

In Ovo Exposure to *o,p'*-DDE Affects Sexual Development But Not Sexual Differentiation in Japanese Medaka (*Oryzias latipes*)

Diana M. Papoulias,^{1,2} Sergio A. Villalobos,³ John Meadows,¹ Douglas B. Noltie,² John P. Giesy,⁴ and Donald E. Tillitt¹

¹U.S. Geological Survey, Columbia Environmental Research Center, Columbia, Missouri, USA; ²University of Missouri, Department of Fisheries and Wildlife, Columbia, Missouri, USA; ³Syngenta, Inc., Greensboro, North Carolina, USA; ⁴Michigan State University, East Lansing, Michigan, USA

Despite being banned in many countries, dichlorodiphenyltrichloroethane (DDT) and its metabolites dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) continue to be found in fish tissues at concentrations of concern. Like *o,p'*-DDT, *o,p'*-DDE is estrogenic and is believed to exert its effects through binding to the estrogen receptor. The limited toxicologic data for *o,p'*-DDE suggest that it decreases fecundity and fertility of fishes. We conducted an egg injection study using the d-rR strain of medaka and environmentally relevant concentrations of *o,p'*-DDE to examine its effects on sexual differentiation and development. The gonads of exposed fish showed no evidence of sex reversal or intersex. However, other gonad abnormalities occurred in exposed individuals. Females exhibited few vitellogenic oocytes and increased atresia. Male testes appeared morphologically normal but were very small. Gonadosomatic index values for both sexes were lower for exposed fish. Our observations of abnormal female and very small male gonads after *in ovo* *o,p'*-DDE exposure may be indicative of effects on early endocrine processes important for normal ovarian and testicular development. **Key words:** egg injection, medaka, *o,p'*-DDE, sexual development, sexual differentiation, xenoestrogen. *Environ Health Perspect* 111:29–32 (2003). [Online 8 November 2002] doi:10.1289/ehp.5540 available via <http://dx.doi.org/>

In ovo exposure of fish to the synthetic estrogen ethinyl estradiol can cause genetic males to sexually differentiate as phenotypic females (Papoulias et al. 1999). Presumably, the binding of estrogen to the estrogen receptor (ER) initiates the little-understood process of establishing the female phenotype in teleost fishes. Because many man-made chemicals bind to the ER, the question arises of whether environmentally exposed male fish are at risk of being feminized.

Although use of dichlorodiphenyltrichloroethane (DDT) is banned in much of the world, the parent compound and its metabolites persist in the global environment, throughout the food chain, and thus remain a concern (Brown 1997; Munn and Gruber 1997; Schmitt et al. 1999; Simonich and Hites 1995). DDT and its metabolites are highly lipophilic and therefore easily bioaccumulate in fish. Ungerer and Thomas (1996) demonstrated that breeding females will accumulate *o,p'*-DDT within the triglyceride-rich oil globule of the oocyte. Here, the *o,p'*-DDT remains sequestered until egg fertilization, after which it becomes available to the developing embryo. The oil globule is generally the last endogenous nutritional source used by the sac fry before they switch to exogenous feeding (Heming and Buddington 1988). Thus, for many species, the greatest exposure to the developing embryo from hydrophobic estrogenic chemicals occurs during sexual differentiation (i.e., shortly after hatch).

Despite the diminished environmental presence of DDT over the last 30 years, DDE

(dichlorodiphenyldichloroethylene) metabolites are still commonly detected (Adeshina and Todd 1991; Brown 1997; Hellou et al. 1995; Schecter et al. 1997). DDE is a breakdown product of DDT, a recognized endocrine system modulator. However, very few laboratory studies of the effects of DDE have been published, and of these, most report on the *p,p'*-DDE isomer. The *p,p'*-isomers are far more common than the *o,p'*-isomers, because technical-grade DDT consists of approximately 80% *p,p'*-DDT and 20% *o,p'*-DDT. The *p,p'*-isomers are considered androgen receptor antagonists and not xenoestrogens (Kelce et al. 1995).

Although *o,p'*-DDT has long been recognized as estrogenic (Bitman et al. 1968; Kupfer and Bulger 1976), relatively little is known about its metabolite *o,p'*-DDE. However, like *o,p'*-DDT, *o,p'*-DDE is believed to work through the ER and to have a similar ER agonist potency (Donohoe and Curtis 1996), and exposure to *o,p'*-DDE has been associated with decreased fecundity and fertility and increased early oocyte atresia (Hose et al. 1989). Recently, Wells and Van Der Kraak (2000) showed that *o,p'*-DDE and *o,p'*-DDT will also bind to the androgen receptor in goldfish (*Carassius auratus*) but not rainbow trout (*Oncorhynchus mykiss*) testes at approximately half the affinity of *p,p'*-DDE. *o,p'*-DDE has even been shown to bind to the progesterone receptor in the eggshell gland mucosa of egg-laying ducks and domestic fowl and to the endometrium of the rabbit uterus

(Lundholm 1988). Furthermore, DDT can be metabolized to DDE by the developing fish embryo (Atchison and Johnson 1975).

The objective of this study was to investigate the effects of *o,p'*-DDE on sexual differentiation and development. We accomplished this through injection of environmentally relevant concentrations of *o,p'*-DDE into early-stage embryos of medaka (*Oryzias latipes*).

Materials and Methods

Test organism. The d-rR strain of medaka was a generous gift of Akihiro Shima (University of Tokyo, Tokyo, Japan). Broodstock were maintained at the Columbia Environmental Research Center (CERC) in well water under an 18:6 hr light:dark photoperiod at 27°C. In this strain, either R (orange-red) or r (white) is carried by the X or Y chromosome, respectively. White females (X^rX^r) crossed with orange-red males (X^rX^R) produce approximately equal numbers of white daughters and orange-red sons. Crossovers or genetic imbalances occur at a rate of about 0.005–0.5%, making coloration a reliable marker of genetic sex (Hishida 1965).

In addition to the genetic marker, there is distinct sexual dimorphism in medaka. Males bear a notched dorsal fin, an anal fin with a convex and serrated posterior margin, and an elongate penultimate anal fin ray that give their anal fins a square appearance. In addition, papillar processes form on the rays of the anal fin in breeding males. Females lack the dorsal notch, and their anal fins are more concave, smooth at the margin, and lack extended rays.

Injection exposure. Preparation. Details for embryo exposure and rearing are provided in Papoulias et al. (1999). Embryos were collected

Address correspondence to D.M. Papoulias, U.S. Geological Survey, Columbia Environmental Research Center, 4200 New Haven Road, Columbia, MO 65201 USA. Telephone: (573) 876-1902. Fax: (573) 876-1896. E-mail: Diana_Papoulias@usgs.gov

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in the morning of the day before exposure and embedded in a solid 1% agarose (Sigma Chemical Co., St. Louis, MO) matrix that had been set up in square 36-grid petri dishes (Falcon, Lincoln Park, NJ). The agarose matrix provided the necessary stability for injecting the embryos. The dish was then filled with well water and stored in a cool place (-20°C) until injection (within 24 hr).

Injection. Immediately preceding injection, dead embryos or embryos that had completed epiboly were replaced, and the well water covering the embryos was replaced with a sterile saline solution. Aluminosilicate capillary tubes (1.0 mm outer diameter and 0.53 internal diameter; Sutter Instrument Co., Novato, CA) were used to make injection needles with 5–10 μm internal-diameter tips. A calculated volume of material ($\sim 0.1\%$ of egg volume; egg weight, 1.0 mg \pm 0.2 mg/egg) was injected directly into the oil globule using a Narashigi picoinjector (PLI-188; Nikon, Inc., Melville, NY) and micromanipulator (MM-3; Stoelting Co., Wood Dale, IL). The injected embryos were left in the agarose at room temperature until the next day.

Incubation. Approximately 24 hr after injection, dead embryos were culled and recorded, and the survivors were placed in well water in 150-mL glass side-arm test tubes (Corning, Corning, NY) for incubation (rolled at 27°C by a gentle stream of air). Dead embryos were removed daily and counted.

Grow-out. Upon hatch, the medaka larvae were moved to $12.7 \times 12.7 \times 16.5$ cm containers suspended in $137.2 \times 35.6 \times 30.5$ cm stainless steel raceways supplied with a constant flow of well water (~ 300 mL/min). An 18:6 hr light:dark photoperiod and 25– 27°C temperatures were maintained throughout rearing. Three times per day, the newly hatched fry were fed day-old brine shrimp (*Artemia salina*) and finely ground flake food (Wardley Spectra IV; Secaucus, NJ). After reaching 2 weeks of age, the medaka were provided 2-day-old brine shrimp and flake food. Feeding continued at three times daily during the week and once per day on weekends. The fish were moved to $12.7 \times 15.2 \times 22.9$ cm stainless steel screen

cages (200 mm² mesh) after 1–2 months and were maintained there until they reached maturity. Research was conducted in accordance with the CERC Animal Welfare Plan and approved by CERC's Animal Care and Use Committee.

Experimental design. *o,p'*-DDE (99.8% pure; Chem Service, Westchester, PA) was dissolved in methylene chloride to the appropriate concentrations via serial dilution. After transfer to sterile triolein (95% pure, Sigma), the carrier of choice for these injections, the methylene chloride was removed by evaporation. Embryos (36 per treatment) were injected with a 0.5 nL volume of triolein alone (control) or one of three concentrations of *o,p'*-DDE (0.5, 0.05, 0.005 ng/embryo) in the carrier triolein. Adult fish were identified as genetic males (XY, orange-red) or genetic females (XX, white) at 107 days after fertilization and were measured (total length) and weighed (blotted dry) to assess growth. All were sacrificed in a concentrated solution of MS-222 (Sigma), and the gonads were dissected from a subsample of five white and five red fish to assess gonadosomatic index (GSI), sex reversal, and gonad histopathology. GSI was calculated as gonad weight divided by somatic weight $\times 100$. Sex reversal was determined by comparing fish color (genetic sex) to the phenotypic sex as determined by inspection of the gonad. Histologic analysis of the preserved gonads (Bouin's solution; Sigma) followed standard procedures for processing, dewaxing, hydration, and dehydration (Luna 1968). All sections were cut at 7 μm and stained using hematoxylin and eosin.

Data analysis. Mean body weight, body length, and GSI were tested for differences among control and *o,p'*-DDE-treated groups. Values for GSI ratios were arcsine transformed before analysis using one-way analysis of variance (ANOVA), testing males and females separately. We used a two-way ANOVA to test the interaction of sex and treatment on weight and length. A general linear model ($\alpha = 0.05$) and least-squares means were used to detect any significant differences. The tests were performed using SAS software (SAS 2000).

Results

Survival and growth. Survival was highest in the triolein-exposed fish (69%) and lowest in the 0.5 ng *o,p'*-DDE/embryo treatment (44%). Fish from the highest *o,p'*-DDE exposure (0.5 ng/embryo) were significantly larger than fish exposed to lower doses or the control (Table 1), and there was no significant interaction between treatment and color (genetic sex) on fish size.

Gonadosomatic index. GSI was inversely related to *o,p'*-DDE exposure for both males and females (Table 1). The lower GSI values for the exposed fish resulted from greatly decreased gonad weights.

Secondary sexual characteristics. Anal fin breeding tubercles or processes were observed on all triolein-injected males but on only one *o,p'*-DDE-injected individual (0.005 ng/embryo). No tubercles were observed on any of the embryos injected with 0.05 or 0.5 ng *o,p'*-DDE. All of the other secondary sexual characteristics (i.e., fin morphology) appeared appropriate for the sex phenotype.

Gonadal histopathology. Histologic examination of the gonads of the *o,p'*-DDE-exposed males and females indicated that no sex reversal or intersexes were caused by *o,p'*-DDE. However, female medaka exposed *in ovo* to *o,p'*-DDE did display gonadal abnormalities. All treated females possessed very few vitellogenic oocytes and exhibited an increased incidence of atresia, whereas untreated female gonads appeared mature, had empty follicles, and contained oocytes at various developmental stages (Figure 1A and B). The testes of both treated and untreated males were mature, but testes were markedly smaller in treated fish (Figure 1C and D).

Discussion

Male-to-female sex reversal was not observed at any dose of *o,p'*-DDE (0.005–0.5 ng/egg) in our study. This differs from the results of Stewart et al. (2000), who did observe male-to-female sex reversal in d-rR medaka exposed *in ovo* to *o,p'*-DDT. This inconsistency may be due to the differences between the parent compound and the metabolite or, more likely, the concentration: in the experiments

Table 1. Total length, weight, and GSI [mean \pm SD (*n*)] of adult males and females exposed to different doses of *o,p'*-DDE *in ovo*.

<i>o,p'</i> -DDE (ng/embryo)	Length (mm)		Weight (g)		GSI		Gonad weight	
	Female	Male	Female	Male	Female	Male	Female	Male
0	26 \pm 1 (16)	27 \pm 2 (8)	0.16 \pm 0.02 (16)	0.18 \pm 0.03 (8)	3.12 \pm 2.11 (5)	0.58 \pm 0.24 (5)	0.0048 \pm 0.003 (5)	0.001 \pm 0.0006 (5)
0.005	27 \pm 2 (9)	27 \pm 1 (12)	0.17 \pm 0.03 (9)	0.18 \pm 0.02 (12)	1.42 \pm 0.21 ^{##} (5)	0.09 \pm 0.07 ^{##} (5)	0.0026 \pm 0.0006 (5)	0.0002 \pm 0.0001 (5)
0.05	26 \pm 1 (12)	27 \pm 2 (10)	0.15 \pm 0.02 (12)	0.17 \pm 0.04 (10)	ND	0.09 \pm 0.05 ^{##} (4)	ND	0.0002 \pm 0.0001 (4)
0.5	29 \pm 1* (9)	29 \pm 1** (7)	0.21 \pm 0.03 [#] (9)	0.23 \pm 0.04** (7)	0.87 \pm 0.11 ^{##} (5)	0.09 \pm 0.04 ^{##} (5)	0.0019 \pm 0.0002 (5)	0.0002 \pm 0.0001 (5)

ND, no data available for gonad weights of females.

*Longer than triolein-injected controls and 0.05-exposed females ($p < 0.05$). **Longer or heavier than triolein-injected controls and lower dose exposed males ($p < 0.05$). # Heavier than triolein-injected controls and lower dose exposed females. ## Lower GSI than triolein-injected controls ($p < 0.05$).

of Stewart et al. (2000), 227 ± 22 ng *o,p'*-DDT/egg was required for sex reversal, an exposure approximately 400 times our highest concentration. Metcalfe et al. (2000) have reported that maternal exposure to *o,p'*-DDT (egg concentrations estimated at 80–102 ng/egg) failed to produce intersex progeny. Although the lack of male offspring produced by treated females might have been indicative of complete feminization of genetic males, these authors did not consider this finding definitive evidence of sex reversal because their experiments were not conducted with fish that had a male genetic marker (e.g., d-rR strain). Carlson et al. (2000) injected groups of mixed-sex and monosex rainbow trout (*Oncorhynchus mykiss*) and mixed-sex chinook salmon (*Oncorhynchus tshawytscha*) embryos with *o,p'*-DDE in ranges of 10–160 mg/kg and 1–80 mg/kg, respectively. In their first set of trout experiments, these authors observed an elevated male:female sex ratio at 80 and 160 mg/kg and a single intersex individual at the lethally toxic 160 mg/kg exposure. Their subsequent experiments with trout and salmon did not indicate an increase in males over females, and no further evidence of intersex was found. The lack of an effect on sexual phenotype after *in ovo* *o,p'*-DDE exposure in the experiments of Carlson et al. (2000) are consistent with our results with medaka.

However, whereas we observed a marked effect on GSI, they reported none for the subsample of females from the mixed-sex trout experiment that were reared to maturity; male GSI was not reported. Carlson et al. (2000) concluded that *o,p'*-DDE-induced mortality was likely to mask the more subtle effects on sexual development.

The observed effects on gonadal development and histopathology in the present study are consistent with some previous studies of effects of DDT exposure on fishes, yet inconsistent with other studies. Female white croakers (*Genyonemus lineatus*) collected from San Pedro Bay, California, with body burdens of ≥ 3.8 ppm Σ DDT (total of all isomers of DDT and its breakdown products) could not be induced to spawn and exhibited only early-stage oocytes, of which high numbers were atretic relative to reference fish (Hose et al. 1989). In contrast to our results, Metcalfe et al. (2000) reported advanced oogenesis in female medaka developed from eggs with *o,p'*-DDT concentrations of approximately 80–102 ng/egg, although both reference and treated groups began producing viable eggs at the same time. Males in this same study did not display small gonads and associated secondary sexual effects (lack of breeding tubercles) that we observed. However, when Metcalfe et al. (2000) exposed these male

progeny to estradiol at 10 months of age, they found that the male offspring showed significantly greater induction of vitellogenin than did controls, suggesting that early DDT exposure may have potentiated vitellogenin induction later in life.

The effects of *o,p'*-DDE we observed on gonadal development in fish are also similar to the effects reportedly caused by estrogen exposure. *In ovo* exposure of medaka to ethinyl estradiol also produced fish with small ovaries with mostly perinuclear and atretic oocytes, and normal, but less mature, testes (Papoulias et al. 1999). Direct comparisons between results of other estrogen exposure studies and our *o,p'*-DDE results may be questionable because of differences in developmental stages at which fish were exposed. Adult fathead minnow (*Pimephales promelas*) males exposed for 21 days to low concentrations of estrogen and estrone showed complete inhibition of testicular growth during recrudescence (Panter et al. 1998). Exposing sexually mature male and female fathead minnows to 17α -estradiol for 14 days caused a reduction in male secondary sexual characteristic development and degenerative testis changes, whereas ovarian development appeared retarded and produced more atretic oocytes than in unexposed females (Miles-Richardson et al. 1999). It is interesting to note that male summer flounder (*Paralichthys denatus*) exposed as juveniles to *o,p'*-DDT displayed degenerative features similar to those in adult fatheads exposed to estrogen (Zarogian et al. 2001), perhaps further supporting the idea that exposure effects will vary depending on the life stage at which exposure occurs.

Estrogen exposure typically has the toxic effect of repressing gonadal and somatic growth; consequently, we do not consider that the larger size of the fish in our highest *o,p'*-DDE treatment was due to anabolic effects of the xenoestrogen (Donaldson et al. 1979; Papoulias et al. 1999). Although we attempted to feed our fish to excess, we cannot be certain that the body size differences we saw were due to an *o,p'*-DDE effect because of the lowered survival of the highest treatment group. Higher mortality resulted in lower tank density, potentially allowing more food per fish in these treatments. Nevertheless, gonad weight relative to somatic weight decreased with increasing *o,p'*-DDE concentration. We suggest that exposure to nonlethal concentrations of (xeno)estrogens early in development may be analogous to chemical castration in mammals, which diverts energetic resources normally used to develop mature gonads toward somatic growth.

Our results indicate that male medaka embryos exposed to ≤ 0.5 ng/embryo of *o,p'*-DDE are not at risk of being feminized. Although estrogens at this concentration can

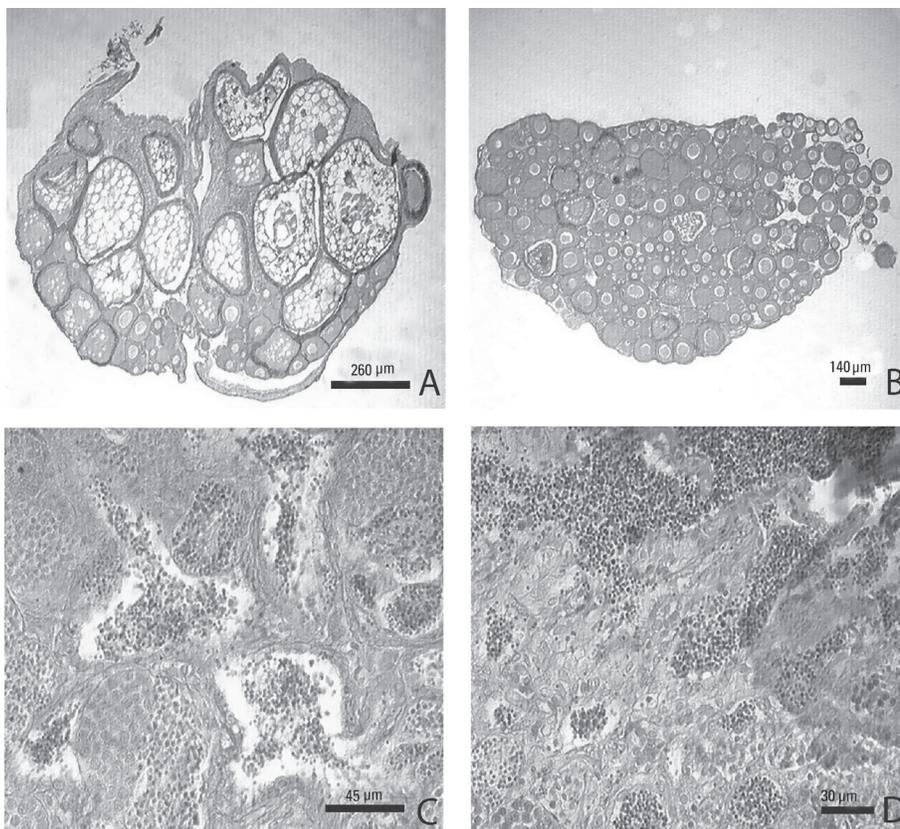


Figure 1. Gonadal sections of female (A and B) and male (C and D) of adult d-rR medaka exposed *in ovo* to triolein solvent (A and C) and 0.5 ng *o,p'*-DDE/embryo (B and D).

cause sex reversal (Papoulias et al. 1999), the comparatively lower affinity of *o,p'*-DDE and *o,p'*-DDT for the ER (Nimrod and Benson 1997) suggests that higher exposures of these compounds are required for sex reversal to occur. Nevertheless, *o,p'*-DDT and *o,p'*-DDE at low concentrations may interfere with the binding of natural ligands to a variety of steroid binding moieties (e.g., receptors and binding proteins) (Danzo 1997; Donohoe and Curtis 1996; Gaido et al. 1997; Kupfer and Bulger 1976; Lundholm 1998; Mason and Schulte 1980; Nimrod and Benson 1997; Shilling and Williams 2000; Wells and Van Der Kraak 2000), thereby allowing for endocrine-disrupting effects.

Whether the effects on gonad development that we observed in the medaka fish result from disruption of the endocrine system specifically or are a consequence of other mechanisms of toxicity was not determined in the present study. Chemically induced genotoxic and nongenotoxic effects during development may manifest at early or later ontogenetic stages (McNabb et al. 1999). Although our method of exposing the developing embryos through injection mimics maternal transfer of contaminants, it also allows for disruption of the development and differentiation of many tissues and processes specifically occurring during embryogenesis. Reproductive toxicity of the *o,p'*-DDE-exposed medaka in our study was evident by the small, underdeveloped gonads and suggests early impairment along the brain–pituitary–gonadal axis.

Few data are available regarding environmental concentrations of *o,p'*-DDE in eggs and embryos from which risks to fish populations can be assessed. Concentrations found in Lake Michigan salmonid eggs have been reported to range from 3 to 150 µg/kg (Giesy et al. 1986; Miller 1993). Data collected in the early 1990s from the northwestern United States indicate that fish tissue concentrations of 8–22 µg/kg may still be encountered (Brown 1997; Munn and Gruber 1997). At these *o,p'*-DDE concentrations, toxic effects were observed in our medaka.

We have shown that the reproductive development of both males and females may be impaired by maternally derived concentrations of *o,p'*-DDE as low as 0.005 ng/embryo (or 5 ng/g egg). Our observations of small but normal male testes and abnormal female ovaries after *in ovo* *o,p'*-DDE exposure may be indicative of disruption of early endocrine

processes that lead to normal ovarian and testicular development. Although *o,p'*-DDE body burdens are generally quite low (parts per billion), our data suggest that *o,p'*-DDE may affect sexual development but is unlikely to affect sexual differentiation in offspring of most exposed fishes.

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