September 24, 2003
Lecture 8: In-Vivo Studies Showing Endocrine System Toxicity

I. Case of the "Less than Manly Alligators"

A. One of the most publicized cases of endocrine disruption at possibly the population levels comes from research on alligators conducted at the Universitiy of Florida in the lab of L. Guillette (review of research in Guillette, L. J. Jr., et al. 2000. Alligators and Endocrine Disrupting Contaminants: A Current Perspective . *American Zoologist:* Vol. 40, No. 3, pp. 438–452).

1. The earliest work leading to the hypothesis that the alligator endocrine system was being affected by contaminants in water was published in 1994 (Guillette et al., 1994, "Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida," Environ. Health Perspectives 102:680);
   a. This study suggests that at high enough environmental levels, feminization of alligator eggs can occur; this study is an example of eco-epidemiology, but over the many years that Guillette et al. have been studying the phenomenon, they have used a combination of experimental lab studies and field monitoring measurements.

B. The Contaminated Environment and Affected Population

1. Guillette et al. found in Lake Apopka, FL declining alligator populations and evidence of an elevated estrogen to testosterone levels compared to Lake Woodruff, a pristine area that is part of a National Wildlife Refuge (Figure 1).

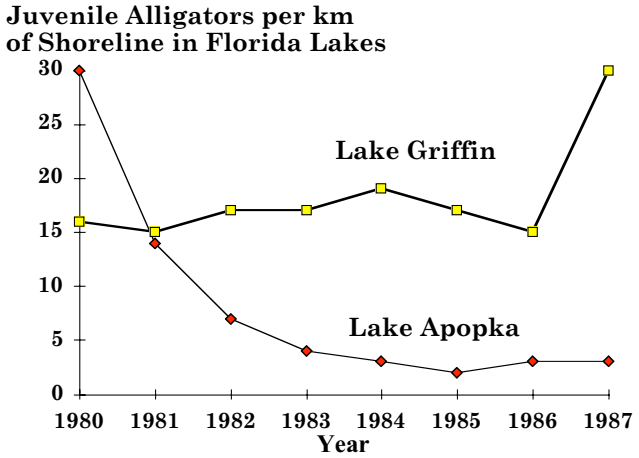


Figure 1. Population abundance of juvenile alligators in two Lakes in central Florida; Lake Griffin is another reference lake (as is Lake Woodruff) where alligator populations seemed "normal" in abundance (Guillette et al. 1994).

2. Lake Apopka, which is in central Florida, is adjacent to a EPA Superfund site ("listing due to a pesticide spill in 1980 from the Tower chemical Company") and receives agricultural drainage.

a. The lake also receives treated sewage outfall from the city of Winter Garden. Note the case of the surfactant degradation product nonylphenol below and its effects on fish.
b. The pesticide spill involved dicofol, an insecticidally active ingredient contaminated with DDT and its isomers. Note the similarity in structure. The DDT is a byproduct of the manufacturing process, and in the environment it rapidly oxidizes to DDE.

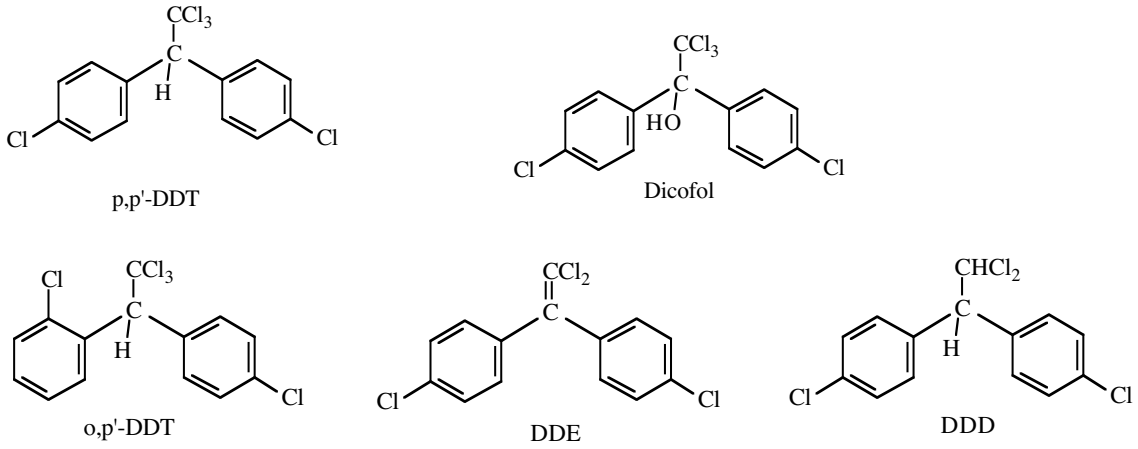


Figure 2. DDT occurs is several isomeric forms (note o,p-DDT). In the environment DDT is rapidly oxidized to DDE. Under reducing conditions, which is characteristic of low oxygen waters, especially in the sediment layer, DDT is reduced to DDD. Dicofol, which is used as an acaricide (rather than an insecticide), is more readily biodegradable than DDT by virtue of being hydroxylated at the bridge carbon atom.

3. In the 1990's alligator populations began to rebound in Lake Apopka, but not to the former levels before the pesticide spill.
   a. The rebound was hypothesized to be due to juvenile alligator recruitment; i.e., greater survival to the juvenile stage as a result of an increase in clutch survivability.
4. Other studies had shown that alligator eggs from Lake Apopka contained comparatively high levels of several DDTr (an indication of DDT plus metabolites and isomers) residues as well as other chlorinated hydrocarbon pesticides (Figure 3).
   a. Heinz et al. (1991, "Contaminants in American alligator eggs from Lake Apopka, Lake Griffin, and Lake Okeechobee, Florida," Environ. Monitoring & Assessment 16:277) reported concentrations of DDE ranging from 3.2-8.1 ppm (wet weight basis).
   b. Bear in mind that alligator eggs from other reference sites (which were supposedly not as contaminated) also had measurable pesticide residues, but there seemed to be no effect on juvenile alligator abundance.

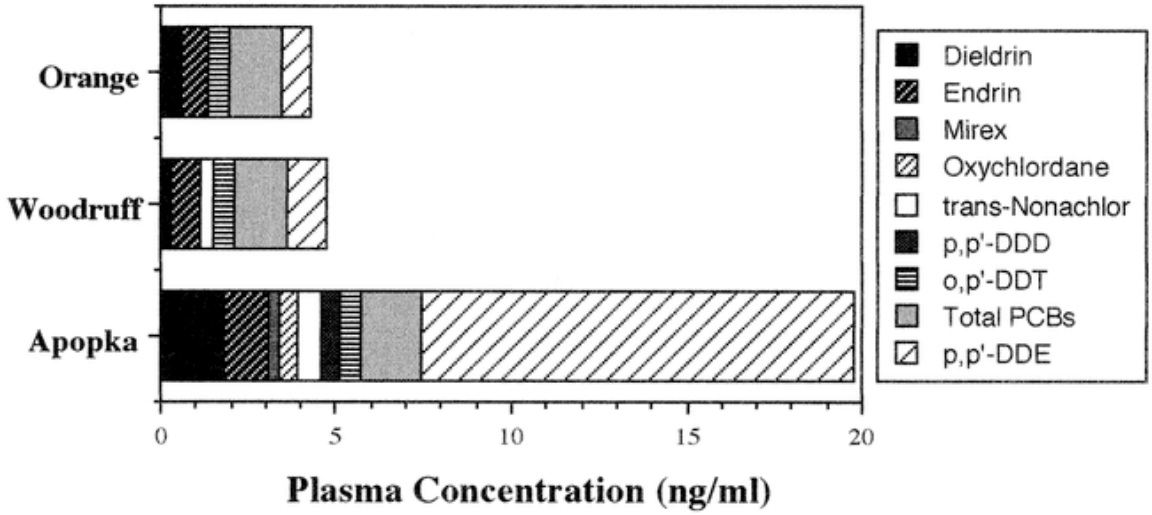


Figure 3. Comparison in levels of pesticide contamination in alligator eggs collected in Florida. Note that trans-nonachlor and oxychlordane are contaminants in the now banned termiticide, chlordane. They are also environmental breakdown products. Be aware that Florida is a state with tremendous termite problems and the environmentally persistent chlordane was used just about everywhere since the 1950's (Graph taken from Guillette et al. 2000).

C. Benchmarks of Toxicity
   1. Guillette et al. observed morphological changes in male reproductive tissue that suggested feminization of male gonads.
   2. One of the morphological signposts was a comparatively reduced "penis" size (Figure 4).

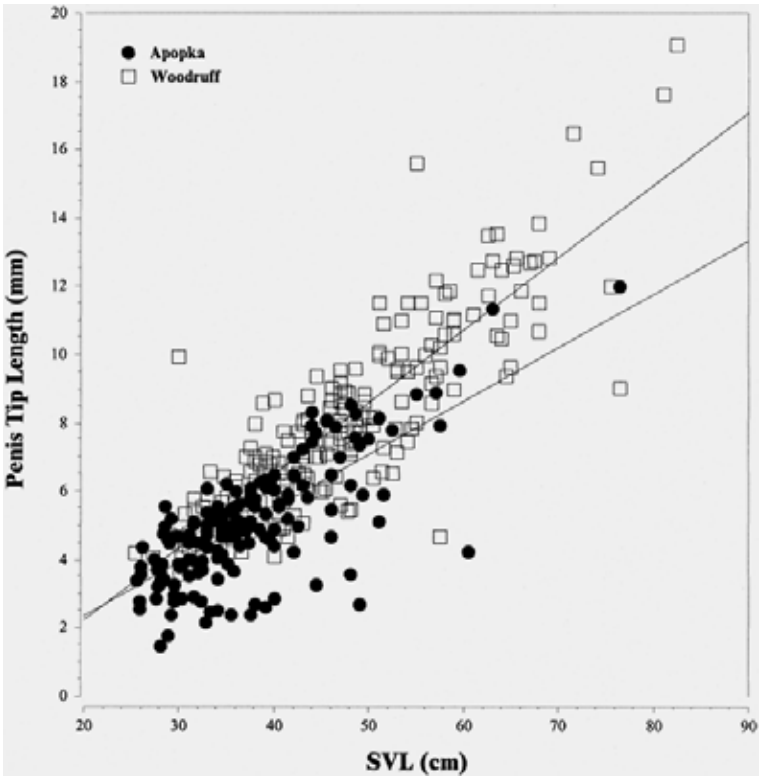


Figure 4. The relationship of penis tip length to snout-vent length (SVL) in juvenile alligators. One would expect that as SVL gets bigger, the penis would get proportionally bigger also. Note that the penis size is proportionally smaller in the Lake Apopka alligators compared to the Lake Woodruff population. (Graph is from Guillette 2000)

3. Another benchmark of toxicity was the level of sex hormones found in the alligator blood.
   a. The potential feminization (when males have female-like characteristics) was an observation consistent with elevated levels of estrogen in males from Lake Apopka compared to "control" lakes; the normal sex hormone ratio for males should be 1:1 (Figure 5);
   b. Thus, one of the first hypothesis was that the lake contaminants, which were found not only in the alligator eggs, but also in the blood, were mimicking estrogen

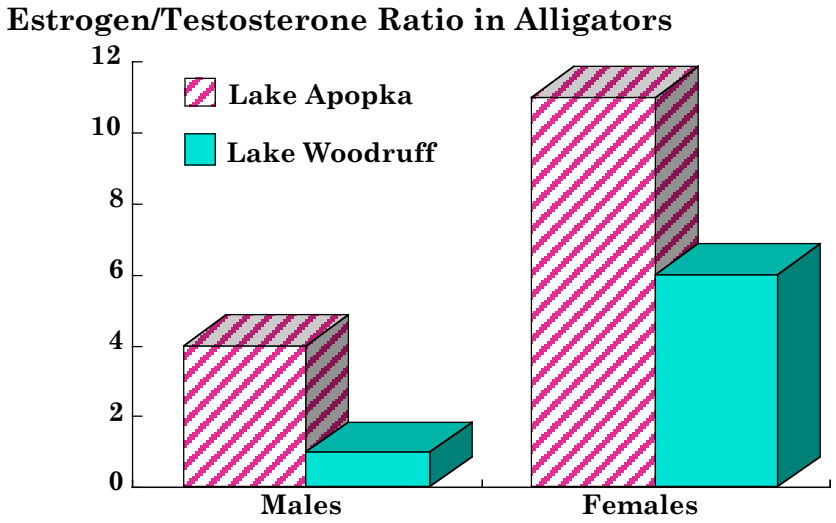


Figure 5. Hormone ratios in alligators collected from Lake Apopka and compared to the reference site, Lake Woodruff. Lake Apopka had about a 4:1 estrogen:testosterone level, but normal for males should be ~1:1

4. Other benchmarks of toxicity come from laboratory studies. Guillette et al. have taken advantage of the temperature dependent sex determination physiology of reptiles (note that this phenomenon also occurs in fish)
   a. When alligators are exposed to cool temperatures (26 – 30°C, females predominate. Above 30°C, the proportion of males increases, and at 32°C, nearly all eggs turn out to be male.
   b. Alligator eggs were experimentally exposed to different concentrations of DDE and o,p-DDE, as well as estradiol (the natural estrogen) as a positive control. The eggs were incubated at a male-producing temperature.

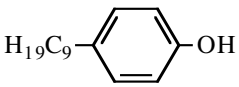
Doses of 0.1 and 0.3 mg/kg egg mass did not affect the sex ratio (all males hatched from the eggs.

1. However, at doses of 1, 3, and 10 mg/kg egg mass, the ratio of male to females was 1:1 (i.e., 50% females hatched at a male producing temperature).

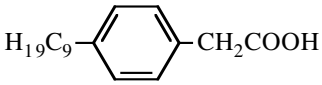
D. In another study, Guillette concluded that other pesticide contaminants, namely EDB (ethane dibromide, an agricultural fumigant with fungicidal and herbicidal properties), could also be a factor in the " shrinking" alligator genitalia and populations.
   1. Furthermore, and perhaps more importantly, Guillette et al. (2000) have emphasized that hormone mimicry may be only one of several other mechanisms for causing endocrine disruption.

II. The Case of the Sexually Confused Fish

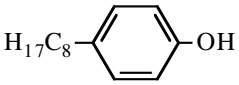
A. Several research groups have shown that male fish caged near sewage outfalls have the unusual female characteristic of vitellogenin in the blood (Jobling, S. and J. P. Sumpter. 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: An in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquatic Toxicology 27:361-372.)
   1. Vitellogenin is an egg protein produced by females in the presence of male fish "ready to mate"
      a. Both females and males are genetically competent to produce vitellogenin, but the biochemical process is not turned on in males (however, males do have a very tiny amount of vitellogenin in the plasma compared to females).
   2. The working hypothesis that explains this odd benchmark of toxicity is the presence of certain contaminants in the treated sewage outfalls. The "final" degradation product of the surfactant group known as alkylphenol polyethoxylates (APEs) has been hypothesized as being the main endocrine disrupting chemical. The product is nonylphenol.



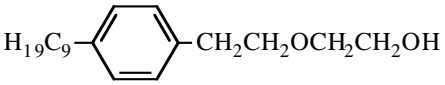
nonylphenol



nonylphenoxycarboxylic acid (NP1EC)



octylphenol

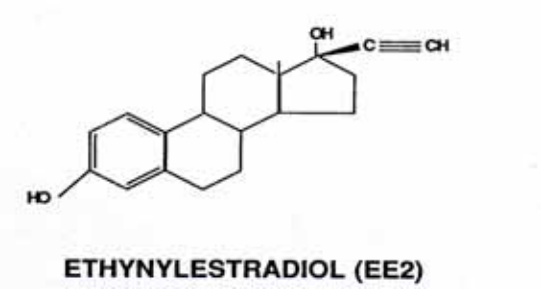


nonylphenoldiethoxylate (NP2EO)

Figure 6. Breakdown products of the surfactant group known as alkylphenol polyethoxylates.

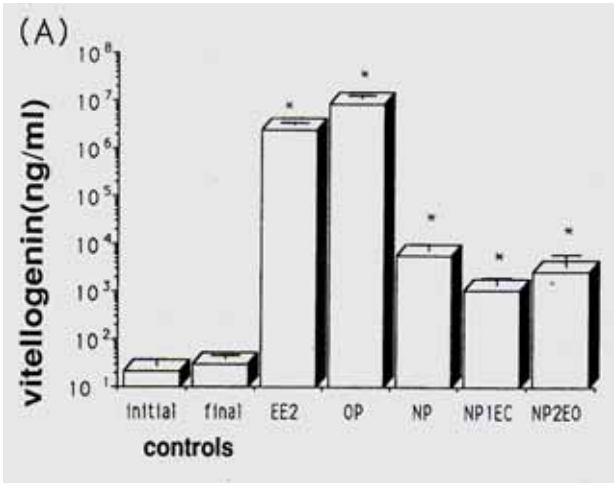
B. Toxicity of APEs
   1. Acute oral LD50 rats range from 1420 to >28,000 mg/kg; dermal in rabbits range from >2000 mg/kg to > 10,000 mg/kg.
   2. Negative in carcinogenicity tests; not mutagenic;
   3. 3. Ecotoxicity
      a. Freshwater fish: LC_{50} ~1.3 - 1000 mg/L
      b. Crustaceans: LC_{50} ~2.9-10,000 mg/L

C. Endocrine Disrupting Effects
   1. At least two groups of researchers, one in the U.S. and one in the U.K., have detected elevated levels of vitellogenin in male fish that have been captured near municipal sewage outfalls;
      a. U.S.: (Folmar, L. C. et al. 1996, Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (Cyprinus carpio) captured near a major metropolitan sewage treatment plant);
      b. U.K.: (Harries et al. 1996, A survey of estrogenic activity in United Kingdom inland waters, Environ. Toxicol. & Chem. 15:1993-2002)
      c. What's the big deal about vitellogenin detection in male serum of fish?
         1. Vitellogenin is a lipophosphoprotein normally synthesized in the female liver under the control of estradiol; it is transported by the blood to the ovary where it is taken up into the oocytes during yolk formation;
            a. In maturing female fish it is found in the plasma in large amounts (up to 100 mg/L, often constituting over 50% of blood protein)
         2. Males have very little estradiol, thus, normally you would not expect to see vitellogenin (also, why would a male produce it when they have no eggs in which to develop yolk proteins); however, there does seem to be a detectable background level of about 10 μg/L (as described in Harries et al. 1996),
         3. Exposure of males to estrogen has been associated with the production of vitellogenin;
         4. Thus, the presence of vitellogenin in the plasma of a male fish is a very sensitive biomarker of exposure to an estrogenic chemical (Sumpter, J. P. and Jobling S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environmental Health Perspectives 103, supplement 7:173-178.).
      d. Dose-response studies and single dose testing of NP, OP, NP1EC, and NP2EO have been studied by Jobling et al. (1996): EE2 (ethynylestradiol), a potent synthetic estrogen, was used as a positive control.



ETHYNYLESTRADIOL (EE2)

1. The potency of the APEO degradation products is perhaps 100,000 times less than that of estradiol in stimulating or causing vitellogenesis (i.e., vitellogenin production) and reduced testicular size;
2. Concentrations of 20.3 μg/L NP and 4.8 μg/L OP were the lowest doses tested that caused vitellogenin production in rainbow trout (i.e., these concentrations were the LOEL);
3. The corresponding NOELs were 5.0 and 1.6 μg/L, respectively, for NP and OP;
4. In these experiments, fish were exposed to the indicated concentrations for 3 weeks; thus, given the actual river water concentrations, the LOELs indicate a good probability for a real environmental effect.
5. Jobling and Sumpter (1993) used cultured hepatocytes (liver cells) to test the effects of various alkyl phenols with various alkyl chain lengths (Aquatic toxicology 27:361-372).
   a. They hypothesized that production of vitellogenin was under control of estrogen;
   b. As in the later studies with fish in-vivo, this in-vitro study also observed increases in vitellogenin production.
   c. The estrogenic control of vitellogenin production was confirmed by incubating the antiestrogenic chemical, tamoxifen, with the hepatocytes in the presence of the alkylphenols. Vitellogenin production was suppressed as a result.
   d. They also noted that the longer the alkyl chain, then the less potent was the alkyl phenol in inducing vitellogenin synthesis (Figure 7A, see OP bar, which is octylphenol).



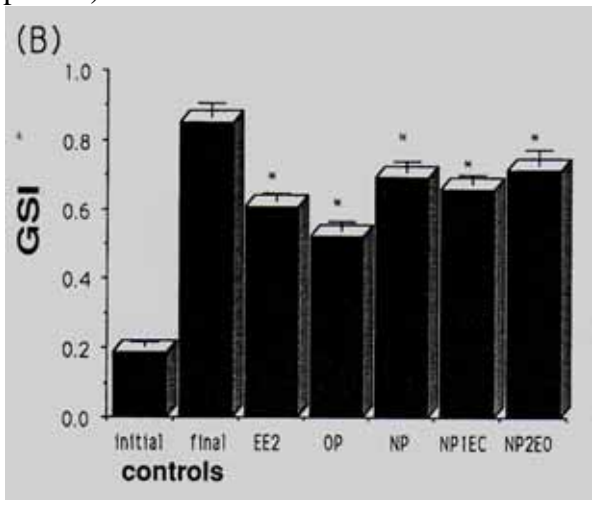


Figure 7. Effect of estrogenic alkylphenolic compounds (30 μg/L) and EE2 (2 ng/L) on the synthesis of vitellogenin and testicular growth in male rainbow trout exposed for 3 weeks. NP1EC is nonylphenoxycarboxylic acid; NP2EO is nonlyphenoldiethoxylate. EE2 (ethynylestradiol) is very potent synthetic estrogen. The latter two compounds are intermediate breakdown products on the pathway leading to nonylphenol. OP is octylphenol. The vitellogenin was measured in the blood plasma, and testicular growth was measured as the gonadosomatic index (GSI) (Jobling et al. 1996, Environ. Toxicol. & Chem. 15:194-202)

2. In-vitro studies have shown both NP and OP to have estrogenic activity
   a. Soto et al., 1991, p-nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene, Environ. Health Perspectives 92:167-173;
   b. White, R. et al. 1994, Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175-182.
3. Sumpter, who was responsible for pointing out the estrogenic activity found in sewage effluents, several years ago hypothesized that the alkyl phenols may not be the main culprit in inducing vitellogenin production in fish.
   a. In papers published in fall of 1998, his group showed that natural estrogens may be the main culprit that cause endocrine effects in fish near sewage outfalls. (Desbrow, C.; Routledge, E. J.; Brighty, G. C.; Sumpter, J. P., and Waldock, M. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. 1998; Environ. Sci. & Technol. 32:1549-1558; Routledge, E. J et al. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. 1998; ES&T 32:1559-1565.)
4. On the other hand, recent research in Spain did not rule out the possible contribution nonylphenol to vitellogenin induction in male carp (Sole et al. Environ. Sci. Technol. 2000, 34:5076-5083).
   a. Note that male carp collected in the vicinity of STPs showed elevated levels of vitellogenin (Compare Table 1 data with data in Figure 8).

Table 1. Alkylphenol concentrations in water upstream and downstream of sewage treatment plants (STPs) in Spain

Site	Distance to STP (km)	NP (μg/L)	NPEO (μg/L)	NPEC
Anola Tributary				
Site 1 (upstream)	5	18	<0.2	<0.08
Site 2 (downstream)	23	644	100	70
Site 3 (downstream)	27	<0.15	<0.2	<0.08
Cardener Tributary				
Site 1 (upstream)	1.5	51	<0.2	<0.08
Site 2 (downstream)	4	398	20	40
Site 3 (downstream)	8	42	<0.2	<0.08

STP, Sewage Treatment Plant; NP, nonylphenol; NPEO, nonyphenol polyethoxylated; NPEC, nonylphenol monoethoxycarboxylate

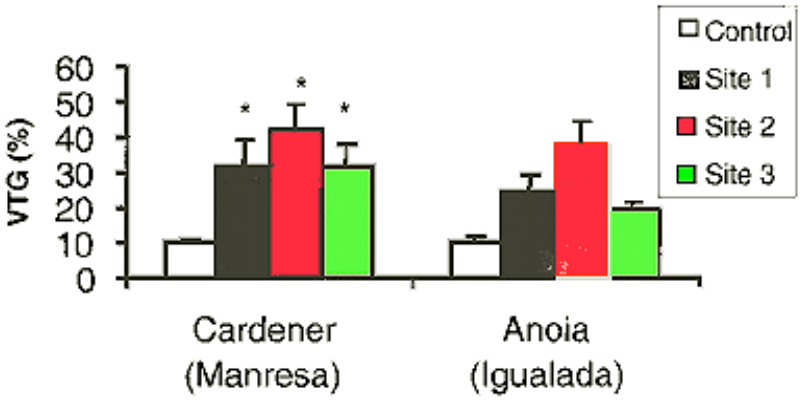


Figure 8. Vitellogenin content is expressed as the percent relative to a positive control (male fish injected with synthetic estrogen. Control fish were not exposed to alkylphenols.

5. Regardless of the cause of "feminization" in carp collected near STPs, Europe, England, France, Germany, and the Scandinavian countries have instituted a voluntary ban on APE use in household cleaning products, and restrictions on industrial cleaning applications are set to follow in 2000 (Renner, 1997, ES&T 31:316A).

D. Is there relevance for human populations?
   1. Nonylphenols may be ubiquitous in food (Guenther et al. 2002, Environmental Science and Technology 36:1676-1680
      a. Found NPs in all food items; concentrations from 0.1 μg/kg – 19 μg/kg
      b. Intake estimated at 7.5 μg/day; infants fed breast milk and formula at 0.2 μg/day and 1.4 μg/day, respectively
      c. Putative sources: formulation residues from pesticide applications; tris(nonylphenol)phosphite antioxidant used in plastic packaging; surfactants in disinfectants

III. The Case of the Declining Amphibians and a "Food Fight" Over the Effects of a Herbicide

A. I refer you to an essay I wrote that is available on the website for the newsletter, Agrichemical and Environmental News.
   1. **Felsot, A. S.** 2002. Pesticides, parasites, and pollywogs: Hazards vs. risks. Agrichemical & Environmental News (December) 200. 12 pp. http://aenews.wsu.edu (scroll down and click on the December 2002 issue)