Technical Underpinnings and Initial Validation of a Fish Assay for Detecting EDCs

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Tier 1 EDC Screening Battery[1]

Uterotrophic Assay (rat)

- estrogens

Hershberger Assy (rat)

- (anti-) androgens

Female "Pubertal" Assay (rat)

- (anti-) estrogens,androgens, thyroid?

Amphibian Metamorphosis Assay

- (anti-) thyroid

Fish Gonadal Recrudescence Assay

- **HPG axis (including estrogens, androgens)**

[1]EDSTAC (1998)

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Test Species: Fathead Minnow

- "White" rat of aquatic toxicology in North America/Europe
- Standard test methods
- History of regulatory use
- Easily handled/cultured in lab
- Small (but not too small)
- Relatively rapid life cycle
- Cyclical spawner (ca., 3 d intervals)

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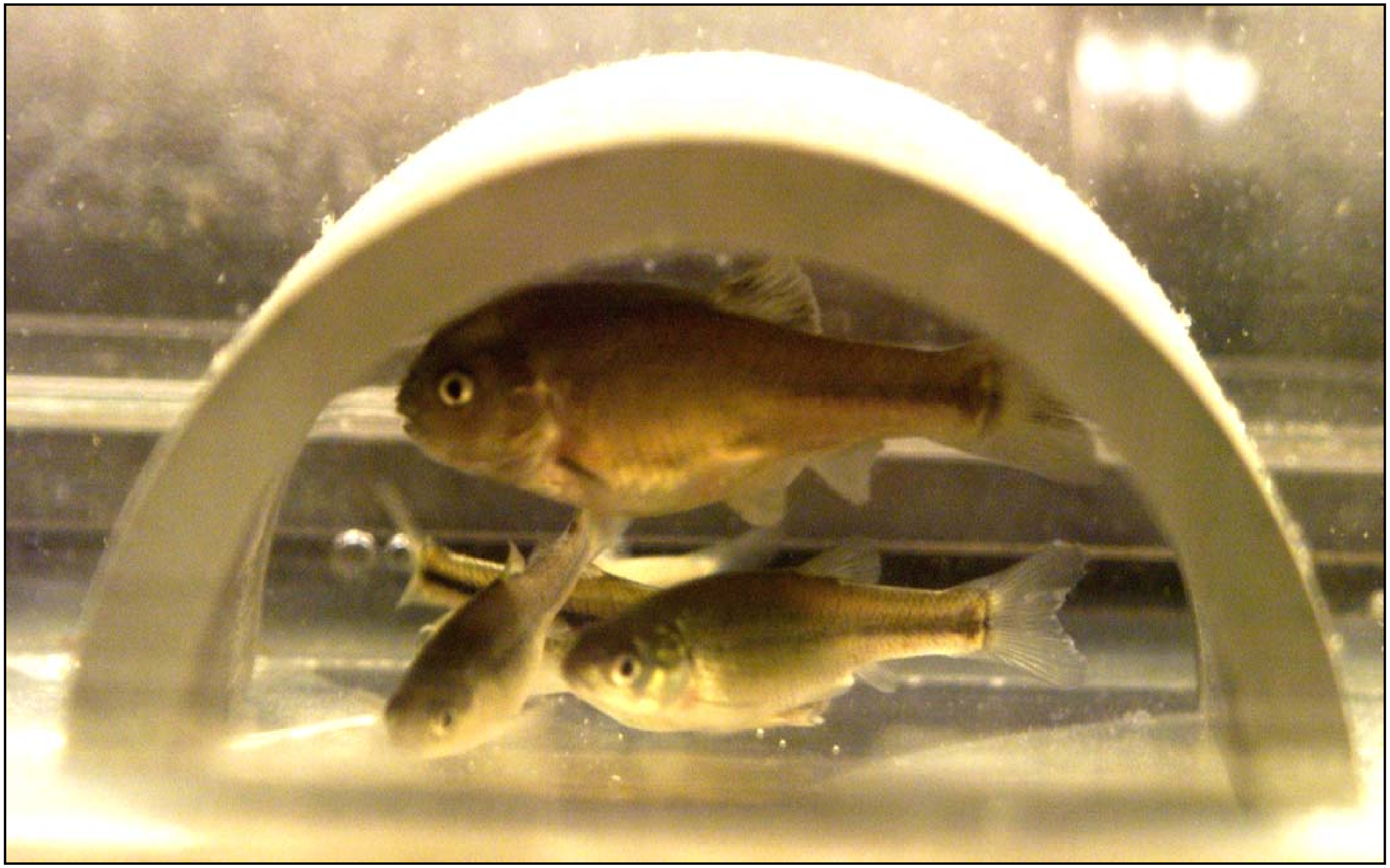

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Proposed Gonadal Recrudescence Assay

- Mature fish maintained under "winter" conditions (regressed gonad, no secondary sex characteristics)
- Photoperiod/temperature increased coincident with chemical exposure
- Endpoints
  - Secondary sex characteristics
  - Ovary/testis development (GSI, histology)
  - Gamete maturation
  - Sex steroids
  - Vitellogenin

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Drawbacks to Recrudescence Assay

- Photoperiod/temperature regime needed to precisely induce recrudescence not well characterized for fathead minnow
- Available studies indicate response may be protracted (weeks to months) and highly asynchronous
- Logistic/practical challenges to simultaneous photoperiod/temperature alteration and chemical exposure

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Alternative: A Short-Term Reproduction Test

- Similar suite of endpoints as recommended for recrudescence assay
  - Secondary sex characteristics
  - Ovary/testis development (GSI, histology)
  - Sex steroids
  - Vitellogenin

Test Overview

- Initiated with mature, spawning fish
- 14-21 day pre-exposure followed by $\leq$ 21 day chemical exposure
  - Behavior
  - Fecundity
  - Fertility
  - Hatch
  - Secondary sex characteristics
  - Gonadal status (GSI, histology)
  - Plasma vitellogenin
  - Plasma steroids (E2, T, KT)

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USEPA. 2002. A Short-Term Method for Assessing the Reproductive and Developmental Toxicity of Encocrine-Disrupting Chemicals Using the Fathead Minnow (Pimephales promelas)

- Test context
- Species background/biology
- Test system(s)/exposure methods
- Conducting assay/evaluation of "apical" endpoints
- Methods for "diagnostic" endpoints (detailed appendices)
- Statistical methods/QA & QC
- Test interpretation

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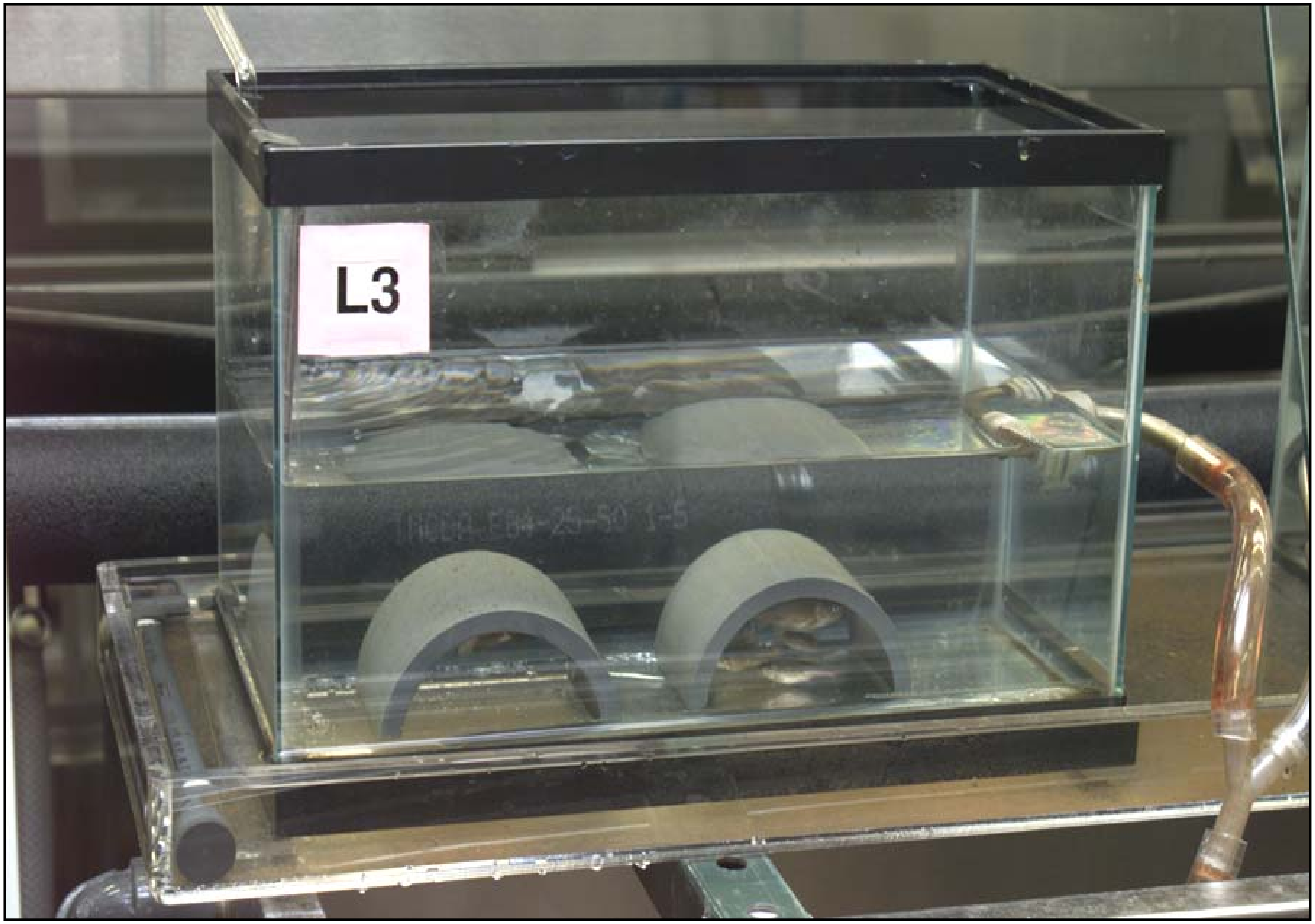

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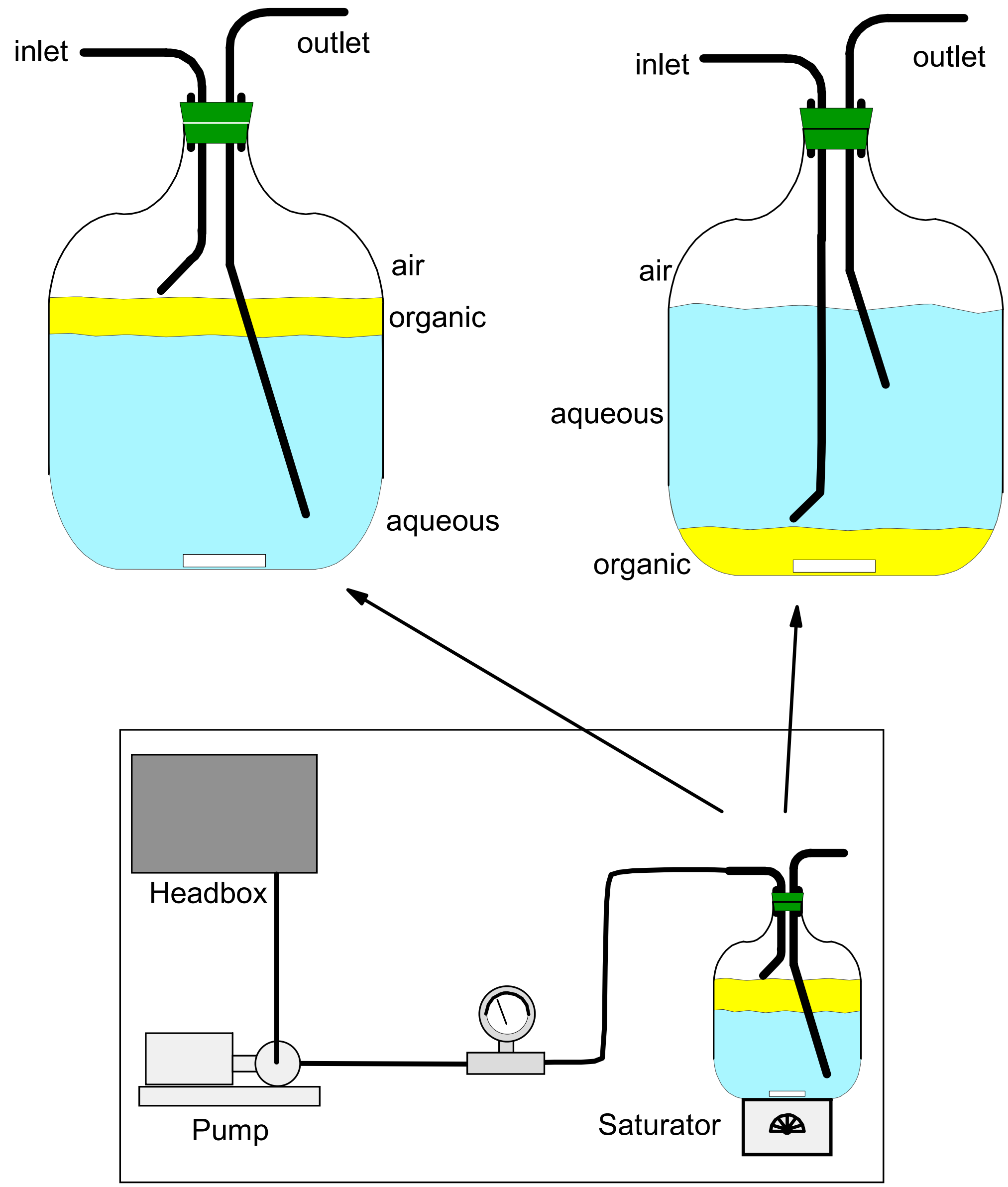
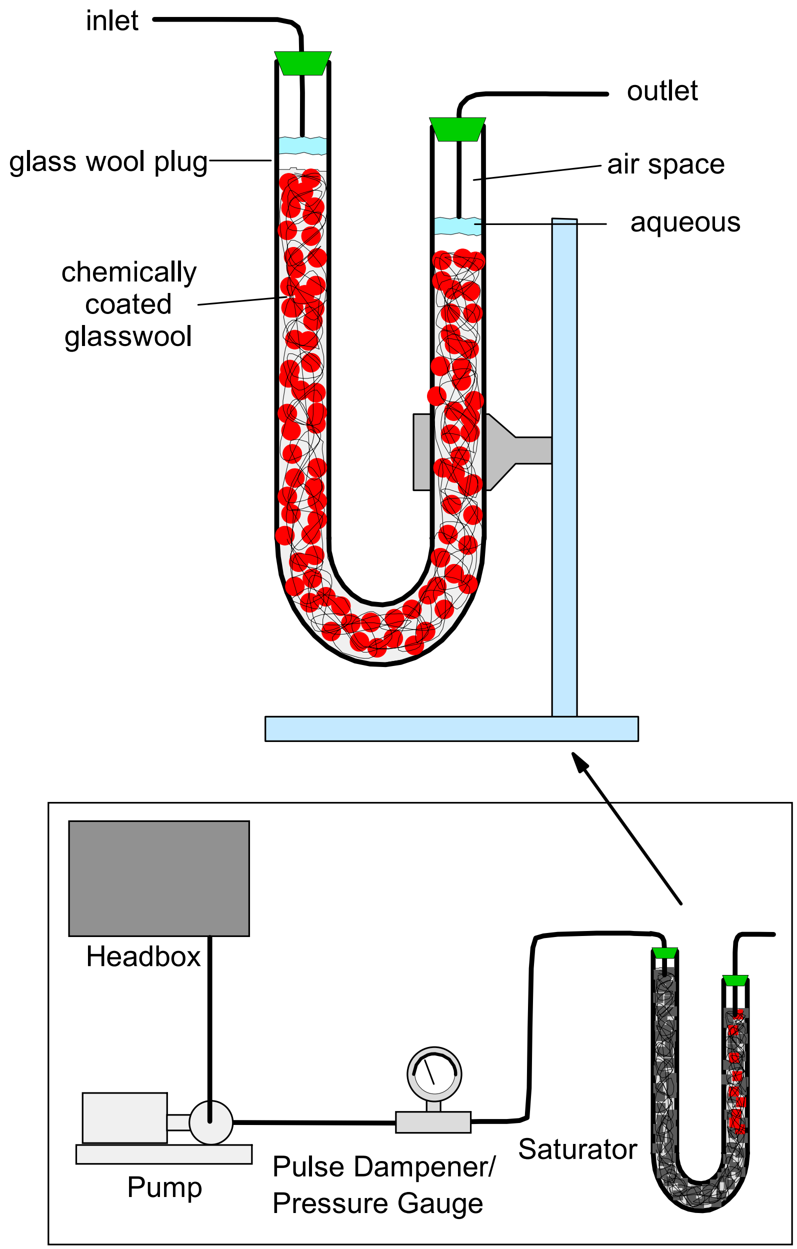
Liquid - Liquid Saturator



Solid - Liquid Saturator



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Alternative Exposure Options

Kahl et al. (2001) J. Fish Biol.

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Endpoints and Test Evaluation/Interpretation

- Development of sensitive and reliable techniques for measurement endpoints
- Documentation of basic reproductive cycle of the fathead minnow
- Characterization of responses/toxicity pathways for HPG-active toxicants
- Evaluation of interlaboratory performance

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ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY

ETAP

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Gonadal histology and characteristic histopathology associated with endocrine disruption in the adult fathead minnow (*Pimephales promelas*)

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Abstract

Examination of gonadal histopathology has been beneficial in understanding and assessing the effects of potential endocrine disrupting chemicals in fish and other organisms. The present study describes the normal gonadal histology of the fathead minnow (*Pimephales promelas*), a widely used test organism, reviews typical effects of endocrine disrupting chemicals with different modes/mechanisms of action on the histological structure of the ovaries and testes, and recommends methods for optimizing histopathological results.

Keywords: Fish; Ovary; Testis; Histology; Endocrine disruptors

1. Introduction

About 80,000 chemicals have been introduced into the environment within the last 50 years (Curtis and Skaar, 2002). There is mounting evidence that some of these chemicals may are an important potential target of environmental endocrine disruption, useful data may be obtained from biomarkers or assays ranging from molecular to ecosystem-wide levels of organization (van der Oost et al., 2003).

In 1996, the US Congress passed legislation requiring

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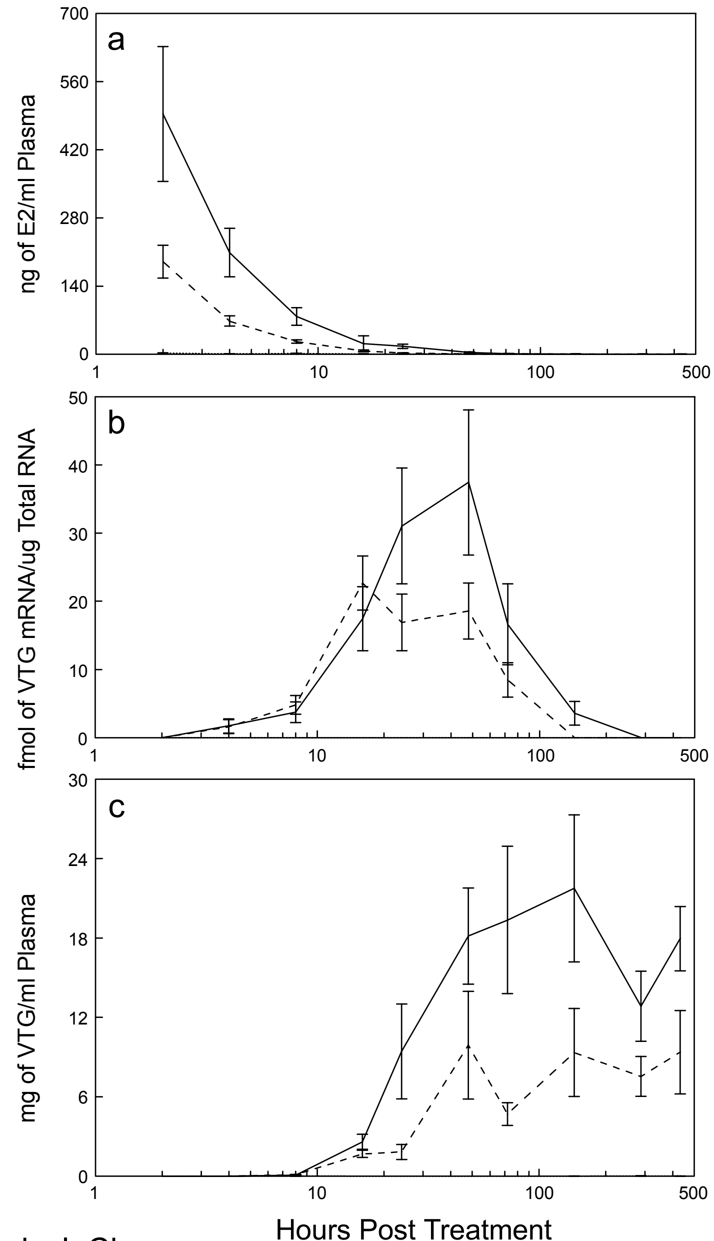
Measuring Vitellogenin: Time-Course Studies



Korte et al. (2000) Environ. Toxicol. Chem.

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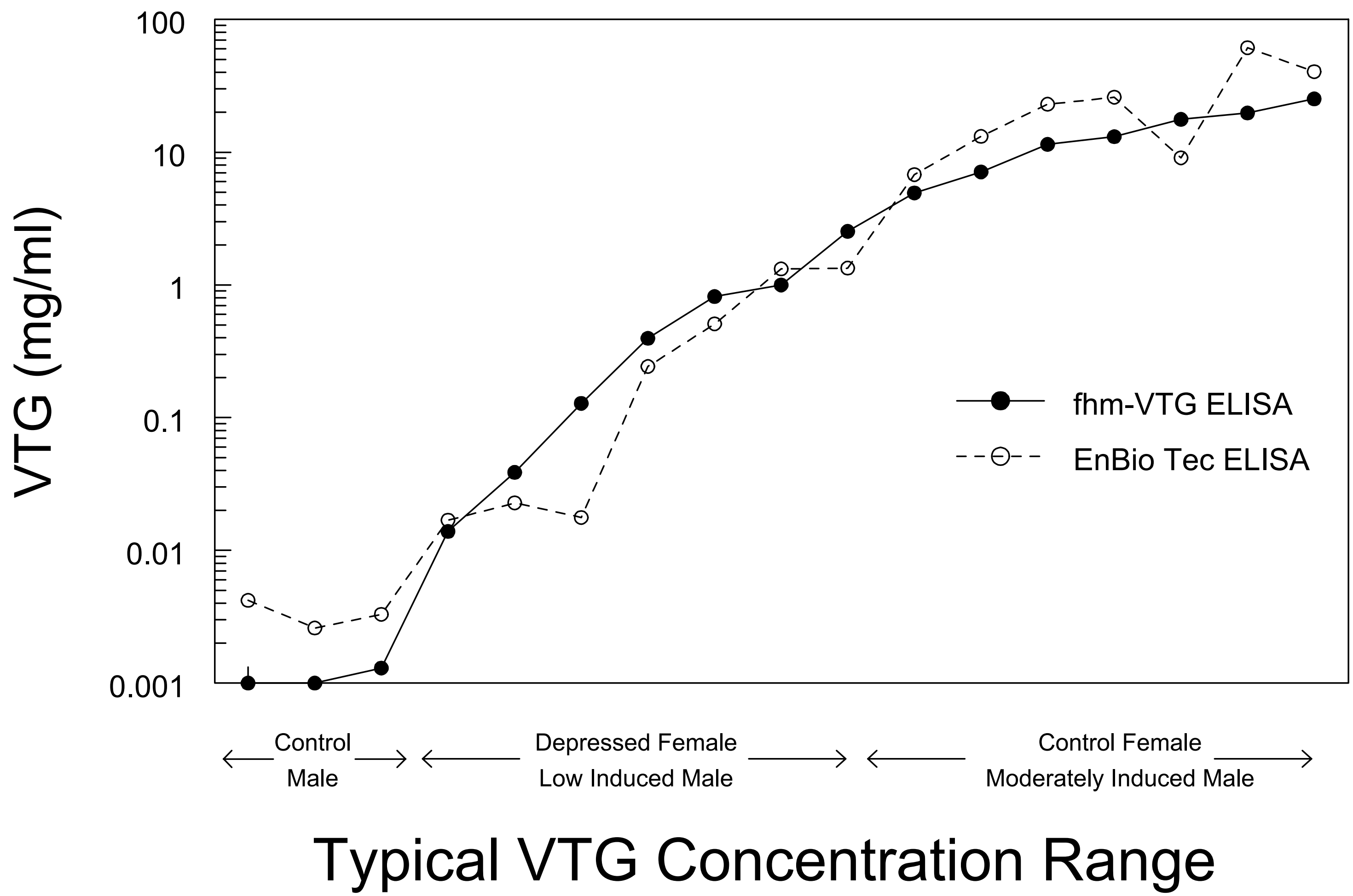
Comparison of Vitellogenin ELISAs



Korte et al. (2004) Comp. Biochem. Physiol.

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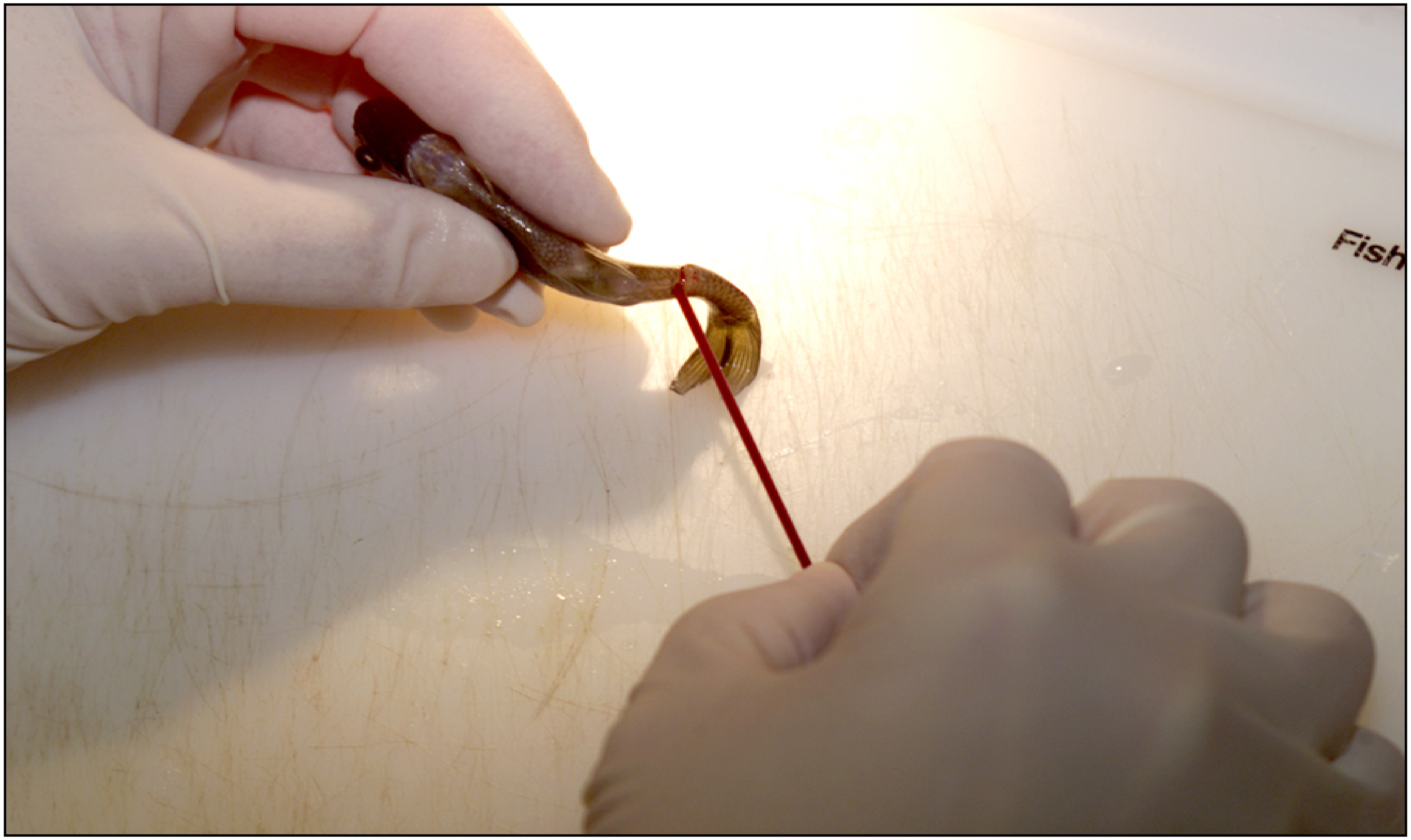
Blood Sampling Technique



USEPA (2000)

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Sex Steroids in the Fathead Minnow

- Plasma volumes in individual fish range from ~20-60 µl (sex-dependent)
- Optimized RIA techniques allow reliable E2, T, KT measurements on sample sizes of ~ 5 µl

Males

- E2, T and KT ~ 0.6, 10 and 35 ng/ml
- T and KT co-correlated

Females

- E2, T and KT ~ 6,3 and ND (0.5) ng/ml
- T and E2 co-correlated

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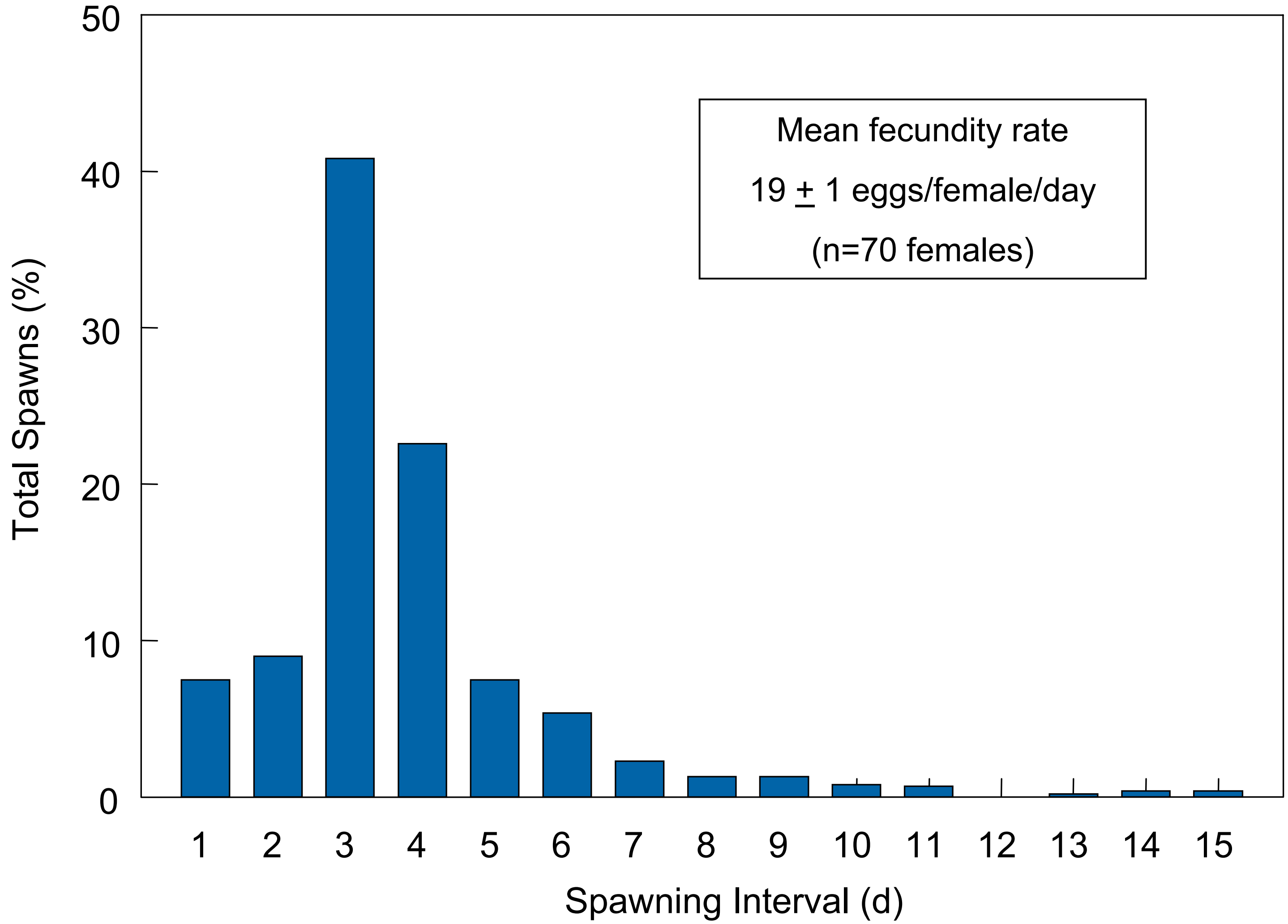
Spawning Frequency of Fathead Minnows



Jensen et al. (2001) Comp. Biochem. Physiol.

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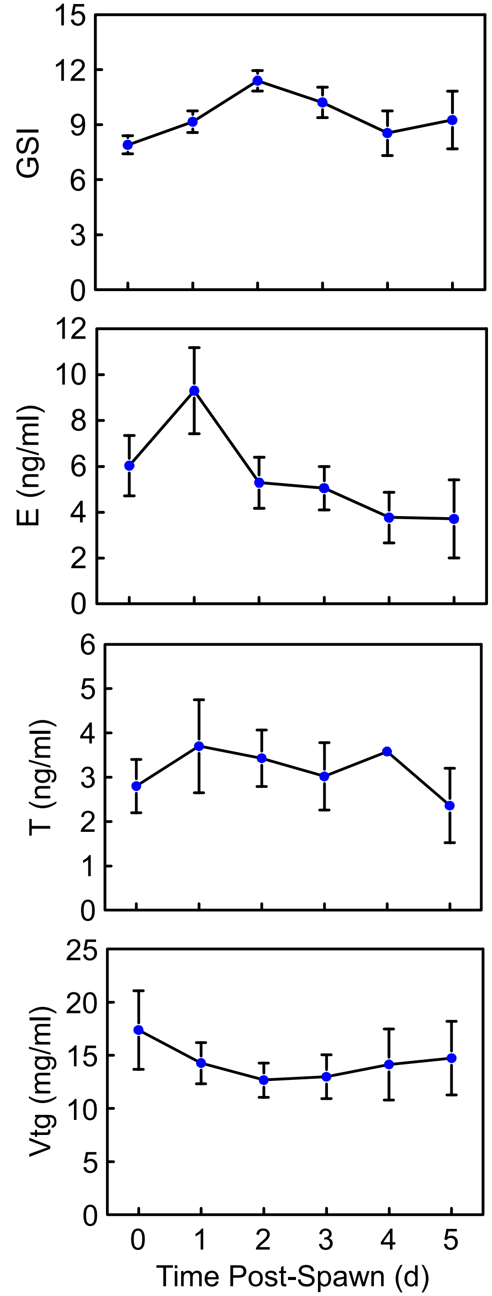
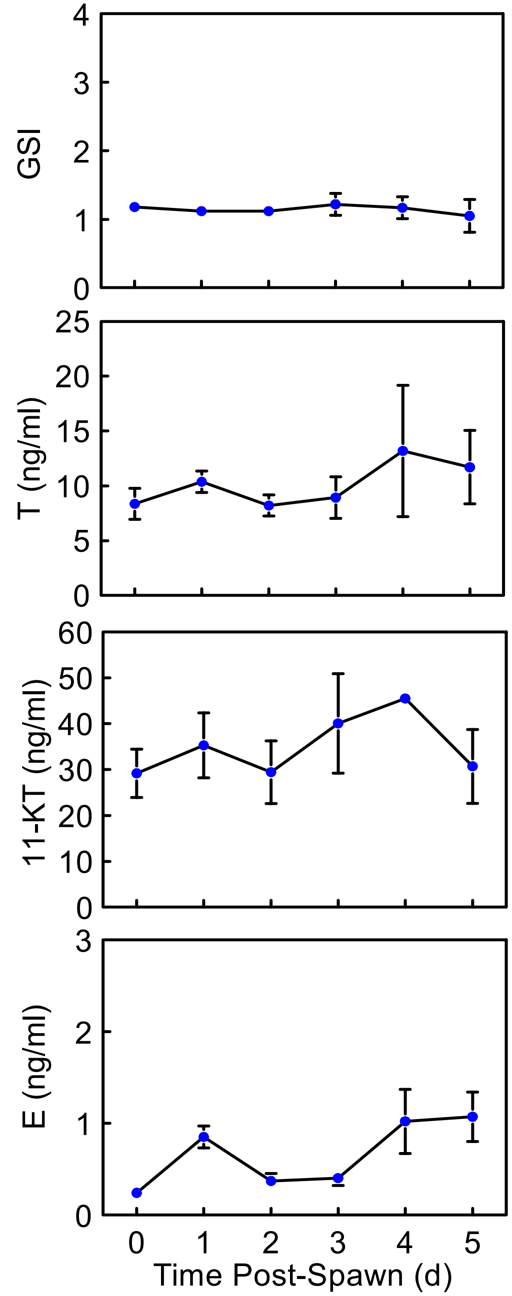
Fathead Minnow Reproductive Cycle: Female



Jensen et al. (2001) Comp. Biochem. Physiol.

Fathead Minnow Reproductive Cycle: Male



Jensen et al. (2001) Comp. Biochem. Physiol.

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Characterization with Known EDCs

- Estrogens (E2, Methoxychlor)
- Androgens (α, β-Trenbolone, Methyltestosterone*)
- Anti-Androgens (Flutamide, Vinclozolin)
- Steroid Metabolism Modulators (Fadrozole, Prochloraz*, Fenarimol*)

*Chemicals w/ mixed MOA

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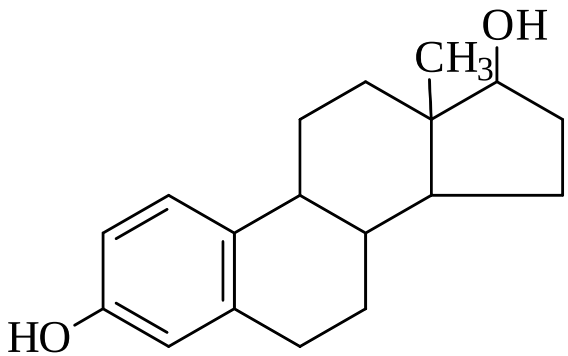
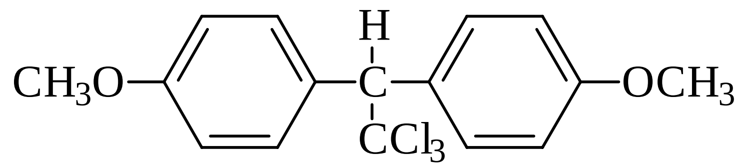
Model ER Agonists

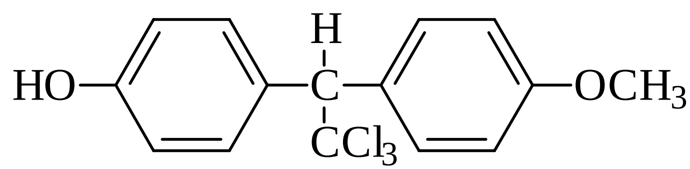


17β-Estradiol [50-28-2]



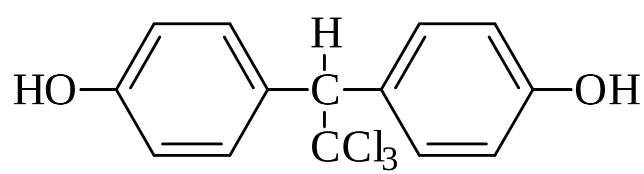
Methoxychlor [72-43-5]

↓



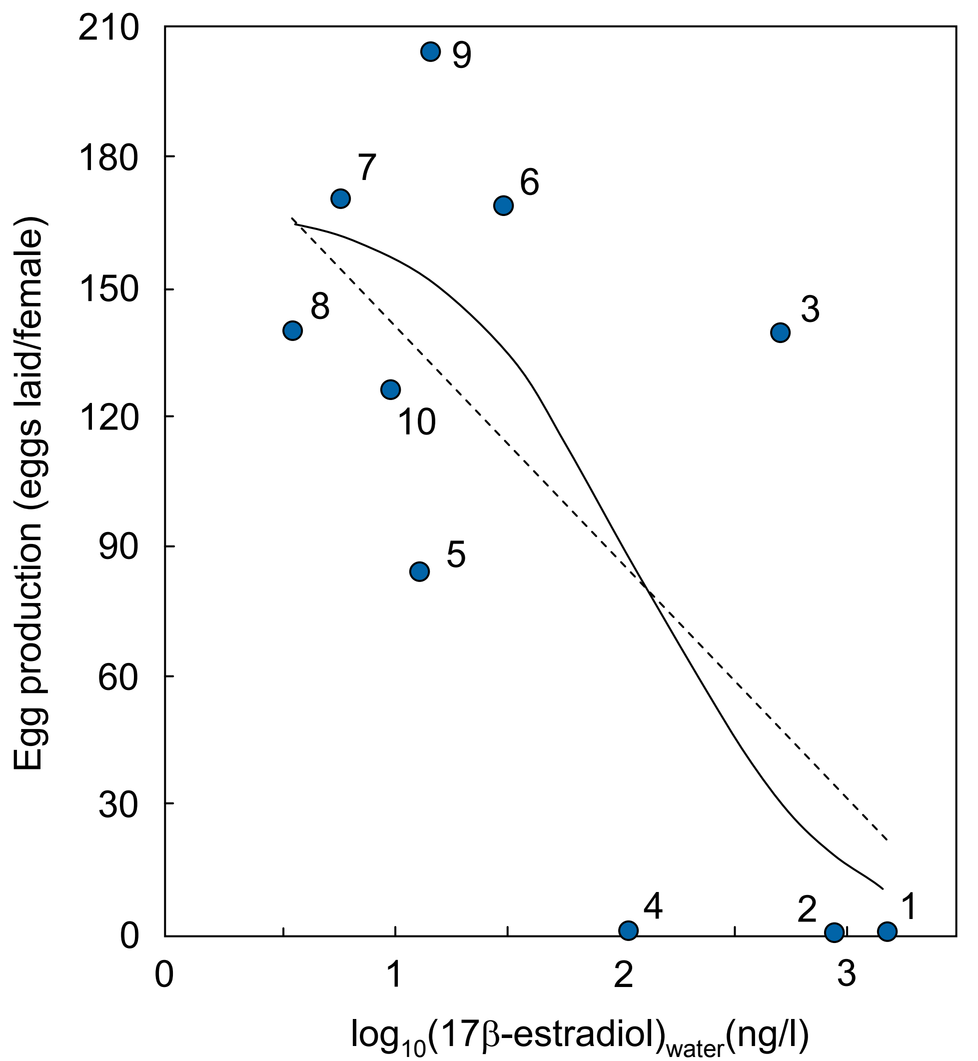
Monohydroxy methoxychlor [75938-34-0]

↓



Dihydroxy methoxychor [2971-36-0]

Effects of E2 on Fathead Fecundity



Kramer et al. (1998) Aquat. Toxicol.

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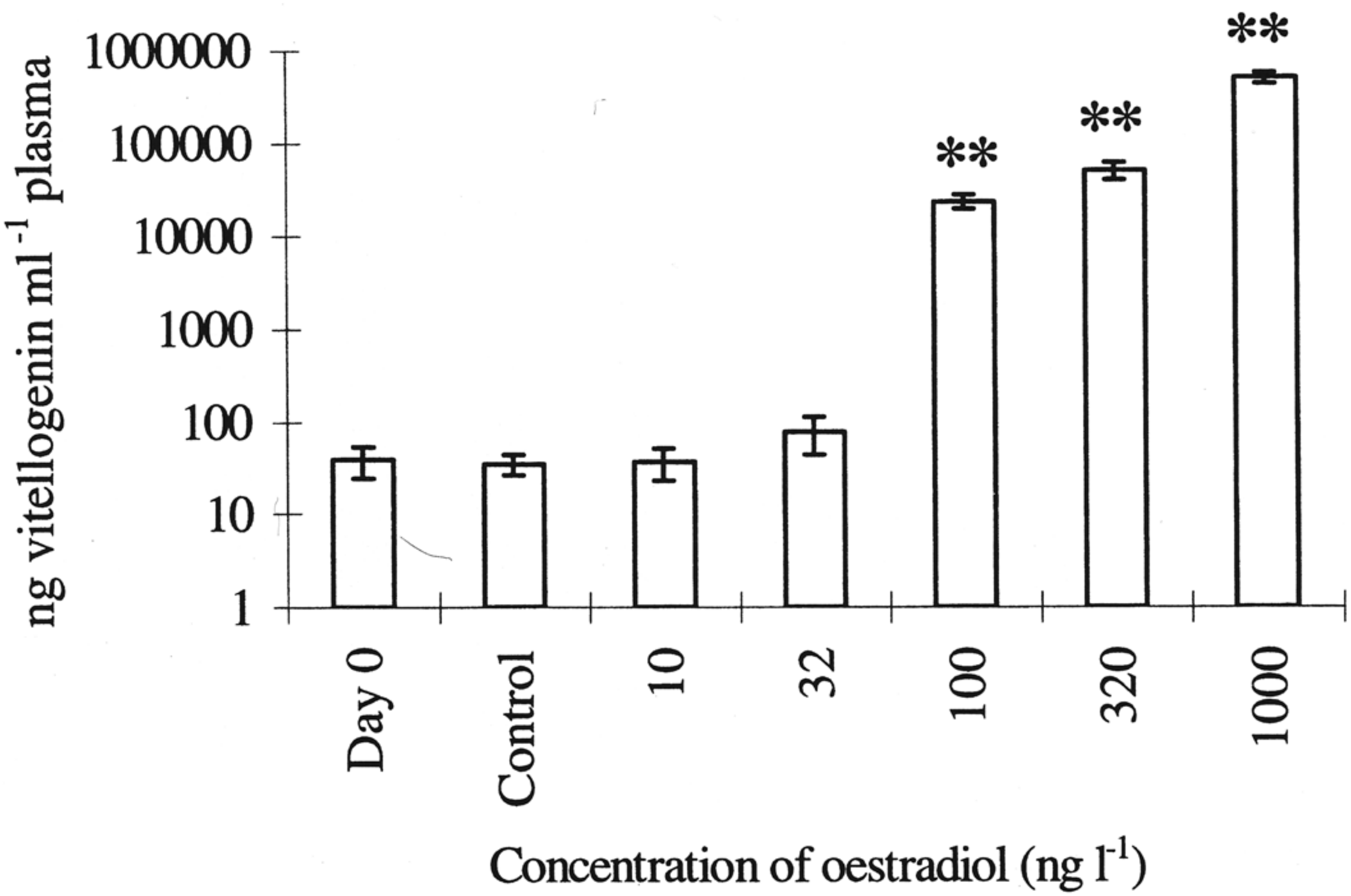
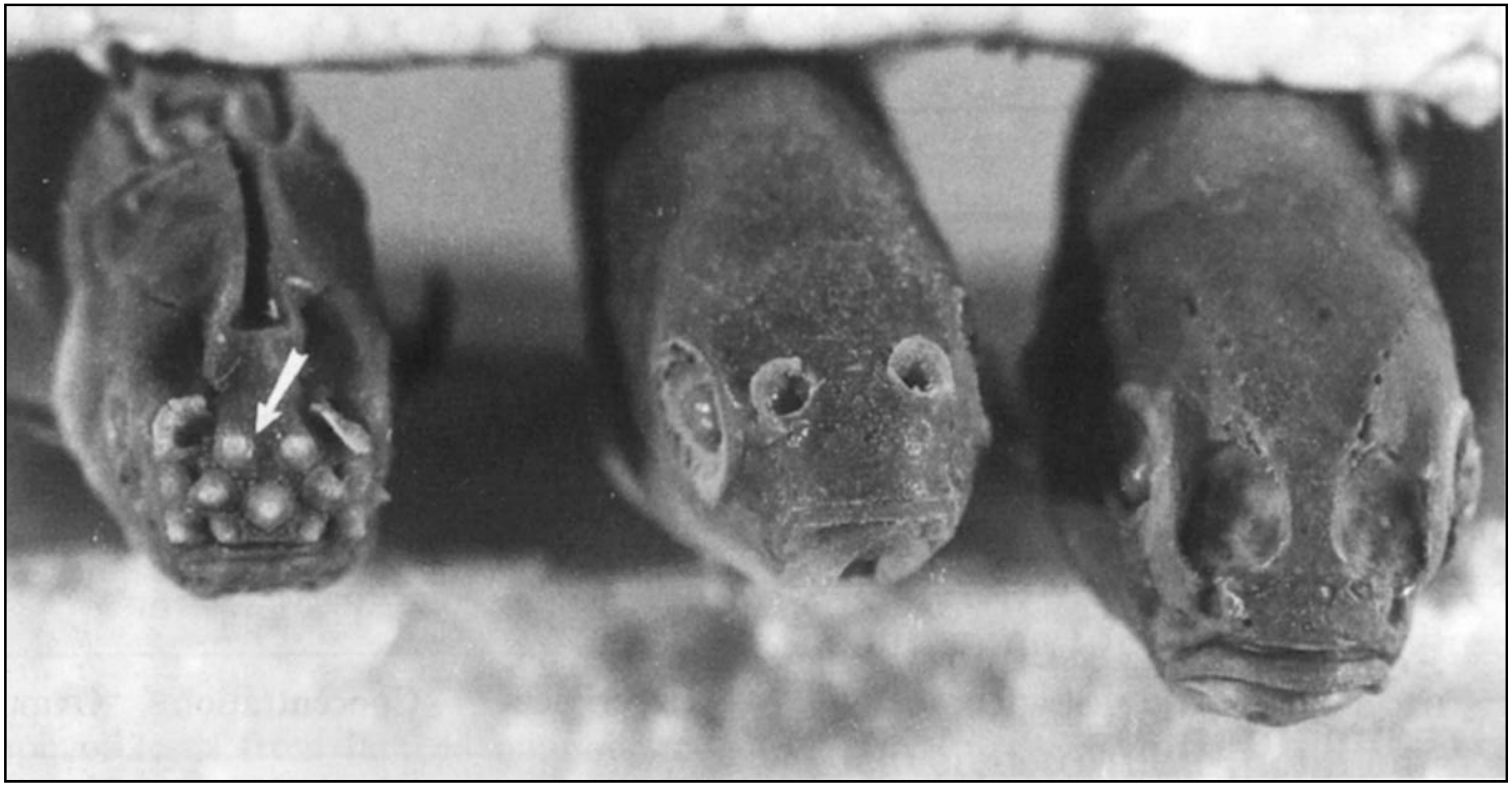
Effects of E2 on Vitellogenin in Males



Panter et al. (1998) Aquat. Toxic.

E2 Effects on Male Tubercles



Miles-Richardson et al. (1999) Aquat. Toxicol.

Effects of Methoxychlor on Fathead Minnow Reproduction

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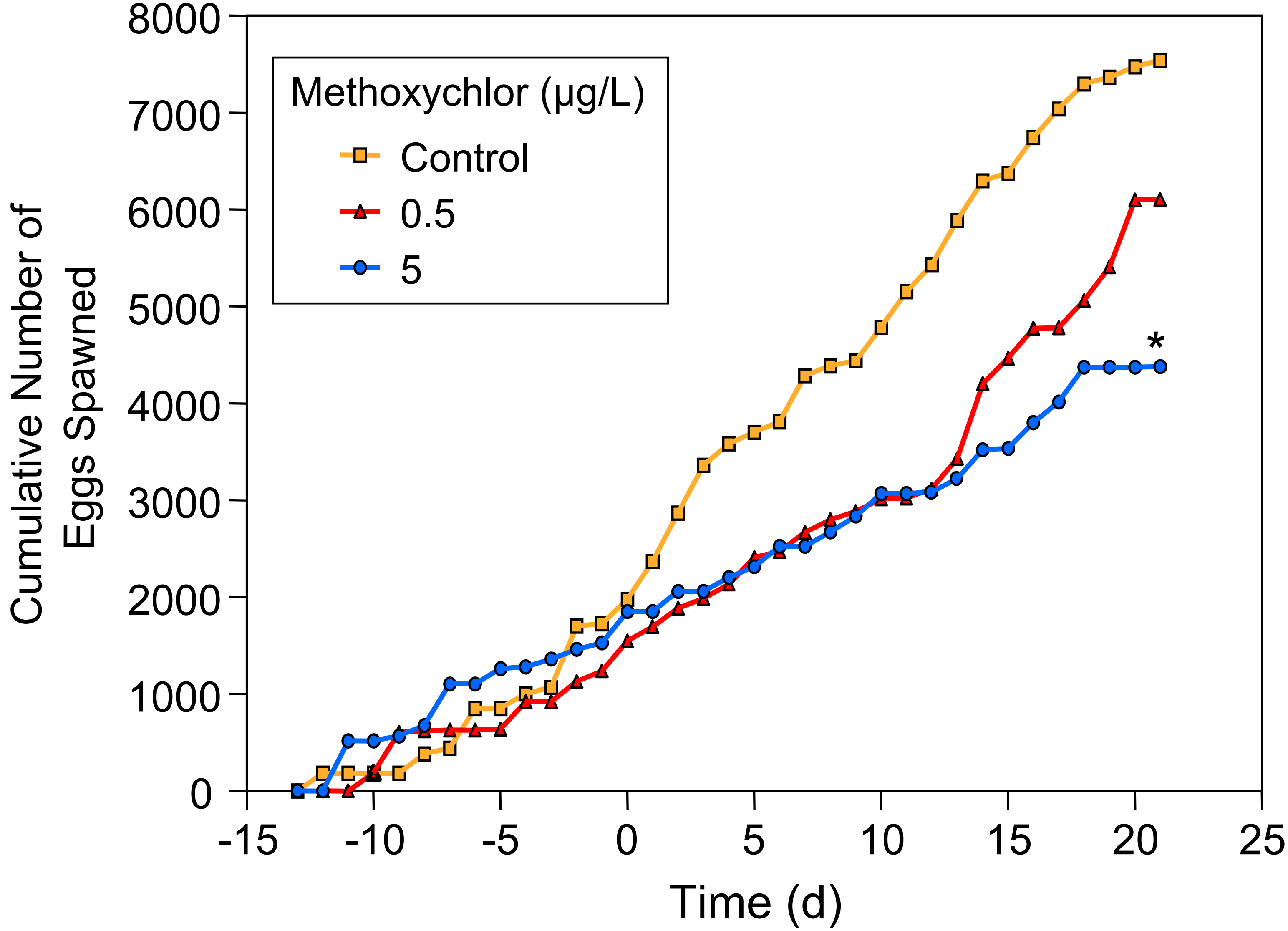

Ankley et al. (2001) Environ. Toxicol. Chem.

Methoxychor Effects on Fathead Minnow

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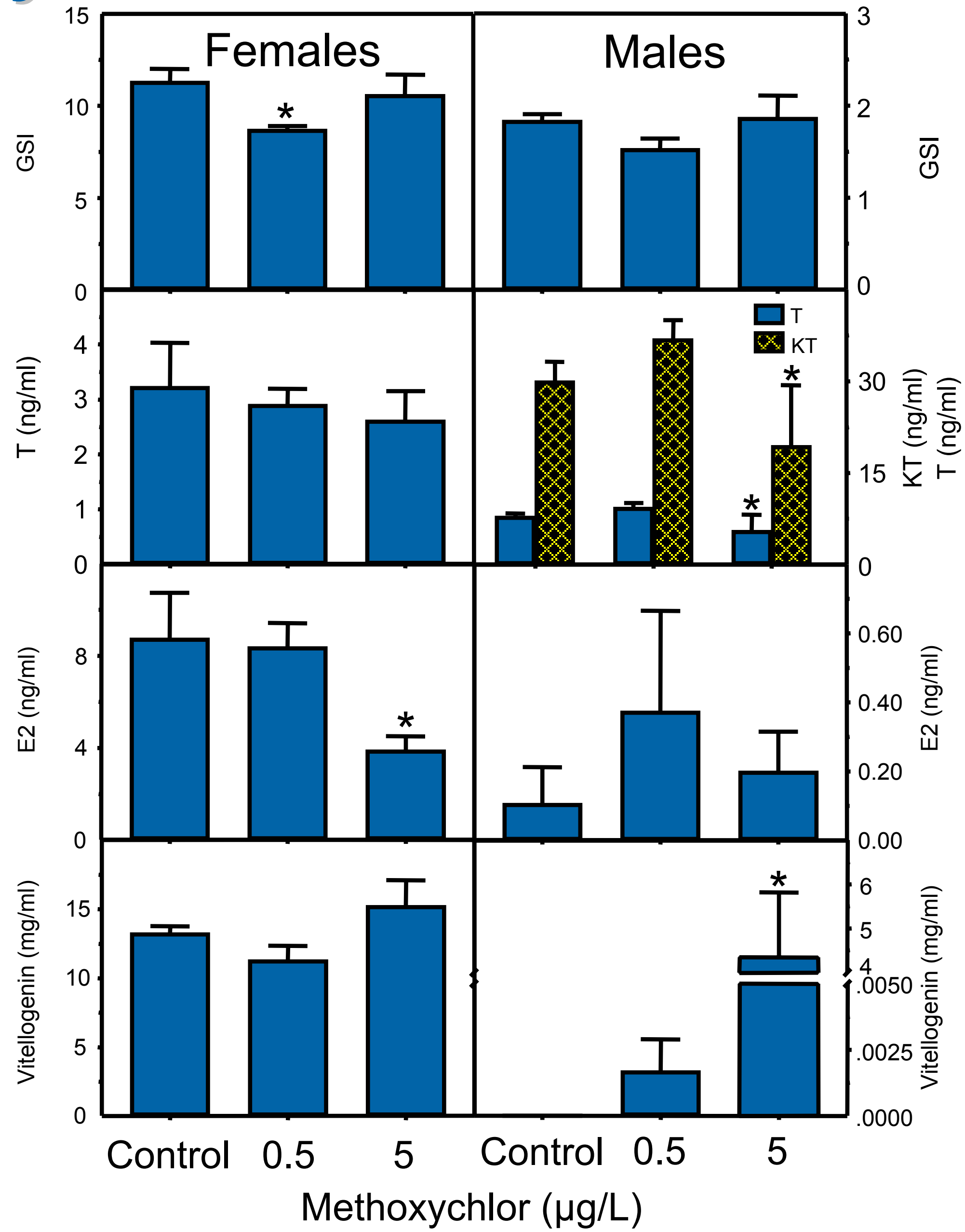
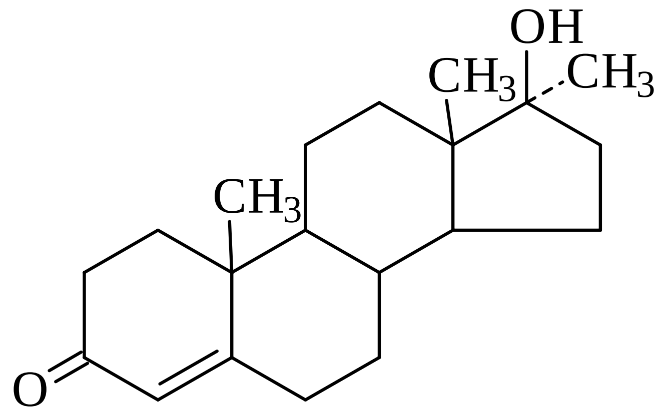
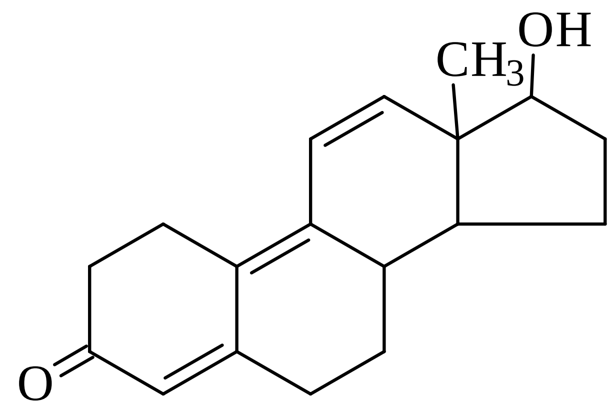


Ankley et al. (2001) Environ.Toxicol. Chem.

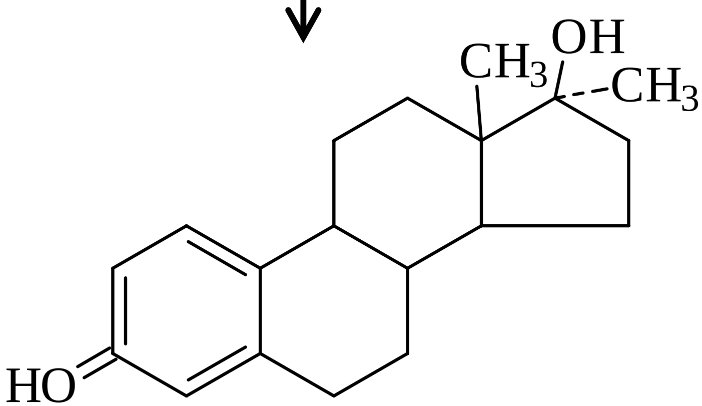
Model AR Agonists



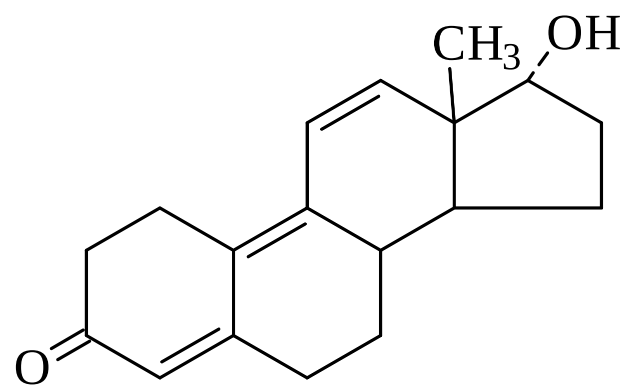
17α Methyl Testosterone [58-18-4]



17β-Trenbolone [10161-33-8]



17α Methyl 17β-Estradiol [302-76-1]



17β-Trenbolone [80657-17-6]

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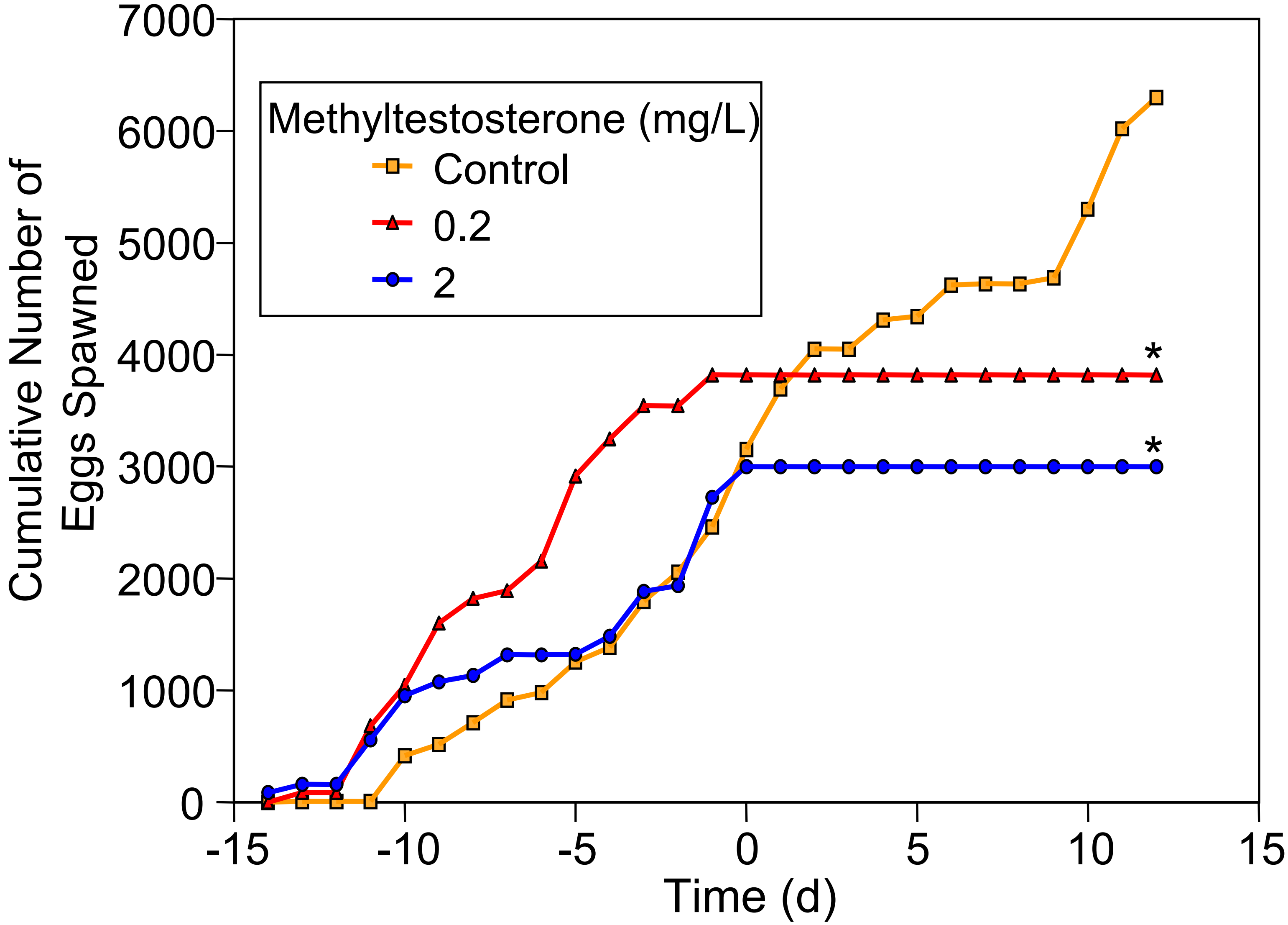
Effects of Methyltestosterone on Fecundity



Ankley et al. (2001) Environ.Toxicol. Chem.

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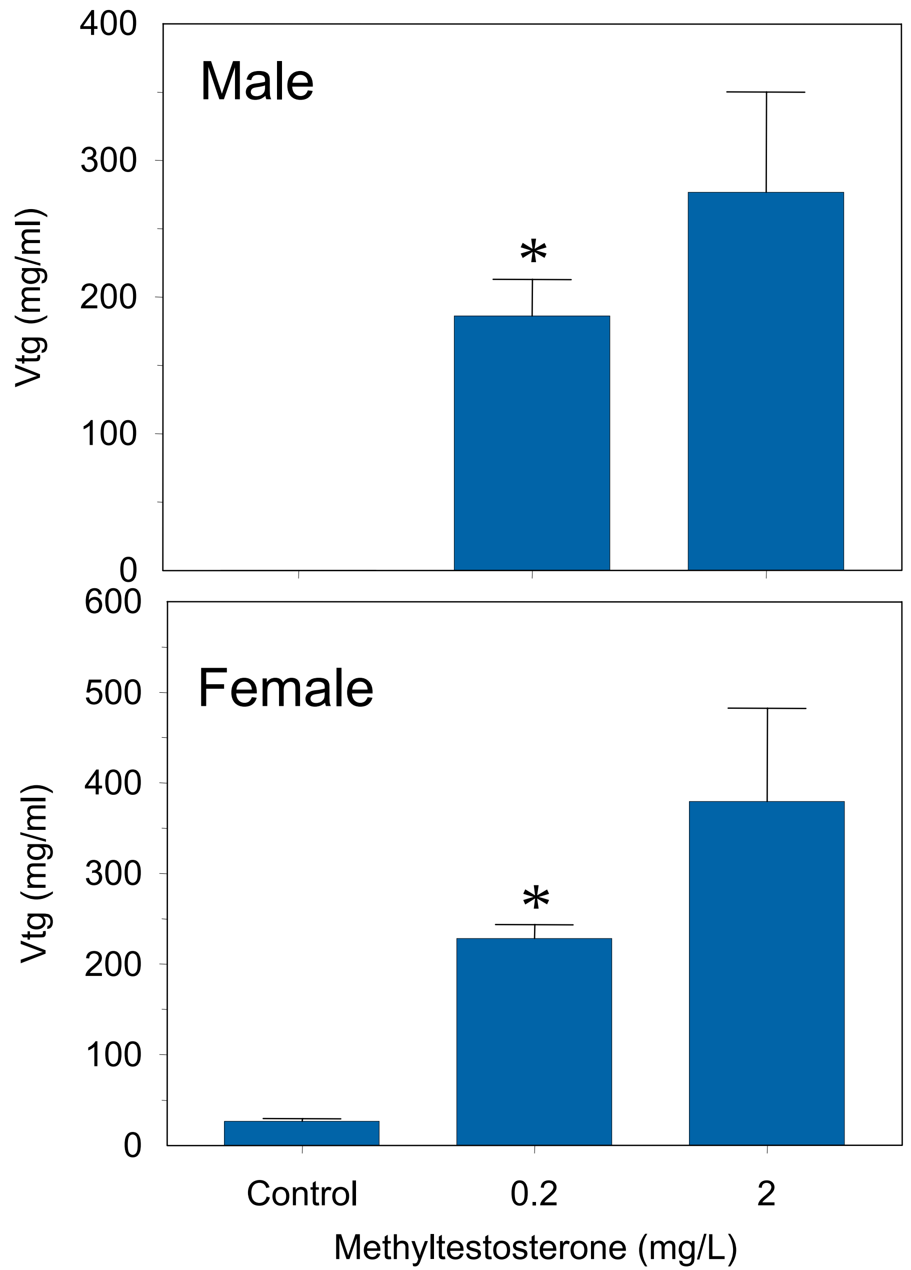
Methyltestosterone Masculinizes Females

Ankley et al. (2001) Environ.Toxicol. Chem.

Effects of Methyltestosterone on Vitellogenin



Ankley et al. (2001) Environ.Toxicol. Chem.

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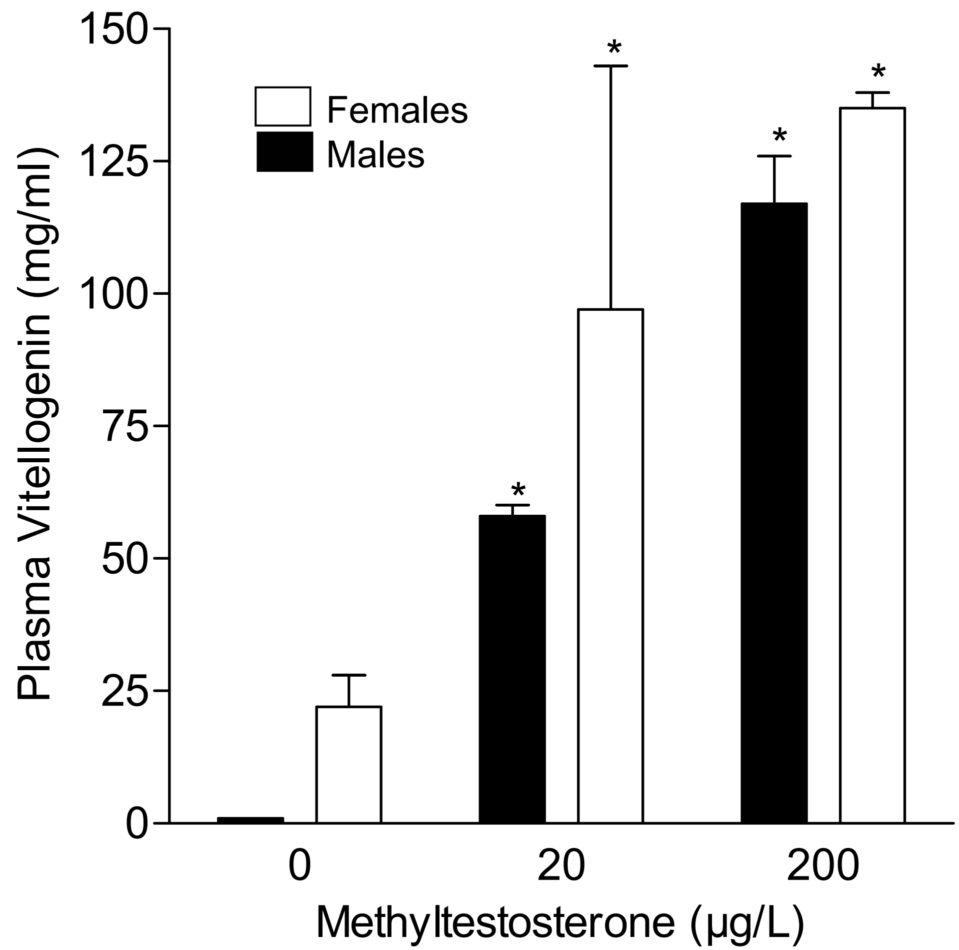
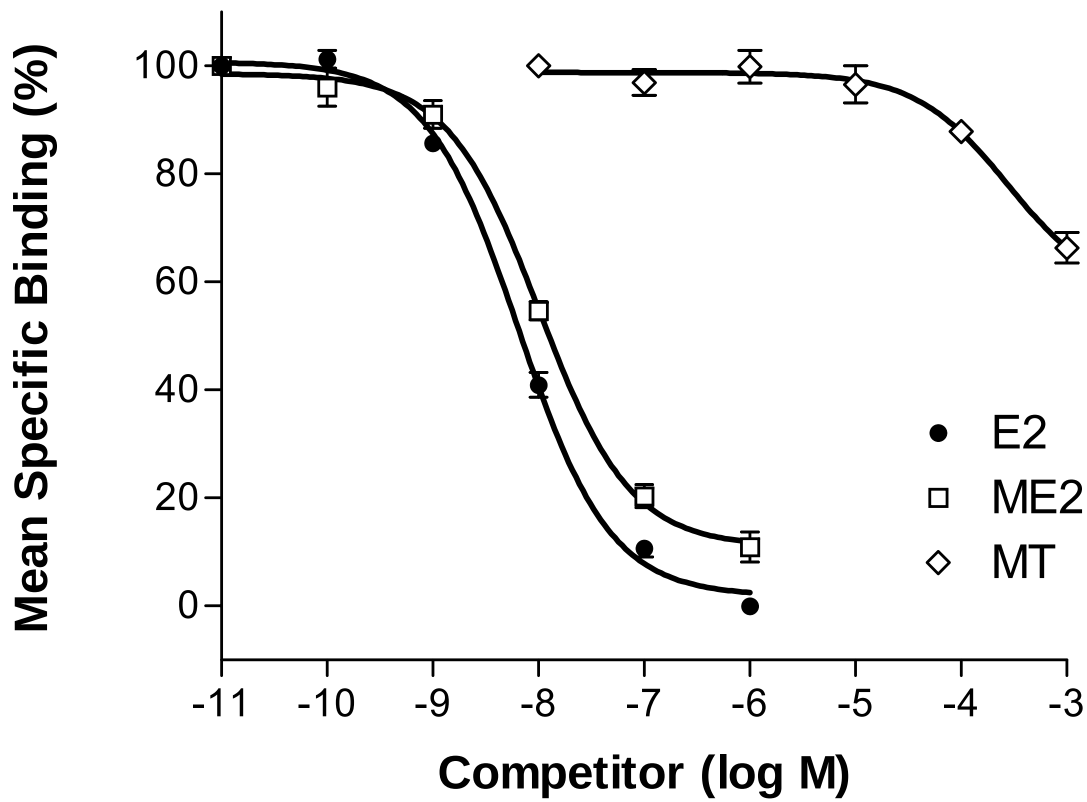
Effects of Methyltestosterone on Vitellogenin



Hornung et al. (2004) Aquat. Toxicol.

MT/ME2 Detection and Binding to Fathead Minnow ER

- Plasma ME2 in exposed fish: 2-3 ng/L (females) and 4-5 ng/L (males)



Hornung et al. (2004) Aquat. Toxicol

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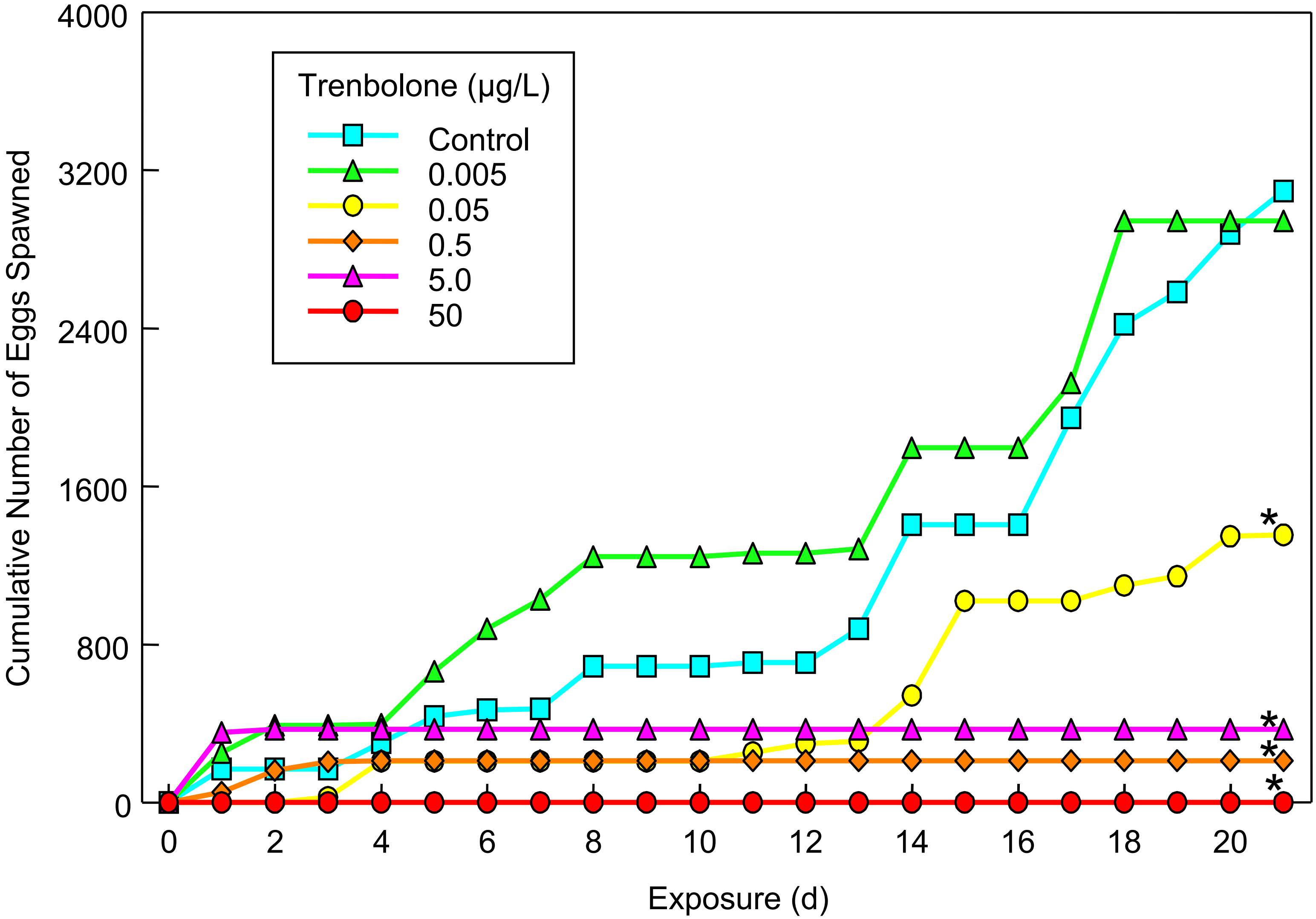
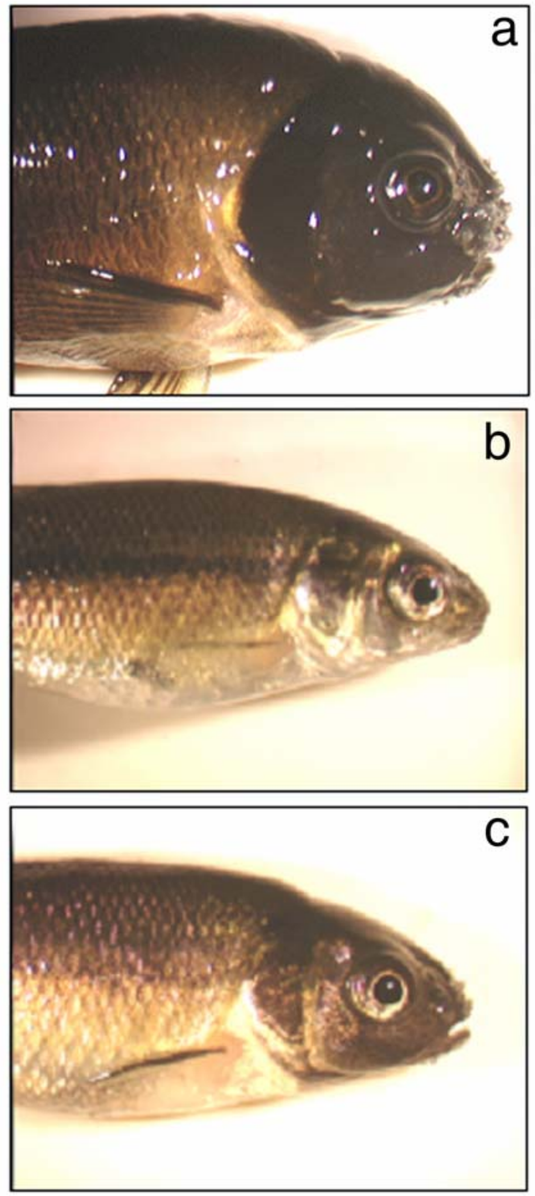
Effects of 17β-Trenbolone on Fathead Minnow Fecundity



Ankley et al. (2003) Environ.Toxicol. Chem.

Masculinization by 17β-Trenbolone



Ankley et al. (2003) Environ.Toxicol. Chem.

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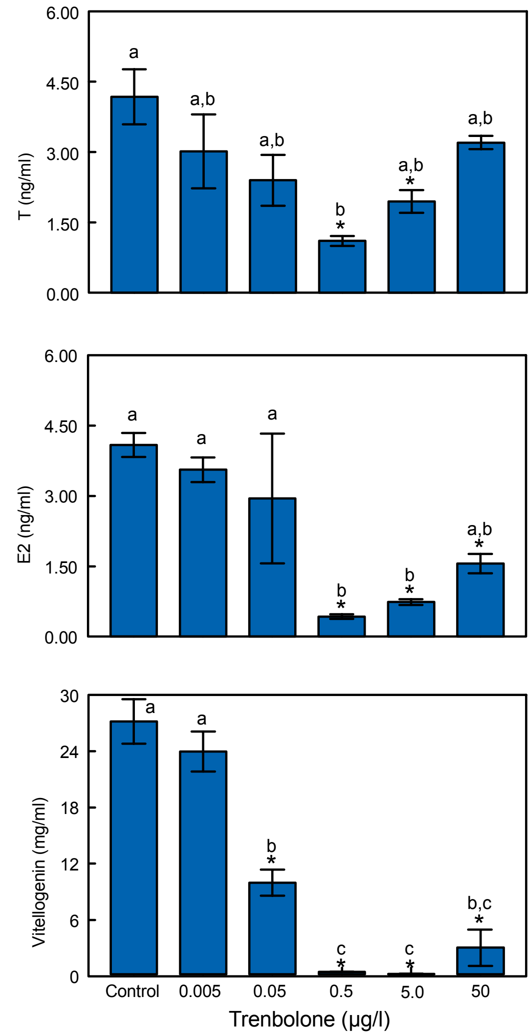
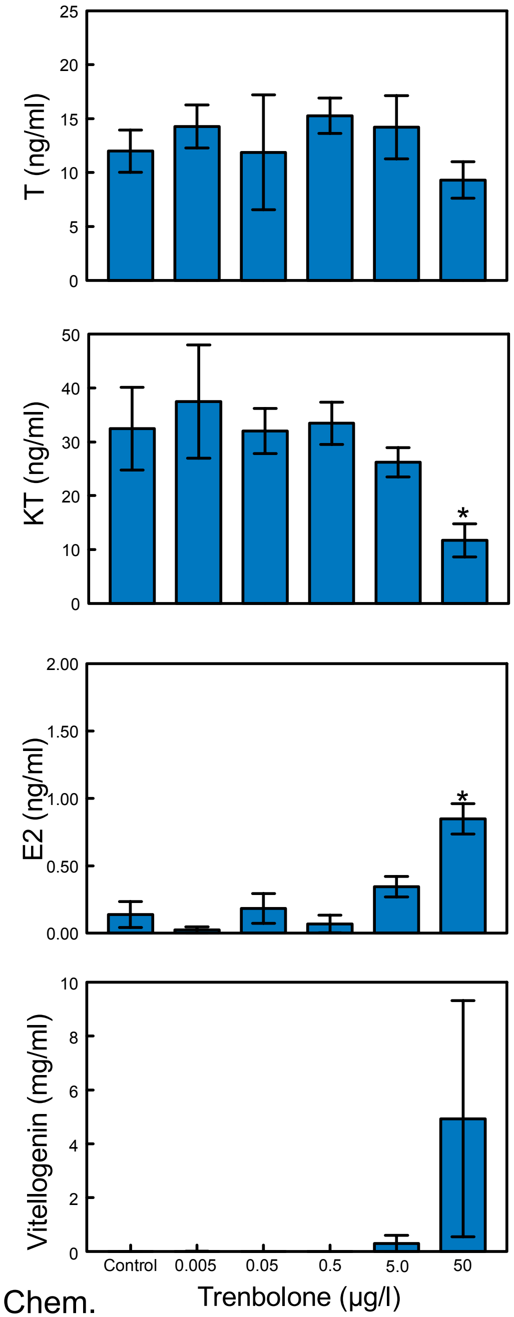
17β-Trenbolone Effects on Females



Ankley et al. (2003) Environ.Toxicol. Chem.

17β-Trenbolone Effects on Males



Ankley et al. (2003) Environ.Toxicol. Chem.

17β-Trenbolone Effects on Ovarian Histopathology

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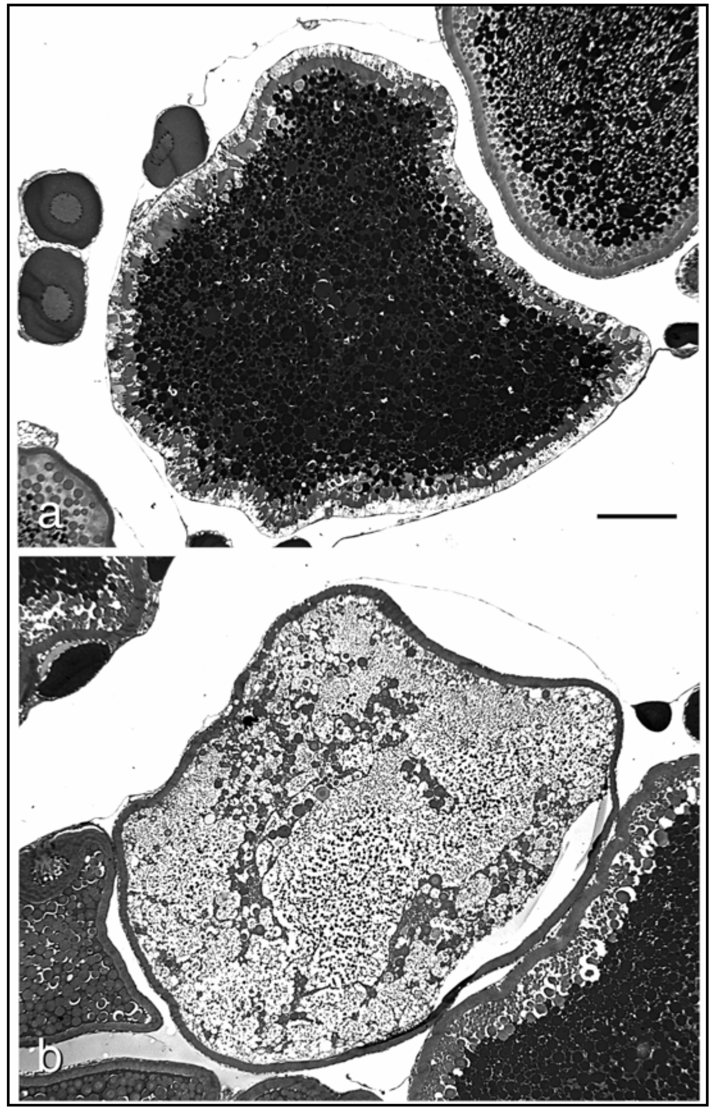

Ankley et al. (2003) Environ.Toxicol. Chem.

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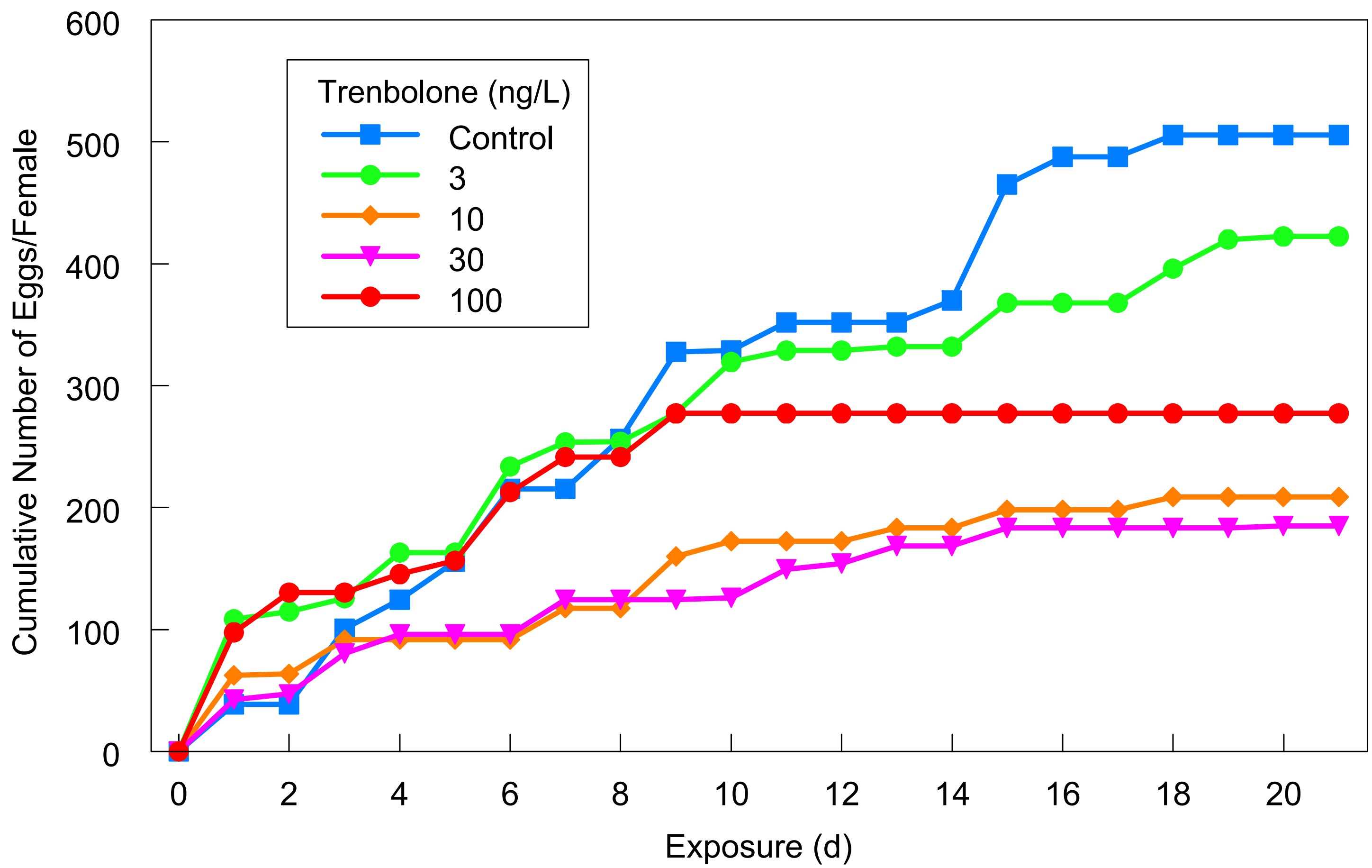
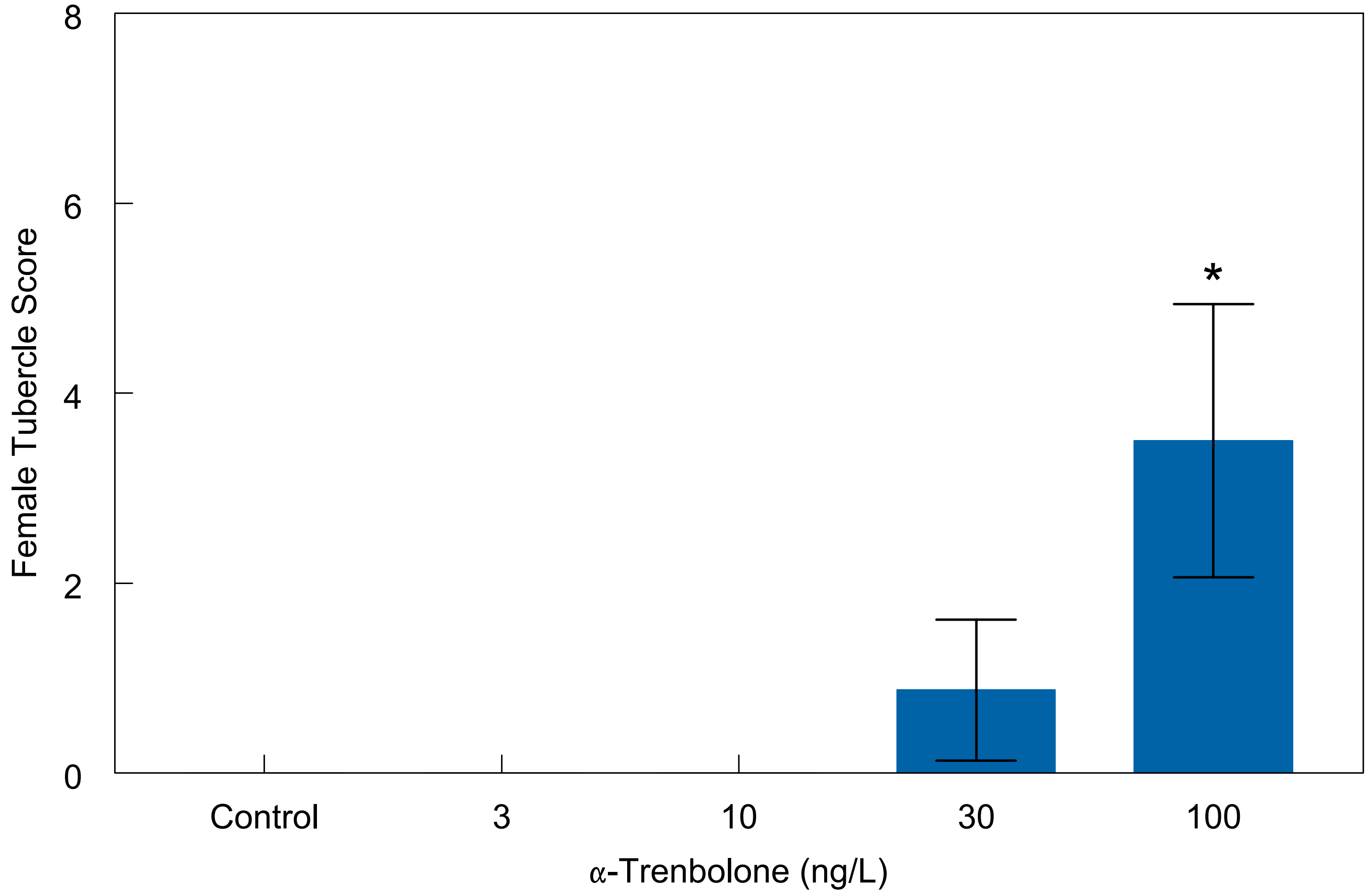
Effects of 17α-Trenbolone on Fathead Minnow Fecundity



Jensen et al. (2005) In preparation

Effects of 17α-Trenbolone on Fathead Minnow Tubercle Score[1]



[1]Scoring system from Jensen et al. (2001)

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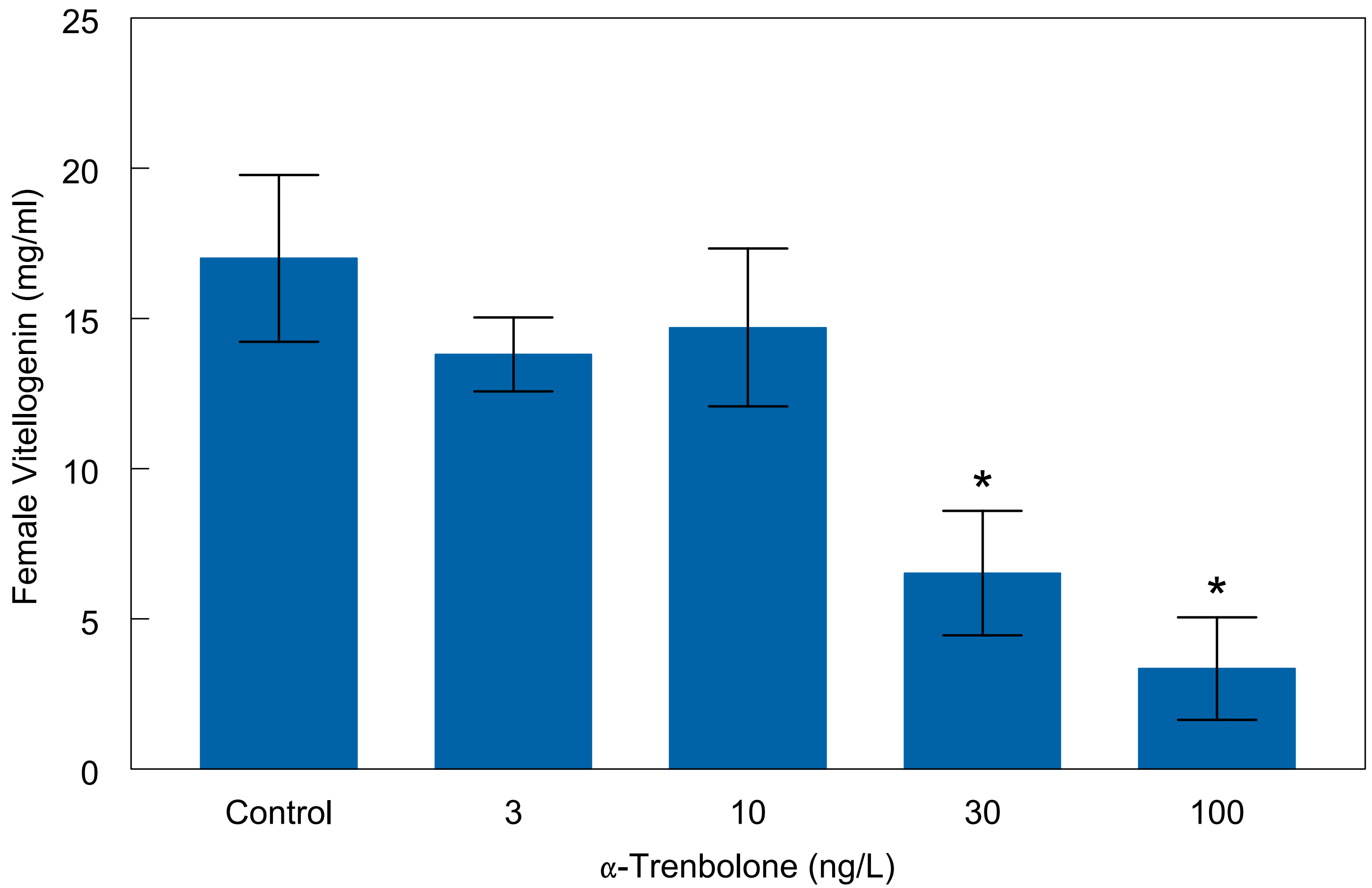
Effects of 17α-Trenbolone on Vitellogenin in Females



Jensen et al. (2005) In preparation

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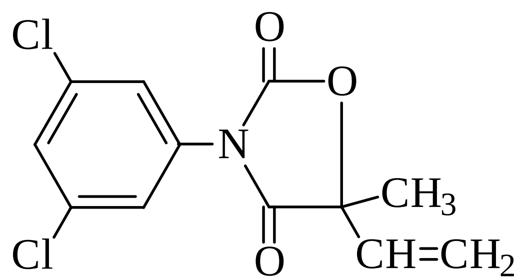
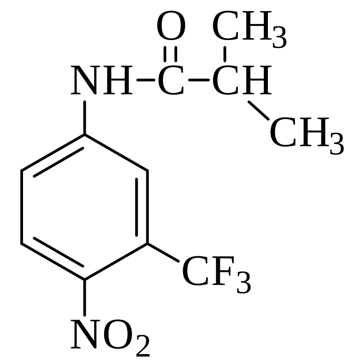
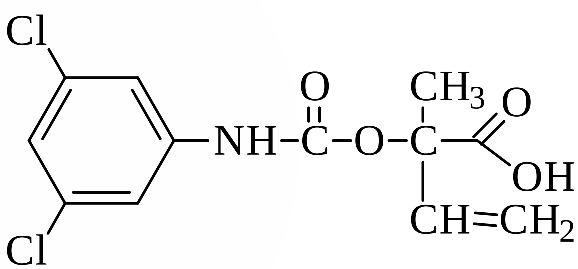
Model AR Antagonists



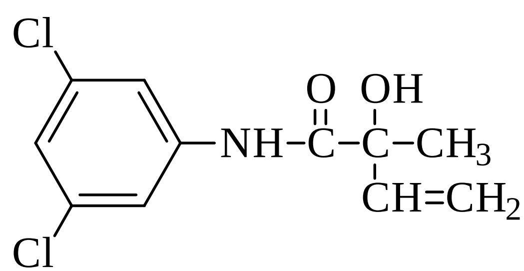
Vinclozolin [50471-44-8]



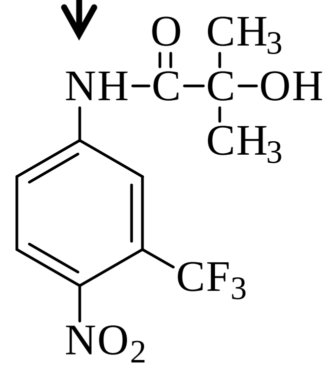
Flutamide
[13311-84-7]



M1



M2



2 Hydroxy Flutamide
[52806-53-8]

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aquatic toxicology

Aquatic Toxicology 48 (2000) 461–475

www.elsevier.com/locate/aquatox

Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*)

E.A. Makynen [a], M.D. Kahl [a], K.M. Jensen [a], J.E. Tietge [a], K.L. Wells [b], G. Van Der Kraak [b], G.T. Ankley [a,*]

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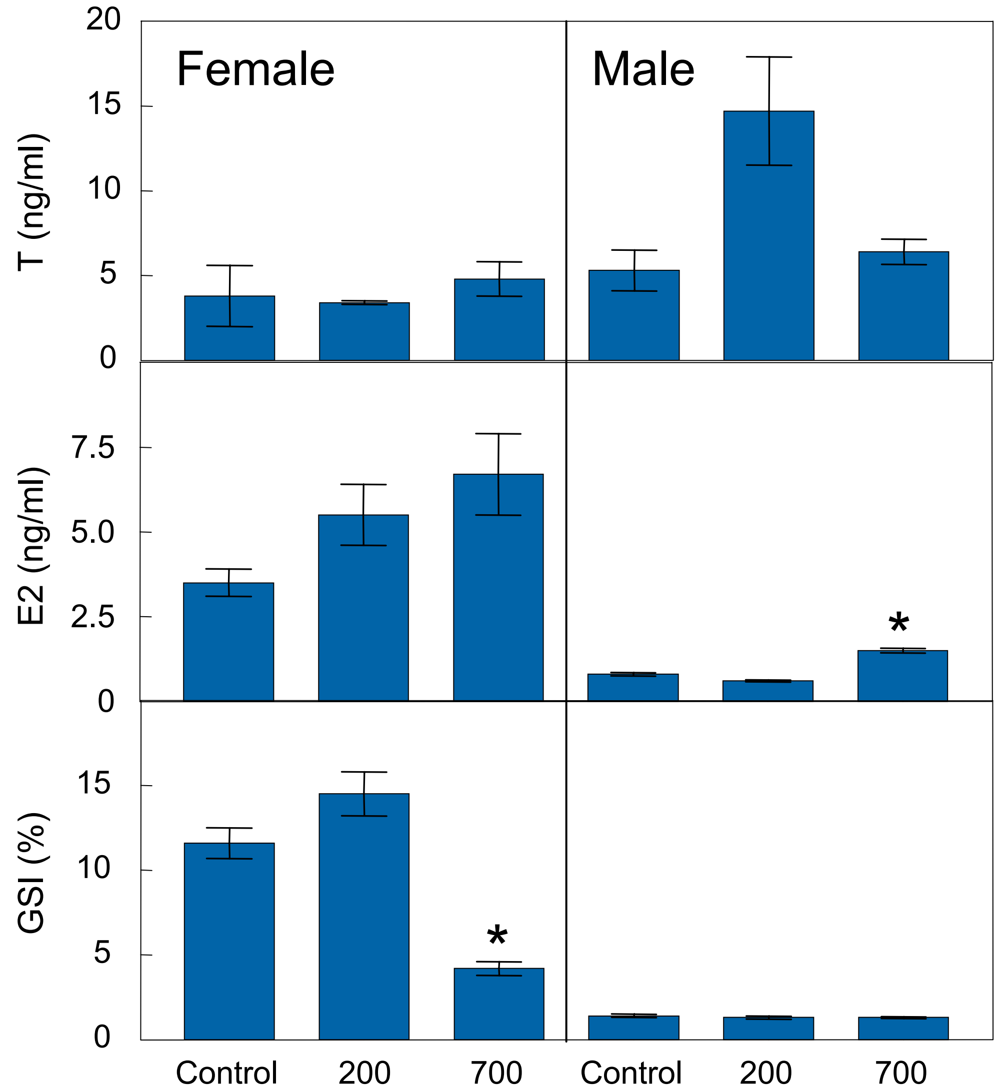
Effects of Vinclozolin on Fathead Minnow Reproductive Endpoints



Makynen et al. (2000) Aquat. Toxicol.

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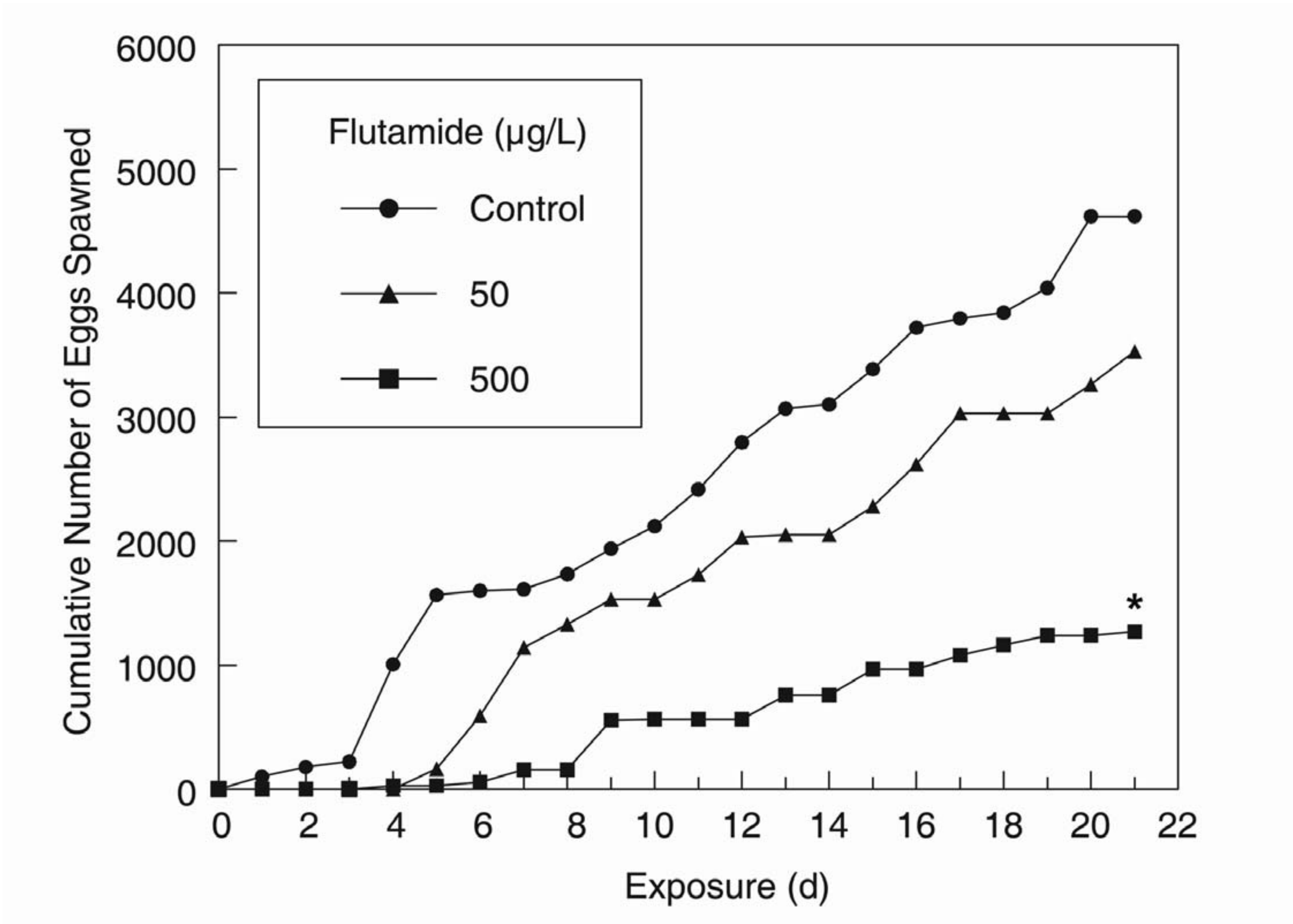
Effects of Flutamide on Fathead Minnow Fecundity



Jensen et al. (2004) Aquat. Toxicol.

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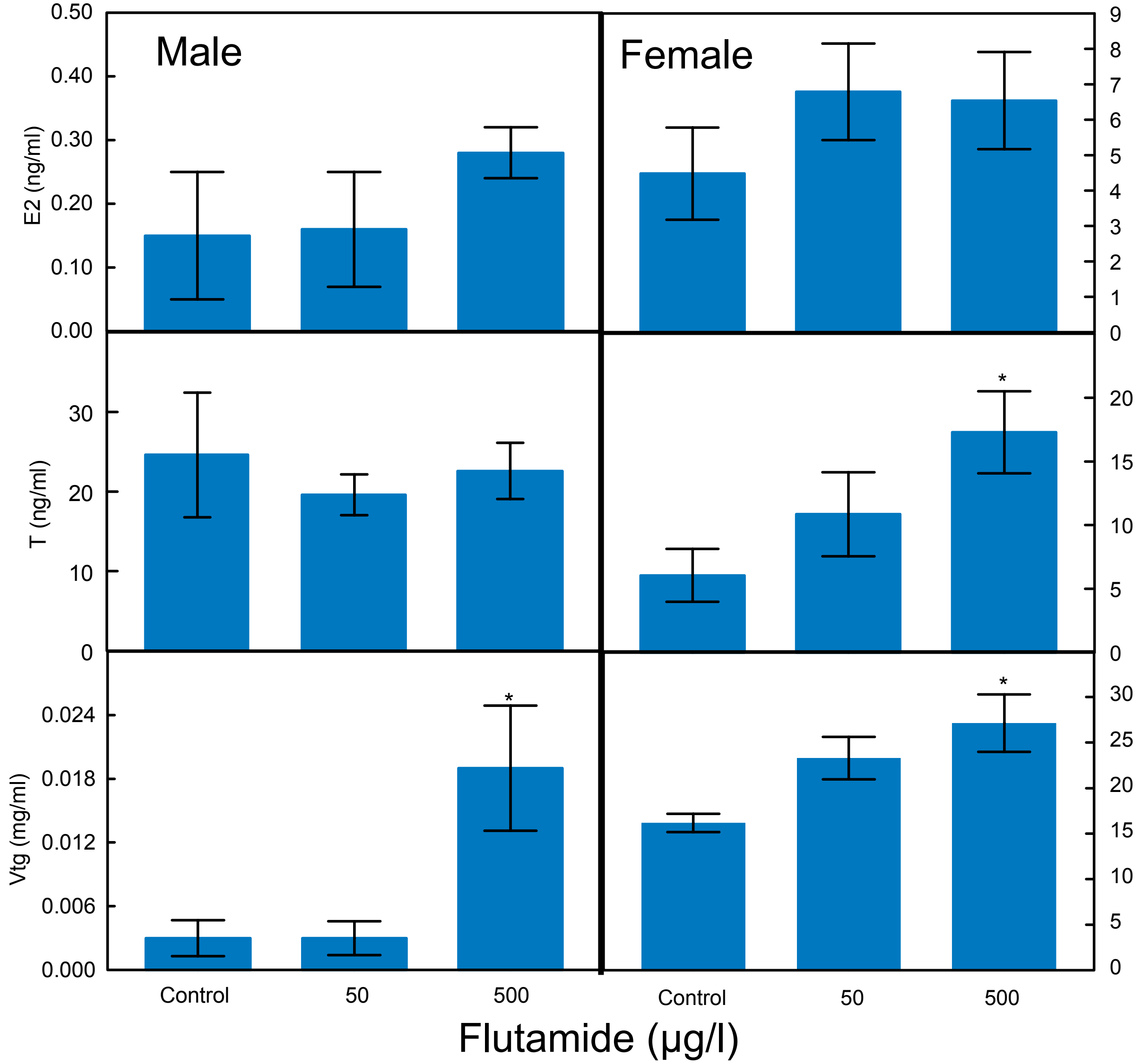
Flutamide Effects on Steroids and Vitellogenin in Fathead Minnow



Jensen et al. (2004) Aquat. Toxicol.

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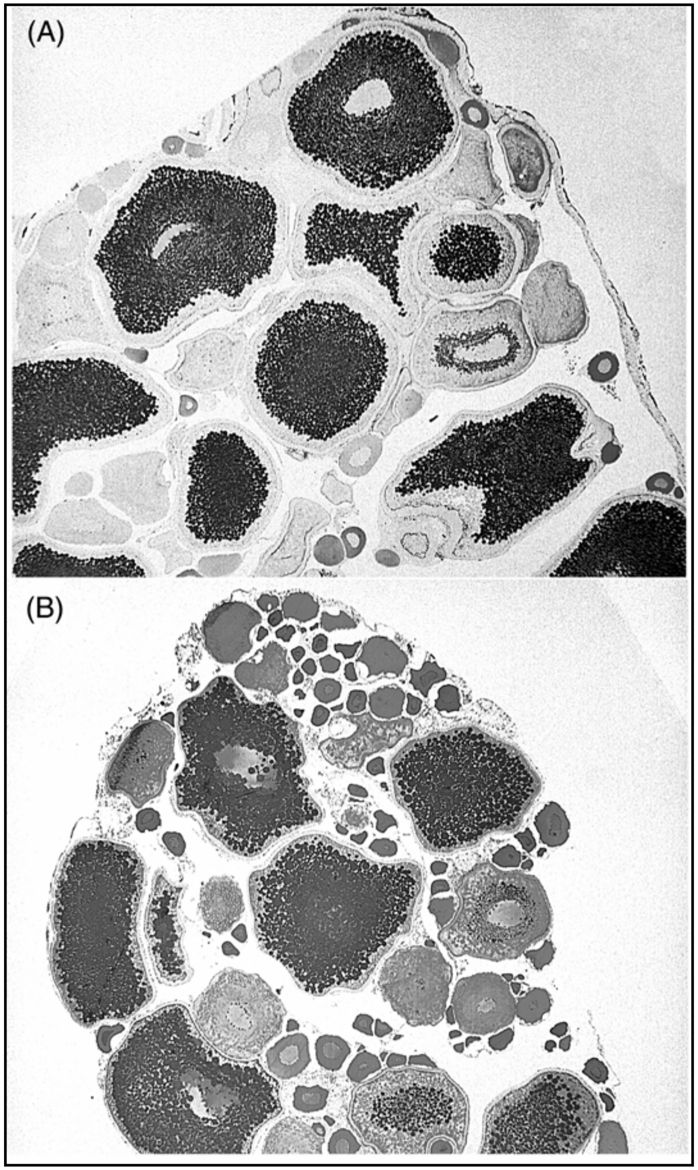
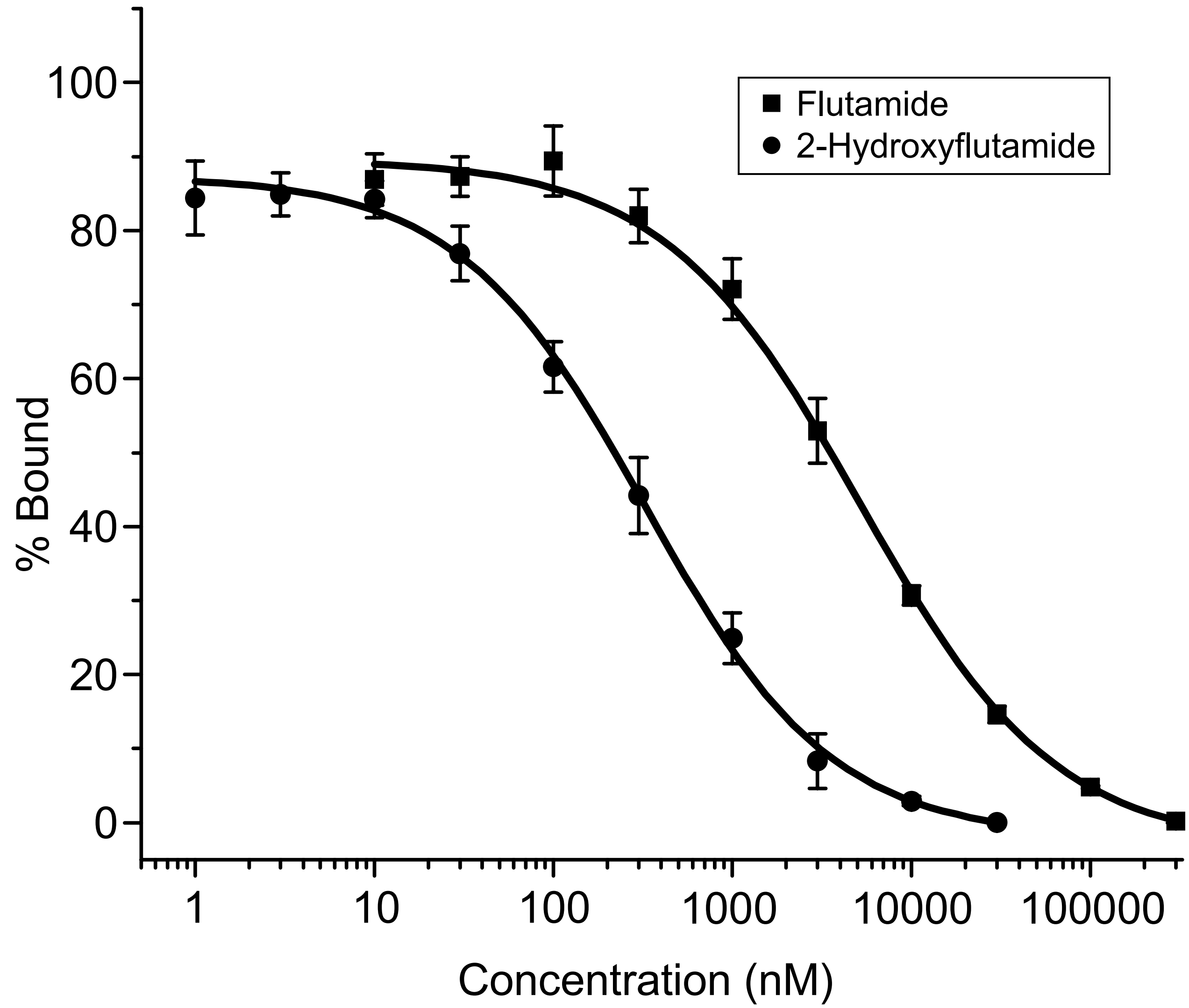
Effect of Flutamide on Fathead Minnow Ovary



Jensen et al. (2004) Aquat. Toxicol.

Flutamide Binding to Fathead Minnow AR



Ankley et al. (2004) Environ. Sci. Technol.

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In vivo Confirmation of Flutamide as AR Antagonist

- Treatment Groups: (a) control; (b) Flutamide (400 µg/L); (c) Trenbolone (0.5 µg/L); (d) Flutamide (400 µg/L) + Trenbolone (0.5 µg/L)
- Endpoint: Tubercle Production (7,14 d) in adult female fathead minnows
- Result: 17β-Trenbolone induced tubercle formation in 13 of 20 (7d) and 19 of 20 (14 d) fish. Co-treatment with flutamide completely blocked response

Ankley et al. (2004) Environ. Sci. Technol.

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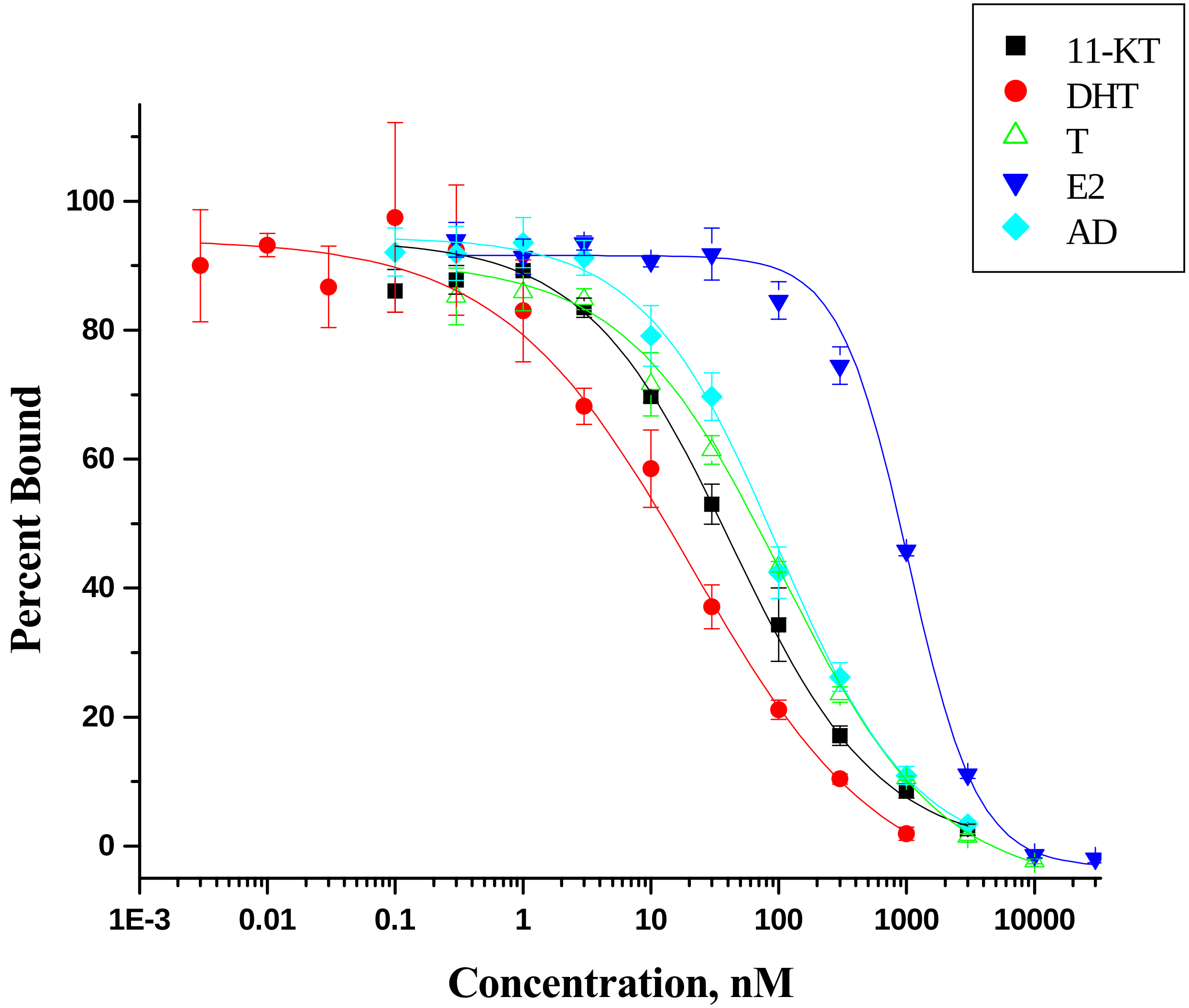
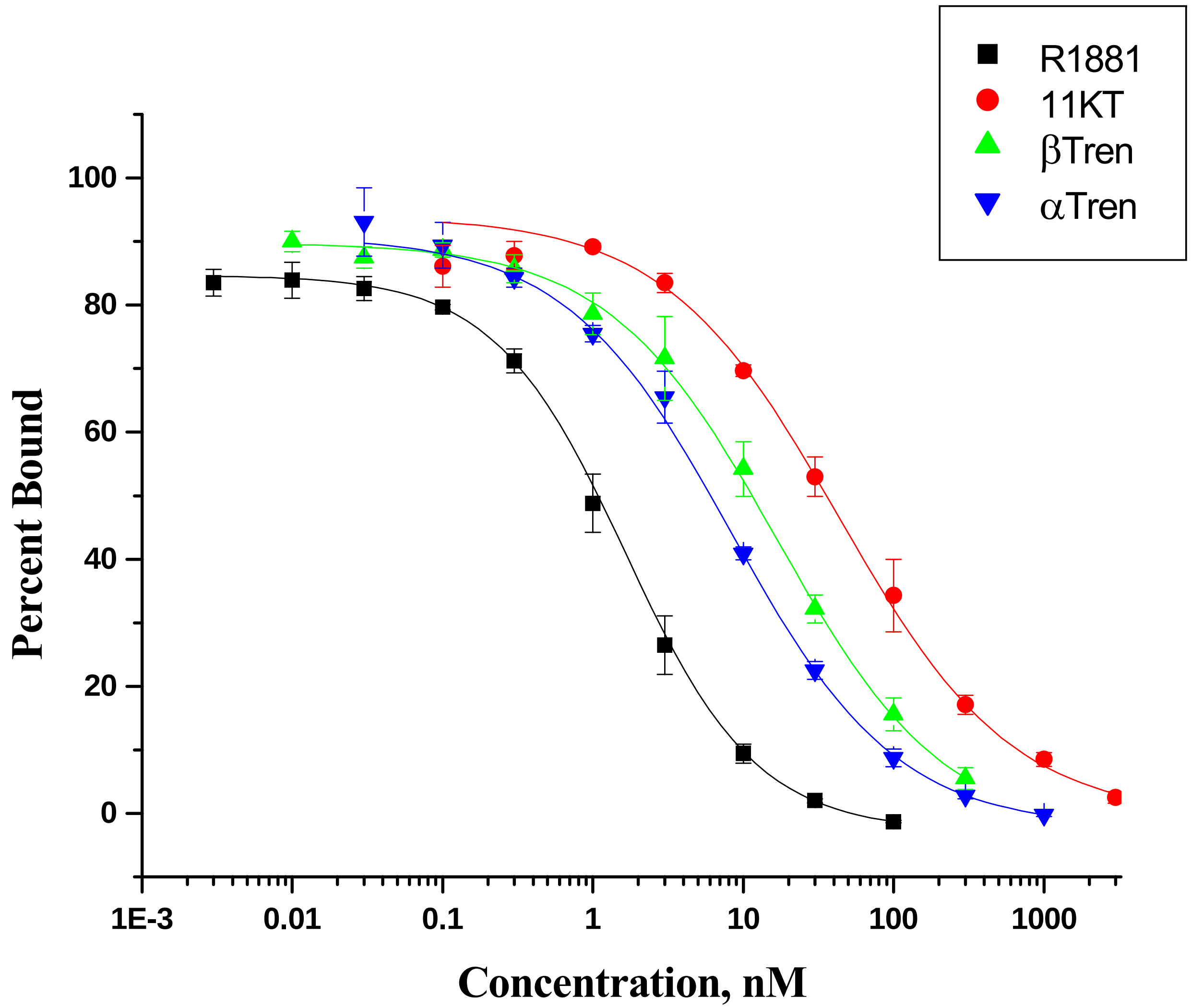
Natural Steroid Binding to Fathead Minnow AR



Wilson et al. (2004) Environ. Sci. Technol.

Binding of Synthetic Androgens to Fathead Minnow AR



Wilson et al. (2004) Environ. Sci. Technol.

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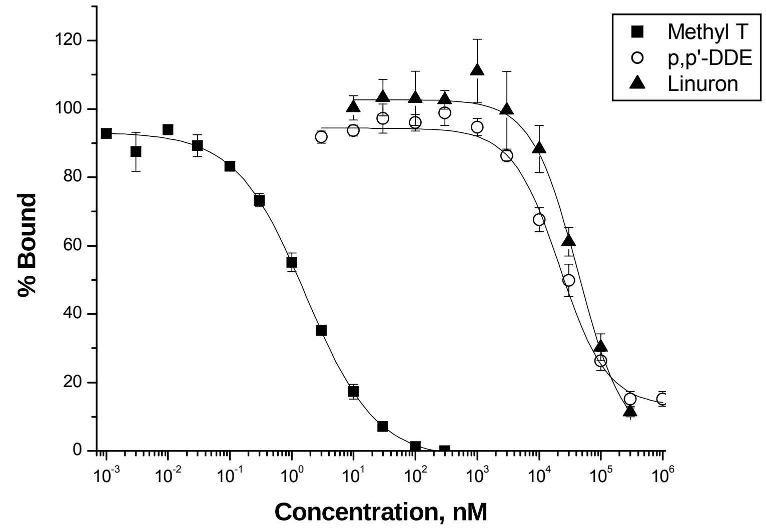
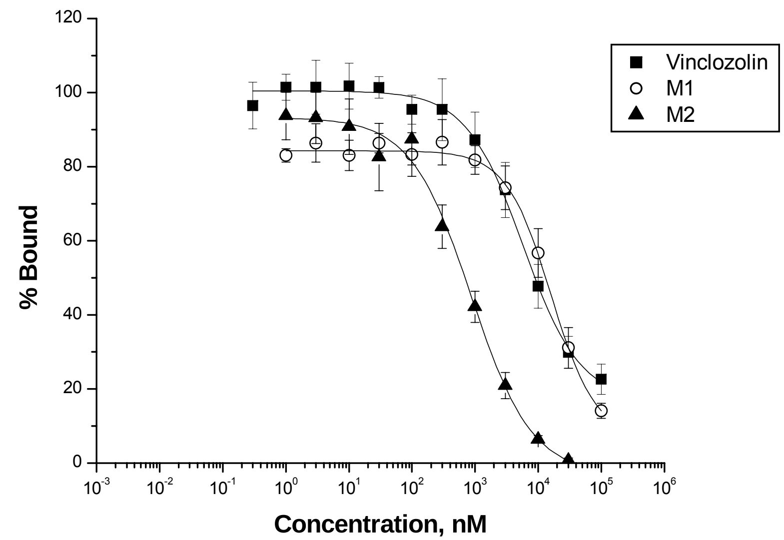
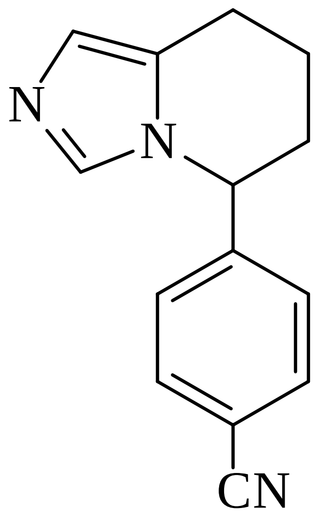
Binding of Antagonists to the Fathead Minnow AR



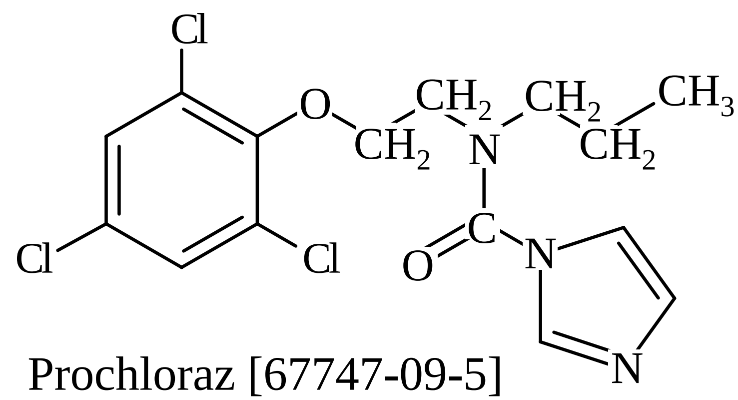


Wilson et al. (2004) Environ. Sci. Technol.

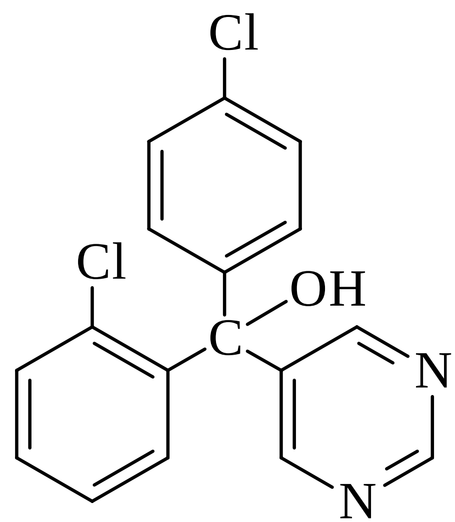
Steroid Metabolism Inhibitors



Fadrozole [102676-31-3]

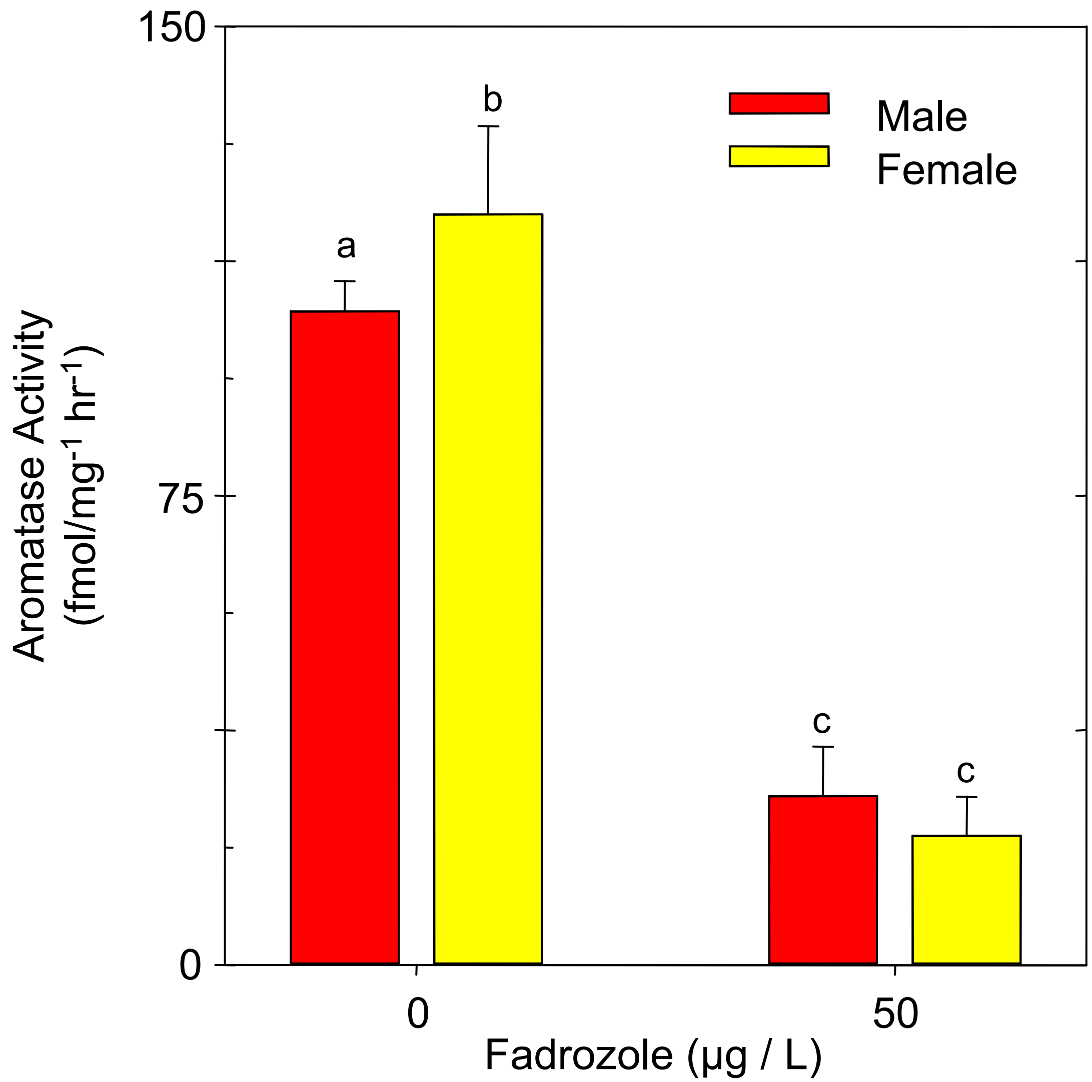


Prochloraz [67747-09-5]



Fenarimol [60168-88-9]

Effect of Fadrozole on Fathead Minnow Aromatase



Ankley et al. (2002) Toxicol. Sci.

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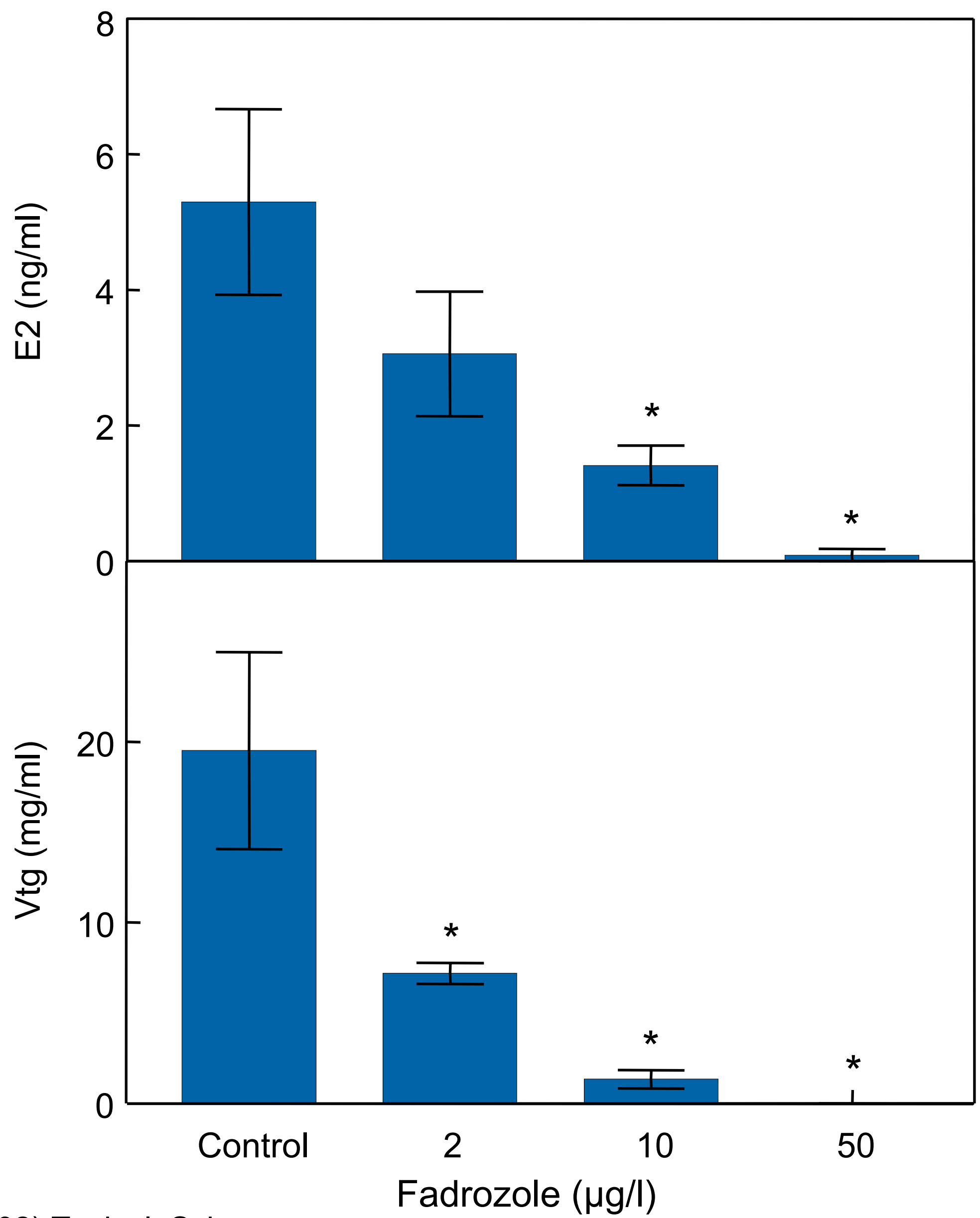
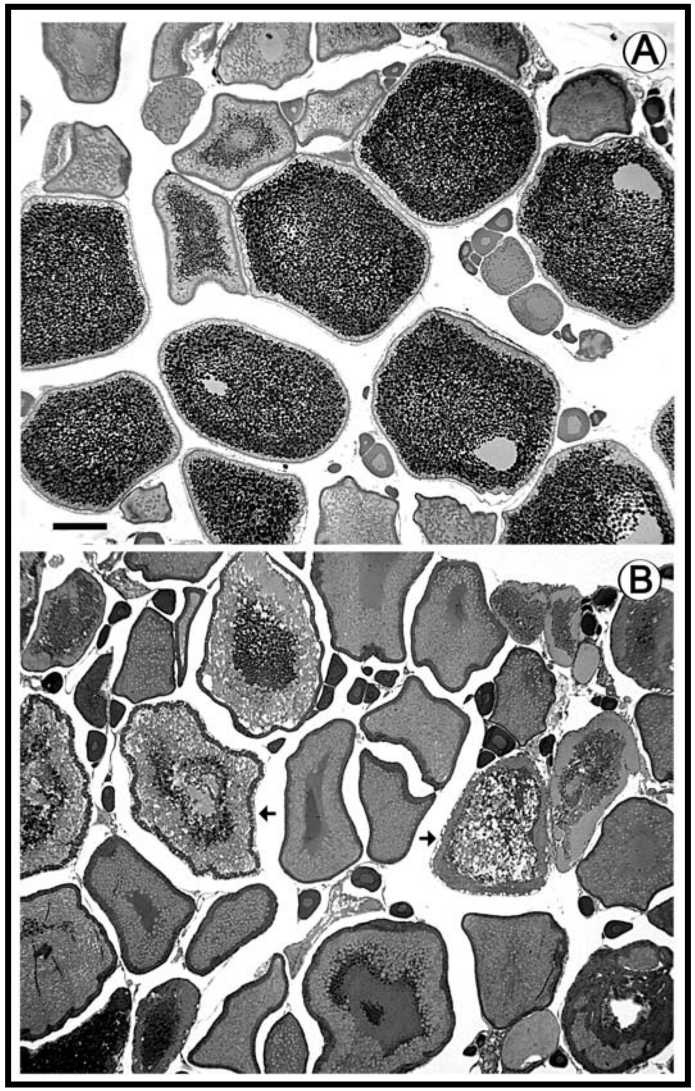
Fadrozole Effects on E2, Vtg



Ankley et al. (2002) Toxicol. Sci.

Fadrozole Effects on Fathead Minnow Ovary



Ankley et al. (2002) Toxicol. Sci.

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Effects of Fadrozole on Fathead Minnow Fecundity

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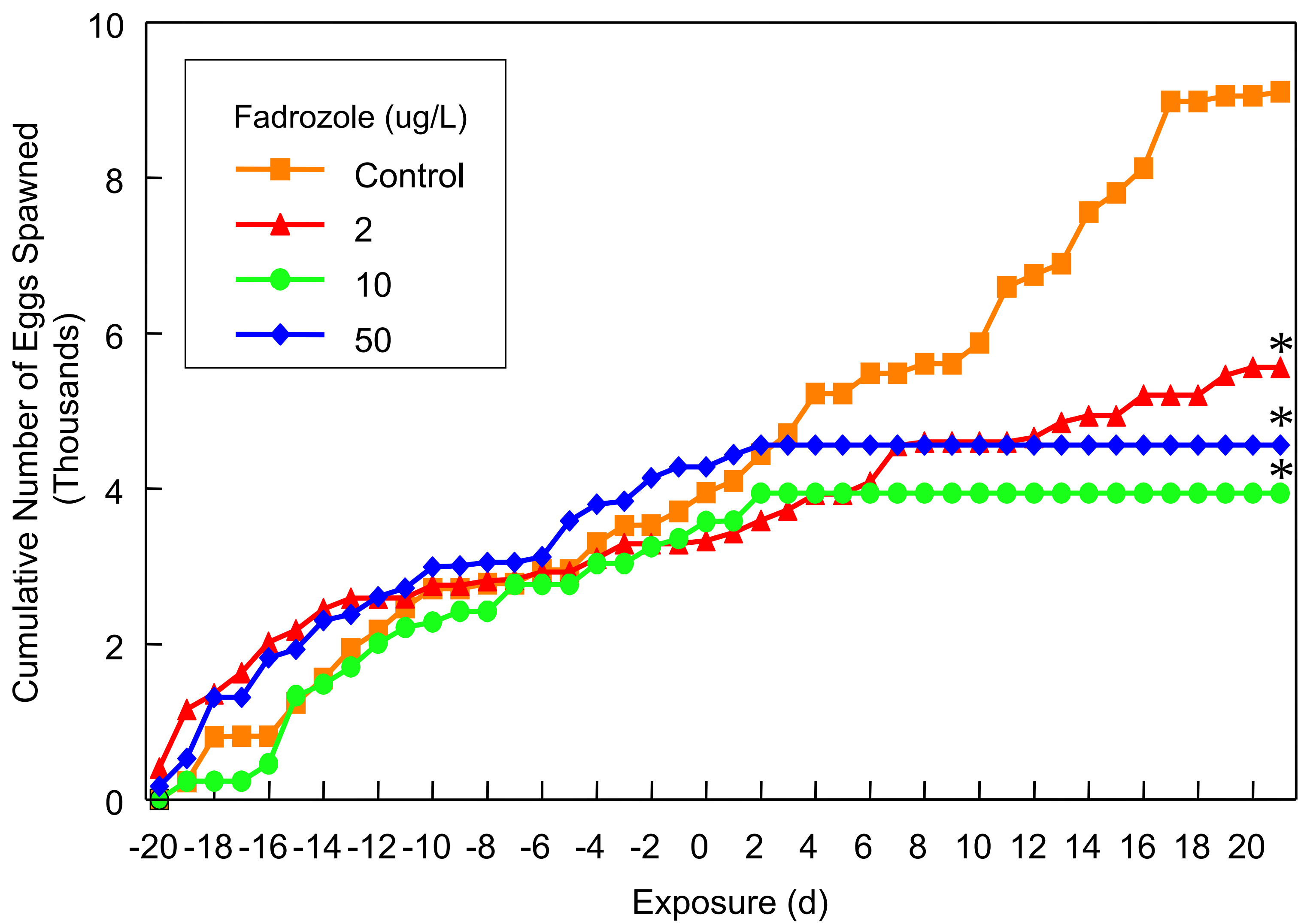
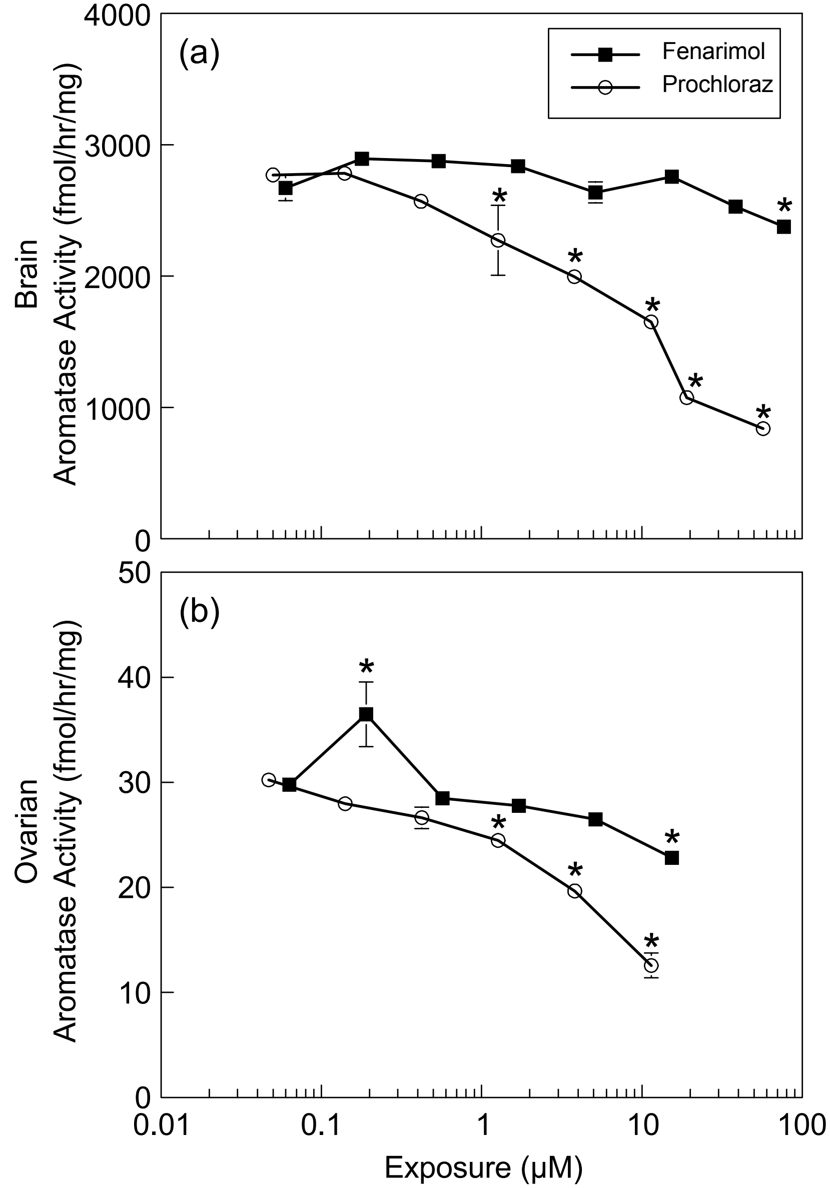


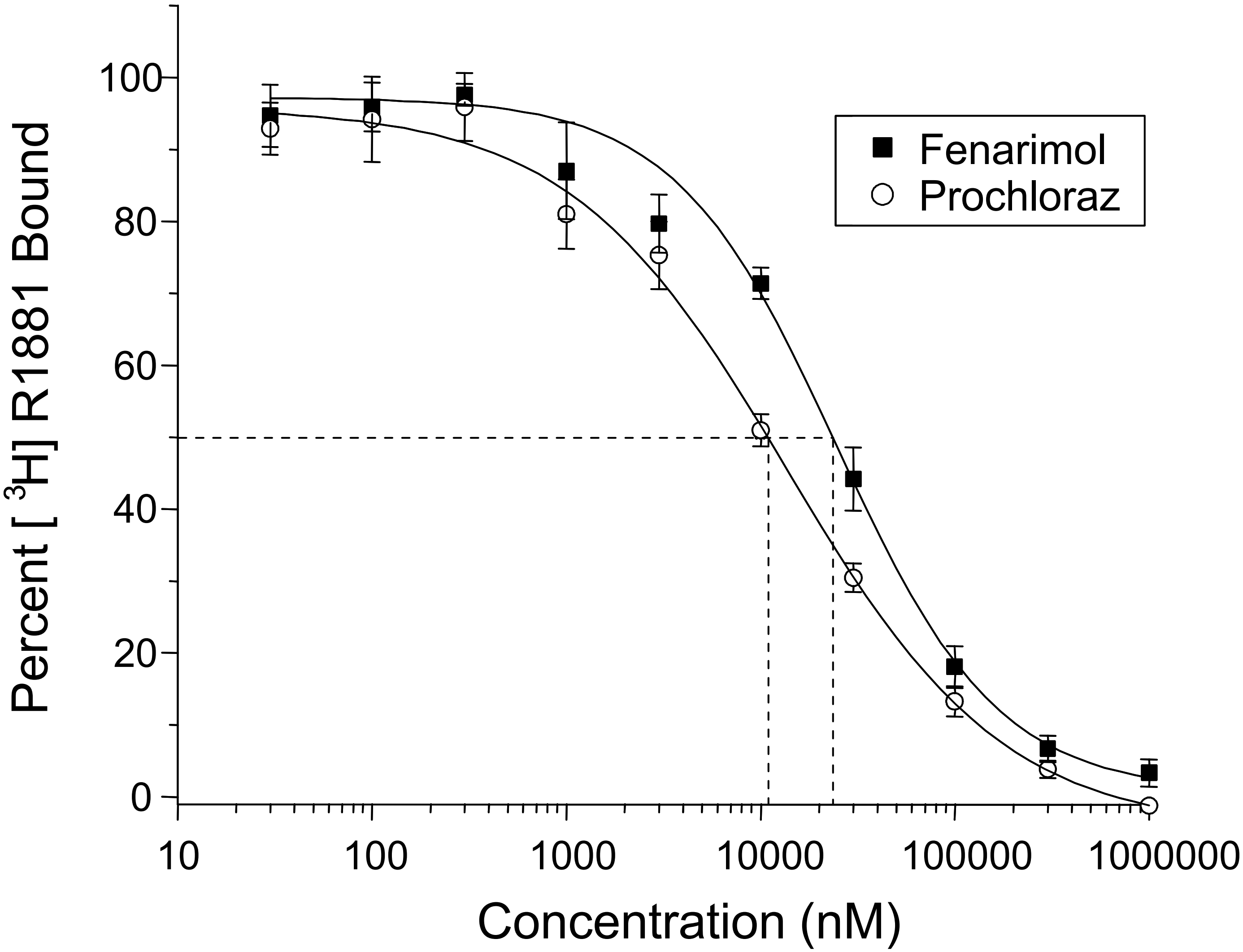
Ankley et al. (2002) Toxicol. Sci.

Inhibition of Fathead Minnow Aromatase by Two Fungicides



Ankley et al. (2005) Toxicol. Sci.

Binding of Two Fungicides to Fathead Minnow AR



Ankley et al. (2005) Toxicol. Sci.

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Effects of Two Fungicides on Fathead Minnow Fecundity

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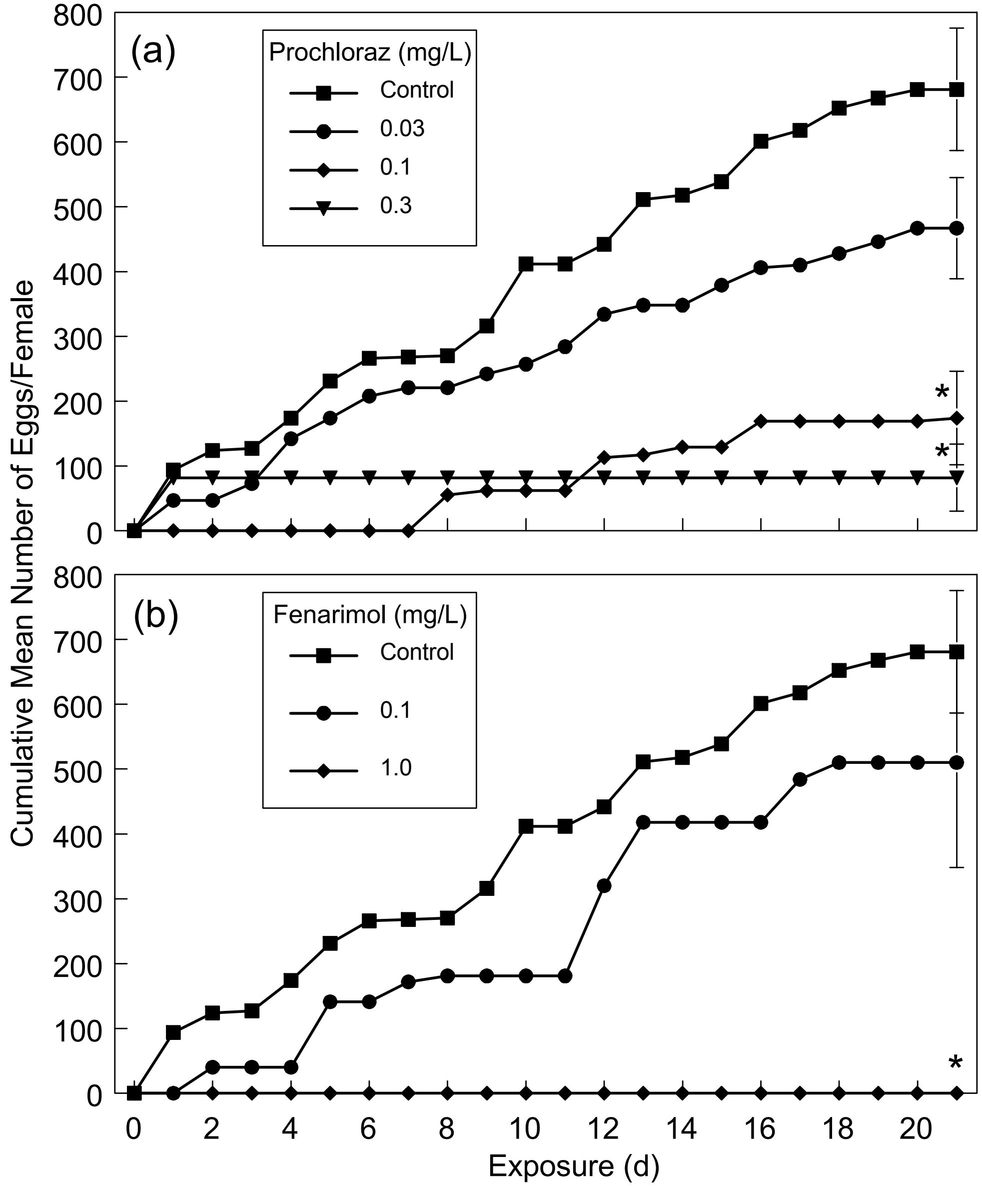

Ankley et al. (2005) Toxicol. Sci.

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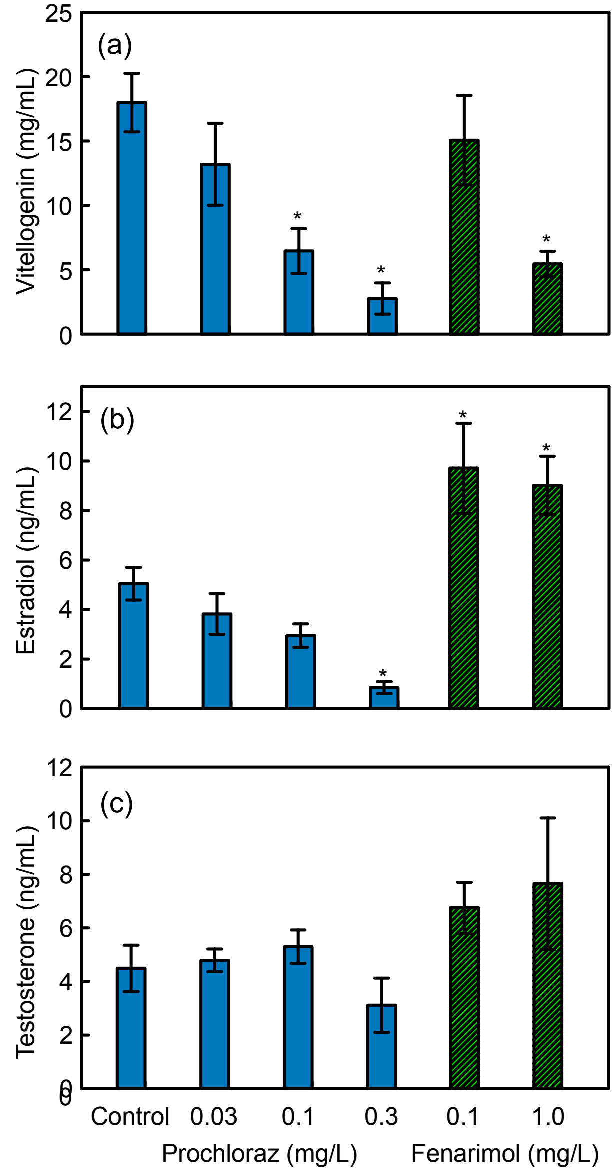
Endocrine Effects of Two Fungicides on Fathead Minnow Females



Ankley et al. (2005) Toxicol. Sci.

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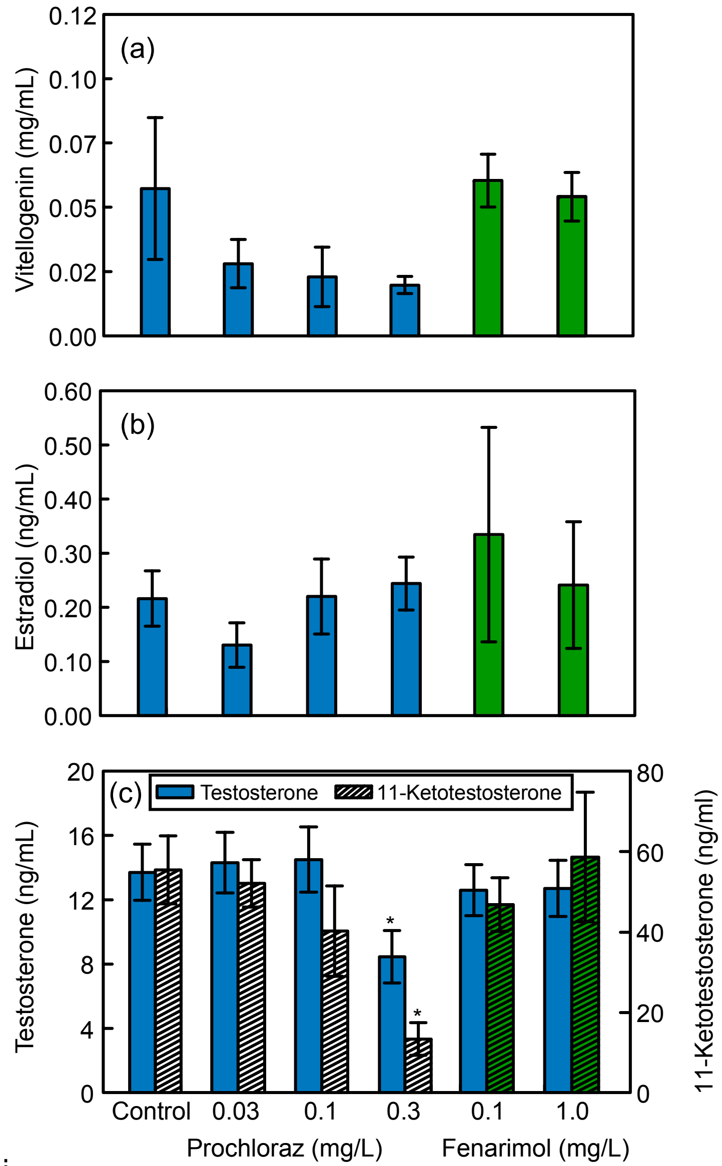
Endocrine Effects of Two Fungicides on Fathead Minnow Males



Ankley et al. (2005) Toxicol. Sci.

External Evalution of 21-d Fathead Minnow Test Designs

Battelle (EPA-supported contractor)

- Assays with four chemicals tested by MED
- Assays with additional five possible EDCs

Organization for Economic Cooperation (OECD)

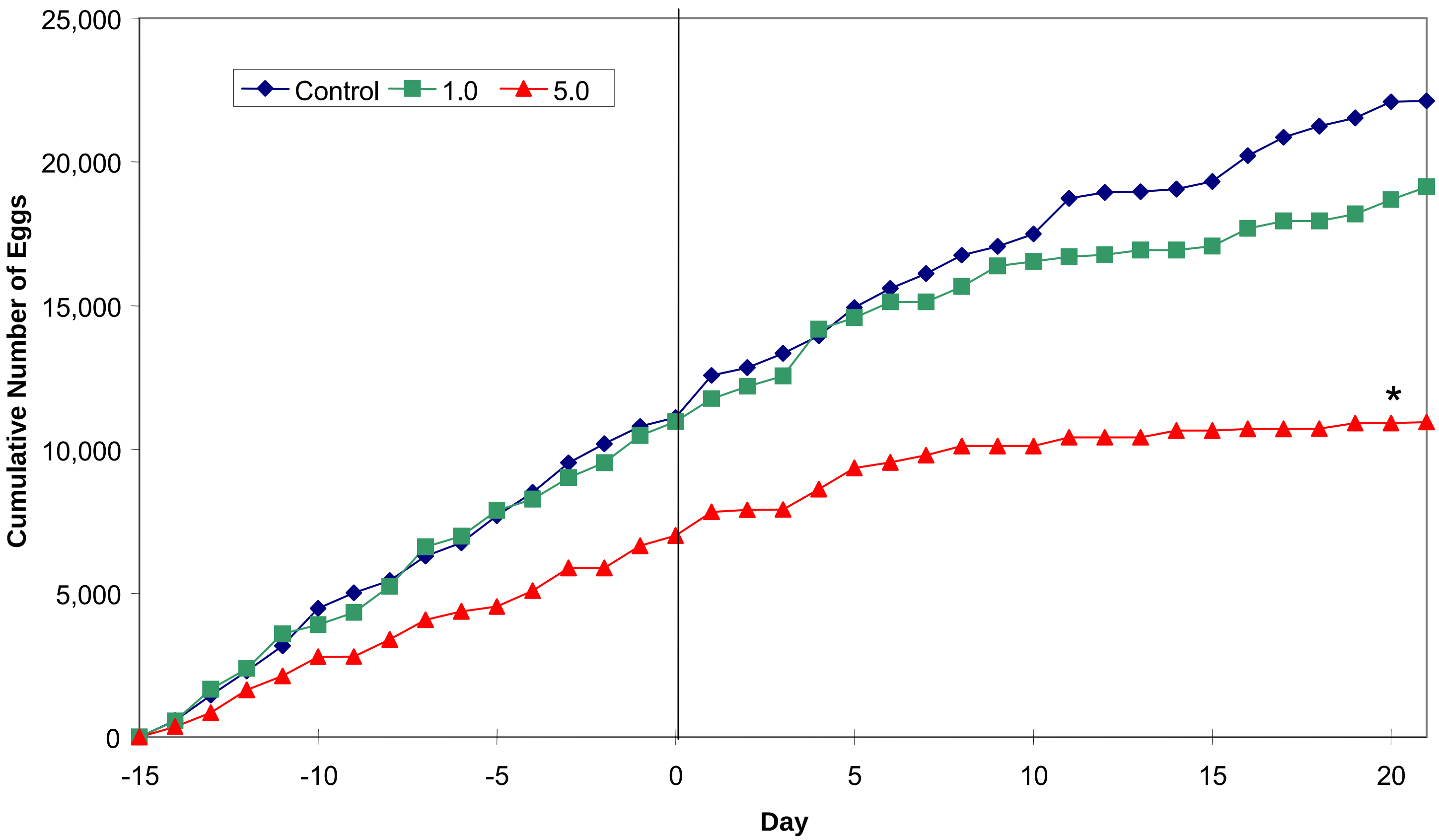
- Ring-test using 21-d nonspawning assay and two potent steroids (Phase1A)
- Ring-test using 21-d spawning assay and “weaker” EDCs (Phase 1B)

Objective: Battelle Study 1

Evaluate responses to four known EDCs using 21-d fathead minnow protocol

Chemical	Nominal Test Concentrations (µg/L)	
Methoxychlor	1.0	5.0
17β-Trenbolone	0.1	1.0
Flutamide	6.0	650
Fadrozole	5.0	50

Methoxychlor Effects in Fathead Minnow Assay



- Treatment-related mortality observed in 5 µg/L males
- Male vitellogenin (mg/ml): Control = <0.001 (<0.001, 7)
  Low = 0.001 (0.001, 7)
  High = 10.89 (1)

Battelle (2003)

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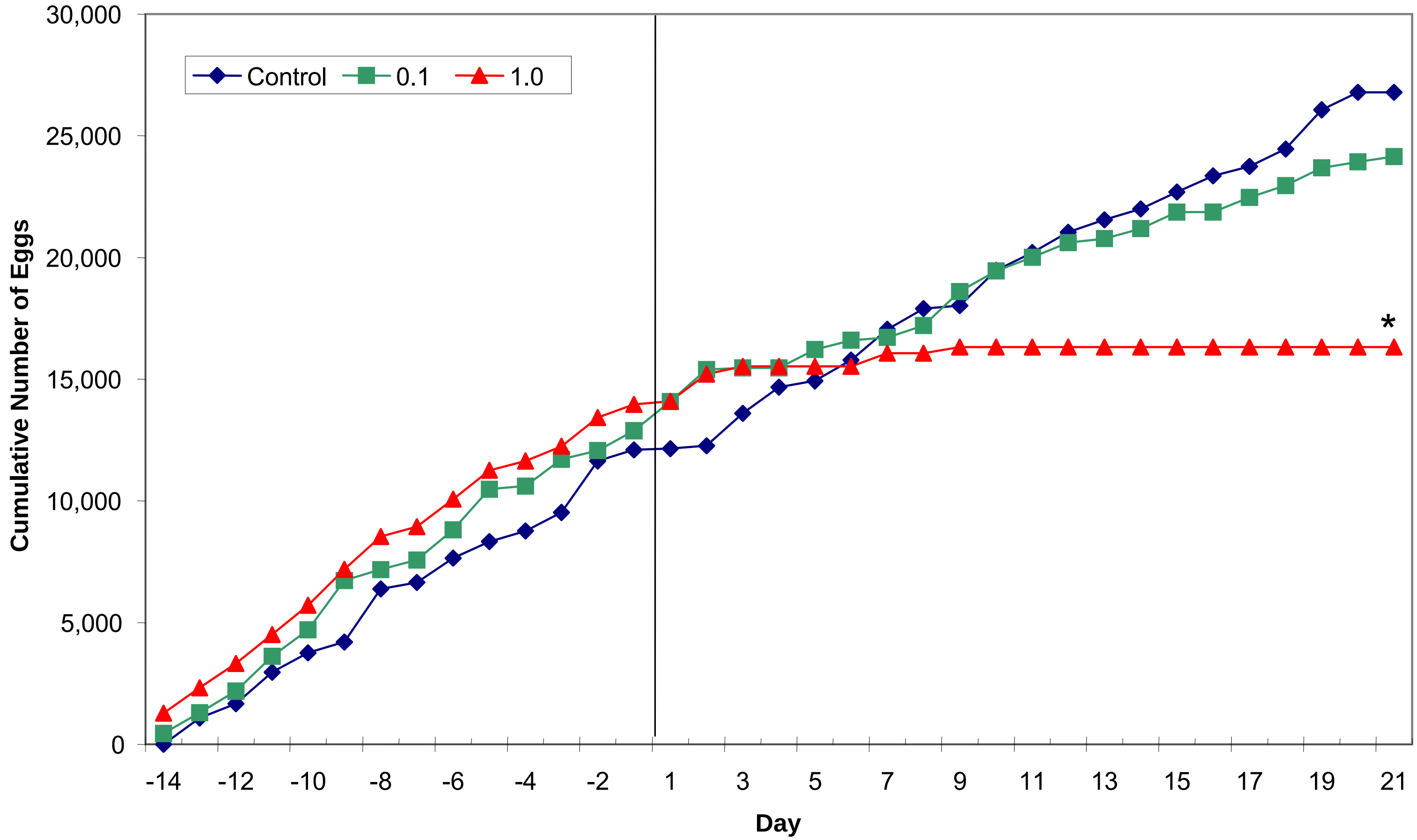
17β-Trenbolone Effects in Fathead Minnow Assay



- Masculinization observed in exposed females, particularly at 1.0 µg/L.
- Significant decrease in female vitellogenin (mg/ml): Control = 1.29 (1.16,8)
  Low = 1.22 (1.09, 10)
  High = 0.11 (0.27,9)

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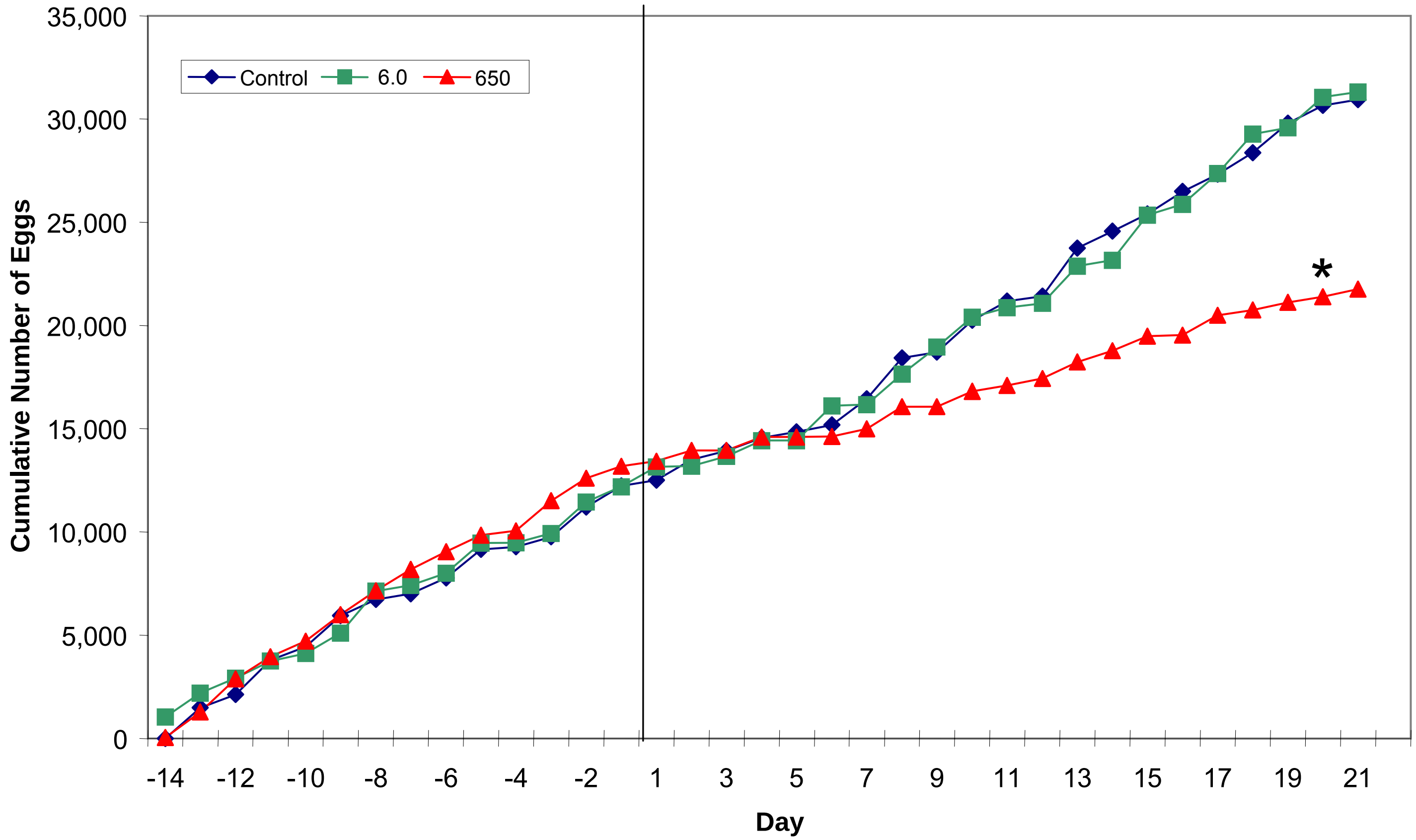
Flutamide Effects in Fathead Minnow Assay



- No significant/dose-dependent were noted for any endpoint other than increase in proportion of artetic follicles in ovaries of fish from high concentration

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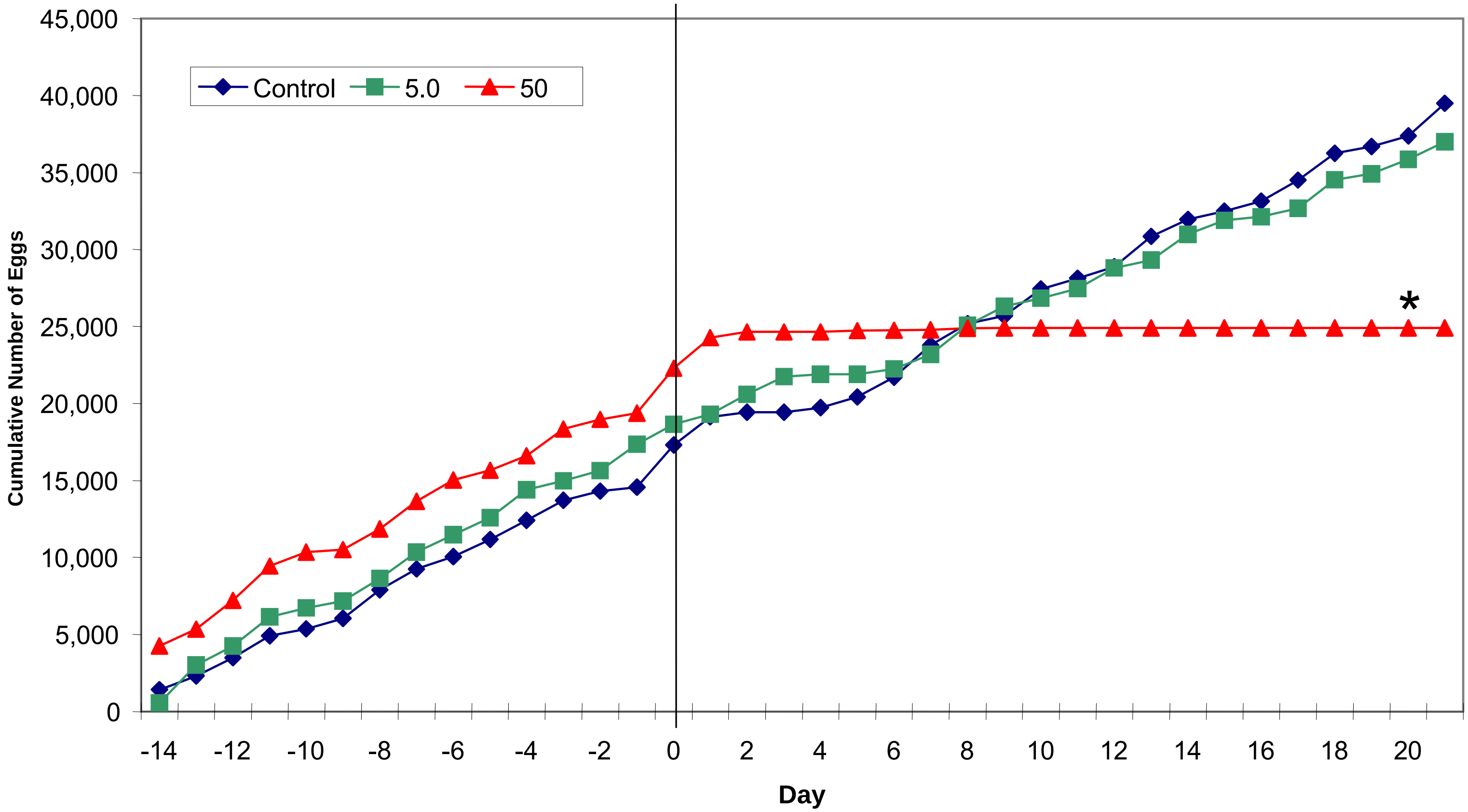
Fadrozole Effects in Fathead Minnow Assay



- Significant decrease in female vitellogenin (mg/ml): Control = 5.95 (2.25,10)
  Low = 1.20 (0.75, 10)
  High = 0.005 (0.011, 9)

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Summary

- Reasonable agreement between MED and Battelle for key apical and diagnostic data generated for four different chemicals
  - Methoxychlor
    - Fecundity reductions comparable
    - Effects on male VTG induction qualitatively similar
  - Trenbolone
    - Fecundity reductions comparable
    - Female masculinization similar
    - Decreases in female VTG similar
  - Flutamide
    - Fecundity reductions comparable
    - Some histological changes in ovary
  - Fadrozole
    - Fecundity reductions comparable
    - VTG reductions in females similar

Objective: Battelle Study 2

Evaluate responses to five suspect/weak EDCs using 21-d fathead minnow protocol

Chemical	Nominal Test Concentrations (µg/L)	
Atrazine	25	250
Bisphenol A	64	640
p,p’-DDE	0.02	0.2
Perchlorate	5,000	50,000
Cadmium	1	10

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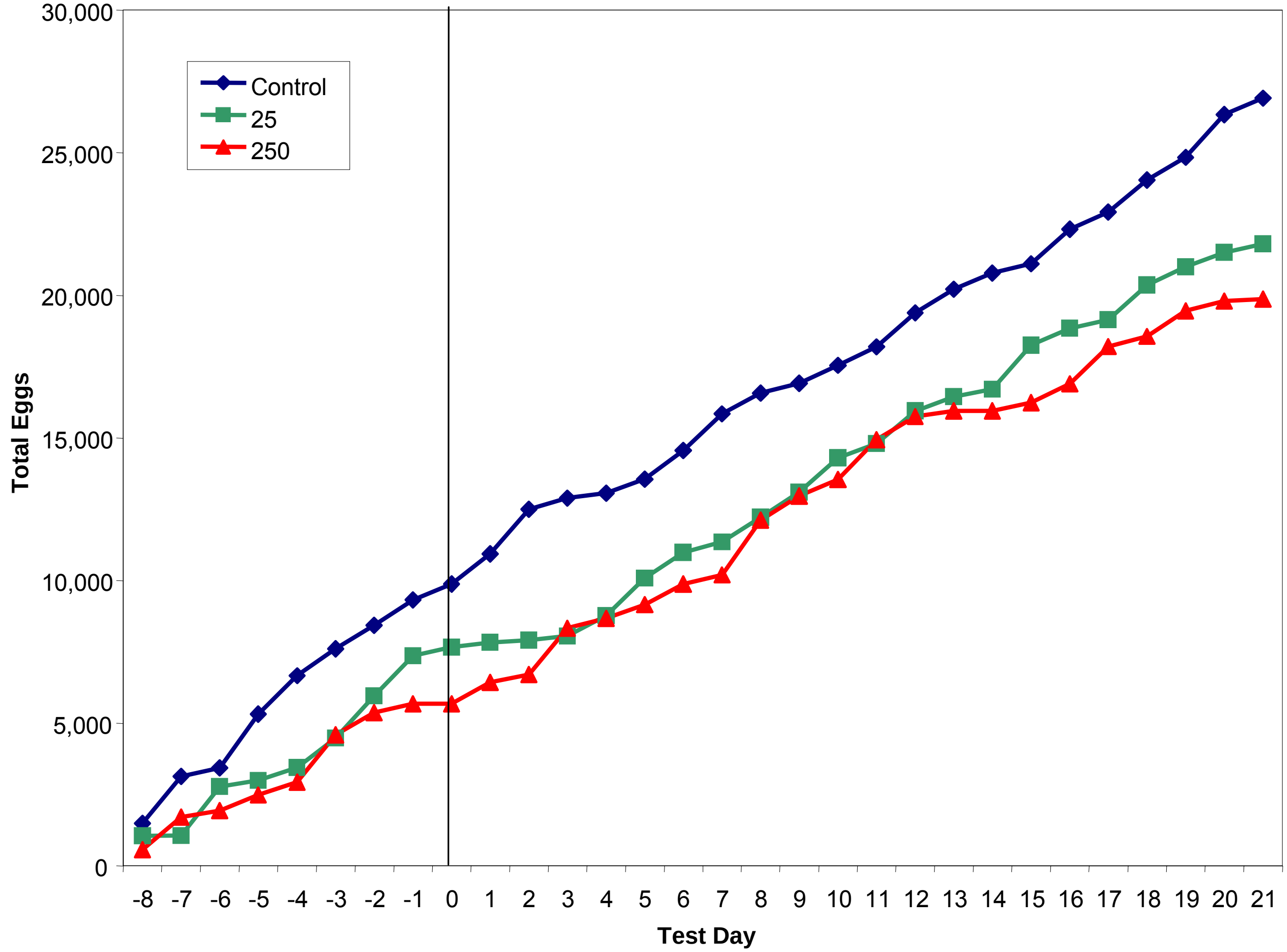
Atrazine Effects in Fathead Minnow Assay



- Fecundity and most other endpoints not significantly affected
- Slight treatment-related effects in testicular histology

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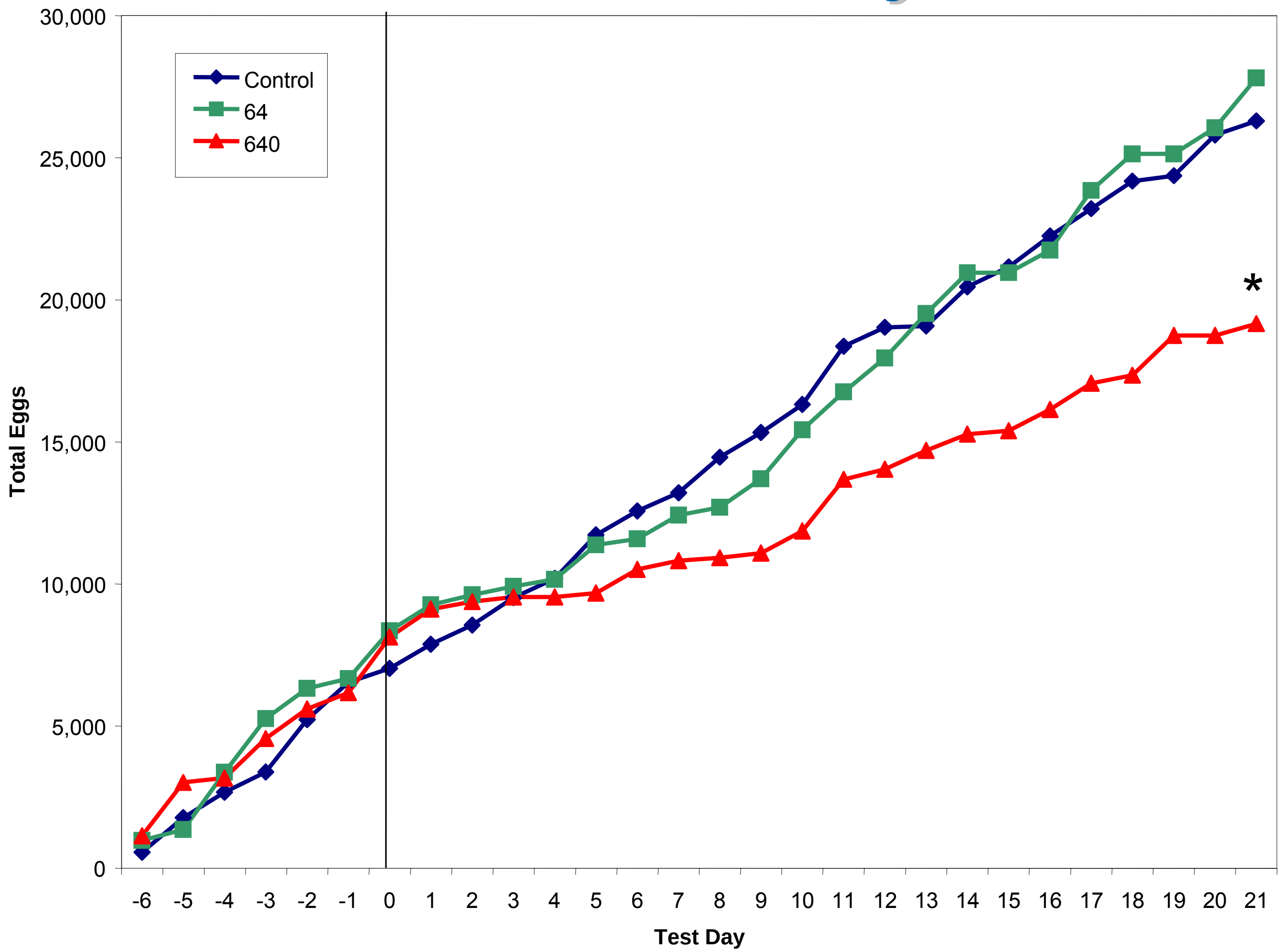
Bisphenol A Effects in Fathead Minnow Assay



- Significant induction of vitellogenin in males (both treatments) and females (high treatment)

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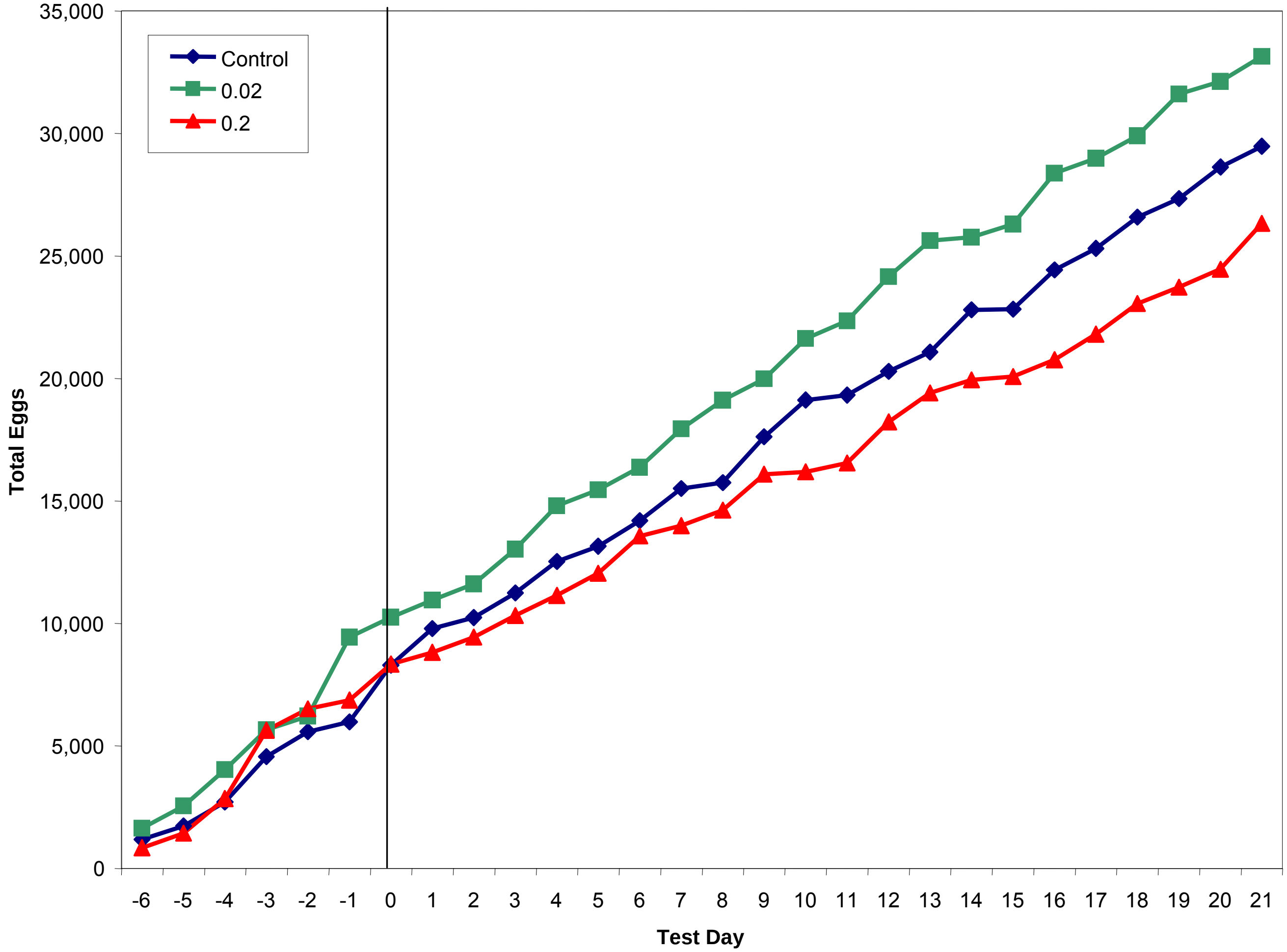
p,p'-DDE Effects in Fathead Minnow Assay



- No significant effects on fecundity and most other endpoints
- Increased incidence of atretic follicles in ovaries of females from both treatment groups

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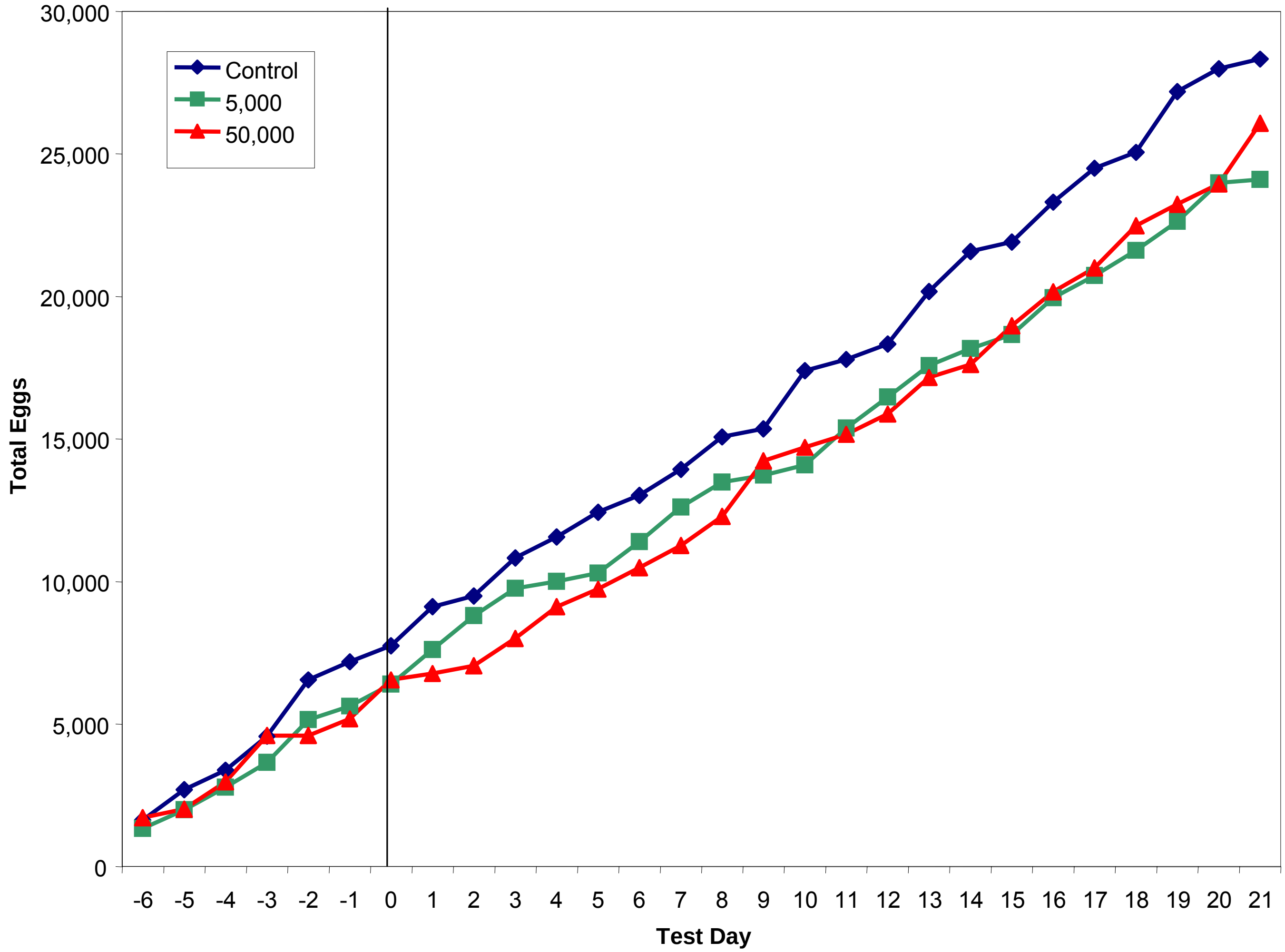
Perchlorate Effects in Fathead Minnow Assay



- No marked effects on any endpoint

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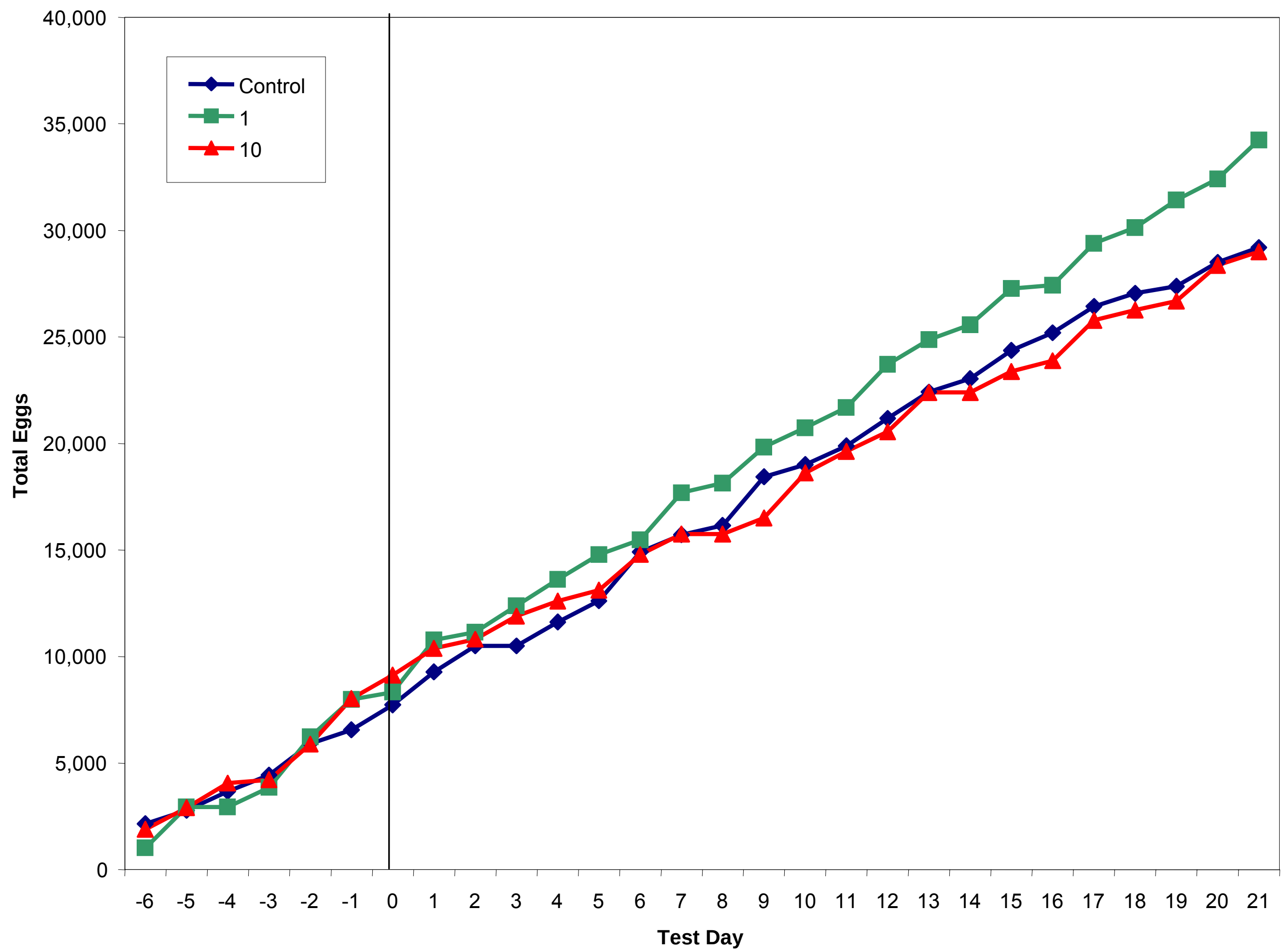
Cadmium Effects in Fathead Minnow Assay



- No marked effects on any endpoint

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Mean values and variability of fecundity & biochemical endpoints in **control females**

Control Treatment Group	Fecundity Eggs/♀/day			Vitellogenin (mg/mL)			Estradiol* (ng/mL)			Testosterone* (ng/mL)		
	Mean	SD[a]	CV[b]	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Atrazine	53.6	13.1	24%	6.6	2.4	36%	2.52	1.46	58%	0.90	0.29	33%
Bisphenol A	57.8	10.6	18%	11.1	3.4	31%	2.29	1.42	62%	0.49	0.47	97%
Cadmium	63.9	18.3	29%	10.7	2.5	23%	2.36	0.84	36%	0.99	0.68	69%
p,p-DDE	65.5	9.1	14%	11.5	2.6	22%	2.92	1.00	34%	0.41	0.31	77%
Perchlorate	61.3	8.3	14%	8.3	2.0	24%	1.79	0.41	23%	0.82	0.24	29%
Study Mean	60.4	11.8	20%	9.6	3.1	32%	2.38	1.10	46%	0.73	0.46	64%

Battelle (2005)

* Determined via ELISA rather than RIA

Role of OECD in EDC Testing

- OECD general mandate to develop internationally-harmonized test guidelines
- Membership comprised of developed countries with business consortium advisory groups
- OECD EDTA (Endocrine Disruptor Testing Advisory) oversight group assembled in 1998
- VMG-eco (Validation Management-Ecology) group formed under EDTA in 2001 to address non-mammalian tests
- Fish Drafting Group (FDG) one of several expert technical groups providing input to VMG-eco on method selection and ring testing

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Protocols Considered by FDG

- Short-term (14 d) vitellogenin induction assay in juvenile fish
- Moderate-term (45-80 d) assays focused on gonad development/differentiation

- Short-term (21 d) non-spawning assay
- Short-term (21 d) spawning assay
  - Initiated with reproductively-active adults
  - Endpoints: secondary sex characteristics, gonad histology, vitellogenin status

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Phase 1A: Evaluation of Non-Spawning Assay

- Mature zebrafish, medaka and fathead minnow
- Animals segregated by sex (screen) in same tank (10♂;10♀)
- Fish exposed to 17β-estradiol (10, 32 and 100 ng/L) or 17β-trenbolone (50, 500 and 5000 ng/L)
- Samples collected after 14 and 21 d for analyses (sex characteristics, vitellogenin, gonad histology)
- Four participating labs (3 Japan, 1 Germany)

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Phase 1A: Overall Results

- Estradiol consistently increased vitellogenin in males of all three species, while trenbolone decreased vitellogenin in females
- Trenbolone induced male secondary sexual characteristics in fathead minnow (tubercles) and medaka (anal fin papillary processes) females (but not zebrafish)
- Gonadal histopathology results compromised by high incidence of abnormalities in controls probably caused by "forced" cessation in spawning

Phase 1B: Evaluation of Spawning Assay

- Mature zebrafish, medaka and fathead minnow
- Replicates comprised of five animals of each sex housed together
- Fourteen participating labs from seven countries
- Endpoints included those from Phase 1A as well as qualitative assessment of spawning
- Exposures conducted for 21 d with chemicals representing three MOA

Chemical	MOA	Concentrations (µg/L)
4-t-Pentylphenol	ER Agonist	100,320,1000
Estradiol (PC)	ER Agonist	0.1
Flutamide	AR Antagonist	100, 500, 1000
Prochloraz	Aromatase Inhibitor	20, 100, 300
Fadrozole (PC)	Aromatase Inhibiter	100

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Phase 1B: Overall Results

- Both estrogens reduced spawning, induced vitellogenin synthesis (in males) of all three species, and inhibited secondary sex characteristics (tubercles) in male fathead minnows
- Prochloraz and fadrozole both reduced spawning, and consistently reduced vitellogenin concentrations (in females) of all three species
- Effects of flutamide on the core endpoints were somewhat ambiguous - the main indication(s) of effects of the anti-androgen were on gonadal histopathology

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Overall OECD Conclusions/Recommendations

- General test design/endpoints reasonable for detecting estrogens, androgens and inhibitors of steroid metabolism
- Anti-androgens more challenging to detect
- Standardized guidance for histological approaches/interpretation desirable (ongoing effort)
- Design may need species-specific optimization for spawning activity

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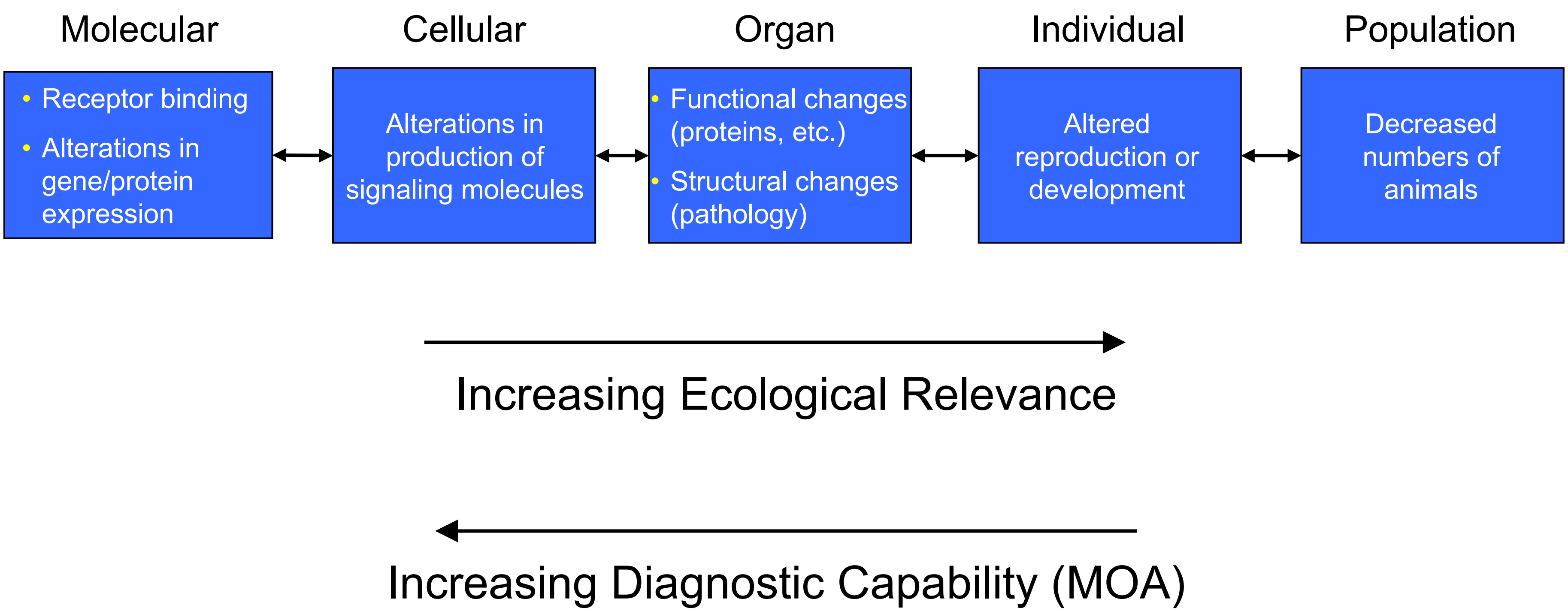
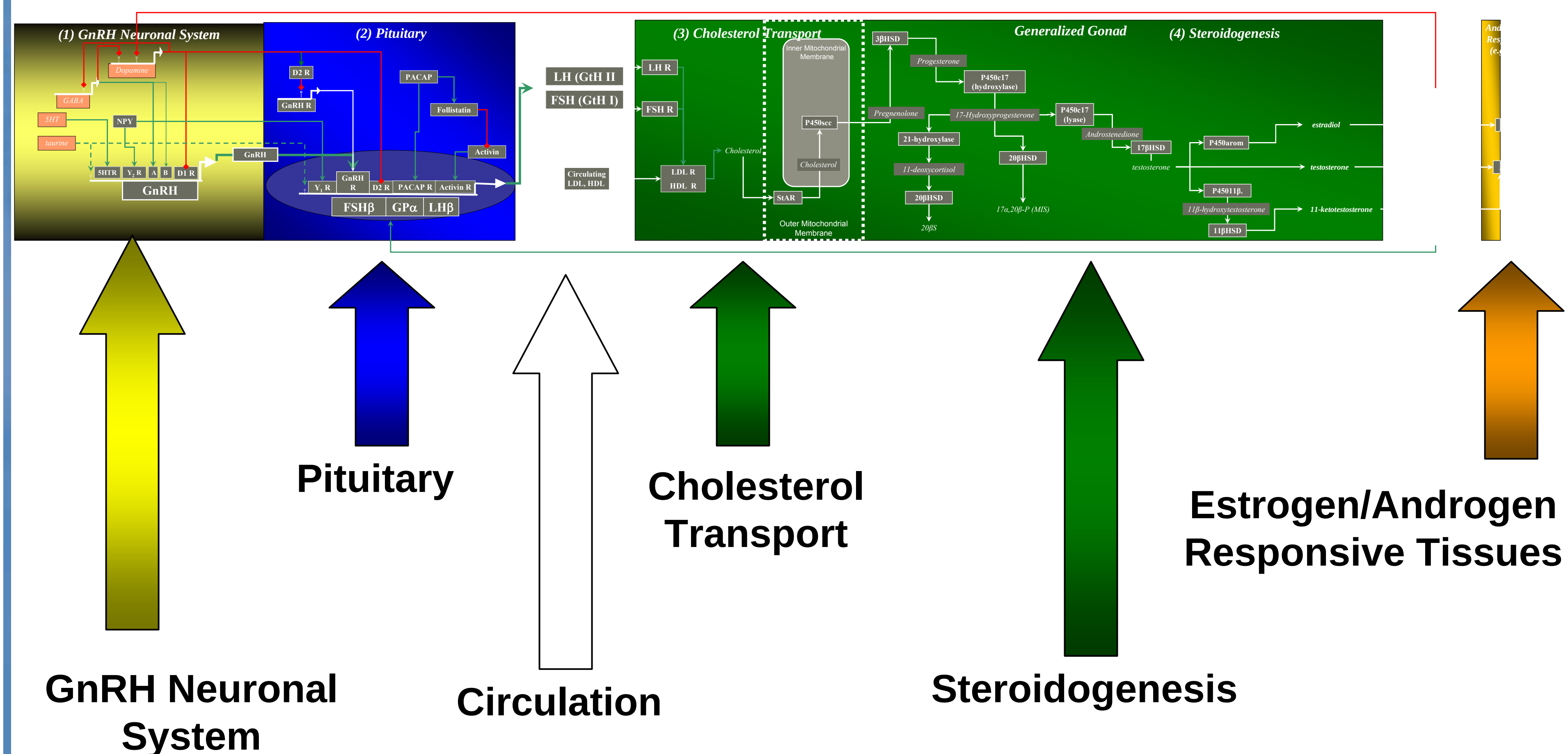
Future Directions

- Enhancing diagnostic capability of the assay via genomics/systems biology approaches
- Understanding linkages across endpoints to adverse outcomes at individual/population levels

Linkages Across Biological Levels of Organization: Toxicity "Pathways" for EDCs



Conceptual model of the teleost HPG axis



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Rapid Communication

Modeling impacts on populations: fathead minnow (*Pimephales promelas*) exposure to the endocrine disruptor 17β-trenbolone as a case study

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Abstract

Evaluation of population-level impacts is critical to credible ecological risk assessments. In this study, a predictive model was developed to translate changes in fecundity of the fathead minnow (*Pimephales promelas*) in a short-term laboratory toxicity test to alterations in population growth rate. The model uniquely combines a Leslie population projection matrix and the logistic equation. Application of the model requires only a life table for the organism of interest, a measure of carrying capacity for the given population, and an estimation of the effect of a stressor on vital rates. The model was applied to investigate population dynamics for fathead minnow exposed to the androgen receptor agonist 17β-trenbolone. Organismal-level responses for fathead minnows exposed to varying levels of 17β-trenbolone were used to determine projected alterations in a population existing in a small body of water containing varying concentrations of the androgen. Fathead minnow populations occurring at carrying capacity and subsequently exposed to 0.027 μg/L of 17β-trenbolone exhibited a 51% projected decrease in average population size after 2 years of exposure. Populations at carrying capacity exposed to concentrations of 17β-trenbolone ⩾0.266 μg/L exhibited a 93% projected decrease in average population size after 2 years of exposure. Overall, fathead minnow populations exposed to continued concentrations of 17β-trenbolone equal to or greater than 0.027 μg/L were projected to have average equilibrium population sizes that approached zero.

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And, lots of others...