From Treatment Plants to Turbots: Data Suggesting Effects From Endocrine Disrupting Chemicals in Wastewater Discharged into the Pacific Ocean

Jeffrey L. Armstrong, Ph.D.
Environmental Assessment Division
Orange County Sanitation District

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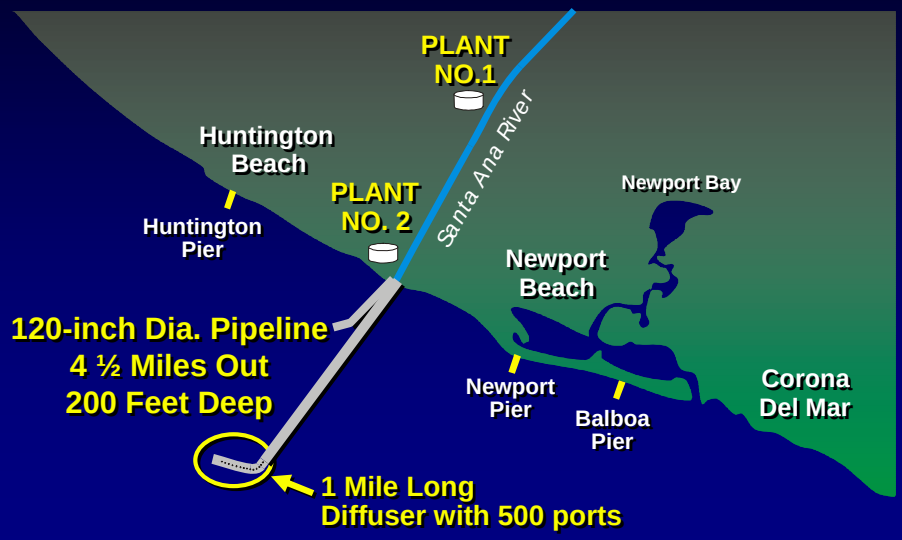
Who is the OCSD?

- Third largest POTW west of the Mississippi River
- 470 sq. mi. service area
- Serves 2.5 million people
- Treats 243 MGD

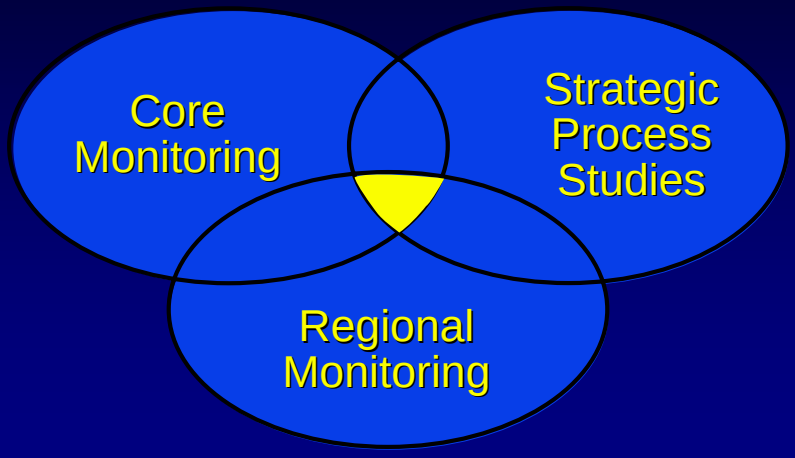
2

Ocean Discharge of Effluent



3

Ocean Monitoring Program



4

Strategic Process Studies

- Studies and OCSD level of effort agreed upon by regulators
- Two purposes:
  - Answer questions raised by core monitoring
  - Address issues of concern
    - e.g., current mapping, sediment toxicity, endocrine disrupting chemicals

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Research Projects

- OCSD is not a research agency
- Three main strategies for conducting research projects:
  - In-house projects
  - Contractors
  - Collaborations with university researchers and others

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Collaborating with Universities and Others on Research Projects

- Areas where OCSD staff lacks expertise
- OCSD provides:
  - Ecological expertise
  - In-kind services (vessel, crew and supplies for field collection)
  - Funding of graduate students

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Shoreline Microbiology: What is the relationship between bacteria concentration in ankle deep water, where most of the monitoring samples are collected, and the surfzone, where much of the water contact recreation occurs?

Water Quality: What is the spatial extent and duration of stormwater plumes in the coastal ocean?
Coastal Ecology: 1) What is the extent and magnitude of contamination and associated biological effects ion the SCB? and 2) What is the mass of pollutants accumulated in the SCB?

Endocrine Disrupting Chemical Exposure to Flatfish

- Cortisol Inhibition
  - Kevin Kelley's lab at CSU Long Beach, OCSD
- Estrogenicity
  - Dan Schlenk's lab at UC Riverside, OCSD
- Sperm DNA Damage
  - Computer Sciences Corp. (San Diego, CA), OCSD
- Correlation of Parasites in Fishes to Cortisol Inhibition
  - Kelley Lab (CSULB), OCSD

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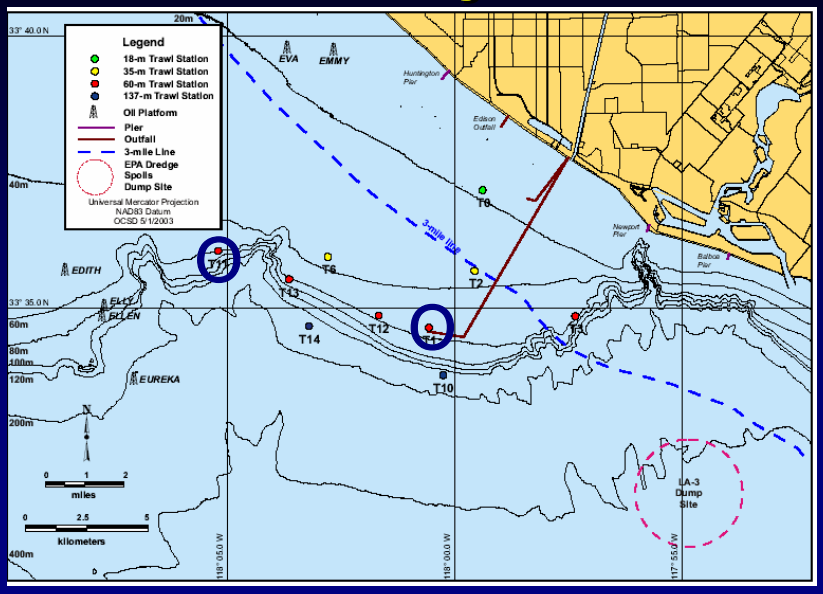
SPS Study Area



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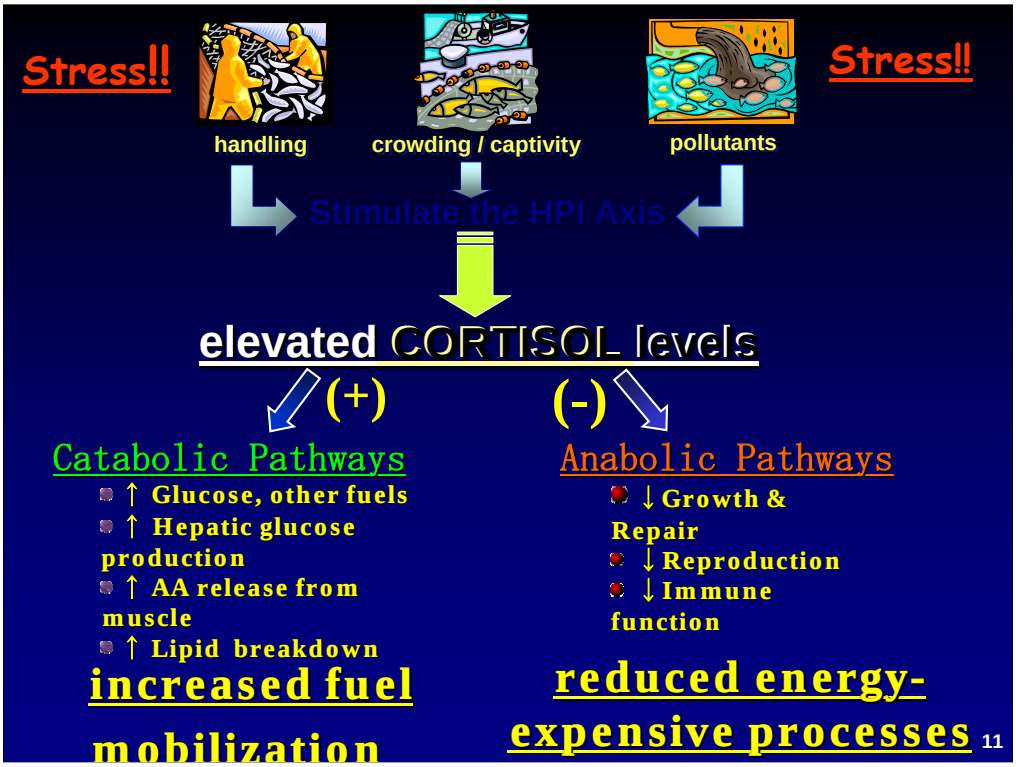
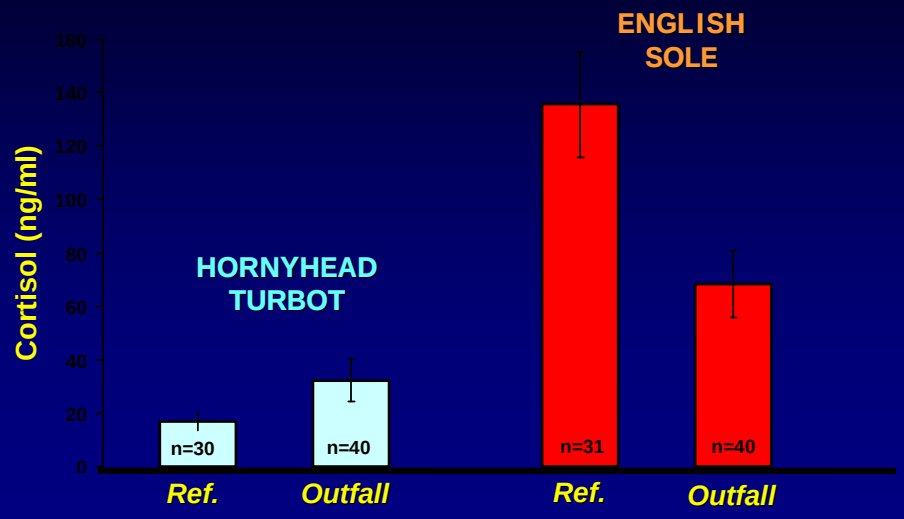
Cortisol Production

- Produced via the HPI Axis
- Cortisol production is inhibited by chronic stress
- Inhibition may be caused by PPCP/EDCs?

10


Stress!!
handling
crowding / captivity
pollutants
Stress!!
Stimulate the HPI Axis
elevated CORTISOL levels
(+)
(-)
Catabolic Pathways
↑ Glucose, other fuels
↑ Hepatic glucose production
↑ AA release from muscle
↑ Lipid breakdown
increased fuel mobilization
Anabolic Pathways
↓ Growth & Repair
↓ Reproduction
↓ Immune function
reduced energy-expensive processes
11

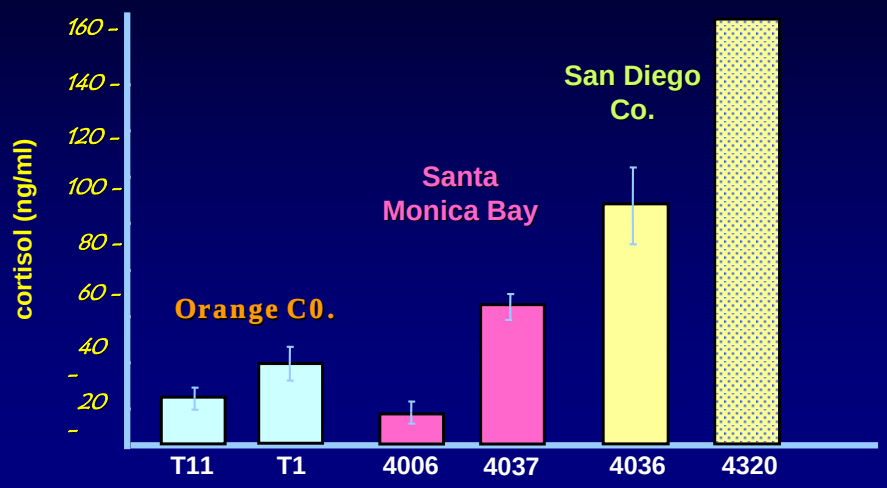
Peak Cortisol Concentrations in Post-trawl Flatfish



12

Peak Cortisol

Hornyhead Turbot from Different Locales



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Insulin-like Growth Factor 1 (IGF-1)

- Mediates the effects of growth hormone
- Depressed in stressed fish
  - Elevated cortisol levels inhibits the production of IGF-1

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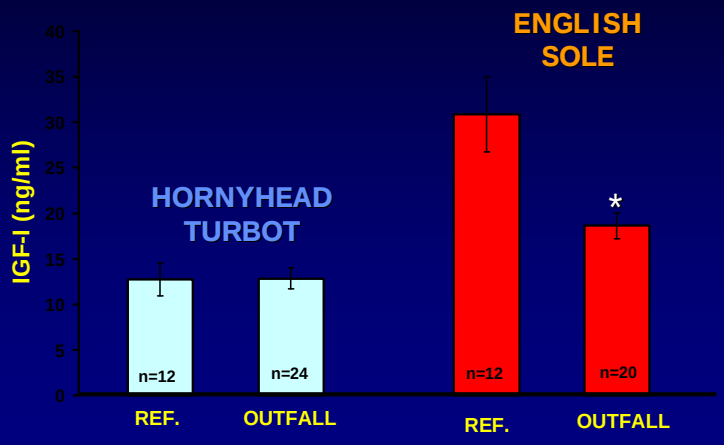
Plasma IGF-I Concentrations in Flatfish



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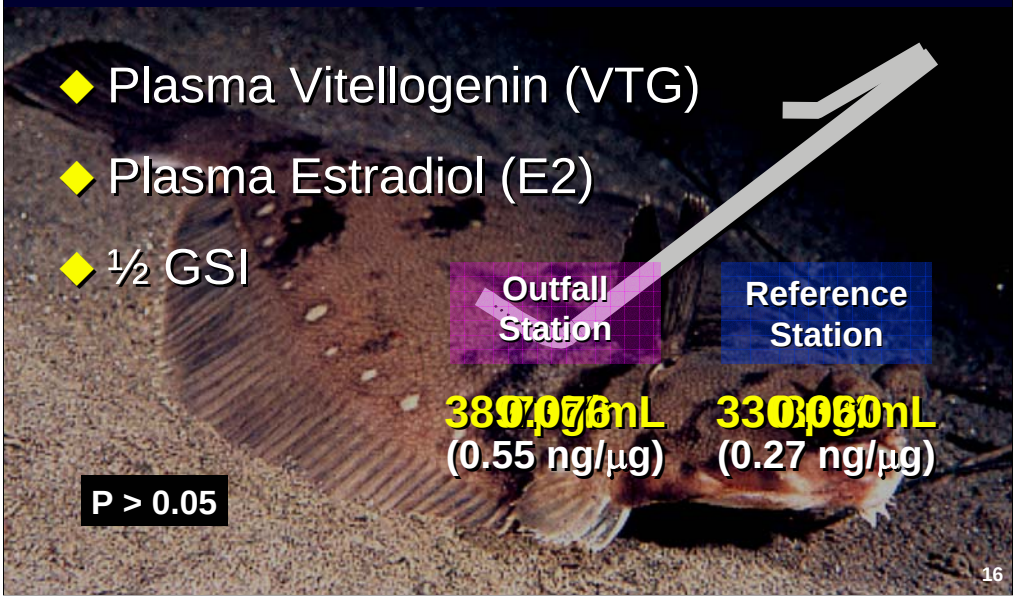
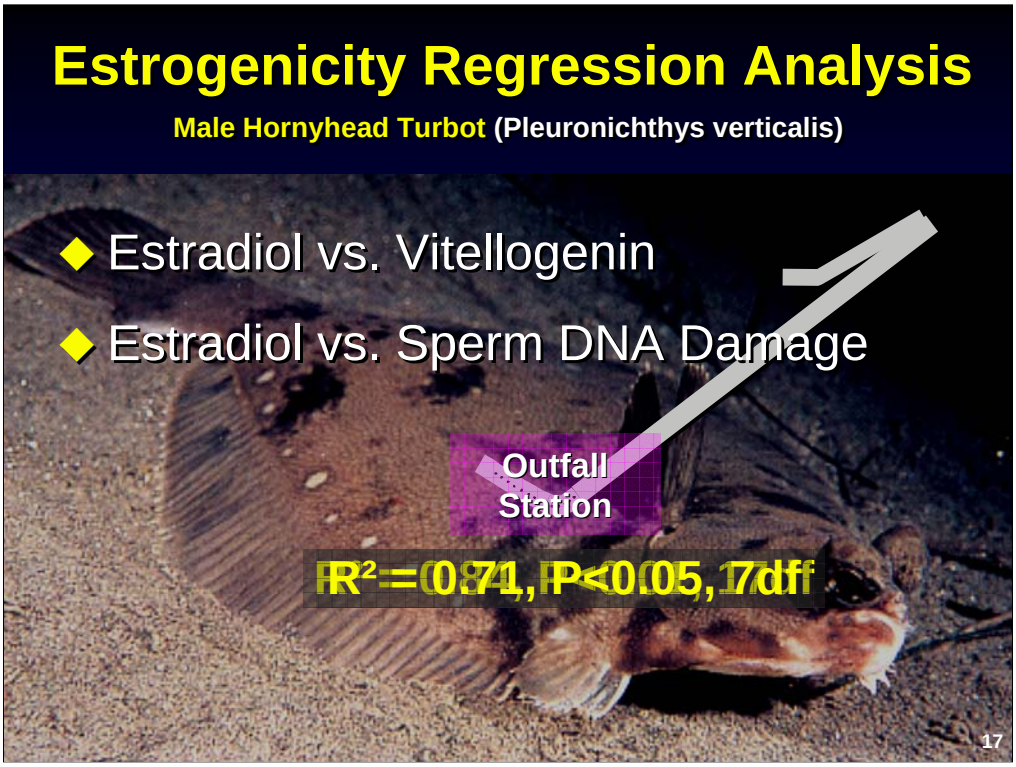
Estrogenicity

Male Hornyhead Turbot (Pleuronichthys verticalis)



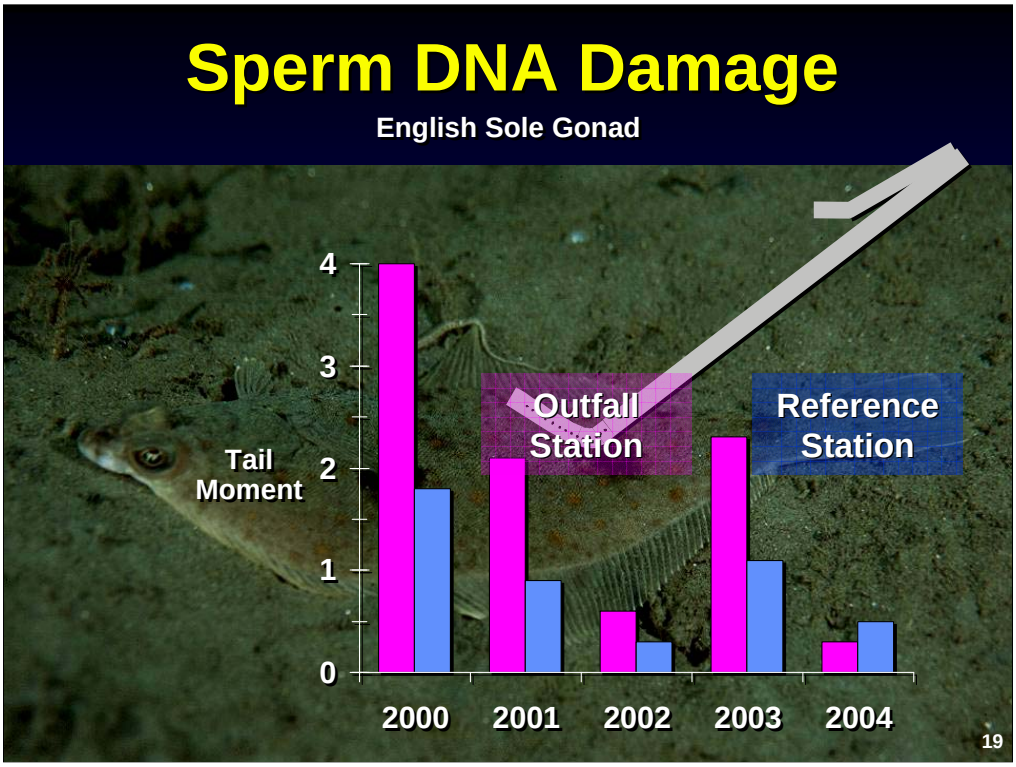


no significant relationship in reference males

Sperm DNA Damage

- Studies conducted 2000–2004
- Comet Assay
- DNA damage consistently greater at Outfall Station over Reference Station

18


Sperm DNA Damage
English Sole Gonad
Tail Moment
4
3
2
1
0
Outfall Station
Reference Station
2000
2001
2002
2003
2004
19

Parasites of Marine Fishes Associated with Wastewater Discharges in the Southern California Bight

- Julianne Kalman's doctoral dissertation at UCLA
- Does parasitization affect fish stress levels hormone levels?
  - An aside to her dissertation done with Dr. Kevin Kelley (CSULB)

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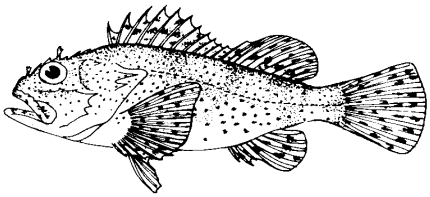
Scorpaena guttata
(California scorpionfish)



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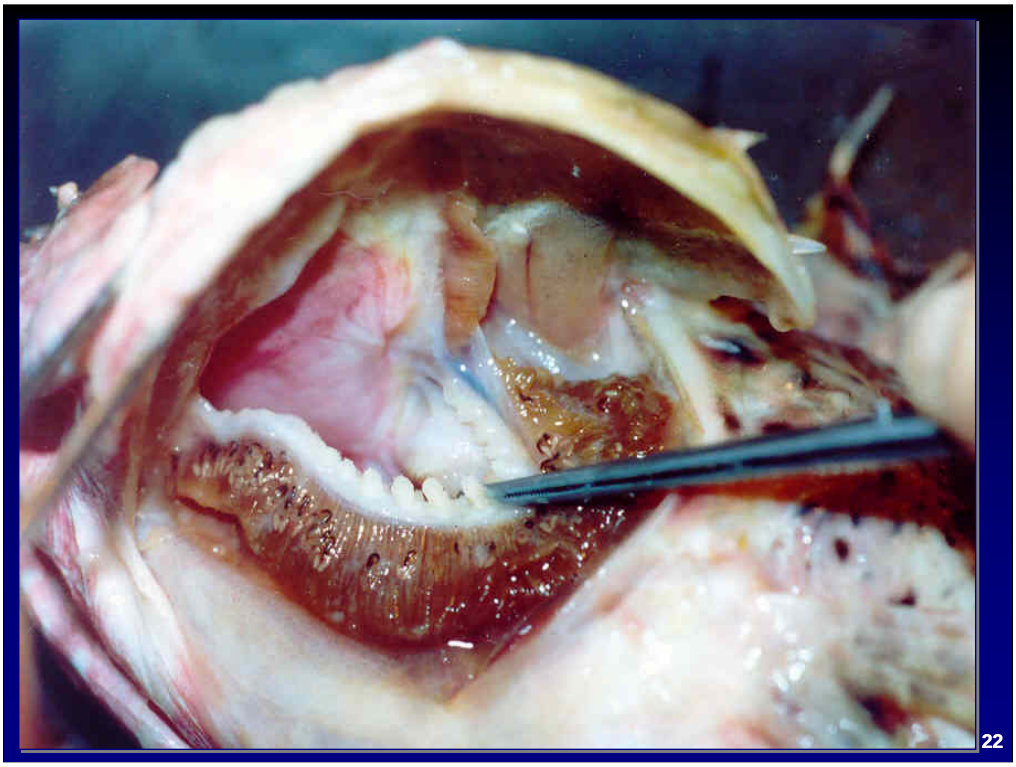
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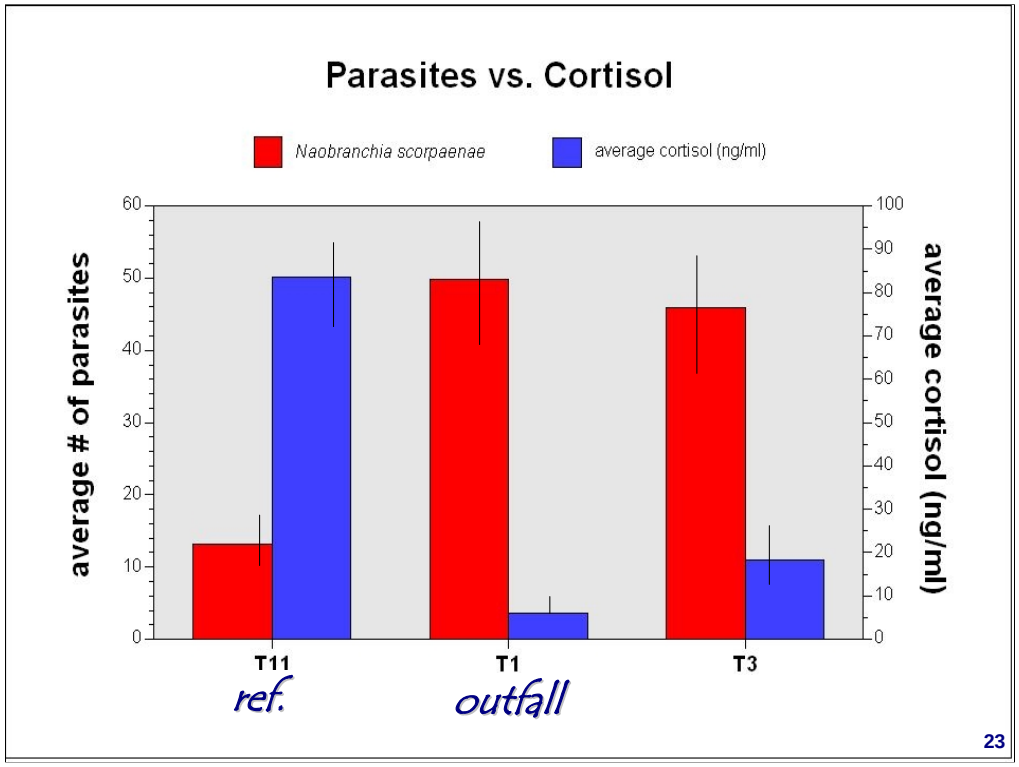
Parasites vs. Cortisol
Naobranchia scorpaenae
average cortisol (ng/ml)
average # of parasites
average cortisol (ng/ml)
0
10
20
30
40
50
60
0
10
20
30
40
50
60
70
80
90
100
T11
T1
T3
ref.
outfall
23

Conclusions of EDC/Stress Studies to Date

- Indications of Feminization
  - Increased [vitellogenin] in males – both species
  - Increased sperm DNA damage in HT
- Indications of Masculinization
  - Increased male GSI – both species
  - Higher proportion of male HT
- Stress and Growth Factor Hormone Production Inhibited at the Outfall

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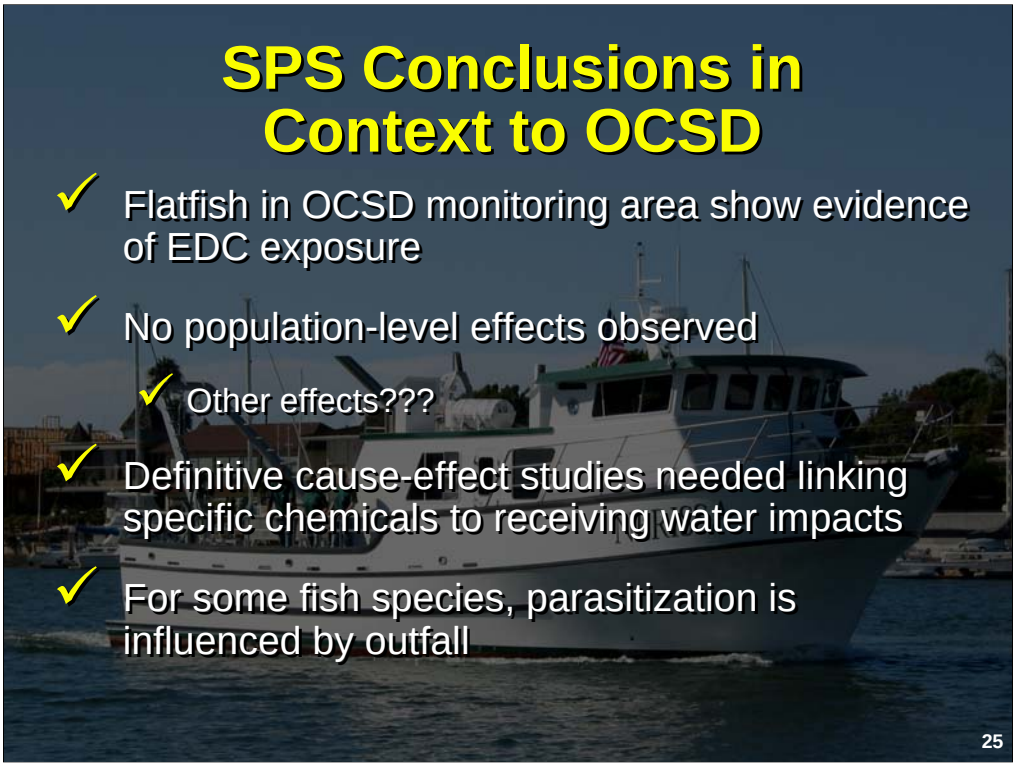

For over 30 years (since discharging at the 120” ocean outfall in 1972), our monitoring and special study data have consistently shown that the we are, and have been, protective of the coastal ocean environment and human health.

Studies in Progress

- Estrogenicity Source Identification
  UC Riverside, Doctoral Student Research
- Correlation of EDCs in Fish Tissues to POTW Effluent, Sediments, and Infauna (Food)
  CSU Long Beach, OCSD, and City of LA
- Cortisol Inhibition and Fish Parasitization
  CSU Long Beach
- Endocrine Disruption in Coastal Flatfish
  SCCWRP, OCSD, LACSD, CLAEMD, CSD, UC Riverside, CSU Long Beach, UC San Diego

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Acknowledgements

- CSU Long Beach, CA
  Dr. Kevin Kelley Professor of Endocrinology
  Dr. Julianne Kalman Post Doctoral Researcher, Lecturer
  Jesus Reyes Masters Student
  Kathy Sak Laboratory Technician

- U.C. Riverside, CA
  Dr. Dan Schlenk Professor of Aquatic Toxicology
  Dr. Luke Roy Masters Graduate (UCR)
  Mary Ann Irwin Doctoral Candidate

- Computer Sciences Corp., San Diego, CA
  Scott Steinert

Jeff Armstrong
(714) 593-7455

jarmstrong@ocsd.com

Visit Our Website: www.OCSD.com

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Toxic Effects of Selective Serotonin Reuptake Inhibitors (SSRIs) on Aquatic Organisms

Marsha C. Black, PhD
Dept. of Environmental Health Science
College of Public Health
University of Georgia
Athens, Georgia, USA

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Outline

- SSRIs - MOA and clinical significance
- Presence in the environment
- Study objectives
- Results and Discussion
  - Acute toxicity (macroinvertebrate, fish)
  - Chronic effects (macroinvertebrate, fish, frog)
- Summary and conclusions
- Future research directions

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Selective Serotonin Reuptake Inhibitors (SSRIs)

- Treat clinical depression, obsessive-compulsive and panic disorders, PMS, etc.
- Clinical MOA: block serotonin reuptake
- Examples:
  - Fluoxetine (Prozac® and Sarafem®)
  - Sertraline (Zoloft®)
  - Citalopram (Celexa® and Lexapro®)
  - Fluvoxamine (Luvox®)
  - Paroxetine (Paxil®)

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Pharmaceuticals are therapeutic agents prescribed in both animal and human health and consist of multiple classes of drugs, including antibiotics, hormones, endocrine disruptors and antidepressants, with a variety of chemical structures and mechanisms of action.

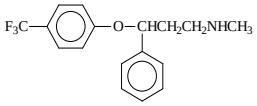
Original sources include precriptions (H+V) and

Of greatest concern however, are the [hormonally active] compounds, which have the potential to cause physiologic imbalances to non-target organisms;

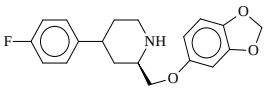
Sources originate from excretion and didposal of pharmaceutic products, including human prescription and OTC drugs, vet drugs including those used as feed additives in livestock and aquaculture operations

Concern because these drugs are designed to have a specific biological effect, usually at low conc.

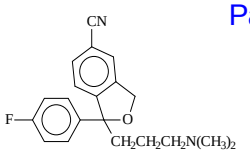
SSRI Structures



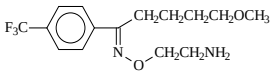
Fluoxetine (Prozac®)



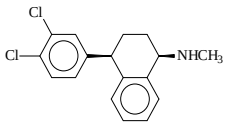
Paroxetine (Paxil®)



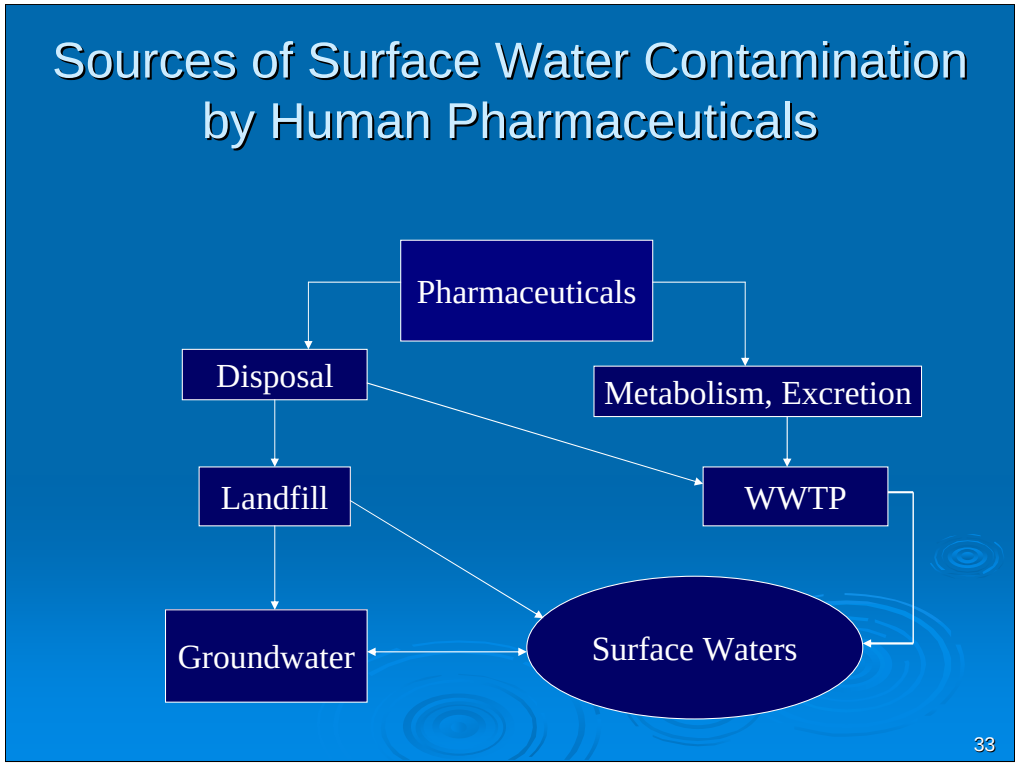
Citalopram (Celexa®)



Fluvoxamine (Luvox®)



Sertraline (Zoloft®)



Human Pharmaceutical compounds basically have two pathways by which they can contaminate the aquatic ecosystem. They can be taken as prescribed, where they enter the WWT facilities as mixtures of metabolites and parent compound, pass through the treatment process and become part of the effluent into surface waters.

Conversely, they can be discarded into the trash or flushed down the toilet, as the parent compound. This pathway allows pharmaceuticals to enter the landfill and become a constituent of the runoff into surface waters or to enter surface waters through wastewater effluents, analogous to the metabolic route.

SSRIs: Detection in the Environment

- Fluoxetine detected in surface waters
  - 0.012 ppb detected in USGS reconnaissance study (Kolpin et al. 2002)
  - 0.030-0.099 ppb in Canada (Metcalfe et al. 2003)
  - 0.031-0.076 ppb in Mississippi (Kwon and Armbrust, unpublished)
- Fluoxetine, sertraline and metabolites detected in fish tissues (Brooks et al., 2005)

Physicochemical Properties of SSRIs

(data from Kwon and Armbrust)

Compound	Log K_{OW}[a]	Log K_{OC}[b]	Photolysis $t_{½}$[c] (d)
Citalopram	1.39	5.63	39
Fluoxetine	1.22	4.65	122
Fluvoxamine	1.21	3.82	0.57; 29
Paroxetine	1.37	4.47	0.67
Sertraline	1.37	4.17	23

[a]Measured on salt form

[b]Average calculated from experiments with 5 different soils and sediments

[c] Average calculated from experiments with 2 different lake water samples

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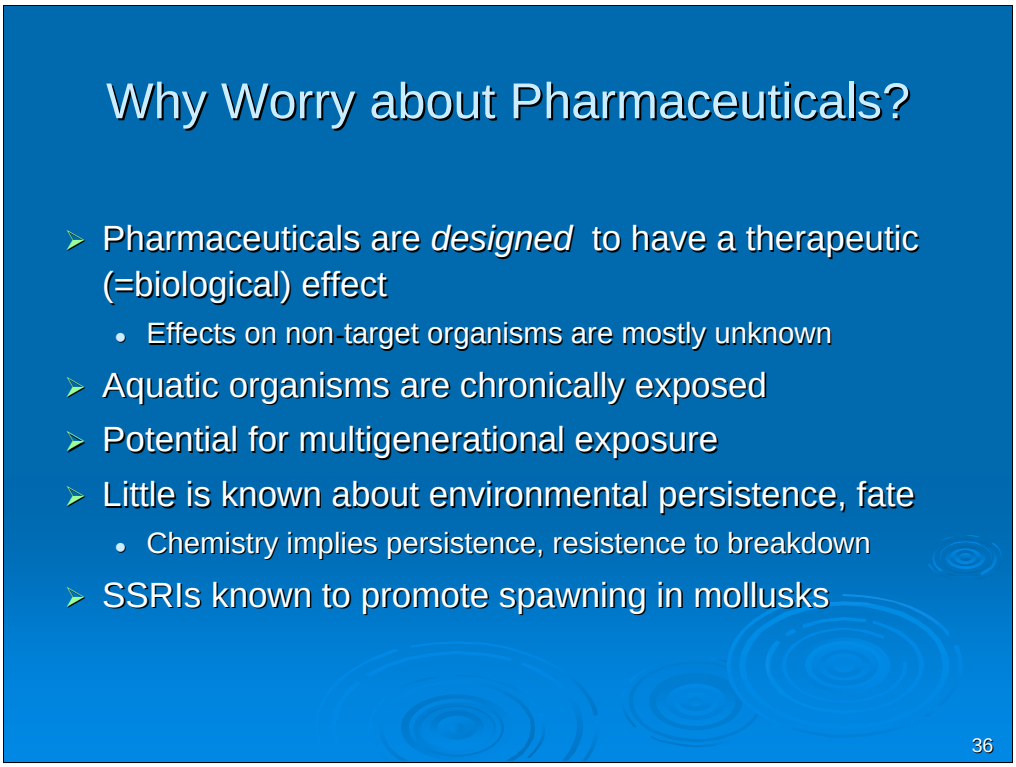

If pharmaceuticals are present at such low concentrations in surface water, then why are we concerned about them?

The main reason is because they are designed to have a biological effect and can be hormonally active, even at very low concentrations.

Since these compounds are present at such low concentrations, we aren't really concerned about acute toxicity, but rather effects that are the result of chronic exposure.

Since pharmaceuticals are entering streams on a regular basis through sewage treatment effluent, exposure is ongoing and would affect multiple generations of aquatic organisms.

Pharmaceuticals also have the potential to effect organisms at all stages of the life cycle.

Overall Research Plan…

- Determine environmental fate of SSRIs
  - Techniques used for pesticide registration
  - Measure hydrolysis, photolysis, metabolism, etc.
- Measure parent and major degradation products
  - Wastewater effluent
  - Downstream receiving water
- Determine acute, chronic impacts to aquatic organisms
  - *Ceriodaphnia dubia* (macroinvertebrate)
  - *Gambusia affinis* (Western mosquito fish)
  - *Xenopus laevis* (frog)

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Toxicity Tests

- Test organism: *Ceriodaphnia dubia*
- Acute toxicity (48 h)
  - Single compound exposures
  - Binary, quaternary mixture exposures
  - Mortality (LC50) as endpoint
- Chronic toxicity
  - 7 day mini-chronic test
  - Brood size, # broods as endpoints
- All tests followed US EPA protocols

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Acute Toxicity (LC50) of SSRIs

SSRI	LC50 ppb[a]
Citalopram (Celexa®)	3180 (220)
Fluvoxamine (Luvox®)	1260 (830)
Paroxetine (Paxil®)	470 (60)
Fluoxetine (Prozac®)	590 (130)
Sertraline (Zoloft®)	140 (20)

[a]Mean (± SD) of 3 tests

Henry et al. 2004, *Environ Toxicol Chem* 23:2229-2233

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Chronic Toxicity of SSRIs

SSRI	NOEC[a] (ppb)	LOEC[a] (ppb)
Citalopram (Celexa®)	800	4000
Fluvoxamine (Luvox®)	366	1466[b]
Paroxetine (Paxil®)	220	440[b]
Fluoxetine (Prozac®)	89	447[b]
Sertraline (Zoloft®)	9	45

[a]Total number of neonates produced over 7-8 d

[b]Number of broods also significantly reduced

(Henry et al. 2004, *Environ Toxicol Chem* 23:2229-2233)

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Acute Toxicity of Fluoxetine to Western Mosquitofish

- 7-d acute tests
- Endpoints:
  - Mortality (LC50)
  - Fish behavior

Western mosquitofish
Gambusia affinis

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Acute Toxicity of Fluoxetine to Western Mosquitofish

- Mortality
  - 7-day LC50 = 614 ppb
- Behavioral effects (0.6 and 6 ppb)
  - Uncoordinated swimming
  - Lethargy, lack of response to stimuli
  - Less aggression, interaction between individuals

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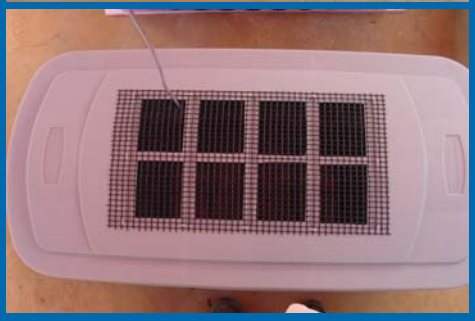
Chronic Exposures in Outdoor Mesocosms



- 110-L plastic tanks
- 50 fish/tank
- 85-d exposure
- Water change 1x/wk

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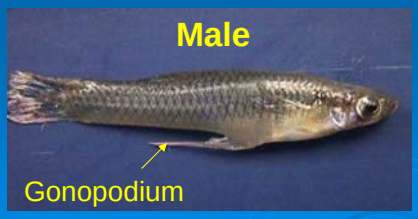
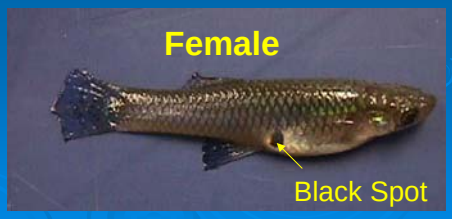
Chronic Tests (140 d) with Mosquitofish

- Time to reproductive maturity
  - Fully developed gonopodium (males)
  - Formation of black spot (females)
- Histological effects on gonads?





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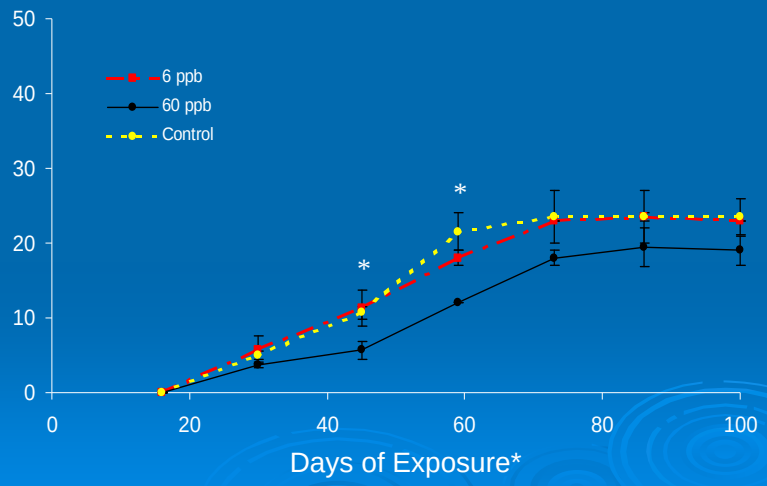
Effect of Fluoxetine on Male Sexual Development



*Fish were 39-d old at t 0

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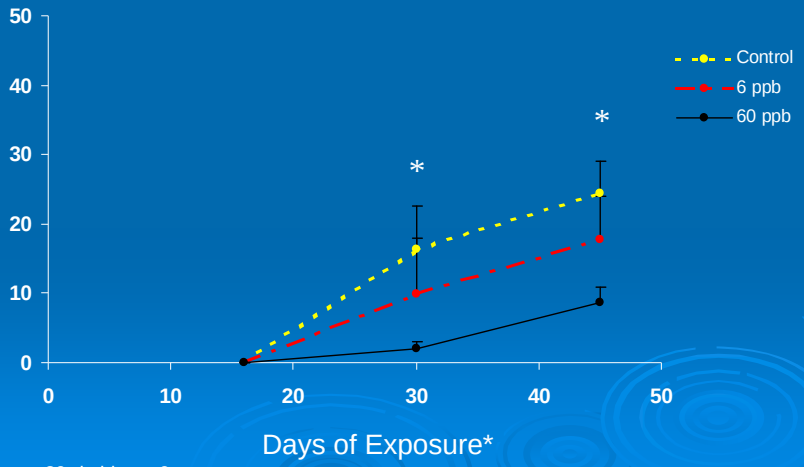
Effect of Fluoxetine on Female Sexual Development



*Fish were 39-d old at t 0

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Research with the African Clawed Frog (*Xenopus laevis*)

- Easy to breed in the lab
  - Inject with HCG
- Tadpole to frog in 60-70 d
- Many measurable endpoints
  - Mortality
  - Developmental malformations
  - Time to metamorphosis

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And the final stage is called metamorphic climax.

Thyroid that were increasing during prometamorphosis reach peak levels during the climax stage.

This period is characterized by rapid morphological change.

The tadpole stops eating, the forelimbs emerge, and the tail starts to be resorbed into the body.

When the tail has been fully resorbed, metamorphosis is complete and the animal becomes a juvenile frog.

Why Study Frogs?

- Thyroid hormones (T_3,T_4) cue metamorphosis
- Tadpoles with no thyroid – metamorphosis inhibited
- Exposure to chemicals that reduce circulating T_3 will delay or inhibit metamorphosis

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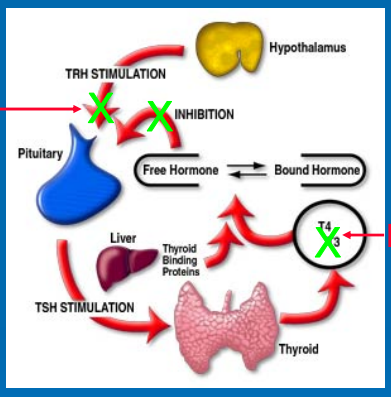
When the tail has been fully resorbed, metamorphosis is complete and the animal becomes a juvenile frog.

Regulation of Thyroid Axis in Mammals



www.dpcweb.com/images/medicalconditions/thyroid/thyroid%20illustration.jpg

- Serotonin inhibits the release of TRH from the hypothalamus in rats
  - Mitsuma et al. 1983; Mitsuma et al. 1996
- Fluoxetine reduces circulating T3 and T4; increases TSH
  - Golstein et al., 1983

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So what does fluoxetine have to do with thyriod hormones?

Thyrotropin releasing hormone causes the thyroid to produce more thyroid hormone.

Mammalian studies show that serotonin inhibits the release of TRH from the hypothalamus.

Since fluoxetine acts by increasing serotonin transmission, this drug should inhibit TRH release and therefore decrease levels of circulating thyroid hormones in tadpoles.

Does Fluoxetine Inhibit Frog Metamorphosis?

- Expose tadpoles from hatch until metamorphosis
  - Fluoxetine (FL): 0.059, 0.295, 2.95, 29.5 ppb (measured)
  - Ammonium perchlorate (AP): 10 ppb
  - Control (clean exposure water)
- Observe daily for limb development until metamorphosis is complete

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As I just discussed, increasing concentrations of thyroid hormones are necessary for metamorphosis to occur.

But if thyriod hormone levels are not high enough, metamorphosis can be delayed or even totally inhibited.

Dodd and Dodd found that tadpoles that were born without a thyroid gland never metamorphosed but continued to grow to abnormally large sizes.

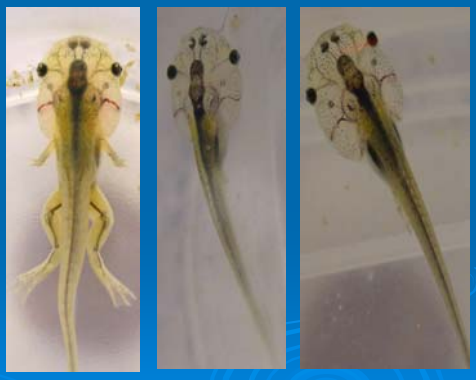
There are also several drugs that can inhibit the thyriod axis. Read them.

Ammonium perchlorate, which is an environmental contaminant, has been shown to delay metamorphosis in frogs.

Effects of Chronic Exposure to Fluoxetine (Xenopus)

- Developmental delays
  - Forelimb formation
  - Tail resorbtion
- Increased time to metamorphosis
- Mortality

Tadpoles at 57 d*



Control 38 ppb FL 9.5 ppb AP

*Data from range-finder experiment. Similar effects at 29.5 ppb in 2nd experiment.

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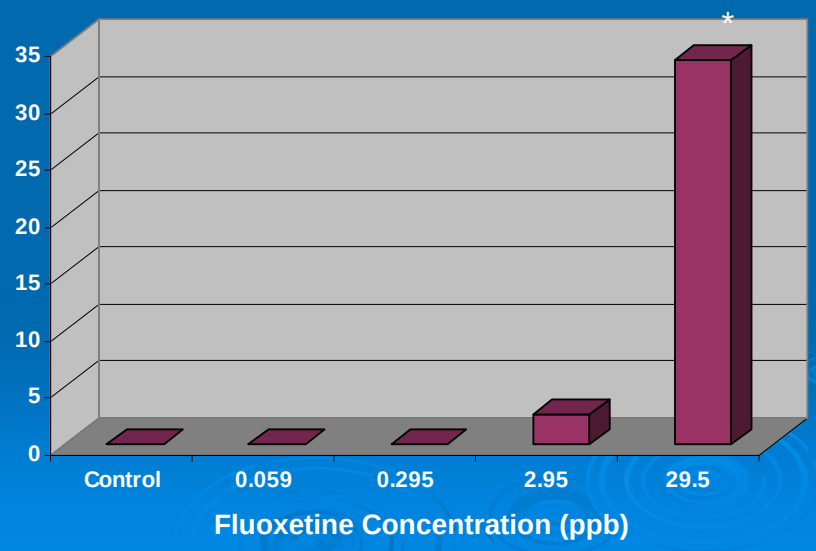
Effect of Chronic Exposure to Fluoxetine on Time to Metamorphosis



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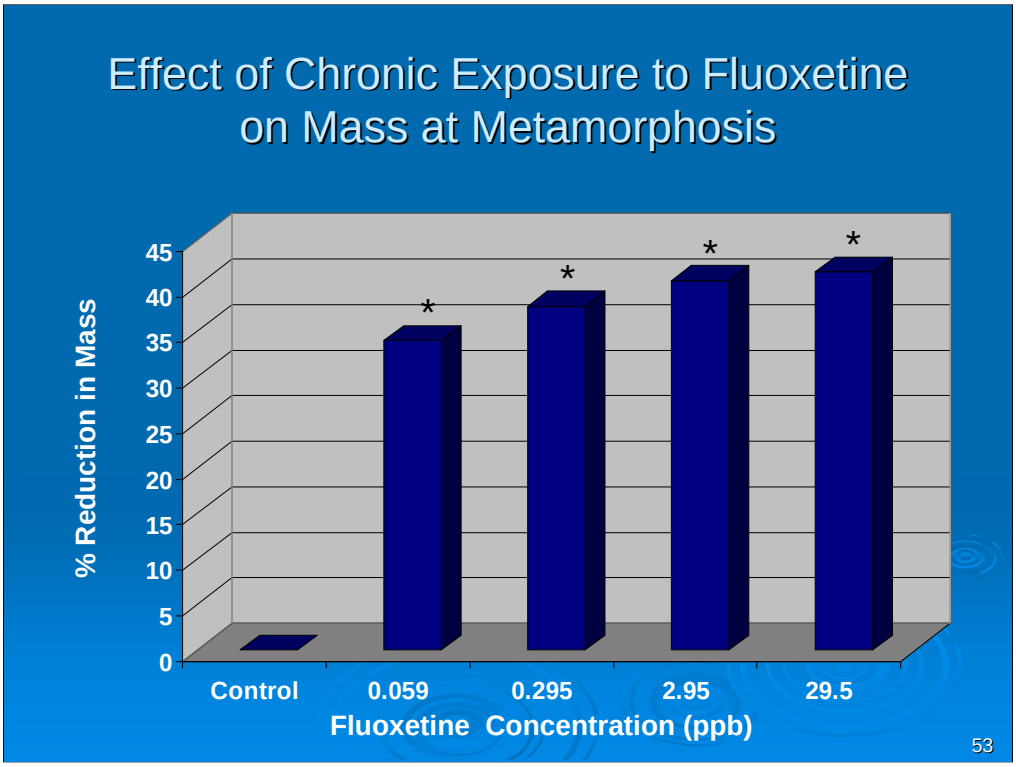
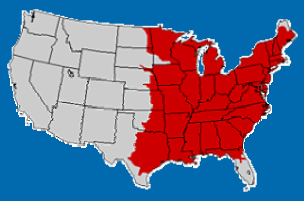

Effect of Chronic Exposure to Fluoxetine on Mass at Metamorphosis
% Reduction in Mass
45
40
35
30
25
20
15
10
5
0
*
*
*
*
Control
0.059
0.295
2.95
29.5
Fluoxetine Concentration (ppb)
53

Exposure with Hyla chrysoscelis



- Cope's gray tree frog
- Native to eastern US
- Predator-induced coloration
  - Bright orange coloration on tail fin
    - Directs strikes away from body
- Predatory stress
  - Stress alone decreases activity
  - Stress + carbaryl increased mortality
    - Relyea et al. 2001

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Laboratory Exposure Methods

- Egg masses collected from field
- Exposure: Gosner stage 25 - Metamorphosis
- Fluoxetine: 0, 0.10, 0.15, 0.20 ppb
  - ± Predator treatment
    - 10 ml of water from dragonfly holding tanks
- Individually exposed in 1 L of solution
  - n = 13 tadpoles per treatment; N = 104
- Static renewal
  - 100% renewal of solutions
  - Fed 3:1 mixture of rabbit chow & Tetramin

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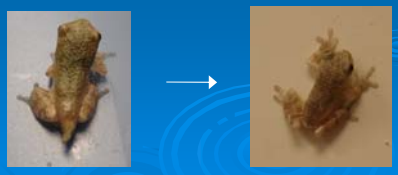
Endpoints Measured

- Mortality
- Malformations
- Time to forelimb emergence
- Time to completion of metamorphosis
- Mass and Length
  - Day 14; metamorphosis

Forelimb Emergence



Tail Resorbtion

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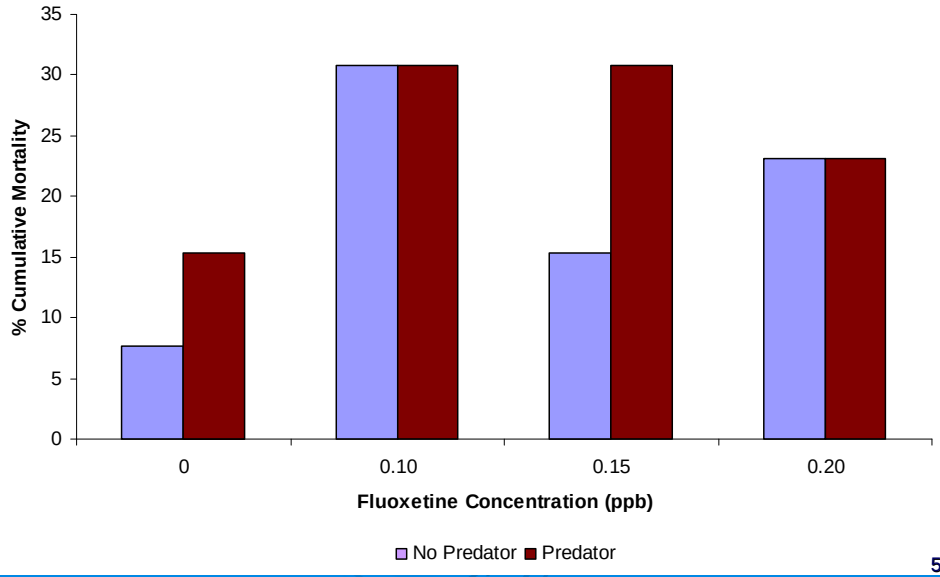
Cumulative Mortality of *H. chrysoscelis*



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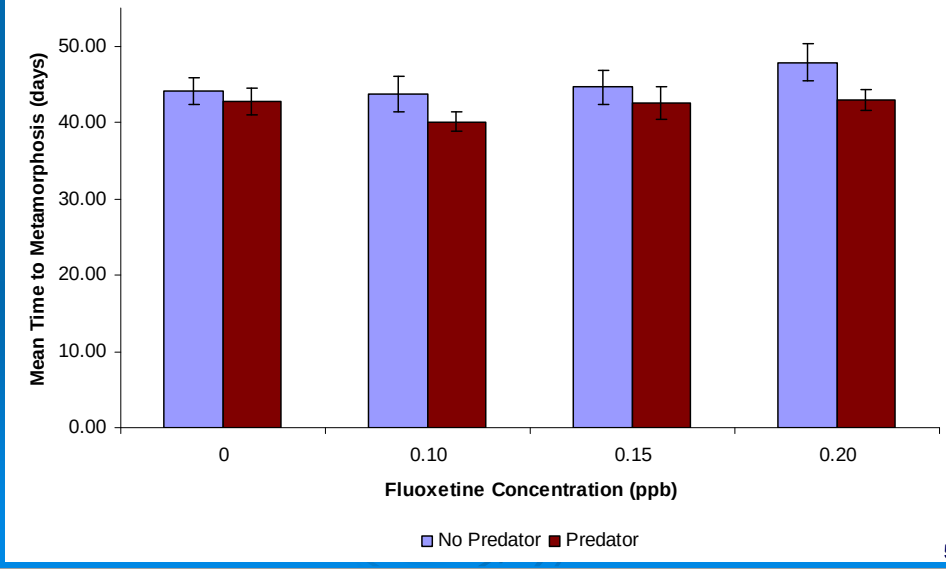
Time to Metamorphosis for *H. chrysoscelis*



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Discussion

- No significant effect of FL on *H. chrysoscelis*
- No significant difference ± predator treatment
  - Fright response dependent on tadpole consumption by dragonflies?
- *H. chrysoscelis* appears less sensitive to fluoxetine than *X. laevis*

Conclusions (so far…)

- SSRIs are acutely toxic to *Ceriodaphnia* and mosquitofish
- Fluoxetine affects fish behavior
- Fluoxetine delays sexual development in fish
- Fluoxetine delays development and metamorphosis in *X. laevis*

None of these effects observed at environmentally-relevant concentrations.

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Conclusions (cont'd)

- Reduced mass with chronic exposure to FL
  - *X. laevis* only

Occurred at environmentally relevant concentrations

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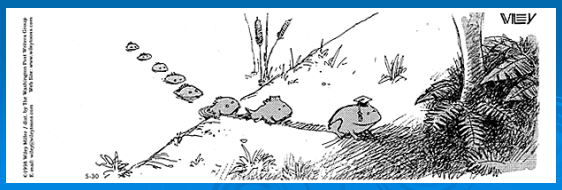
Implications of the Research

- Behavioral effects (lethargy in fish)
  - ↑ Predation, ↓ reproductive success, population decline?
- Delayed development (fish, frogs)
  - ↑ Predation, dessication (frogs), population decline?
- Reduced mass and limb malformations (frogs)
  - ↑ Predation, ↓ reproductive success, population decline?



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So we found that exposure to fluoxetine delays metamorphosis. Why is this important?

Tadpoles of course are a source of food for fish and other predators. Tadpoles that take longer to metamorphose and enter the terrestrial environment are subject to becoming fish food.

Most eggs are laid in ephemeral ponds or wetlands. If metamorphosis is delayed, tadpoles may die from desiccation before they have time to complete metamorphosis.

Death by predation and desiccation both decrease recruitment to the terrestrial environment. If the delay in metamorphosis is significant enough or if exposure occurs over multiple generations, population declines may eventually result.

Future Research Questions Generated by Research

- Conduct additional SSRI exposures with *Xenopus*
- Validate apparent impact of FL on the thyroid axis by measuring TH, TSH during frog development (+/- FL)
- What is the toxicity of mixtures of SSRIs in the amphibian model?
- What are environmentally-relevant SSRI concentrations?

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I would like to thank the College of Agriculture and Environmental Sciences for providing the funding for my research.

My research advisors, Dr. Theodore Henry and Dr. Marsha Black

Patricia Smith, Deanna Conners and Emily Rogers for their lab assistance

And Drs. Armbrust and Wook-Kwon of Mississippi State for providing chemical analyses.

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Thank You

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Links to Additional Resources

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