Experimental Evaluation of Vitellogenin as a Predictive Biomarker for Reproductive Disruption

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Vitellogenin (VTG) synthesis in male oviparous vertebrates is used as an indicator of environmental estrogen exposure, but the relationship between elevated VTG levels and the effects of environmental estrogens on reproductive success are poorly understood. To examine whether altered VTG expression predicts reproductive impairment, we exposed medaka (*Oryzias latipes*) for 2 or 8 weeks posthatch to 0, 0.5, 1.0, 2.5, and 7.5 ppb of the environmental estrogen *o,p´*-DDT. Fish were sampled 2, 4, and 8 weeks after hatch to examine VTG expression and gonad development. After exposure, fish were transferred to clean water, grown to sexual maturity, and placed in mating pairs. We collected eggs for 7 days and scored them for fecundity (number of eggs), fertility (percent fertilized), and hatching success (percent hatched). DDT had no effect on VTG expression after a 2-week exposure, whereas all doses induced VTG after 8 weeks. At both exposure durations, the highest doses of DDT caused a female-skewed sex ratio in adults. Gonadal feminization appeared to be progressive: some ovotestes were observed after 2- or 4-week exposure to the two highest doses, but the proportion of ovaries increased after 8 weeks. Both 2- and 8-week exposures significantly reduced fertility and hatching success at all doses, with lower doses having a greater effect after longer exposure. Fertility and hatching success were more sensitive to estrogenic disruption than were gonad differentiation and vitellogenin expression. We suggest that VTG expression may be interpreted as a warning of reproductive consequences, but absence of expression cannot be interpreted as absence of consequences. *Key words*: endocrine disruption, fish, medaka, reproductive success, sex differentiation, vitellogenesis. *Environ Health Perspect* 109:681–690 (2001). [Online 25 June 2001]
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The significance of endocrine disruption for population and ecosystem health is an important issue in contemporary environmental science. Endocrine disruption is defined as the perturbation of endogenous hormone function by exogenous chemicals. Endogenous sex steroid hormones may have two broad categories of influence on physiologic systems: organization and activation (*1,2*). Organizational effects are usually permanent changes in morphology that persist after the hormone stimulus is removed and that affect subsequent function and behavior. Activational effects are usually transient changes in morphology, function, and behavior that disappear when the hormone stimulus is removed. Classically, the organizational effects of sex steroid hormones include sex-specific morphologic differentiation of the gonads and brain. Activational effects of sex steroids in vertebrates include seasonal gonadal recrudescence and onset of breeding coloration and behavior (*1,2*). Both categories of action are essential for normal reproduction.

Endocrine disruption can alter both the organizational and activational effects of reproductive hormones, possibly having a profound effect on an organism's ability to reproduce and therefore on its fitness. Environmental contaminants can have organizational effects on sex differentiation and on expression of secondary sex characteristics in a variety of vertebrates (*3–17*). In addition, numerous studies have documented the activational effects of exogenous chemicals on levels of sex steroid hormones and on hormone-mediated protein synthesis (*12, 18–36*). The implicit proposition in many of these studies is that the more easily measured activational effects such as hormone and protein levels can be used as biomarkers of the potential organizational effects such as altered gonad morphology. However, in most cases, the effects of contaminants on reproductive capability are not known, nor are the relative sensitivities of the organizational and activational responses known. To understand the impact of endocrine disruption on natural populations, we must begin to analyze the relative sensitivity of these responses, giving particular attention to quantifying the consequences for individual fitness, that is, offspring production.

Although several classes of endocrine-disrupting agents have been identified, including environmental estrogens, antiestrogens, androgens, antiandrogens, antiprogestins, and retinoid mimics (*37–39*), environmental estrogens have been most thoroughly studied. Fish have been a particularly popular model for studying estrogenic endocrine disruption because both males and females produce vitellogenin (VTG), the precursor to egg yolk proteins, in response to estrogen or estrogen mimics (*40*). VTG production is nonexistent to low in males and immature females, whereas mature female fish have a seasonal cycle of serum VTG levels, with peak values reaching tens of milligrams per milliliter (*40*). In mature females, vitellogenesis is under hormonal regulation by estradiol, the major endogenous estrogen in all vertebrates. Increasing titers of estradiol stimulate the liver to produce VTG, which is then transported in the bloodstream to the ovary. VTG enters the oocyte via specific receptor-mediated endocytosis (*41*). Once inside the oocyte, VTG is cleaved into the smaller yolk proteins (phosvitin, lipovitellin, and beta-component), which accumulate in yolk globules or granules (*42*).

Because of the specific association between VTG synthesis and estrogen stimulation and because of the low background production of this protein in all but mature females, VTG is a highly specific biomarker for estrogen exposure in fish. Exposure to several classes of chemicals and chemical mixtures is known to alter fish vitellogenesis *in vivo*, including the alkylphenols nonylphenol and octylphenol, the steroidal estrogen ethinylestradiol, the pesticides methoxychlor and *o,p´*-DDT and their metabolites, phytoestrogens, and sewage effluent (*34,35,39,43*). Direct activation of vitellogenesis in hepatic cell culture also occurs in response to some environmental estrogens, including alkylphenols, *o,p´*-DDT, Aroclor 1254 (a polychlorinated biphenyl mixture), bisphenol A, chlordecone, lindane, and phytoestrogens,

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although sensitivity appears to vary depending upon the donor species (*40,44–46*).

Although alterations in vitellogenesis have been well documented *in vivo* and *in vitro*, the effects of environmental estrogens on sex differentiation in fish have only recently been investigated. Alkylphenol exposure caused feminization of the testis in medaka [*Oryzias latipes* (*8,9*)] and carp [*Cyprinus carpio* (*7*)] and reduced testis and ovary growth in juvenile rainbow trout [*Oncorhynchus mykiss* (*47,48*)]. *o,p´*-DDT also caused feminization in medaka, either by immersion exposure of fry (*11*) or by injection into fertilized eggs (*10*). However, neither the *o,p´* nor *p,p´* isomer of DDE altered sex differentiation in juvenile rainbow trout (*49*). Vinclozolin, a fungicide with antiandrogen action in mammals, showed no effect on fathead minnow (*Pimephales promelas*) ovarian or testicular development (*50*).

Recently, the effects of natural and environmental estrogens on reproductive success (number of offspring produced) have been investigated in laboratory exposures of adult males. Estradiol exposure of adult males caused decreased hatching success in medaka (*51*) and in fathead minnows (*52*). Octylphenol and bisphenol A also reduced male fertility (percent fertilized) and embryo survivorship in medaka mating trials (*51,53*). Very few studies in fish have examined the effects of developmental exposure to environmental estrogens on subsequent reproductive success, and no studies have yet addressed the link between vitellogenin induction, sex differentiation of exposed fish, and reproductive success.

This study addresses two issues: the effects of developmental estrogen exposure on individual fitness (number of offspring) and the utility of VTG as a predictive biomarker for altered sexual development and impaired reproduction. We predict that developmental exposure to environmental estrogens will have an organizational or permanent effect on gonad morphology and reproductive function, but that it will have an activational or transitory effect on VTG production (i.e., exposed animals will produce VTG only while the stimulus is present). If this is the case, VTG serves as an excellent biomarker of current estrogenic exposure, but it may not indicate organizational effects such as altered sex differentiation and impaired reproductive function.

We exposed developing medaka to *o,p´*-DDT for 2 or 8 weeks posthatch and examined the effects of exposure on VTG synthesis, sex differentiation, and reproductive success. We used medaka as a model because they are easily maintained in the laboratory and are known to be sensitive to environmental estrogens (*8–11,53,54*). We chose to use *o,p´*-DDT as a model environmental estrogen for several reasons. First, although DDT use was banned in the United States in 1973, DDT isomers and metabolites are extremely stable and are globally distributed at concentrations ranging from 0 to 10 ppm (*55*). Second, *o,p´*-DDT is known to be a relatively potent environmental estrogen in frogs (*56*), turtles (*57*), birds (*58*), and mammalian cells (*59*). Finally, recent work has shown that *o,p´*-DDT feminizes developing male medaka (*10,11*).

In this study we show that *o,p´*-DDT is estrogenic in medaka: it stimulates VTG synthesis in juveniles and adults, feminizes developing males, and reduces reproductive success. However, VTG expression was the least sensitive physiologic response to estrogenic endocrine disruption.

Methods

Animals. Adult medaka were taken from the broodstock at the University of Southern Mississippi Institute of Marine Sciences. Broodstock were maintained in glass aquaria submerged in a central water bath and held at 25°C with a 16 hr light:8 hr dark photoperiod. Fish were fed dry flake food three times daily and brine shrimp nauplii twice weekly. Mating groups were maintained at 27°C in individual mating chambers within a raceway receiving a continuous flow of well water. Females were examined daily, and egg clusters were collected and placed in embryo rearing medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2 \cdot 2H_2O$, and 0.016% $MgSO_4 \cdot 7H_2O$; Carolina Biological, Burlington, NC). Upon hatching, fry were immediately transferred to exposure aquaria.

Experimental design. Gonadal differentiation in medaka occurs within the first 2 weeks after hatch and is sensitive to exogenous steroid hormones, including estrogens (*60*). To determine whether exposure to an environmental estrogen during the period of sex differentiation is sufficient to alter gonadal development and reproductive capacity, we treated fry throughout the period of sex differentiation (2 weeks posthatch) and then transferred them to well water. At 2, 4, and 8 weeks posthatch, 12 fish were sacrificed from each treatment, 6 for analysis of VTG expression and 6 for examination of gonad histology. Because sampling required several hours, treatments were processed in random order, and time of sampling was recorded in order to avoid confounding time-of-day effects with treatment effects. At sexual maturity (16 weeks posthatch), fish were placed in mating pairs. Three categories of mating pairs were established for each dose of DDT: exposed female × solvent control male (n = 6 pairs per dose), exposed male × solvent control female (n = 6 pairs per dose), and exposed male × exposed female (n = 6 pairs per dose). At the end of a 7-day mating trial, adults were sacrificed for analysis of VTG expression and gonad histology. As above, treatments were processed in random order.

To more closely approximate the lifelong exposure that nonmigratory fish potentially experience in the natural environment, fry were treated from hatch through early puberty (8 weeks posthatch), then transferred to well water. As in the previous experiment, 12 fish were sacrificed at 2, 4, and 8 weeks posthatch for analysis of VTG expression and gonad histology. At sexual maturity (12 weeks posthatch), adults were placed in mating pairs as described above. Adults were sacrificed for analysis of VTG and gonad histology at the end of the mating trial.

Fish care and treatment were in accordance with all guidelines of the Southeastern Louisiana University and University of Southern Mississippi Animal Care and Use Committees.

Exposure conditions. In both treatment paradigms, fry were exposed to well water, solvent (triethylene glycol), or 0.5, 1, 2.5, or 7.5 mg/L (ppb) *o,p´*-DDT in a flow-through system. Water was particle and carbon filtered, temperature adjusted, and aerated before entering test aquaria. The flow rate was maintained at 100 L/aquarium/day in an intermittent flow-through chamber similar to that described by Walker et al. (*61*). Aquaria were housed in a heated recirculating water bath at 27 ± 1°C on a 16 hr light:8 hr dark photoperiod. The time weighted mean and SD of temperature, dissolved oxygen, and pH were 27.1 ± 0.5°C, 6.5 ± 1.7 mg/L, and 9.0 ± 0.2, respectively.

Treated and water control fry were exposed in duplicate 20-L aquaria (45 fry/aquarium), and solvent control fry were exposed in quadruplicate 20-L aquaria (45 fry/aquarium). Within each aquarium, fry were housed in a 1.5-L cylindrical mesh container to protect against physical damage and to allow more efficient foraging until 3 weeks of age, when they were released into the aquarium. Fry were fed microworms until day 3, microworms once and brine shrimp nauplii twice daily until day 6, and AquaTox Special dry flakes (Ziegler Bros, Gardner, PA) once and brine shrimp nauplii twice daily from day 7 to day 58. From day 57 to termination, fish were fed dry flakes three times and brine shimp nauplii once daily.

Chemical analysis. We purchased DDT from Lancaster Synthesis Inc. (Windham, NH) and measured DDT concentrations using gas chromatography with electron capture detection (GC/ECD). DDT was concentrated by solid phase extraction before analysis. Extraction efficiency ranged from

91 to 104%. Actual doses were 50–80% of the nominal dose (Table 1) and are shown in all figures and tables.

Vitellogenin analysis. Fish smaller than 90 mg were euthanized in ice-cold MS-222 (0.1 mg/mL) and homogenized in ice-cold phosphate-buffered saline (pH 7.3) supplemented with 20 µM leupeptin, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 5 mM dithiothreitol, and 5 µg/mL aprotinin. The homogenate was immediately mixed with aprotinin [0.021 trypsin-inhibiting units (TIU)/100 µL homogenate]. Fish larger than 90 mg were bled by cutting a gill arc and collecting the blood into a heparinized microcapillary tube. Aprotinin (1 µL of 4.2 TIU/mL) was immediately added to whole blood samples. Both homogenates and blood were centrifuged at 13,800 × *g* for 5 min at 4°C, and the supernatant was aspirated and stored at –80°C until analysis.

We diluted homogenate and plasma samples in Laemmli sample buffer (100-fold for plasma samples and to 0.5 µg total protein per well for homogenates) and separated proteins by SDS-PAGE at 120 V for 2 hr. Proteins were electroblotted (45 V for 2 hr) to a polyvinylidene difluoride membrane. We detected VTG using a monoclonal fish vitellogenin antibody obtained from Nancy Denslow (University of Florida, Gainesville, FL) (*62*). VTG was visualized using the Vectastain ABCAmP kit (Vector Laboratories, Burlingame, CA) and quantified by densitometry using a BioRad gel documentation system and Quantity One software (BioRad, Hercules, CA). The density of all samples was normalized to micrograms protein per well (homogenates) or microliter equivalents of plasma per well. We then calculated the relative VTG score as the ratio of normalized sample density to normalized standard density. The standard consisted of pooled plasma from vitellogenic female medaka. Both microliter equivalents and microgram protein were known for the standard, allowing comparison with plasma samples and homogenate samples.

Histologic examination. Fish were fixed in 10% buffered formalin, embedded in paraffin, sectioned longitudinally, and stained with hematoxylin and eosin for analysis of gonad structure. We categorized a gonad as an ovotestis if both sperm tubules and oocytes were present. Gonad maturity scores were assigned to adult male and female gonads. Scores were based on stages of oogenesis and spermatogenesis defined by Wallace and Selman (*42*) and Grier (*63*), respectively. Males were scored as follows: 1 if only spermatogonia were present; 2 if mostly spermatogonia were present, with some cysts of spermatids; 3 if sperm-filled cysts and tubules occupied < 50% of sectioned area; or 4 if sperm-filled cysts and tubules occupied > 50% of section area. Females were scored as follows: 1 if only primary oocytes were present; 2 if primary oocytes and early yolk vesicle stage oocytes were present; 3 if primary oocytes, early yolk vesicle oocytes, and approximately 50% late yolk vesicle oocytes were present; or 4 if mostly late yolk vesicle oocytes and vitellogenic oocytes were present.

Table 1. Nominal and measured doses of *o,p'*-DDT in exposure aquaria (mean ± SEM).

Nominal dose (µg/L)	Actual dose (µg/L) 2-Week exposure	8-Week exposure
0.5	0.23 ± 0.02	0.30 ± 0.02
1.0	0.50 ± 0.03	0.69 ± 0.03
2.5	1.37 ± 0.12	1.94 ± 0.09
7.5	4.32 ± 0.23	5.19 ± 0.18

Quantification of reproductive success. The two groups of fry used in the 2- and 8-week exposures differed in time to reach sexual maturity, taking 16 and 12 weeks, respectively. Maturity was judged by the presence of distinct secondary sex characteristics. In both experiments, sex ratio at maturity was determined by examination of secondary sex characteristics, and fish were divided into three types of mating groups: female exposed × control male (female), male exposed × control female (male), and female exposed × male exposed (both). This design allowed us to determine whether DDT had differential effects on female and male reproductive success and whether exposure of both partners caused more or less damage than exposure of only one partner.

We quantified reproductive success of each pair during a 7-day mating trial. We used three parameters to assess reproductive success: number of eggs per pair, percentage of eggs fertilized, and percentage of embryos that hatched and survived 72 hr. Number of eggs is a measure of fecundity and serves as an estimate of the effects of both male and female exposure on female reproductive success. Male exposure could potentially alter male pheromone production and responsiveness, thereby altering the pheromonal and behavioral cues received by females. Females could possibly respond by changing egg production. The percentage of fertilized eggs serves as a measure of male fertility (when the male is exposed) and female fertility (when the female is exposed). Finally, the percentage of embryos that hatch serves as an estimate of individual fitness (number of offspring produced). At the end of the reproductive trials, adults were sacrificed for analysis of VTG and examination of the gonads.

Statistics. We compared survival (percent that survived) of treated fish to survival in triethylene glycol and water control groups by χ^2 analysis. We analyzed size (milligrams wet weight) relative to dose and age and VTG score relative to dose and age by two-way analysis of variance (ANOVA). To examine time-of-day effects, we analyzed adult female VTG scores over the course of the sampling day by one-way ANOVA. To evaluate the effects of developmental DDT exposure on adult VTG synthesis, adult male and female VTG scores relative to dose were analyzed by separate one-way ANOVAs. Because of the small subsample taken at each time point (n = 6), statistical analysis of the ratio of ovaries, testes, undifferentiated gonads, and ovotestes could not be performed. However, this cross-sectional sampling allowed investigation of the process of gonad differentiation in 2- to 8-week-old fish. These results were supported by histologic and statistical analyses of adult sex ratio. Differences between the sex ratio of exposed adults and the expected 1:1 sex ratio were identified by χ^2 analysis. We analyzed changes in gonad maturity scores relative to time of day and dose by the Kruskal-Wallis test. Changes in reproductive parameters within a type of mating group (female only treated, male only treated, both treated) were analyzed by one-way ANOVA (main effect = DDT dose) followed by Dunnett's post hoc test to identify treatments that differed significantly from solvent controls. Correlations between percent hatched and VTG score were examined via simple regression. All values expressed as percentages were arcsine (square root) transformed before analysis. In all tests, α was set at 0.05.

Results

Survival and growth. Solvent (triethylene glycol) had no effect on survival, but DDT treatment significantly reduced survival to sexual maturity in both experiments (Figure 1). DDT doses 0.50 µg/L significantly reduced survival in the 2-week exposure, whereas doses of 0.30, 1.94, and 5.19 µg/L, but not 0.69 µg/L, reduced survival in the 8-week exposure. DDT treatment appeared to significantly alter growth in both the 2- and 8-week exposures (two-way ANOVA, 2-week F_{time} = 862.36, p < 0.0001; 8-week F_{time} = 471.66, p < 0.0001; 2-week F_{DDT} = 5.49, p < 0.0001; 8-week F_{DDT} = 12.18, p < 0.0001; 2-week $F_{time \times DDT}$ = 2.876, p = 0.0003; 8-week $F_{time \times DDT}$ = 5.112, p < 0.0001). In both exposures, fish were larger in DDT treatment groups where survivorship was lower, suggesting that density, not DDT, directly affects size. However, the highest dose of DDT in the 8-week exposure drastically decreased both survivorship and size, suggesting that long-term exposure to 5.19 µg/L DDT is extremely toxic.

Vitellogenin synthesis. DDT did not cause any significant change in VTG production in juvenile (2 to 8 weeks posthatch) or adult fish

(17 weeks posthatch) exposed for 2 weeks posthatch (Figure 2). VTG expression did increase over time, as expected with the onset of sexual maturity. When fry were continuously exposed to DDT for 8 weeks posthatch, VTG production was induced in a dose- and time-dependent manner (Figure 3). Once exposure ended at 8 weeks posthatch, VTG production was attenuated. Sexually mature fish (13 weeks posthatch) produced VTG, but not at the same levels as during DDT stimulation. Neither 2- nor 8-week developmental exposure to DDT induced significant VTG production in adult males, nor did it alter adult female VTG production (Figure 2B and Figure 3B). VTG synthesis in adult females did not change significantly over the 8-hr sampling period, indicating that vitellogenesis is a constant process in this daily spawning fish.

Sex differentiation. Developmental exposure to DDT caused a female-skewed sex ratio in adults (Figure 4). When exposure occurred during the first 2 weeks posthatch, only the highest dose (4.32 µg/L) caused feminization (Figure 4). When fry were exposed for 8 weeks posthatch, both the 1.94 and 5.2 µg/L doses caused a significantly female-skewed sex ratio (Figure 4). Cross-sectional sampling of exposed fry (n = 6 individuals per dose per sampling time) suggested that feminization was a progressive process. In fry exposed for 2 weeks posthatch (Figure 5), gonadal differentiation was not clearly distinguishable by light microscopy until 4 weeks posthatch. By 4 and 8 weeks, we observed a disproportionate number of ovaries in fry exposed to the highest doses of DDT (Figure 5). In some fry exposed for 8 weeks posthatch (Figure 6), ovotestes appeared after 2 and 4 weeks of exposure to the highest doses of DDT, whereas after 8 weeks only ovaries were observed (Figure 6). However, two males exposed to 1.94 µg/L DDT and sampled at sexual maturity (13 weeks) had ovotestes, suggesting that incomplete feminization occurred in these fish. These observations strongly suggest that *o,p´*-DDT-induced feminization is due to gonadal reorganization and not to differential mortality of DDT-exposed male fish.

Gonadal condition of adults. Although DDT caused a significantly female-skewed sex ratio at the highest doses, ovarian and testicular morphology of adults did not differ between DDT-treated fish and control fish. Neither testis nor ovary maturity scores varied significantly with DDT treatment in either the 2- or 8-week exposure (data not shown). Essentially, adult ovaries and testes appeared to be normal. However, two "males" exposed to 1.94 µg/L DDT for 8 weeks had ovotestes. In one of these individuals, a few primary oocytes were scattered throughout the testis, but no mature sperm were present and this fish did not fertilize any eggs. In the other animal, approximately 50% of the gonad contained both primary and early yolk vesicle oocytes, but a few cysts of mature sperm were present (Figure 7) and this individual was able to fertilize eggs.

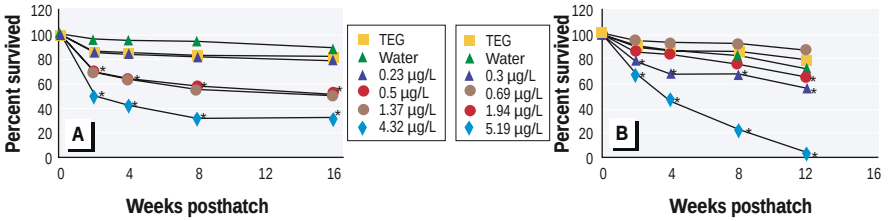

Figure 1. Survivorship in response to (*A*) 2- and (*B*) 8-week posthatch exposure to DDT (percent survived).In the 2-week exposure, most deaths occurred during the 2-week exposure period, whereas in the 8-week exposure, most deaths occurred during the first 4 weeks of exposure. The actual dose of 5.19 µg/L DDT proved to be extremely toxic, killing almost all fish after 8 weeks.

*Treatments for which survivorship differed significantly from the solvent control (triethylene glycol) and the no treatment control (water).

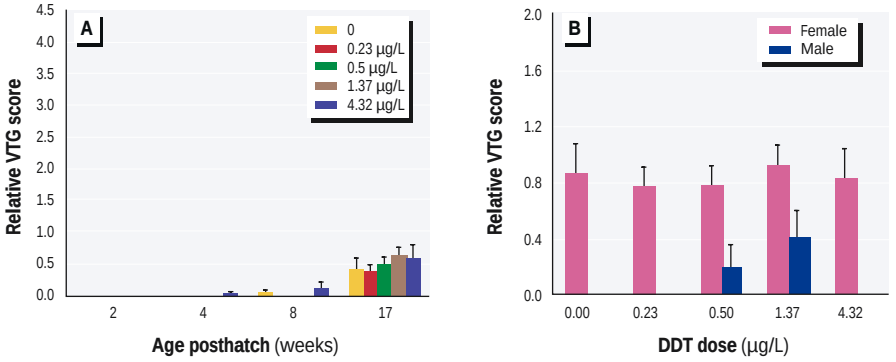

Figure 2. VTG expression in (*A*) developing and (*B*) sexually mature fish in response to 2-week developmental exposure to DDT (mean ± SEM). Relative VTG score is defined in the text. In (*A*), bars represent mean VTG score for each treatment, regardless of sex. Sex could be determined only in a few fish at 8 weeks posthatch and in mature fish (17 weeks). DDT does not alter VTG expression (two-way ANOVA, F_{DDT} = 0.134, p = 0.98), but VTG expression does increase with age (F_{age} = 19.82, p < 0.0001), as expected. No DDT × age interaction occurred ($F_{DDT \times age}$ = 0.13, p = 0.99). n = 6 individuals per bar for all doses at 2, 4, and 8 weeks; at 17 weeks, n = 12 (0 µg/L), n = 22 (0.23 µg/L), n = 23 (0.5 µg/L), n = 22 (1.37 µg/L), n = 7 (4.32 µg/L). In (*B*), VTG production of sexually mature males and females (17 weeks) is not altered by developmental exposure to DDT [one-way ANOVA; males, F = 0.56, p = 0.73, n = 6 (0 µg/L), n = 11 (0.23 µg/L), n = 11 (0.5 µg/L), n = 12 (1.37 µg/L), n = 2 (4.32 µg/L); females, F = 0.16, p = 0.97, n = 6 (0 µg/L), n = 11 (0.23 µg/L), n = 12 (0.5 µg/L), n = 10 (1.37 µg/L), n = 5 (4.32 µg/L)].

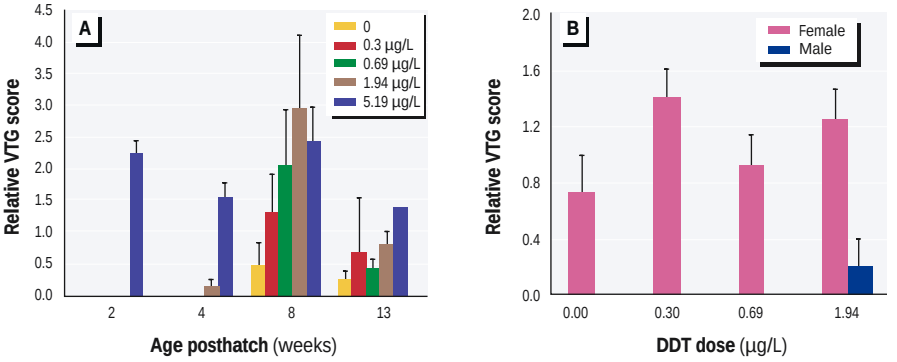

Figure 3. VTG expression in (*A*) developing and (*B*) sexually mature fish in response to 8-week developmental exposure to DDT (mean ± SEM). In (*A*), bars represent mean VTG score for each treatment, regardless of sex. DDT altered VTG expression in a dose- and age-dependent manner (F_{DDT} = 11.76, p < 0.0001; F_{age} = 12.65, p < 0.001; $F_{DDT \times age}$ = 2.07, p = 0.01), with higher doses inducing VTG after short-term exposure and lower doses inducing VTG expression after longer exposure. VTG expression attenuates once the DDT stimulus is removed at 8 weeks posthatch. For all doses at 2, 4, and 8 weeks, n = 6 individuals per bar; at 13 weeks, n = 16 (0 µg/L), n = 22 (0.3 µg/L), n = 22 (0.69 µg/L), n = 15 (1.94 µg/L), n = 1 (5.19 µg/L). In (*B*), VTG production of sexually mature males and females (13 weeks) is not altered by developmental exposure to DDT (one-way ANOVA; males, F = 1.71, p = 0.17, n = 9 (0 µg/L), n = 11 (0.3 µg/L), n = 11 (0.69 µg/L), n = 6 (1.94 µg/L); females, F = 0.85, p = 0.52, n = 7 (0 µg/L), n = 11 (0.3 µg/L), n = 11 (0.69 µg/L), n = 9 (1.94 µg/L)).

Reproductive success. Developmental exposure to DDT had a relatively small impact on female fecundity (egg number). After a 2-week developmental exposure, the highest doses of DDT (1.37 and 4.32 µg/L) reduced fecundity of exposed females, but not of control females mated with exposed males (Table 2). The highest dose also reduced fecundity of pairs with both partners exposed, indicating that the effect on female fecundity is probably due to a direct action of DDT on ovaries. After an 8-week developmental exposure, so few individuals (n = 2 females) survived the highest DDT dose (5.19 µg/L) that no mating pairs could be tested. Regardless of whether the female, male, or both partners were exposed, doses 1.94 µg/L did not reduce female fecundity (Table 2). Instead, 0.69 µg/L DDT significantly enhanced egg number in exposed females mated with control males.

Female and male fertility (percent fertilized) were significantly reduced by developmental exposure to DDT. After 2- or 8-week developmental exposure, female fertility was significantly reduced by doses of DDT 0.23 µg/L (Figures 8 and 9). Male fertility was significantly decreased by doses 0.5 µg/L after a 2-week developmental exposure and by doses 0.3 µg/L after an 8-week developmental exposure (Figures 8 and 9). Interestingly, when both partners were exposed, fertility of fish exposed for 2 weeks was only reduced at the highest dose (4.32 µg/L), whereas fertility of fish exposed for 8 weeks was reduced at doses 0.69 µg/L (Figures 8 and 9).

Female and male fitness (percent hatched) were drastically reduced by developmental exposure to DDT (Figures 8 and 9). After a 2-week developmental exposure, doses from 0.23 to 1.37 µg/L significantly reduced hatching of embryos from exposed females. Oddly, the highest dose, 4.32 µg/L, had no effect on hatching. Hatching was also reduced for males exposed to doses 0.5 µg/L and mated with control females (Figure 8). When both parents were exposed, hatching was significantly reduced at all doses except 0.5 µg/L. After an 8-week developmental exposure of females or males or both to any dose of DDT, embryo hatching was catastrophically reduced (Figure 9). In most cases, < 20% of embryos hatched. The pronounced reduction in hatching success and the significant reduction in fertility suggest that DDT has genotoxic effects on germ cells.

Because hatching success was most sensitive to DDT exposure, we examined correlations between hatching success and adult VTG expression. Hatching success was not significantly correlated with VTG expression in adult fish in either the 2- or 8-week exposed fish (data not shown).

Discussion

Exposure of developing medaka to the environmental estrogen *o,p´*-DDT caused both time- and dose-dependent effects. Short-term developmental exposure (2 weeks) caused feminization at the highest dose and reduced fertility and hatching success at much lower doses, but it did not alter VTG synthesis in juveniles or in adults (Table 3). Long-term developmental exposure (8 weeks) feminized males at doses 1.94 µg/L and drastically reduced fertility and hatching success at doses 0.3 µg/L (Table 3). Long-term exposure also induced VTG synthesis in juveniles and adults at the lowest dose, but VTG production was attenuated once the estrogenic stimulus was removed so that the adult pattern of VTG synthesis in males and females was unchanged. Both experiments indicate that DDT had permanent organizational

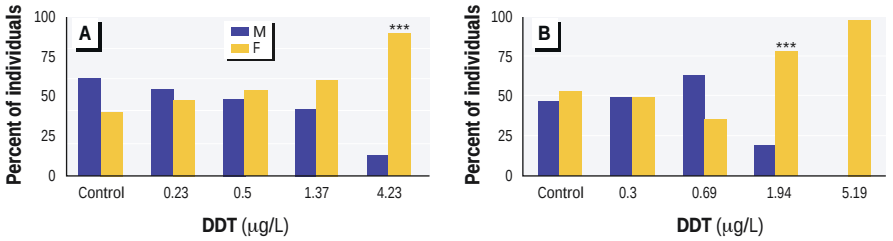

Figure 4. Adult sex ratio of medaka exposed to DDT for (*A*) 2 weeks or (*B*) 8 weeks during development. DDT feminized fish in a dose- and time-dependent manner: feminization occurred at a lower dose when exposure was longer. No statistical test could be performed for 8-week exposure to 5.2 µg/L DDT due to the small sample size. The total numbers per dose were as follows: for the 2-week group, n = 104 (0 µg/L), n = 50 (0.23 µg/L), n = 32 (0.5 µg/L), n = 32 (1.37 µg/L), n = 19 (4.23 µg/L); 8-week, n = 134 (0 µg/L), n = 36 (0.3 µg/L), n = 56 (0.69 µg/L), n = 41 (1.94 µg/L), n = 2 (5.2 µg/L).
***Ratios significantly different from 1:1 (χ^2 test, p 0.0010).

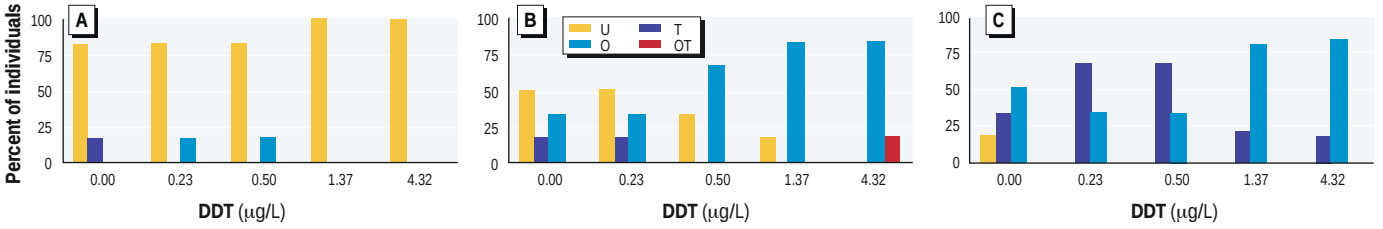

Figure 5. Sex differentiation in medaka exposed to DDT for 2 weeks posthatch and sampled at (*A*) 2, (*B*) 4, and (*C*) 8 weeks of age. Abbreviations: O, ovary; OT, ovotestis; T, testis; U, undifferentiated. DDT exposure increases the proportion of fry with ovaries at 4 and 8 weeks of age. Six fish per treatment were sampled at each time point; therefore, no statistical analysis of sex ratio was possible.

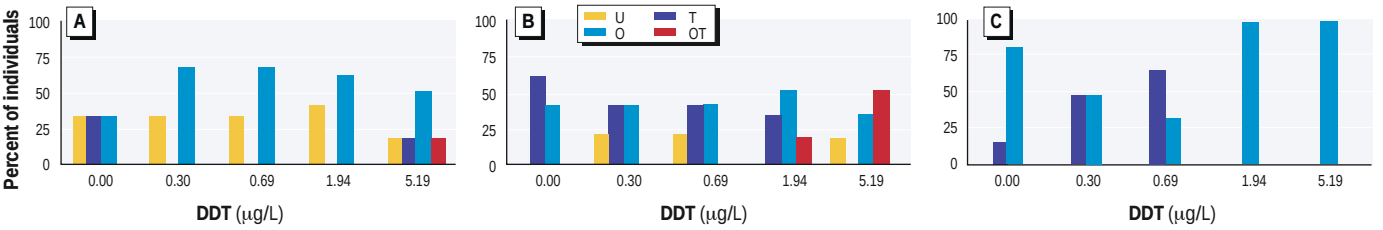

Figure 6. Sex differentiation in medaka exposed to DDT for 8 weeks posthatch and sampled at (*A*) 2, (*B*) 4, and (*C*) 8 weeks of age. Abbreviations: O, ovary; OT, ovotestis; T, testis; U, undifferentiated. DDT exposure dramatically increased the proportion of fry with ovaries at 8 weeks of age. The female-skewed sex ratio in control fish sampled at 8 weeks is probably a stochastic occurrence due to the small sample size. Control adults did not show a skewed sex ratio (Figure 4). As in Figure 4, six fish per treatment were sampled at each time point, so no statistical analysis of sex ratio was possible.

effects on sexual development and reproductive function, but transitory activational effects on VTG production. Organizational responses were more sensitive to disruption than were activational responses; relative sensitivities were: hatching success > fertility >> gonad differentiation >> VTG production.

Our data show that VTG, as detected by Western blotting with a highly specific antibody, is a moderately sensitive indicator of current exposure to an environmental estrogen. Although ELISA quantification of VTG concentration is frequently considered the most sensitive method, Western blotting was chosen as the most appropriate and consistent means to provide accurate, specific detection in both types of sample matrices—plasma and whole body homogenates. In our detection system, VTG-immunoreactive bands migrated identically in plasma, homogenates, and the female standard plasma. This detection system proved to be quite sensitive, requiring 100-fold dilution of plasma samples and dilution of whole body homogenates to 0.5 µg total protein per well.

The sensitivity of the vitellogenic response to environmental estrogens may be species and compound specific. For instance, DDT stimulates VTG synthesis *in vivo* in mosquitofish [*Gambusia affinis* (*64*)], medaka (*11*), and rainbow trout (*65*), but not in catfish [*Ictalurus punctatus* (*66*)]. *In vitro*, DDT stimulates VTG synthesis in rainbow trout hepatocytes (*40*), but fails to bind the seatrout (*Cynoscion nebulosus*) estrogen receptor (*67*). Crews et al. (*68*) suggested that such species-typical sensitivities to endocrine-disrupting compounds may depend on differences in circulating levels of sex steroid hormones and on receptor affinities.

Our data clearly show that developmental exposure to an environmental estrogen can have profound negative impacts on individual fitness. Most obviously, sexual development can be redirected from a male to female pathway, changing the population sex ratio and thus the number of available partners. More insidiously, testes may be partially feminized, reducing spermatogenic capacity. Most insidiously of all, fertility of eggs and sperm and hatching success of embryos can be severely impaired, even in individuals with histologically normal ovaries and testes.

Other studies that exposed medaka to DDT during development have also shown pronounced effects on gonadal development and sex ratio. Injection of a 227-ppm dose into eggs caused 86% sex reversal of genetic males (*10*), and immersion doses as low as 2.3 ppb (micrograms per liter) caused a significantly female-skewed sex ratio in medaka exposed for 3 months posthatch (*11*). In the present study, immersion doses of 2–4 ppb caused a female-skewed sex ratio, with efficacy depending on exposure duration. In contrast, sex differentiation in juvenile rainbow trout was completely unaltered by injection exposure to the DDT metabolites *o,p´*-DDE or *p,p´*-DDE (*49*). As mentioned above, sensitivity to particular environmental estrogens may depend on the hormone and receptor physiology of each species.

In medaka, other environmental estrogens appear to have less potent effects on sex differentiation than does DDT. Nonylphenol induced ovotestes in males exposed to approximately 25 ppb and induced sex reversal in fish exposed to 50 ppb for 3 months posthatch (*69*). In a separate study, lower, more environmentally relevant doses of nonylphenol (0.54–2 ppb) had no feminizing effect, but did appear to cause a male-skewed sex ratio at 0.77 ppb (*70*). β-Hexachlorocyclohexane (β-HCH; an isomer of the pesticide lindane) was less potent still, requiring a 3-month exposure to doses > 0.18 ppm to feminize medaka (*71*). Finally, methoxychlor at low doses (0.18–2.31 ppb) was completely ineffective, failing to alter sex ratio after developmental exposure (*70*).

Alterations in sex differentiation have not been as thoroughly examined in other fish species. In a laboratory study, Gimeno et al. (*7*) found that genetically male carp were feminized by exposure to relatively high doses of 4-*tert*-pentylphenol (0.1–1ppm) for 90 days posthatch. In field collections, Jobling et al. (*33*) observed a high proportion of intersex roach (*Rutilus rutilus*, a cyprinid fish) downstream from sewage treatment facilities. The authors defined intersex fish as having both male and female gonadal characteristics and assumed that the fish were feminized males because the percentage of males sampled at a site decreased as the percentage of intersex fish increased. Although alkylphenols and synthetic steroidal estrogens are possible feminizing agents in sewage effluent, the specific estrogenic component(s) of these effluents is currently unknown. Currently, analysis of the reproductive capacity of these feminized wild fish has not been reported.

In the present study, fertility and hatching success were quite sensitive to estrogenic disruption in both sexes, with significant impairment occurring at doses as low as 0.23 ppb DDT. DDT is probably transferred from the parent to the gametes as are other lipophilic contaminants in fishes (*72–75*). Parentally transferred DDT may directly or indirectly inhibit the ability of sperm to fertilize eggs and the capacity of eggs to be fertilized. Female fertility was more sensitive to developmental DDT exposure than was male fertility. The reduction in female fertility was not due to decreased egg production

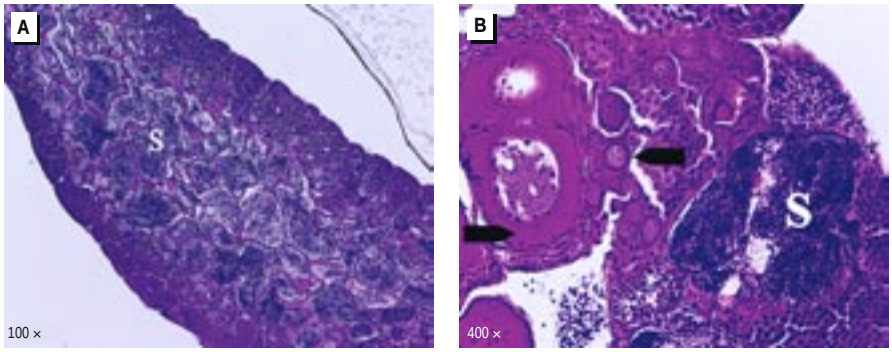

Figure 7. (*A*) Testis in an adult male medaka (13 weeks posthatch) exposed to triethylene glycol for 8 weeks posthatch. (*B*) Ovotestis in an adult male medaka (13 weeks posthatch) exposed to 1.94 µg/L *o,p´*-DDT for 8 weeks posthatch. S, sperm. Arrowheads indicate primary oocytes and oocytes in the yolk vesicle stage (*42*).

Table 2. Effect of DDT exposure of females and males on female fecundity (number of eggs).

Exposure duration	DDT (µg/L)	No. of eggs: Exposed female	Exposed male	Both exposed
2 Weeks	0	59.0 ± 6.3	59.0 ± 6.3	59.0 ± 6.3
	0.23	52.8 ± 5.5	81.7 ± 11.5	34.2 ± 5.6
	0.50	41.3 ± 5.2	44.8 ± 8.7	59.5 ± 10.9
	1.37	34.0 ± 9.3*	46.7 ± 5.6	45.7 ± 10.3
	4.32	35.8 ± 4.7*	—[a]	9.0 ± 6.0*
8 Weeks	0	33.3 ± 4.6	33.3 ± 4.6	33.3 ± 4.6
	0.30	52.7 ± 5.4	32.3 ± 4.7	45.3 ± 6.2
	0.69	58.0 ± 7.8*	32.0 ± 4.0	34.3 ± 6.7
	1.94	47.6 ± 8.1	16.5 ± 8.5	21.5 ± 2.8

Exposed females were mated with control males, and exposed males were mated with control females.
[a]There were no pairs due to small number of individuals. *Significantly different from the control (ANOVA followed by Dunnet's post hoc test, *p* 0.05).

because fecundity was only slightly reduced by DDT exposure (Table 2).

Because both male and female fertility were compromised, one would predict that exposure of both parents would catastrophically reduce fertilization. Interestingly, exposure of both parents appeared to rescue the fertility reduction (Figures 8 and 9). For instance, 2-week exposure to 1.37 µg/L DDT reduced fertilization when either the female or the male alone was exposed, but it did not reduce fertility when both parents were exposed (Figure 8). Although intriguing, the biological basis of these results is unclear. Several possibilities exist, including changes in fertilization systems at the molecular level and alterations in courtship initiation and consummation. Alternatively, the rescue effect observed may be a statistical artifact due to the relatively small sample size (n = 6 pairs per mating group). However, the consistency of the effect across both experiments suggests a biological basis.

Other environmental estrogens reduce fertility and hatching success in medaka, but with apparently lower potency. Developmental exposure to di(n-butyl) phthalate (776 mg/kg/day) reduced fecundity at doses approximately 1,000-fold greater than the effective dose of DDT (*76*), whereas doses of nonylphenol and methoxychlor in the same range as DDT (0.2–2 ppb) had no effect on fecundity or hatching success (*70*). Exposure of adult male medaka to DDT also reduces reproductive success, but much higher doses are required in adults than those that were effective during development. Octylphenol and bisphenol A significantly reduced fertility and hatching success at doses of approximately 20 ppb and 2.28 ppm, respectively (*51,53*). Neither nonylphenol nor di(2-ethylhexyl)phthalate affected male fertility or hatching success (*51,70*).

Very little work has examined the effects of environmental estrogens or other endocrine-disrupting compounds on reproductive success in other fish species. In fathead minnows, exposure of paired adult males and females to estradiol reduced egg production (*52*), and exposure of sexually mature males reduced the size of the fat pad and the breeding tubercles, a prominent male secondary sex characteristic (*77*). Adult exposure to nonylphenol (1–10 ppb) also reduced fat pad size in males and egg production in females, whereas exposure to butylbenzyl phthalate (100 ppb) had no effect (*78*). Developmental exposure to the presumed antiandrogen vinclozolin and its metabolites had no effect on sex ratio or fecundity in fathead minnows (*50*).

The differential potency of estrogenic compounds is probably due to differences in bioaccumulation. Medaka readily accumulate DDT: eggs collected from females exposed to 2.5 ppb DDT for 3 or 6 weeks contained an average of 3,600 ppm DDT (*11*). Although bioaccumulation studies of alkylphenols have not been performed in medaka, nonylphenol is rapidly metabolized and excreted via the bile in rainbow trout (*79*), suggesting that bioaccumulation is limited. Little is known about the kinetics of bisphenol A and phthalate metabolism in fish, but these compounds may be metabolized more rapidly or accumulated more slowly than DDT.

Conclusion

The purpose of this study was to examine the effects of an environmental estrogen on

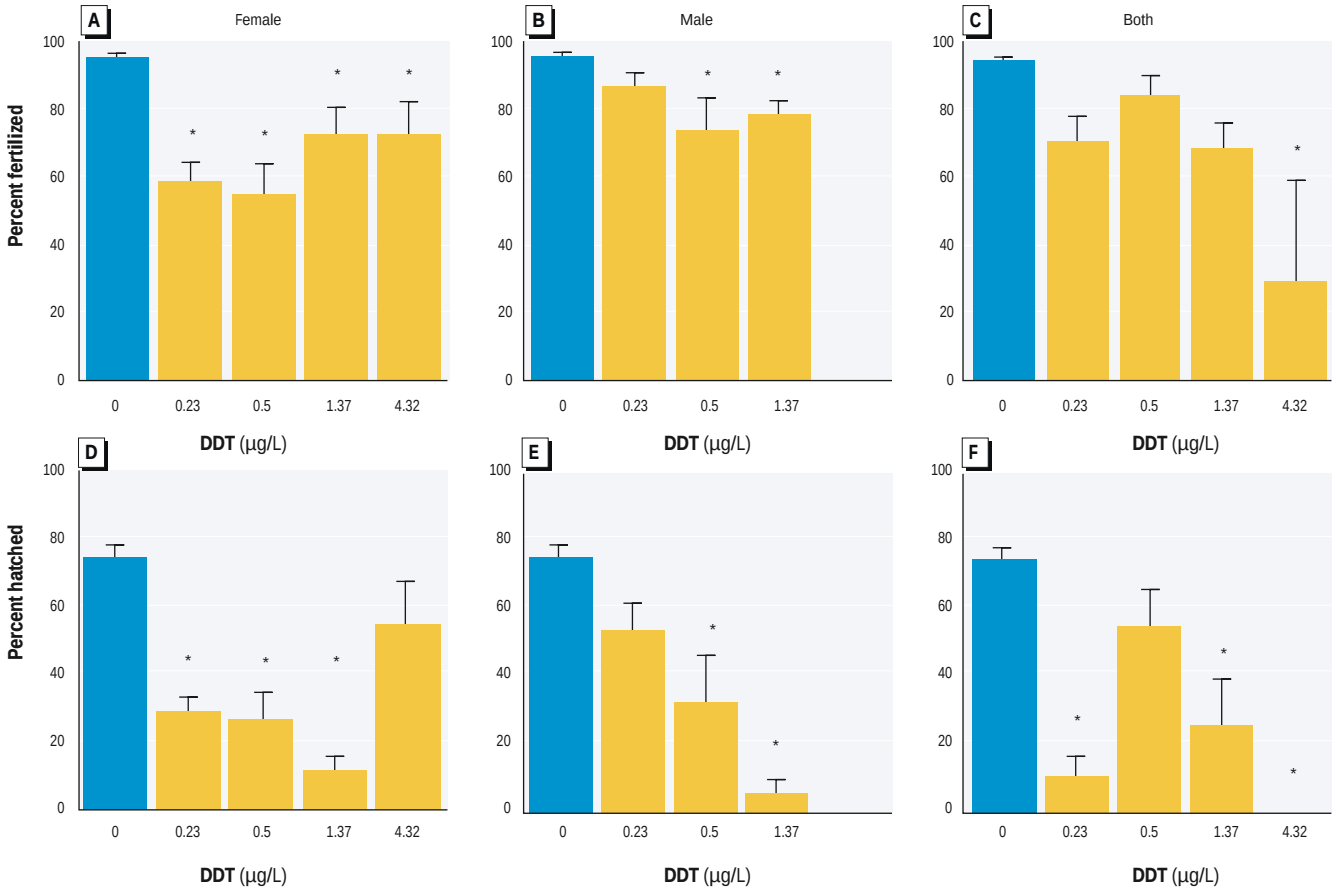

Figure 8. Reproductive success of medaka exposed to DDT for 2 weeks during development. Bars represent mean ± SEM for six pairs of fish for which one or both partners were exposed. DDT significantly reduced male and female fertility; hatching sucess was significantly reduced by developmental exposure of male, female, or both parents.

*Significantly different from the control (ANOVA followed by Dunnett's post hoc comparisons, $p < 0.05$).

individual fitness and to evaluate the effectiveness of VTG as a predictor of impaired reproduction in fish. Because a short-term developmental exposure to DDT feminized medaka and impaired reproductive function in the absence of abnormal VTG expression at any age, we suggest that VTG measurement cannot serve as the the definitive test for predicting reproductive impairment. VTG synthesis is an activational response to estrogen or environmental estrogen stimulation and disappears once the stimulus is removed. Therefore, absence of VTG expression can be a false negative if fish have not been recently exposed. However, because VTG expression is less sensitive to environmental estrogen action than gonadal development and reproductive function, its presence in adult males can reasonably be interpreted as a signal that embryos produced in a similar environment may suffer organizational effects such as gonadal feminization and reduced fertility as adults.

References and Notes

1. Arnold AP, Breedlove SM. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm Behav 19:469–498 (1985).
2. Moore MC. Application of organization-activation theory to alternative male reproductive strategies: a review. Horm Behav 25:154–179 (1991).
3. Crain D, Spiteri I, Guillette L. The functional and structural observations of the neonatal reproductive system of alligators exposed in ovo to atrazine, 2,4-D, or estradiol. Toxicol Ind Health 15:180–185 (1999).
4. Howell WM, Black DA, Bortone SA. Abnormal expression of secondary sex characters in a population of mosquitofish, *Gambusia affinis holbrooki*: evidence for environmentally-induced masculinization. Copeia 4:676–681 (1980).
5. 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).
6. Fry DM. Reproductive effects in birds exposed to pesticides and industrial chemicals. Environ Health Perspect 103(suppl 7):165–171 (1995).
7. Gimeno S, Gerritsen A, Bowmer T, Komen H. Feminization of male carp. Nature 384:221–222 (1996).
8. Gray MA, Metcalfe CD. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to *p*-nonylphenol. Environ Toxicol Chem 16:1082–1086 (1997).
9. Gray MA, Niimi AJ, Metcalfe CD. Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. Environ Toxicol Chem 18:1835–1842 (1999).
10. Edmunds JSG, McCarthy RA, Ramsdell JS. Permanent and functional male-to-female sex reversal in d-rR strain medaka (*Oryzias latipes*) following egg microinjection of *o,p*'-DDT. Environ Health Perspect 108:219–224 (2000).
11. Metcalfe TL, Metcalfe CD, Kiparissis Y, Niimi AJ, Foran CM, Benson WH. Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to *o,p*'-DDT in water or through maternal transfer. Environ Toxicol Chem 19:1893–1900 (2000).
12. Munkittrick KR, Van Der Kraak GJ. Receiving water environmental effects associated with discharges from Ontario pulp mills. Pulp Pap Canada 95:57–59 (1994).
13. Bergeron J, Willingham E, Osborn C III, Rhen T, Crews D. Developmental synergism of steroidal estrogens in sex determination. Environ Health Perspect 107:93–97 (1999).
14. Willingham E, Crews D. Sex reversal effects of environmentally relevant xenobiotic concentrations on the red-eared

Table 3. Sensitivity of various physiologic parameters to estrogenic disruption by *o,p*'-DDT. Doses (µg/L) represent the lowest dose that caused significant alteration at any age.

	2-Week		8-Week	
Parameter	Male	Female	Male	Female
VTG	—[a]	—[a]	0.30	0.30
Gonadal feminization	4.32	—[b]	1.94	—[b]
No. of eggs	—[a]	1.37	—[a]	—[a]
Percent fertilized	0.50	0.23	0.30	0.30
Percent hatched	0.23	0.23	0.30	0.30

Gonadal feminization refers to both ovotestis formation and complete feminization resulting in a female-skewed sex ratio. [a]No dose had an effect. [b]No change in ovarian structure.

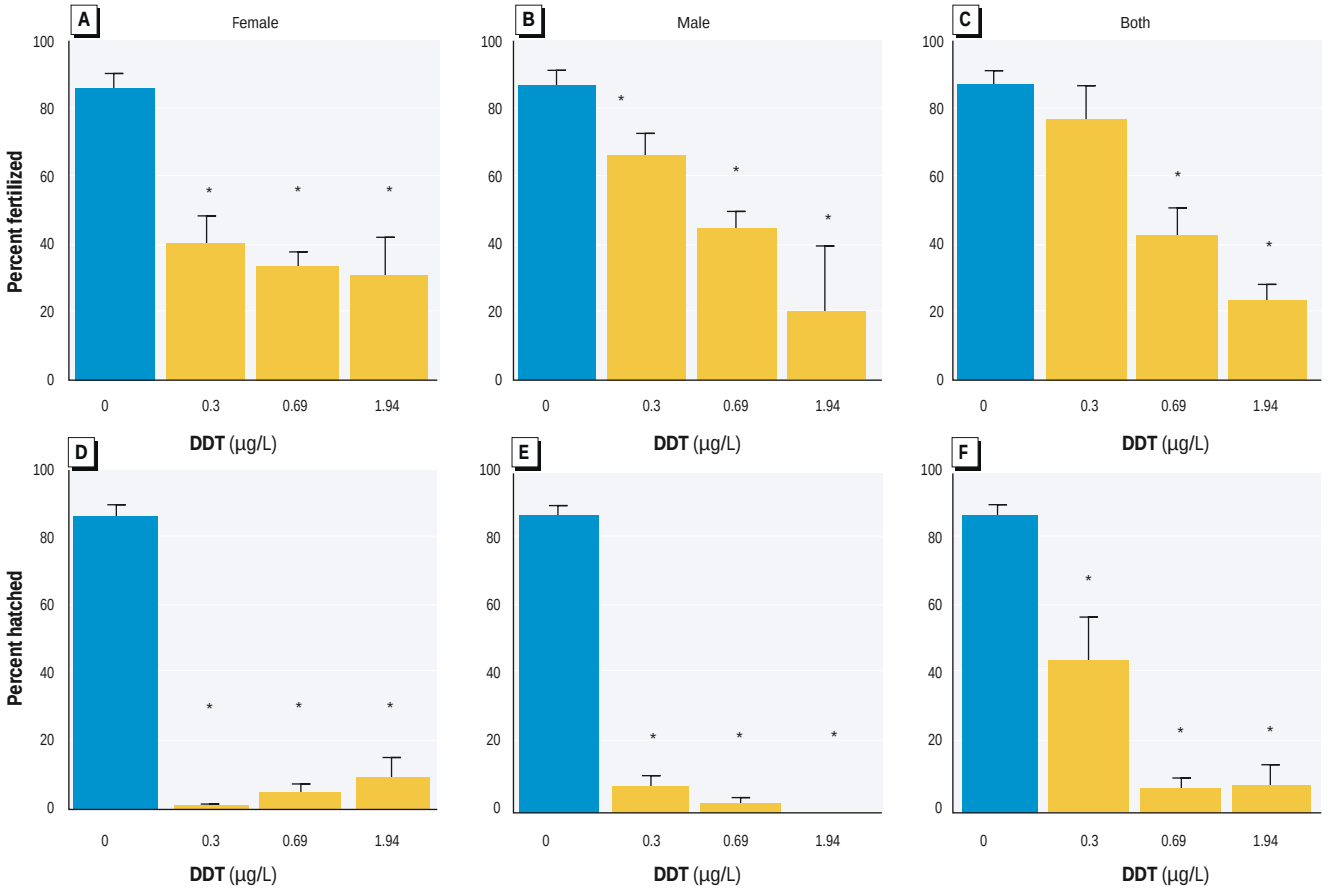

Figure 9. Reproductive success of medaka exposed to DDT for 8 weeks during development. Bars represent mean ± SEM for six pairs of fish for which one or both partners were exposed. DDT significantly reduced male and female fertility and catastrophically reduced hatching success, regardless of which parent was exposed. *Significantly different from the control (ANOVA followed by Dunnett's post hoc comparisons, $p < 0.05$).

slider turtle, a species with temperature dependent sex determination. Gen Comp Endocrinol 113:429–435 (1999).
15. Bergeron JM, Crews D, McLachlan JA. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Envron Health Perspect 102:780–781 (1994).
16. Matter JM, Crain DA, Sills-McMurry C, Pickford DB, Rainwater TR, Reynolds KD, Rooney AA, Dickerson RL, Guillette LJ Jr. Effects of endocrine disrupting contaminants in reptiles: alligators. In: Principles and Processes for Evaluating Endocrine Disruption in Wildlife (Kendall R, Dickerson R, Giesy J, Suk W, eds). Pensacola, FL:SETAC Press, 1998;267–289.
17. Clark EJ, Norris DO, Jones RE. Interactions of gonadal steroids and pesticides (DDT, DDE) on gonaduct growth in larval tiger salamanders, *Ambystoma tigrinum*. Gen Comp Endocrinol 109:94–105 (1998).
18. Crain DA, Guillette LJ, Pickford DB, Percival HF, Woodward AR. Sex-steroid and thyroid hormone concentrations in juvenile alligators (*Alligator mississippiensis*) from contaminated and reference lakes in Florida, USA. Environ Toxicol Chem 17:446–452 (1998).
19. Folmar LC, Denslow ND, Rao V, Chow M, Crain DA, Enblom J, Marcino J, Guillette LJ Jr. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. Environ Health Perspect 104:1096–1101(1996).
20. Guillette LJ, Woodward AR, Crain DA, Pickford DB, Rooney AA, Percival HF. Plasma steroid concentrations and male phallus size in juvenile alligators from seven Florida lakes. Gen Comp Endocrinol 116:356–372 (1999).
21. Guillette LJ Jr, Brock JW, Rooney AA, Woodward AR. Serum concentrations of various environmental contaminants and their relationship to sex steroid concentrations and phallus size in juvenile American alligators. Arch Environ Contam Toxicol 36:447–455 (1999).
22. Willingham E, Rhen T, Sakata J, Crews D. Embryonic treatment with xenobiotics disrupts steroid hormone profiles in hatchling red-eared slider turtles (*Trachemys scripta elegans*). Environ Health Perspect 108:329–332 (2000).
23. Spies R, Thomas P. Reproductive and endocrine status of female kelp bass from a contaminated site in the Southern California Bight and estrogen receptor binding of DDTs. In: Chemically Induced Alterations in Functional Development and Reproduction of Fishes (Rolland RR, Gilbertson M, Peterson RE, eds). Pensacola, FL:SETAC Press, 1997;113–134.
24. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR, Rokosh DA. Impact of bleached kraft mill effluent on population characteristics, liver MFO activity, and serum steroid levels of a Lake Superior white sucker (*Catostomus commersoni*) population. Can J Fish Aquat Sci 48:1371–1380 (1991).
25. Van Der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. Exposure to bleached kraft mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol Appl Pharmacol 115:224–233 (1992).
26. MacLatchy DL, Van Der Kraak GJ. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. Toxicol Appl Pharmacol 134:305–312 (1995).
27. Munkittrick DR, McMaster ME, Portt CB, Van Der Kraak GJ, Smith IR, Dixon DG. Changes in maturity, plasma sex steroid levels, hepatic mixed function oxygenase activity, and the presence of external lesions in lake whitefish (*Coregonus clupeaformis*) exposed to bleached kraft mill effluent. Can J Fish Aquat Sci 49:1560–1569 (1992).
28. McMaster ME, Van Der Kraak GJ, Munkittrick KR. An epidemiological evaluation of the biochemical basis for steroid hormone depressions in fish exposed to industrial wastes. J Gt Lakes Res 22:153–171 (1996).
29. Munkittrick KR, Van Der Kraak GJ, McMaster ME, Portt CB. Response of hepatic MFO activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. Environ Toxicol Chem 11:1427–1439 (1992).
30. de Solla SR, Bishop CA, Van Der Kraak G, Brooks RJ. Impact of organochlorine contamination on levels of sex hormones and external morphology of common snapping turtles (*Chelydra serpentina serpentina*). Environ Health Perspect 106:253–260 (1998).
31. Van der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, Chang JP. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. Toxicol Appl Pharmacol 115:224–233 (1992).
32. Harries JE, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. Environ Toxicol Chem 16:534–542 (1997).
33. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. Widespread sexual disruption in wild fish. Environ Sci Technol 32:2498–2506 (1998).
34. Allen Y, Scott A, Matthiessen P, Haworth S, Thain J, Feist S. Survery of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. Environ Toxicol Chem 18:1791–1800 (1999).
35. Allen Y, Matthiessen P, Scott A, Haworth S, Feist S, Thain J. The extent of oestrogenic contamination in the UK estuarine and marine environments–further surveys of flounder. Sci Total Environ 233:5–20 (1999).
36. Hashimoto S, Bessho H, Hara A, Nakamura M, Iguchi T, Fugita K. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*). Mar Environ Res 49:37–53 (2000).
37. Oberdorster E, Cheek AO. Gender benders at the beach: endocrine disruption in marine and estuarine organisms. Environ Toxicol Chem 20:23–36 (2001).
38. Cheek AO, Vonier PM, Oberdörster E, Burow BC, McLachlan JA. Environmental signaling: a biological context for endocrine disruption. Environ Health Perspect 106(suppl 1):5–10 (1998).
39. Kime DE. Endocrine Disruption in Fish. Norwell, MA:Kluwer Academic Publishers, 1998.
40. Sumpter JP, Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103(suppl 7):173–178 (1995).
41. Specker J, Sullivan C. Vitellogenesis in fishes: status and perspectives. In: Perspectives in Comparative Endocrinology (Davey K, Peter R, Tobe S, eds). Ottawa, Ontario, Canada:National Research Council of Canada, 1994;304–315.
42. Wallace RA, Selman K. Cellular and dynamic aspects of oocyte growth in teleosts. Am Zool 21:325–343 (1981).
43. Sumpter JP. Xenoendocrine disrupters—environmental impacts. Toxicol Lett 102–103:337–342 (1998).
44. Pelissero C, Flouriot G, Foucher JL, Bennetau B, Dunogues J, LeGac F, Sumpter JP. Vitellogenin synthesis in cultured hepatocytes; an in vitro test for the estrogenic potency of chemicals. J Steroid Biochem Mol Biol 44:263–272 (1993).
45. White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182 (1994).
46. Flouriot G, Pakdel F, Ducouret B, Valotaire Y. Influence of xenobiotics on rainbow trout liver estrogen receptor and vitellogenin gene expression. J Mol Endocrinol 15:143–151 (1995).
47. Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. Environ Toxicol Chem 15:194–202 (1996).
48. Ashfield L, Pottinger T, Sumpter J. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. Environ Toxicol Chem 17:679–686 (1998).
49. Carlson DB, Curtis LR, Williams DE. Salmonid sexual development is not consistently altered by embryonic expsoure to endocrine-active chemicals. Environ Health Perspect 108:249–255 (2000).
50. Makynen E, Kahl M, Jensen K, Tietge J, Wells K, Van Der Kraak G, Ankley G. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimephales promelas*). Aquat Toxicol 48:461–475 (2000).
51. Shioda T, Wakabayashi M. Effect of certain chemical on the reproduction of medaka (*Oryzias latipes*). Chemosphere 40:239–243 (2000).
52. Kramer V, Miles-Richardson S, Pierens S, Giesy J. Reproductive impairment and induction of alkaline-labile phosphate, a biomarker of estrogen exposure, in fathead minnows (*Pimephales promelas*) exposed to waterborne 17β-estradiol. Aquat Toxicol 40:335–360 (1998).
53. Gronen S, Denslow N, Manning S, Barnes S, Barnes D, Brouwer M. Serum vitellogenin levels and reproductive impairment of male Japanese medaka (*Oryzias latipes*) exposed to 4-*tert*-octylphenol. Environ Health Perspect 107:385–390 (1999).
54. Yamamoto T, Matsuda N. Effects of estradiol, stilbestrol, and some alkyl-carbonyl androstanes upon sex differentiation in the medaka, *Oryzias latipes*. Gen Comp Endocrinol 3:101–110 (1963).
55. Simonich SL, Hites RA. Global distribution of persistent organochlorine compounds. Science 269:1851–1854 (1995).
56. Palmer BD, Huth LK, Pieto DL, Selcer KW. Vitellogenin as a biomarker of xenobiotic estrogens in an amphibian model system. Environ Toxicol Chem 17:30–36 (1998).
57. Palmer BD, Palmer SK. Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog. Environ Health Perspect 103(suppl 4):19–25 (1995).
58. Fry DM, Toone CK. DDT-induced feminization of gull embryos. Science 213:922–924 (1981).
59. Soto AM, Chung KL, Sonnenschein C. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ Health Perspect 102:380–383 (1994).
60. Yamamoto T. Sex differentiation. In: Fish Physiology, Vol 3 (Hoar WS, Randall DJ, eds). New York:Academic Press, 1969;117–175.
61. Walker W, Overstreet R, Manning C, Hawkins W. Small aquarium fishes as models for environmental carcinogenesis: exposure methodology. J Appl Toxicol 5:255–260 (1985).
62. Denslow N, Chow M, Folmar L, Bonomelli S, Heppell S, Sullivan C. Development of antibodies to teleost vitellogenins: potential biomarkers for environmental estrogens. In: Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment, Vol 5 (Bengston DA, Henshel DS, eds). Philadelphia:American Society for Testing and Materials, 1996.
63. Grier HJ. Cellular organization of the testis and spermatogenesis in fishes. Am Zool 21:345–357 (1981).
64. Denison MS, Chambers JE, Yarbrough JD. Persistent vitellogenin-like protein and binding of DDT in the serum of insecticide-resistant mosquitofish (*Gambusia affinis*). Comp Biochem Physiol 69C:113–116 (1981).
65. Donohoe R, Curtis L. Estrogenic activity of chlordecone, *o,p*'-DDT, and *o,p*'-DDE in juvenile rainbow trout: induction of vitellogenesis and interaction with hepatic estrogen binding sites. Aquat Toxicol 36:31–52 (1996).
66. Nimrod AC, Benson WH. Estrogenic responses to xenobiotics in channel catfish (*Ictalurus punctatus*). Mar Environ Res 42:155–160 (1996).
67. Thomas P, Smith J. Binding of xenobiotics to the estrogen receptor of spotted seatrout: a screening assay for potential estrogenic effects. Mar Environ Res 35:147–151 (1993).
68. Crews D, Willingham E, Skipper J. Endocrine disruptors: present issues, future problems. Q Rev Biol 75:243–260 (2000).
69. Metcalfe C, Gray M, Metcalfe T. Induction of testis-ova in medaka by exposure to estrogenic compounds [Abstract]. In: 18th Annual Meeting of SETAC, San Francisco, CA, 16–20 November 1997. Pensacola, FL:Society of Environmental Toxicology and Chemistry, 1997;137.
70. Nimrod A, Benson W. Reproduction and development in Japanese medaka following an early life stage exposure to xenoestrogens. Aquat Toxicol 44:141–156 (1998).
71. Wester P, Canton J. Histopathological study of *Oryzias latipes* (medaka) after long-term β-haxachlorocyclohexane exposure. Aquat Toxicol 9:21–45 (1986).
72. Vodicnik M, Peterson R. The enhancing effect of spawning on elimination of a persistent polychlorinated biphenyl from female yellow perch. Fundam Appl Toxicol 5:770–776 (1985).
73. Monod G. Egg mortality of Lake Geneva charr (*Salvelinus alpinus* L) contaminated by PCB and DDT derivatives. Bull Environ Contam Toxicol 35:531–536 (1985).
74. Miller M, Amrhein J. Maternal transfer of organochlorine compounds in Lake Superior siscowet (*Salvelinus namaycush siscowet*) to their eggs. Bull Environ Contam Toxicol 55:96–103 (1995).
75. Guiney PD Melancon MJ Jr, Lech JJ, Petersen RE. Effects of egg and sperm maturation and spawning on the distribution and elimination of a polychlorinated biphenyl in rainbow trout (*Salmo gairdneri*). Toxicol Appl Pharmacol 47:261–272 (1979).
76. Cooper K, Patyna S. A multi-generation assay evaluating phthalates in Japanese medaka (*Oryzias latipes*) and

biochemical imprinting. In: International Symposium on Endocrine-Disrupting Substance Testing in Medaka, Nagoya, Japan, 17–20 March 2000. Tokyo: Environment Agency, Government of Japan, 2000;44–46.

77. Miles-Richardson S, Kramer V, Fitzgerald S, Render J, Yamini B, Barbee S, Giesy J. Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). Aquat Toxicol 47:129–145 (1999).

78. Tyler C, Harries J, Runnels T, Maddix S, vanAerle R, Harris C, Sumpter J. Reproductive effects of environmental estrogens in the fathead minnow (*Pimephales promelas*) [Abstract]. In: SETC 20th Annual Meeting, Philadelphia, PA, 14–18 November 1999. Pensacola, FL:Society of Environmental Toxicology and Chemistry, 1999;95.

79. Coldham NG, Sivapathasundaram S, Dave M, Ashfield LA, Pottinger TG, Goodall C, Sauer MJ. Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout (*Oncorhynchus mykiss*). Drug Metab Dispos 26:347–354 (1998).