

September 21, 2005

Lecture 8: Endocrine System Effects—Part II: Tests for Hazard ID

I. Chemicals Shown to Have Effects on the Endocrine System

A. Chemicals Most Frequently Studied

1. Pesticides
 - a. DDT, DDE
 1. Anti-androgenic activity
 - b. Vinclozolin
 1. Anti-androgenic activity
 - c. Methoxychlor
 1. Estrogenic agonist
 - d. Atrazine
 1. Induces aromatase
2. PCBs (polychlorinated biphenyls)
 - a. Actually the PCBs must be hydroxylated before they exhibit estrogenic activity
3. Dioxins (especially 2,3,7,8-TCDD [tetrachlorodibenzodioxin])
 - a. Anti-estrogenic activity
4. Bisphenol
 - a. Estrogenic activity
5. Nonylphenol
 - a. Estrogenic activity
6. Tributyl tin
 - a. Associated with imposex condition in mollusks (animal has morphological characteristics of both genders)
7. Phthalates (plasticizers)
 - a. anti-androgenic activity
8. Metals
 - a. Lead
 - b. Mercury (metalloid)

B. Chemicals Recently Implicated as Having Hormonal Activity By Virtue of Interaction with Estrogen Receptors in-vitro or Putative Interactions with Thyroid Hormone Receptors and/or Metabolism

1. Benzophenone derivative (UV stabilizers and sunscreens)
2. Pyrethroid insecticides
3. Polybrominated diphenyl ethers (flame retardants)
4. Perchlorate (rocket fuel contaminant; naturally occurring chemical)

C. Natural Biochemicals

1. Phytoestrogens (includes a number of flavonoids that have antioxidant activity and thus believed beneficial properties as 'nutraceuticals').
 - a. Genistein
 - b. Chrysin
 - c. Coumestrol
 - d. Chalcone
 - e. Kaempferol

- f. Daidzein
- g. Apigenin
- 2. Fungal mycotoxins
 - a. Zearlenone

II. Testing for Hormonally Active Agents (HAAs): In Vitro Tests

A. Biochemical Basis for In-Vitro Tests of Estrogenic Activity

1. Interactions of HAAs with the estrogen receptor are one of the several mechanisms believed responsible for causing endocrine system related effects note, however, that there are other possible mechanisms.
 - a. For example, the interaction could be with testosterone mimics, and therefore androgen receptors.
 - b. The interaction could be with enzymes that metabolize endogenous hormones and therefore reduce or change the titer in the body.

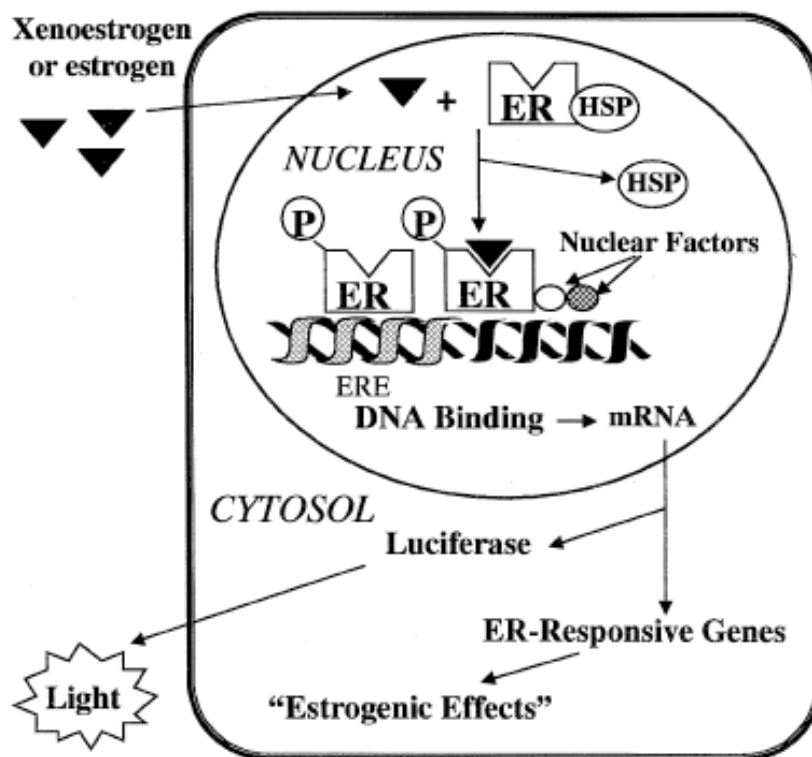


Figure 1. Model for interaction of estrogen or estrogen mimics (xenoestrogens) with cell and receptors and the development of an assay (from Giesy et al. 2002).

- B. For those compounds that interact with the estrogen receptor (ER) the following discussion describes the hypothetical mechanism of action (Information from Giesy, J. P., K. Hilscherova, P. D. Jones, K. Kannan, and M. Machala. 2002. Cell bioassays for detection of aryl hydrocarbon (AhR) and estrogen receptor (ER) mediated activity in environmental samples. Marine Pollution Bulletin 45:3-16.) (See Figure 1)

1. The ER is a nuclear receptor protein known as a transcription factor; it is associated with a protein called the Heat Shock Protein (HSP), and these two macromolecules cover up a DNA binding domain for estrogen.

- a. The binding domain is known as the ERE (estrogen responsive element), and it is located in the regulatory regions of estrogen-inducible genes.
 2. Estrogen (estradiol) and estrogen mimics (also called xenoestrogens) diffuse into the nucleus and bind to the ER. The HSP disassociates from the ER and the ER/estrogen or ER/mimic complex dimerizes (two units associate with one another and bind to the specific DNA coding region, or ERE).
 3. ER complexes bound to an ERE recruit additional transcription factors, leading to increased gene transcription (i.e., synthesis of messenger RNA or mRNA, and thus synthesis of proteins required for expression of hormonal action).
 4. Based on the biochemical principles discussed above, several *in-vitro* assays have been developed using recombinant DNA techniques that allow transfection of wild type cells (for example, a breast cancer cell line known as the MCF-7; yeast cells) with reporter genes that are under transcriptional control of the ERE.
 - a. Reporter genes are genes that are turned on to produce a substrate that is easily measured, usually spectrophotometrically
- C. One example of an in-vitro test relying on transcribed reporter genes utilizes a gene encoding for galactosidase, an enzyme whose activity is easily measured in the presence of the sugar (monosaccharide) substrate, galactose.
1. One system involves the use of yeast cells and is called the YES system (Yeast Estrogen System) (Figure 2).
 - a. The human estrogen receptor has been cloned into yeast cells (a.k.a. the Yeast Estrogen System); the cloned gene also contains reporter genes; (Arnold, S. F. et al. 1996, A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. Environ. Health Perspectives 104:544-548)
 - b. When the human estrogen receptor is turned on, the reporter genes (which code for galactosidase enzyme, a.k.a. LAC Z gene) are also turned on
 - c. By measuring the amount of galactosidase, one can estimate the degree of estrogenic activity a chemical might have
 - d. All potential endocrine disrupting (i.e., estrogen mimic) chemicals are compared to estradiol (the positive control).
 2. The YES system has been used by many research groups to reliably predict which chemicals can interact with the ERE via interaction with the ER. One study, however, came under controversy when it attempted to determine whether mixtures of weakly interacting chemicals can synergize an estrogenic response (Arnold et al., 1996, Synergistic activation of estrogen receptor with combinations of environmental chemicals. Science 272:1489-1492) (Figure 3)
 - a. When endosulfan or dieldrin were tested in this system alone, their potency was virtually nil compared to estradiol; however, when added together in equal amounts, each of which had little activity alone, their potency increased by nearly 1000-fold; this response was synergistic (i.e., greater than simply additive).

1. However, note that the synergistic response was still ~ 1000 fold less potent than the natural estrogen; furthermore, the data shows evidence of a threshold (or NOEL) (Figure 3).

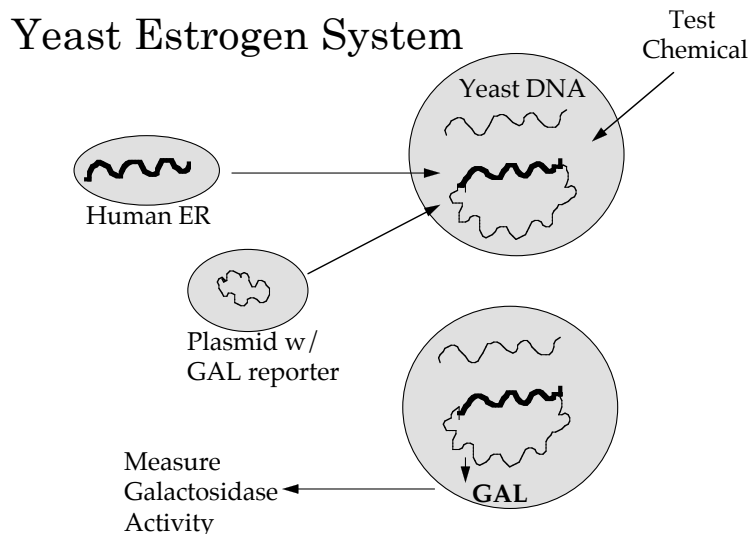


Figure 2. Yeast Estrogen Assay System for Estrogen Mimics. ER is the human estrogen receptor; a bacterial plasmid containing the galactosidase gene is cloned into a yeast cell along with the human ER gene. HAAs that interact with the human ER will cause transcription of GAL, which can be detected as a color change in the presence of the sugar substrate, galactose.

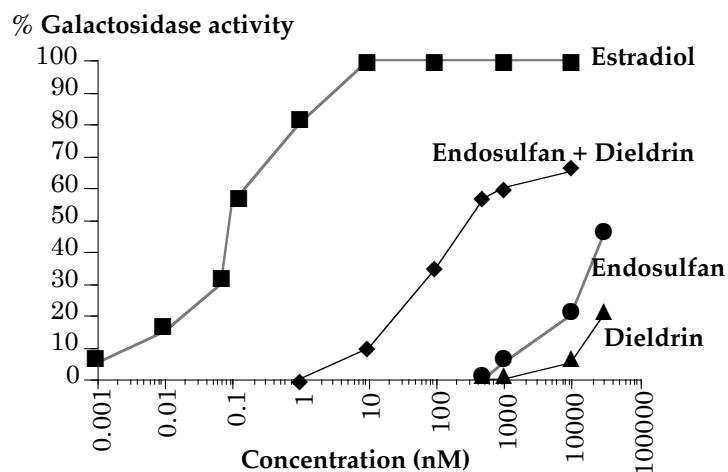


Figure 3. Results from Arnold et al. 1996, suggesting synergism between estrogen mimics. Note that endosulfan and dieldrin basically have no activity (unless very extreme concentrations are used).

- The following summer (1997), in a letter to Science, McLachlan [research leader of the lab at Tulane Univ. in which Arnold et al. were working] retracted their work showing synergistic interactions. They claimed they

could not repeat the experiments. Others have confirmed that the interactions are additive rather than synergistic.

- D. Another example of an in vitro system includes the gene for luciferase, which emits phosphorescence when turned on (i.e., synthesized in response to the binding of the ERE and estrogen or estrogen mimic),
1. However, this assay was originally used for examining androgenic rather than estrogenic effects.
 - a. The human androgen receptor is cloned into a cell line and linked to a luciferase reporting gene;
 1. **“Luciferase** is a generic name for [enzymes](#) commonly used in nature for [bioluminescence](#). The name itself is derived from *Lucifer*, which means *light-bearer*. The most famous one is firefly **luciferase** ([EC 1.13.12.7](#)). In luminescent reactions, light is produced by the [oxidation](#) of a [luciferin](#) (a pigment), sometimes involving [Adenosine triphosphate](#) (ATP).” (Definition from Wikipedia, URL: <http://en.wikipedia.org/wiki/Luciferase>)
 - b. The receptor (i.e., the cells) is probed with the target chemicals, including testosterone as the control;
 - c. When the receptor is activated by binding of the hormone (known as transcriptional activation), luciferase activity increases; luciferase catalyzes a chemical reaction that produces light, so light output can be measured by a spectrophotometer; light output is compared between treatments. The greater the light, then the greater the receptor induction (Figure 4).

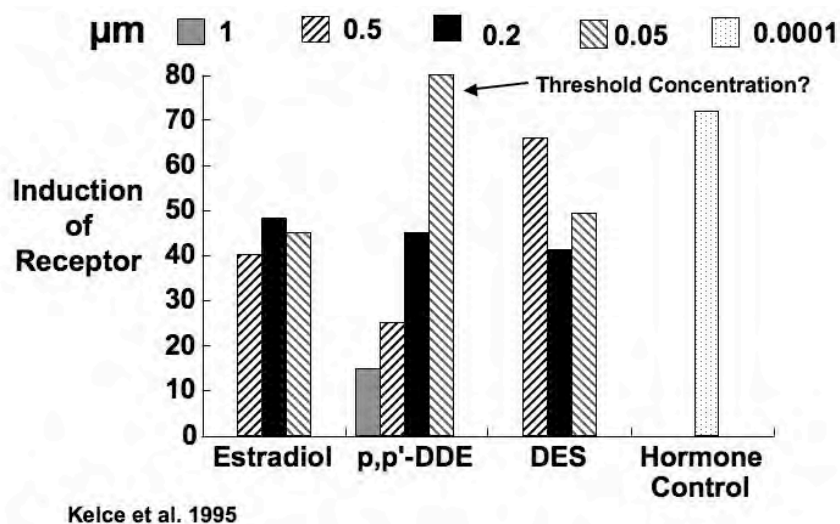


Figure 4. Results from the luciferase reporter assay testing different concentrations of estrogen mimics and androgen antagonists. Concentration units are micromolar. The control hormone is testosterone. Note that DDE seems to have a NOAEL in this assay at 0.05 micromolar (the equivalent of 16 ppb).

1. Using this assay, Kelce et al. 1995 (Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist,” Nature 375:581)

reported that DDE, which was shown to have poor estrogenic activity, actually has moderately potent anti-androgenic activity.

- a. In other words, DDE binds to the testosterone receptor blocking the binding of testosterone; however, unlike testosterone, the receptor is not activated.
- b. Thus, in the graph, note that the induction of receptor as measured by light output is lowest for the highest dose of DDE; DDE would be considered a competitive inhibitor to testosterone.
- c. Is there a threshold? Clear thresholds were found; i.e., no effect on transcription @ 0.05 μM (~16 ppb)

- E. The MCF-7 Breast Cancer Cell Line is a reporter system that relies on cell proliferation as an endpoint. It has been used as a screen for estrogen mimics as well as a test to determine whether synergism is operational.
1. The E-Screen--one of Theo Colborn's co-authors, Ana M. Soto (Tufts University) and her colleagues published a paper investigating the estrogenic effects of DDT, endosulfan, toxaphene, and dieldrin on human estrogen-sensitive cells using the E-Screen (1994, "The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells," Environ. Health Perspectives 102:380)
 2. They used cultured human breast cancer estrogen-sensitive MCF-7 cells; the biological response measured was cell proliferation in response to dosing
 3. Thus, when estradiol is put in the nutrient medium (which is actually filtered human blood serum), the cells divide; similarly, a response can be observed if a chemical acting like estrogen causes the cells to divide; in such a case the chemical would be considered a potential hormone mimic, xenoestrogen, or an endocrine disrupter (note that each of these words has been used in the literature and popular press)

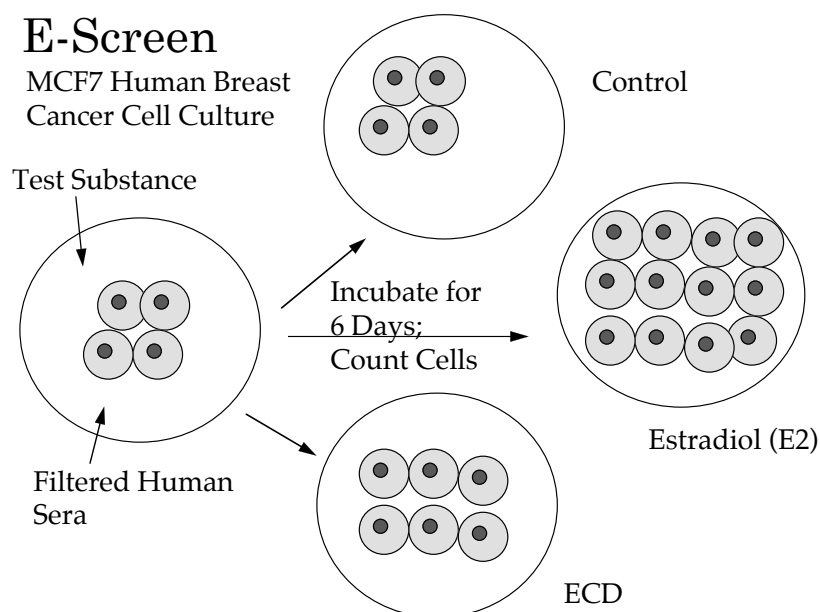


Figure 5. Schematic of the E-Screen assay

- a. The data for cell numbers are plotted relative to dose of estradiol or the suspected EDC. The further toward the y-axis a curve is, then the relatively more potent it is; thus in the next graph, estradiol is about 1 million times more potent than the pesticides

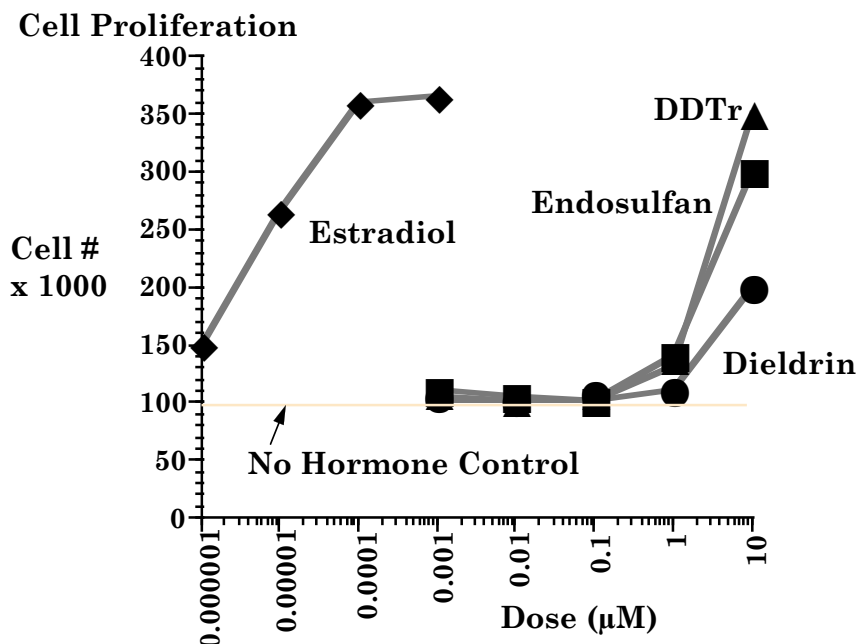


Figure 6. Dose-response curve for results from the E-Screen assay (modified from Soto et al. 1994).

- b. The results in the next table below show effective minimum doses to achieve a measurable effect and proliferative efficiency (ratio between highest cell number in the presence and absence of the “estrogen”):

Table 1. Effective minimum dose and proliferative efficiency of estrogen mimics in comparison to estradiol based on responsiveness in the E-Screen assay (after Soto et al. 1994).

Chemical	Effective Minimum Dose	Proliferative Efficiency
estradiol	10 pM	3.68
o,p'-DDT	10 μM	3.17
endosulfan	10 μM	2.99
DDT	10 μM	2.93
dieldrin	10 μM	2.02
toxaphene	10 μM	1.91

The poliferative efficiency (or effect) is the ratio between the highest cell yield obtained with the test chemical and the hormone-free control.

- c. Furthermore, a clear dose-response (threshold) was observed. For example, 10 nM of o,p'-DDT did not have a significantly different response than the hormoneless control

- d. When a mixture of each of the pesticides at a concentration of $1 \mu\text{M}$ (for a total of $10 \mu\text{M}$) was tested, the response was about $2/3$ of the response of the estradiol treatment and about $2X$ the response of the no hormone control; this result suggests additivity, perhaps by action at the same biochemical receptor.

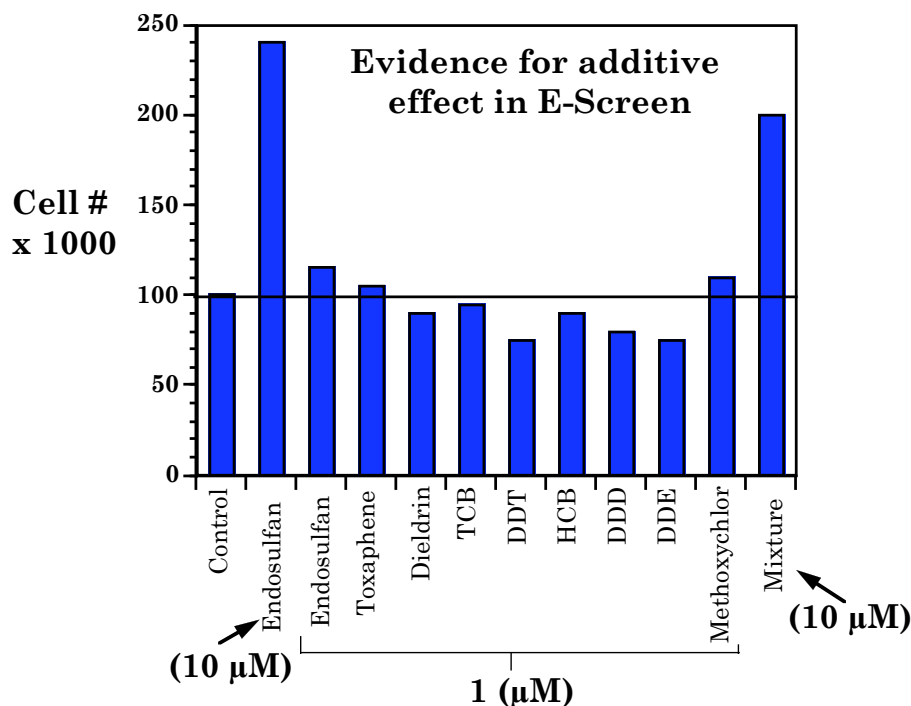


Figure 7. Additivity of estrogen mimics in the E-Screen assay.

III. Testing for Hormonally Active Agents (HAAs): In Vivo Tests

- A. Some examples of **in vivo systems** for HAA effects include:
 1. Rodent uterine wet weight changes after feeding mice or rats with suspected endocrine active chemicals
 2. Sperm quality and quantity assays
 3. Reproductive toxicity studies
 4. Feeding compound over two or more generations to rodents and examining number of litters and effects on fertility
 5. Behavioral assays that putatively measure gender specific behaviors
 6. Morphological examination to check for skewed sex ratio or “wrong gender” tissues/organs
 7. Measure biomarkers in plasma; e.g., vitellogenin (egg yolk protein in male plasma); hormone titers
- B. Usually the in vivo tests have unusually high doses compared to those likely to be encountered in the environment.
- C. Juvenile or adult organisms can be dosed directly with high levels of a suspected HAA.
 1. For example, Kelce et al. (1995) dosed 25 day old rats daily with 100 mg/kg bw DDE until day 57.

- a. The onset of puberty was delayed by 5 days.
 - b. No effect was observed on whole body weight nor on the testosterone levels in blood (suggesting an anti-androgenic effect).
2. For example, Kelce et al. (1995) dosed adult rats (120 days old) with 100 mg/kg bw DDE daily for 4 days.
 - a. The seminal vesicle weight was 85% of the control, and the ventral prostate weight was 70% of the control.
 - b. The blood levels of testosterone were not affected (average concentration ~6.6 ppb).
3. The magnitude of the doses in the Kelce et al. (1995) in vivo studies sharply contrast with known intake levels of DDT (total DDT residues, including the parent and the metabolites, like DDE).
 - a. According to the FDA (Food & Drug Administration) Total Diet Study (a program for monitoring pesticide residues in foods consumed as they are prepared at home, the mean daily intake of DDT by a 6-11 month old human infant was 0.00005 mg/kg bw/day. These data were calculated from analyses conducted during 1986-1991.
 1. Thus, Kelce et al. (1995) had given pregnant rats on a daily basis over a million times the dose humans were normally exposed to nearly 20 years ago.
- D. A pregnant female can be dosed and then the effects on the offspring monitored.
 1. For example, Kelce et al. (1995) dosed pregnant rats daily with 100 mg/kg bw DDE from day 14-18 of gestation.
 - a. The neonate males retained their thoracic nipples (an anti-androgenic effect).
- E. Fish, amphibians, and reptiles have been used to test for HAAs, specifically estrogenic compounds, by monitoring vitellogenin production in males.
 1. Vitellogenin is an egg yolk protein encoded by a gene in both male and females. However, only the female gene is turned on in response to estradiol signaling. It's induction in males is a sign of exposure to an estrogenic substance.
 2. One group of researchers has investigated using mixed sex fish (specifically, fathead minnows) to use in vivo for assessing estrogenicity or anti-estrogenicity of contaminants as well as natural products. (Panter, G. H., T. H. Hutchinson, R. Lange, C. M. Lye, J. P. Sumpter, M. Zerulla and C. R. Tyler. 2002. Utility of a juvenile fathead minnow screening assay for detecting (anti-)estrogenic substances. *Environ. Toxicol. Chem.* 21 (2):319-326.)
 - a. Mixed sexes of juvenile fathead minnows are exposed to various concentration of chemicals dissolved in water in a flow-through system over a period of 21 days.
 - b. The induction of vitellogenin (VTG) is measured on a whole body basis using an enzyme linked immunoassay type kit (ELISA).
 - c. In addition to measuring VTG, the fish are examined for weight, length, and condition changes in comparison to the controls.
 1. The negative controls consist of water only, water with the solvent used to dissolve the contaminants, and a positive control of water spiked with EE2 (the synthetic estrogen, ethynylestradiol).

2. Condition is a measure of the relationship between fish weight and fish length.
- d. Figures 8 and 9 show the results from one experiment wherein juvenile fathead minnows were exposed in water to different concentrations of methoxychlor and the plant biochemical genistein for up to 21 days.

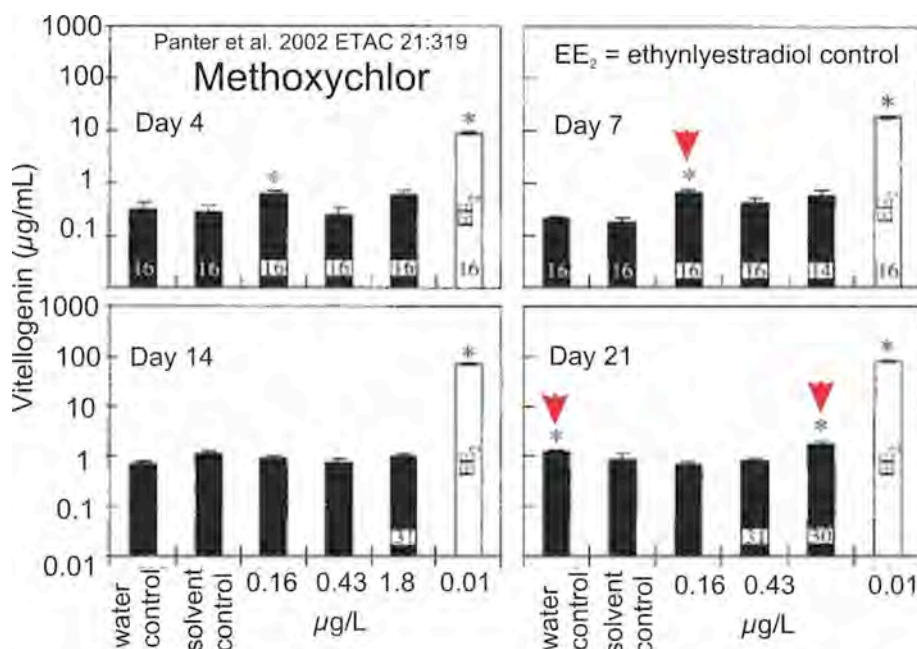


Figure 8. Vitellogenin levels in whole bodies of juvenile fathead minnows after 4, 7, 14, and 21 days of exposure to different concentrations of methoxychlor insecticide in water (Panter et al. 2002).

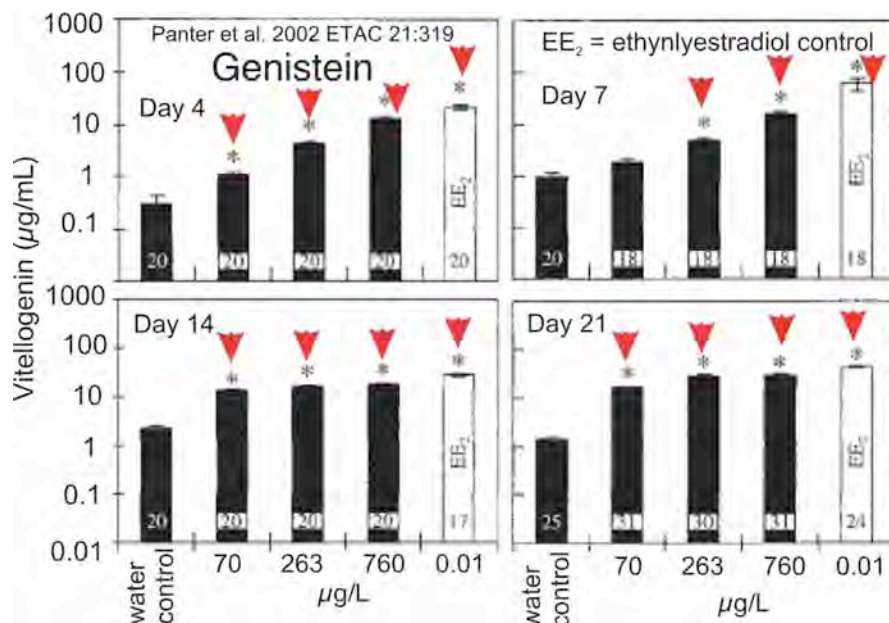
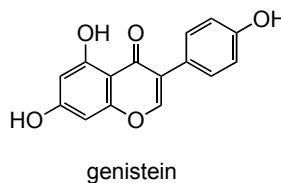
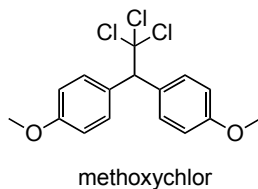
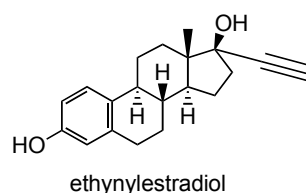
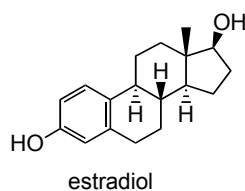
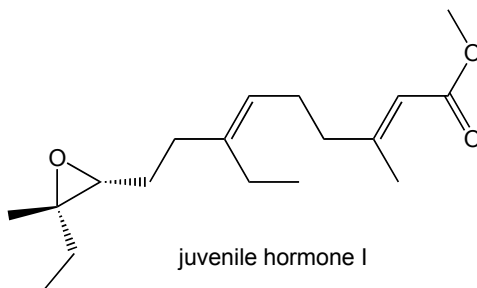
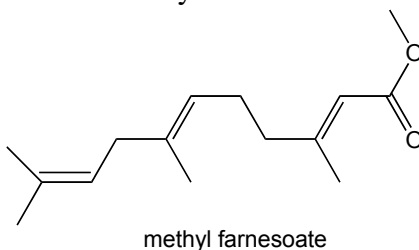


Figure 9. Vitellogenin levels in whole bodies of juvenile fathead minnows after 4, 7, 14, and 21 days of exposure to different concentrations of genistein insecticide in water (Panter et al. 2002).



F. Recently, researchers have reported success in using the crustacean *Daphnia magna* (the water flea) to screen chemicals for juvenoid-related endocrine activity (Wang, H. Y., A. W. Olmstead, H. Li and G. A. LeBlanc. 2005. He screening of chemicals for juvenoid-related endocrine activity using the water flea daphnia magna. *Aquatic Toxicology* 74 (3):193-204).

1. Recall that juvenile hormone (JH) and derivatives maintain the juvenile stage of development in Arthropods (insects, crustaceans, spiders); in the absence of JH development proceeds to the adult stage. JH is also necessary in adult females for deposition of egg protein (vitellogenesis).
 - a. In contrast to insects, which depend on juvenile hormone itself, crustacea depend on the derivative methyl farnesoate.



2. In aquatic culture, the water flea female will produce only females in the absence endogenous methyl farnesoate (MF). Oocytes exposed to MF during ovarian oocyte maturation develop in males.
 - a. It's been hypothesized that MF regulates the expression of male sex-determining genes during oocyte maturation.
 - b. There is a critical 12 h developmental window for manifestation of the masculinization effect.
3. In the assay, male sex determination in broods is used as the endpoint to indicate hormonal activity of a contaminant.
 - a. Figure 10 shows the experimental design of the assay.

Wang et al. 2005 Aquatic Toxicol. 74:193

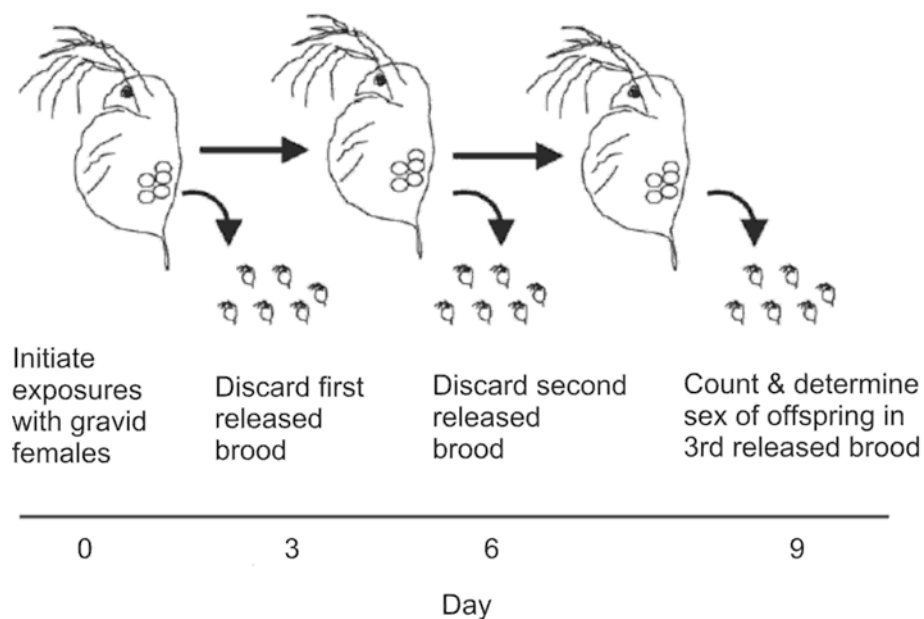
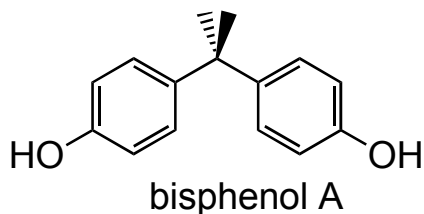


Figure 10. In the *Daphnia* juvenoid hormone activity assay, the first two broods are discarded and measurements made on the third brood. The presence of a single male in a brood is counted as a "male brood".

4. Figure 11 shows the results with one of the tested compounds. Note that the experiment was set up to show potentiation (i.e., enhancement) of the effect of bisphenol A on methyl farnesoate activity. In other words, more male-containing broods (i.e., percentage of broods produced that had males in them) were produced when MF was mixed with BPA than with MF alone. BPA at the concentration tested seemed to have no activity.



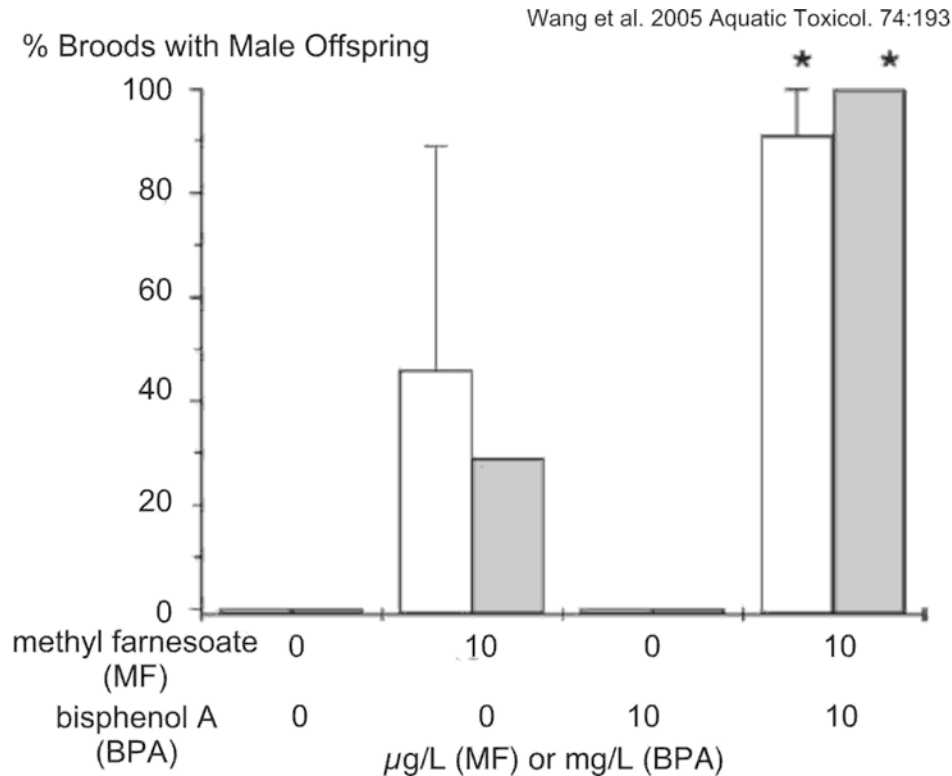
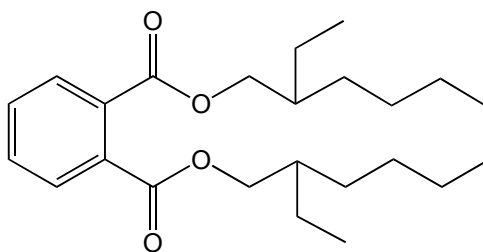


Figure 11. Percentage of broods containing males after exposure to either MF alone, BPA alone, or a mixture of MF and BPA. Note that BPA alone has no activity, but it does potentiate (enhance) the effect of MF. The white bars represent the percentage male offspring in male-containing broods, and the gray bars represent the percentage broods that contained male offspring.

IV. Testing for Hormonally Active Agents (HAAs): Epidemiological (Epi) Tests

- A. Epi tests can take many forms, but one that is increasingly common, especially with human subjects, is to measure residues of a contaminant in body fluids (if living) or tissues (if dead or biopsied) in an attempt to relate the magnitude of the residues to some condition (or pathology).
- B. A recent example is the research by Swann et al. that examined phthalates concentration in prenatal urine samples and the incidence of reduced anogenital distance in male babies. The following is the abstract from Swann et al. 2005.
 1. Prenatal phthalate exposure impairs testicular function and shortens anogenital distance (AGD) in male rodents. We present data from the first study to examine AGD and other genital measurements in relation to prenatal phthalate exposure in humans. A standardized measure of AGD was obtained in 134 boys 2–36 months of age. AGD was significantly correlated with penile volume ($R = 0.27$, $p = 0.001$) and the proportion of boys with incomplete testicular descent ($R = 0.20$, $p = 0.02$). We defined the anogenital index (AGI) as AGD divided by weight at examination [$AGI = AGD/weight$ (mm/kg)] and calculated the age-adjusted AGI by regression analysis. We examined nine phthalate monoester metabolites, measured in prenatal urine samples, as predictors of age-adjusted AGI in regression and categorical analyses that included all participants with prenatal urine samples ($n = 85$). Urinary concentrations of four phthalate metabolites [monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), and monoisobutyl phthalate (MiBP)] were inversely related to AGI. After adjusting for age at examination, p -values for regression

coefficients ranged from 0.007 to 0.097. Comparing boys with prenatal MBP concentration in the highest quartile with those in the lowest quartile, the odds ratio for a shorter than expected AGI was 10.2 (95% confidence interval, 2.5 to 42.2). The corresponding odds ratios for MEP, MBzP, and MiBP were 4.7, 3.8, and 9.1, respectively (all p -values < 0.05). We defined a summary phthalate score to quantify joint exposure to these four phthalate metabolites. The ageadjusted AGI decreased significantly with increasing phthalate score (p -value for slope = 0.009). The associations between male genital development and phthalate exposure seen here are consistent with the phthalate-related syndrome of incomplete virilization that has been reported in prenatally exposed rodents. The median concentrations of phthalate metabolites that are associated with short AGI and incomplete testicular descent are below those found in one-quarter of the female population of the United States, based on a nationwide sample. These data support the hypothesis that prenatal phthalate exposure at environmental levels can adversely affect male reproductive development in humans. *Key words:* anogenital distance, benzylbutyl phthalate, dibutyl phthalate, diethyl phthalate, monobenzyl phthalate, monoethyl phthalate, monoisobutyl phthalate, mono-*n*butyl phthalate, phthalates, prenatal exposure. *Environ Health Perspect* 113:1056–1061 (2005).



di(2-ethylhexyl) phthalate

- C. One of the problems of just examining residues in body fluids is that one cannot know how much is actually at the target receptor, especially during the critical period of growth or development. More study is needed of the relationship between toxicokinetics and toxicodynamics.
1. With the Swann et al. (2005) study in particular, the significance of anogenital distance in males is not known presently. It may be a condition that actually “clears” up with growth.
 2. Furthermore, studies with the plasticizer phthalates in rodents have shown very high doses (10’s of mg/kg bw) are required to produce any effect on sexual development. Exposure of humans to plasticizers seems very low compared to these doses. Thus, there are still questions about the plausibility of the effect.
 3. Nevertheless, studies like those of Swann et al. are hypothesis generating, not validating, and require further investigation, especially considering the low levels of exposure in humans.