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 is 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 gonadal 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.
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 (Oncorhynus 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 antian- drogen 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% CaCl2 · 2H2O, and 0.016% MgSO4 · 7H2O; Carolina Biological, Burlington, N.C.), frozen upon hatching, and then transferred to exposure aquaria.

**Experimental design.** Gonadal differentiation in medaka occurs within the first 2 weeks after hatching 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. untreated control adults were sacrificed 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.

**Chemical analysis.** We purchased DDT from Lancaster Synthesis Inc. (Windham, N.H.) 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...
Vitellogenin analysis. Fish smaller than 90 mg were euthanized in ice-cold M S-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 (PM SF), 5 mM dithiothreitol, and 5 µg/mL aprotinin. The homogenate was immediately mixed with aprotinin [0.021 tryptase-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 Laemmlı sample buffer (100-fold for plasma samples and to 0.5 µg total protein per well for homogenates) and separated proteins by SD-S-PAGE at 120 V for 2 hr. Proteins were electroblotted (45 V for 2 hr) to a polyvinylidenefluoride 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 ABC Amper 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 micrograms 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 microgram 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 Walleck 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.

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. Males were scored as follows: 1 if only spermatogonia were present, with some cysts of spermatids and sperm-filled cysts and tubules occupied > 50% of sectioned area; 2 if sperm-filled cysts and tubules occupied < 50% of section 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 aquarium (mean ± SEM).

| Nominal dose (µg/L) | Actual dose (µg/L) 2-Week exposure | Actual dose (µg/L) 8-Week exposure |
|--------------------|-----------------------------------|----------------------------------|
| 0.5                | 0.23 ± 0.02                      | 0.33 ± 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                      |

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 0.05 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 Ftime = 471.66, p < 0.0001; 8-week Ftime = 862.36, p < 0.0001; 2-week FDDT = 5.49, p < 0.0001; 8-week FDDT = 12.18, p < 0.0001; 2-week Ftime × DDT = 2.876, p = 0.0003; 8-week Ftime × 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.
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.

**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), ovoocytes 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 ovoocytes, 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 testes 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 ovoocytes. 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, FDDT = 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 (FDDT × 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 (FDDT = 11.76, p < 0.0001; Fage = 12.65, p < 0.0001; FDDT × 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 (0.23 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 (Table 2). The highest dose also reduced fecundity of exposed females, but reduced fertility of pairs with both partners exposed, indicating that the effect on female fecundity (egg number) 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 = 50 (0.23 µg/L), n = 32 (0.50 µg/L), n = 32 (1.37 µg/L), n = 19 (4.23 µg/L); 8-week, n = 134 (0 µg/L), n = 36 (0.23 µg/L), n = 56 (0.69 µg/L), n = 43 (1.94 µg/L), n = 2 (5.2 µg/L). ***Ratios significantly different from 1:1 (χ² test, p < 0.001).

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 (0.50 µg/L), n = 32 (1.37 µg/L), n = 19 (4.23 µg/L); 8-week, n = 134 (0 µg/L), n = 36 (0.23 µg/L), n = 56 (0.69 µg/L), n = 43 (1.94 µg/L), n = 2 (5.2 µg/L). ***Ratios significantly different from 1:1 (χ² test, p < 0.001).

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 vitro 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 to 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, tests 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). p,β-Hexachlorocyclohexane (p,β-HCH; one of the pesticides 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

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

| Exposure duration | DDT (µL) | Exposed female | Exposed male | Both exposed |
|-------------------|----------|----------------|--------------|--------------|
| 2 Weeks           |          |                |              |              |
| 0                 | 59.0 ± 6.3 | 59.0 ± 6.3     | 59.0 ± 6.3   |
| 0.02              | 52.8 ± 5.5 | 81.7 ± 11.5    | 59.5 ± 10.9  |
| 0.50              | 41.3 ± 5.2 | 44.8 ± 8.7     | 34.2 ± 5.6   |
| 1.37              | 34.0 ± 9.3* | 46.7 ± 5.6     | 45.7 ± 10.3  |
| 4.32              | 35.8 ± 4.7* | 30.6 ± 5.8     | 9.0 ± 6.0*   |
| 8 Weeks           |          |                |              |              |
| 0                 | 33.3 ± 4.6 | 33.3 ± 4.6     | 33.3 ± 4.6   |
| 0.03              | 52.7 ± 5.4 | 32.3 ± 4.7     | 45.3 ± 6.2   |
| 0.69              | 58.0 ± 1.8* | 32.0 ± 4.0     | 34.3 ± 6.7   |
| 1.94              | 47.6 ± 8.1 | 16.5 ± 2.85    | 21.5 ± 2.8   |

Exposed females were mated with control males, and exposed males were mated with control females. *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 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 alkylphenols 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...
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 definitive test for predicting reproductive impairment. VTG synthesis is an activation 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 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 activation 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 Boltini: evidence for environmentally-induced masculinization. Copeia 4576–461 (1980).
5. Guillette LJJ, Gross TS, Masson GR, Matter J, 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. Gimel 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 18:1833–1842 (1999).
10. Edmunds JG, McCarthy RA, Ramsdell JS. Permanent and functional male-to-female sex reversal in d-rR strain medaka (Oryzias latipes) following egg microinjection of 4,4′-DDT. Environ Health Perspect 108:219–224 (2000).
11. Metcalfe TL, Metcalfe CD, Kiparisssy I, Niimi AJ, Foran CM, Benson WH. Gonadal development and endocrine responses in Japanese medaka (Oryzias latipes) exposed to 4,4′-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 4,4′-DDT.** Doses (µg/L) represent the lowest dose that caused significant alteration at any age.

| Parameter                  | 2-Week Male | 2-Week Female | 8-Week Male | 8-Week Female |
|----------------------------|------------|--------------|------------|--------------|
| VTG                        | 0.30       | 0.30         | 0.30       | 0.30         |
| Gonadal feminization       | 0.30       | 0.30         | 0.30       | 0.30         |
| No. of eggs                | 0.30       | 0.30         | 0.30       | 0.30         |
| 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.

*No dose had an effect. **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).
sider turtle, a species with a temperature-dependent sex determination, and its exposure to PCBs has been studied.

31. Van der Kraak GJ, Munkittrick KR, McMaster ME, Portt CB, de Solla SR, Bishop CA, Van Der Kraak G, Brooks RJ.

30. de Solla SR, Bishop CA, Van Der Kraak G, Brooks RJ.

29. Munkittrick KR, Van Der Kraak GJ, McMaster ME, Portt CB.

28. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR, Egeberg S, Heird W.

27. Clark NJ, Norris NM, Dames RE. Interactions of gonadal steroids and PCBs on differences in growth and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ Toxicol Chem 17:446–452 (1998).

26. Folmar LC, Desniew N, D'Arcy MA, Chester MA, Crain DA, Enblom J, Marino J, Guillette LJ Jr. Vitellogenin induction and reduced serum testosterone concentrations in male carp (Cyprinus carpio) captured near a major petrochemical plant. Environ Health Perspect 104:1096-1100 (1996).

25. Guillette LJ Jr, Woodward AR, Crain DA, Pickford DB, Woodward AR, Desniew N. Plasma steroid concentrations and male phallic size in alligators: implications for sex steroid concentrations and phallic size in juvenile American alligators. Arch Environ Contam Toxicol 36:447–455 (1999).

24. Willingham E, Rhem T, Sakata J, Crews D. Embryonic development of rainbow trout liver estrogen receptor and estrogenic contamination of the aquatic environment. Environ Health Perspect 103(suppl 1):5–10 (1998).

23. Speirs R, Thomas P. Reproductive and endocrine status of feral male carp (Cyprinus carpio) from seven Florida lakes. Gen Comp Endocrinol 116:336–372 (1999).

22. Willingham E, Rhen T, Sakata J, Crews D. Embryonic and larval growth in larval tiger salmanders. Ambystoma tigrinum. Gen Comp Endocrinol 109:94-110 (1998).

21. Crain DA, Guillette LJ Jr, Pickford DB, Percival HF, Woodward AR. Sex-stereotypy and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ Toxicol Chem 17:446–452 (1998).

20. Folmar LC, Desniew N, D'Arcy MA, Chester MA, Crain DA, Enblom J, Marino J, Guillette LJ Jr. Vitellogenin induction and reduced serum testosterone concentrations in male carp (Cyprinus carpio) captured near a major petrochemical plant. Environ Health Perspect 104:1096-1100 (1996).

19. Guillette LJ Jr, Woodward AR, Crain DA, Pickford DB, Woodward AR, Desniew N. Plasma steroid concentrations and male phallic size in alligators: implications for sex steroid concentrations and phallic size in juvenile American alligators. Arch Environ Contam Toxicol 36:447–455 (1999).

18. Speirs R, Thomas P. Reproductive and endocrine status of feral male carp (Cyprinus carpio) from seven Florida lakes. Gen Comp Endocrinol 116:336–372 (1999).

17. Crain DA, Guillette LJ Jr, Pickford DB, Percival HF, Woodward AR. Sex-stereotypy and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ Toxicol Chem 17:446–452 (1998).

16. Folmar LC, Desniew N, D’Arcy MA, Chester MA, Crain DA, Enblom J, Marino J, Guillette LJ Jr. Vitellogenin induction and reduced serum testosterone concentrations in male carp (Cyprinus carpio) captured near a major petrochemical plant. Environ Health Perspect 104:1096-1100 (1996).

15. Bergeron J, McCue T, McLaughlin JAC. PCBs as environmental endotoxins: turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 107(suppl 1):701–103 (1999).

14. Mather J, Crain DA, Sills-Mc Curr Y, Pickford DB, Rainwater TR, Reynolds KD, Rooney AA, Dickerson RL, Guillette LJ Jr. Effects of endocrine disrupting contaminants on sex determination in reptiles: Principles and consequences for Evaluating Endocrine Disruption in Wildlife (Kendall R, Dickerson R, Giesy J, Suk W, eds). Pensacola, FL:SETAC Press, 1998:267-289.

13. Clark NJ, Norris NM, Dames RE. Interactions of gonadal steroids and PCBs on differences in growth and thyroid hormone concentrations in juvenile alligators (Alligator mississippiensis) from contaminated and reference lakes in Florida, USA. Environ Toxicol Chem 17:446–452 (1998).

12. Folmar LC, Desniew N, D’Arcy MA, Chester MA, Crain DA, Enblom J, Marino J, Guillette LJ Jr. Vitellogenin induction and reduced serum testosterone concentrations in male carp (Cyprinus carpio) captured near a major petrochemical plant. Environ Health Perspect 104:1096-1100 (1996).

11. Guillette LJ Jr, Woodward AR, Crain DA, Pickford DB, Rooney AA, Percival HF. Plasma steroid concentrations and male phallic size in alligators: implications for sex steroid concentrations and phallic size in juvenile American alligators. Arch Environ Contam Toxicol 36:447–455 (1999).

10. Speirs R, Thomas P. Reproductive and endocrine status of feral male carp 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, 1997:113-114.

9. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

8. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

7. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

6. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

5. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

4. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

3. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

2. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).

1. Munkittrick KR, Portt CB, Van Der Kraak GJ, Smith IR. Xenobiotic exposure to endocrine-active chemicals. Environ Health Perspect 110:103-114 (2002).
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 M. Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout (Oncorhynchus mykiss). Drug Metab Dispos 26:347–354 (1998).