

Short-Term Exposure of Chinook Salmon (*Oncorhynchus tshawytscha*) to *o,p'*-DDE or DMSO during Early Life-History Stages Causes Long-Term Humoral Immunosuppression

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We evaluated the effect of short-term exposures to a xenobiotic chemical during early life-history stages on the long-term immune competence of chinook salmon (*Oncorhynchus tshawytscha*). Immersion of chinook salmon eggs in a nominal concentration of *o,p'*-dichlorodiphenyldichloroethylene (*o,p'*-DDE; 10 ppm) for 1 hr at fertilization followed by immersion in the same dose for 2 hr at hatch resulted in a significant reduction in the ability of splenic leukocytes from fish 1 year after treatment to undergo blastogenesis upon *in vitro* stimulation with lipopolysaccharide. We also observed that the vehicle, dimethyl sulfoxide (DMSO), caused a significant reduction in the ability of the splenic leukocytes to express surface immunoglobulin M (SIgM) at this time. The concentration of *o,p'*-DDE in a pooled sample of whole fry from this treatment was 0.53 µg/g lipid 1 month after first feeding but was undetectable in all other treatments. Mortality rate, time to hatch, fish length, and weight were unaffected by treatment with *o,p'*-DDE. Similarly, sex ratios, gonadal development, and concentrations of plasma estradiol and 11-ketotestosterone were not affected by the treatment. In addition, we found no evidence that plasma lysozyme concentrations or the mitogenic responses of splenic leukocytes to concanavalin A or polyinosinic-polycytidylic acid were influenced by the treatment. In this experiment, a brief period of exposure to *o,p'*-DDE or DMSO during early development was able to induce long-term effects on humoral immune competence of chinook salmon. Such immunosuppression may increase susceptibility to disease, which may in turn be critical to regulating the population. **Key words:** chinook salmon, DDE, DMSO, endocrine-disrupting chemical, immunosuppression, organochlorine, xenobiotic. *Environ Health Perspect* 111:1601–1607 (2003). doi:10.1289/ehp.6171 available via <http://dx.doi.org/> [Online 12 June 2003]

The status of salmonid stocks in the Pacific Northwest is of growing concern. As of 2003, 13 of the 17 evolutionary significant units (ESUs) in the Columbia River basin have been listed as threatened or endangered under the Endangered Species Act (National Oceanic and Atmospheric Administration 2003). In addition, several other ESUs from other basins have been listed or are under consideration for listing. To date, most research into the decline has focused on the decline in habitat and the effect of overharvesting, hydropower dams, and hatchery production. However, in many fish populations, including salmonids, substantial mortality occurs during the early life-history stages. Incidences of disease can be critical in population regulation at these stages (Freeland 1983), and increased susceptibility to disease due to immunosuppression may contribute to the decline of fish populations exposed to chemical contaminants (Arkoosh et al. 1998a). One such contaminant is the organochlorine 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), a pesticide widely used in the United States until 1972 (Cooper 1991). Despite evidence that DDT impairs developmental and reproductive success in fish and wildlife species (Donohoe and Curtis 1996; Fry 1995; Guillette et al. 1994,

1996), it is still used in some countries. Furthermore, global atmospheric transport results in ubiquitous contamination in the environment. Dichlorodiphenyldichloroethylene (DDE) is the primary metabolite of DDT. Its lipophilic nature is such that it tends to accumulate in sediments and has been measured in several estuaries on the Pacific Coast of Oregon and detected in several benthic species in the Pacific Northwest (Foster et al. 2001; Oregon Department of Environmental Quality 2000). To our knowledge, wild salmonids in the Pacific Northwest have not been examined for the presence of DDE; however, it has been detected in salmonids in the Great Lakes (Merna 1986; Norstrom et al. 1978) and in the Danube River (Vojinovic et al. 1990). Furthermore, Waldichuk (1979) suggested that the sublethal effects of such organochlorine contaminants may be a factor in the survival of Atlantic salmon stocks. Similarly, a number of studies have linked contamination within Pacific Coast estuaries and a lowered immune response of salmonids (Arkoosh et al. 1994, 1998b, 2000; Collier et al. 2000). These studies also suggest that immunodysfunction resulting from migration through, or residence in, such contaminated estuaries may

affect the survival of populations that are threatened.

Two isomers of DDE are present in the environment: *p,p'*-DDE and *o,p'*-DDE. The former has been reported to act as an anti-androgen in rats (Kelce et al. 1995), whereas *o,p'*-DDE has estrogenic activity in tissue and biologic assays (Colborn et al. 1993; Donohoe and Curtis 1996; Soto et al. 1994). Displacement of estrogen from its receptor by *o,p'*-DDE has been demonstrated in a number of vertebrate organisms, including humans and fish (Chen et al. 1997; Kramer and Giesy 1999; Matthews et al. 2000; Nelson 1974; Nelson et al. 1978).

The immune effects of DDT and its metabolites are well documented in mammals. Lahvis et al. (1995) demonstrated an inverse correlation between the lymphocyte responses to concanavalin A (ConA) and blood concentrations of *p,p'*-DDT, *p,p'*-DDE, and *o,p'*-DDE in free-ranging bottlenose dolphins (*Tursiops truncatus*). In addition, exposure of beluga whale (*Delphinapterus leucas*) leukocytes to 25–100 ppm *p,p'*-DDT *in vitro* caused a significant reduction in leukocyte proliferation when stimulated with phytohemagglutinin (De Guise et al. 1998). In rats, humoral immunosuppression occurred after oral administration of DDT (Koner et al. 1998), and in humans, prenatal exposure to DDE has been correlated to incidences of disease (Dewailly et

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We thank R. Chitwood and L. Siddens for their technical assistance.

The Oregon Cooperative Fish and Wildlife Research Unit is supported cooperatively by the U.S. Geological Survey, Oregon State University, and the Oregon Department of Fish and Wildlife. This work was supported by grant NA76RG0476 from National Oceanic and Atmospheric Administration (NOAA) to the Oregon State University Sea Grant College Program and by appropriations made by the Oregon state legislature.

The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies.

The authors declare they have no conflict of interest. Received 20 December 2002; accepted 1 July 2003.

al. 2000). Furthermore, in mammalian epidemiologic studies, DDE is increasingly linked to the risk of cancer (Cocco et al. 2000; Porta et al. 1999; Romieu et al. 2000; Wolff et al. 1993), although the findings are often contradictory and inconclusive (Snedeker 2001).

The mechanism for chemically induced immunomodulation is unclear. One possible mode of action is from direct toxicity to the immune cells and organs. Immunomodulation may also result from interactions between the immune and endocrine systems (Dunier 1996). Interactions between the immune system and the hypothalamic–pituitary–gonadal (HPG) axis in mammals (Ablin et al. 1979; Grossman 1984; Waltman et al. 1971; Yohn 1973) and in fish (Cross and Willoughby 1989; Pickering and Christie 1980; Slater and Schreck 1993) suggest that exposure of fish to a contaminant with estrogenic activity, such as *o,p'*-DDE, may induce effects on the immune system via the endocrine system. It is widely accepted that endogenous estrogen suppresses cell-mediated immunity but, paradoxically, stimulates the humoral response (Grossman 1984). Xenobiotics may also affect the immune system at a number of levels of complexity, and different immune cells and processes have a range of sensitivities to pollutants (Dunier 1996; Wester et al. 1994). Cell-mediated immunity includes antigen-specific immune responses against intracellular pathogens that do not directly involve antibodies. In tissue culture, polyclonal mitogens such as ConA or polyinosinic-polycytidylic acid (polyI:C) are able to induce proliferation of T lymphocytes of many specificities or clonal origins. Humoral immunity involves the recognition of extracellular antigen by B lymphocytes bearing antigen-specific antibodies on their surface. Some antigens, such as bacterial lipopolysaccharides (LPSs), can trigger polyclonal activation of B lymphocytes in the absence of helper T cells. The nonspecific or innate immune system includes cells and other substances that respond to infectious microorganisms in a nonspecific manner. An example of such a substance in fish is lysozyme, an antibacterial enzyme able to lyse gram-positive bacteria. A suite of tests, including a variety of mitogens, is therefore required to evaluate immunomodulatory mechanisms that may be influenced by xenobiotics (Wester et al. 1994).

Given that organochlorine contaminants have been found in many of the spawning/rearing areas of salmonids, and that these contaminants have known effects on the immune system, we were interested in determining whether a mechanism exists for such contaminants to interfere with the development and/or function of the immune system.

The goal of this study was to determine if brief exposures to *o,p'*-DDE during embryonic development can have long-term effects

on immune function in chinook salmon (*Oncorhynchus tshawytscha*). We chose to expose fish to the compound immediately after fertilization (during the water hardening period) and then later during hatch, because *a*) these life-history stages are particularly sensitive to environmental perturbations (Rosenthal and Alderdice 1976); *b*) development of the immune system occurs during and after these stages (Ellis 1977; Grace and Manning 1980; Tatner 1996); *c*) developing fish embryos are particularly sensitive to hormonal signals during these stages, evidenced by manipulation of sex ratios by exposure to exogenous estrogens or androgens (Feist et al. 1995; Piferrer and Donaldson 1989); *d*) eggs may receive high contaminant loads by transgenerational exposure through maternal transfer of mobilized lipids; and *e*) these fish are in closest contact with potentially contaminated sediments during these periods of development.

Materials and Methods

Fish gametes. We obtained fall chinook salmon gametes from Fall Creek Hatchery (Aalsea, OR) in November 1998. Five mature females and four mature males were removed from their holding pen and sacrificed. Eggs were stripped from the abdomen of the females, pooled, placed in an insulated plastic bag, and kept on ice. Milt was extracted from the males, pooled, placed in an O₂-enriched plastic bag on ice, and held in the dark. Eggs and milt were transported to the Fish Performance and Genetics Laboratory (FPGL) at Oregon State University (OSU) within 5 hr. All animals were treated in accordance with OSU's Care of Laboratory Animals guidelines (Oregon State University Institutional Animal Care and Use Committee 2003).

Experimental design. On arrival at FPGL, batches of pooled eggs (90 g, equivalent to ~250–300 eggs, three batches per treatment) were fertilized by the addition of 1 mL of pooled milt followed by a 2-min immersion in a bath of well water at 11 ± 0.5°C. Immediately after fertilization, all eggs within a batch were randomly assigned to one of four triplicated immersion treatments prepared in 2.5 L of well water: *a*) well-water control; *b*) vehicle control, 0.04% dimethyl sulfoxide (DMSO); *c*) 10 ppm *o,p'*-DDE in 0.04% DMSO; and *d*) 100 ppm *o,p'*-DDE in 0.04% DMSO. Eggs were immersed in treatments for 1 hr. Immersion temperature before and after exposure was 11 ± 0.5°C.

After treatment, eggs were maintained undisturbed in Heath trays supplied with a constant flow of well water (12.5 ± 0.5°C at 9 L/min) until 240°C temperature units (CTU) had accumulated. In accordance with standard hatchery practice, after 240 CTU, eggs were treated twice weekly with 0.5 mL/min formalin to control fungus. Mortality was recorded

and unfertilized eggs were removed at 420 CTU. Random subsamples of viable eggs (*n* = 200 eggs per replicate) were placed in a monolayer in holding containers (10 cm diameter × 5 cm high polyvinyl chloride pipe with mesh base) within the Heath trays (all remaining eggs were removed from the trays). When 50% of the well-water control group had hatched, all replicates were immersed for 2 hr in a later treatment of the same nominal dose as at fertilization: *a*) well-water control; *b*) vehicle control, 0.04% DMSO; *c*) 10 ppm *o,p'*-DDE in 0.04% DMSO; *d*) 100 ppm *o,p'*-DDE in 0.04% DMSO. Oxygen was supplied to the treatments for 5 sec every 15 min.

After treatment, fry were maintained in Heath trays until the yolk sac was absorbed, at which time a random subsample of fry from each replicate (*n* = 55 fry per replicate) was placed in individual 0.6-m-diameter circular tanks. The remaining fish within each replicate were transferred to alternate rearing tanks (0.6 m diameter) and reared in the same manner. Mortality was recorded, and expired fish were replaced by like-treated fish from the alternate tanks (to maintain a standard rearing density) for approximately 1 month after first feeding.

One month after first feeding, a random sample of whole fry was netted from each replicate tank (*n* = 5) and killed by a lethal dose (200 mg/L) of tricaine methane sulfonate (MS-222) buffered with sodium bicarbonate. Whole fry were pooled from replicates within a treatment and frozen at –20°C until analysis for *o,p'*-DDE tissue concentration.

Treated fish were raised under regular hatchery conditions of 12.5 ± 0.5°C flow-through well water and natural photoperiod and fed a commercial diet of Semi-moist Pellets (BioOregon, Warrenton, OR) daily. When size and density dictated, all fish were moved to and maintained in 1-m-diameter circular tanks. One year after first feeding, fish were sampled for endocrine and immune parameters.

Sampling. In April 2000, fish from the four treatments were netted from the holding tanks and immediately immersed in a lethal dose (200 mg/L) of buffered MS-222. Weight and fork length were recorded. A mixed ateri-venous sample of blood was collected ventral to the spinal column using EDTA-coated vacuum tubes with a 21-gauge needle. After collection, blood was centrifuged at 1,800 × *g* for 5 min, and plasma was collected and stored at –80°C until assayed for 17β-estradiol (E₂), 11-ketotestosterone (11-KT), and lysozyme levels. Residual blood was drained from the fish by severing the caudal peduncle. Fish were returned to the laboratory on ice for dissection of the gonads and the spleen. From these tissues, we conducted gonadal histology and determined sex, splenic leukocyte blastogenesis,

and surface immunoglobulin M (SIgM) expression in response to LPS. A second sampling event took place in May 2000 to assess the mitogenic response of splenic leukocytes to ConA and polyI:C.

Sampling was conducted over 3 days for each sampling event. Eight fish per replicate ($n = 24$ per treatment) were sampled for first sampling event; six fish per replicate were sampled for the second ($n = 18$ per treatment).

Assay procedures. Concentration of o,p' -DDE in fish. To assess the efficacy of the immersion treatments, a pooled sample of whole fry ($n = 15$) from each treatment was collected for analysis of o,p' -DDE concentration and percentage lipid 1 month after exogenous feeding. Chemical analyses were carried out by gas chromatography following the methods described by Gunderson et al. (1998) to a detectable limit of 0.01 ppm. Additionally, 1 year later, muscle tissue from one juvenile fish per treatment (selected randomly from the replicates) was analyzed for o,p' -DDE concentration and percentage lipid.

Blastogenesis and surface immunoglobulin expression. To quantify the effect of o,p' -DDE on humoral immunity, we determined the ability of splenic leukocytes to undergo blastogenesis and express SIgM upon *in vitro* mitogenic stimulation with LPS. The method used quantifies the fluorescein isothiocyanate (FITC)-labeled anti-rainbow trout surface immunoglobulin monoclonal antibody (anti-RBT SIgM-FITC; the original antibody hybridomas were a generous gift from G. Warr, Medical University of South Carolina, Charleston, SC) (DeLuca et al. 1983) bound to individual leukocytes by flow cytometry. The assay was optimized and validated for juvenile chinook salmon by Milston et al. (2003). Briefly, splenic leukocytes of fish from the four immersion treatments were cultured with either tissue culture media (TCM) or *Escherichia coli* LPS 055:B5 (final concentration 100 $\mu\text{g}/\text{mL}$; Sigma Chemical Co., St. Louis, MO) at 17°C for 4 days under blood gas. Before and after cell culture, leukocytes were labeled with anti-RBT SIgM-FITC on ice, in the dark, for 30 min. Cells were washed and analyzed by flow cytometry (CellQuest; BD Biosciences, San Jose, CA) for the percentage of cells ($n = 10,000$) undergoing blastogenesis, determined by changes in the cells' granularity and size and the percentage of SIgM-bearing lymphoblasts (e.g., Figures 1 and 2). During analysis, the quadrants in CellQuest were defined from a representative cell sample from control (water)-treated fish. The position of the quadrant was set to delineate between different major cell populations based on size (forward scatter) and fluorescence intensity. The position of the quadrant was the same for all subsequent cell samples.

Cell viability within stimulated and nonstimulated cultures was determined using the Trypan blue exclusion test.

Plasma steroid concentrations. Plasma concentrations of E_2 and 11-KT were assayed by radioimmunoassay following the methods of Sower and Schreck (1982) as modified by Fitzpatrick et al. (1986).

Sex identification and gonadal histology. The sex of each fish was identified visually by examining gross gonadal morphology under a dissecting microscope. In addition, gonads were inspected for developmental abnormalities by histologic analysis. Gonad samples were fixed in 10% buffered formalin and imbedded in paraffin, sectioned (10- μm transverse sections), and stained with eosin and hematoxylin. The sections were examined under a light microscope and compared with descriptions for normal reproductive development as described by Piferrer and Donaldson (1989) and Feist et al. (1990).

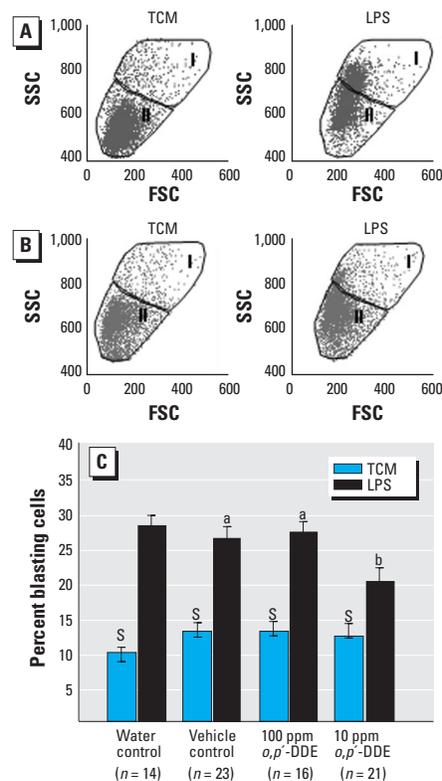


Figure 1. Representative forward-scatter (FSC) and side-scatter (SSC) cytograms from chinook salmon from water-control (A) and 10-ppm o,p' -DDE treatments (B). (C) Activation quantified as the percentage (mean \pm SE) of the population of splenic leukocytes (I and II) ($n = 10,000$ total cells) undergoing blastogenesis after a 4-day *in vitro* incubation with TCM or LPS. See "Materials and Methods" for details. Columns with the same superscript are not significantly different ($p > 0.05$, ANOVA comparing vehicle control with 10- and 100-ppm treatments).

Lysozyme activity. To quantify the effect of o,p' -DDE on nonspecific immunity, plasma lysozyme activity was measured following a modified method of Litwack (1955), as applied by Sankaran and Gurnani (1972). Briefly, the ability of plasma lysozyme to break down the peptidoglycan layer of the gram-positive bacterium *Micrococcus lysodeikticus* was established. Ten microliters of plasma were incubated in triplicate in a 96-well microplate with 200 μL of *M. lysodeikticus* (0.025% wt/vol suspension in 0.02 M acetate buffer) for a 20-min period at room temperature. The change in optical density over the incubation period was measured at 450 nm and compared with a standard curve of hen egg-white lysozyme over the linear range of 3–15 mg/mL.

Mitogenic response to ConA or polyI:C. To quantify the effect of o,p' -DDE on cell-mediated immunity the ability of splenic leukocytes from treated fish to be stimulated in response to T-lymphocyte-specific mitogens

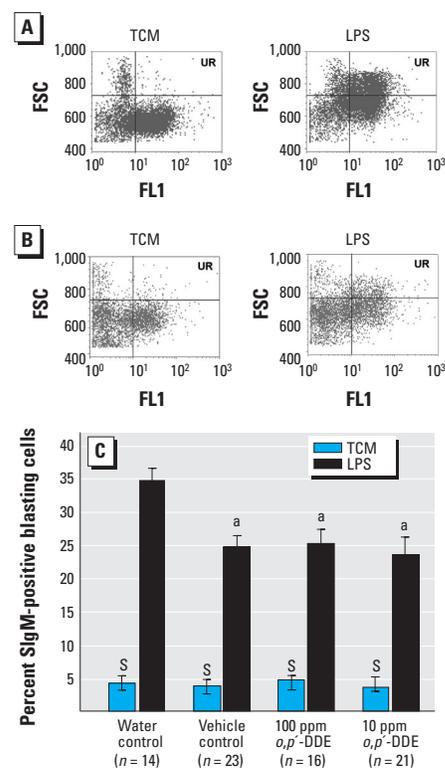


Figure 2. Representative forward-scatter (FSC) and fluorescence intensity (FL1) cytograms from chinook salmon from water-control (A) and 10-ppm o,p' -DDE treatments (B); these correspond to the blastogenesis cytograms in Figure 1. UR, upper right quadrant (SIgM-positive blasting cells); all quadrants, population ($n = 10,000$ total cells). (C) Activation quantified as the percentage (mean \pm SE) of the population of blasting splenic leukocytes expressing SIgM after a 4-day *in vitro* incubation with TCM or LPS. See "Materials and Methods" for details. Columns with the same superscript are not significantly different ($p > 0.05$, ANOVA comparing vehicle control with 10- and 100-ppm treatments).

was measured based on methods adapted from Krishnan (1975). Cell cycle progression was determined and quantified by flow cytometric analysis of DNA of individual cells labeled with propidium iodide (PI) (Sigma). Splenic leukocytes were stimulated *in vitro* with ConA (250 µg/mL) or polyI:C (500 µg/mL) or were placed in TCM alone for 4 days at 17°C under blood gas. After incubation, cells were fixed with methanol, washed, resuspended in phosphate-buffered saline plus EDTA and RNase A, and then labeled with PI for 30 min at room temperature before flow cytometric analysis. Quantification of the percentage of cells ($n = 10,000$) within the population that were undergoing mitosis was determined, as defined by DNA content reflected by PI fluorescence. A stimulation index was calculated as the ratio of the percentage of cells in S and G₂M phases in the mitogen-stimulated cultures divided by the percentage of cells in S and G₂M phases in the unstimulated cultures.

Statistical analyses. We used analysis of variance (ANOVA) to determine differences between replicates. If there were no differences between replicates, data were pooled within a treatment. Groups were tested for normality and variance homogeneity, and, where detected, parametric statistical analyses were applied (*t*-test comparing two means, or ANOVA followed by Tukey's multiple comparison test). If variances were not homogeneous, nonparametric tests (Kruskal-Wallis or Mann-Whitney *U* test) were applied with Dunn's posttest where differences were detected. For percentage blasting and SIgM data, the vehicle control was first compared with the water control using a *t*-test. Treatment effects were then analyzed using ANOVA followed by Tukey's multiple comparison test.

Percentage values were transformed by arcsine of the square root of the value for further analysis by ANOVA and subsequent multiple range tests. Treatment effects on the discrete responses of mortality and hatching times were analyzed using a chi-squared test for differences between treatments in mortality and hatch rates over several time points. For all statistical tests, differences were considered significant below $p = 0.05$.

Results

Concentration of *o,p'*-DDE in tissues. The concentration of *o,p'*-DDE in whole fry (pooled sample of 15 fish) treated with 10 ppm *o,p'*-DDE was 0.53 µg/g lipid 1 month after first feeding but was undetectable in all other treatments (Table 1). One year later, the level of *o,p'*-DDE in a muscle sample taken from a single fish in this treatment group was 0.51 µg/g lipid. At this time, DDE was detected in the muscle of single fish taken from each of the control groups and the 100-ppm treatment, albeit at a lower

level than that detected in the 10-ppm treatment group ($n = 1$ per treatment).

Percentage of cells undergoing blastogenesis after 4 days of *in vitro* activation with TCM or LPS. For all treatments, cells immunized *in vitro* with LPS had significantly more cells undergoing blastogenesis than did those that were cultured in the presence of TCM alone (Mann-Whitney *U* test, $p < 0.001$; Figure 1). DMSO had no effect on the percentage of blasting after stimulation with LPS ($p = 0.181$, *t*-test comparing vehicle control with water control). Fish that were treated with 10 ppm DDE had a significantly lower percentage of cells undergoing blastogenesis by day 4 of the *in vitro* activation with LPS than did fish from the vehicle control and the 100 ppm DDE treatment groups (one-way ANOVA, $p = 0.007$, followed by Tukey's multiple comparison test).

There were no significant differences between treatments for the percentage of cells undergoing blastogenesis after incubation with TCM alone (one-way ANOVA, $p > 0.05$). Cell viability did not differ between cells incubated with LPS or with TCM alone.

Percentage SIgM-positive blasting cells. For all treatments, cells immunized *in vitro* with LPS had a significantly higher percentage of SIgM-positive blasting cells within the population than those that were cultured in the presence of TCM alone (Mann-Whitney *U* test, $p < 0.001$; Figure 2). DMSO significantly reduced the percentage of SIgM-positive blasting cells after stimulation with LPS ($p = 0.0041$, *t*-test comparing vehicle control with water control). Treatment with 10 or 100 ppm DDE had no significant effect on the percentage of SIgM-positive blasting cells when compared with the vehicle control (one-way ANOVA, $p = 0.922$, followed by Tukey's multiple comparison test).

The percentage of SIgM-positive blasting cells within the leukocyte population did not differ significantly between treatments for cells incubated in TCM alone (one-way ANOVA, $p > 0.05$).

Fish size. At the time of sampling for blastogenesis and SIgM, the median weight of fish in the 10-ppm treatment group was significantly lower than that of the controls for this sampling event (Mann-Whitney *U* test,

$p = 0.0394$; data not shown). However there were no differences in the weight of fish from all treatments at the time of the second sampling event (mitogenic assay) 1 month and 10 days later. There was no evidence of a relationship between weight at the first sampling and the percentage of blasting cells or the percentage of SIgM-positive blasting cells (linear regression, $R^2 = 0.0086$ and 0.003 , respectively). There were no significant differences in fish length between treatments for either sampling event.

There was no effect of *o,p'*-DDE on mortality, time to hatch, sex ratio, gonadal development, plasma lysozyme concentration, or leukocyte mitogenesis in response to ConA or polyI:C, or plasma E₂ and 11-KT concentration (Table 2).

Discussion

This study provides the first evidence that very brief exposures to a low dose of *o,p'*-DDE during early life stages can have a long-term effect on the immune function of chinook salmon. In a pooled sample of 15 whole Chinook salmon fry exposed as eggs to a nominal dose of 10 ppm *o,p'*-DDE by immersion for 1 hr at fertilization, followed by a 2-hr exposure at hatch, the *o,p'*-DDE concentration was 0.53 µg/g lipid weight at the time of first feeding. The treatment was sufficient to significantly reduce the humoral response of juvenile fish (1 year later) to a mitogen. The ability of splenic leukocytes to undergo blastogenesis, when stimulated *in vitro* for 4 days with LPS, was significantly reduced in fish that showed uptake of DDE during the exposure.

The concentration of DDE in fish from the 100-ppm *o,p'*-DDE treatment was nondetectable at the time of first feeding, suggesting that there was no uptake of the compound during the immersions. This is likely attributable to the low solubility of the compound in water. During the 100-ppm *o,p'*-DDE immersions, after addition of the stock solution to the water bath, the compound precipitated out of solution. It is likely that the precipitate was too large to be absorbed across the membrane of the eggs and fry, thus preventing uptake of the compound into the fish. The absence of *o,p'*-DDE in fry of the 100-ppm treatment would explain the lack of a response with

Table 1. Concentration of *o,p'*-DDE (lipid adjusted) and percent lipid values measured in fry (whole-body pooled sample of 15 fish per treatment) 1 month after first feeding or in juveniles (muscle tissue sample from a single fish) 1 year after first feeding.

Treatment	Whole fry		Juvenile muscle	
	µg DDE/g lipid (lipid adjusted)	Percent lipid ^a	µg DDE/g lipid (lipid adjusted)	Percent lipid ^a
Well-water control	< MDL	2.4	0.34	11.7
Vehicle control	< MDL	3.3	0.19	15.9
10 ppm <i>o,p'</i> -DDE	0.53	3.8	0.51	13.6
100 ppm <i>o,p'</i> -DDE	< MDL	5.1	0.29	10.3

MDL, minimum detectable level.

^aPercentage of lipids in whole body (fry) or muscle tissue (juveniles).

respect to blastogenesis for this treatment. The precipitate was not observed during the preparation of the 10-ppm treatments. In this regard, the 100-ppm treatment most likely represents the vehicle control conditions.

It is noteworthy that 1 year after first feeding, *o,p'*-DDE was detected in the muscle tissue of juveniles for all treatments, including those that were not intentionally exposed to the compound. Because this compound is considered ubiquitous, it is possible that it may be present at low levels in the fish feed. We did not test this in the present study, but we feel that this also would be an interesting avenue to pursue.

Guillette et al. (1995) proposed that the long-term effects of endocrine-disrupting chemicals are a result of exposure to chemicals during critical periods of development. The results of the present study are in agreement with this. It is during the period of embryonic development in fish that the appearance of immune organs and the onset of functional maturity occur. Generally, the thymus is the first lymphoid organ to develop, followed by the kidney. Both tend to develop before hatching, whereas the spleen and the gut-associated lymphoid tissue tend to develop after hatching (Castillo et al. 1993; Ellis 1977). Our results indicate that exposure to DDE during the time of development resulted in a decrease in the ability of leukocytes to undergo blastogenesis in fish 1 year later. However, we were not able to distinguish between the effects of exposure at fertilization and at hatch. We hypothesize that the mechanism for such an effect on the development of the immune system is due to the estrogenic action of *o,p'*-DDE (Donohoe and Curtis 1996; Nelson 1974; Nelson et al. 1978; Soto et al.

1994). Grossman (1984) reviewed a number of studies that show evidence suggesting that gonadal steroids, including estrogen, play a significant role in the regulation of the mammalian immune function. Furthermore, recent studies have shown the presence of estrogen receptors on lymphoid cells, and endocrine-immune interaction has been suggested as a possible mechanism for estrogen-induced immunomodulation in mature humans (Cutolo et al. 1995). Estrogen receptors have also been detected on the leukocytes of channel catfish in preliminary studies (Patino R. Personal communication), suggesting that such a mechanism may be highly conserved.

However, it is important to distinguish between the effect of xenobiotics during early development and effects on the mature immune system. A number of observations suggest that the developing embryo is particularly sensitive to hormonal signals. In many nonmammalian vertebrates, estrogens are apparently essential for sexual differentiation and early exposure to endocrine-disrupting chemicals can influence this process (Bull et al. 1988; Fry 1995; Guillette et al. 1994, 1995, 1996). In salmonids, steroid hormones are produced by the embryo during the period around hatching (Feist et al. 1990), and exposure to exogenous estrogens or androgens around this period can alter the sex of the individuals (Feist et al. 1995; Piferrer and Donaldson 1989). Organizational roles of steroids during early development may thus affect not only cells designed to have reproductive function, but other steroid dependent systems such as the immune system as well.

Alternately, the observed immunosuppression may be due to a toxicologic effect during development of the immune system or to the

perception of DDE as a stressor. Although we cannot rule out the possibility of immunosuppression due to direct toxicity, it is unlikely that the immunosuppression is a result of a stress response to the presence of DDE because juvenile salmonids are unable to elicit a corticosteroidogenic response until 1 week after hatch (Barry et al. 1995a, 1995b; Feist and Schreck 2001).

Interestingly, we also found that of the cells that did undergo blastogenesis, significantly fewer expressed SIgM in fish that were exposed to DMSO (vehicle) compared with fish from the water control treatment. This suggests that DMSO may in itself have an immunosuppressive effect at this level. Previously, van't Erve et al. (1998) showed that treatment with DMSO led to a reduction in IgG production in mice. Similarly, Pestronk and Drachman (1980) showed that DMSO significantly reduced antibody production in rats. These results contrast with those of Caren et al. (1985), who found that DMSO had no effect on the humoral immune response in mice. The lack of response in their study may be due to the sampling design, because Pestronk et al. (1985) found that the effect of DMSO was dependent on the stage of the immune response and the strength of the antigenic stimulation. Similarly, Nash et al. (1983) found that the degree of immune inhibition increased relative to the time of DMSO ingestion before immunization in mice. The same authors also found that DMSO depressed IgG and IgA, but not IgM, within the first 7 days. In all these studies, the effect was measured soon after treatment with DMSO; however, in the present study, the decline in SIgM was observed 1 year after treatment with DMSO, suggesting that DMSO may have altered the development of the immune system. The mechanism by which DMSO exerts its immunosuppressive effect is unclear. Strong et al. (1972) and Hwan Mook et al. (1996) report that DMSO appears to be specific for the T-dependent B-cell response to a mitogen, and not T-independent or polyclonal B-cell responses. Our results are not in agreement with these findings and may relate to differences in the effect of DMSO during development of the immune system versus its effect on cells in mature animals. The immunosuppressive effect of DMSO is of note because it is often used as a cryopreservant during the freezing of mammalian embryos and ova (Friedler et al. 1988; Yu and Quinn 1994). In addition, the use of DMSO as a cryoprotectant in fish has been investigated (Bart 2000; Cabrita et al. 2003). Given these uses and our own findings that DMSO may have immunosuppressive effects during early development that are long lasting, this would be an interesting and important avenue for future research.

The results of the present study support the many studies showing immunosuppressive

Table 2. Measures of nonspecific and cell-mediated immune function, hatching, and survival in fish from the four treatment groups.

Treatment	Plasma lysozyme ^a µg HEWL/mL (SE)	Mean stimulation index ^b		Hatch (%) ^c	Mortality (%) ^d
		PolyI:C (SE)	ConA (SE)		
Water control					
Replicate 1	41.88 (1.42)	2.38 (0.34)	2.03 (0.34)	39	39
Replicate 2	39.14 (1.56)	—	—	30	30
Replicate 3	36.26 (1.20)	1.49 (0.12)	1.74 (0.13)	29	19
Vehicle control					
Replicate 1	45.95 (1.58)	2.14 (0.27)	2.10 (0.30)	47	23
Replicate 2	40.37 (1.12)	—	—	48	25
Replicate 3	37.10 (1.33)	1.58 (0.20)	1.34 (0.04)	40	16
10 ppm <i>o,p'</i> -DDE					
Replicate 1	41.88 (1.42)	1.77 (0.37)	1.69 (0.22)	36	18
Replicate 2	39.14 (1.56)	—	—	34	22
Replicate 3	36.26 (1.20)	1.96 (0.38)	1.72 (0.5)	50	23
100 ppm <i>o,p'</i> -DDE					
Replicate 1	41.88 (1.42)	2.78 (0.33)	2.31 (0.27)	64	23
Replicate 2	39.14 (1.56)	—	—	33	22
Replicate 3	36.26 (1.20)	1.73 (0.30)	1.86 (0.14)	50	12

—, insufficient viable leukocytes to conduct analysis; leHEWL, hen egg-white lysozyme.

^aFor each replicate, $n = 8$. ^bRatio of the percentage of the splenic leukocyte population in S and G₂M phases of cell cycle after culture with mitogen [polyI:C (500 µg/mL) or ConA (250 µg/mL)] to the percentage of the splenic leukocyte population in S and G₂M phases of cell cycle after incubation with tissue culture media; $n = 6$ for each replicate. ^cPercentage hatched at 40 days after fertilization based on $n = 200$ per replicate. ^dPercentage of mortality between fertilization and 1 month after first feeding.

effects of organochlorines through *in vitro*, laboratory, and environmental exposures. What is particularly alarming about our results is that such a short period of exposure was able to induce long-term effects on humoral immune competence. In the present study, DDE was administered via aqueous exposure, although in the environment DDE is, in general, bound to the sediment. Because of this, the most likely route of uptake would be via the diet and through deposition of lipid into eggs before spawning. Miller (1993) found that the concentration of DDE in the muscle tissue of gravid chinook salmon was significantly correlated with the concentration of DDE in the eggs. It is also possible that contaminants may be released from the sediments during spawning and taken up by the eggs during water hardening because there is considerable disturbance of sediments by the adults. Aqueous exposure may also occur during flood events that expose bound contaminants, or contaminants may enter the water in pulses from agricultural or industrial sites, particularly during heavy runoff events in the late fall as many fish are spawning. In recent decades, there has been an increase in urban, industrial, and agricultural development in coastal and lakeside regions. As a consequence, effluent and surface runoff are often channeled directly into water-courses. For example, DeVault (1985) found high ratios of *o,p'*-DDE to *p,p'*-DDE in fish from the Great Lakes harbors and tributaries. Composite samples of indigenous fish from various sites had concentrations of *o,p'*-DDE ranging from 0.1 to 0.32 mg/kg whole body wet weight. Furthermore, these values were not corrected for extraction efficiency, so they may, in fact, be higher. In our study, fry with whole-body concentrations of *o,p'*-DDE similar to this exhibited long-term humoral immunosuppression. Further research is needed to determine how much exposure eggs, or recently hatched fish living within the sediments, have to contaminants such as DDE. The lipophilic nature of many of these contaminants results in their bioaccumulation and subsequent transfer to developing eggs via mobilization of lipids. Such observations have been made in mammals: Rehana and Rao (1992) found that dietary exposure of female mice to DDT led to humoral immunosuppression of the offspring, suggesting that the suppression occurred during development of the immune system as a result of maternally transferred DDT or its metabolites. The present results may also apply more widely given the similarities among the immune system of many vertebrates. Of particular concern are populations of fish in nations that still employ DDT as an agent for vector disease control.

The decline of salmonids in the Pacific Northwest is likely due to a combination of factors, of which contaminant exposure may be

one (National Research Council 1996). Based on our present results, it is clear that a mechanism for immunosuppression due to chemical exposure from the environment or maternal transfer during early periods exists for salmonids. This immunosuppression may increase the susceptibility of fish to disease, leading to a reduction in recruitment of juveniles, which would be critical to regulating the population. It is not clear, however, whether such a problem exists. Given the persistence of organochlorines and other contaminants in several of the spawning/rearing areas of salmonids in the Pacific Northwest and in other areas, we feel that this is an important avenue of research to continue. Research on exposure of salmonids at various sites to contaminants during spawning is needed to establish whether contaminant-induced immunosuppression may play a role in the decline of these species.

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