

Permanent and Functional Male-to-Female Sex Reversal in d-rR Strain Medaka (*Oryzias latipes*) Following Egg Microinjection of *o,p'*-DDT

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Complete sex reversal of fish is accomplished routinely in aquaculture practices by exposing fish to exogenous sex steroids during gonadal differentiation. A variety of environmental chemicals are also active at sex steroid receptors and theoretically possess the potential to alter normal sexual differentiation in fish. However, in controlled environmental chemical exposures to date, only partial alterations of fish sexual phenotype have been observed. Here we report complete, permanent, and functional male-to-female sex reversal in the Japanese medaka (*Oryzias latipes*, d-rR strain) after a onetime embryonic exposure to the xenoestrogen *o,p'*-DDT. d-rR strain medaka are strict gonochorists that possess both sex-linked pigmentation, which distinguishes genotypic sex, and sexually dimorphic external secondary sexual characteristics, which distinguish phenotypic sex. We directly microinjected the xenoestrogen *o,p'*-DDT into the egg yolks of medaka at fertilization to parallel the maternal transfer of lipophilic contaminants to the embryo. At 10 weeks of age, microinjected medaka were examined for mortality and sex reversal. A calculated embryonic dose of 511 ± 22 ng/egg *o,p'*-DDT (mean \pm standard error) resulted in 50% mortality. An embryonic exposure of 227 ± 22 ng/egg *o,p'*-DDT resulted in 86% (6 of 7) sex reversal of genetic males to a female phenotype (XY females). XY females were distinguished by sex-linked male pigmentation accompanying female secondary sexual characteristics. Histologic examination of the gonads confirmed active ovaries in 100% of the XY females. In 10-day breeding trials in which XY females were paired with normal XY males, 50% of the XY females produced fertilized embryos; this represents a comparable breeding success rate to normal XX females. Fertilized eggs produced from XY females hatched to viable larvae. These results clearly indicate that a weakly estrogenic pesticide, *o,p'*-DDT, when presented during the critical period of gonadal development, can profoundly alter sexual differentiation. **Key words:** endocrine disruptors, medaka, *o,p'*-DDT, sex reversal, xenoestrogen. *Environ Health Perspect* 108:219–224 (2000). [Online 21 January 2000]

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Teleost sexual differentiation is sensitive and responsive to manipulation by exogenous estrogen and androgen steroid hormones (1–3). A variety of environmental chemicals demonstrate intrinsic activity at teleost steroid receptors (4–7) and steroidogenic pathways (8–9) and therefore could potentially alter normal sexual differentiation and development in feral and laboratory fish populations. Although the induction of the egg yolk lipoprotein vitellogenin in male fish has received attention as a sensitive biomarker for estrogen exposure (10–12), relatively few studies have focused on long-term permanent adverse reproductive effects of xenobiotic exposure. Adverse effects noted in the wild include masculinized mosquito fish discovered downstream of kraft mill effluent discharge in a Florida creek (13) and an increased rate of hermaphroditism in roach (*Rutilus rutilus*) from English riverways receiving sewage treatment water effluent (11). In the laboratory, feminized testes and formation of a permanent oviduct resulted from aqueous exposure to the xenoestrogen 4-*tert*-pentylphenol (TTP) before and during the period of sexual differentiation in a genetically

all-male population of common carp (14). The formation of testis–ova was demonstrated in male medaka after continuous aqueous exposure to the xenoestrogen 4-nonylphenol from hatching until sexual maturity (15). In the present study, we test the hypothesis that a onetime exposure to the xenoestrogen pesticide *o,p'*-DDT administered before gonadal differentiation would permanently alter fish sex differentiation.

We chose the direct microinjection of contaminants into the embryonic yolk as a route of exposure to parallel the maternal transfer of persistent lipophilic contaminants. The significant bioaccumulation and transfer of contaminant loads of organochlorines (DDT and polychlorinated biphenyls) from maternal stores to eggs and subsequent embryos and larvae has been well documented in feral populations of freshwater (16,17) and saltwater fish (18,19) and in controlled feeding experiments (20). The work of Ungerer and Thomas (21) strengthens our choice of an exposure model; these authors orally administered *o,p'*-DDT to adult Atlantic croaker (*Micropogonias undulatus*) and examined the subsequent distribution of

the lipophilic pesticide. The highest percentage of the administered *o,p'*-DDT accumulated in the ovaries, as compared to the liver, muscle, and brain. In addition, within the oocytes 95% of the *o,p'*-DDT compartmentalized to the triglyceride-rich oil droplet. On analysis of the plasma, it was determined that the *o,p'*-DDT fractionated with the triglyceride rich very-low-density lipoprotein fraction, suggesting transport by the protein through the blood and subsequent receptor-mediated uptake by the oocyte.

We chose the d-rR strain of medaka (*Oryzias latipes*) to uniquely identify sex reversal after contaminant exposure. Medaka are strict differentiated gonochorists with XX females and XY males (22). Medaka have secondary sexual characteristics that make external differentiation of males and females possible in sexually mature fish (Figures 1 and 2). The d-rR strain exhibits sex-linked pigmentation (orange/red males, white females), which serves as a reliable marker for genotypic sex. All males have the orange/red phenotype with a genotype of $X^R Y^R$, where R is xanthic pigmentation. Females express a white phenotype with a genotype of $X^X X^X$ (I). This marker for sexual genotype can be observed microscopically at the time of hatching, and the coloration remains

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permanent throughout the life of the fish. Yamamoto (1,2) developed this strain of medaka to study the effects of natural steroids on vertebrate sexual differentiation. The advantage of this model is that the discrepancy between sexual genotype and phenotype can be discerned at the level of the individual fish, and subsequent studies are not dependent on experimental sex ratios for identifying sex reversal (23).

Exposure of medaka embryos to the estrogenic contaminant *o,p'*-DDT (24–28) during this earliest window of sexual differentiation resulted in permanent alteration of sexual phenotype.

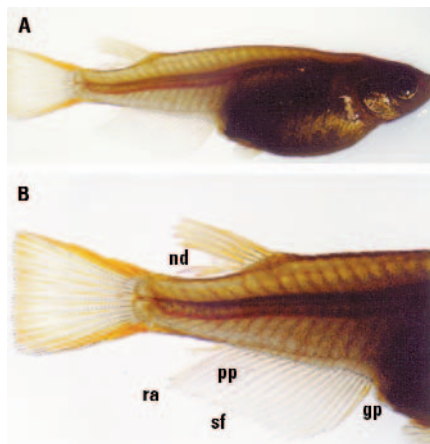


Figure 1. d-rR strain male medaka. (A) Male medaka (X^Y^m) are identified genotypically by sex-linked paternal inheritance of orange/red pigmentation and phenotypically by development of secondary sexual characteristics. (B) Secondary male sexual characteristics include notched dorsal fin (nd), rectangular anal fin (ra), serrated anal fin margin (sf), papillary processes (pp), and reduced genital papillae (gp).

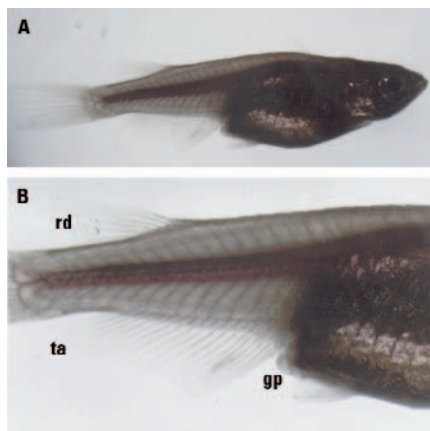


Figure 2. d-rR strain female medaka. (A) Female medaka (X^X) are identified genotypically by sex-linked inheritance of white pigmentation and phenotypically by development of secondary sexual characteristics. (B) Secondary female sexual characteristics include rounded dorsal fin (rd), triangular anal fin (ta), and bilobed bulbous genital papillae (gp).

Materials and Methods

Medaka culture. Fertilized eggs of the d-rR strain of medaka were provided by Y. Wakamatsu, Nagoya University, Nagoya, Japan, and a population of fish was maintained under breeding conditions of 25°C and 16 hr light:8 hr dark cycle. The fish were housed in 2.5 gallon aquaria containing an aerated balanced salt solution (1g/L NaCl, 0.08 g/L KCl, 0.03 g/L CaCl₂, 0.03 g/L MgSO₄) and fed flake food (OSI Vivid Color; Ocean Star International, Hayward, CA) in the morning and live artemia in the afternoon. Fifty percent water changes were performed every week. Ten to 12 fish were housed in each tank, with a 1:1 male:female ratio. Each morning eggs were collected directly from breeding females, culled for fertilization under a stereoscope, and placed in an isosmotic solution (Yamamoto's rearing solution: 133 mM NaCl, 2.7 mM KCl, 2.1 mM CaCl₂, 0.2 mM NaHCO₃) (29).

Chemicals and biochemicals. The organochlorine pesticide *o,p'*-DDT ([4-chlorophenyl]ethane) used in this study was obtained neat from Ultra Scientific (North Kingston, RI) and dissolved in 95% pure triolein (Sigma, St. Louis, MO). 17β-Estradiol was purchased from Sigma.

Microinjections. For the microinjection technique, we followed a protocol for fish embryo nanoinjection outlined by Walker et al. (30) and modified for medaka (31). Approximately 30 medaka eggs were collected in the morning, and microinjections were performed 6 to 8 hr after fertilization. The fertilized eggs were placed in an injection cassette and stabilized with 0.5% agarose in 12.5% (wt/vol) Hank's solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃). Micropipettes (outer diameter 1 mm; Narishige, Tokyo, Japan), pulled with a vertical puller (Narishige), were back loaded with triolein oil vehicle or triolein oil with reconstituted *o,p'*-DDT or estradiol. Microinjections were performed with a micromanipulator (MO-155; Narishige) under 30× magnification with a

compound microscope (Nikon, Tokyo, Japan). The stage micromanipulator was used to advance the egg onto the injection needle, piercing the chorion and resting in the egg yolk. A drop of triolein was injected into the egg yolk adjacent to the natural oil droplets. The triolein oil was injected into the egg yolk with the aid of a pressure injector system (MPPI-2; Applied Scientific Instrumentation, Eugene, OR).

The *o,p'*-DDT dose ranges and number of eggs injected per dose are provided in Table 1. We injected vehicle at equal volumes to experimental doses into 25 eggs. Sex reversal of medaka has been demonstrated upon microinjection of approximately 1 ng/egg estrone-16-¹⁴C (32); therefore, injection of 2 ng/egg of 17 β-estradiol was chosen as a positive estrogenic control and injected into 14 eggs. The diameter of each microinjected oil droplet was measured microscopically to calculate the volume injected (1.0–3.0 nL). Although we tried to inject the same volume per egg, there was some variability in injection volumes; this resulted in variability in masses of injected contaminant. Therefore, we pooled similar masses within a dose range and calculated a standard error.

After injections, the eggs were washed for 1 min in 12.5% Hank's buffer, transferred to 24-well plates containing 1 mL Yamamoto's rearing solution, and maintained in controlled conditions. The embryos were maintained at 25°C on a 16 hr light:8 hr dark cycle and monitored daily until hatching. Upon hatching, larvae were fed powdered fry food (Tetramin; Tetra, Mèlle, Germany) until they were able to feed on flake food and artemia at about 2 weeks of age.

Quantification and verification of retention of microinjected contaminants. We used medaka eggs microinjected at fertilization with a nominal concentration of *o,p'*-DDT for quantification and verification of retention of the contaminant. At 8 days after fertilization, the *o,p'*-DDT was extracted from the eggs and measured using gas chromatography. Briefly, the eggs were individually placed in 50-mL glass extraction tubes containing 5 g Na₂SO₄ in 25 mL dichloromethane. The

Table 1. Dose regime for *o,p'*-DDT microinjection into medaka eggs, with hatch and survival success.

Compound/dose (ng/egg) ^a	No. of eggs ^b	Hatch (%)	Survival (%) ^c
<i>o,p'</i> -DDT			
19.6 ± 2.7	23	87	74
33 ± 5.2	21	69	76
227 ± 22	18	95	67
580 ± 62	6	100	33
1,079 ± 87	8	86	0
Estradiol (2.1 ± 0.3)	14	92	71
Vehicle (1–3 nL/egg)	25	90	76

^aInjection masses were pooled (see "Materials and Methods"). ^bNumber of eggs indicates the number of eggs that survived the injection process; embryos that died within 24 hr were considered to have sustained lethal mechanical injury through the microinjection process and were not counted. ^cSurvival was determined by viability to 14 days posthatching; survival to 10 weeks (Table 2), when sex determination was made, was often reduced due to fish death while rearing.

eggs were homogenized with a Teflon pestle. An internal standard (D8 *p,p'*-DDT; Cambridge Isotope, Andover, MA) was gravimetrically added to each sample to later determine the contaminant concentration by ratio comparison. The extraction tubes were placed in a bath sonicator for 12 min and then the dichloromethane was decanted into turbo evaporating tubes. Two more 25-mL dichloromethane extractions were performed with a 12-min sonication each time. The extraction rinses were combined in the evaporating tubes for a final volume of 75 mL. A solvent exchange was performed by the addition of 5 mL of hexane and by nitrogen evaporating the dichloromethane. The solvent was reduced to 0.5 mL. An additional 5 mL hexane was added and again reduced to 0.5 mL. The hexane solvent was transferred by Pasteur pipette to a 15-mL glass centrifuge tube. To hydrolyze the extracted lipids, 1 mL H_2SO_4 was added and the tube was vortexed 1 min. The sample was diluted approximately 10 times by the addition of 10 mL hexane and vortexed 30 sec. A 1-mL sample of the hexane fraction was transferred to an amber autosampler vial and analyzed by gas chromatography-electron capture detection (HP6890; Hewlett Packard, Wilmington, DE).

Sex determination. Ten weeks after hatching, we examined medaka from each group (experimental groups, vehicle controls, and noninjected controls) for pigmentation to determine genotype and secondary sexual characteristics to determine phenotype. Each fish was fully anesthetized in 0.1% MS-222 (ethyl m-aminobenzoate; Sigma). We then examined fish with a stereoscope microscope (M5; Wild, Heerbrugg, Switzerland) to determine pigmentation and to score for male and female external secondary sexual characteristics (Figures 1 and 2). After scoring, the midsection of each fish was dissected for gonad analysis. (Dissections of fish from the dose that exhibited sex reversal were delayed until breeding trials were conducted.) Briefly, scissors were used to perform a transverse cut directly posterior to the pectoral fins and a second transverse cut was made directly posterior to the anal pore. The dissected trunk section (approximately 5 mm) was placed in a 10-mL scintillation vial containing 3 mL Bouin's solution (Sigma). The tissue was fixed for 48 hr in Bouin's solution and then replaced by 70% ethanol (v/v). After 24 hr, the 70% ethanol was replaced with an equal volume of 70% ethanol to dilute the Bouin's solution, which continued to leach out of the tissue. The tissue were stored in 70% ethanol until they were embedded in paraffin.

Prior to paraffin embedding, the fish midsections were placed in a 1.0% nitric

acid solution (v/v) for 1 hr to decalcify bony tissue. Tissues were cut with a scalpel one-fourth of the length of the tissue section from the anus. The two cut faces were placed face down and mounted in paraffin, allowed to cool, and sectioned on a microtome (American Optics, Aliso Viejo, CA). This tissue arrangement allowed for sections to be cut anteriorly and posteriorly simultaneously. Tissues sections (5 μ m) were mounted and stained with hematoxylin and eosin.

Breeding trials. After sex determination, a breeding trial was initiated for the experimental group in which sex reversal was observed. In the breeding trial, 11-week-old phenotypic females (X^Y^R females and X^X^F females) were paired individually with normal X^Y^R males of the same age. Pairs were placed in watch bowls filled with 250 mL Yamamoto's solution. Breeding conditions were maintained at 27°C with a 16L:8D photoperiod. Female fish were monitored for egg production every morning for 10 days. Fish that produced fertilized eggs during this 10-day interval were qualified as egg producers. The fecundity of each egg producer was not quantified; however, fertilized eggs from each fish were raised to hatching and all hatchlings appeared normal.

As a control breeding experiment, the breeding success of three control breeding stock groups was evaluated. The breeding success of fish in three breeding tanks were monitored for 10 days. The tanks contained 10–12 fish in a 1:1 male:female ratio (16 total females). The fish were 2–3 months old. The 2.5-gallon tanks were maintained at 27°C with a 16L:8D photoperiod. Individual females were identified by external characteristics; if they produced eggs within a 10-day period, they were qualified as egg producers. Breeding percentages were calculated for three separate 10-day periods.

Results

Retention of microinjected contaminants. We analyzed a subsample of microinjected eggs to verify retention of microinjected contaminants. Three eggs were microinjected with nominal concentration of *o,p'*-DDT in a triolein vehicle. Eight days after fertilization (2 days before hatching) the microinjected *o,p'*-DDT was extracted individually from each egg and measured by gas chromatography. The *o,p'*-DDT (mean \pm standard error) recovered at day 8 (601 ng \pm 49 ng; $n = 3$) was 118% of the calculated injection mass of 511 ng. We detected no breakdown products of *o,p'*-DDT. We found no detectable *o,p'*-DDT in either a vehicle-injected egg or in a control noninjected egg. The recovery experiments indicate that the calculation method used to determine microinjected contaminant mass was accurate within 20% and that

the microinjected contaminant remains within the chorion of the embryo following injection and throughout the development of the embryo. Visual observation also indicated that the injected oil droplet remains intact through hatching.

Determination of lethal dose of *o,p'*-DDT to hatching success and larval survivability. The microinjection of a triolein oil droplet containing dissolved *o,p'*-DDT into medaka eggs shortly after fertilization resulted in a dose-dependent reduction in medaka larvae survivability (Table 1). A median lethal dose (LD_{50}) of 511 ± 28 ng/egg *o,p'*-DDT was calculated by least squares regression. Hatching success was not reduced by *o,p'*-DDT over this range.

Contaminant alteration of sexual phenotype. Medaka treated with sublethal concentrations of microinjected *o,p'*-DDT were grown to an age of 10 weeks (≈ 20 mm in length), when the fish display mature external secondary sexual characteristics. Male-to-female sex reversal occurred in 86% of genetic males (6 of 7) after exposure to 227 ± 22 ng/egg *o,p'*-DDT (Table 2). We observed no sex reversal of phenotype in either genotypic males or females after exposures at concentrations $< 227 \pm 22$ ng/egg *o,p'*-DDT. Sex-reversed fish (X^Y^R -females) clearly exhibited the orange/red xanthic pigmentation distinguishing genetic males concomitant with distinctive female secondary sexual characteristics including enlarged genital papillae, small triangular anal fin, and rounded dorsal fin (Figure 3A, B). Microinjection of 2.0 ng/egg 17β -estradiol resulted in male-to-female sex reversal (X^Y^R females) in 1 of 5 genetic males (Table 2). No sex reversal was evident after vehicle injection ($n = 25$).

To determine functional sex reversal, we paired *o,p'*-DDT sex-reversed fish (X^Y^R females) with normal males (X^Y^R males) for 10 days. Three out of six pairs produced

Table 2. Sex reversal of genetic males after microinjection of vehicle, 17β -estradiol (1–2 ng/egg), and *o,p'*-DDT.

Treatment	X^Y^R males ^a	X^Y^R females ^b
Vehicle (triolein)	19	0
17β -Estradiol	4	1*
<i>o,p'</i> -DDT (ng/egg)		
20 \pm 3	6	0
33 \pm 5	5	0
227 \pm 22	1	6**
580 \pm 62	All males dead	
1,079 \pm 87	All males dead	

^a X^Y^R males (normal males) were identified by orange/red sex-linked pigmentation, male external secondary sexual characteristics, and the presence of a testis. ^b X^Y^R females (sex-reversed females) were identified by orange/red sex linked pigmentation, female external secondary sexual characteristics, and the presence of an ovary. *Significantly different from vehicle at $p = 0.04$ (Pearson's chi-square analysis). **Highly significantly different from vehicle at $p < 0.0001$ (chi-square analysis).

fertilized eggs (Table 3; Figure 3C), a breeding success rate that did not differ from XX^r females. Fertilized eggs from X^rY^R females hatched, producing viable larvae; this indicates a completely functional male-to-female sex reversal. Genotypic females showed no alteration in sexual phenotype, exhibiting both normal female secondary sexual characteristics and successful breeding.

Gonad histology. We histologically examined the gonads of experimental fish to confirm phenotypic sex. In all fish examined, the

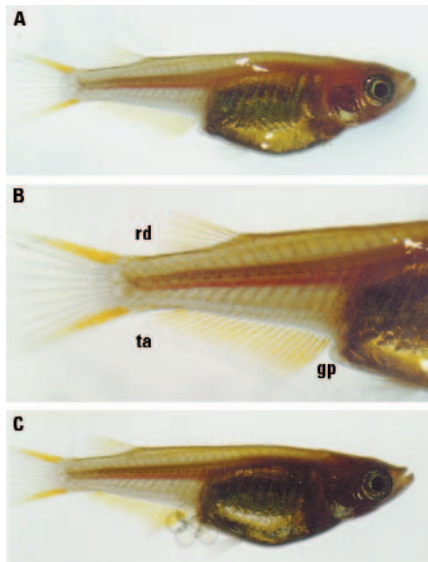


Figure 3. Male-to-female sex reversal (X^rY^R female) after o,p' -DDT microinjection. (A) A representative medaka microinjected with 227 ± 22 ng/egg o,p' -DDT within 8 hr of fertilization that exhibits male-to-female sex reversal; the fish displays orange/red pigmentation indicative of the male genotype and has distinctive female secondary sexual characteristics. (B) An enlargement shows the phenotypic female characteristics of rounded dorsal fin (rd), triangular anal fin (ta), and bilobed bulbous genital papillae (gp). (C) Definitive functional sex reversal demonstrated in a male-to-female sex-reversed (X^rY^R female) medaka exhibiting oviposition of fertilized eggs after pairing with an X^rY^R male.

Table 3. Results of breeding trials in which phenotypic females were placed individually with normal XY males for 10 days and monitored daily for egg production.

Treatment/genotype	Phenotypic females	Egg producers	Breeding success (%)
Control			
XX females	16	4	25
XX females	16	9	56
XX females	16	7	44
o,p' -DDT ^a			
XY females	6	3	50
XX females	4	2	50

Three 10-day observation periods of the breeding stock medaka were used as control groups. No statistical differences in breeding success were observed between groups (chi-square analysis).

^a o,p' DDT dose was 227 ± 22 ng/egg.

sexual phenotype determined by external secondary sexual characteristics matched the phenotypic sex determined by gonad histology. Vehicle-injected XX^r females presented normal ovaries with actively maturing oocytes (Figure 4A), and vehicle-injected X^rY^R males had normal testes that actively produced sperm (Figure 4B). All (6 of 6) o,p' -DDT-treated sex-reversed females (X^rY^R females) had ovaries that actively produced maturing oocytes (Figure 5A). The one 17 β -estradiol-treated male-to-female sex-reversed fish (X^rY^R female) also had a functional ovary (Figure 5B).

Discussion

Here we report evidence to support the hypothesis that a contaminant with *in vitro* endocrine activity alters sex differentiation in the intact organism. We observed complete, permanent, and functional male-to-female sex reversal of the Japanese medaka (d-rR strain) after a onetime egg exposure to the xenoestrogen o,p' -DDT. The exposure paralleled the maternal transfer of lipophilic contaminants by the microinjection of the contaminant directly into the egg yolk following fertilization. Sex reversal (X^rY^R females) was detected in sexually mature medaka by the presence of female external secondary sexual characteristics in genetically male fish. We confirmed sex reversal by the presence of ovarian tissue in all genetically

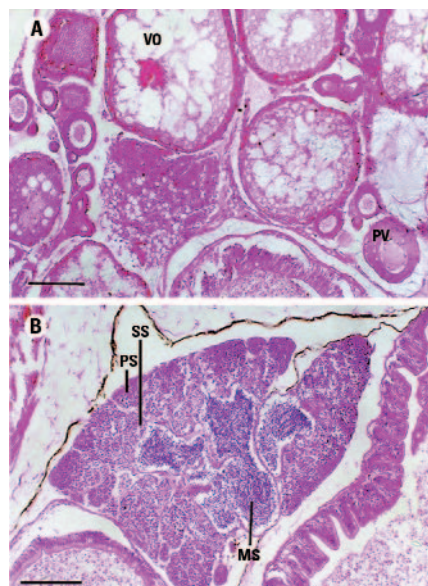


Figure 4. Cross sections (5 μ m) through normal gonad of sexually mature d-rR strain medaka (*Oryzias latipes*) stained with hematoxylin and eosin and magnified 100 \times . Bar = 100 μ m. (A) Normal ovary from 10-week-old X^rX^r female with previtellogenic oocytes (PV) and vitellogenic oocytes (VO). (B) Normal testis from 10-week-old X^rY^R male with primary spermatocytes (PS), secondary spermatocytes (SS), and mature sperm (MS).

male fish that expressed external female morphology. We determined functional sex reversal using mating experiments in which sex-reversed females (X^rY^R females) paired with normal males (X^rY^R males) produced fertilized eggs that hatched into viable larvae. The X^rY^R females had the same breeding success as normal XX^r females. Other studies have shown feminization of male fish after aqueous exposures to xenoestrogens during sexual differentiation (14,15,33), but to our knowledge this is the first evidence of functional sex reversal in a fish after xenoestrogen exposure. These adverse reproductive effects occurred at a concentration two times less than the LD₅₀, indicating that the developing larvae are more sensitive to the endocrine effects of o,p' -DDT than to the lethal effects on development. Microinjection of 17 β -estradiol also produced male-to-female sex reversal, using the above criteria of external female morphology in a genetic male medaka (X^rY^R female) and the presence of an active ovary. The dose of 17 β -estradiol chosen was anticipated to result in a larger percentage of sex reversal. That only 1 of 5 males demonstrated sex reversal was probably because the 17 β -estradiol-triolein mixture was microinjected into the perivitelline space rather than into the egg yolk, with subsequent poor absorption and exposure.

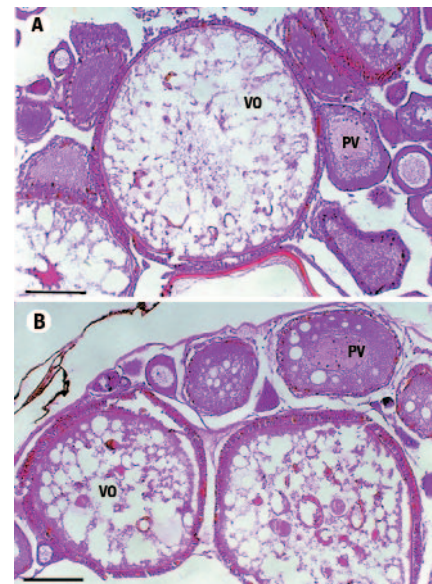


Figure 5. Cross sections (5 μ m) through gonad of male-to-female sex-reversed gonad from d-rR strain medaka (*Oryzias latipes*) stained with hematoxylin and eosin and magnified 100 \times . Bar = 100 μ m. (A) Ovary from 10-week-old male-to-female sex-reversed (X^rY^R female) medaka microinjected with 227 ± 22 ng/egg o,p' -DDT at fertilization; previtellogenic oocytes (PV) and vitellogenic oocytes (VO) are present. (B) Ovary from 10-week-old male-to-female sex-reversed (X^rY^R female) medaka microinjected with 1–2 ng/egg 17 β -estradiol at fertilization; PV and VO are present.

However, the identical responses induced by 17β -estradiol and *o,p'*-DDT suggest that *o,p'*-DDT microinjected into freshly fertilized eggs promotes male-to-female sex reversal by mimicking the effects of exogenous estrogens (1,32). We suggest that *o,p'*-DDT mimics or promotes the action of an ovarian determinant which signals somatic cells in the genital ridge to differentiate into granulosa cells. Egg microinjection of *o,p'*-DDT caused no alteration of normal female sex differentiation. Genotypic females developed normal female secondary sex characteristics, with active ovaries that produced viable eggs upon mating. Therefore, microinjecting *o,p'*-DDT into medaka eggs before gonadal differentiation skewed the sex ratio to a 91% female phenotype. *o,p'*-DDT is not the major component of technical grade DDT or the major organismal metabolite; therefore, natural concentrations in fish eggs may not reach the levels of this compound used in this study. However, the data clearly indicate that a sublethal concentration of a weakly estrogenic contaminant may profoundly alter gonadal organization in developing fish and how these alterations can have effects on population sex ratios.

Medaka, at a length of 4–5 mm upon hatching, have a bipotential gonad that does not differentiate until the fish reaches 6–11 mm (29). Unlike mammals, in which testis differentiation precedes ovarian differentiation, the medaka ovary differentiates before the testis (1). This later differentiation of the testis as compared to the ovary may point to a window, before differentiation of Sertoli cells, when the undifferentiated gonad of the developing male may be sensitive to estrogenic cues from maternally transferred xenoestrogens.

o,p'-DDT has been demonstrated to be weakly estrogenic in several fish species. Juvenile rainbow trout demonstrate a significant increase in plasma vitellogenin after chronic dietary exposure; however, the response is minimal as compared to the estradiol response, even when a high liver burden (14 $\mu\text{g/g}$; 14 ppm) was reached (7). Weak estrogenic activity of *o,p'*-DDT has also been demonstrated through binding of cytosolic hepatic estrogen receptors in various fish. In rainbow trout, *o,p'*-DDT competes for hepatic estrogen binding sites, but requires concentrations 156,000 times those required of the synthetic estrogen moxestrol to achieve a comparable response (7). This estrogen receptor affinity is much lower than in mammalian studies in which *o,p'*-DDT competitively inhibits binding of labeled estradiol at concentrations 2,000 times that of the synthetic estrogen diethylstilbestrol (26). Nimrod and Benson (34) showed *o,p'*-DDT to have no greater competitive activity than testosterone at the catfish estrogen

receptor; they also found no increase in serum vitellogenin levels after intraperitoneal injection. Sumpster and Jobling (11) found vitellogenin induction in trout hepatocytes after exposure to 5 μM *o,p'*-DDT. Thomas and Smith (4) found *o,p'*-DDT to have no competitive activity for the spotted seatrout estrogen receptor. It is surprising that although *o,p'*-DDT demonstrates such weak estrogen activity in fish models, we observed such potent effects on sexual development and organization in our study. One explanation is that during the period of gonadal differentiation, which our model targeted, the larvae are more sensitive to the presence of exogenous estrogens than the adult fish and tissues used in other studies. The potent effects on sexual differentiation demonstrated by the “weak” xenoestrogen indicate that other weak xenoestrogens may have potent endocrine effects *in vivo* when administered through a maternal route of exposure and that more potent lipophilic xenoestrogens would be expected to show similar dramatic effects at even lower concentrations.

High doses of *o,p'*-DDT (227 ± 22 ng/egg or 227 ± 22 ppm) were required to achieve permanent male-to-female sex reversal in medaka. Background DDT levels in perch in the Baltic sea have been measured up to 0.8 ppm total DDT and 0.019 ppm *o,p'*-DDT in whole fish homogenates. In polluted areas of the Baltic sea, measured levels of DDT in perch were 1.4 ppm total DDT and .035 ppm *o,p'*-DDT (35). The doses required to achieve lethal effects were higher in our experiments than those reported in wild fish. For a feral population of lake trout exposed to DDT in the wild, a 10-ppm concentration of total DDT in the eggs correlated to a 50% lethal effect (11). This is 50 times less than the LD_{50} (511 ppm) that we calculated for medaka exposure to *o,p'*-DDT in this study. Technical grade DDT is a mixture of 65–70% *p,p'*-DDT and 30–35% *o,p'*-DDT. *p,p'*-DDT has been shown to be 6 times more toxic to fish than the other DDT isomers and twice as toxic as technical grade DDT (36,37). Therefore, the difference in LD_{50} values is in part attributable to the use of the less toxic isomer *o,p'*-DDT. Another contributing factor to the reduced toxicity could be the experimental bioavailability of the contaminant. Although we chose to model the absorption of *o,p'*-DDT through the natural oil droplet in which 95% of maternally transferred *o,p'*-DDT is sequestered (21), the bioavailability of the contaminants through absorption of the triolein oil droplet may not have perfectly paralleled absorption from the natural oil droplet. Although both the natural and injected droplets are composed of triglycerides (38), the triolein oil droplet never fused with the

natural oil droplet, indicating some differences in physical properties that could result in different rates and extents to which the contaminant load within the oil droplet becomes bioavailable. The natural oil droplet appeared to be metabolized more rapidly, as seen by a measurement in reduction of diameter by day 5 posthatch, whereas the triolein droplet diameter remained unchanged at day 5 posthatch. Due to the difference in the oil metabolism, a smaller fraction of the *o,p'*-DDT may have been actually bioavailable to the developing embryo; this could contribute to an apparent reduced toxicity.

The time course of the availability of the contaminant to the embryo/larvae correlated well with experimental and wild observations. In maternally transferred DDT in wild trout, no toxicity occurred before hatching (11). In wild trout, a toxic syndrome developed upon hatching at the time of complete yolk sac absorption. The last stages of yolk sac absorption coincide with metabolism of the triglyceride oil droplets in the yolk. The toxic syndrome manifests itself as a distended air bladder, which caused the trout fry to float upside down on the surface and to eventually die. This syndrome occurred in the last stages of yolk sac absorption immediately before initiation of feeding. No reduction in hatching success was noted throughout the experimental *o,p'*-DDT dose range. The majority of mortality in the medaka *o,p'*-DDT experiments occurred between day 6 and day 8 when the yolk sac had been completely absorbed and the natural oil droplet was beginning to be metabolized, as evidenced by a decreasing diameter. By correlating the time to death with the injected oil droplet absorption, we believe that egg microinjection parallels well the dynamics of maternal embryo transfer seen in the wild.

In summary, direct oocyte microinjection provides an *in vivo* approach that parallels the maternal transfer of lipophilic contaminants and exposes embryos and larvae to contaminants during a sensitive developmental window for sexual differentiation. Sublethal concentrations of the xenoestrogen *o,p'*-DDT induced permanent and functional male-to-female sex reversal. The data present clear evidence how a weakly estrogenic compound, bioconcentrated and transferred to an embryo, can have significant effects on sex differentiation.

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