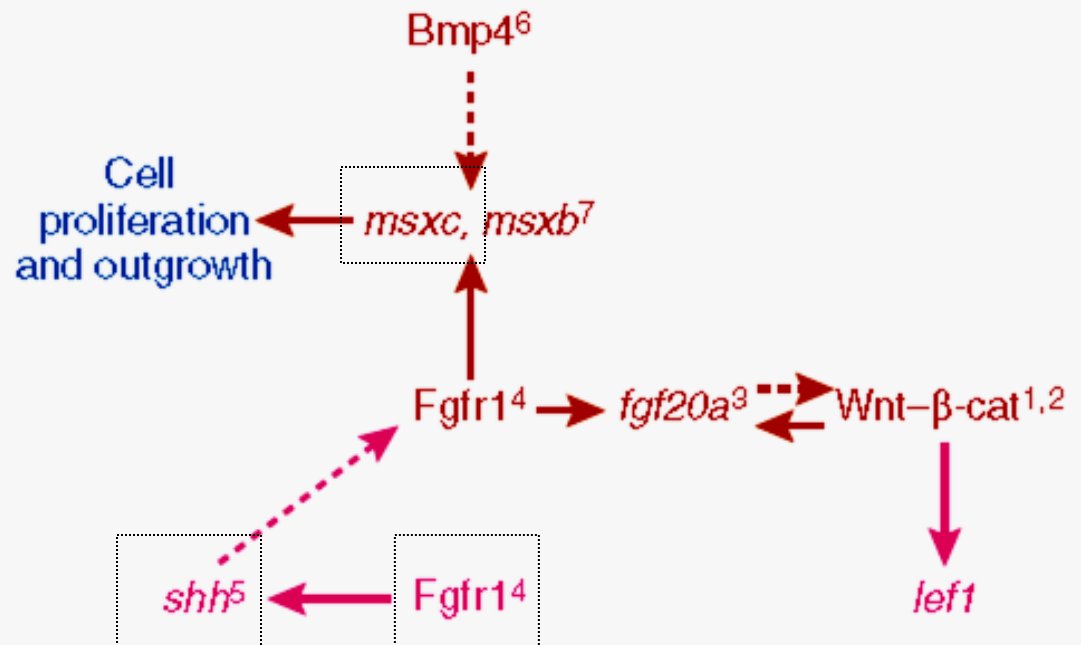
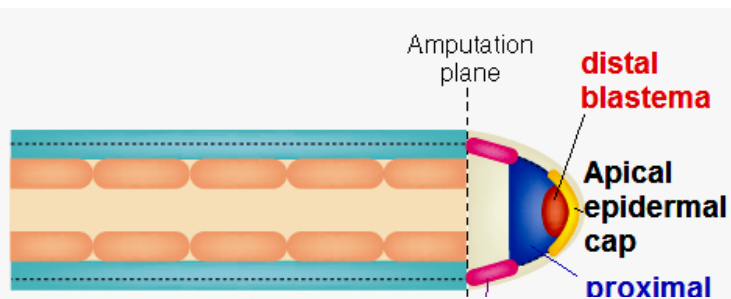
FEMALE



Howell 1980

(Erica Brockmeier)⁴²

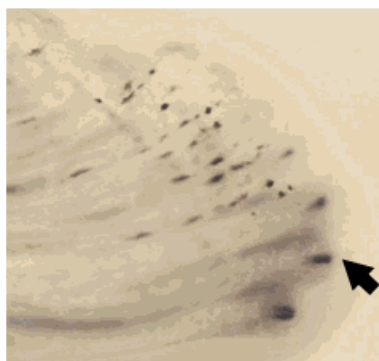
Growth of gonapodium



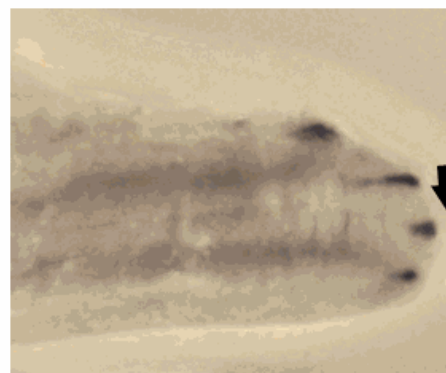


Sonic
hedgehog

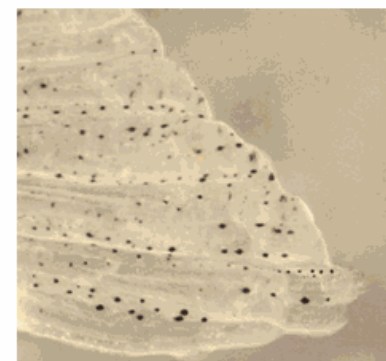
Day 7



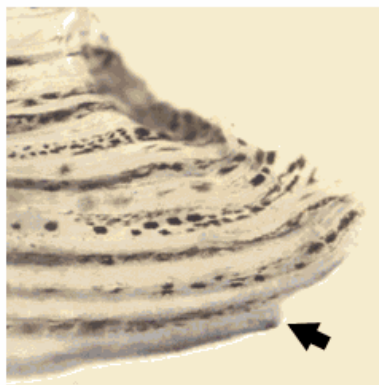
Day 14



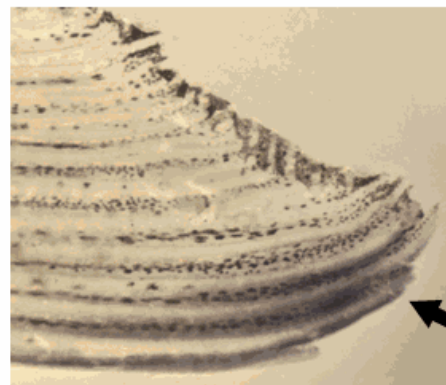
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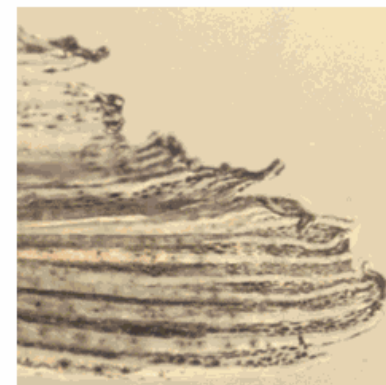
Day 5



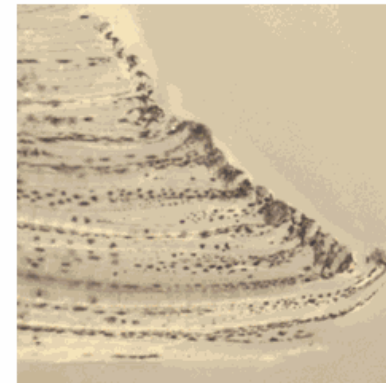
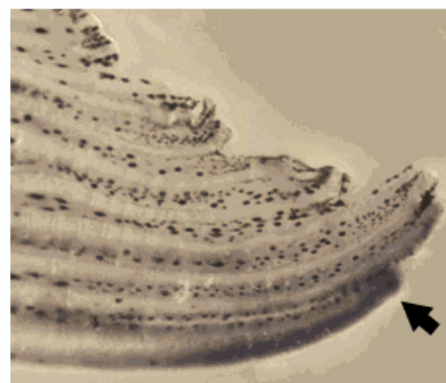
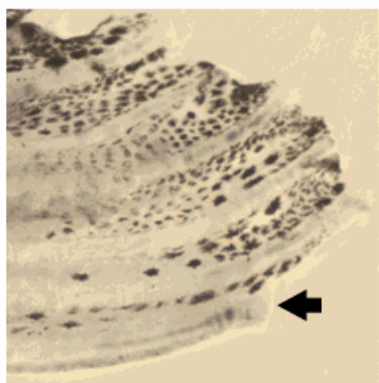
Day 10



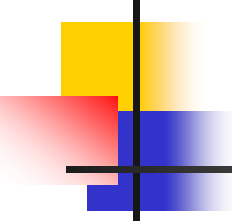
control



Muscle
segment
homeobox
C

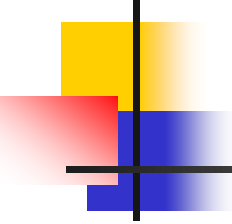


Fibroblast
growth
factor
receptor 1



Conclusions

- Bioanalytical approaches are complementary to analytical chemical approaches and can be used for monitoring
- Bioanalytical approaches distinguish the bioactive fraction from the total
- Biomarkers can be linked to higher order population level effects
- Systems biology approach gives a holistic interpretation of issues
- Florida has issues with some of its water resources



Environmental Assays

In vivo Fish assays

- Plasma Vtg
- Plasma hormones
 - E2, T, 11-KT
- Microarray analysis
- In vivo toxicity bioassays
- FHM cumulative egg production

In vitro assays

- ER binding assay
- ER/AR/PR/GR transactivation assays
- TIE

Toxicity Bioassays

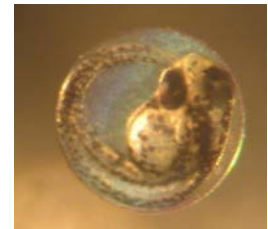
- Measuring acute toxicity to freshwater organisms (Static Renewal)

- Larval fathead minnow survival(96 hrs)
- *C. dubia* survival (48 hrs)
- *D. magna* survival(96 hrs)



- Short term tests for estimating chronic toxicity to freshwater organisms

- Larval fathead minnow survival and growth (7 days)
- *C. dubia* survival and reproduction (7 days)



- Short term tests for estimating embryonic survival and teratogenicity

- Zebrafish embryo toxicity test (5 days)



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