Salmonid Sexual Development Is Not Consistently Altered by Embryonic Exposure to Endocrine-Active Chemicals

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Fish sexual development is sensitive to exogenous hormone manipulation, and salmonids have been used extensively as environmental sentinels and models for biomedical research. We simulated maternal transfer of contaminants by microinjecting rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) embryos. Fish were reared for 6 months and sexed, and gonads were removed for histology and measurement of *in vitro* steroid production. Analysis of fat samples showed that dichlorodiphenylethylene (DDE) levels, *o,p′*-DDE and *p,p′*-DDE isomers, were elevated 6 months after treatment. A preliminary study showed an increased ratio of males to females after treatment with 80 mg/kg and 160 mg/kg of the xenoestrogen *o,p′*-DDE. One fish treated with 160 mg/kg *o,p′*-DDE had gonads with cells typical of both males and females. A follow-up study, using more fish and excluding the highly toxic 160 mg/kg *o,p′*-DDE dose, showed no effect on sex ratio or gonadal histology. Embryonic exposure of monosex male trout, monosex female trout, and mixed sex salmon to *o,p′*-DDE, *p,p′*-DDE, mixtures of DDE isomers, and octylphenol failed to alter sexual development. We observed no treatment-dependent changes in *in vitro* gonadal steroid production in any experiments. Trout exposed *in ovo* and reared to maturity spawned successfully. These results suggest that mortality attributable to the xenoestrogens *o,p′*-DDE, chlordecone, and octylphenol, and the antiandrogen *p,p′*-DDE, is likely to occur before the appearance of subtle changes in sexual development. Because trout appeared to be sensitive to endocrine disruption, we cannot dismiss the threat of heavily contaminated sites or complex mixtures to normal sexual development of salmonids or other aquatic organisms. *Key words:* chlordecone, DDE, endocrine disruption, maternal transfer, octylphenol, *Oncorhynchus*, rainbow trout, sex differentiation, xenoestrogens. *Environ Health Perspect* 108:249–255 (2000). [Online 3 February 2000]

http://ehpnet1.niehs.nih.gov/docs/2000/108p249-255carlson/abstract.html

Many anthropogenic and naturally occurring chemicals are known to interact with endocrine systems of animals. Organochlorine (OC) pollutants and alkylphenolic detergents are two classes of persistent endocrine active chemicals (EACs). OCs include industrial chemicals and contaminants such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins (PCDDs), and insecticides such as DDT (and metabolites) and chlordecone (Kepone). The production and use of OCs has ceased in most industrialized nations; however, their use continues in developing countries. Global atmospheric transport results in ubiquitous contamination, including Arctic regions, marine mammals, and humans (*1–3*). Unlike OCs, production and use of alkylphenol ethoxylate surfactants, particularly in sewage treatment works, is common and ongoing. The persistence and estrogenicity of alkylphenols has been well documented (*4*).

Laboratory and field data have implicated OCs in impaired reproductive success and abnormal sexual development in fish and wildlife species. DDT and its metabolites have caused egg shell thinning and endocrine and reproductive toxicity in wild birds, altering population structure (*5*). Sexual abnormalities reported in Florida alligators are thought to be caused by the demasculinizing effects of DDT metabolites, including *p,p′*-DDE (*6,7*). There have been reviews that discussed the role of OC exposure in reproductive and sexual abnormalities in Great Lakes salmon (*8*) and in marine mammals (*2*). Feminization of trout in United Kingdom rivers has been observed and is likely due to contaminants in sewage outfalls, which include alkylphenols and pharmacologic estrogens (*9,10*). Laboratory studies with OCs and alkylphenols, including *o,p′*-DDE, chlordecone, and octylphenol, have documented the estrogenicity of these chemicals (*4,11–13*).

Because hormones are involved in the etiology of various human cancers, EACs have been predicted to increase cancer risks. Selected human populations are exposed to high levels of OCs from chemical manufacturing, heavy pesticide use, point-source pollution, and diets high in contaminated fish. Correlations have been suggested between xenoestrogens and breast cancer (*14*) and declining sperm counts (*15*); however, causal links with adverse human health effects have not been established (*16,17*). *In utero* exposure to diethylstilbestrol (DES), a potent nonsteroidal pharmacologic estrogen, has been linked to rare vaginal cancers in women and to sex organ alterations in men (*18,19*). Developmental exposure of EACs to humans *in utero* and from breast milk has been documented (*20*) and implicated in permanent cognitive dysfunction (*21*).

Rainbow trout, *Oncorhynchus mykiss*, have been studied extensively in fisheries, environmental, biomedical, and endocrine research. Lipophilic OCs and alkylphenols are resistant to metabolism, resulting in bioaccumulation in fish and subsequent human exposure from fish consumption. Female fish transfer persistent chemicals to eggs, effectively clearing contaminant burdens, but exposing developing embryos to EACs (*22,23*). These chemicals may alter sexual development of embryos, which has been documented in laboratory studies with other chemicals in fish and reptiles (*24–26*). There is a great deal of sexual plasticity in teleost fish, but sex steroid hormones appear to control sexual characteristics in all species (*27–29*). Complete and partial feminization of trout has been achieved by various estrogen treatments to eggs and fry (*30,31*), and androgens can be used to create functional all-male populations of salmonids (*24,32*). Hormone receptor expression, endocrine feedback loops, and steroid-metabolizing enzymes can be altered by chemical exposure (*11,33–35*). Although physiologic consequences may differ, biochemical and molecular functions of steroid receptors in fish seem to be homologous to humans (*36–38*).

In this study, we examined the effects of the xenoestrogens *o,p′*-DDE, chlordecone,

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Monosex trout embryos were provided by G. Thorgaard. We thank C. Schreck, M. Fitzpatrick, and R. Chitwood for providing laboratory facilities, assistance with steroid radioimmunoassays, aquaculture, and critical scientific discussions; J. Spitzbergen for providing expert histopathology assistance; R. Inman for analysis of DDE residues; and the staff of the Food Toxicology and Nutrition Laboratory for technical assistance.

Partially supported by NIH grants ES-07060, ES-03850, ES-04766.

Received 25 May 1999; accepted 18 October 1999.

and octylphenol, and the antiandrogen *p,p′*-DDE, on various end points of sexual development in rainbow trout. We chose to study DDE metabolites of DDT because of their persistence and residual prevalence *in vivo* in all vertebrates. A general time line of trout sexual development is shown in Figure 1. Rainbow trout sexual differentiation probably occurs between hatch and yolk sac absorption (*24,29,30*). Salmonids are generally sensitive to complete reversal of genetic sex before the completion of sexual differentiation (*9,24,29,32*); however, partial sex changes are possible after gonadal differentiation (*24,30*). At early stages of development, including egg hardening [0–2 days postfertilization (DPF)] and organ formation (7–21 DPF), embryos are fragile, and handling can result in high mortality. Embryos that have completed organ development are better able to withstand chemical and physical manipulation. The completion of organogenesis is marked by the appearance of recognizable embryo eyes, at which time chemicals can be microinjected into the yolk to ensure accurate dosing, as previously described by Black et al. (*39*). The injections, timed to precede sexual differentiation, allow elevated body burdens of persistent contaminants throughout sexual development to model exposure to maternally transferred chemicals. We determined sex and examined gonadal morphology and histology approximately 6 months after treatment. We studied immature gonads *in vitro* to determine the steroid synthesizing potential of exposed fish. We reared subsamples of fish to sexual maturity and assessed gonadal maturity, fertility, and egg viability. We conducted experiments with mixed-sex populations, as well as single-sex populations of male and female fish. We also treated chinook salmon (*Oncorhynchus tshawytscha*), which are sensitive to complete feminization by steroid hormones (*40*), with *o,p′*-DDE to facilitate species comparisons of sexual development.

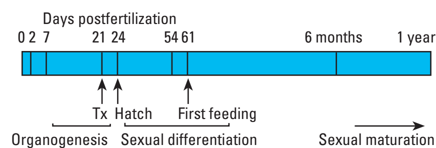

Figure 1. Timeline of rainbow trout sexual development.[a] DPF, days postfertilization: 0–2 DPF, egg hardening (early embryogenesis); 7–21 DPF, organogenesis (eye pigmentation indicates development stage/rate); 21 DPF, injection (Tx); 24–28 DPF, hatch (period of high stress and mortality); 54 DPF, yolk-sac absorption (assimilation and redistribution of yolk products); 61 DPF, first feeding (period of high stress and mortality); 4–6 months, gonads become recognizable; 1–2 years, gonadal maturation and spawning.
[a]All time estimates are for trout reared at 12°C; the development rate is slowed by cooler water temperatures. Chinook salmon have the same developmental stages, but growth is slower.

Materials and Methods

Chemicals. We obtained *o,p′*-DDE [100% pure; 1,1-dichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethylene] and *p,p′*-DDE [100% pure; 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene], two persistent metabolites of 1,1-dichlorodiphenyl-trichloroethane (DDT), from AccuStandard (New Haven, CT). We purchased 4-*tert*-octylphenol (OP; 97% pure) from Aldrich Chemical Co. (Milwaukee, WI); chlordecone (99.5% pure) from ChemService (West Chester, PA); and ring-labeled [^{14}C]*p,p′*-DDE (> 95% pure; 12.7 µCi/µmol), [^{14}C]chlordecone (96% pure; 6.1 µCi/µmol), and salmon pituitary powder from Sigma Chemical Co. (St. Louis, MO). We prepared a mixture of *p,p′*-DDE and *o,p′*-DDE (5.4:1, mass:mass) based on the ratio of technical grade DDT isomers and residual environmental levels (*40–42*). All chemicals were dissolved in menhaden oil for microinjection of embryos. We purchased sterile RPMI-1640 culture medium (without glutamine) and gentamicin sulfate (50 mg/mL) from Whitakker Bioproducts (Baltimore, MD).

Embryo microinjections. We obtained Mt. Shasta strain rainbow trout (*Oncorhynchus mykiss*) eggs from the Marine/Freshwater Biomedical Sciences Center aquaculture facility at Oregon State University and chinook salmon (*Oncorhynchus tshawytscha*) eggs from the Fish Genetics and Performance Laboratory at Smith Farm, Oregon State University. All-male and all-female rainbow trout were provided by Gary Thorgaard (Washington State University, Pullman, WA). Fish were maintained and euthanized with the approval of the Institutional Animal Care and Use Committee at Oregon State University. Trout were maintained in tanks with continuously running well water, 12–14°C, on a 12 hr light:dark photoperiod, except for experiment 1 and the maturation stage of experiment 2, which were subjected to the natural photoperiod.

We microinjected 1 µL menhaden oil containing the chemical (or vehicle only) into the yolk of eyed embryos (21 DPF; average weight 110 mg/egg) using a Hamilton MicroLab 900 automated syringe pump (Hamilton Co., Reno, NV) fitted with a 31 gauge needle. This type of microinjection was first used successfully in trout to administer accurate carcinogen doses (*39*). In preliminary range-finding experiments, we injected 6-month-old trout intraperitoneally (ip) with DDE and chlordecone to identify approximate lowest-observed-adverse-effect level (LOAEL) doses for embryo injections. Doses of OP were below embryo-lethal levels (*43*). We performed five microinjection experiments as follows:

- Experiment 1: 100 eggs/treatment (in duplicate) from a mixed-sex rainbow trout population were microinjected with 40, 80, or 160 mg/kg *o,p′*-DDE or 7.5, 15, or 30 mg/kg chlordecone
- Experiment 2: 150 eggs/treatment (in duplicate) from a mixed-sex rainbow trout population were treated with 10, 40, or 80 mg/kg *o,p′*-DDE; 10, 40, or 80 mg/kg *p,p′*-DDE; or 10, 40, or 80 mg/kg DDE mixture
- Experiment 3: monosex male trout were microinjected with 1, 40, or 80 mg/kg *o,p′*-DDE (100 eggs/treatment) or 0.01, 0.1, or 1 mg/kg OP (65 eggs/treatment)
- Experiment 4: monosex female trout (50 eggs/treatment, in duplicate) were microinjected with 1, 10, 40, or 80 mg/kg *o,p′*-DDE or 0.01, 0.1, or 1 mg/kg OP
- Experiment 5: chinook salmon (85–100 eggs/treatment) were microinjected, in duplicate, with 1 or 80 mg/kg *o,p′*-DDE (in 3 µL vehicle), or with 13.33 mg/kg (in 1 µL vehicle) or 40 mg/kg *o,p′*-DDE (in 3 µL vehicle) for which there were no duplicates.

Mortality was monitored over time and fish were reared for approximately 6 months, until gonads were large enough for the sex to be easily determined and for gonads to be removed. We used analysis of variance (ANOVA) to determine mortality differences after hatch and time of first feeding after yolk absorption, two critical windows of fish development (Figure 1). We compared sex ratios by chi-square analysis.

Gonad incubations and histology. After approximately 6 months, we anesthetized the fish in a 50 mg/L solution of tricaine-methanesulfonate (MS222; Argent Chemicals Laboratories, Redmond, WA). We recorded weights and determined the sex by gross examination of gonad morphology of each fish. Gonads from at least 10 fish/treatment were removed and placed in 24-well culture plates containing ice cold medium (RPMI-1640). Fish were then killed by severing the spinal cord. *In vitro* gonadal steroid production was measured using a modification of the method of Fitzpatrick et al. (*30*). Gonads were washed twice in 1 mL RPMI-1640 containing 50 µg gentamicin/mL for 1 hr. In preliminary experiments, we had determined that the addition of 10 µg salmon pituitary extract per milliliter of medium stimulated maximal, linearly increasing steroid production by gonads for up to 24 hr. Thus, steroid hormone production was induced by addition of 12.5 µg salmon pituitary powder into 1.25 mL medium. Gonads were incubated for 24 hr at 4°C in a 95% O_2:5% CO_2 gas mixture in sealed chambers. After incubation, media was removed and stored at

-80°C until sex steroids were measured by radioimmunoassay (*30*). We detected no sex steroids in media alone or when gonads were incubated in the absence of pituitary powder. Histology of gonads confirmed that tissues were viable after 24-hr incubations. We compared steroid production between treatments by ANOVA and the nonparametric Kruskal-Wallis and Wilcoxon Tests (StatView v4.5, Abacus Concepts, Berkeley, CA).

At least 10 pairs of gonads from each treatment were fixed in 10% buffered formalin for histologic examination. After fixation, tissues were embedded in paraffin and sectioned into 5 µm slices. Sections (three to six per gonad pair) were stained in hematoxylin and eosin and examined under light microscopy for sex-specific gonad structures and germ cells. Results of histologic sex evaluations were compared to gross observations. With the exception of salmon in experiment 5, gross sexing was confirmed to be > 99% accurate, and data represent the gross sex of all individual fish. Salmon sex ratio data consisted only of histologically confirmed sexes.

Residue analysis and half-life. We used radiochemicals to estimate the persistence of DDE isomers and chlordecone in fish. We injected embryos (21 DPF) with 10 or 80 mg/kg [^{14}C]*p,p′*-DDE or 7.5 or 15 mg/kg [^{14}C]chlordecone. Eggs were maintained in an aerated, static water bath for 20 days; subsamples were removed and snap frozen in liquid N_2 at 0, 1, 7, and 20 days. Embryos or fry (hatch occurred at 25 DPF) were solubilized in 1 mL soluene and shaken overnight in a 37°C water bath. Solutions were decolorized with 30% H_2O_2 and cooled, and radioactivity was measured by liquid scintillation counting (Beckman LS6500, Beckman Instruments, Fullerton, CA), employing automatic quench and chemiluminescence correction. Estimations of residue half-life were determined by least squares regression using Microsoft Excel, Version 7.0 (Microsoft, Redmond, WA) (*22,23*).

We also measured DDE residues in trout from experiment 2. Fat samples collected at 283 days after egg injections were extracted with hexane and separated by column chromatography on deactivated alumina. DDE isomers were measured on a gas chromatograph equipped with electron capture detector (Varian 3740; Varian, Inc., Palo Alto, CA) in the Environmental Chemistry and Toxicology Laboratory at Oregon State University. Detection limits for *o,p′*-DDE and *p,p′*-DDE isomers were 0.02 and 0.01 µg/g fat, respectively.

Sexual maturation. A subset of mixed-sex trout from experiment 2 were reared for 2.5 years to monitor sexual maturation. Fish were maintained under a natural photoperiod for the final 6 months before the winter spawning season. At maturation, fish were anesthetized in MS222 and eggs were removed from ripe females. We compared total egg weight to total fish weight to determine the gonadal somatic index (GSI) for individual females. We compared total egg weight to individual egg weight (average weight of 30–50 eggs) to estimate fecundity (total eggs released). Eggs from each female were fertilized with a mixture of sperm from at least three stock (control) males. Sperm from experimental males was used to fertilize eggs from two separate control females. From each cross, 100 eggs were removed immediately after fertilization and reared in incubation cups (PVC pipe with fine mesh bottoms) with continuously running water. We determined fertilization success after 24 hr. Timing of hatch, hatch success, and egg mortalities were recorded until 1 week posthatch. Gonads of all mature fish, including those not used for spawning, were examined grossly to determine if fish were maturing normally.

Results

Early life stages of salmonids were more sensitive to *o,p′*-DDE and chlordecone than older fish. Mortality (96 hr) of 2-month-old fish injected ip with *o,p′*-DDE (160 mg/kg highest dose) was 5%; with chlordecone (7.5–30 mg/kg doses), mortality was from 10 to 35% (range-finding data not shown). Mortality of mixed-sex trout injected as embryos with equivalent doses of *o,p′*-DDE was up to 49% above oil-injected controls (Table 1). Chlordecone (experiment 1) mortality ranged from 20 to 45% above oil-injected controls ($p < 0.05$), which increased in a dose–dependent manner. Mortality in *o,p′*-DDE-treated salmon (experiment 5)

Table 1. Mortality (%) of salmonid fry treated as embryos with endocrine active chemicals.

Embryo treatment	Exp 1 (mixed sex)		Exp 2 (mixed sex)		Exp 3 (males)		Exp 4 (females)		Exp 5 (salmon)	
(mg/kg)	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]
Uninjected	17[c]	19[c]	17[c]	19[c]	8	12	1	11	23	36
Oil injected	3	22	18	45	56[d]	56[d]	5	20	26	46
o,p′-DDE										
1	–	–	–	–	42	44	3	13	27	57
10	–	–	17	58*	–	–	8	22	–	–
40	14*	29*	17	54*	55	62	8	28	10	23
80	10*	38*	32	75**	47	47	3	29	30	50
160	13*	71**	–	–	–	–	–	–	–	–
Chlordecone										
7.5	27*	42*	–	–	–	–	–	–	–	–
15	15*	51*	–	–	–	–	–	–	–	–
30	22*	67**	–	–	–	–	–	–	–	–
p,p-DDE										
10	–	–	12	48	–	–	–	–	–	–
40	–	–	11	44	–	–	–	–	–	–
80	–	–	13	48	–	–	–	–	–	–
DDE mix										
10	–	–	15	74*	–	–	–	–	–	–
40	–	–	13	51	–	–	–	–	–	–
80	–	–	8	55	–	–	–	–	–	–
Octylphenol										
0.01	–	–	–	–	57	62	4	14	–	–
0.1	–	–	–	–	68	69	5	8	–	–
1	–	–	–	–	25	29	3	6	–	–

Exp, experiment. Mortality from Exp 3 and Exp 4 genetically manipulated populations should not be used to estimate male and female mortality in stock population.
[a]Mortality measured approximately 7 days post-hatch. [b]Mortality measured approximately 14 days after fish were fed for the first time. [c]Estimated mortality in single stock fish spawn, which corresponds well with historic levels of mortality in this partially inbred population (personal observation). [d]Higher mortality than uninjected controls ($p < 0.05$). *Higher mortality than oil-injected controls of the same experiment ($p < 0.05$). **Higher mortality than lower dose treatments and oil-injected controls ($p < 0.05$).

was no different than oil-injected controls (Table 1). In monosex trout populations, mortality in treatment groups did not differ from oil-injected controls; however, the injection itself increased mortality in males (experiment 3, Table 1). Octylphenol exposure did not affect survival in a dose–dependent manner in monosex male or female trout (Table 1). Although mortality was lower in treated monosex female fish as compared to monosex males (Table 1), data were confounded because fish were genetically manipulated during fertilization and because high male mortality was treatment independent. Approximately 50% of monosex males and 80–90% of monosex females died before chemical treatment, which resulted in varying degrees of selection for hearty fish.

Xenoestrogen treatment in experiment 1 resulted in increased ratios of males:females at all doses. Results were consistent between duplicate treatments, and final analyses were performed on pooled data from tanks containing replicate treatments. There were significantly more males than females in the treatment groups for 80 and 160 mg/kg *o,p′*-DDE (Figure 2). Trends from chlordecone treatments suggested an increased male:female ratio (1.9, 1.25, and 2.25 at 7.5, 15, and 30 mg/kg, respectively), but the sex ratios were not statistically different from the control ratio of 1:1 (Figure 2). Similar experiments using rainbow trout (experiment 2) and chinook salmon (experiment 5) failed to replicate the changes in sex ratio observed after *o,p′*-DDE treatment in experiment 1. Neither *p,p′*-DDE, DDE mixtures, nor octylphenol treatment altered sex ratios in trout or salmon. Monosex populations of rainbow trout were also treated with *o,p′*-DDE and octylphenol, but no sex reversal was observed; all fish in experiment 3 were male and all fish in experiment 4 were female, regardless of treatment. Histologic examination confirmed that gross observations of sexual phenotype were correct for trout. However, gross determination of salmon sex often resulted in incorrectly scoring males as females. Consequently, salmon sex ratios reflect only information obtained histologically.

We observed one pair of unusual gonads from a fish treated with 160 mg/kg *o,p′*-DDE in experiment 1. Under gross examination these gonads were phenotypically female, but under light microscopy, distinct male and female germ cells were apparent within each gonad (Figure 3). Germ cells were not mixed throughout the gonad, rather two distinct portions of the gonad existed, each containing cells indicative of male or female gonads. All other gonads were normal in all experiments, containing either male or female organization and cells.

DDEs and chlordecone were successfully administered to eggs and persisted throughout the period of sexual differentiation in developing fish. The elimination of radiolabeled [^{14}C]chlordecone and [^{14}C]*p,p′*-DDE from eggs provided estimates of the chlordecone half-life (19–29 days for 7.5 and 15 mg/kg doses) and *p,p′*-DDE half-life (347–408 days; 10 and 80 mg/kg doses). We also measured DDE residues in fat samples taken from fish 283 days after injection in experiment 2. Mean residues were 0.033 and 0.15 ppm after treatment with 10 and 40 mg/kg *o,p′*- DDE respectively, and 0.402, 1.4, and 2.7 ppm after treatment with 10, 40, and 80 mg/kg *p,p′*-DDE, respectively. Table 2 shows that both *o,p′*-DDE and *p,p′*-DDE were present in treated fish, but not in oil-injected fish. Detection limits were 0.02 and 0.01 mg/g fat for *o,p′*-DDE and *p,p′*-DDE, respectively. We estimated the total body burden of DDE isomers based on the fact that approximately 6.98% of total body weight is fat in juvenile rainbow trout (*44*). Residues of *p,p′*-DDE predicted by half-life after 283 days were 61.3% and 72.1% for low and high doses, respectively; these levels were similar to the observed levels of 78.3% and 57% for the original dose (Table 2). Two things probably contribute to higher observed environmental levels of *p,p′*-DDE: *p,p′*-DDE is more persistent in fish than *o,p′*-DDE (shown here), and larger amounts of *p,p′*-isomers are used in technical mixtures.

Treatment with these known and putative xenoestrogens and antiandrogen did not alter *in vitro* steroid production by gonads in a dose-dependent manner in any experiments. However, gonadal steroid production proved to be sensitive to embryonic manipulation, as evidenced by approximately 50% decreased androgen production (testosterone and 11-ketotestosterone) by gonads from male fish injected with chemicals or vehicle alone in experiment 3, as compared to uninjected controls ($p < 0.05$; data not shown). This response seemed to be related to the injection itself because androgen levels did not vary between any treatment group and oil-injected controls.

Subsets of fish from mixed-sex (experiment 2) and all-male (experiment 3) treatments were reared for 2–2.5 years, until sexual maturation. We performed gross examinations of at least 10 fish from each treatment, and all fish had normal gonads. Ripe males and females from experiment 2 spawned successfully with control fish. Fertilization success was virtually 100%, and most eggs survived until well-developed embryos were visible within eggs (14 DPF). Mortality increased, in general, after 14 DPF. Survival of progeny from males was greater than from females, although survival did not correlate well with *in ovo* toxicant exposure (Table 3). Mortality may have increased in males treated with increasing *p,p′*-DDE, but statistical measures and trends could not be established from the subsample of fish spawned (Table 3). We observed no treatment-dependent differences in female GSI or egg production. Crosses of *in ovo* exposed males and females also developed and hatched successfully (data not shown). Although these data do not represent a comprehensive study of reproductive effects on population dynamics, they show that treated fish seemed to be maturing normally.

Discussion

Doses of *o,p′*-DDE and chlordecone that had little effect on trout fry and juveniles were lethal to embryos and yolk-sac fry. Lethality varied little between 4 days and 14 days in fry injected ip, suggesting that absorption of toxicants did not increase appreciably after 4 days (range-finding experiment). Embryos were injected with DDEs and chlordecone at what proved to be maximally tolerable doses, which are not likely to occur in nature except perhaps at highly contaminated sites. That developing embryos were more susceptible to toxicants than older fish was not surprising and suggests that maternal transfer of chemicals from females living in heavily contaminated waters could affect embryo survival and local fish populations.

There were two unintended consequences of microinjection of eggs with vehicle alone. Injection of eggs decreased time to hatch in both trout and salmon (personal observation), which may increase background mortality rates. The mortality of oil-injected controls in our experiments was not higher than historical levels or higher than comparable uninjected controls except in experiments 2 and 3. In those experiments, increases in mortality may have been because injections altered development or because of genetic variations in brood fish (experiment 2) or genetically manipulated offspring (experiment 3). Injection with oil or chemicals also

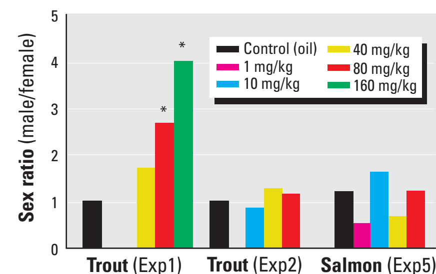

Figure 2. Ratio of males:females in response to embryonic *o,p′*-DDE treatment in rainbow trout and salmon. Exp, experiment.

*$p < 0.05$ for 80 mg/kg ($n = 33$) and 160 mg/kg ($n = 15$) treatments.

caused a dose-independent decrease in androgen production by males in experiment 3. In *in vitro* steroid production experiments performed 6 months after exposure, male gonads produced approximately 50% less testosterone and 11-ketotestosterone than uninjected fish. No differences were observed between treatment groups and oil-injected controls, suggesting an association with the injection itself. Thin layer chromatography of menhaden oil vehicle revealed few components other than triacylglycerides; chromatography plates appeared identical in composition to a canola oil control. Changes in steroid production could be a result of an unidentified contaminant or steroid in the oil vehicle, altered development due to injection, or a stress response due to egg manipulation. Topical application in DMSO resulted in approximately 30% incorporation of toxicants into eggs (*45*). Although toxicant absorption was lower after DMSO applications than after injections, topical application may be a less stressful dosing technique (*46*).

There were no obvious explanations why sex ratios were affected in experiment 1 but not in other experiments. Fish came from different spawns, and light cycles (natural vs. 12 hr light:dark) and water temperatures (12–14°C) may have varied slightly, but those conditions varied between unaffected treatments (experiments 2–4) as well. Mortality rates may have been higher in females than in males in experiment 1, but results should have been similar in experiment 2. No data from the current studies or from the literature suggest a mechanism for increased female mortality for the chemicals tested. Unfortunately, attempts to compare mortality in all-male (experiment 3) and all-female (experiment 4) treatments were confounded by high background mortality rates (approximately 50% in males and 80–90% in females) before experimental treatment. Background mortality was probably due to genetic manipulation of fish. The all-female population was produced by gynogenesis from eggs of a single female, which included ultraviolet irradiation of sperm and subsequent thermal shock of eggs (*47*). All-male fish were produced by fertilizing eggs from a single female with milt of a single androgenetic male that had previously been used to sire all-male offspring. High mortality of embryos before chemical treatment may have had no bearing on post-injection mortality (experiment 3, all-males); however, genetic differences in parent fish, combined with genetic manipulation, make it difficult to compare mortality rates between single-sex populations and natural populations.

Histologic observation of male and female germ cells in one of two fish examined from the 160 mg/kg *o,p′*-DDE exposure group in experiment 1 provides compelling evidence that trout are susceptible to xenoestrogen treatment. Observations of unusual gonads in rainbow trout exposed to PCBs (*48*) and 17β-estradiol (*30*), as well as similar field observations in cricket frogs (*49*), support our assumption that gonadal abnormalities were caused by chemical treatment. We examined gonads from hundreds of fish; although morphologic variability was common, we saw no other gonads that contained

Table 2. DDE residues in fish approximately 9 months (283 days) after embryo exposures.

Embryo treatment (mg/kg)	DDE residue analysis (% original dose) *o,p′*-DDE	*p,p′*-DDE
Oil (vehicle)	BDL[a]	BDL
o,p′-DDE		
10	6.5 ± 0.1	BDL
40	3.8 ± 1.1	< 2.6[b]
p,p′-DDE		
10	BDL	78.3 ± 15.0
40	< 2.5[b]	58 ± 6.8
80	< 1.5[b]	57 ± 14.5
DDE mix[c]		
10	44.2 ± 4.1	109 ± 11.9
40	16.6 ± 5.3	56.9 ± 2.7
80	7.6 ± 2.0	58.8 ± 4.7

Values represent mean ± SE of six fish, with the exception of 10 mg/kg *o,p′*-DDE (n = 2) and 40 mg/kg *p,p′*-DDE (n = 5). The percent of original dose recovered was estimated based on 6.98% total body weight as fat in rainbow trout (*44*).
[a]Below detection limit (0.02 and 0.01 µg/g fat for *o,p′*- and *p,p′*-DDE, respectively). [b]Isomer was detected in some samples. [c]DDE mix was 5.4:1 *p,p′*-DDE:*o,p′*-DDE.

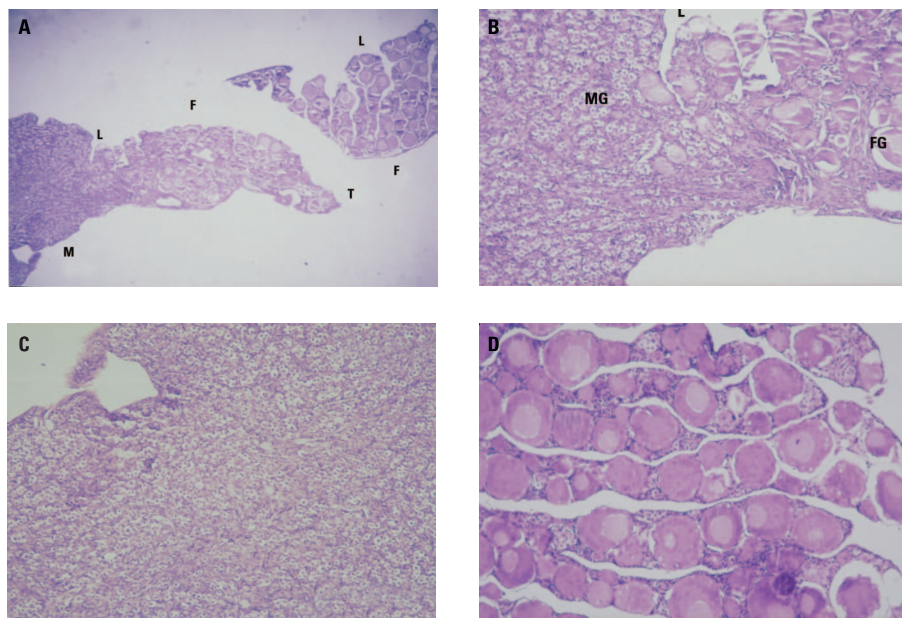

Figure 3. Gonad of 6-month-old morphologic female rainbow trout treated with 160 mg/kg *o,p′*-DDE *in ovo* (experiment 1). Abbreviations: T, tear in gonad, probably not related to treatment; F, characteristic female gonad structure; M, characteristic male gonad structure; L, lamellae structures characteristic of female gonads. (*A*) The gonad appears to have distinct female and male portions, with no apparent mixing of germ cells except around the transition boundary shown here (8× magnification). (*B*) High magnification (132×) of the transition point between the female and male gonad structures. Note the distinct membrane characteristic of female lamellae (L) (appears to be a final lamellar structure rather than a tear in the gonad, as characterized by the intact membrane); a typical female germ cell (FG) at 6 months of age; and the typical cluster arrangement of male germ cells (MG) at this stage of development. This is the only portion of the gonad where male and female germ cells can be seen together. (*C*) Male portion of the gonad representative of a normal male gonad in fish of this age (32× magnification). (*D*) Female portion of the gonad representative of normal female gonad in fish of this age (32× magnification).

Table 3. Reproductive performance in trout exposed as embryos to DDE isomers.

P_1 Treatment (mg/kg)	F_1 Survival (%)[a] P_1 Male (n)	P_1 Female (n)
Untreated	88 (2)	44[b] (2)
Oil (vehicle)	76 (1)	ND
o,p′-DDE		
10	72 (1)	71 (1)
40	85 (2)	ND
80	82 (2)	33 (1)
p,p′-DDE		
10	82 (2)	35 (3)
40	77 (1)	24[b] (2)
80	69 (1)	47 (1)
DDE mix[c]		
10	87 (2)	54 (1)
40	94 (1)	10 (2)
80	78 (2)	ND

Abbreviations: ND, not determined; P_1, parental generation. Trout injected with chemicals as embryos were reared to maturity. Treated fish were crossed with untreated stock fish, and embryo survival was monitored. Gonads were normal in all fish by gross examination.
[a]Survival is represented as the mean of offspring that survived until 1 week after hatch; small samples prevented meaningful SE calculations. [b]Eggs from one female failed to hatch after successful fertilization. [c]DDE mix was 5.4:1 *p,p′*-DDE:*o,p′*-DDE.

both male and female germ cells, suggesting that our observations were not attributable to random genetic variations. Because the 160 mg/kg *o,p′*-DDE treatment was acutely toxic to embryos and is unlikely to be observed in natural waters, we did not repeat studies using that dose. It is possible that gonadal abnormalities are caused by a threshold dose which is higher than all other doses used in our experiments. Similarly, the absence of endocrine abnormalities in OP-treated fish in experiments 3 and 4 may be because doses were below the threshold for adverse effects.

It is possible that injected chemicals may be sequestered away from target tissues or somehow metabolized and cleared from fish. We did not include estrogen treatments as positive controls because trout embryos have the ability to metabolize and excrete exogenously administered estrogens within 24 hr (*50*); thus, trout are not feminized by short-term estrogen exposure. Evidence from radiochemical studies and residue analyses confirmed that DDEs and chlordecone were present in fish throughout the period of sexual differentiation (*24,29,30*). No residue analysis of octylphenol was performed, but its lipophilic nature suggests a long half-life in fish (*51*).

We injected eggs at 21 DPF, after the completion of organogenesis and the most sensitive stages of embryo development. EACs may affect early neuroendocrine and sexual development in trout before the injection times in these experiments, but sexual differentiation in salmonids is incomplete and sensitive to manipulation subsequent to exposure periods tested (*24,29,30*). A recent study in rats provided *in vitro* evidence that dopamine-responsive neurons under estrogen control could be altered by xenoestrogens, suggesting a mechanism for disruption of neuroendocrine development in early central nervous system organization (*52*). The major advantage to studying endocrine development in early fish embryos, either by maternal exposure or early topical application, is that all stages of development would be exposed to EACs. Disadvantages to early fish exposure include the potential for extensive mortality (similar to genetic manipulation), inefficient chemical dosing, and variability of chemical exposure between eggs.

In this study we interpreted normal gonadal steroid production *in vitro* as an indication of normal sexual development in immature fish. Fitzpatrick et al. (*30*) observed decreased *in vitro* steroid production in juvenile trout fed 17β-estradiol and methyltestosterone, which suggests that abnormal development of gonads in our studies would have produced anomalous steroid profiles. Sex steroid production in fish increases during maturation and, similar to human responses to DES (*18,19*), exposure to hormones during development can result in sexual abnormalities that are not apparent until maturation. Feist et al. (*24*) showed that morphologic changes in gonads of functional, sex-reversed male rainbow trout were observed only after sexual maturation. The absence of sexual abnormalities observed in mature males and females suggests that gonads responded normally to natural increases in sex steroids during maturation.

Gametes from a subset of males and females from experiment 2 crossed with either control fish or other treated fish resulted in viable offspring. Genetic variability played a large role in reproductive success, as evidenced by the failure of all crosses with one of two female stock fish (Table 3). Consequently, quantitative measurements of egg viability in treated females were impossible. Successful spawning of treated fish from experiment 2 provided further evidence that fish were maturing normally; however, changes in reproductive indices may have been masked by the small subsample of spawned fish. We monitored embryo development between 14 and 21 DPF by observing the size and intensity of eye pigments; hatch occurred at 24–25 DPF (in 13°C water). Mortality increased between 14 and 21 DPF, and hatch was delayed 1–3 days in eggs spawned from various xenoestrogen-exposed mothers (personal observation). Data from individual males spawned after treatment with the antiandrogen *p,p′*-DDE were also suggestive of decreased offspring survival, relative to increasing embryonic antiandrogen exposure (Table 3). Although fish seemed to be maturing normally and reproducing successfully, offspring survival may have been affected by parental EAC exposure. These observations should be substantiated in future experiments that focus on reproductive indices because subtle differences in egg mortality and hatching time in offspring of EAC-exposed parents could have detrimental effects on fish populations.

We observed significant abnormalities in sexual development in rainbow trout in only one experiment in which fish were treated with *o,p′*-DDE, an estrogenic metabolite of DDT. Embryonic exposure of trout to *p,p′*-DDE, DDE mixtures, chlordecone, and octylphenol failed to significantly alter any of the parameters of sexual development tested. A single experiment with *o,p′*-DDE in chinook salmon, a species sensitive to complete feminization, also failed to cause developmental abnormalities. Our conclusions are consistent with a recent report that *p,p′*-DDE failed to alter sex differentiation in a marine turtle (*53*). With the exception of heavily contaminated sites (*21,54*), environmental residues are unlikely to approach levels that would expose fish to doses used in this study. Such contamination, if present, would probably alter fish populations because of increased lethality before more subtle endocrine disrupting effects could occur. Although salmonid fishes are advantageous models for biomedical research, long life spans and low sensitivity to environment levels of EACs point to aquarium fish as better models for multigenerational endocrine toxicity studies. We cannot dismiss the potential hazards of complex mixtures or variations due to species differences, but we can conclude that average environmental levels of the chemicals tested are unlikely to have profound effects on endocrine development in salmonids.

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