

Effects of Natural Steroids from Poultry Litter on Aquatic Organisms

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Funding Sources:

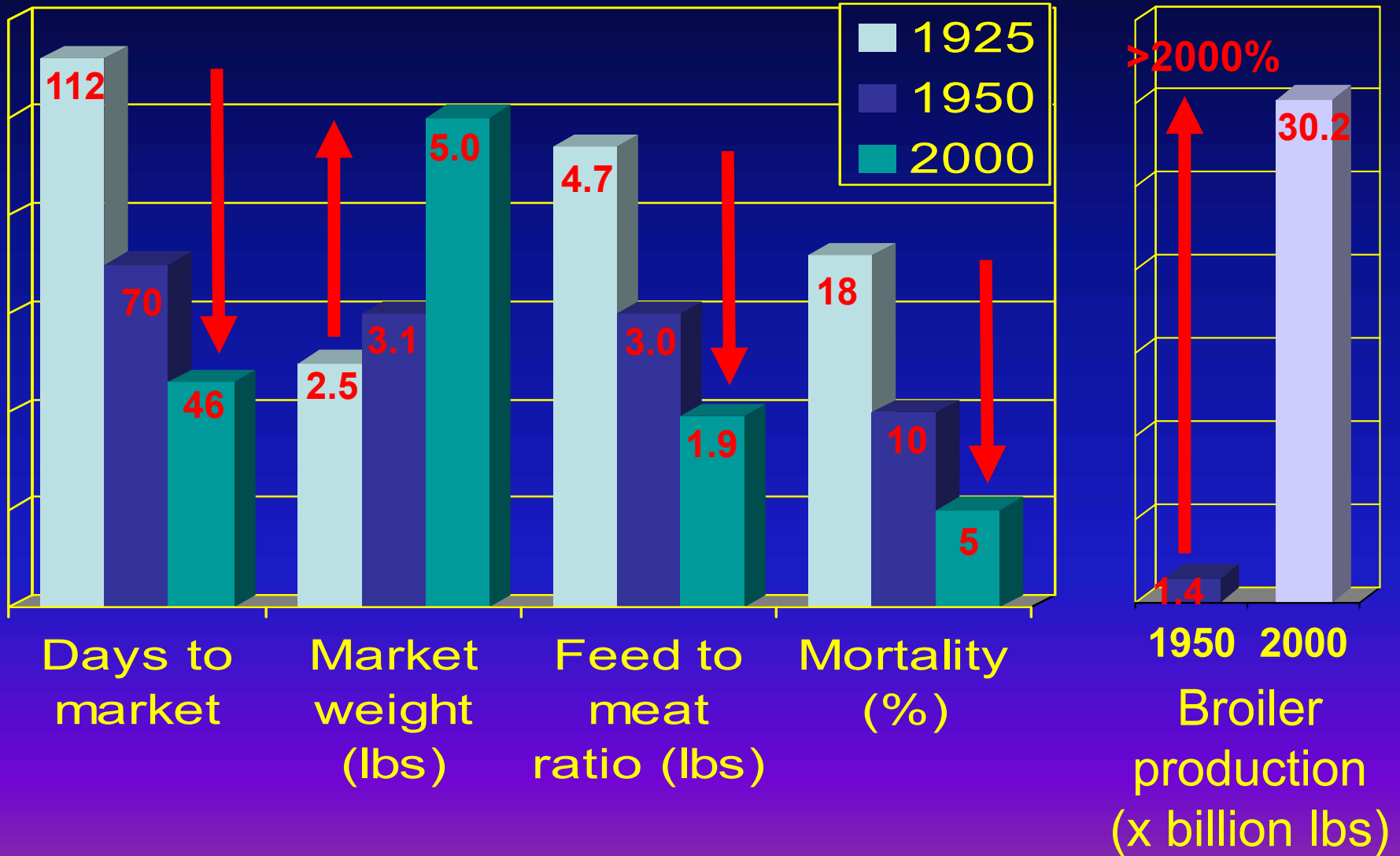
Current Funding

- **US EPA STAR Grant (2007-2010)**
 - Converted to a EPA Cooperative Agreement to expand research effort with different EPA labs

Previous Funding

- **Harry R. Hughes Center for *Agro-Ecology*, Inc.**
 - Funding through USDA
- **NOAA National Sea Grant College**

US Poultry Facts



Delmarva Poultry Facts

- ~600 millions birds are produced annually (~7% of US total)
- ~700,000 to 800,000 tons of poultry litter annually
- Litter is predominately land applied (primarily to satisfy nutrient requirements of corn production)
- Excessive land application can have impacts on regional surface and ground water quality
- Chickens Excrete Nitrogen and Phosphorous!!!
Excessive nutrient impacts are most frequently cited pollution issue

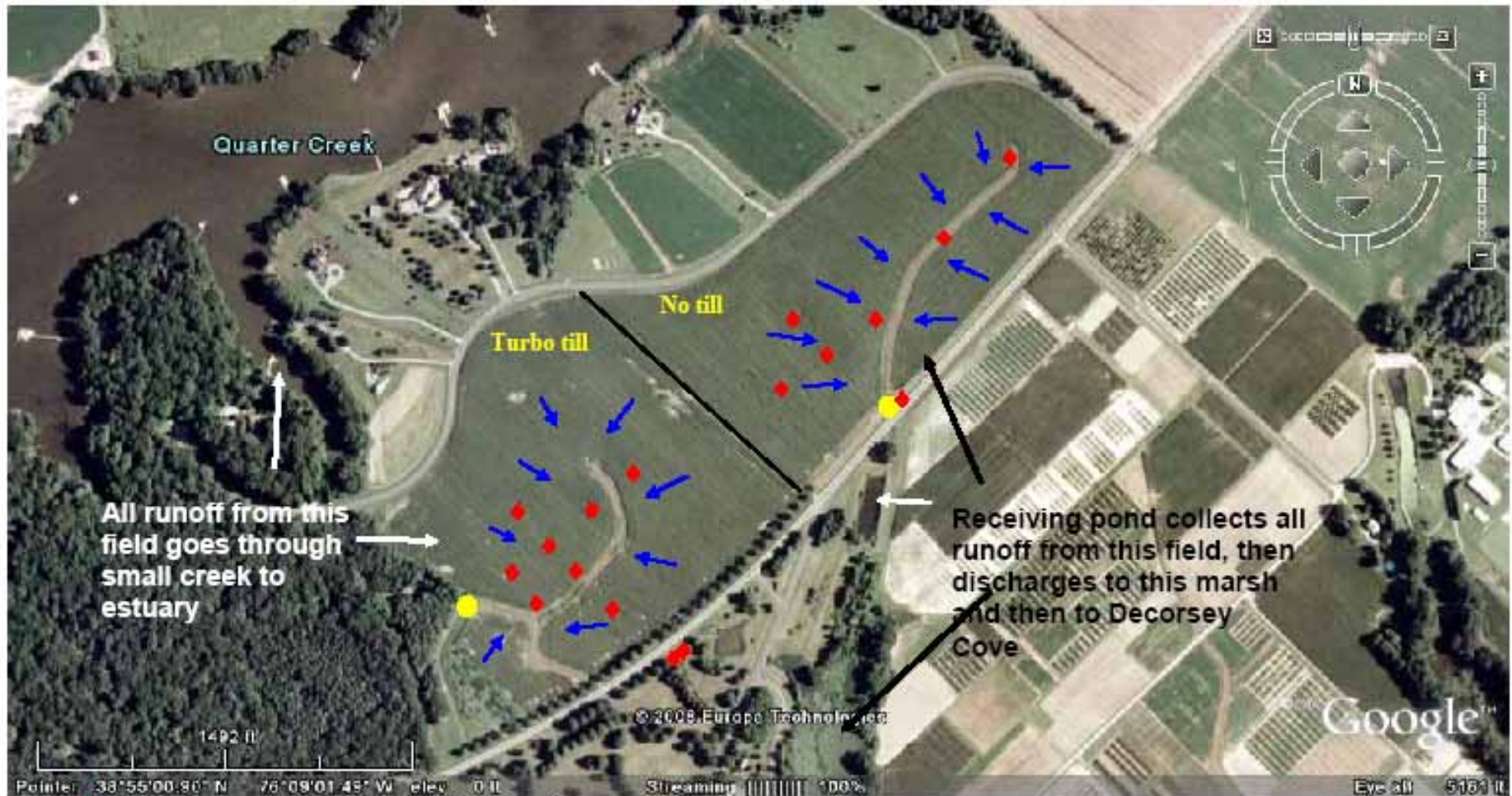
Chemical Analysis of Poultry Litter

- **Nutrients** ~ Nitrogen (4% by weight) and Phosphorous (1.5% by weight)
- **Bacterial and viral pathogens**
- **Historic & Current-Use Pesticides**
 - DDT & metabolites (~0.4 – 2.9 ng/g)
 - Lindane (0.6 – 4.6 ng/g)
 - Chlordanes (0.5 – 13 ng/g)
 - Metolachlor (3.1 – 14 ng/g)
 - λ -Cyhalothrin (3.7 – 28 ng/g)
 - Atrazine (0.3 – 1.3 $\mu\text{g/g}$)
- **PAHs** ~ Benz[a]anthracene (0.21 – 0.27 $\mu\text{g/g}$)
- **Antibiotics** ~ Chlortetracycline; Sulfadimethoxine; Lincomycin
- **Metals** ~ Copper (400 $\mu\text{g/g}$); Zinc (430 $\mu\text{g/g}$); Arsenic (29 $\mu\text{g/g}$)
- **Steroids (from 8 litter sources)**
 - 17 β -Estradiol (~ 125 ng/g dry litter); Testosterone (~50 ng/g dry litter)

Poultry Waste Issues Investigated in this Project

- STEROIDS –Emerging Contaminants of Concern - EPA, USDA, NOAA, USGS, etc. Endocrine Disruptors!
- Chickens excrete reproductive hormones, specifically estradiol, estrone and testosterone. Natural byproduct – **Not fed to Chickens.**
- Build up in chicken houses. Manure plus bedding material (**litter**) removed approximately every two years. Made available to farmers as **fertilizer**. Major disposal method.
- Free except for trucking expenses!
- Because litter is a complex mixture of contaminants we refer to exposures to aqueous litter solutions as **Poultry Litter Associated Contaminants (PLAC) exposures.**

Research Fields – 33 acre



- ◆ Groundwater wells. Study vertical then lateral movement.
- surface flow to collection channels
- Automatic runoff collection. All surface runoff passes through these points.

200 tons





Spread at 3 tons/acre



Run off from 2008 Litter Application





Aquatic Toxicology Lab

Fish Exposures – Lab and Caged – Adult & Larvae

Endpoints

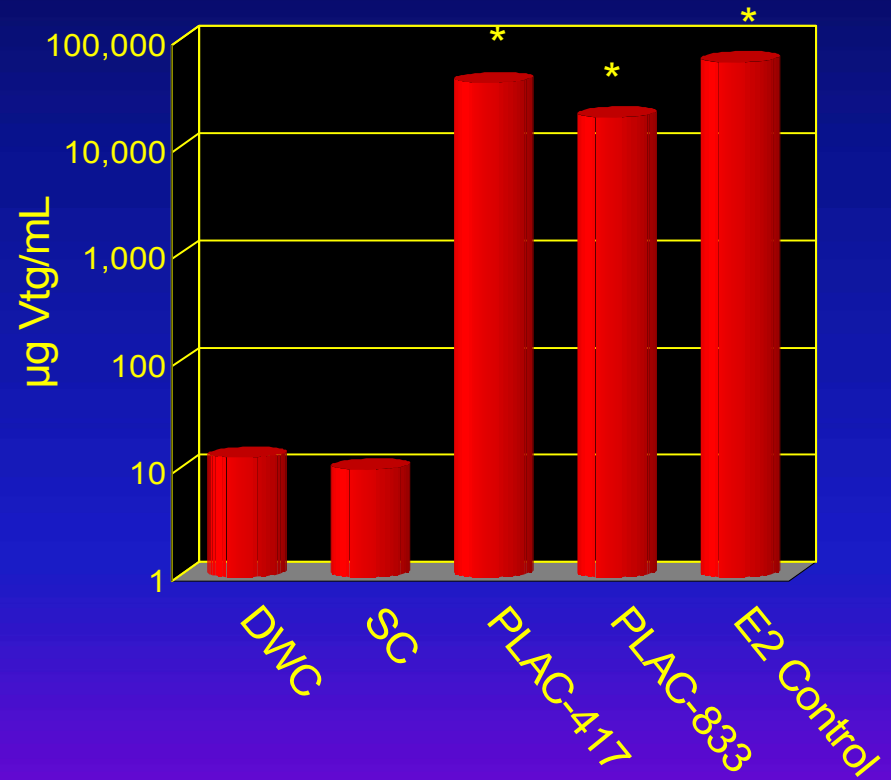
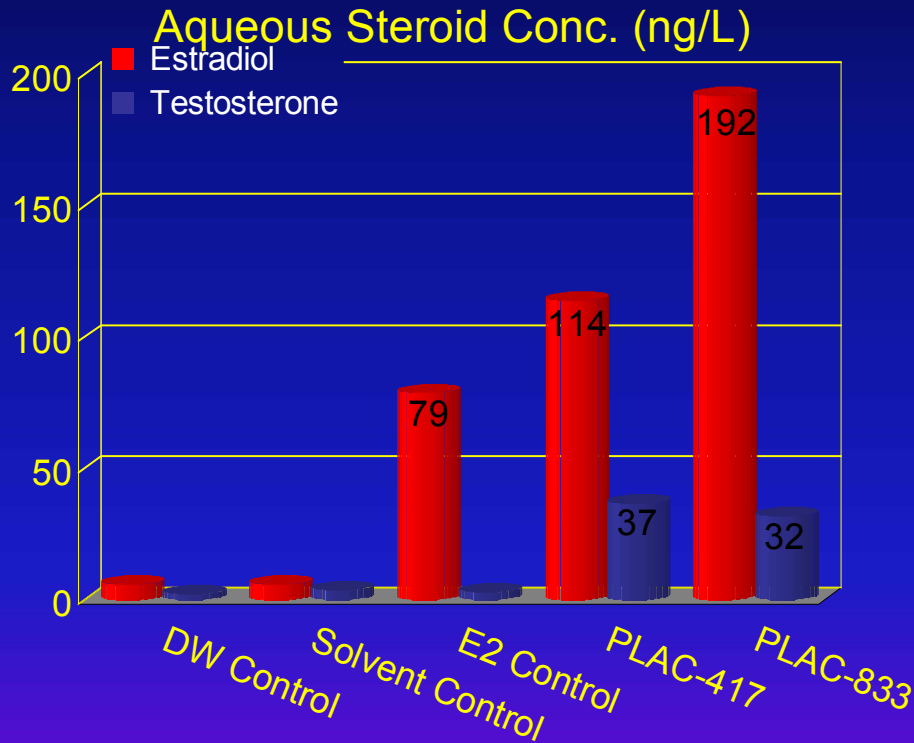
- Estrogens and Testosterone (RIA); now GC/MS/MS
- Vitellogenin (VTG) induction in males (ELISA) and gel electrophoresis - (egg yolk precursor protein should only be in females) – blood plasma level
- VTG induction (mRNA) in larval and adult. **New!**
- Estrogenicity - in-vitro estrogen-inducible reporter-gene assay - human mammary cell line
- Reproductive success during exposure
- Gender distribution of larval fish after grow out
- Gonad abnormalities (intersex) - ♂ testicular oocytes
- Nutrient & steroid runoff comparison between tillage practices – No till vs Turbo Till vs conventional till

Eastern Shore Field Collections and Fish Field Caging Experiments

Work with MD Department of Natural Resources and its Maryland Biological Stream Survey Program

- **Fish Community Structure** - Electroshock streams to determine whether fish and amphibian communities in areas known to have measurable levels of steroids have been impacted compared to reference streams.
- **Benthic Community Structure** - Collect benthic (bottom) samples to see if these communities have been impacted.
- **Largemouth bass** - VTG and/or exhibit intersex. Electroshock largemouth bass from lakes.
- **Cage adult fish** in streams and examine for VTG.

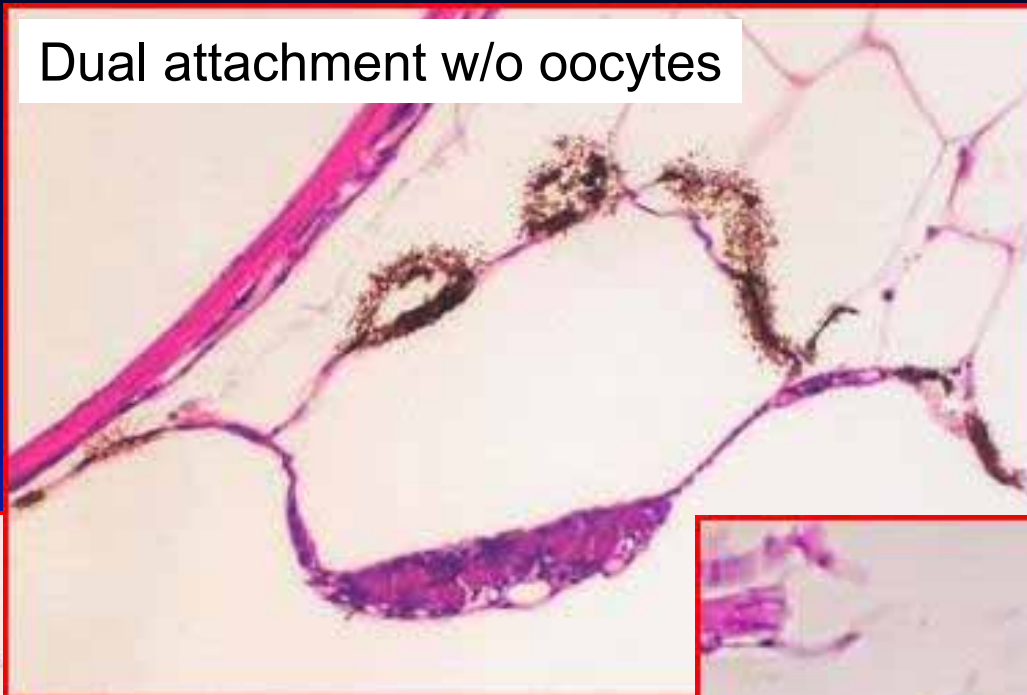
Plasma Vtg in Mature Male Fathead Minnows (21-d lab exposure)



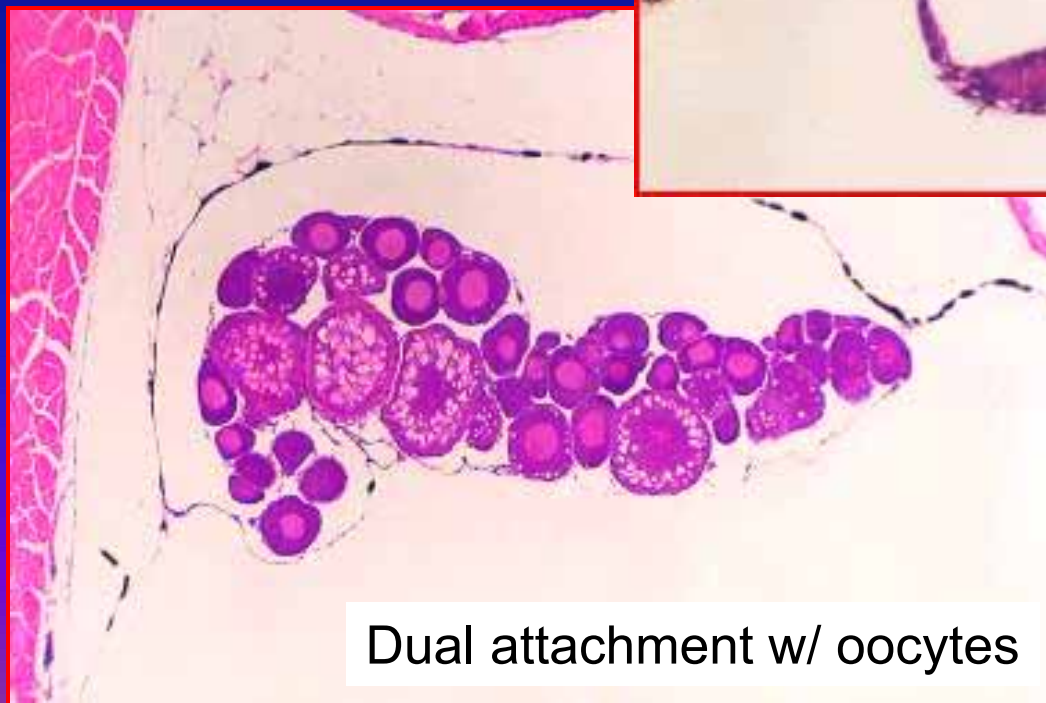
PLACs are as mg poultry litter per L test water

Gonad Development and Gender Differentiation

Dual attachment w/o oocytes



Dual attachment w/ oocytes

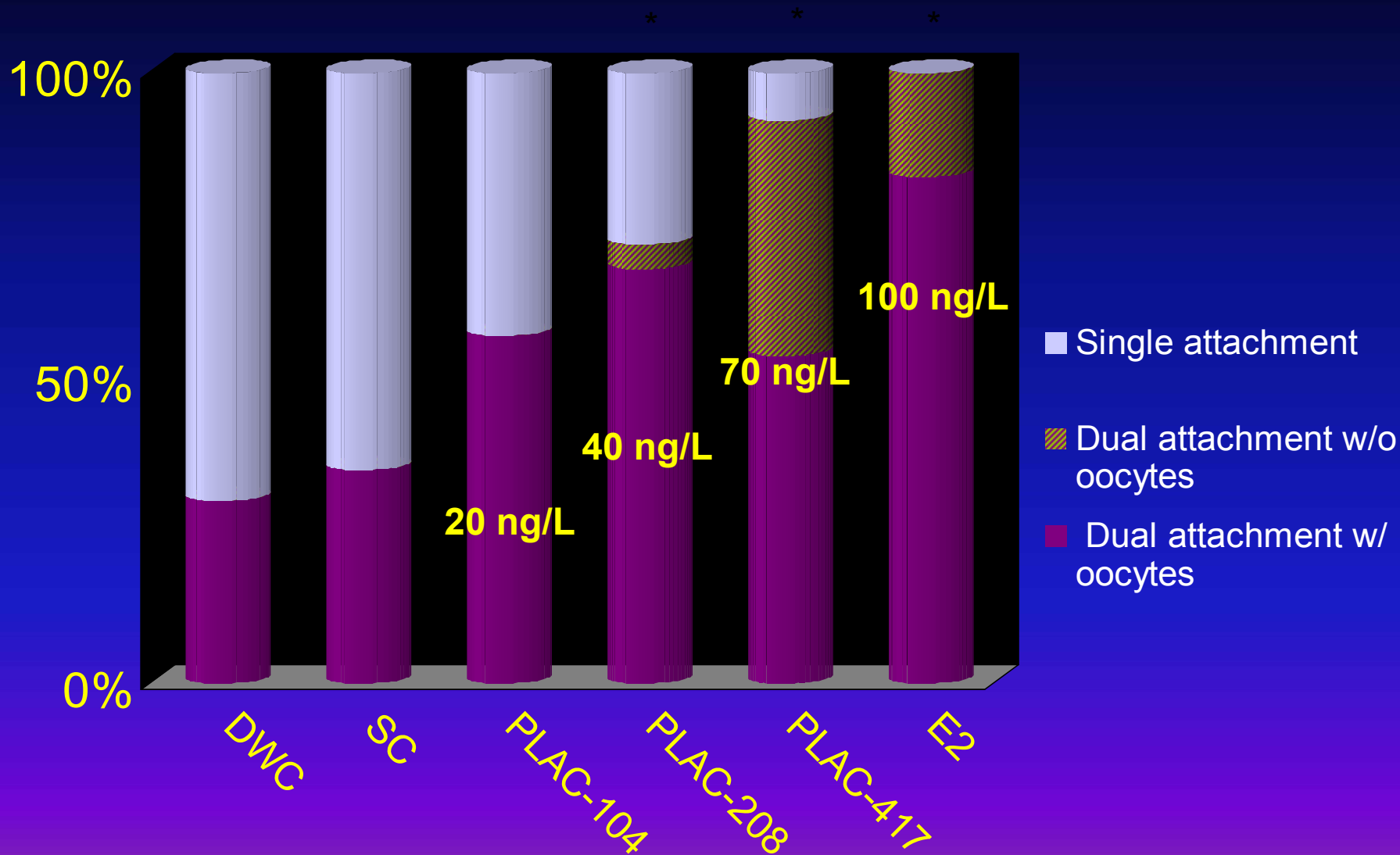


Single attachment



Gonad Development and Gender Differentiation

(Larval fatheads exposed 21-d and held to 60-dph)



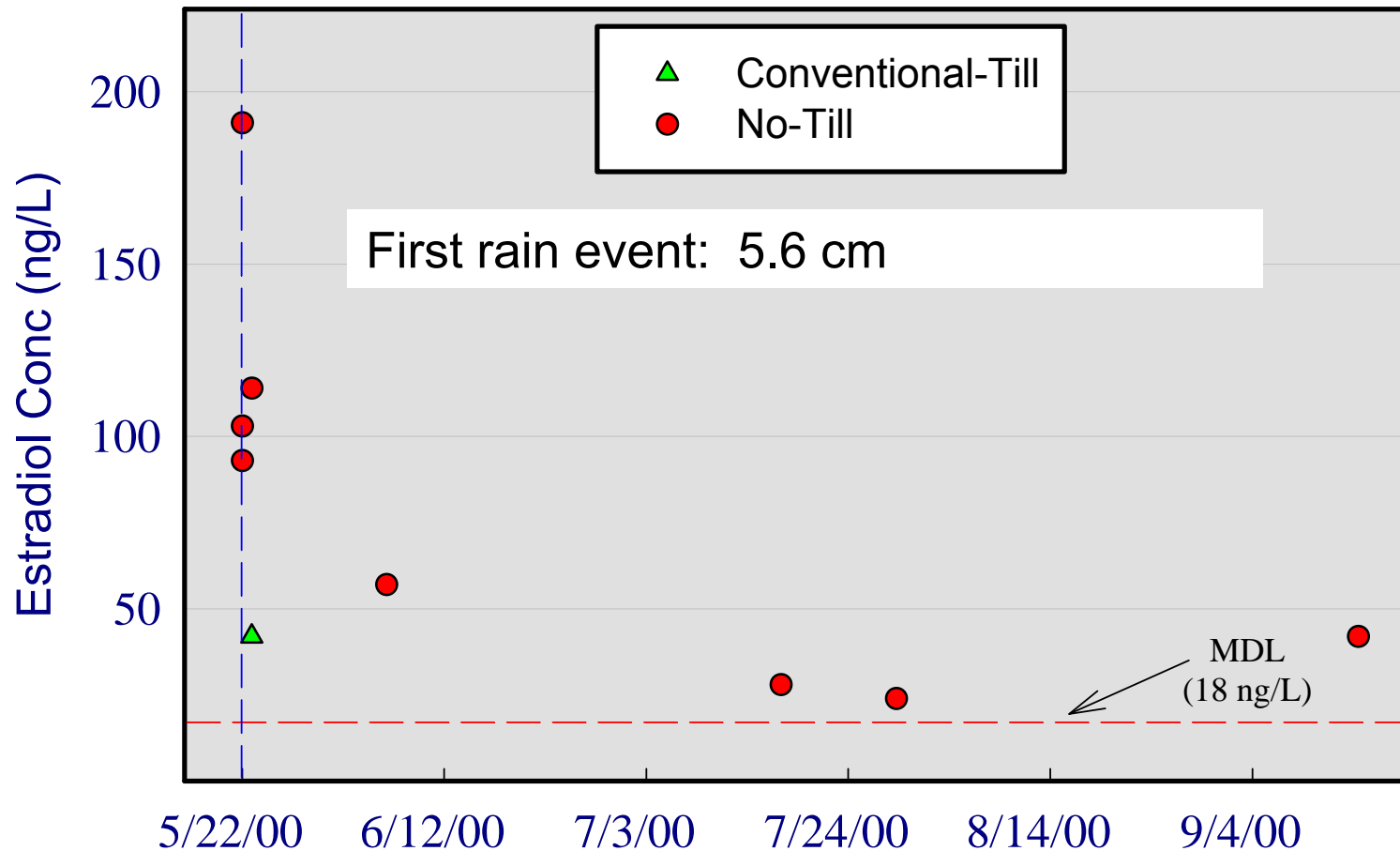
Yellow = average E2 exposure concentration

Summary of Lab Results:

- Steroids have consistently been measured in poultry litter. Estradiol and testosterone are water soluble and have been consistently measured in aqueous solutions.
- PLACs are clearly capable of inducing ED in fish
- Vitellogenesis is a sensitive indicator of poultry litter-associated estrogenicity
- Gender differentiation is equally (or possibly more) sensitive
- No intersex found
- Environmental range of concern for litter-derived E2: ~40 ng/L

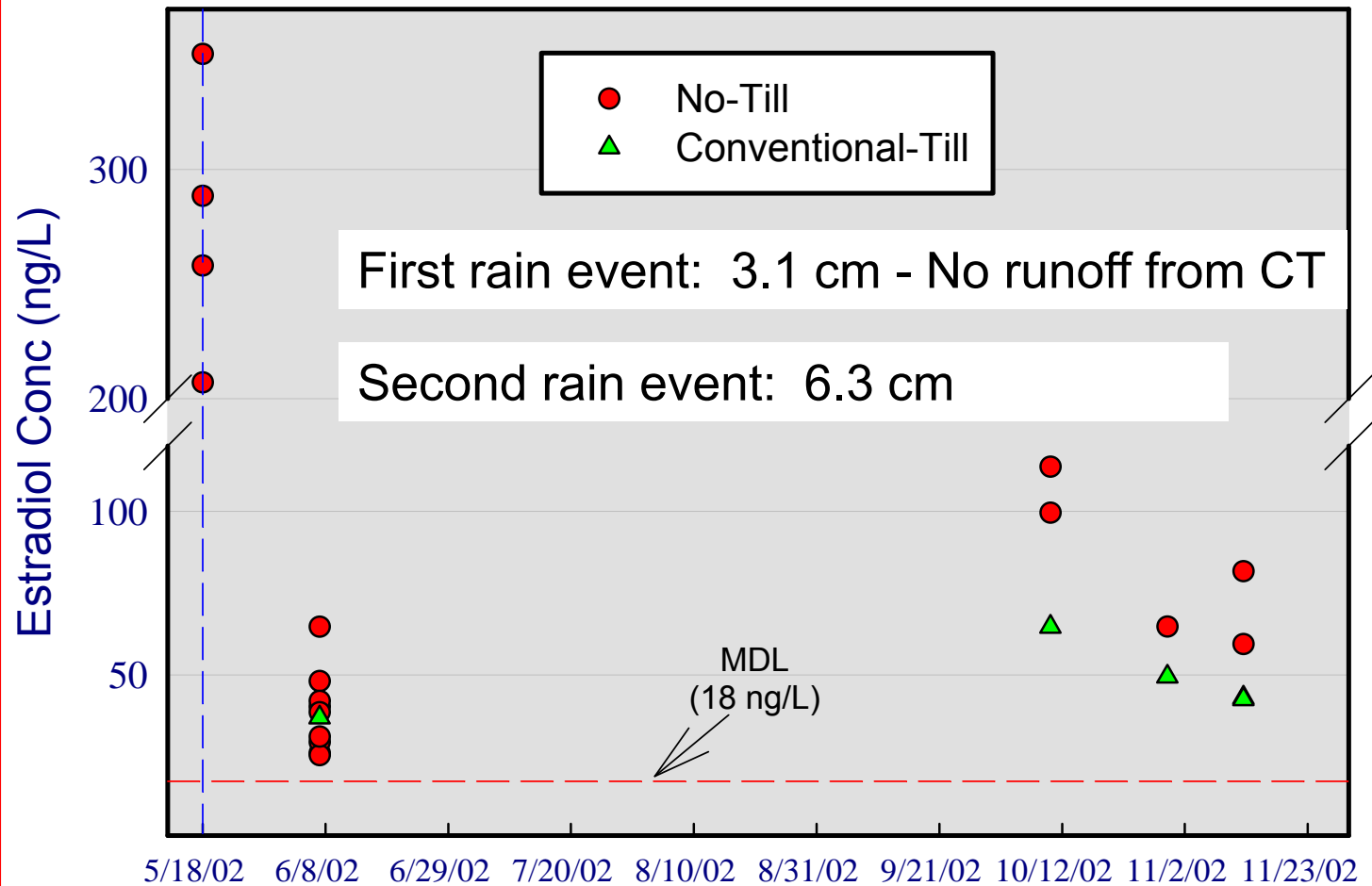
Controlled Field Investigation

Estradiol in runoff following *Spring 2000* litter application



Controlled Field Investigation

Estradiol in runoff following *Spring 2002*
litter application

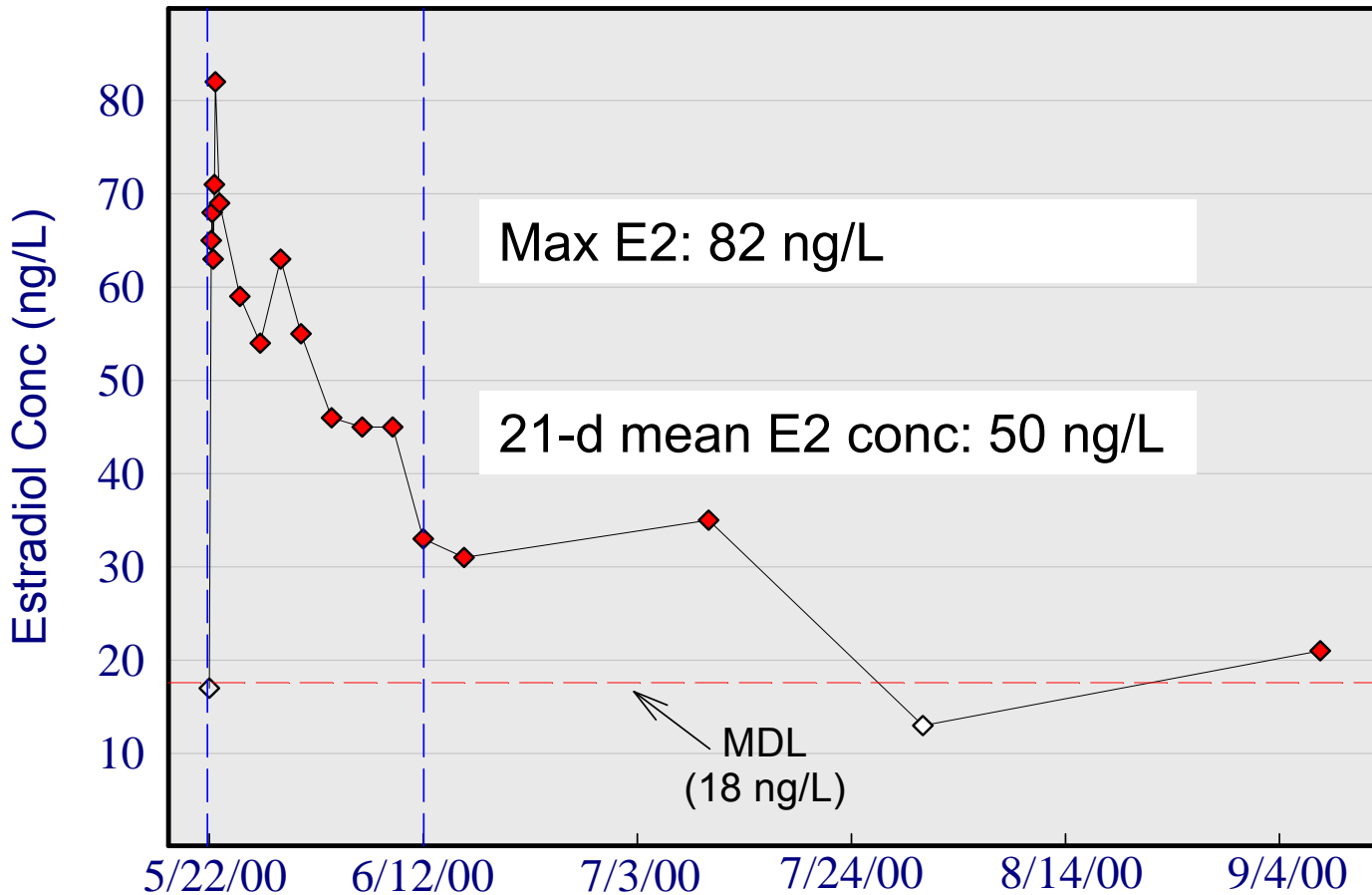


Controlled Field Investigation

- Fecal steroids readily transport from litter-amended fields to surface waters via runoff
- Amount transported was a function of precipitation frequency/intensity and of tillage practice employed
- Steroid transport under NT practices was 2x - 10x greater than under CT
- Additionally we have found, with Ken Staver, that the same trend holds for N, P, As, and Cu. That is, greater transport from field from No-till than Conventional till, especially after the first heavy rain event.

Controlled Field Investigation

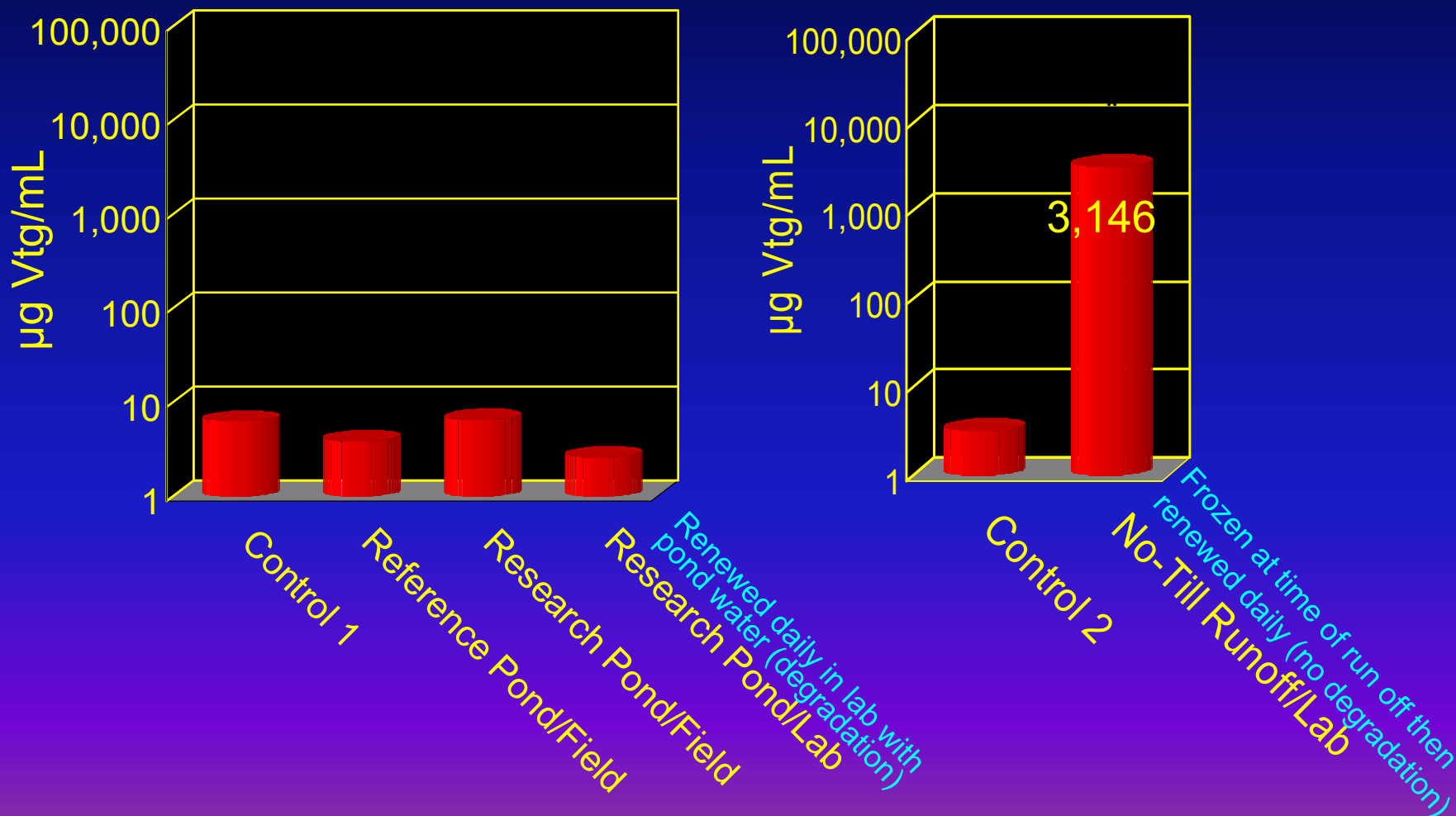
Estradiol in the retention pond following
Spring 2000 litter application



Runoff / Retention Pond Exposures

(Exposed for 21-d either in receiving ponds or lab)

Plasma Vtg in Adult Males



Controlled Field Investigation

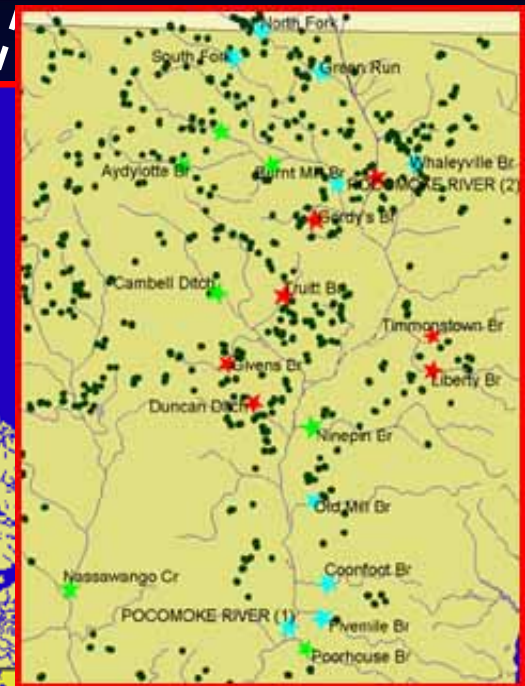
- Persistence of litter-derived E2 in surface waters on the order of weeks to months
- Sustained E2 levels greater than 40 ng/L definitely possible
- Environmental range of concern for litter-derived E2: ~40 ng/L from our laboratory work (Pretty close to this in pond)
- Runoff from litter-amended fields capable of inducing ED in fish (frozen sample)
- However, dilution and natural “aging” seemed to reduce estrogenicity

Field Investigation

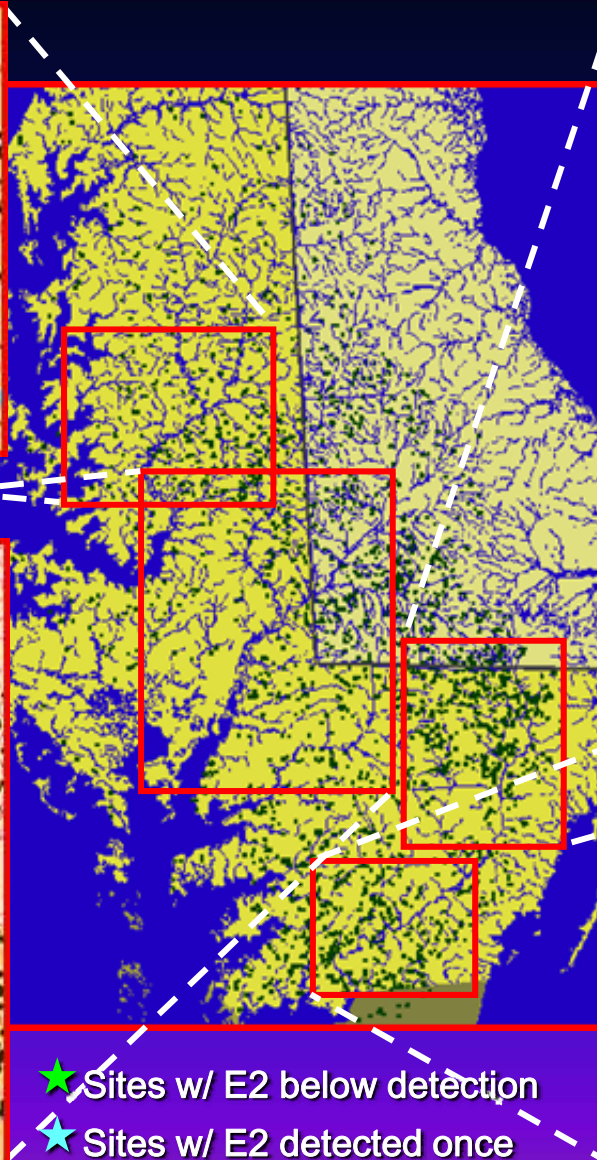
Choptank



Upper Pocomoke



Nanticoke



Lower Pocomoke

- ★ Sites w/ E2 below detection
- ★ Sites w/ E2 detected once
- ★ Sites w/ E2 detected more than once
- Poultry houses (within Chesapeake watershed)

Field Investigation

- Of sites sampled during **SPRING RAIN EVENTS** shortly after poultry litter application:
 - 60% had detectable E2 (≥ 18 ng/L)
 - Flowing streams: 18 – 45 ng/L
 - Ephemeral ponds: < 245 ng/L
- Only 15% of sites sampled during **LATE SUMMER LOW-FLOW CONDITIONS** had detectable E2
- WWTP receiving waters had high E2 concentrations during both high- and low-flow periods
 - Range: 70 -112 ng/L

Field Investigation

- Fish and frogs were collected from 15 sites in Pocomoke and Choptank watersheds during the fall of 2005 / 8 sites during the spring and summer of 2006 (Electroshocking)
- Gonads (♂ & ♀) were examined histologically for ED-related pathology
- Plasma samples (♂ only) were analyzed for Vtg
- One of problems is that we don't get smallmouth bass in shore streams (not natural habitat) and we were able to only sample small numbers of largemouth bass because streams are so small.

CURRENT RESEARCH

EPA STAR CONTRACT



Fish kill in receiving pond after 2008 runoff event





Vickie Wilson – US EPA/NHEERL Research Triangle Park

Developed an **in-vitro estrogen-inducible reporter-gene assay** for measuring compounds that are **estrogen agonist (mimics)** or **antagonist (inhibitory)**
Quick screening tool for endocrine disruption of estrogen system (hours)

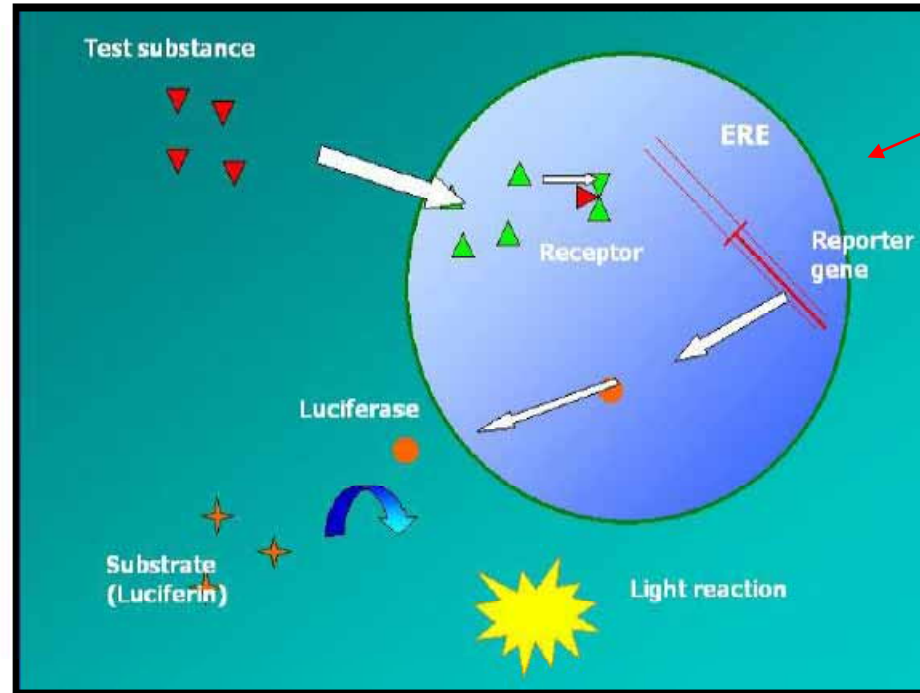


Figure 2: Schematic representation of the estrogen-inducible reporter-gene system in the T47D-Kbluc cell line.

T47D-Kbluc cells were stably transfected with a triplet ERE (estrogen-responsive elements)-promoter-luciferase reporter gene construct. Binding of the test substance to the estrogen receptor leads to gene transcription of the luciferase reporter gene and production of the luciferase enzyme. After addition of the Luciferase Assay reagents, light production is measured with a luminometer as a measure for the receptor binding of the tested substance/compound.

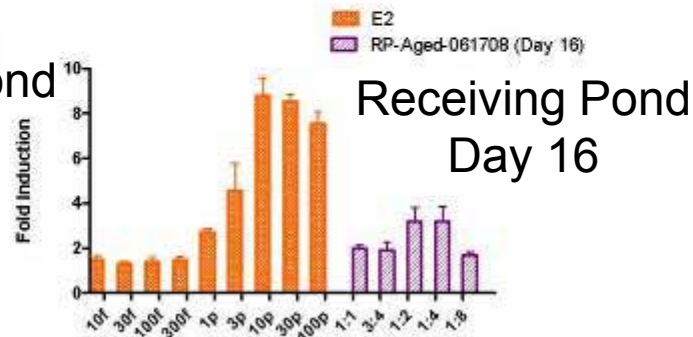
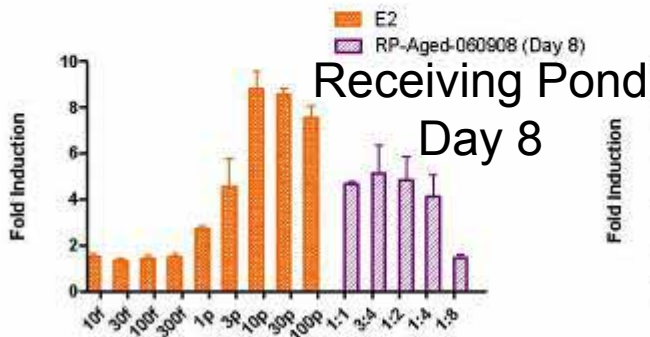
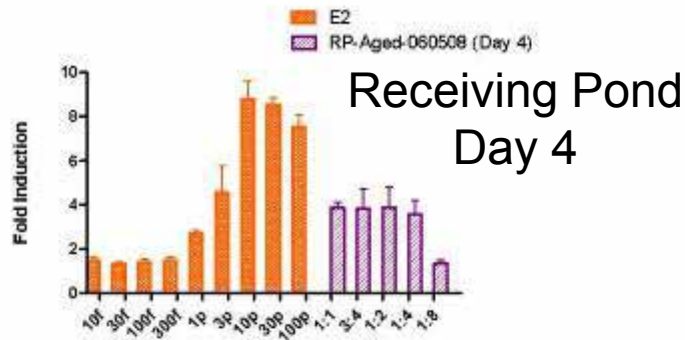
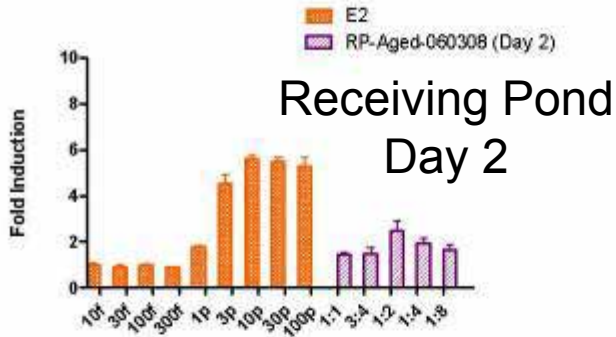
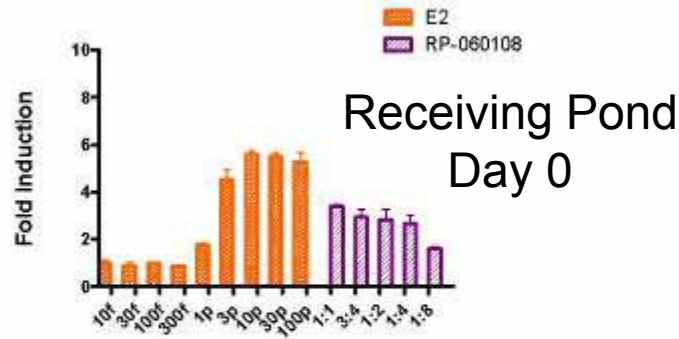
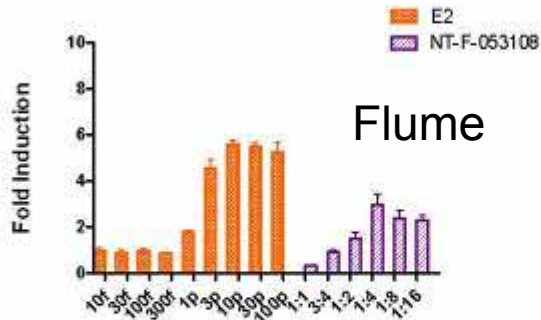
T47D-KBluc Assay

U of MD samples

Batch 2

Methods similar to that described on previous slide.

Some evidence of cytotoxicity at higher concentrations. As samples were diluted cytotoxicity was reduced or eliminated but some estrogenicity could still be detected.

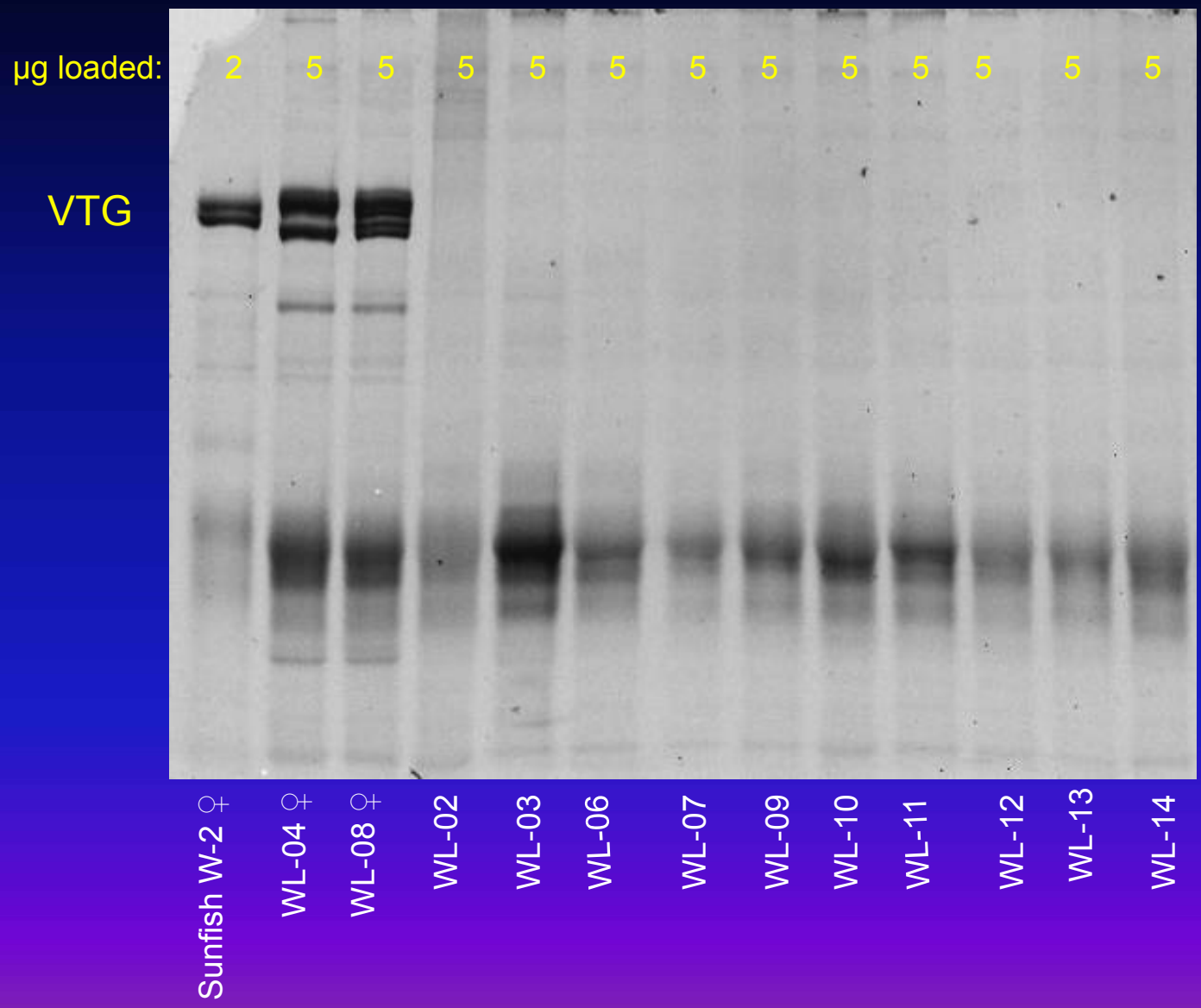




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Large Mouth Bass Plasma – Gel Electrophoresis



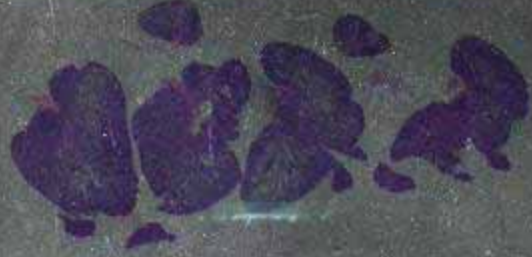
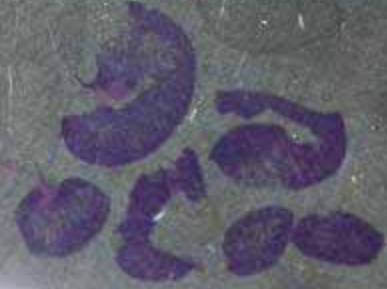
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TL-G04B TL-G04A

SPECIMEN

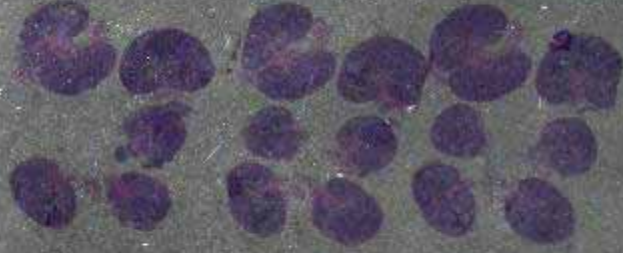
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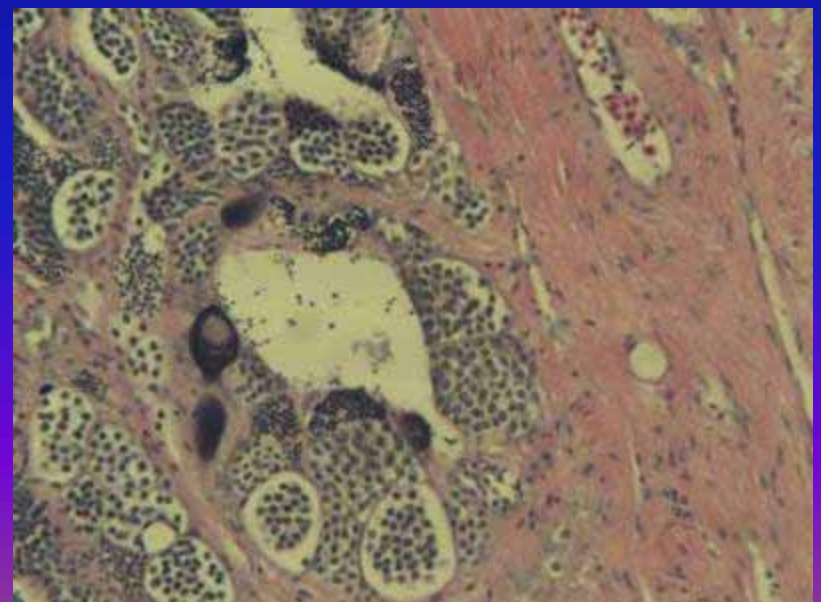
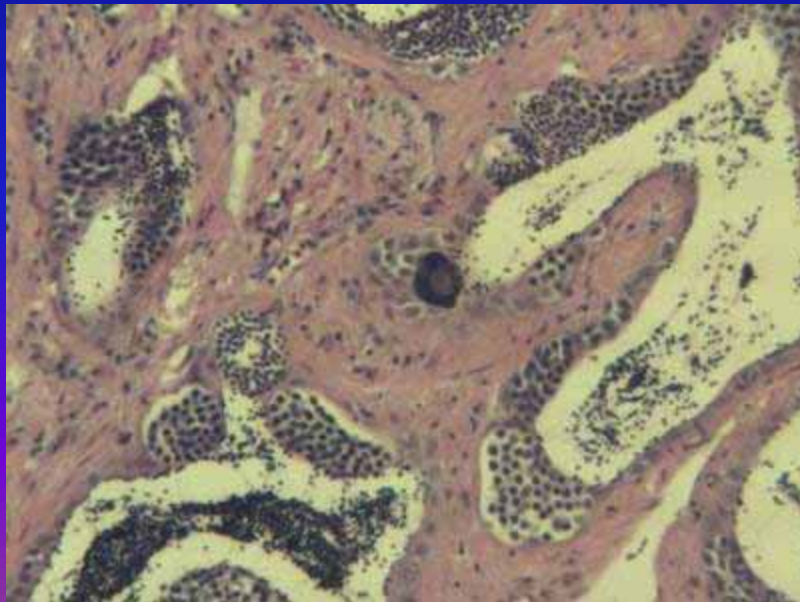
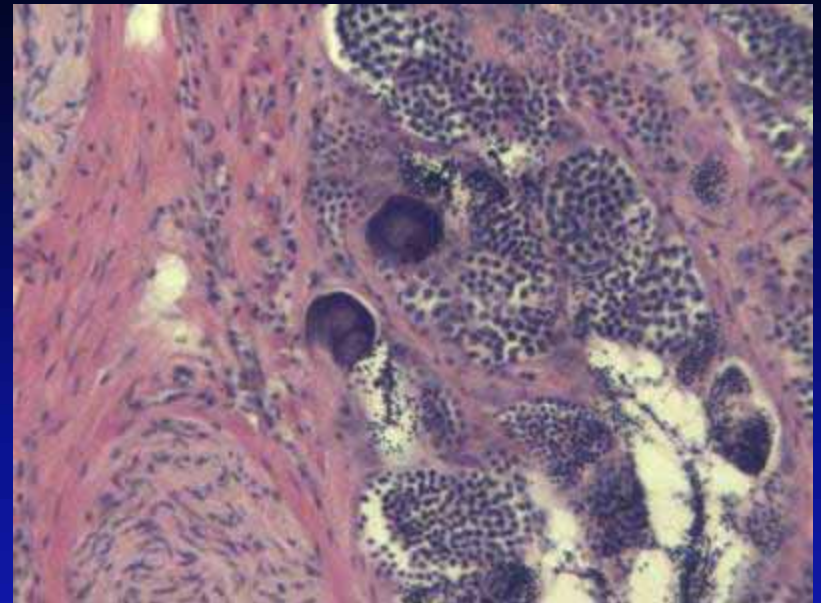
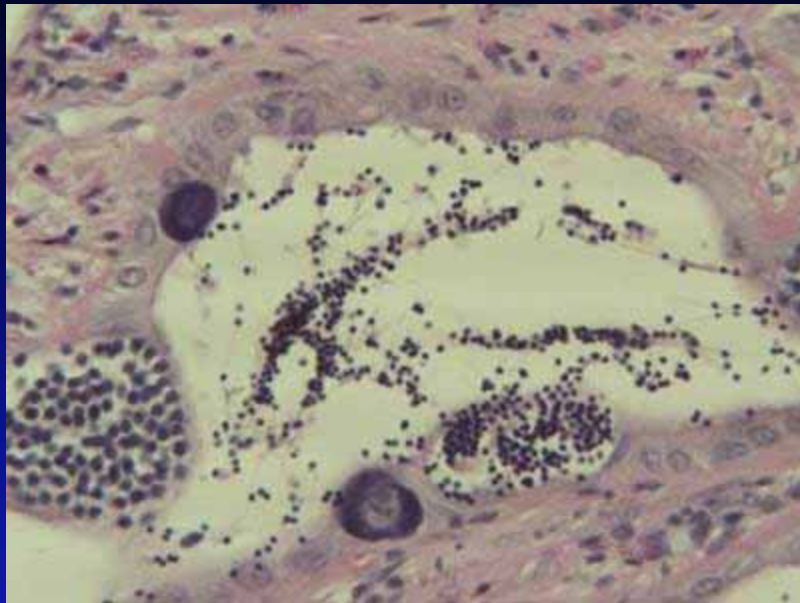
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SPECIMEN



Testicular Oocytes in Largemouth Bass

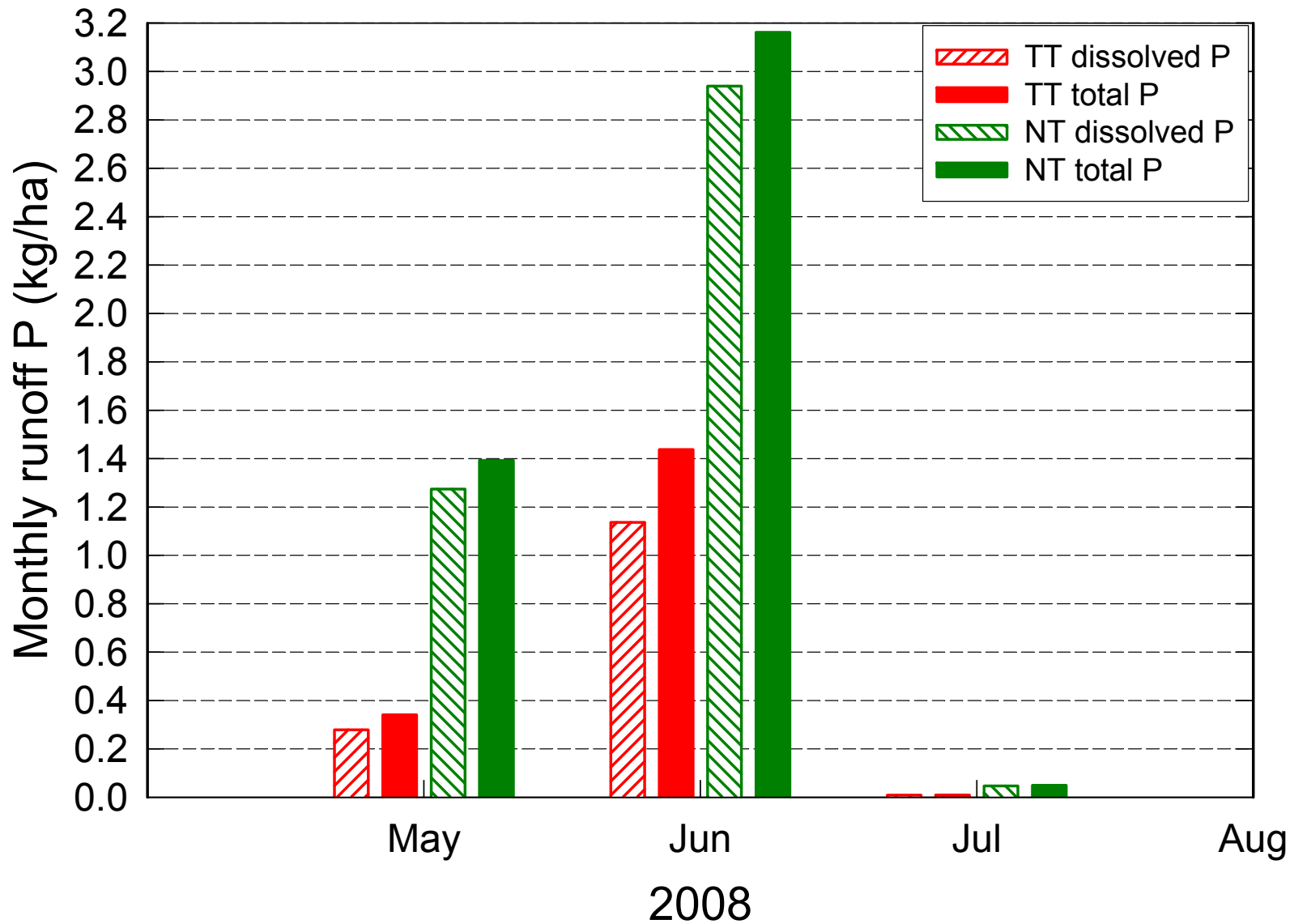


Testicular Oocytes in LMB

Maryland			Delaware		
Smithville Lake	Tuckahoe Lake	Williston Lake	Hearns Pond	Moores Lake	McColley Pond
2	0	0	8	9	4
1	0	0	5	1	5
4	0	2	3	2	0
1	34	1	4	0	8
4	0	5	3	2	0
0	6	1	1	10	5
0	0	0	3	0	6
0	0	1	2	7	0
0	2	4		0	1
0	2	3		0	
	0	4			
	0				
5/10 (50%)	4/12 (33%)	8/10 (80%)	8/8 (100%)	6/10 (60%)	6/9 (67%)

Concern or background ??? (12 to 18 sections)

Nutrient runoff comparison



THANK YOU

Methods

- **Gel Electrophoresis VTG** – samples electrophoresed on 8% polyacrylamide gels, stained with Pro-Q Diamond phosphoprotein stain and quantified against a standard curve using purified *Fundulus heteroclitus* or *Pimephales promelas* VTG
 - VTG confirmation – proteins transferred from gels onto PVDF membranes and stained with coomassie blue. VTG bands N-terminal amino acid sequenced. Resulting sequences compared to VTG sequences deposited in GenBank using BLAST (basic local alignment search tool)
- **ELISA VTG** - competitive (enzyme linked immunosorbent assay) using appropriate mono- and poly-clonal antibodies for the species of interest.
- Samples, standards, and blanks incubated with a primary antibody, mouse monoclonal anti-carp Vtg. Introduced to a 96-well plate pre-coated with fathead minnow Vtg and blocked with 5% BSA in carbonate buffer.

Methods (Cont.)

- ELISA (cont.) Primary antibody attached to Vtg from samples was sequestered while remaining unbound antibody in solution was free to react with the Vtg well-coating. Only antibody attached to this plate-bound Vtg remained after three washings with a PBS/Tween 20 solution. Goat anti-mouse antiglobulin conjugated w/ horseradish introduced to wells and bound with the primary antibody/Vtg complex, where available. After a final wash only this Vtg antibody-antiglobulin complex remained attached to wells. A TMB substrate, designed to degrade in the presence of horseradish peroxidase and cause a color change from clear to brilliant blue, was introduced to the wells. Appearance of an intense color change indicated the presence of significant conjugate and, therefore, a lack of Vtg in the original sample. Conversely, absence of a color change indicated a lack of conjugate and, therefore, the presence of significant Vtg in the original sample.

Methods (Cont.)

- **STEROID RIA** (radioimmunoassay) depends on the competition between unlabeled and radiolabeled [^3H] steroid for limited steroid-specific antibody. Following incubation in tubes, centrifugation in the presence of a dextran/carbon solution pelletizes unbound steroid, allowing supernatant, containing antibody bound steroids (labeled and unlabeled), to be decanted into scintillation vials for determination of radioactivity via beta-counter. Resulting radioactivity is inversely proportional to the concentration of hormone in the sample.