Vitellogenin Induction by Xenobiotic Estrogens in the Red-eared Turtle and African Clawed Frog

Brent D. Palmer and Sylvia K. Palmer

Laboratory of Reproductive Ecology, Department of Biological Sciences, College of Osteopathic Medicine, Ohio University, Athens, Ohio

Many environmental pollutants have estrogenic activity in animals. Xenobiotic estrogens include many pesticides and industrial chemicals that bioaccumulate. The impact of these common pollutants on the reproductive success of wildlife may be considerable, particularly in threatened or endangered species. This research examined the use of plasma vitellogenin in males as a biomarker for estrogenic xenobiotics in reptiles and amphibians. Adult male turtles (Trachemys scripta) and frogs (Xenopus laevis) were given ip injections of estradiol-17β (E2), diethylstilbestrol (DES), or α,p′-DDT (1-chloro-2,2,4-trichloro-1,1-bis(chlorophenyl)ethane) daily for 7 days, and plasma was collected on day 14. The estrogenic activity of each compound was determined by measuring the induction of plasma vitellogenin. Vitellogenin was identified by precipitation, electrophoresis, Western blot, and enzyme-linked immunosorbent assay (ELISA). In both species, estradiol and DES treatments induced the most vitellogenin, whereas DDT treatments induced smaller amounts of vitellogenin in a dose-dependent fashion. These data indicate that induction of plasma vitellogenin in males may be a useful biomarker of xenobiotic estrogen activity in wild populations of reptiles and amphibians. — Environ Health Perspect 103 (Suppl 4):19-25 (1995)

Key words: estrogen, xenobiotic, diethylstilbestrol, DDT, turtle, frog, vitellogenin

Introduction

Xenobiotic estrogens in the environment pose an insidious risk to both wildlife and humans. By disrupting reproduction and developmental processes, they may impair not only the individuals exposed, but have lasting influence on their offspring as well. Xenobiotic estrogens include many herbicides, fungicides, insecticides, and industrial chemicals. At least 45 chemicals or their metabolites have been identified as endocrine disrupters (1). The impact of these common pollutants on the reproductive success of wildlife may be considerable. Due to their lipophilic nature and persistence in the environment, many of these compounds bioaccumulate (2). Therefore, those most affected will be those in the upper food chain or aquatic species that are exposed to high doses due to toxic accumulation in aquatic ecosystems.

A rapid, sensitive, and inexpensive test to screen wildlife for the effects of estrogenic chemicals is greatly needed. This test should be one that demonstrates not merely the presence of a compound in the tissues or body fluids, but measures a biological effect. An ideal test should meet several criteria, specifically a) have widespread applicability across many animal groups; b) require a small and easily obtainable sample without causing undue harm or distress to the animals; c) measure a physiologic response to xenobiotic compounds; d) measure a response through known biochemical pathways; e) be responsive to a large class of estrogenic chemicals; and f) be quantifiable.

One of the most important and sensitive responses to estrogen is the induction of protein transcription and translation (3,4). Particularly well known among these responses is estrogenic induction of the lipoprotein vitellogenin, expressed in all oviparous and ovoviviparous vertebrates (5,6). This makes testing for vitellogenin useful as an indicator of estrogenic activity over a wide range of vertebrate groups. Further, the mechanism has been studied in detail as a model of estrogen action (4-13). Vitellogenin is produced by cells in the liver in response to estrogen. The response is only elicited if an estrogenic compound binds to and activates estrogen receptors in hepatocytes. In females, vitellogenin is transported in the circulatory system to the ovaries, where it is incorporated into the developing ovarian follicles as yolk.

Males normally have no detectable production of vitellogenin due to their normally low levels of endogenous estrogens (13-18). However, their liver is capable of synthesizing and secreting vitellogenin into the blood in response to exogenous estrogen stimulation (6,17-25). The response is not as rapid or as strong as in females that are exposed to the same concentrations of estrogen (18). However, since males normally have no vitellogenin, the expression of any vitellogenin serves as an ideal biomarker for xenobiotic estrogenic stimulation. Vitellogenin expression has been used successfully for identification of exposure to environmental estrogens in fish, including wild populations (26,27), and under laboratory (28) and in vitro conditions (29,30).

This study was undertaken to determine if plasma vitellogenin is inducible by xenobiotic estrogens in male turtles and frogs, therefore making it a potential biomarker of xenobiotic estrogen exposure in reptiles and amphibians. Turtles and frogs were exposed to a) natural estrogen, estradiol-17β (E2); b) a well-known artificial estrogen, diethylstilbestrol (DES); and c) an important environmental estrogen, the pesticide α,p′-DDT. Measurable vitellogenin was induced in both turtles and frogs, indicating that vitellogenin may be
useful as a biomarker for xenobiotic estrogen exposure in amphibians and reptiles.

**Materials and Methods**

**Species**

Adult red-eared turtles (*Trachemys scripta*) were purchased from a commercial dealer (Lemberger; Oshkosh, WI). The turtles were housed in large Rubbermaid tubs (1.25 m long×0.75 m wide×0.75 m deep) at 21°C. Each tub was supplied with a basking ramp mounted 0.3 m below 150-watt flood lamps. This allowed the animals to thermoregulate their body temperature. Food (Wardley Pond-Ten; Secaucus, NJ) was provided *ad libitum* 3 days per week.

Adult male African clawed frogs (*Xenopus laevis*) were purchased from a commercial dealer (Sullivan; Nashville, TN). The frogs were divided randomly into groups of 5 and housed in 5-gal glass aquaria in 10 cm of water maintained at room temperature (21°C). Food (Wardley Pond-Ten) was provided 3 days per week, and the water was changed the following day.

**Chemicals**

Estradiol-17β (E2; Sigma, St. Louis, MO), diethylstilbestrol (DES; Sigma), and o,p'-DDT (1-chloro-2-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene; AccuStandard, New Haven, CT) were dissolved in corn oil. Placebos consisted of pure corn oil. Volumes of all injections were equilibrated across treatment groups for each species.

**Production of Turtle Vitellogenin for Antibody Production**

Six adult female turtles were injected daily with 1 μg/g E2, for 7 days, and on alternate days until day 21. Blood was collected from the caudal vein of each turtle using a 1-ml syringe and a 26-gauge needle (31). Whole blood was centrifuged in heparinized tubes for 2 min at 14,000 g, and the plasma was separated and frozen at −20°C. Vitellogenin was purified by precipitation and checked for purity by electrophoresis.

**Purification of Vitellogenin**

The protocol for purification of vitellogenin was modified from that described by Wiley (32). Two-hundred fifty microliters of plasma sample were gently mixed with 1 ml of 20 mM Na2EDTA and 80 μl of 0.5-M MgCl2 and centrifuged at 2500 g for 15 min. The supernatant was removed to another centrifuge tube, and then vitellogenin was precipitated with 1.24 ml distilled H2O and centrifuged at 2500 g for 15 min. The resulting pellet of purified vitellogenin was redissolved in 1 M NaCl, 50 mM Tris-HCl (pH 7.5) and then centrifuged at 2500 g for 30 min. The supernatant was removed to another centrifuge tube, and then vitellogenin was precipitated with 1.24 ml distilled H2O and centrifuged at 2500 g for 15 min. The resulting pellet of purified vitellogenin was redissolved in 1 M NaCl, 50 mM Tris-HCl (pH 7.5) and stored at −20°C. Purification of frog vitellogenin was performed using 50 μl of plasma and proportionately reduced quantities of other reagents. Quantification of vitellogenin was accomplished using Lowry reagent (BioRad, Melville, NY).

**Polyclonal Antibody Production and Purification**

Turtle (*T. scripta*) vitellogenin was purified as above and used to produce polyclonal antibodies in rabbits. Turtle vitellogenin (0.50 mg in 1 ml of 1 M NaCl, 50 mM Tris-HCl, pH 7.5) was mixed with 1 ml of Freund’s complete adjuvant and injected intradermally at four to six sites along the back of rabbits using a 20-gauge 1.5-inch needle. Using the above protocol the rabbits were boosted twice with Freund’s incomplete adjuvant at 3-week intervals. The rabbits were bled by cardiac puncture under deep anesthesia. The blood was allowed to clot at 4°C overnight to separate the serum, which was then stored at −80°C.

Antivitellogenin antibodies were purified using a procedure modified from Harlow and Lane (33). A 1-ml column was prepared by swelling 0.3 g Sepharose-4B-CNBr in 1 mM HCl for 15 min, rinsing with 70 ml of 1 mM HCl, and washing 3 times with 1 ml coupling buffer (0.5 mM NaPO4, pH 7.5). Precipitation-purified vitellogenin was dissolved in coupling buffer (20 mg/2 ml), added to the Sepharose column, agitated overnight, and rinsed twice with coupling buffer. The column was washed once with 1 M NaCl, 0.05 M NaPO4, pH 7.5 and incubated overnight, with gentle rocking, at room temperature with 10 vol of blocking buffer (100 mM ethanolamine, pH 7.5). The column was washed twice with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) and mercaptoethanol added to 0.01% and stored at 4°C until use.

Before use, the column was rinsed with 10 ml of 10 mM Tris buffer (pH 7.5) and flushed with 10 ml of 100 mM glycine buffer, pH 2.5, followed by 10 ml of 10 mM Tris buffer pH 8.8. The column was then washed with 10 ml of 100 mM triethylamine (pH 11.5), followed by 10 ml of 10 mM Tris, pH 7.5.

Following column preparation, polyclonal antiserum specific to vitellogenin was diluted to 10% in 10 mM Tris, pH 7.5 and passed through the column 3 times with a flow rate of 1 ml/min. The column was washed with 20 ml of 10 mM Tris, pH 7.5 and then 20 ml of 500 mM NaCl, 10 mM Tris-buffered saline (pH 7.5). The column was eluted with 20 ml of 100 mM glycine buffer, pH 2.5, and the antibodies collected in a tube containing 1 ml of 1 M Tris, pH 8.8. The column was washed with 10 mM Tris, pH 8.8, the column eluted again with 10 ml of 100 mM triethylamine, pH 11.5. Antibodies were collected in a tube containing 1 ml of 1 M Tris, pH 8.8. The vitellogenin antibody fractions were combined and dialyzed against PBS with 0.02% sodium azide.

Specificity of purified antivitellogenin antibody was tested against whole plasma, precipitation-purified vitellogenin, a protein of 214 kDa (the presumptive vitellogenin identified electrophoretically) electroeluted from 5% PAGE, and bovine serum albumin (BSA). Antivitellogenin antibodies were tested by enzyme-linked immunosorbent assay (ELISA) and Western blot to determine cross-reactivity. Controls included omission of protein antigen, omission of primary antibody, and omission of secondary antibody. Only batches of purified antivitellogenin antibody that exhibited high affinity for vitellogenin and minimal cross-reactivity with other antigens were used in immunodetection.

**One-dimensional Polyacrylamide Gel Electrophoresis**

Whole plasma or purified vitellogenin samples were solubilized in 10 mM Tris buffer (pH 7.4) containing 2% (w/v) sodium dodecyl sulfate (SDS) and 10% (v/v) 2-mercaptoethanol and separated by molecular weight using one-dimensional SDS polyacrylamide gel electrophoresis (1D-SDS-PAGE). Separation gels were made from 5% T (total acrylamide) to facilitate analyses of high molecular weight proteins (34). Plasma samples or purified vitellogenin were loaded onto a discontinuous PAGE apparatus, then run at 200 V for 35 min or until complete. The gels were fixed and stained with Coomassie blue (34) and silver (35). Molecular weights were determined using BioRad high molecular weight standards and calculation of Rf values (36). Vitellogenin was quantified from 1D-SDS-PAGE of plasma using a BioRad GS-670 imaging densitometer to digitize the images. The concentrations of vitellogenin (band densities)
were calculated by plotting the band absorbance and integrating the area under the curve for vitellogenin (BioRad Molecular Analyst software). Comparisons of treatment groups were performed using Kruskal-Wallis one-way analysis of variance on ranks (ANOVA: \( p < 0.05 \)). Multiple means comparisons were performed using the Student-Newman-Keuls method (37).

**Western Blotting**

To identify vitellogenin immunologically, whole plasma proteins or precipitation purified vitellogenin were transferred to polyvinylidene difluoride (PVDF) membranes immediately following 1D-SDS-PAGE. Briefly, the gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol (38)) immediately following electrophoresis, and the proteins were transferred to a PVDF membrane under a 15 V electrical field for 1 hr. After transfer, the PVDF membrane was equilibrated in Tris-buffered saline (TBS; 25 mM Tris, 0.3M NaCl, pH 7.4) for 15 min, blocked with 5% (w/v) powdered milk in TBS (2 hr), and incubated overnight with primary antibodies (diluted 1:50,000 in 5% (w/v) powdered milk in TBS) specific for vitellogenin. The transfer membrane was washed in TBS (3×5 min) and incubated for 2 hr with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in 5% powdered milk in TBS. The PVDF membrane was washed again in TBS (3×5 min), and incubated at 37°C in 0.1 g/l nitro blue tetrazolium (NBT), 0.05 g/l 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 2 mM MgCl₂ in 0.1M Tris buffer (pH 8.8 for 15 min) for localization of reactive proteins.

**Induction of Vitellogenin in Turtles with \( E_2 \), DES, and \( o,p'\)-DDT**

Adult male turtles were ranked by mass and divided into experimental and control groups to equalize specimen sizes among treatments. The treatment groups consisted of animals treated with 1 µg/g \( E_2 \), 1 µg/g DES, 250 µg/g \( o,p'\)-DDT, 1 µg/g \( o,p'\)-DDT, and oil only as control. Each compound was dissolved in 0.1 ml corn oil and injected ip. The treatments were given daily for 7 days. Plasma was collected on day 14 from the caudal vein of each turtle using a 1-ml syringe and a 26-gauge needle (31). Whole blood was spun in heparinized tubes for 2 min at 14,000g, and plasma was separated and frozen at \(-20^\circ C\). This treatment protocol with exogenous \( E_2 \) has been shown to produce circulating concentrations of serum \( E_2 \) in turtles of approximately 333 pg/ml (Selcer and Palmer, unpublished data), which is equivalent to \( E_2 \) concentrations in females during natural vitellogenesis.

**Induction of Vitellogenin in Frogs with \( E_2 \), DES, and \( o,p'\)-DDT**

Twenty-five adult male frogs of matched size were randomly assigned into treatment groups of five and housed in separate 5 gal aquaria with 10 cm of water. The treatment groups consisted of animals treated with 1 µg/g \( E_2 \), 1 µg/g DES, 250 µg/g \( o,p'\)-DDT, 1 µg/g \( o,p'\)-DDT, and oil only as control. Compounds were dissolved in 40 µl of corn oil and injected ip. Injections were given daily for 7 days, and frogs were bled by cardiac puncture on day 14. Whole blood was centrifuged in heparinized tubes for 2 min at 14,000g and plasma separated and frozen at \(-20^\circ C\).

**ELISA**

ELISA (enzyme-linked immunosorbant assay) was used to determine relative levels of vitellogenin in plasma samples. Ten-microliter plasma samples and 10 µl excess BSA were each diluted with 40 µl TBS (25 mM Tris, 0.3M NaCl, pH 7.4). The diluted plasma solutions were added to individual wells of a polystyrene microtiter plate, and the BSA solution was added to a single well as a control; and the plate was incubated overnight at 4°C. Plates were washed three times with TBS-Tween (TBS plus 0.2% Tween 20) and blocked with 150 µl Blotto-Tween (5% nonfat dry milk, 0.2% Tween 20, and 0.02% sodium azide in TBS) for 2 hr. The plates were washed three times with TBS-Tween, and 50 µl antivitellogenin antibodies diluted 1:20,000 in Blotto-Tween were added to each well. The plates were then incubated for 2 hr at room temperature. The plates were thoroughly washed four times with Blotto-Tween and incubated for 2 hr with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma) diluted 1:1,000 in Blotto-Tween. After thoroughly washing each well with TBS-Tween 4 times, the plates were developed in 0.1% (w/v) p-nitrophenyl phosphate (PNPP) in developing buffer (0.1 M NaCl, 0.1 M Tris, 5 mM MgCl₂, pH 9.5) and incubated for 15 min at room temperature. The reaction was stopped with 50 µl of stop buffer (20 mM Tris, 5 mM EDTA, pH 7.5), and the plate densities were read on a Bio Tek Microplate Reader at 405 nm. The well coated with BSA served as the reagent blank, and its absorbance was subtracted from that of the other wells. Statistical comparisons among treatment groups were performed using Kruskal-Wallis one-way analysis of variance on ranks (ANOVA: \( p < 0.05 \)). Multiple means comparisons were performed using the Student-Newman-Keuls method (37).

**Results**

**Induction of Vitellogenin in Turtles**

A protein of 214 kDa was extractable from \( E_2 \), DES, and both \( o,p'\)-DDT treatment groups. This protein exhibited cross-reactivity with antivitellogenin antibodies in Western blots (Figure 1) and was identified as vitellogenin. ELISA analyses of day 14 turtle plasma indicated significant (\( p < 0.001 \)) induction of vitellogenin production from \( E_2 \), DES, and both DDT treatments (Figure 2). \( E_2 \) and DES, a well known artificial estrogen, induced the highest concentrations of vitellogenin. The persistent pesticide \( o,p'\)-DDT induced smaller amounts of vitellogenin in a dose-dependent manner. No vitellogenin was extractable from the control plasma or detectable using 1D-SDS-PAGE and Western blot analyses, indicating that the slight absorbance of control plasma in ELISA analyses is attributed to nonspecific binding of the polyclonal antibody (Figure 2).
Induction of Vitellogenin in Frogs

A protein of approximately 200 kDa was extractable from the plasma of frogs treated with E₂, DES, and both DDT regimes (Figure 3), but was not extractable from the plasma of control specimens. This protein was purified as described above and identified as vitellogenin. However, this protein demonstrated no cross-reactivity with the antiturtle vitellogenin antibodies. Computerized image analysis of 1D-SDS-PAGE was used to quantify relative concentrations of vitellogenin induced by the treatment regimes. The pattern of vitellogenin production in frogs mirrored that seen in turtles, with significant variation (p < 0.001) in relative quantities of vitellogenin produced by treatments (Figure 4). E₂ induced the most vitellogenin, followed by DES and o,p'-DDT in a dose-dependent fashion. No extractable vitellogenin was detectable by 1D-SDS-PAGE from control samples.

Discussion

DES was developed as a synthetic estrogen that was used to prevent premature births in women beginning in 1948. Over 1 million women took DES between 1960 and 1970 (1). DES was shown to have significant detrimental effects on embryonic development in humans and animal models, and was subsequently banned from use by pregnant women in 1971. Daughters of women who took DES suffer reproductive organ dysfunction, reduced fertility, abnormal pregnancies (39), and increased incidence of vaginal clear-cell adenocarcinomas (40). Similar reproductive and developmental problems have been seen in female laboratory animals (39,41–43). Exposure of male humans and male laboratory animals to DES during development leads to lesions of the reproductive system (44,45) and an increased incidence of reproductive tract cancers in laboratory animals (46). The mechanism of DES action has been well studied. It is an agonist of estrogen receptors, binding to them and eliciting transcription of estrogen-induced proteins (39,47).

The use of the organochlorine pesticide DDT was restricted in the United States in 1972; however, elevated levels of DDT and its metabolites are still encountered in the tissues of wildlife in this country (48). Although some of this can be due to DDT’s long half-life (57.5 years) in temperate soils (49), this does not entirely account for the continuing high levels. A likely source is the continued manufacture and use of DDT in developing countries (1). DDT used in developing countries is readily vaporized and transported globally via the atmosphere (50,51), providing a continuing rain of DDT throughout the world. This indicates that DDT is still an important environmental hazard and serves as a good model for physiological studies due to the extensive studies on its mechanism of action.

DDT is well known for its effects on reproduction in avian species, most notably causing eggshell thinning (52). However, the role of DDT as an estrogen has been more insidious (53). DDT has two isomers, p,p’-DDT and o,p’-DDT, which break down into their respective metabolites, p,p’-DDE and o,p’-DDE (53). The o,p’ isomers of DDT and DDE bind to estrogen receptors agonistically, mimicking endogenous estrogen (54,55).

The induction of vitellogenin by a compound indicates that it is an agonist of hepatic estrogen receptors, stimulating them to induce transcription of the vitellogenin genes. Both the artificial estrogen, DES and the pesticide DDT were able to stimulate male turtles and frogs to produce significant levels of vitellogenin. DES has been shown to bind to nuclear estrogen receptors in turtles, inhibiting 96.7% of specifically bound E₂ (56). In the environment, DDT is known to accumulate in large concentrations in turtles and frogs. The softshell turtle has been recorded with 732 ppm DDT in adults (57). Adult toads have exhibited 0.13 ppm DDT (57).

An insidious aspect of lipophilic compounds is that they are also deposited in eggs during vitellogenesis. It is likely that as lipids are mobilized from body stores, lipophilic contaminants also are mobilized and become incorporated into the growing ovarian follicles. Thus the adult’s burden of lipophilic contaminants may be passed on to their offspring, where they can influence the fertilizability of the egg and development of the embryo, the most sensitive period of the life cycle. Eggs of loggerhead sea turtles exhibited approximately 0.1 ppm DDE (58), equal to the highest levels detected in the adult turtles (59). In the snapping turtle, up to 864 ppb DDT has been reported in their eggs (60). The levels of organochlorine contaminants in reptilian eggs correlates with increases in deformities and lowered rates of hatching success (61). In species that
exhibit temperature-dependent sex determination (TSD), such as many turtles, the implications for effects by environmental estrogens are enormous. The sex of the offspring in species that exhibit TSD is regulated by levels of sex steroids (62,63). By increasing levels of perceived estrogen, xenobiotic estrogens may adversely affect sexual development in these species. It has been shown that exposure of turtle eggs to xenobiotics induces oyster-like characteristics in hatchlings incubated at male-producing temperatures (64). Vitellogenin may prove useful as a biomarker for exposure to xenobiotic estrogens due to its numerous advantages. First, it is applicable to a wide range of species, potentially any oviparous or ovoviparous vertebrate. The criterion for using vitellogenin as a biomarker in a particular species is that males do not normally express vitellogenin, but are capable of doing so in response to estrogenic compounds. Second, the test is relatively non-invasive, requiring only small (microliter) quantities of plasma or serum. This eliminates the necessity of acquiring tissue biopsies of organs such as liver, muscle, and fat. Third, it indicates a physiological response to an environmental challenge, indicating that the animal is being influenced by environmental estrogens. This eliminates the confusion often encountered when contaminants are detected in wildlife, but without knowing if the contaminant is causing any physiological effects. Fourth, the expression of vitellogenin is through known physiological and biochemical pathways. Indeed, the induction of vitellogenin has been studied intensively as a model for steroid regulation of gene expression and protein synthesis (4–13). Fifth, the induction of vitellogenin is sensitive to any estrogenic contaminant. In fact, the vitellogenic response has been used to screen unknown compounds for estrogenic properties (29,30). Sixth, the response is quantifiable. Finally, the assay for vitellogenin can be performed relatively easily and inexpensively, especially compared to the exorbitant cost of screening for a wide range of specific contaminants. The vitellogenic response has been used to test for exposure to estrogenic xenobiotics in fishes (26–30).

There are, however, several drawbacks regarding vitellogenin analysis that must be considered. It is unlikely that the production of vitellogenin in males represents a seriously deleterious physiological response. This assay also provides no direct information regarding the female or developing embryo. However, if estrogen receptors are being stimulated in the liver of males, receptors in other organs such as the testes and prostate gland of males and reproductive tissues of females and embryos may likewise be affected. In fact, since females and embryos often show greater responses to estrogen, they may be affected equally or even more so. Further, vitellogenin production does not indicate what compound may be causing the effect. However, it may be used as a rapid, sensitive, and economical initial screen, followed (as indicated by positive vitellogenic responses) by more costly screens to identify the specific contaminating compounds. This assay assumes that males do not produce vitellogenin but are capable of doing so. This requires that some physiology of the species be known, such as comparing contaminated populations to clean control populations. These limitations considered, vitellogenin may serve as an excellent biomarker for xenobiotic estrogen exposure in reptiles and amphibians or other oviparous and ovoviparous vertebrates.

Increasingly more evidence of the estrogenic effects of contaminants on wildlife is appearing. In the late 1970s, male herring gull embryos and chicks from Lake Ontario showed the development of oviducts and ovarylike gonads. Laboratory studies have since shown that estrogenic pesticides such as dicofol, kethane, and methoxychlor produce these effects in California gulls, western gulls, and kestrels (65). Herring gull populations contaminated with estrogenic compounds such as DDT, DDE, and PCBs have been shown to form female-female pairs (66). In Florida, the occurrence of feminized and masculinized male alligators is associated with the presence of the estrogenic pesticides (67). Hermaphroditic fish populations have been reported in the lagoons of sewage treatment works (27).

Are these examples of wildlife effects isolated incidents or just the tip of the iceberg? It is unclear at this point. Some populations, such as bald eagles surrounding the Great Lakes, are still in trouble. Adult bald eagles that migrate to the lake shore develop reproductive difficulties associated with contaminated food (48). The Great Lakes is acting as a sink for bald eagles migrating from productively fit inland populations. Alligators in Lake Apopka, Florida, have drastically reduced reproductive success (67,68). These local populations of long-lived species may be heeding for extinction, because reproduction on the site is insufficient to maintain the population size, requiring migration from surrounding populations to sustain them. It is possible that the decline of other species around the globe, such as amphibians (69–74), is partially linked to reproductive impairment by endocrine-disrupting compounds (BD Palmer, unpublished data). The assay of vitellogenin in males of these and other species in the wild would indicate whether they are being physiologically influenced by the estrogenic properties of environmental contaminants.

REFERENCES

1. Colborn T, Vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384 (1993).

2. Peterle TJ. Wildlife Toxicology. New York: Van Vostand Reinhold, 1991.

3. Gorski J, Furlow JD, Murdock FE, Fritsch M, Kaneko K, Ying D, Malayer JR. Perturbations in the model of estrogen receptor regulation of gene expression. Biol Reprod 48:8–14 (1993).

4. Tata JR, Smith DF. Vitellogenesis: a versatile model for hormonal regulation of gene expression. Recent Prog Horm Res 35:47–95 (1979).

5. Wallace RA. Vitellogenesis and oocyte growth in nonmammalian vertebrates. In: Developmental Biology (Browder LW, ed). New York: Plenum Press, 1985;127–177.

6. Ho S-M. Endocrinology of vitellogenesis. In: Hormones and Reproduction in Fishes, Amphibians and Reptiles (Norris DO, Jones RE, eds). New York: Plenum Publishing, 1987.

7. Tata JR. The expression of the vitellogenin gene. Cell 9:1–14 (1976).

8. Callard IP, Ho S-M, Gapp DA, Taylor S, Danko D, Wulczyn G. Estrogens and estrogenic actions in fish, amphibians and reptiles. Dev Toxicol Environ Sci 5:213–237 (1980).

9. Wahl W, Dawid IB, Jagg I, Wyler T, Riffel GU. Vitellogenin in Xenopus laevis is encoded in a small family of genes. Cell 16:535–549 (1979).

10. Moomsen TP, Walsh PJ. Vitellogenesis and oocyte assembly.
In: Fish Physiology, Vol 11A (Hoar WS, Randall DJ, eds). San Diego: Academic Press, 1988:347–406.

11. Riegel AT, Atitken SC, Martin MB, Schoeberg DR. Differential induction of hepatic estrogen receptor and vitellogenin gene transcription in Xenopus laevis. Endocrinology 120:1283–1290 (1990).

12. Pakdel F, Feon S, Le Gac R, Le Menn F, Valotaire Y. In vivo estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol Cell Endocrinol 75:205–212 (1991).

13. Ho S-M, Klein R, McPherson GJ, Heiserman GJ, Callard IP. Regulation of vitellogenin in reptiles. Herpetologica 38:40–50 (1982).

14. Ho S-M, Taylor S, Callard IP. Effect of hypophysectomy and growth hormone on estrogen-induced vitellogenesis in the fresh water turtle, Chrysemys p. picta. Gen Comp Endocrinol 48:354–260 (1982).

15. Ho S-M, Wangh LJ, Callard IP. Sexual differences in the in vitro induction of vitellogenesis in the turtle: role of the pituitary and growth hormone. Comp Biochem Physiol 81:467–472 (1985).

16. Hara A, Takanoka K, Hira H. Immunochemical identification of female specific serum protein, vitellogenin, in the medaka Oryzias latipes (teleost). Comp Biochem Physiol 76A:135–142 (1983).

17. LeGuillec K, Lawless K, Valotaire Y, Kross M, Tenniswood M. Vitellogenin gene expression in male rainbow trout (Salmo gairdneri). Gen Comp Endocrinol 71:359–371 (1988).

18. Ho S-M, Danko D, Callard IP. Effect of exogenous estradiol-17β on plasma vitellogenin levels in male and female Chrysemys and its modulation by testosterone and progesterone. Gen Comp Endocrinol 43:413–421 (1981).

19. Wang LJ, Knowland J. Synthesis of vitellogenin in culture of male and female frog liver regulated by estradiol treatment in vitro. Proc Nat Acad Sci USA 72:3172–3175 (1975).

20. Sundararaj BI, Nath P. Steroid-induced synthesis of vitellogenin in the catfish, Heterophyes floschii (Bloch). Gen Comp Endocrinol 57B:201–210 (1981).

21. Chen TT, Howard DA, Agellon LB, Lin CM, Davies SL. Estrogen-controlled gene expression: induction of two estrogen-responsive genes in the liver of rainbow trout (Salmo gairdneri). Physiol Zool 62:25–37 (1988).

22. Bradley JT, Grizzle JM. Vitellogenin induction by estradiol in channel catfish, Ictalurus punctatus. Gen Comp Endocrinol 73:28–39 (1989).

23. Vaillant C, LeGuillec C, Padel F, Valotaire Y. Vitellogenin gene expression in primary culture of male trout hepatocytes. Gen Comp Endocrinol 70:284–290 (1988).

24. Pakdel F, LeGuillec C, Vaillant CM, LeRoux G, Valotaire Y. Identification and estrogen induction of two estrogen receptors (ER) messengers in the rainbow trout liver: sequence homology with other ERs. Mol Endocrinol 31:47–51 (1989).

25. Morales MH, Osuna R, Sanchez E. Vitellogenesis in Anolis pulchellus induction of VTG-like protein in liver explants from male and immature lizards. J Exp Zool 260:50–58 (1991).

26. Goodwin AE, Grizzle JM, Bradley JT, Estridge BH. Monoclonal antibody-based immunoassay of vitellogenin in the blood of male channel catfish (Ictalurus punctatus). Comp Biochem Physiol 101B:441–446 (1992).

27. Burgon CE, Hardiman PA, Byre VJ, Eno NC, Tyler CR, Sumpter JP. Estrogenic effects of effluents from sewage treatment works. Chem Ecol 8:275–285 (1994).

28. Chen TT, Peid PC, Van Beneden R, Sonstegard RR. Effect of acrolein 1254 and mirex on estradiol-induced vitellogenin production in juvenile rainbow trout, Salmo gairdneri. Can J Fish Aquat Sci 43:169 (1986).

29. Schaeffer S, Bonning JP. Detergent components in sewage effluent are weakly oestrogenic to fish: an in vitro study using rainbow trout (Oncorhynus mykiss) hepatocytes. Aquat Toxicol 27:361–372 (1993).

30. Pelisser C, Flouriot B, Fouger JL, Bennetau B, Dunogues J, Le Gac F, Sumpter JP. Vitellogenin synthesis in cultured hepatoctyes: an in vitro test for the estrogenic potency of chemicals. J Steroid Biochem Mol Biol 44:263–272 (1993).

31. Powell SC, Kneef JA. Blood collection from Macrolepsis tenimix (Troost). Herpetol Rev 23:19 (1992).

32. Wiley HS, Opresko L, Wallace RA. New methods for the purification of vertebrate vitellogenin. Anal Biochem 97:145–152 (1979).

33. Harlow E, Lane D. Antibodies: A Laboratory Manual. New York:Cold Spring Harbor Laboratory, 1988.

34. Lappi MI, CL. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685 (1970).

35. Roberts RM, Baumber GA, Buhi WC, Denny JB, Fitzgerald LA, Babelin SF, Horst MN. Analysis of membrane polypeptides by two-dimensional polyacrylamide gel electrophoresis. In: Molecular and Chemical Characterization of Membrane Receptors (Venter JC, Harrison LC, eds). New York:Alan R Liss, 1984:61–113.

36. Shapiro AL, Vinuela D, Maizel JV Jr. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem Biophys Res Commun 28:815 (1967).

37. Glantz SA. Primer of Biostatistics, 3rd ed. New York:McGraw Hill, 1992.

38. Towbin H, Dastelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Nat Acad Sci, USA 76:4350–4354 (1979).

39. Takasugi N, Bern HA. Introduction: abnormal genital tract development in mammals following early exposure to sex hormones. In: Toxicity of Hormones in Perinatal Life (Mori T, Nagasawa H, eds). Boca Raton, FL:CRC Press, 1988:1–7.

40. Herbst A, Ulfelder H, Poskanzer C. Adenocarcinoma of the vagina: association of maternal stilbestrol therapy and tumor appearance in young women. N Engl J Med 284:878–881 (1971).

41. Bern HA, Talamantes FJ. Neonatal mouse models and their relation to disease in the human female. In: Estrogens in the Environment (Herbst A, Bern A, eds). New York:Thieme Stratton, 1981:129–147.

42. Bern HA, Edery M, Mills KT, Kohrman AP, Mori T, Larson L. Long-term alteration in histology and steroid levels of the genital tract and mammary gland following neonatal exposure of female BALB/cCrjg mice to various doses of diethylstilbestrol. Cancer Res 47:4165–4172 (1987).

43. Bern HA, Mills KT, Edery M. Estrogen-associated defects in rodent mammary gland development. In: Estrogens in the Environment II. Influences on development (McLachlan J, ed). New York:Elsevier, 1981:29–32.

44. McLachlan J, Newbold R, Bullock B. Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science 190:991–992 (1975).

45. Driscoll S, Taylor S. Effects of prenatal maternal estrogen on the male urogenital system. J Am Coll Obstet Gynecol 56:537–542 (1980).

46. Ari Y, Chen C-Y, Nishizuka Y. Cancer development in male reproductive tract in rats given diethylstilbestrol at neonatal age. Ann 69:861–862 (1978).

47. Robinson AK, Mukku VR, Stancel GM. Analysis and characterization of estrogenic xenobiotics and natural products. In: Estrogens in the Environment II. Influences on Development (McLachlan JA, ed). New York:Plenum, 1985:107–115.

48. Colborn TM, Davidson A, Green SN, Hodge RA, Jackson CI, Liroff RA. Great Lakes, great legacy? Washington:The Conservation Foundation, 1990.

49. Cooke BK, Stringer A. Distribution and breakdown of DDT in orchard soil. Pestic Sci 13:545–551 (1982).

50. Eisenreich SJ, Looney BB, Thornton JD. Airborne organic contaminants in the Great Lakes ecosystem. Environ Sci Technol 15:30–38 (1981).

51. Rapport RA, Urban NR, Capel PD, Baker JE, Looney BB, Eisenreich SJ, Gorham E. New DDT inputs to North America: atmospheric deposition. Chemosphere 14:1167–1173 (1985).

52. Faber R, Hickey J. Eggshell thinning, chlorinated hydrocarbons, and mercury in inland aquatic bird eggs, 1969 and 1970.
Pestic Monit J 7:27–36 (1975).

53. Bulger WH, Kupfer D. Estrogenic action of DDT analogs. Am J Ind Med 4:163–173 (1983).

54. McBlain WA. The levo enantiomer of \(a,p'-\text{ DDE} \) inhibits the binding of 17 beta-estradiol to the estrogen receptor. Life Sci 40:215–221 (1987).

55. McLachlan JA, Newbold RR. Estrogens and development. Environ Health Perspect 75:25–27 (1987).

56. Riley D, Callard IP. Characterization of turtle liver nuclear estrogen receptors, seasonal changes, and pituitary dependence of cytosolic and nuclear forms. J Exp Zool 245:277–285 (1988).

57. Edwards CA. Persistent Pesticides in the Environment. 2nd ed. Cleveland: CRC Press, 1973.

58. Clark DR Jr, Krynitshy AJ. DDE residues and artificial incubation of loggerhead sea turtle eggs. Bull Environ Contam Toxicol 34:121–125 (1985).

59. McKim JM Jr, Johnson KJ. Polychlorinated biphenyls and \(p,p'-\text{ DDE} \) in loggerhead and green postyearling Atlantic sea turtles. Bull Environ Contam Toxicol 31:53–60 (1983).

60. Environment Canada, Department of Fisheries and Oceans, Health and Welfare Canada. Toxic chemicals in the Great Lakes and associated effects. Volume 1, Contaminant Levels and Trends. Toronto, Ontario: Health and Welfare Canada, 1991.

61. Bishop CA, Brooks RJ, Carey JH, Ng P, Norstrom RJ, Lean DRS. The case for a cause-effect linkage between environmental contamination and development in eggs of the common snapping turtle (Chelydra serpentina serpentina) from Ontario, Canada. J Toxcol Environ Health 33:521–548 (1991).

62. Crews D, Bull JJ, Wibbels T. Estrogen and sex reversal in turtles: a dose-dependent phenomenon. Gen Comp Endocrinol 81:357–364 (1991).

63. Tousignant A, Crews D. Effect of exogenous estradiol applied at different embryonic stages on sex determination, growth, and mortality in the leopard gecko (Eublepharis macularius). J Exp Zool 268:17–21 (1994).

64. Crews D. Temperature, steroids and sex determination. J Endocrinol 142:1–8 (1994).

65. Fry DM, Toone DK. DDT-induced feminization of gull embryos. Science 231:919–924 (1981).

66. Fox G. Epidemiological and pathological evidence of contaminant-induced alterations in sexual development in free-living wildlife. In: Chemically Induced Alterations in Sexual and Functional Development: the Wildlife/Human Connection (Colborn T, Clement C, eds). Princeton, NJ: Princeton Scientific Publishing, 1992:147–158.

67. Guillette LJ Jr, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ Health Perspect 102:680–688 (1994).

68. Heinz GH, Percival HF, Jennings ML. Contaminants in American alligator eggs from Lake Apopka, Lake Griffin, Lake Okeechobee, Florida. Environ Monit Assess 16:277–286 (1991).

69. Hayes MP, Jennings MR. Decline of ranid frogs in western North America: Are bullfrogs (Rana Catesbeiana) responsible? J Herpetol 20:490–509 (1986).

70. Blaustein AR, Wake DB. Declining amphibian populations: A global phenomenon? Trends Ecol Evol 5:203–204 (1990).

71. Phillips K. Where have all the frogs and toads gone? Bioscience 40:422–424 (1990).

72. Vitt LJ, Caldwell JP, Wilbur HM, Smith DC. Amphibians as harbingers of decay. Bioscience 40:418 (1990).

73. Wake DB. Declining amphibian populations. Science 253:860 (1991).

74. Blaustein AR. Chicken little or Nero's fiddle? A perspective on declining amphibian populations. Herpetologica 50:85–97 (1994).