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Persistent organochlorines (OCs) bioaccumulate in aquatic biota and potentially impair reproduction via endocrine disruption. The present research evaluated estrogenic effects of the OCs, chlordecone (CD) and o,p'-DDT/DDE, and the antiestrogenicity of 3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB) in juvenile rainbow trout, utilizing vitellogenesis as a biomarker. Vitellogenesis, estrogen regulated hepatic production of the yolk protein precursor, vitellogenin (Vg), served as an indicator of estrogenic or antiestrogenic actions relevant to reproductive toxicity or hepatocarcinogenesis. In an estrogen-dose response experiment, plasma Vg was the most sensitive biomarker of vitellogenesis induction, compared to changes in plasma lipids, hepatic estrogen receptor (ER) concentrations or liver somatic index (%LSI). Vitellogenesis was weakly induced by chronic dietary CD exposure (0.4 mg/kg/day) and was associated with relatively high hepatic CD concentrations (15 mg/kg) but was not correlated to the observed mild promotion of hepatocarcinogenesis. *In vitro*

binding assays confirmed CD was weakly estrogenic as CD exhibited low binding affinity for trout hepatic ER (1000-fold less than the synthetic estrogen, moxestrol). A second group of xenoestrogens, o,p'-DDT and o,p'-DDE, demonstrated very weak *in vitro* trout ER binding affinity (156,000-fold less than moxestrol) and p,p'-DDE was inactive. *In vivo*, plasma Vg and hepatic ER concentrations were significantly elevated by o,p'-DDT and o,p'-DDE, but not p,p'-DDE, (total dose 45 to 90 mg/kg via ip injection) in trout at mg/kg hepatic concentrations. Co-planar PCBs such as 345-HCB may act as antiestrogens by decreasing ER or plasma estradiol concentrations or by altering estrogen regulated gene expression. However, results demonstrated 345-HCB (0.25 to 100 mg/kg via ip injection) did not antagonize estrogen induced increases in hepatic ER, plasma Vg or %LSI but significantly elevated CYP1A protein and mRNA content and depressed plasma estradiol. We concluded vitellogenesis modulation in trout was a less sensitive 345-HCB exposure biomarker than CYP1A induction and plasma estradiol suppression by 345-HCB did not significantly influence plasma Vg. Collectively, results indicated vitellogenesis was a suitable biomarker for assessing weak estrogenic effects of OCs in juvenile rainbow trout but was not responsive to potential antiestrogenic effects of 345-HCB.

Vitellogenesis: A Biomarker for Estrogenic and Antiestrogenic Effects of Organochlorines in Rainbow Trout

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CHAPTER 1

Introduction

LITERATURE SUMMARY AND RESEARCH OBJECTIVES

Organochlorines (OCs) such as polychlorinated biphenyls (PCBs), DDT and cyclodiene-type insecticides (*e.g.* chlordecone) persist in aquatic environments throughout the United States due to their stability and trophic transfer (Huggett and Bender, 1980; Baumann *et al.*, 1988; Kennicutt *et al.*, 1988, O'Conner & Huggett, 1988; Hargrave *et al.*, 1992). However, few studies have related OC body burdens to specific toxicological effects (Malins and Ostrander, 1991). Recent evidence has suggested one potential effect of bioaccumlated OCs (and other contaminants) is disruption of normal endocrine function by estrogenic or antiestrogenic actions (Colborn and Clement, 1992). In the broadest sense, estrogenic xenobiotics mimic the physiological functions of estrogen. Generally, these agents produce estrogenic effects by binding to the estrogen receptor (ER) which initiates a cascade of intracellular events such as gene transcription and protein synthesis. Examples of environmental estrogen agonists in mammals include: chlordecone (CD; Eroschenko, 1981), o,p'-DDT (Bulgur and Kupfer, 1985), methoxychlor (Kupfer and Bulger, 1987), some PCB metabolites (Korach *et al.*, 1988), alkylphenols (Mueller and Kim, 1978) and phytoestrogens (Makela *et al.*, 1994). Other agents inhibit estrogen-mediated physiological processes (*i.e.* antiestrogens) by directly binding to the ER

without activating gene transcription (*e.g.* tamoxifen; Jordan, 1984) or by indirectly interfering with estrogen action (*e.g.* co-planar PCBs and polychlorinated dibenzo-p-dioxins; PCDDs; Safe *et al.*, 1991). Although many studies have examined estrogenic and antiestrogenic actions of OCs in mammals, few have investigated these responses in fish. The present research evaluated the estrogenicity of CD and o,p'-DDT/DDE and the antiestrogenicity of 3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB) in juvenile rainbow trout.

A primary concern associated with estrogenic or antiestrogenic OC bioaccumulation is interference with reproduction or development.
In mammals, estrogen influences or controls all aspects of female reproduction and mediates normal development and maturation (Clark and Mani, 1994). Exposure to the xenoestrogens, CD and o,p'-DDT, produces female reproductive dysfunction involving persistent vaginal estrus, increased uterine weight in immature animals, altered estrous cycling and reduced reproductive success (Guzelian, 1982; Bulger and Kupfer, 1985). Symptoms of PCB/PCDD-induced female reproductive toxicity include inability to maintain pregnancy, reduced litter size and altered estrous cycling (Peterson *et al.*, 1993). Estrogen also plays a vital role in sexual differentiation, ovarian development and maintenance of the adult female sexual cycle in fish (Fostier *et al.*, 1983) but few studies have directly evaluated the female reproductive effects of estrogenic/antiestrogenic OCs. Field evidence of reduced reproductive success and declining fish populations, in conjunction with OC bioaccumulation, suggests reproductive toxicity may already exist in aquatic environments (Donaldson, 1990;

Spies *et al.*, 1990; Mac & Edsall, 1991). However, the limited number of meaningful biomarkers of fish reproductive dysfunction and the incomplete understanding of OC mechanisms of action in fish have limited evaluation of this environmental problem. A central goal of the present study was to develop a biomarker of estrogenic or antiestrogenic action relevant to reproductive toxicity in fish.

An additional aim of this research was to examine the role of estrogenic OCs in cancer modulation. It has been hypothesized that xenoestrogen bioaccumlation may elevate cancer risk (Davis *et al.*, 1993). Hepatocarcinogenesis was enhanced by estrogen, CD and DDT in rats (Peraino *et al.*, 1975; Mastri and Lucier, 1985; Sirica *et al.*, 1989) and by estrogen and DDT in rainbow trout (Nunez *et al.*, 1988). We were interested in developing a biomarker of hepatic estrogenic action to evaluate mechanisms of hepatocarcinogenesis in trout.

Chemicals with diverse structures have estrogenic or antiestrogenic effects and assays are needed to screen agents for these responses. Recent strategies have included the use of transfected molecular constructs to assess gene activation (*e.g.* ER response element plus a reporter gene) or the use of cell lines to examine estrogen regulated growth or protein synthesis *in vitro* (McLachlan, 1993). More traditional testing methods have inferred estrogenic activity for a compound by its ability to inhibit specific [^{3}H]estradiol binding to target tissues *in vitro* (Jordan *et al.*, 1985). Others have examined changes in uterine weight or vaginal cornification in immature or castrate animals to demonstrate estrogenicity or antiestrogenicity for a chemical

(Jordan *et al.*, 1985). However, monitoring for potential estrogenic/antiestrogenic effects in feral organisms favors the use of biomarkers; biochemical, physiological, or histological indicators of exposure or effects of a xenobiotic (Huggett *et al.*, 1992). Several indicators of fish reproductive dysfunction such as gonad histopathology, fertilization rate, fecundity, or hatching success require time-consuming, labor intensive efforts and are often insensitive, non-specific indicators of estrogenic or antiestrogenic effects (Donaldson, 1990). We developed a biomarker for juvenile rainbow trout that has received limited attention; induction of (for estrogenic agents) or inhibition (for antiestrogens) of estrogen-induced vitellogenesis. Vitellogenesis, hepatic production of the egg-yolk protein precursor, vitellogenin (Vg), seemed to be a suitable, specific estrogen regulated process to evaluate OC induced reproductive toxicity.

A general model of vitellogenesis in oviparous vertebrates has been developed (Tata and Smith, 1979; Wahli *et al.*, 1981). Briefly, estradiol interacts with hepatic ERs which regulate transcription of Vg and other genes. Subsequent Vg mRNA processing is followed by translocation to the rough endoplasmic reticulum. Here translation, processing of pre-Vg subunits and phosphorylation occurs. Glycosylation (mannose, N-acetylglucosamine, N-acetylneuraminic acid) and phosphorylation take place in the Golgi apparatus. Lipids are incorporated into Vg in the Golgi and/or the endoplasmic reticulum. Signal peptides are cleaved, the protein dimerizes and then Vg is secreted into the bloodstream. Pinocytotic uptake is mediated by a Vg receptor on the oocyte membrane of mature females. Once inside the oocyte, proteolytic

enzymes cleave Vg into the yolk proteins, lipovitellin and phosvitin. These yolk proteins provide a reservoir of amino acids, lipids and inorganic phosphate necessary for embryonic development.

Female rainbow trout exhibit seasonal variation in plasma Vg levels, ranging from 0.5 to 50 mg/ml, two months prior to spawning (Scott and Sumpter, 1983). Vg is also inducible in immature and male rainbow trout and is augmented in females by estradiol treatment (Campbell and Idler, 1980). Trout Vg (470 kDa) is a lipophosphoprotein (18% lipid; 0.6% alkali-labile phosphorus) that contains carbohydrate and binds calcium (Campbell and Idler, 1980). The lipid composition is 11% phospholipids, 4% triglycerides and 2% cholesterol (Norberg and Haux, 1985). Vg mRNA has been isolated from rainbow trout and consists of 6300 nucleotides (Pakdel *et al.* 1991). Additionally, the structure and regulation of the rainbow trout hepatic ER gene has been investigated (LeDrean *et al.*, 1994). Estrogen stimulation kinetics in adult male rainbow trout were temperature sensitive and showed rapid (within 24 hours) induction of ER mRNA and nuclear ER levels, preceding Vg mRNA transcription (Pakdel *et al.*, 1991; Mackay and Lazier, 1993). Serum Vg levels were detected within 72 hours and rose to 70 mg/ml after 10 days (Mackay and Lazier 1993). Vg mRNA accumulation continued until 15 days after estrogen injection when estrogen plasma levels, receptor and receptor mRNA levels had recovered to basal levels. Since Vg is rapidly secreted by the liver, hepatic Vg concentrations are lower than in plasma (Copeland and Thomas, 1988). Relatively large increases in liver-to-body weight ratios (liver somatic index; %LSI), resulting

from hypertrophy and hyperplasia have also been noted during vitellogenesis (Mommsen and Walsh, 1983). Additionally, the liver produces very low density lipoprotein (VLDL) and plasma hyperlipidemia is observed (Wallaert and Babin, 1992). Thus, there are several endpoints in vitellogenesis that can be monitored; liver somatic index, hepatic ER, plasma lipids and plasma Vg. The multi-component nature of Vg synthesis makes it an attractive model for study of ER-mediated transcription, translation and post-translational modification.

Recently the vitellogenesis model has been used to study the effects of various contaminants in fish. Estrogenic compounds have induced vitellogenesis. Denison *et al.* (1981) reported DDT-resistant, non-reproductive mosquitofish had continuously elevated plasma Vg. Phytoestrogens also elevated plasma Vg without an accompanying rise in plasma estradiol *in vivo* (Pelissero *et al.*, 1991) and in hepatocyte cultures (Pelissero *et al.*, 1993). Evidence for alkylphenol estrogenic effects in the environment was presented by Purdom *et al.* (1994). The study demonstrated caged male rainbow trout exposed to river outfalls from 30 sewage plants produced Vg (500 - 100,000 fold induction) within three weeks. Further research suggested alkylphenols were the causative agent. Jobling and Sumpter (1993) utilized synthesis of Vg in rainbow trout hepatocytes to illustrate that a series of p-alkylphenolic compounds were weakly estrogenic.

OCs with potential antiestrogenic effects may reduce Vg levels. Thomas (1989) fed Arochlor 1254 (5mg/kg) for 17 days to mature Atlantic croaker and found impaired ovarian growth, decreased plasma Vg, estradiol and gonadotropin levels.

Chen *et al.* (1986) also studied the effect of Arochlor 1254 (3-200 mg/kg) and mirex (0.05-50 mg/kg) diets on estradiol-induced juvenile rainbow trout. All treatments but the lowest mirex dose significantly lowered plasma Vg levels. In San Francisco Bay, female starry flounders with higher hepatic concentrations of PCBs exhibited decreased plasma Vg when compared to control sites (Spies *et al.*, 1990). However, a variety of other pollutants have also impaired estrogen-induced Vg synthesis. Plasma Vg in mature trout was depressed when exposed to acidic conditions (Tam *et al.*, 1987; Roy *et al.*, 1990), endosulfan (Chakraborty and Singh, 1992) or 3-methylcholanthrene (Singh, 1989). Polvsen *et al.* (1990) noted a 40% decrease in plasma Vg following acute cadmium doses (2 mg/kg) to estradiol-induced flounders.

Vg transcription regulation by steroid hormones continues to be an active research area. Recent *in vitro* evidence suggests there is heterosynergism between estrogen and glucocorticoid response elements in the promoter region of chicken and *Xenopus* Vg genes (Slater *et al.*, 1991). Supportive *in vivo* work by Sundararaj *et al.* (1982) showed cortisol potentiated estrogen stimulation of Vg synthesis in catfish. A progesterone response element was also identified in the chicken Vg gene (Cato *et al.*, 1988). Wangh and Schneider (1982) determined thyroid hormones were necessary for *in vitro* induction of *Xenopus* Vg synthesis but the mechanism was unknown. In fish, thryoxine modulated estrogen stimulated protein synthesis and lipid metabolism (Cyr *et al.*, 1988) but no evidence of a direct effect on Vg synthesis is available. Synergistic activation of a Vg promoter by protein kinase C activators indicated cross

talk between other signal transduction pathways, via ER phosphorylation, might also modulate Vg transcription (Cho and Katzenellenbogen, 1993). Whereas estrogen is the dominant regulator of Vg transcription, other hormones may act in concert. Thus, a suite of responses such as plasma steroids, Vg and hepatic ER levels may be monitored to elucidate specific mechanisms of antiestrogenic action.

The initial phase of the present research involved development of an enzyme-linked immunosorbent assay to quantify plasma Vg (adapted from Goodwin *et al.*, 1992; Kishida *et al.*, 1992; Kwon *et al.*, 1993) and a hepatic estrogen binding assay to estimate ER concentrations in trout (adapted from Pasmanik and Callard, 1988; MacKay and Lazier, 1993; and Patnode and Curtis, 1994). An estrogen dose-response and time course experiment was then conducted to characterize the sensitivities of a suite of vitellogenesis biomarkers. These methods and results are described in Chapter 2.

Secondly, we explored two mechanisms by which the xenoestrogen, CD might modulate 7,12-dimethylbenz[*a*]anthracene (DMBA) induced carcinogenesis in trout; via altered carcinogen disposition or estrogenic actions (Chapter 2). We characterized CD, cholesterol and DMBA disposition after CD pretreatment to determine if CD bioaccumulation in trout modified xenobiotic transport as previously described in our laboratory in mice (Carpenter and Curtis, 1989). CD estrogenicity was evaluated by monitoring plasma Vg and hepatic ER concentrations in chronically exposed trout and via *in vitro* competitive binding assays.

We also assessed the ability of a second group of estrogenic OCs (o,p'-DDT, o,p'-DDE and p,p'-DDE) to induce vitellogenesis and to competitively bind to the hepatic ER in juvenile rainbow trout (Chapter 3). Target organ doses associated with Vg production were estimated by conducting a disposition study.

Finally, we utilized vitellogenesis to examine the antiestrogenic effects of the co-planar PCB, 345-HCB, in juvenile rainbow trout (Chapter 4). Several markers of vitellogenesis (liver somatic index, hepatic ER, plasma Vg and plasma estradiol concentrations) in estrogen induced fish were monitored. Additionally, we evaluated 345-HCB CYP1A induction potential and its modulation by estradiol to compare the relative sensitivity of this marker to vitellogenesis. A preliminary evaluation of the antiestrogenic effects of ßnaphthoflavone was also conducted.

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CHAPTER 2

Modulation of 7,12-Dimethylbenz[*a*]anthracene Induced Cancer Incidence and Hepatic Vitellogenin Synthesis by Chlordecone in Rainbow Trout

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ABSTRACT

Chlordecone (CD) bioaccumulation may modulate carcinogenesis in rainbow trout via estrogenic actions or altered carcinogen disposition. We developed an *in vivo* marker of hepatic estrogenic action, synthesis of the yolk-protein precursor, vitellogenin (Vg), by conducting an estrogen dose-response experiment. Juvenile trout were injected with 17ß-estradiol (0 - 10 mg/kg) on day 0 and 3 and sampled on days 3 to 12. Estradiol (5 mg/kg) increased plasma Vg (2400%), liver somatic index (LSI, 200%) and hepatic cytosolic estrogen receptor levels (ER, 300%) at day 6. The results suggested plasma Vg was a sensitive marker of estrogen exposure.

Previous work demonstrated CD pretreatment altered disposition of a subsequent [^{14}C]CD or [^{14}C]cholesterol dose in rodents. We proposed CD bioaccumulation in trout might modify tumor incidence by producing similar changes in the disposition of CD and the genotoxin, 7,12-dimethylbenz[*a*]anthracene (DMBA). [^{14}C]CD disposition experiments were conducted following 2 ip (0 - 15 mg/kg) and 2 dietary (0 - 0.4 mg/kg/day) pretreatment regimes. In the carcinogenesis experiment, trout were fed CD (0, 0.1, 0.4 mg/kg/day) for 9 weeks, received a water-borne [^{3}H]DMBA exposure (1 ppm, 20 hr), and resumed CD diets for 33 weeks. CD pretreatment did not influence [^{14}C]CD, [^{14}C]cholesterol or [^{3}H]DMBA hepatic concentrations, hepatic [^{3}H]DMBA-DNA adduct levels, or hepatic/stomach tumor incidence.

However, CD weakly promoted DMBA-induced hepatic tumor incidence at the low but not the high CD dose. CD bioaccumulation, following exposure to the high CD dose, resulted in hepatic CD concentrations of 15 mg/kg and was associated with detectable plasma Vg levels but not elevated hepatic ER concentrations. *In vitro* competitive binding assays confirmed CD had weak affinity (1000-fold less than estradiol) for the trout hepatic ER. We conclude CD weakly promoted hepatic tumor incidence and produced mild estrogenic effects in trout but the two responses were not positively correlated in this study. These results indicate vitellogenesis may be a useful model to further study estrogenic effects of organochlorine bioaccumulation.

INTRODUCTION

Estrogenic organochlorine (OC) bioaccumulation has been associated with enhanced human cancer risk in some studies (Wolff *et al.*, 1993; Davis *et al.*, 1993) but not in others (Krieger *et al.*, 1994). The present study explored two mechanisms by which the xenoestrogen, chlordecone (CD), might modulate 7,12-dimethylbenz[*a*]anthracene (DMBA) induced carcinogenesis in juvenile rainbow trout; via estrogenic actions or altered carcinogen disposition. CD was a promoter in both sexes of mice and rats, producing a significantly higher incidence of hepatic carcinoma in female rats compared to males (Reuber, 1978; Sirica *et al.*, 1989). The mechanism by which CD enhanced tumor formation has not been determined but it may be related to its estrogenic properties (Eroschenko, 1981). Estrogenic steroids acted as promoters of hepatocarcinogenesis in rats (Yager *et al.*, 1984; Lucier *et al.*, 1991) and rainbow trout (Nunez *et al.*, 1989).

The first objective of our study was to evaluate the role of CD estrogenic actions in tumor promotion. Towards this end, we developed an *in vivo* marker of estrogen regulated gene expression in the liver and related marker responses in CD exposed juvenile rainbow trout to tumor promotion. We chose to study vitellogenesis, a process during which fish synthesize large amounts of hepatic lipoproteins for egg production. Vitellogenesis is analogous to lactation in mammals and has been utilized as an end-point in the study of steroid-regulated gene expression in several species (Tata and Smith, 1979). Under the control of estrogen, the liver produces and exports very low density lipoprotein (VLDL) and a special high density lipoprotein,

vitellogenin (Vg; Ng and Idler, 1983; Mommsen and Walsh, 1988). Additional responses to estradiol treatment include; elevation of hepatic estrogen receptor (ER) levels (MacKay and Lazier, 1993), liver hyperplasia and hypertrophy (Mommsen and Walsh, 1988), and plasma hyperlipidemia (*i.e.* triglycerides and cholesterol; Wallaert and Babin, 1992). In contrast to mature females, estrogenized juvenile fish accumulate Vg in the plasma due to the lack of or the immature state of the ovaries (Mackay and Lazier, 1993). Estrogenic compounds such as DDT, phytoestrogens and nonylphenol have induced Vg synthesis in fish (Denison *et al.*, 1981; Pelissero *et al.*, 1991; 1993; White *et al.*, 1994). We characterized the sensitivity of the vitellogenesis markers [liver somatic index (%LSI), hepatic ER concentrations, plasma lipid levels and Vg synthesis] by conducting an estrogen-dose response and time course experiment. Additionally, we determined the *in vitro* relative binding affinity of CD for trout hepatic ER to assess the estrogenic potency of CD. Hepatic ER and plasma Vg concentrations in chronically CD exposed trout were then evaluated to determine the potential relationship of estrogenic actions to cancer promotion.

A second aim of the present study was to examine the effect of CD exposure on carcinogen disposition and subsequent tumor formation. To adequately assess cancer risk, it is important to understand mechanisms of OC disposition and bioaccumulation. Although OCs generally distribute to lipid rich tissues, recent research indicates bioaccumulation is more complex than simple lipid partitioning and involves protein binding (Curtis *et al.*, 1990; Gilroy *et al.*, 1993; Kedderis *et al.*,

1993). CD distributes preferentially to the liver of humans (Cohn *et al.*, 1978) and rats (Egle *et al.*, 1978) primarily due to its affinity for plasma high density lipoproteins, which transport cholesterol from peripheral tissues to the liver (Maliwal and Guthrie, 1982). Research in our laboratory demonstrated that CD pretreatment (5-15 mg/kg, ip) altered subsequent disposition of [^{14}C]CD and [^{14}C]cholesterol in male mice (Carpenter and Curtis, 1989; 1991) and rats (Gilroy *et al.*, 1994). In mice, the response was characterized by a decline in hepatic [^{14}C]CD and [^{14}C]cholesterol levels and an increase in non-hepatic tissues such as fat. Changes were not due to differences in total lipid content and were consistent with modified hepatic cholesterol transport involving a specific, high affinity, low capacity system. Previous research by others suggested sterol carrier proteins were important for CD intrahepatic transport (Soine *et al.*, 1984). While the mechanism remains unidentified, evidence suggests CD may alter protein-mediated lipid/xenobiotic transport.

Other environmental carcinogens, such as polycyclic aromatic hydrocarbons (PAHs), may also be carried intrahepatically by lipid binding proteins (Soues *et al.*, 1989; Zhong *et al.*, 1989). Thus, modified lipid transport, resulting from CD pretreatment, might alter target organ doses of genotoxic (PAH) and non-genotoxic (CD) carcinogens. Changes in bioaccumulation might be reflected in different tumor incidence or organospecificity. We assessed whether CD pretreatment would alter disposition of a subsequent dose of [^{14}C]CD, [^{14}C]cholesterol, or the PAH, [^{3}H]-DMBA, in rainbow trout. Hepatic DMBA-DNA adduct levels and tumor incidence

were used to quantify CD/PAH genotoxic interactions. We selected rainbow trout because DMBA is a potent, multi-site carcinogen in this animal model (Fong *et al.*, 1993). Additionally, reports of PAH-induced hepatic neoplasia in feral populations suggest fish may be an important indicator species for environmental carcinogenesis (Baumann, 1989).

MATERIALS AND METHODS

Chemicals

CD was purchased from ChemService (West Chester, PA; 99.5% pure) and [^{14}C]CD from Sigma Chemical Co. (St. Louis, MO; 6.1 mCi/mmole, 96% pure). Chlordecone alcohol (CD-OH) was generously supplied by M.W. Fariss and J.D. Smith (Medical College of Virginia, Dept. of Pathology, Richmond VA). DMBA was obtained from Aldrich Chemical Co. (Milwaukee, WI; 98% pure) and [^{3}H]DMBA from Amersham Corp. (Arlington Heights, IL; 63 Ci/mmole, 98% pure). 17ß-Estradiol 3-benzoate (Sigma Chemical Co.) was used for estradiol treatments. Moxestrol, [^{3}H]moxestrol (86 Ci/mmole; 97.7% pure) and [^{14}C]cholesterol (53 mCi/mmole; 99% pure) were obtained from New England Nuclear (Wilmington, DE).

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) were provided by the Marine and Freshwater Biomedical Research Center core facility at Oregon State University. Fish were fed a growth ration (4-8% dry weight diet/dry weight fish) of Oregon Test

Diet (Lee *et al.*, 1991) and held in flow-through circular tanks (13°C) on a 12-hr light-dark photoperiod.

Vitellogenesis Model Development

In order to assess the sensitivity of selected vitellogenesis markers to estradiol, an estrogen dose-response and time-course experiment was conducted. We injected fish (≈18 g) ip with 0, 0.1, 1, 5 or 10 mg/kg 17ß-estradiol, dissolved in peanut oil, on day 0 and again on day 3. Three, 6, 9 and 12 days after the initial injection, fish were killed and liver and blood were sampled (see below). Livers were frozen in liquid nitrogen and stored at -80°C for later ER analysis. Plasma estradiol was quantified by radioimmunoassay as described by Fitzpatrick *et al.* (1986). Plasma total cholesterol and triglyceride levels were determined by commercial kits (Sigma Diagnostic Procedures 352 and 334; Sigma Chemical Co., St. Louis, MO).

Vitellogenin Enzyme Linked Immunosorbent Assay (ELISA)

Blood samples were collected from the caudal vein in heparinized syringes, transferred to vials containing phenylmethylsulfonylfluoride (0.3 μM) and centrifuged for 2 min at 1500 g. Plasma was stored at -80°C. Vg plasma levels were quantified by a competitive ELISA. Purified rainbow trout Vg and anti-Vg rabbit immunoserum (polyclonal, titre 165,000x) were obtained from David R. Idler (Memorial University of Newfoundland; St. John's, Newfoundland, Canada; prepared as described in So *et al.*, 1985). High-affinity ELISA plates (Immulon 4; Dynatech, Chantilly, VA) were coated with 100 μl 0.1 M carbonate buffer (pH 9.6) containing 25 ng Vg for 24 hr at

4°C. Vg standards (60μl, 12-1600 ng/ml) or plasma samples diluted in assay buffer [phosphate buffered saline (PBS), 0.125 M NaCl and 0.1 M sodium phosphate; 1% bovine serum albumin; 0.1% Triton X-100 and 0.1% Tween-20] and antibody (1:60,000; 60 μl) were combined in each well of low binding affinity plates (Costar, Cambridge, MA) and incubated at 4°C for 24 hr. ELISA plates were washed with PBS/0.1% Tween-20 four times and blotted dry. Wells were blocked with 100 μl assay buffer for 1 hr at 34°C and washed. Contents of low binding affinity wells (100 μl) were transferred to ELISA plate wells and incubated at 4°C for 24 hr. After washing, 100 μl anti-rabbit IgG (1/1500, biotinylated from donkey; Amersham Corp., Arlington Heights, IL), was added to each well, followed by incubation for 2 hr at 34°C. Washing was followed by addition of 100 μl strepavidin-horseradish peroxidase (1/600; Amersham Corp., Arlington Heights, IL), incubation for 2 hr at 34°C, then washing. Plate wells were developed by adding 100 μl of freshly prepared chromagen [1.5 mg 3,3,5,5-tetramethylbenzidine, dissolved in 150 μl DMSO, 15 ml 0.1 M sodium acetate buffer (pH 6.0), and 0.01% H_2O_2]. The plates were incubated at room temperature for 10 min and the reaction was stopped by addition of 100 μl 2 M H_2SO_4. Absorbances were read at 450 nm on a Titertek Multiskan Plus MKII plate reader.

Estrogen Binding Assay

Livers were homogenized [(1:2, w/v) in TEMS buffer (10 mM Tris-HCl, 1.5 mM EDTA, 20 mM sodium molybdate, 12 mM monothioglycerol, 10% glycerol; pH 7.4)] with a motor-driven Teflon/ground glass homogenizer on ice. Homogenates

were centrifuged at 2000 g for 20 min at 4°C, and the supernatant was decanted for additional preparation of cytosol. The nuclear pellet was washed three times in 10 mM Tris-HCl, 3 mM $MgCl_2$, 2 mM monothioglycerol and 0.25 M sucrose (pH 7.5; 1:10 w/v) and centrifuged at 2000 g for 15 min. The washed pellet was extracted with nuclear extraction buffer (NEB; 50 mM Tris-HCl, 1.5 mM EDTA, 12 mM monothioglycerol, 0.7 M KCl and 30% glycerol; pH 7.5; 1:2 w/v) in an ice-bath for 1 hour, mixing at 15 min intervals. Both the low-speed supernatant (cytosolic fraction) and nuclear pellet extract were centrifuged at 100,000 g for 1 hr at 4°C. Resultant cytosolic and nuclear supernatants were stripped with dextran-coated charcoal (2 vol supernatant/1 vol TEMS or NEB containing 5% charcoal and 0.5% dextran, w/v) for 10 min in an ice bath. The nuclear pellet was resuspended and DNA concentration determined by the method of Burton (1956). Charcoal-treated cytosol and nuclear extracts were centrifuged at 100,000 g for 1 hr at 4°C and supernatants were removed and stored at -80°C. Protein concentrations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Cytosolic or nuclear extracts (150 μl, protein concentration 5-6 mg/ml) were incubated for 24 hr (2°C) with the synthetic estrogen, [^{3}H]moxestrol (0.3 - 10 nM; 50μl), in the absence or presence of 200-fold excess radioinert moxestrol (50 μl) to determine total and non-specific binding. To separate bound from free moxestrol, 0.5 ml TEMS or NEB containing 2.5% charcoal and 0.25% dextran were added and the samples were incubated in an ice bath for 10 min. Tubes were centrifuged at 1200 g

for 15 min (2°C) and 0.5 ml of the resulting supernatant was used to determine amounts of bound radioactivity. Specific binding (total minus non-specific binding) was calculated and K_d and estrogen binding site numbers were determined from Scatchard plots (Scatchard, 1949) by least-squares regression.

We also assessed the affinity of CD, relative to estradiol, for trout hepatic ER by means of an *in vitro* competitive binding assay. Trout (≈ 25 g, n=29) were injected on day 0 and 3 with 0.9 mg/kg 17ß-estradiol and killed on day 6. Duplicate cytosol preparations were obtained from pooled liver homogenates. Cytosol was incubated with 5 nM [^{3}H]moxestrol in the presence or absence of radioinert moxestrol, estradiol, CD or CD-OH (5 nM - 50 uM) for 24 hr at 4°C. Radioinert compounds were dissolved in 99% ethanol and diluted to appropriate final concentrations which contained <1% ethanol. Two assays, run in duplicate, were performed on separate days.

CD and Cholesterol Disposition Following CD Pretreatment

Four [^{14}C]CD disposition experiments were conducted following 2 ip and 2 dietary CD pretreatment regimes. Sexually immature fish, within the initial and final weight ranges of those in the carcinogenesis experiment, were utilized. In Experiment 1, CD pretreatment and tracer doses were equivalent to those tested by Carpenter and Curtis (1989) in mice. Trout received an ip CD pretreatment dose (1, 5 or 15 mg/kg; ≈200 g fish) followed by a 5 mg/kg [^{14}C]CD ip injection (or 10 nmol/g; 0.5 μCi dissolved in corn oil) given 3, 7 or 14 days later. Lower doses were selected in Experiment 2 where ip injection of CD (1 mg/kg) was followed 3 days

later by a [^{14}C]CD tracer dose (0.1 or 1 mg/kg ip; ≈15 g fish). Due to acute toxicity, higher CD ip doses could not be tested. To test greater cumulative CD doses, 2 dietary exposure experiments were conducted. In Experiment 3, trout (≈40 g, initial weight) were fed CD (0, 0.1 or 0.3 mg CD/kg/day) for 3, 6, 9 or 12 weeks followed by a [^{14}C]CD ip injection (5 mg/kg). Disposition of cholesterol and lower CD tracer doses were evaluated in Experiment 4. Fish (≈100 g) were challenged with [^{14}C]CD after 3 and 7 days (0.5 mg/kg) and after 3 and 9 weeks (5 mg/kg) dietary exposure to 0, 0.1 or 0.4 mg CD/kg/day. Another group of fish received [^{14}C]cholesterol (0.5 μCi dissolved in corn oil) after 7 (5 mg/kg) or 21 (0.5 mg/kg) days of CD dietary pretreatment (0, 0,1 and 0.4 mg CD/kg/day). Fish were killed 24 hr after [^{14}C]CD injection, blood samples were drawn from the caudal vein and viscera (gallbladder/bile, liver, visceral fat, kidney, gonads, and gastrointestinal tract) were removed. Tissues were homogenized, digested overnight with Soluene 350 tissue solubilzer (Packard, Donners Grove, IL), decolorized (isopropanol + H_2O_2) and counted in duplicate with a Packard Tricarb scintillation counter. Total liver lipid content was determined in Experiment 4 according to Bligh and Dyer (1959).

DMBA Disposition Following CD Pretreatment

The initiation protocol involved exposing trout to [^{3}H]DMBA, following CD pretreatment, in order to assess the effects of CD on DMBA disposition. Trout (≈15 g) were pretreated with dietary CD (0, 0.1 or 0.4 mg/kg/day) for 9 weeks (estimated total dose; 0, 6 and 25 mg/kg). DMBA-initiation was conducted by immersion in

water containing 0 or 1 ppm [^{3}H]DMBA (dissolved in DMSO; final DMSO concentration < 0.04%; see Table 3 for treatment groups) for 20 hr at 14°C. Fish were transferred to uncontaminated water containing submersed charcoal filters and after 4 hr, 8 fish per group were killed and livers, gallbladder/bile and stomach were extracted to determine [^{3}H]DMBA uptake. Twenty-four hr after the end of the immersion period, pooled liver samples (4 groups of 4 livers) were analyzed for [^{3}H]DMBA-derived DNA adducts using the method of Zhang *et al.* (1992).

Carcinogenesis Experiment

One month after DMBA-initiation (described above), fish, housed in duplicate tanks, received diet containing CD [0 (n=100/tank), 0.1 or 0.4 mg/kg/day (n=40/tank)] for 33 weeks. Fish were anesthetized with MS-222, blood samples were taken from a subset of fish (treatment groups 1, 2, 3) for Vg determination and fish were killed by exsanguination. Livers and stomachs were immediately examined under a dissecting microscope for surface tumors (Hendricks *et al.*, 1984). Tissues were preserved in Bouin's solution and livers were hand sliced to detect internal tumors. Tumors and at least ten liver samples per treatment group were examined histologically to classify the neoplasms according to the criteria of Hendricks *et al.* (1984) and to assess hepatotoxicity. A subset of livers (treatment groups 1, 2, 3) were immediately frozen (-80°C) for ER analysis.

Total hepatic CD levels in fish receiving CD for 33 weeks were determined by EC-GLC using the method of Blanke *et al.* (1977) as modified by Carpenter and Curtis (1989). Analysis was conducted on a Varian 3700 gas chromatograph

equipped with a capillary column (Alltech Econo-Cap SE-54, 15 m x 0.54 mm x 1.2 μm; Alltech Assoc., Deerfield, IL). Carrier gas (He) and makeup gas (N_2) flow rates were 30 ml/min and temperatures were; injector, 260°C; column, 200°C; and detector 350°C. Samples were spiked with [^{14}C]CD as an internal standard which was used to correct for recoveries. The mean CD recovery in liver samples was 68%.

Statistics

Liver somatic index (%LSI) was determined by multiplying the liver to body weight ratio by 100. Estrogen dose-response data were evaluated for statistical significance via ANOVA and Newman-Keuls multiple comparison tests. For the competitive binding assay, relative binding affinity was estimated as the ratio of the concentration of radioinert moxestrol to the concentration of competitor required for 50% displacement of [^{3}H]moxestrol binding, when displacement curves were parallel (determined by a test for homogeneity of slopes available on SYSTAT, Systat Inc., Evanston IL). Tracer disposition data was analyzed by 2-way ANOVA (pretreatment time x dose). One-way ANOVA and Newman-Keuls multiple comparison tests, at a given pretreatment time, were performed when significant ($p \leq 0.05$) dose or time effects were observed. Tumor incidence (# tumor-bearing fish/total # examined) data from duplicate treatment tanks were compared statistically by the Fisher's Exact probability test. Liver tumor results from duplicate tanks were not significantly different, and data were pooled and analyzed by Fisher's Exact probability test. For stomach tumor data, individual tanks were the experimental unit. Tank stomach tumor percent incidence (arc-sine transformed) and stomach tumor number/fish were

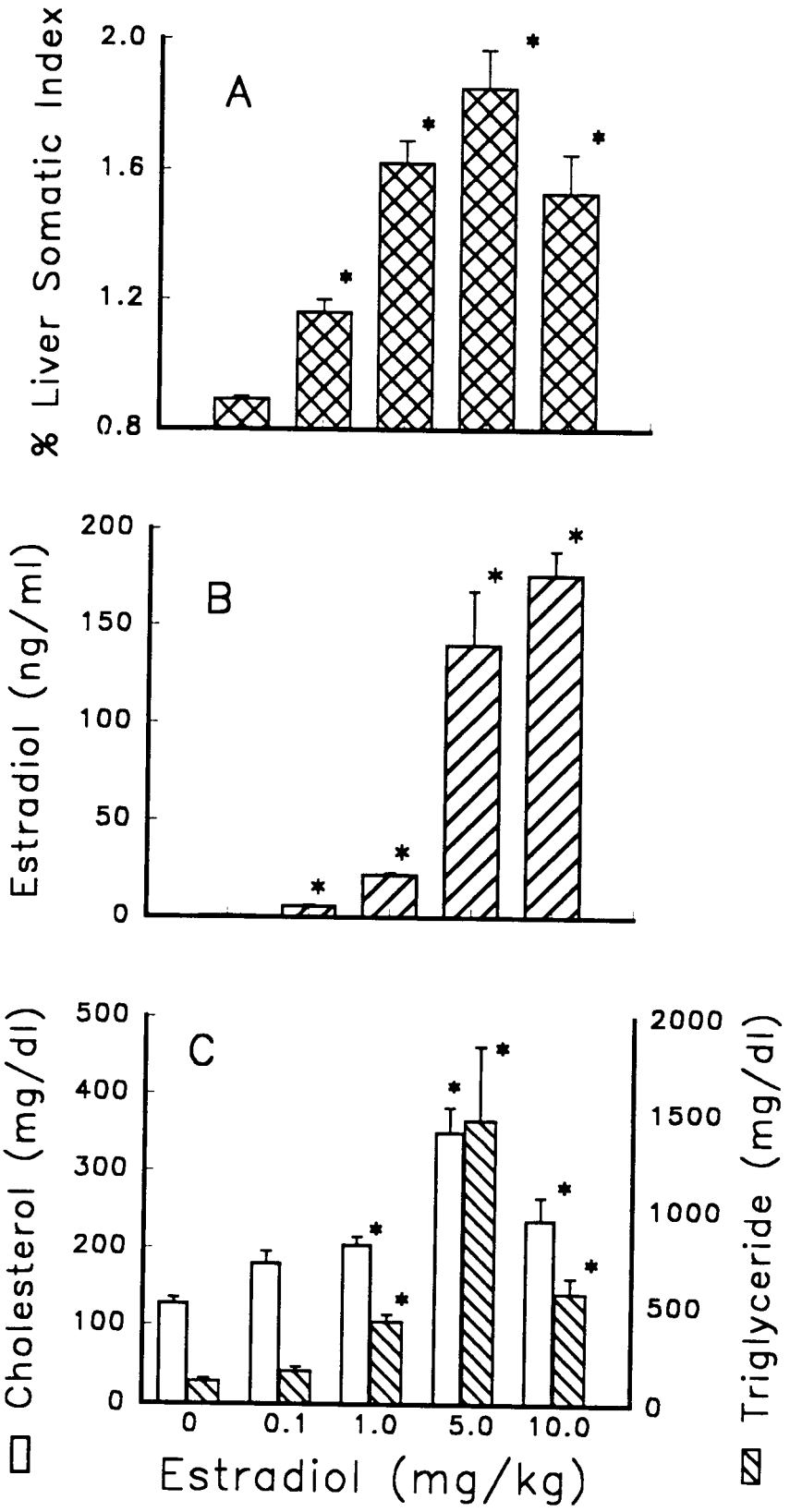
examined by ANOVA. Vg levels in CD-treated fish were evaluated for treatment differences by Kruskal Wallis non-parametric ANOVA and Vg incidence above detection limit data were analyzed by Chi-Square analysis. The %LSI and hepatic ER data from CD-treated fish were analyzed by ANOVA. Mean $\pm$ SE were calculated unless otherwise noted.

RESULTS

Estradiol-Induced Vitellogenin Synthesis

The estrogen-dose response relationship and time course of vitellogenesis was characterized to determine the potency of estradiol. Estradiol treatment doubled %LSI, compared to controls, at the 5 mg/kg dose on day 6 (Figure 2.1A). Mean plasma estradiol on day 6 ranged from $<$ 1 ng/ml in controls to 176 ng/ml at the 10 mg/kg estrogen dose (Figure 2.1B). Estradiol produced hyperlipidemia as shown by plasma total cholesterol levels which rose to 300% (350 mg/dl) and triglycerides which increased to 1200% (1460 mg/dl) of controls at the 5 mg/kg estrogen dose on day 6 (Figure 2.1C). Plasma Vg increased from the detection limit of 0.25 μg/ml in controls to 600 μg/ml at the 5 mg/kg dose (day 6; Figure 2.2A). Vg continued to accumulate in the plasma from day 0 to day 3, reaching 2000 μg/ml by day 12 (5 mg/kg dose; Figure 2.2B). Additionally, hepatic cytosolic ER levels were 300% of control values at the 1 mg/kg estrogen dose (154 fmol/mg protein or 1.0 fmol/μg DNA; day 6, Figure 2.3A). Apparent K_d values also increased from 0.89 nM in

Figure 2.1. **Estrogen elevated liver somatic index (%LSI; A) plasma estradiol (B) and plasma cholesterol and triglyceride levels (C).** Trout ($\approx$18 g) were injected ip with 17ß-estradiol (0, 0.1, 1, 5, 10 mg/kg) on day 0 and 3, sampling on day 6 (* significantly different from control, $p \leq 0.05$).


% Liver Somatic Index
2.0
1.6
1.2
0.8
A
Estradiol (ng/ml)
200
150
100
50
0
B
Cholesterol (mg/dl)
500
400
300
200
100
0
C
2000
1500
1000
500
0
Triglyceride (mg/dl)
0
0.1
1.0
5.0
10.0
Estradiol (mg/kg)

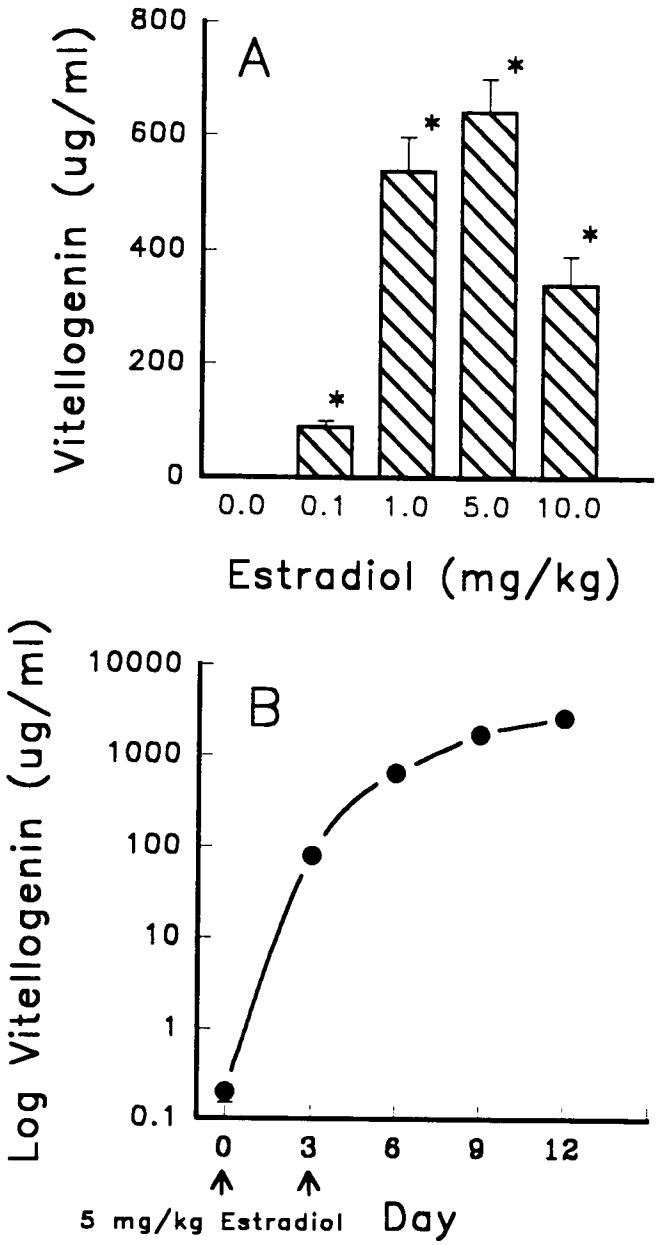
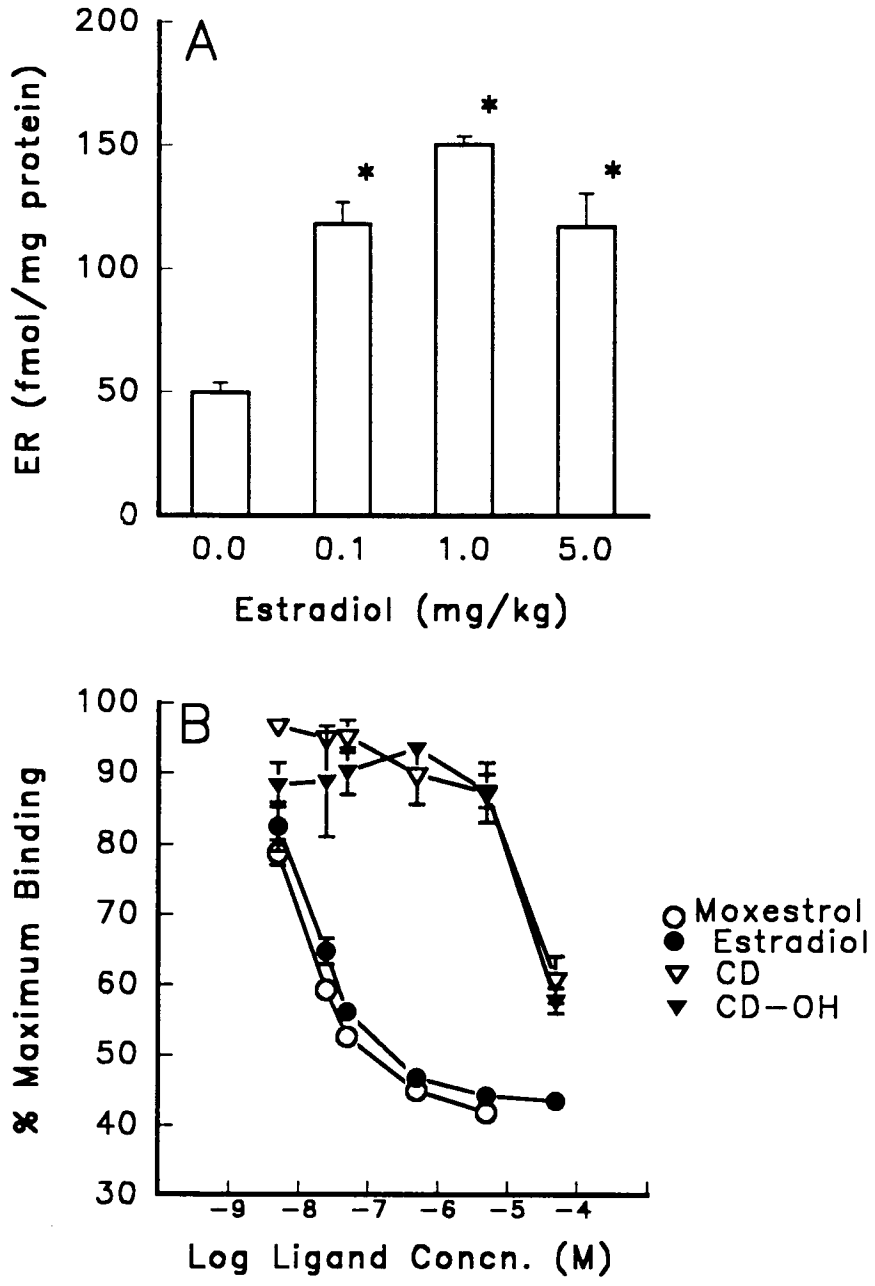


Figure 2.2. **Plasma vitellogenin estrogen dose-response relationship (A) and time course (B).** Trout (≈ 18g) were injected ip with 17ß-estradiol (0, 0.1, 1, 5, 10 mg/kg) on day 0 and 3, sampling on day 3, 6, 9, 12. A) Plasma Vg, determined by ELISA on day 6, was significantly elevated above control levels following estrogen treatment (* significantly different, $p \leq 0.05$). B) Vg accumulation in plasma after ip administration of 5 mg/kg estradiol on day 0 and 3.

Figure 2.3. **Cytosolic hepatic estrogen receptor (ER) number increased with estrogen (A) and chlordecone (CD) showed affinity for estradiol binding sites (B).** A) Hepatic ER levels were determined on day 6 using an equilibrium binding assay (6 pooled livers assayed in duplicate; estradiol-treated differed from control, $p \leq 0.05$). Cytosolic fractions were incubated with [^{3}H]moxestrol (0.3-10 nm) in the presence or absence of 200-fold excess radioinert moxestrol for 24 hr at 4°C, determining specific binding by Scatchard analysis. B) Competitive binding of CD and chlordecone alcohol (CD-OH) was evaluated by incubating cytosol for 24 hr at 4°C with 5 nM [^{3}H]moxestrol and indicated concentrations of competitors, measuring decline in maximum specific binding. Values are means ($\pm$ SD) of two assays run in duplicate.


A
ER (fmol/mg protein)
200
150
100
50
0
0.0
0.1
1.0
5.0
Estradiol (mg/kg)
B
% Maximum Binding
100
90
80
70
60
50
40
30
−9
−8
−7
−6
−5
−4
Log Ligand Concn. (M)
Moxestrol
Estradiol
CD
CD−OH

controls to 7.46 nM at 5 mg/kg estrogen (data not shown). Comparison of ligandconcentrations required to reduce maximum [^{3}H]moxestrol binding by 50% *in vitro* indicated estradiol had equivalent affinity to moxestrol. CD and CD-OH had approximately 1000-fold less affinity than estradiol for estrogen binding sites (Figure 2.3B).

[^{14}C]CD and [^{14}C]Cholesterol Disposition Following CD Pretreatment

CD dietary or ip pretreatment did not alter disposition of subsequent [^{14}C]CD or [^{14}C]cholesterol (data not shown) tracer doses in trout, as previously described in rodents (Carpenter and Curtis, 1989; Gilroy *et al.*, 1994). Tissue [^{14}C]CD distribution was characterized by high levels in liver, visceral fat/gonad and gallbladder/bile (Table 2.1; data from Experiment 1 and 4 not shown). Positive correlations ($r^2 > 0.90$) between [^{14}C]CD tracer dose (Experiment 1-4; 0.1, 0.5, 1.0 and 5.0 mg/kg) and resultant [^{14}C]CD equivalents in plasma, liver and bile of control fish suggested a similar mechanism of distribution, regardless of tracer dose (representative data shown, Table 2.1). Total liver lipid content was not different between CD pretreatment groups (4.7% $\pm$ 0.4, n=15; Experiment 4, Day 63). There was a 50% decrease in total [^{14}C]CD recovery in Experiment 4 (0.4 mg/kg/day, 9 weeks, 25 mg/kg total dose, data not shown) but a similar decline was not observed in Experiment 3 (0.3 mg/kg/day, 12 weeks, 25 mg/kg total dose). Observable acute toxicity occurred only following the 15 mg/kg ip CD pretreatment (Experiment 1) where 50% of the animals died within 7 days.

Table 2.1. **Chlordecone (CD) ip or Dietary Pretreatment Did Not Influence Disposition of a Subsequent [^{14}C]CD Tracer Dose**[a]

Tracer Dose:	0.2 nmol/g [^{14}C]CD[b]		2 nmol/g [^{14}C]CD[b]		10 nmol/g [^{14}C]CD[c]	
CD Pretreatment:	Control	1 mg/kg ip	Control	1 mg/kg ip	Control	25 mg/kg diet
			[^{14}C]CD Equivalents (nmol/g):			
Liver	0.47 ± 0.05	0.55 ± 0.04	4.7 ± 0.4	5.9 ± 0.4	19.4 ± 3.1	19.8 ± 0.8
Plasma	0.09 ± 0.01	0.12 ± 0.01	1.0 ± 0.1	1.2 ± 0.2	6.0 ± 0.9	5.9 ± 0.4
V.Fat/Gonad	0.36 ± 0.05	0.38 ± 0.05	4.1 ± 0.7	5.9 ± 0.4	24.3 ± 7.6	19.2 ± 5.2
Kidney	0.25 ± 0.02	0.31 ± 0.03	3.1 ± 0.3	3.4 ± 0.3	11.1 ± 1.6	10.6 ± 1.0
Gallbladder/Bile	0.48 ± 0.06	0.46 ± 0.05	4.6 ± 0.6	6.1 ± 0.9	13.8 ± 4.3	17.9 ± 1.9
G.I. Tract	0.33 ± 0.04	0.39 ± 0.03	3.3 ± 0.3	3.4 ± 0.2		

[a] Values are mean ± SE; n = 5-6. There were no significant differences between control and CD pretreated groups ($p > 0.05$).

[b] Fish (≈15 g) were pretreated with CD (0 or 1 mg/kg, ip) and 3 days later received a [^{14}C]CD tracer dose (0.2 or 2 nmol/g, 0.1 or 1 mg/kg, ip) in Experiment 2. Tissues were removed and prepared for analysis 24 hr after the tracer dose.

[c] Fish (≈110 g final weight) were pretreated with dietary CD for 12 weeks (0 or 0.3 mg/kg/day, estimated total dose 0 and 25 mg/kg) and received a 10 nmol/g (5 mg/kg, ip) [^{14}C]CD tracer dose in Experiment 3. Tissues were removed and prepared for analysis 24 hr after the tracer dose.

[^{3}H]DMBA Accumulation and Hepatic DNA Adduction

Consistent with [^{14}C]CD disposition results, hepatic and stomach [^{3}H]DMBA equivalents and hepatic [^{3}H]DMBA-derived DNA adducts, following 20 hr immersion in 1 ppm [^{3}H]DMBA, were not significantly different between CD dietary pretreatment groups (0, 0.1 or 0.4 mg/kg/day for 9 weeks, estimated total dose 0, 6, 25 mg/kg; Table 2.2). However, CD pretreatment significantly enhanced [^{3}H]DMBA biliary excretion. The significant increase in biliary [^{3}H]DMBA equivalents in CD exposed groups (Table 2.2) was not accompanied by increased gallbladder/bile or liver weights.

Carcinogenesis Experiment

DMBA water-borne exposure significantly increased stomach tumor incidence (42-66%) in all groups compared to respective non-initiated control fish (Table 2.3). Liver tumor incidence was much lower (1-8%) and differed significantly from non-initiated controls in only two treatments (groups 6 and 9, Table 2.3). Tumor types were identified as hepatocellular carcinomas (HCC), mixed hepatocellular or cholangiocellular carcinomas (MC), hepatocellular adenomas (HCA) and stomach adenomas. CD treatment prior to DMBA-initiation did not alter tumor incidence, in consonance with [^{3}H]DMBA accumulation and DNA adduction results. Additionally, CD was not carcinogenic in fish not exposed to DMBA.

The low dietary CD dose (0.1 mg/kg/day, 33 weeks), following DMBA-initiation, weakly promoted liver tumor incidence (8 vs 2%), compared to controls, but not the high CD dose (0.4 mg/kg/day, Table 2.3). Stomach tumor incidence in duplicate

Table 2.2. **[^{3}H]-7,12-Dimethylbenz[*a*]anthracene (DMBA) Accumulation and Hepatic DNA Binding Following Chlordecone (CD) Dietary Pretreatment**[a]

PRETREATMENT:	Total CD Dietary Dose		
	Control	6 mg/kg	25 mg/kg
[^{3}H] DMBA Equivalents (nmol/g tissue)[b]			
Liver	150 ± 8	148 ± 12	176 ± 12
Gallbladder/Bile	1500 ± 195	2325 ± 222[c]	2497 ± 195[c]
Stomach	44 ± 19	12 ± 1	20 ± 4
DNA Binding (nmol [^{3}H]DMBA/g DNA)[d]			
Liver	20 ± 1	24 ± 4	20 ± 2

[a] Fish (15 g) received dietary CD (0, 0.1 or 0.4 mg/kg/day) for 9 weeks (total dose; 0, 6 and 25 mg/kg) before immersion in [^{3}H]DMBA (1 ppm or 3.9μM) for 20 hours (n=4).

[b] Following immersion, fish were placed in uncontaminated water for 4 hours, killed, and quantities of [^{3}H]DMBA in tissues were determined (n=8).

[c] Significantly different from control, $p \leq 0.05$.

[d] [^{3}H]DMBA-DNA adduction measured in 4 groups of 4 pooled livers.

Table 2.3. **Liver and Stomach Tumor Incidence and Stomach Tumor Multiplicity in 7,12-Dimethylbenz[*a*]anthracene (DMBA)-Initiated Trout Receiving Chlordecone (CD) during Pretreatment or Promotion.**[a]

Treatment	Liver Tumor[b]		Stomach Tumor[c]		
	Incidence[c]	Pooled % Incidence[d]	Incidence	Pooled % Incidence	Tumors/ Fish[f]
Non-Initiated:					
1. C-C-C	0/97; 0/91	0.0	0/97; 0/91	0.0	
2. C-C-CDL	0/37; 0/35	0.0	0/37; 0/35	0.0	
3. C-C-CDH	0/37; 0/29	0.0	0/37; 0/29	0.0	
4. CDL-C-C	0/35; 0/35	0.0	0/35; 0/35	0.0	
5. CDH-C-C	0/36; 0/39	0.0	0/36; 0/39	0.0	
DMBA Initiation:					
6. C-DMBA-C	4/100; 0/97	2.0[g]	62/100; 38/97[g]	52.3	6.5 ± 2.1
CD Pretreatment					
7. CDL-DMBA-C	1/37; 1/37	2.7	15/37; 16/37[g]	41.8	5.8 ± 0.7
8. CDH-DMBA-C	1/37; 0/38	1.3	15/37; 20/38[g]	46.7	10.4 ± 0.2
CD Promotion					
9. C-DMBA-CDL	4/35; 2/39	8.1[g,h]	20/35; 29/39[g]	66.2	12.4 ± 1.3[h]
10. C-DMBA-CDH	1/36; 1/37	2.7	17/36; 18/37[g]	47.9	8.5 ± 0.1

[a] One month after DMBA-initiation, fish, housed in duplicate tanks, received CD promotional diets of 0 (C), 0.1 (CDL) or 0.4 (CDH) mg/kg/day for 33 weeks (total dose; 25 or 92 mg/kg).

[b] Pooled tumor incidence data were analyzed by Fisher's Xact probability test.

[c] Incidence: # of tumor bearing fish/# fish examined in each duplicate treatment tank.

[d] Pooled % Incidence: percent of tumor bearing fish per total number of fish examined

[e] Tank % tumor incidence, after arc-sine transformation, was analyzed by ANOVA.

[f] Mean ± SD of tumor number in tumor-bearing fish (n=2 tanks).

[g] Significantly different from corresponding non-initiated group ($p \leq 0.05$).

[h] Significantly different from C-DMBA-C group ($p \leq 0.05$).

tanks differed significantly in one treatment group (group 6). According to our a priori decision-tree for statistical analysis, stomach tumor incidence replicate data were not pooled. Thus, while pooled stomach tumor incidence was slightly elevated above control in DMBA-initiated fish receiving the low CD dose (66 vs 52%) there were no statistically significant differences between DMBA-exposed groups. In fish bearing stomach tumors, the average number of tumors doubled at the low CD dose but was not different from controls at the high CD dose. Survival and final tank mean body weights (152 g $\pm$ 4, n=6; groups 6, 9, 10) were not affected by CD promotional diets. Mean CD liver concentration in fish receiving 0.4 mg/kg/day for 33 weeks (estimated total dose, 92 mg/kg) was 15.8 $\pm$ 1.1 mg CD/kg wet weight (n=4).

Mild estrogenic effects were observed in trout receiving dietary CD for 33 weeks at doses above those shown to promote liver tumor formation. Vg was detected more frequently and mean levels were significantly elevated above controls (3-fold) in the high CD diet, non-DMBA-initiated group (Table 2.4). There was also a subtle but significant increase in %LSI in CD treated groups compared to controls (Table 2.4). Histological examination showed hepatocellular changes were more indicative of hypertrophy than hyperplasia because enlarged nuclei and mild fat vacuolation were observed in trout livers at the low and high CD doses. Evidence of hepatic necrosis or other cellular structural abnormalities were not observed. CD did not affect hepatic cytosolic ER levels (Table 2.4) or K_d values (2.2 nM $\pm$ 0.1 nM, n=18). Specific nuclear estrogen binding was below non-specific binding levels.

Table 2.4. **Plasma Vitellogenin, Liver Somatic Index (%LSI) and Hepatic Cytosolic Estrogen Receptor Number Following Chlordecone (CD) Dietary Exposure**[a]

	Plasma Vitellogenin			Liver Somatic Index	Hepatic ER[d]
	No. > D.L.[b]	Range (μg/ml)	Mean (95%CI)[c]	(%)	(fmol/mg protein)
Control	4/15	<0.25 - 3.3	0.38 (0.23-0.62)	0.62 ± 0.02	61.7 ± 7.5
CD-Low	3/16	<0.25 - 3.2	0.32 (0.19-0.51)	0.69 ± 0.01[e]	72.5 ± 4.3
CD-High	11/15[e]	<0.25 - 12.0	0.90[e] (0.56-1.50)	0.73 ± 0.02[e]	62.3 ± 8.3

[a] Fish received CD dietary doses of 0, 0.1 (CD-Low) or 0.4 (CD-High) mg/kg/day for 33 weeks. Fish from treatment groups 1, 2 and 3 were sampled (see Table 2.3, n=15-16).

[b] Number of fish where vitellogenin was above the detection limit (0.25 μg/ml)/total number of fish examined.

[c] For mean calculation, 0.25 was substituted for values below D.L.

[d] Values are means ± SE (n=6 groups of 2 pooled livers).

[e] Significantly different from controls, $p \leq 0.05$.

DISCUSSION

We evaluated several responses associated with estrogen induced vitellogenesis in rainbow trout to identify potential markers for estrogenic actions of CD. In our estrogen dose-response study, plasma Vg was the most sensitive indicator of estrogen stimulation. Vg increased 2400% above control values in response to estrogen administration that resulted in physiologically relevant plasma estradiol levels (< 50 ng/ml; Scott and Sumpter, 1983). Plasma Vg concentrations were within the range reported by MacKay and Lazier (1993) for rainbow trout receiving similar estrogen doses, indicating adequate biomarker reliability. Other markers were less sensitive; plasma triglyceride (1200% of control) > hepatic ER levels ≈ plasma total cholesterol (300%) > %LSI (200%). These indicators were dose responsive at estrogen doses of 0.1 to 5 mg/kg. However, at the highest dose tested, 10 mg/kg, there was reduced responsiveness. High dietary estrogen exposure has produced hepatotoxicity in trout (Herman and Kincaid, 1988; Nunez *et al.*, 1989) and toxicity may have been responsible for the observed decline in %LSI, plasma Vg and lipid levels in our study. Many environmental pollutants are capable of exerting estrogenic actions and require screening to assess their potency and possible effects (McLachlan, 1993). *In vitro* tests, such as trout hepatocyte Vg synthesis, have been developed (Vaillant *et al.*, 1988; Pelissero *et al.*, 1993; White *et al.*, 1994). However, monitoring vitellogenesis *in vivo* complements and completes the toxicological profile of a suspected xenoestrogen because it integrates metabolic, hormonal and

dispositional influences. Our *in vivo* estrogen dose response study indicates plasma Vg may be a suitable marker of exposure to estrogenic compounds in juvenile rainbow trout.

We examined whether CD pretreatment might alter carcinogen disposition and subsequently modulate carcinogenesis. Changes in [^{14}C]CD and [^{14}C]cholesterol disposition, following CD preexposure (5-15 mg/kg, ip), have been reported in male rodents (Carpenter and Curtis, 1989; 1991; Gilroy *et al.*, 1994). Despite the range of pretreatment duration, routes of exposure and doses (ip, 1-15 mg/kg; total dietary dose, 0.3 - 25 mg/kg) in this study, CD pretreatment did not produce a similar alteration in [^{14}C]CD or [^{14}C]cholesterol disposition in juvenile trout. The greater acute toxicity of CD to trout prevented the testing of higher doses (trout $LD_{50} \approx 15$ mg/kg vs rat LD_{50} 126 mg/kg; Guzelian, 1982). Rodent dispositional changes were consistent with the involvement or the induction of lipid-transport binding proteins. Our inability to find parallel changes in trout indicated the profile of lipid-binding proteins or their induction potential differed from rodents. CD distribution patterns were similar between species except that trout had consistently lower liver:plasma ratios (3-5:1) than non-CD pretreated rodents (15-34:1; Carpenter and Curtis, 1989; Gilroy *et al.*, 1994). The difference was not suggestive of dissimilar lipid partitioning because total liver lipid content was comparable between rat and trout (4-5%; Gilroy *et al.*, 1994). Our results suggest trout liver may have different affinity for CD than rodents, potentially reflecting dissimilar cholesterol metabolism or constitutive lipid-binding protein profiles. Fish lipid uptake and transport mechanisms parallel

mammals but dynamics vary because fish depend on lipids, rather than carbohydrates, as their primary energy source, (Black and Skinner, 1986; Sheridan, 1988). Other species differences in CD-induced lipid changes have been reported, including observed hypocholesterolemia and hypotriglyceridemia in rodents but not in occupationally-exposed humans receiving similar CD doses (Guzelian, 1992).

Fish may also be refractory to putative CD induction of lipid binding proteins associated with altered CD disposition (Gilroy *et al.*, 1994). CD, a "phenobarbital-like" agent, induced CYP2B1 and CYP2B2 in rodents (Kocarek *et al.*, 1991) but not trout (Vodicnik *et al.*, 1981). Recent evidence linking regulation of CYP2B1/2 to oxysterols (Kocarek *et al.*, 1993; Sudjana-Sugiaman *et al.*, 1994) provides a mechanism whereby CD might alter cholesterol metabolism in rodents but not trout.

We proposed CD pretreatment would alter lipid/xenobiotic transport and consequently change DMBA-induced cancer incidence by modifying DMBA target organ levels. However, consistent with trout [^{14}C]CD disposition results, CD preexposure did not alter liver or stomach [^{3}H]DMBA uptake, liver DNA adduct levels or liver or stomach tumor incidence. The mechanism by which CD pretreatment enhanced [^{3}H]DMBA biliary excretion requires further exploration. CD did impair biliary excretion in rodents (Rochelle and Curtis, 1994). Additionally, biliary efflux of PAHs via the canalicular active transporter, P-glycoprotein, has been enhanced following induction by xenobiotics (LeBlanc, 1994).

Water-borne exposure of juvenile trout to DMBA produced predominantly stomach adenomas whose incidence was not affected by CD pretreatment. Chronic

CD dietary treatment weakly promoted DMBA-initiated hepatic tumors in trout at the low CD but not the high CD dose (total estimated dose at 33 weeks; 25 and 92 mg/kg). Hepatic concentrations at the high CD dose (15 mg/kg) were slightly higher than the minimum concentration required to detect a hepatic tumor promoting effect in male rats (Sirica *et al.*, 1989; 1-5 mg/kg). Stronger promotion by CD occurred when rats received a total dose of 162-486 mg/kg (3-9 mg/kg s.c., 2x/week, 27 weeks) and attained hepatic CD concentrations greater than 30 mg/kg (Sirica *et al.*, 1989). Further work is required to address whether CD doses in our study approximated the lower limit of detection for CD tumor promotion in juvenile trout. Additionally, the ability to detect hepatic tumor promotion by CD may be amplified by the use of sexually mature female trout or a more potent hepatocarcinogen, such as aflatoxin (Nunez *et al.*, 1988).

Similar to the minimal degree of CD tumor promotion, CD appeared to be weakly estrogenic in juvenile trout. We detected Vg synthesis (0.9 μg/ml), in the absence of estrogen stimulation, after chronic dietary exposure to the high CD dose (0.4 mg/kg/day, 33 weeks) but not the low CD (0.1 mg/kg/day) dose. When detected, plasma Vg levels were substantially lower than Vg concentrations resulting from low estrogen doses in the estrogen dose-response experiment (0.1 mg estradiol/kg; 88 μg Vg/ml) and lower than reported in sexually mature female trout (50 mg/ml; Scott and Sumpter, 1983). *In vitro* competitive binding assays confirmed CD's potential to weakly bind to trout hepatic ER. CD binding affinity, 1000-fold less affinity than estrogen, was similar to values reported for rat uterine tissue

(Hammond *et al.*, 1979; Williams *et al.*, 1989). We have also shown that CD-OH, a minor metabolite in fish (Van Veld *et al.*, 1984) and a major metabolite in man (Fariss *et al.*, 1980) retained ER binding affinity comparable to the parent compound. *In vivo* CD estrogenic effects in rats, such as maintenance of pregnancy (Johnson *et al.*, 1992) and altered uterine ER dynamics (Williams *et al.*, 1989), occurred following acute doses of 50-75 mg/kg, similar in range to cumulative doses in the present study. It is difficult to compare trout and mammalian estrogenic marker sensitivities due to the absence of rat target organ CD levels in these reports (Williams *et al.*, 1989; Johnson *et al.*, 1992). However, our results indicated CD was very weakly estrogenic in the juvenile trout vitellogenesis model when hepatic concentrations approximated 15 mg/kg.

Bioaccumulation of estrogenic OCs has the potential to result in high liver burdens, hepatotoxicity and hepatocarcinogenicity. A unique aspect of the rainbow trout model is the ability to combine hepatocarcinogenesis bioassays and vitellogenesis monitoring to examine the role of estrogenic actions in hepatic cancer promotion. We conclude CD minimally promoted hepatic tumor incidence and produced mild estrogenic effects in trout but the two responses were not positively correlated in the present study. Results indicate vitellogenesis is a useful model to further study estrogenic effects of OC bioaccumulation.

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CHAPTER 3

Induction of Vitellogenesis and Hepatic Estrogen Receptor Binding by o,p'-DDT and o,p'-DDE, but not p,p'-DDE, in Juvenile Rainbow Trout

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ABSTRACT

The mechanisms of DDT-induced reproductive impairment in fish have not been fully established but may involve interaction with the estrogen receptor (ER). We examined the ability of o,p'-DDT, o,p'-DDE and p,p'-DDE to induce vitellogenesis (estrogen regulated hepatic production of the yolk-protein precursor, vitellogenin; Vg) and to competitively bind to the hepatic ER in juvenile rainbow trout. Trout were injected ip with single or triplicate doses of o,p'-DDT, o,p'-DDE or p,p'-DDE (0, 5, 15 or 30 mg/kg) and vitellogenesis was monitored 42 days later. We determined plasma Vg and hepatic ER concentrations were significantly elevated by o,p'-DDT and o,p'-DDE (total dose 45 to 90 mg/kg) but not p,p'-DDE. Target organ doses were estimated by conducting a disposition study in which trout were injected ip with [^{14}C]p,p'-DDE (30 mg/kg x 3 injections) and tissue radioactivity was quantified. Hepatic [^{14}C]p,p'-DDE equivalent concentrations resulting from a total dose of 90 mg [^{14}C]p,p'-DDE/kg averaged 14 mg/kg. The relative affinity of DDT analogs for trout hepatic ER was evaluated by *in vitro* competitive binding assays. o,p'-DDT and o,p'-DDE, but not p,p'-DDE were weakly estrogenic, with a binding potency of 6.4×10^{-6} the activity of moxestrol, a synthetic estrogen. These data indicate o,p'-DDT and o,p'-DDE were mildly estrogenic and may induce vitellogenesis in juvenile rainbow trout when hepatic concentrations reach mg/kg quantities.

INTRODUCTION

The organochlorine insecticide, DDT, was widely used in the United States from the 1940s until it was banned in 1972 because of its persistence and toxicity. However, some Central and South American countries and India still produce DDT and its use continues in various tropical regions (World Health Organization, 1989). DDT may be atmospherically transported from these regions, distributed globally and introduced into aquatic ecosystems even where usage is restricted (Rappaport *et al.*, 1985). As a result, residues of DDT and its degradation products (DDE), persist in aquatic biota worldwide (World Health Organization, 1989). In the United States, DDT and DDE occur in at least 300 hazardous waste sites, indicating areas of elevated contamination exist as well (U.S. Dept. of Health and Human Services, 1993).

Widespread concern in the United States was ignited in the 1950s when DDT exposure was associated with documented declines and reproductive impairment in fish and wildlife populations (Burdick *et al.*, 1964; Blus *et al.*, 1995). Reports of decreased reproductive success have continued to the present, especially in areas of considerable DDT contamination. For example, recent dramatic declines in an American alligator population in Lake Apopka, Florida has been linked to impaired embryonic sexual development associated with high egg DDE concentrations (Guillette *et al.*, 1994). In fish, Hose *et al.*, (1989) concluded white croaker,

collected from a contaminated site in San Pedro Bay, California, were unable to spawn in the laboratory when ovarian total DDT concentrations exceeded 4 mg/kg. Additionally, they observed decreases in fecundity, fertility and an increase in early oocyte atresia, compared to a reference site.

The mechanisms of reproductive impairment by DDT in fish have not been fully established. Many reports have indicated the presence of DDT (2-4 mg/kg) in eggs was related to fry mortality and deformities (Burdick *et al.*, 1964; Burdick *et al.*, 1972; Smith and Cole, 1973). However, maternal reproductive dysfunction and sublethal developmental toxicity have received little attention (von Westernhagen, 1988). In female mammals and birds, DDT-induced reproductive toxicity has been associated with estrogenic effects such as increased uterine or oviduct weight, persistent vaginal estrus and initiation/maintenance of pregnancy (Clement and Okey, 1972; Bulger and Kupfer, 1985; Johnson *et al.*, 1988). Developmental abnormalities in o,p-DDT exposed avian embryos have also mimicked the effects of estrogen (Fry *et al.*, 1987).

DDT is a chemical mixture composed primarily of p,p'-DDT ($\approx$ 77%) but contains other isomers (o,p'-DDT, $\approx$ 15%) and constituents (p,p'-DDE, $\approx$ 4%; World Health Organization, 1989). The estrogenic effects of DDT have been attributed to the ability of the o,p'-isomer to competitively bind to the estrogen receptor (ER) in mammals and birds (Nelson, 1974; Turner and Eliel, 1978). Additionally, the environmental degradation product or metabolite, o,p'-DDE, retained ER affinity but p,p'-DDE was inactive (Nelson, 1974; Nelson *et al.*, 1978).

The estrogenicity of DDT analogs has not been evaluated in fish. The objective of the present study was to examine the ability of o,p'-DDT and o,p'-DDE, relative to p,p'-DDE, to produce estrogenic effects in juvenile rainbow trout.

We studied vitellogenesis, an estrogen regulated process in which female fish and other oviparous vertebrates synthesize the egg yolk protein precursor, vitellogenin (Vg), in the liver (Tata and Smith, 1979; Mommsen and Walsh, 1988). Estrogenized male and juvenile fish also produce large amounts of Vg that accumulate in the plasma. In response to estrogen treatment, hepatic ER concentrations were also elevated in adult male rainbow trout (Pakdel *et al.*, 1991). Limited evidence suggests xenoestrogens may induce vitellogenesis. Denison *et al.* (1981) showed chronically DDT exposed feral mosquitofish produced a Vg-like protein. Other estrogenic compounds, phytoestrogens (Pelissero *et al.*, 1993) and alkylphenols (White *et al.*, 1994), were also capable of Vg induction in fish. Thus, we monitored Vg synthesis and hepatic ER levels in juvenile trout exposed to o,p'-DDT, o,p'-DDE and p,p'-DDE to compare the estrogenic potencies of these compounds *in vivo*. In addition, hepatic DDT/DDE concentrations associated with Vg induction were estimated by conducting a [^{14}C]p,p'-DDE disposition study. Induction of Vg synthesis by estrogenic compounds may serve as a surrogate marker for other reproductive or developmental toxicological endpoints because estrogen plays a critical role in the reproductive cycle of female fish (Fostier *et al.*, 1983) and exposure to exogenous estrogen has produced reproductive and developmental toxicity in fish (Donaldson *et al.*, 1979; Hunter and Donaldson, 1983). The *in vitro* ability of o,p'-DDT, o,p'-DDE

and p,p'-DDE to competitively bind to hepatic ER sites was also evaluated in our study to further assess the ability of DDT analogs to mimic estrogen in fish.

MATERIALS AND METHODS

Chemicals

o,p'-DDT [99.8% pure; 1,1,1-trichloro-2-(p-chlorophenyl)-2-(o-chlorophenyl)ethane], 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] were purchased from AccuStandard (New Haven, CT). Ring labeled [^{14}C]p,p'-DDE was obtained from Sigma Chemical Co. (St. Louis, MO; >95% pure; 12.7 μCi/μmol). The synthetic estrogen, moxestrol (and [^{3}H]moxestrol; 86 Ci/mmol; 97% pure), was supplied by New England Nuclear (Wilmington, DE) for estrogen binding assays. Reagents for the Vg ELISA (biotinylated donkey anti-rabbit IgG and strepavidin-horseradish peroxidase) were obtained from Amersham Corp. (Arlington Heights, IL). Purified rainbow trout Vg and anti-Vg rabbit immunoserum were generously supplied by David R. Idler (Memorial University of Newfoundland; St. John's Newfoundland, Canada).

Experimental Animals

Juvenile rainbow trout (*Oncorhynchus mykiss*) were provided by the Marine and Freshwater Biomedical Research core facility at Oregon State University. Fish were fed a maintenance ration (3% dry weight food/dry weight fish) of Oregon Test

Diet (Lee *et al.*, 1991) and held in flow-through circular tanks (13 ± 1°C) on a 12-hr light-dark photoperiod.

Experimental Design and Sampling

Juvenile trout (81 ± 2 g) were injected ip with o,p'-DDT, o,p'DDE or p,p'-DDE (0, 5, 15 mg/kg; dissolved in corn oil, 4 μl/g fish; n = 12 per chemical per dose) in Experiment I. Fourteen days after the initial injection, 6 fish per chemical per dose were killed. The remaining fish received a second (day 14) and a third (day 28) injection and were killed 42 days after the first injection. Blood samples were collected from the caudal vein in heparinized syringes, transferred to vials containing phenylmethylsulfonylfluoride (0.3 μM) and centrifuged for 2 min at 1500 g. Plasma was stored at -80°C until assayed for Vg content. Livers were weighed, immediately frozen in liquid nitrogen and stored at -80°C prior to ER determination.

Higher doses were selected in Experiment II to further elucidate the dose-response relationships between DDT/DDE exposure and induction of vitellogenesis. Trout (72 ± 2 g; n = 6 per dose per chemical) were injected ip with o,p'-DDT (30 mg/kg), o,p'-DDE (0, 15, 30 mg/kg) and p,p'-DDE (30 mg/kg) on day 0, 14 and 28 and were killed on day 42. Plasma and liver samples were collected as described above.

Disposition Study

A disposition study was conducted to estimate target organ concentrations resulting from the ip dosing regime. Trout (66 ± 6g, n=3) received an ip injection

of [^{14}C]p,p'-DDE (30 mg/kg; 0.25 μCi dissolved in corn oil) on day 0, 14 and 28 and were killed on day 42. Fish were not fed 4 days prior to sampling. Blood was sampled from the caudal vein and tissue samples (skin, muscle, gill filaments, gallbladder/bile, liver, spleen, stomach, visceral fat, pyloric caeca, intestine, gonads, kidney, heart and brain) were collected and rinsed with phosphate buffered saline (0.145 M NaCl, 0.1 M phosphate, pH 7.2) to remove external contamination. Any undigested food was removed from the stomach and intestinal contents were removed and counted as fecal material. Prior to tissue removal, the ip cavity was swiped with a Kimwipe® tissue and counted to estimate the unabsorbed fraction of [^{14}C]p,p'-DDE. Tissues were homogenized, digested overnight with Soluene 350 solubilizer (Packard, Donners Grove, IL), decolorized (0.5 ml isopropanol and 0.2 ml 30% H_2O_2) and counted in duplicate by liquid scintillation counting (LSC; Packard Tricarb LS counter). The hepatic subcellular distribution of [^{14}C]p,p'-DDE was determined by LSC of fractions prepared from liver homogenates as described below. The nuclear/plasma membrane, cytosolic (prior to charcoal stripping), microsomal and nuclear pellet wash fractions were resuspended or brought to constant volume, digested, decolorized and analyzed by LSC.

Vg Assay

Plasma Vg was quantified by a competitive enzyme linked immunosorbent assay (ELISA) as described in Chapter 2. Values represent the mean of at least four determinations per sample.

Estrogen Binding Assay

Cytosol was prepared from liver homogenates within 30 days of sampling as described in Chapter 2 and protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Homogenates were pools of two livers in Experiment I and individual livers in Experiment II. Briefly, homogenates were centrifuged at 2000 g for 20 min at 4°C and the supernatant was decanted from the nuclear/plasma membrane pellet. This supernatant was centrifuged at 100,000 g for 1 hr at 4°C, separating the microsomal pellet from the cytosolic fraction (supernatant). The cytosol was stripped with dextran-coated charcoal, centrifuged at 100,000 g for 1 hr decanted and stored at -80°C. The binding of [^{3}H]moxestrol to liver cytosol was quantified by saturation analysis using a charcoal adsorption assay (Chapter 2). Cytosolic extracts (150 μl; protein content 6-8 mg/ml extract) were incubated in triplicate for 24 hr (2°C) with [^{3}H]moxestrol (0.3 to 10 nM) in the absence or presence of 200-fold excess moxestrol. Free moxestrol was adsorbed to charcoal and bound [^{3}H]moxestrol was quantified by LSC to determine total and non-specific binding. Specific binding was calculated (total minus non-specific binding) and the equilibrium dissociation constant (K_d) and maximum binding site number were determined from Scatchard plots.

Competitive Binding Assay

Displacement of [^{3}H]moxestrol from hepatic cytosolic binding sites by DDT analogs was assessed by variation of either competitor or [^{3}H]moxestrol concentrations. Relative binding affinity was evaluated by incubating cytosol, in

triplicate, with a single, saturating concentration of [^{3}H]moxestrol (5 nM) in the presence or absence of moxestrol (5 nM - 5 μM), o,p'-DDT, o,p'-DDE or p,p'-DDE (1 nM - 1 mM). Radioinert compounds were dissolved in 99% ethanol and diluted to appropriate final volume concentrations which contained $<$ 1% ethanol. The assay was repeated four times using different cytosol preparations obtained from pooled juvenile trout livers (4 - 13 fish ranging from 50 - 350 g).

In the second type of competitive binding assay, hepatic cytosol (obtained from a pool of 4 trout livers, 350 $\pm$ 25 g fish) were incubated, in duplicate, for 24 hr (2°C) with varying concentrations of [^{3}H]moxestrol (0.3 to 10 nM) and o,p'-DDT or o,p'-DDE (0.1 or 1.0 μM) or p,p'-DDE (1.0 μM) which determined total binding. Non-specific binding was quantified by parallel incubations with 200-fold molar excess moxestrol. Specific binding (total minus non-specific) was calculated and maximum binding site number and apparent K_d values were estimated from Scatchard plots.

Statistics

Liver somatic index (%LSI) was determined by multiplying the liver-to-body weight ratio by 100. Plasma Vg data were analyzed by a Kruskal-Wallis ANOVA, substituting 0.25 μg/ml for values below the detection limit. When significant differences were detected ($p \leq 0.05$), means were compared by Wilcoxon rank tests. ER data were analyzed by one-way ANOVA and Newman-Keuls multiple comparison tests. Relative binding affinity was calculated as the ratio of the concentration of radioinert moxestrol to the concentration of competitor required for 50% displacement

of [^{3}H]moxestrol binding, when displacement curves were parallel (determined by a test for homogeneity of slopes available on SYSTAT, Systat Inc., Evanston IL). For binding assays where competitor concentrations were constant, significant differences ($p \leq 0.05$) between slopes ($-1/K_d$) of Scatchard plot lines were evaluated using a test for homogeneity of slopes. Total binding site concentrations, determined from Scatchard plots, were not considered statistically different when 95% confidence intervals for linear regressions overlapped at the x-intercept.

RESULTS

In Vivo Studies

Survival, final body weights or %LSI were not significantly altered, compared to control trout, in Experiment I and II by o,p'-DDT, o,p'-DDE or p,p'-DDE (data not shown). Plasma Vg was not detected (< 0.25 μg/ml) in juvenile trout exposed to a total dose of 5 or 15 mg/kg o,p'DDT, o,p'-DDE or p,p'-DDE in Experiment I (Figure 3.1). However, o,p'-DDE and o,p'-DDT, but not p,p'-DDE, increased plasma Vg concentrations at the 45 mg/kg total dose (15 mg/kg x 3). Plasma Vg levels in o,p'-DDE trout (45 mg/kg total dose) were not significantly different between Experiment I and II (6.5 ± 1.8 μg/ml; $n = 12$) and were pooled. A total dose of 90 mg o,p'-DDT or o,p'-DDE/kg (30 mg/kg x 3) substantially elevated plasma Vg, compared to controls. In both experiments, Vg concentrations

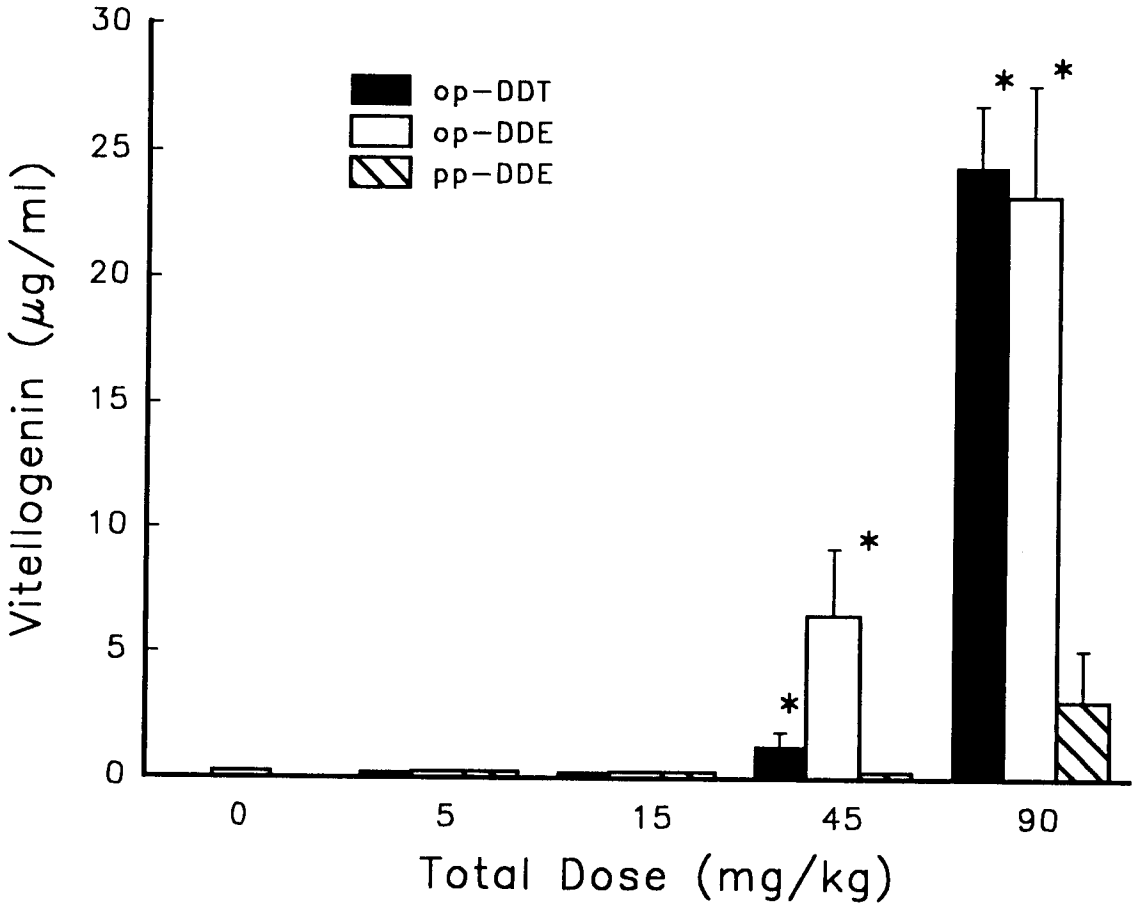

Figure 3.1. **Plasma vitellogenin was induced by o,p'-DDT and o,p'-DDE but not p,p'-DDE.** Juvenile trout were injected ip [total dose; 0, 5, 15 (5 mg/kg x 3), 45 (15 mg/kg x 3), 90 mg/kg (30 mg/kg x 3); n = 6] and plasma was sampled 42 days after the initial injection (* significantly different from control; $p \leq 0.05$; similar dose data from Experiment I and II pooled). The detection limit of the plasma vitellogenin ELISA was 0.25 μg/ml.

were not significantly different between o,p'-DDE and o,p'-DDT treatments. There was an indication of Vg induction in p,p'-DDE treated fish (90 mg/kg total dose) but levels were not significantly different from control.

In agreement with plasma Vg results, hepatic cytosolic ER levels were not affected by exposure to 5 or 15 mg o,p'-DDT, o,p'-DDE or p,p'-DDE/kg (data not shown). Cytosolic ER concentrations, less responsive than plasma Vg, were not significantly elevated at 45 mg/kg (total dose of o,p'-DDT, o,p'-DDE, p,p'-DDE; Figure 3.2). However, ER values were comparably increased (156-181% control) by exposure to 90 mg/kg o,p'-DDT and o,p-DDE but not p,p'-DDE. Hepatic ER concentrations in control and o,p-DDE (45 mg/kg) treated fish were not significantly different between experiment I and II. In both experiments, there was no effect of DDT/DDE treatment on ER K_d which averaged 1.8 $\pm$ 0.1 nM (n = 41).

Tissue distribution of [^{14}C]p,p'-DDE (90 mg/kg total dose) estimated hepatic target organ doses (Table 3.1). Highest tissue concentrations were measured in visceral fat and gonads. Liver contained an estimated average [^{14}C]p,p-DDE concentration of 14 $\pm$ 2 mg/kg. Hepatic subcellular fraction [^{14}C]p,p'-DDE concentrations indicated total liver burden was distributed as follows: cytosol, 1%; microsomes, 13%; nuclear/plasma membrane fraction, 25%; nuclear wash/surface lipid layer, 61%. Radioactivity recovered from the ip cavity averaged 30% ($\pm$ 9%) of the injected dose.

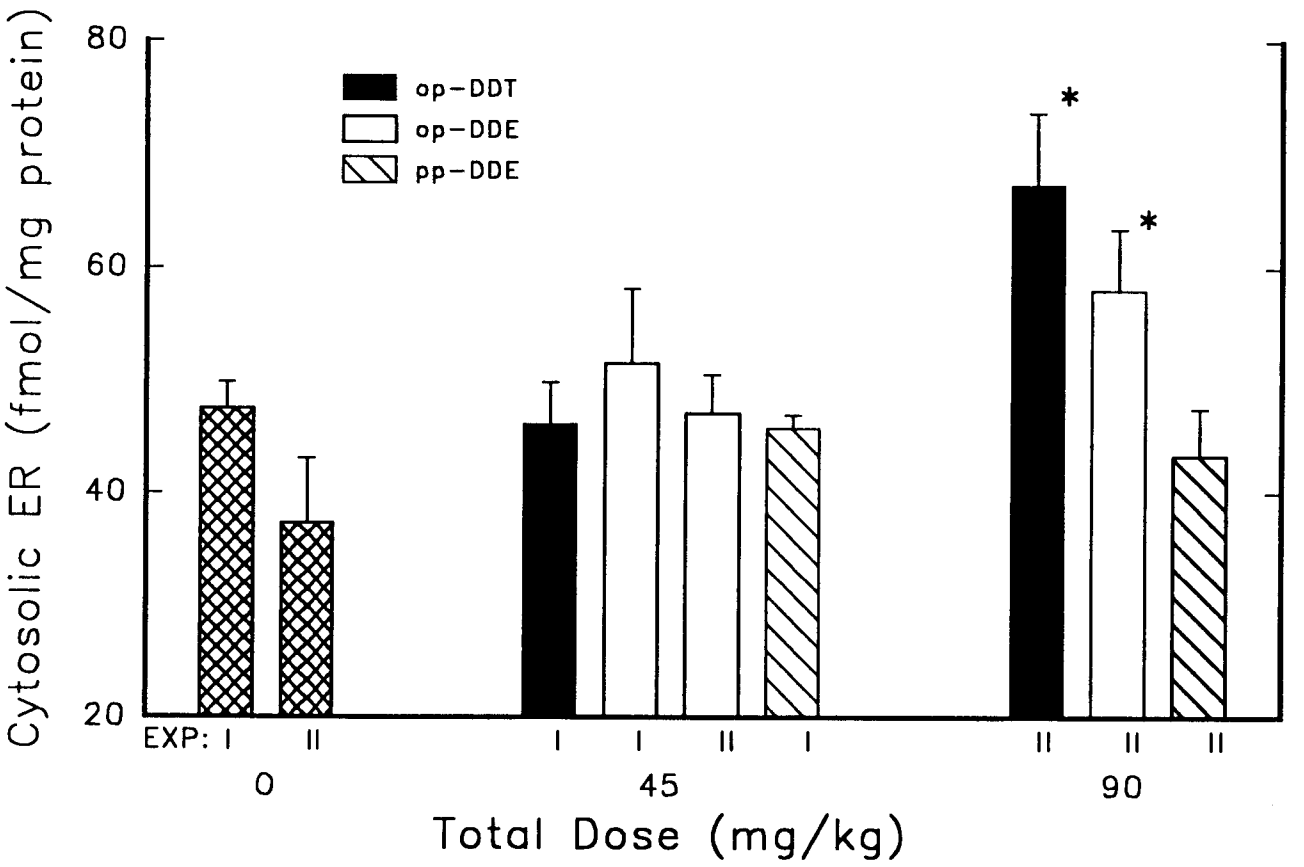

Figure 3.2. **Hepatic cytosolic estrogen receptor concentrations were elevated by o,p'-DDT and o,p'-DDE but not p,p'-DDE.** Juvenile trout were injected ip (total dose; 0, 45, 90 mg/kg) and were sampled 42 days after the initial injection in Experiment (EXP) I and II (* significantly different from control; $p \leq 0.05$).

Table 3.1. **Disposition of [^{14}C]p,p'-DDE in Juvenile Rainbow Trout**[a]

Tissue	[^{14}C]p,p'-DDE Equivalents (mg/kg)[b]
Plasma	3.8 ± 0.4
Gill Filaments	4.7 ± 2.2
Muscle	8.4 ± 0.7
Brain	11.3 ± 4.0
Liver	14.1 ± 2.2
Heart	17.7 ± 2.1
Kidney	18.2 ± 3.4
Spleen	28.7 ± 8.7
Stomach	35.9 ± 3.1
Skin	50.0 ± 8.0
Feces	76.1 ± 19.5
Gallbladder/Bile	90.8 ± 49.4
Pyloric Caeca/Intestine	148.5 ± 13.4
Gonad	327.8 ± 67.3
Visceral Fat	686.1 ± 56.8

[a] Fish (66 ± 6 g) were injected ip with [^{14}C]p,p'-DDE (30 mg/kg on day 0, 14 and 28) and tissues were sampled on day 42. Values are mean ± SE (n = 3).

[b] Plasma expressed as mg/l.

In Vitro Studies

We tested the ability of varying concentrations of o,p'-DDT, o,p'-DDE and p,p'-DDE to inhibit [^{3}H]moxestrol (5 nM) binding to trout hepatic cytosolic estradiol receptors (Figure 3.3). The relative binding affinity of o,p'-DDT and o,p'-DDE for hepatic ER was approximately 6.4 x 10^{-6} that for moxestrol. In comparison, p,p'-DDE did not significantly inhibit [3H]moxestrol binding despite a subtle depression in binding at the 1 μM concentration. When DDT/DDE concentrations were held constant (0.1 or 1 μM) under conditions of varying [3H]moxestrol concentration (0.3 to 10 nM), the level of non-specific binding was not substantially altered (Figure 3.4). Specific binding was reduced by addition of o,p-DDT, o,p-DDE and to a lesser extent p,p'-DDE at saturating concentrations of free [3H]moxestrol (Figure 3.4). Scatchard plots of specific binding data showed o,p'-DDT, o,p'-DDE and p,p'-DDE significantly elevated apparent K_d values (180 -313% control) but had no significant effect on apparent total binding site number (Figure 3.5). This indicated reduced total ER binding was associated with competitive specific receptor binding.

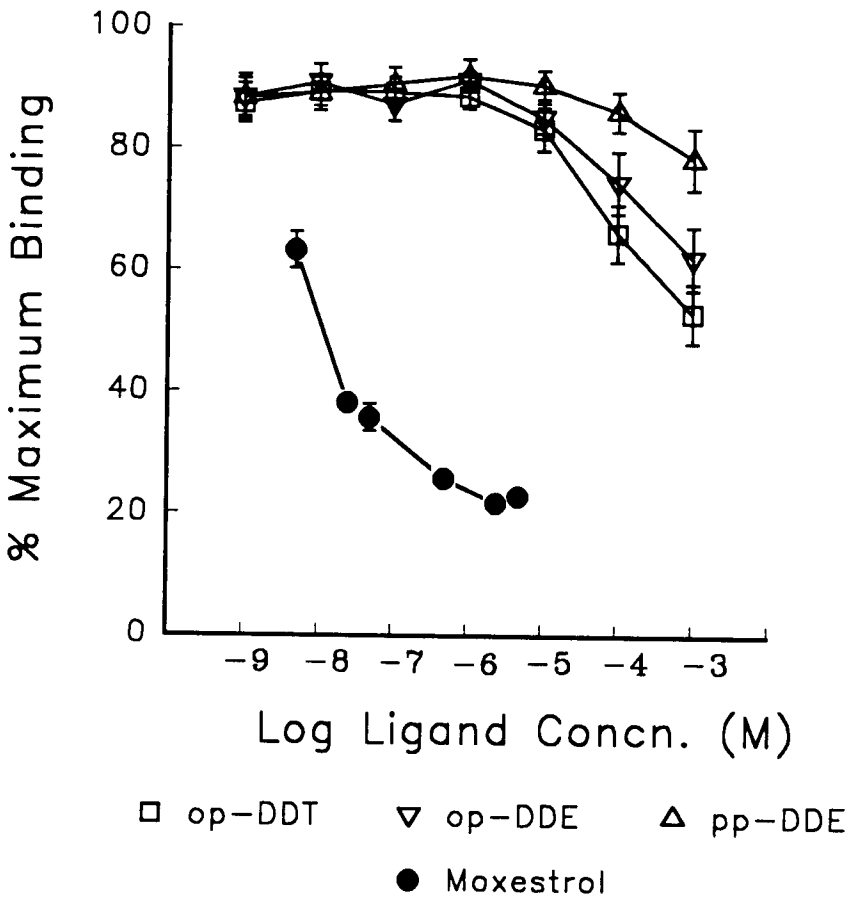

Figure 3.3. **Competitive displacement of [^{3}H]moxestrol from the hepatic estrogen receptor by moxestrol, o,p'-DDT, o,p'-DDE and p,p'-DDE.** Cytosol was incubated, in triplicate, with a saturating concentration of [^{3}H]moxestrol (5 nM) in the presence or absence of moxestrol (5 nM - 5 μM), o,p'-DDT, o,p'-DDE or p,p'-DDE (1 nM - 1 mM). Results expressed as percentage of total [^{3}H]moxestrol binding and represent the mean ($\pm$ SE) of four assays.

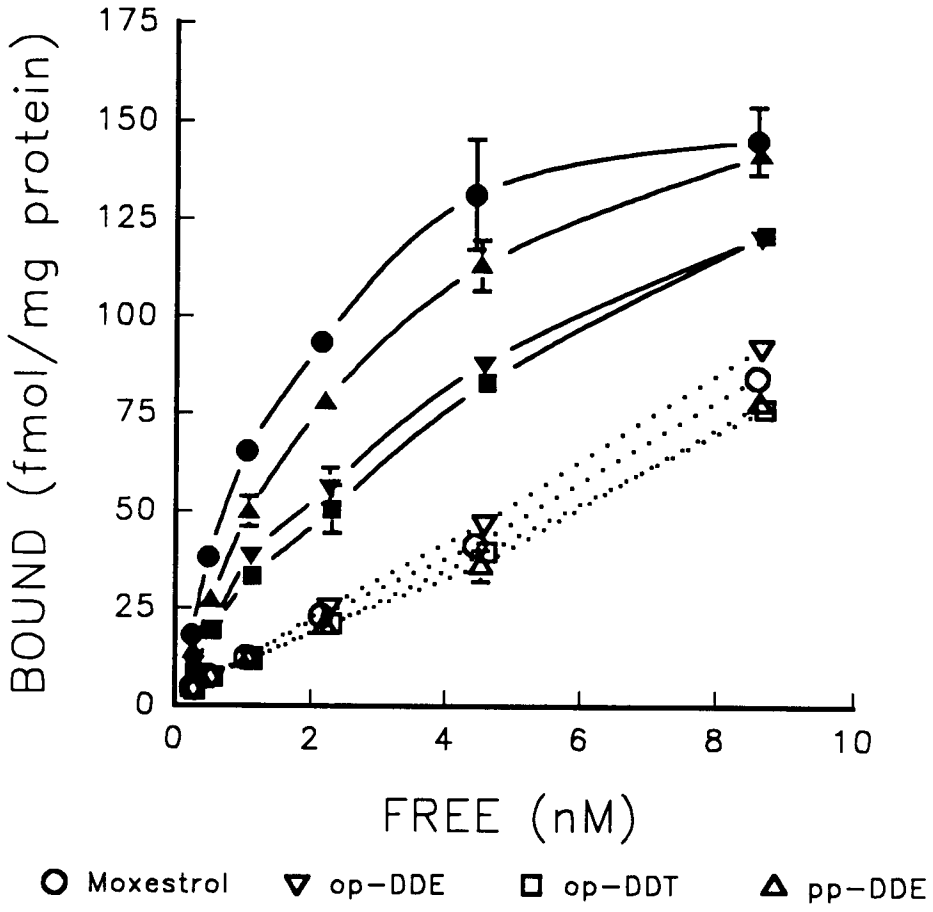

Figure 3.4. **Specific and non-specific binding of [^{3}H]moxestrol (0.3 to 10 nM) to trout hepatic cytosol in the absence (control) or presence of 1 mM o,p'-DDT, o,p'-DDE or p,p'-DDE.** Cytosol was incubated, in duplicate, with indicated concentrations of [^{3}H]moxestrol (total binding). Non-specific binding was determined by parallel incubations with 200-fold molar excess radioinert moxestrol. Specific binding (filled symbols) was estimated by subtracting non-specific (open symbols) from total binding.

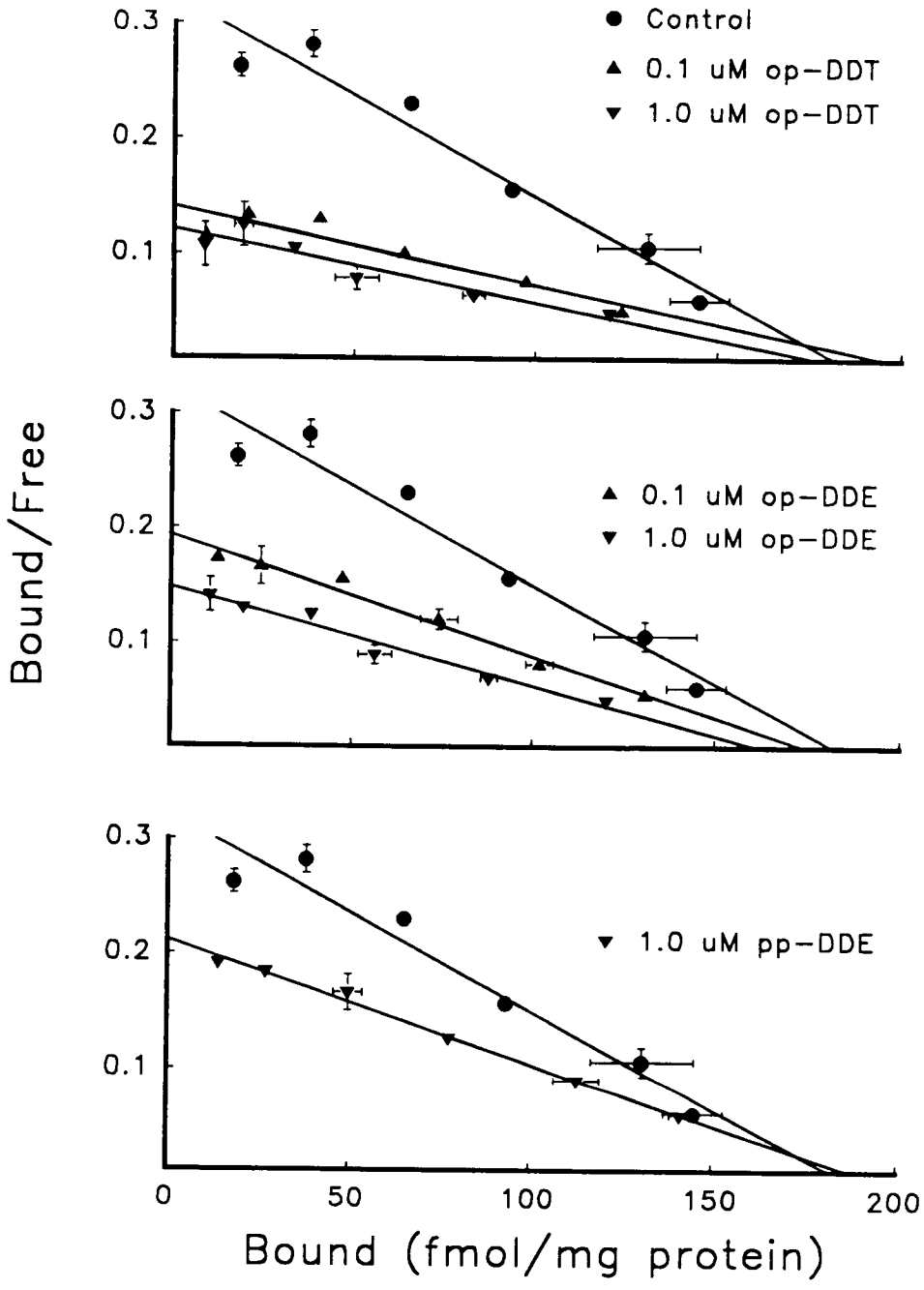

Figure 3.5. **Scatchard plots of the competitive inhibition of [^{3}H]moxestrol specific binding to hepatic cytosol estrogen receptor by increasing concentrations of o,p'-DDT, o,p'-DDE and p,p'-DDE.** Cytosol was incubated as described in Figure 3.4.

DISCUSSION

In the present study, induction of Vg synthesis and elevation of hepatic ER concentrations assessed estrogenicity of o,p'-DDT, o,p'-DDE and p,p'-DDE in juvenile rainbow trout. We also determined the relative binding affinity of these three compounds for the trout hepatic ER via *in vitro* competitive binding assays. We concluded o,p'-DDT and o,p'-DDE were estrogenic in trout because Vg synthesis was significantly induced, hepatic ER levels were elevated and moxestrol binding to hepatic ER was inhibited by these compounds. In contrast, p,p'-DDE was not significantly estrogenic. These results were consistent with Nelson (1974) who reported o,p'-isomers exhibited affinity for rat uterine ER *in vitro* but the p,p'-isomers were relatively inactive. *In vivo* studies have also concluded p,p'-DDT, unlike o,p'-DDT, was unable to increase immature rat uterine or avian oviduct weights (Bitman *et al.*, 1968; Bulger and Kupfer, 1985).

Although p,p'-DDE insignificantly elevated plasma Vg or inhibited moxestrol binding, subtle estrogenicity was suggested at the highest doses tested. There was evidence that p,p'-DDT interfered with binding of endogenous ligands for progesterone and androgen receptors in mammals and birds (Wakeling and Visek, 1973; Lundholm, 1991). A recent report indicated low p,p'-DDE affinity for the ER but higher affinity for the androgen receptor in mammals and suggested high concentrations competed with several steroid receptors (Laws *et al.*, 1995). This observation was supported by the work of LeMenn (1979) which demonstrated pharmacological doses of androgens induced vitellogenesis in fish. Thus, stimulated

vitellogenesis by high doses of a chemical may indicate estrogen agonism but may incompletely represent the chemical's potential to disrupt functions mediated by other steroid receptors.

Whereas o,p'-DDT and o,p'-DDE were estrogenic in trout *in vivo* and *in vitro* studies, they were relatively weak estrogens. Sexually mature female rainbow trout attained plasma Vg levels up to 50 ng/ml when plasma estradiol ranged from 40 to 60 ng/ml (Scott and Sumpter, 1983). In comparison, we estimated significantly higher plasma DDE concentrations (approximately 1000-fold greater) were associated with substantially lower plasma Vg concentrations (20 - 25 μg/ml) in our study. Hepatic ER binding assays confirmed o,p'-DDT and o,p'-DDE were weak competitors, requiring mM concentrations to effectively compete with moxestrol (156,000 fold less potent than moxestrol which displays ER affinity equivalent to 17ß-estradiol; Chapter 2). These results suggest trout hepatic ER may have less affinity for o,p'-DDT than previously reported in mammalian or avian *in vitro* studies. Rat uterine ER had 2,000-fold (Nelson, 1974) and Japanese quail oviduct ER had at least 20,000-fold less affinity (Turner and Eliel, 1978) than the potent estrogen, diethylstilbesterol. Reduced affinity in fish was also reported by Thomas and Smith (1993) who showed hepatic ER from mature female spotted seatrout exhibited no affinity for o,p'-DDT (up to 1 mM). Nevertheless, o,p-DDT doses of 10 to 1000 mg/kg produced estrogenic effects (increased uterine weight) in immature female rats, within the dose range of the present study (45 - 90 mg/kg; Bitman *et al.*, 1968; Robison and Stancel, 1982; Bulger and Kupfer, 1985). Due to the absence of o,p'-DDT target organ

concentrations in rodent studies, it is difficult to compare the sensitivities of mammalian and fish estrogenic responses. Further research is required to assess the responsiveness of different tissue types (hepatic versus uterine) and species to the estrogenic effects of o,p'-DDT, o,p'-DDE and metabolites.

The disposition of [^{14}C]p,p'-DDE in trout was similar to the tissue distribution profile reported for [^{14}C]p,p'-DDT in other fish species; high levels in adipose tissue and lower concentrations in muscle and liver (Grzenda *et al.*, 1970; Macek *et al.*, 1970; Pritchard *et al.*, 1973). This was consistent with the finding that p,p'-DDT was metabolized to p,p'-DDE which was retained in fish tissues (Addison and Willis, 1978). In the present study, gonad [^{14}C]p,p'-DDE concentrations, relative to liver, were higher than levels reported by Grzenda *et al.* (1970) for immature goldfish exposed to dietary [^{14}C]DDT. The disposition of [^{14}C]p,p'-DDE has not been extensively studied in fish but our results suggest specific distribution patterns may vary according to the species, route of administration, dose or time course tested.

We utilized disposition of [^{14}C]p,p'-DDE to approximate target organ concentrations of p,p'-DDE, o,p'-DDE and o,p'-DDT after ip dosing associated with induction of vitellogenesis. The accuracy of the assumption that p,p'-DDE and o,p'-DDE distributed similarly in trout cannot be directly determined because the disposition of [^{14}C]o,p'-DDE has not been evaluated in fish, to the authors knowledge. In rodents, o,p'-DDT was excreted more rapidly than p,p'-DDT due to enhanced hydroxylation (Feil *et al.*, 1973; Smith, 1991). Bitman *et al.* (1968) reported adipose and uterine p,p'-DDT residue levels were 4 to 10 fold higher than

o,p'-DDT. However, in avian species, o,p'-DDT and p,p'-DDT accumulated to an equivalent extent in oviduct and adipose tissues (Bitman *et al.*, 1968). DDT isomers induced their own metabolism, via cytochrome P450 enzyme induction, in rodents and birds (Fawcett *et al.*, 1987). In contrast, fish were refractory to DDT-induced enzyme induction (Buhler and Rasmusson, 1968; Addison *et al.*, 1977), suggesting differential excretion of o,p'-DDT and p,p'-DDT may be less dramatic in fish. Based on these data, [^{14}C]p,p'-DDE tissue concentrations in trout were likely to represent o,p'-DDE concentrations, within an order of magnitude. Data from the present study suggest o,p'-DDT or o,p'-DDE may stimulate vitellogenesis in juvenile rainbow trout when hepatic burdens reach mg/kg levels.

Several monitoring studies in the United States have determined DDT residue levels in feral fish whole-body homogenates, muscle samples and rarely in liver samples. In studies where specific DDT isomers were quantified, o,p'-DDE tissue burdens were consistently lower than p,p'-DDE due to its variable but minor presence in the DDT technical mixture. For example, Giesy *et al.* (1994) reported p,p'-DDE in fish whole body homogenates from Lake Michigan ranged from 3 to 738 ug/kg and o,p'-DDE ranged from 0.15 to 38 μg/kg. In a nationwide survey of 112 sampling stations, fish whole-body homogenates contained less than 1 mg/kg of p,p'-DDT homologs in 90% of the samples (Schmitt *et al.*, 1990). Residue levels were higher (1 - 5 mg/kg) in areas with a history of intensive farming or pesticide manufacturing (Schmitt *et al.*, 1990). These results were consistent with hepatic total DDT residue levels (1.5 mg/kg) measured in white croaker from San Pedro Bay, California, a

DDT-contaminated site (Hose *et al.*, 1989). White croaker ovary total DDT levels were also elevated and concentrations greater than 4 mg/kg were associated with reproductive impairment (Hose *et al.*, 1989). Thus, we suggest it is unlikely that o,p'-DDE or o,p'-DDT hepatic residues reach the mg/kg quantities suspected to induce vitellogenesis in juvenile trout in all but the most contaminated sites.

In summary, we conclude o,p'-DDT and o,p'-DDE, but not p,p'-DDE, were weakly estrogenic in juvenile rainbow trout *in vivo* and *in vitro* studies. Results indicated induction of vitellogenesis was a suitable biomarker for the estrogenic effects of these organochlorines. Further work is required to assess the sensitivity of mature female gonads and developing embryos to the estrogenic effects of o,p'-DDT/DDE in order to define the contribution of this isomer to DDT induced reproductive impairment. Mature female gonads and eggs (via maternal transport; Plack *et al.*, 1979) may be susceptible targets because of their propensity to accumulate DDT.

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CHAPTER 4

Comparative Response of Cytochrome P4501A and Estrogen Induced Vitellogenesis to 3,4,5,3',4',5'-Hexachlorobiphenyl in Rainbow Trout

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ABSTRACT

Co-planar polychlorinated biphenyls (PCBs) are persistent contaminants in aquatic biota and possibly contribute to the majority of PCB mixture toxicity, including reproductive toxicity. In mammals, co-planar PCBs may act as antiestrogens by decreasing estrogen receptor (ER), plasma estradiol concentrations or by altering estrogen regulated gene expression. We utilized vitellogenesis, estrogen regulated synthesis of the yolk-protein precursor, vitellogenin (Vg), to explore the antiestrogenic effects of the co-planar PCB, 3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB) in juvenile rainbow trout. Additionally, we evaluated 345-HCB CYP1A induction potential and its modulation by estradiol. We injected juvenile rainbow trout with 345-HCB (0, 0.25, 2.5, 25, 50 or 100 mg/kg) and after 10 weeks, injected fish with 17ß-estradiol (0, 0.1 mg/kg). Markers of vitellogenesis (liver somatic index (%LSI), hepatic ER, plasma Vg and plasma estradiol concentrations) were monitored and hepatic CYP1A and CYP2K1 induction was assessed by ethoxyresorufin-O-deethylase (EROD) activity, CYP1A1 protein, and CYP1A1 and CYP2K1 mRNA levels. Hepatic ER, EROD and CYP1A1 protein levels were determined following exposure to ßnaphthoflavone (ßNF; 100 mg/kg, ip, sampling 3 days later) in order to compare responses to 345-HCB. 345-HCB did not antagonize estrogen induced increases in %LSI, hepatic ER or plasma Vg and did not alter hepatic ER in vehicle controls. However, a similar dose of ßNF reduced hepatic ER to 33% of control values. The ED_{50} for CYP1A protein and mRNA induction occurred below 2.5 mg 345-HCB/kg with maximal induction (2000 to 2500% control) at doses above 2.5

mg/kg. Maximal CYP1A protein induction was comparable between ßNF and 345-HCB and estradiol reduced CYP1A protein content at a single dose (0.25 mg 345-HCB/kg). EROD was induced by low doses of 345-HCB (0.25 - 2.5 mg/kg) but not higher doses suggesting 345-HCB suppressed CYP1A catalytic activity. Plasma estradiol, in 345-HCB/estrogen treated fish, was significantly reduced at 100 mg 345-HCB/kg but the depression was not associated with CYP1A induction or other antiestrogenic effects. Collectively, these results indicated CYP1A induction was a more sensitive marker of 345-HCB exposure than modulation of estrogen induced vitellogenesis. In the present study, juvenile rainbow trout were refractory to the potential antiestrogenic effects of 345-HCB but appeared to be responsive to ßNF.

INTRODUCTION

Polychlorinated biphenyls (PCB) contaminate aquatic biota throughout the world (World Health Organization, 1993). While the highly toxic, co-planar PCB congeners occur at lower concentrations than the widely distributed, less toxic nonplanar congeners, they may contribute to the majority of PCB mixture toxicity (Tanabe, 1987; Smith *et al.*, 1990; Elskus *et al.*, 1994). Reduced reproductive success has been associated with elevated PCB residues in feral fish (Casillas *et al.*, 1991; Mac and Edsall, 1991). While direct causation cannot be established in ecosystems, PCBs are known reproductive toxins. In depth evaluation of this environmental problem is restricted by the limited number of sensitive reproductive bioindicators and the incomplete understanding of PCB congener-specific dose-response relationships and mechanisms of action in fish.

Many studies have assessed the reproductive toxicity of PCBs in mammals. Symptoms of female reproductive dysfunction have included reduced fertility and litter size, altered estrous/menstrual cycling, and inability to maintain pregnancy (Safe, 1994). Co-planar PCBs contributed to reproductive impairment by operating through the aryl hydrocarbon receptor (AhR) signal transduction pathway as antiestrogens (Safe *et al.*, 1991). The antiestrogenic effects of AhR ligands, such as decreased uterine weight, have been related to reduced estrogen receptor (ER) numbers. Several investigators have reported significant depression of uterine and hepatic ER levels in rodents exposed to the prototypical Ah ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; Romkes *et al.*, 1987; Lin *et al.*, 1991; DeVito *et*

al., 1992). *In vitro* experiments with MCF-7 breast cancer cells showed polynuclear aromatic hydrocarbons (PAHs; Chaloupka *et al.*, 1992), PCBs and polychlorinated dibenzo-*p*-dioxins (PCDDS; Krishnan and Safe, 1993) downregulated ER levels or suppressed estrogen-induced protein secretion, supporting an AhR-mediated mechanism for antiestrogenicity. In addition to ER downregulation, a role for enhanced estradiol metabolism, resulting from enzyme induction, in Ah-ligand antiestrogenicity was proposed (Spink *et al.*, 1994). However, the contribution of this mechanism has remained unresolved because others have not correlated depressed plasma estradiol concentrations with antiestrogenic effects (Shiverick and Munther, 1983; DeVito *et al.*, 1992).

The mechanism of PCB reprotoxicity in fish has received much less attention. Analogous to mammals, laboratory studies demonstrated exposure to PCB mixtures delayed or impaired spawning in several fish species (reviewed in World Health Organization, 1993). The AhR has been detected in fish (Hahn *et al.*, 1994) and responsiveness to AhR-mediated effects such as cytochrome P450 1A (CYP1A) induction (Andersson and Forlin, 1992) and embryotoxicity (Walker and Peterson, 1991) has been established. However, the contribution of co-planar PCB antiestrogenic effects, such as ER downregulation or induced estradiol catabolism, to fish reproductive impairment has been addressed to a very limited extent. The

present study utilized vitellogenesis as a model in juvenile rainbow trout to explore the antiestrogenic effects of the co-planar PCB, 3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB).

A widely accepted general model of vitellogenesis, yolk protein precursor production, in oviparous vertebrates has been developed (Wahli *et al.*, 1981). Briefly, estradiol interacts with the hepatic ER which regulates transcription of the vitellogenin (Vg) gene(s). Subsequent mRNA processing is followed by translation, and post-translational glycosylation, lipidation and phosphorylation. The final protein product in trout, Vg (470 kDa) is secreted into the bloodstream and incorporated into oocytes of sexually mature fish (Campbell and Idler, 1981). Male and juvenile fish have been induced by estrogen to produce large amounts of Vg that accumulates in the plasma. Estrogen stimulation kinetics in adult male rainbow trout showed induction of ER mRNA and ER levels preceded Vg mRNA accumulation (Pakdel *et al.*, 1991). Relatively large increases in liver-to-body weight ratio (liver somatic index; %LSI), resulting from hypertrophy and hyperplasia, have also been noted when vitellogenesis was induced by estradiol in fish (Mommsen and Walsh, 1988). Consequently, studying vitellogenesis in estrogenized juvenile fish provided a means of simultaneously examining co-planar PCB antiestrogenic effects on %LSI, hepatic ER dynamics, estrogen regulated gene expression and estradiol catabolism. Vg production may be a suitable bioindicator of reproductive dysfunction because adequate yolk reserves must be available to nourish the developing embryo.

Very few investigators have examined the effects of PCBs on vitellogenesis. Depressed plasma Vg levels were reported following exposure to Arochlor 1254 in estradiol-induced juvenile rainbow trout (Chen *et al.*, 1986) and in sexually mature Atlantic croaker (Thomas, 1989). Anderson *et al.* (1993) recently reported the AhR ligand, ß-naphthoflavone (ßNF), reduced Vg synthesis in an *in vitro* trout hepatocyte culture system. However, Monosson *et al.* (1994) did not observe a decrease in plasma Vg or estradiol in mature white perch exposed to the co-planar PCB congener, 3,3',4,4'-tetrachlorobiphenyl. Exposure to PCB mixtures has been associated with depressed plasma estradiol levels and hepatic estrogen receptors (Thomas, 1989) but specific co-planar congener effects remain largely undetermined. Our study contributed dose-response information on the effects of 345-HCB on plasma estradiol, Vg and hepatic ER concentrations in estrogenized juvenile rainbow trout.

Additionally, we evaluated the CYP induction potential of 345-HCB and its modulation by estradiol. Toxic equivalent factors (TEFs), based on CYP1A *in vitro* induction, derived for PCDD congeners in mammalian systems did not accurately predict fish TEF values (Clemons *et al.*, 1994). These results suggested *in vivo* congener specific CYP1A induction potencies were required for fish. Estradiol effects on induction in fish were of interest because previous research demonstrated estrogens altered CYP1A induction responsiveness by an undefined mechanism (Andersson and Forlin, 1992) and sex hormones were believed to play a role in CYP2K1 expression (Buhler *et al.*, 1994). Hepatic CYP1A induction following short term exposure to 345-HCB by intraperitoneal (ip) injection was reported in trout

(Kleinow *et al.*, 1990; Miranda *et al.*, 1990). However, Brown *et al.* (1993) suggested hepatic 3,3'4,4',5-pentachlorobiphenyl concentrations required at least six weeks to reach maximal levels following ip injection in lake trout. Therefore, we injected rainbow trout with 345-HCB (0, 0.25, 2.5, 25, 50 or 100 mg/kg, ip), allowed ten weeks for absorption and distribution, and then sampled or injected fish with 17ß-estradiol (0.1 mg/kg). A single dose of 345-HCB was deemed appropriate because trout elimination of hexachlorobiphenyls has been reported to be minimal (Niimi and Oliver, 1983). Vitellogenesis was monitored and hepatic CYP induction was assessed by ethoxyresorufin-O-deethylase (EROD) activity, CYP1A protein and CYP1A and CYP2K1 mRNA levels. Hepatic ER, EROD and CYP1A protein levels were determined following exposure to ßNF to compare its potency to 345-HCB.

MATERIALS AND METHODS

Chemicals

The 345-HCB was obtained from AccuStandard (New Haven, CT; 100% pure by GC/MS). 17ß-Estradiol-3-benzoate and resorufin were purchased from Sigma Chemical Co. (St. Louis, MO) and ßNF was obtained from Aldrich Chemical Co. (Milwaukee, WI). The synthetic estrogen, moxestrol (and [^{3}H]moxestrol, 86 Ci/mmol; 97% pure) was supplied by New England Nuclear (Wilmington, DE) for estrogen binding assays. Reagents for the Vg ELISA (biotinylated donkey anti-rabbit IgG and strepavidin-horseradish peroxidase) were obtained from Amersham Corp. (Arlington Heights IL). Purified rainbow trout Vg and anti-Vg rabbit immunoserum were generously supplied by David R. Idler (Memorial University of Newfoundland; St. John's, Newfoundland, Canada). 7-Ethoxyresorufin was purchased from Molecular Probes, Inc. (Eugene, OR). [^{125}I]-Labeled protein A was from ICN Radiochemicals (Irvine, CA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and nitrocellulose membranes were obtained from Bio-Rad (Hercules, CA). Chemicals and supplies for Northern blot analysis were obtained from sources previously indicated (Buhler *et al.*, 1994). Other chemicals were of the highest quality available commercially.

Experimental Animals

Rainbow trout (*Oncorhynchus mykiss*) were provided by the Marine and Freshwater Biomedical Research Center core facility at Oregon State University. Fish were fed a maintenance ration (3% dry weight food/dry weight fish) of Oregon Test Diet (Lee *et al.*, 1991) and held in flow-through circular tanks (13 ± 1°C) on a 12-hr light-dark photoperiod.

Experimental Design and Sampling

Juvenile trout (46 ± 2 g) were injected ip with 0, 0.25, 2.5, 25, 50 or 100 mg/kg 345-HCB (dissolved in corn oil, 4 μl/g; n=12/dose) and 6 fish per dose were killed after 10 weeks. The remaining fish were injected ip with 0.1 mg 17ß-estradiol/kg (dissolved in peanut oil, 5 μl/g) and were killed 3 days later. Previous research showed this estrogen dose produced sub-maximal increases in plasma Vg and hepatic ER concentrations (see Chapter 2). Blood samples were collected from the caudal vein in heparinized syringes, transferred to vials containing phenylmethylsulfonylfluoride (0.3 μM) and centrifuged for 2 min at 1500 g. Plasma was stored at -80°C until assayed for estradiol and Vg content. Livers were removed, immediately frozen in liquid nitrogen and stored at -80°C.

In a second experiment, juvenile trout (76 ± 6 g) were injected ip with ßNF (100 mg/kg, dissolved in corn oil, 4 μl/g; n=8) or vehicle control (n=8) and were killed 3 days later. Livers were removed and immediately homogenized to prepare cytosolic and microsomal fractions as described below.

Estrogen Binding Assay

Cytosol was prepared from livers within 30 days of sampling as described in Chapter 2 and protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. DNA content of nuclear salt extracts was quantified according to Burton (1956). Individual livers were homogenized except in 345-HCB treated fish which received no estradiol where two livers were pooled prior to homogenization. In the ßNF experiment, duplicate cytosol preparations were obtained from four pooled livers. The binding of [^{3}H]moxestrol to liver cytosol was quantified by saturation analysis using a charcoal adsorption assay (Chapter 2). Briefly, cytosolic extracts (150 μl; protein content 6-8 mg/ml) were incubated for 24 hr (2°C) with [^{3}H]moxestrol (0.3 to 10 nM; 50 μl), in the absence or presence of 200-fold excess radioinert moxestrol (50 μl). Free moxestrol was separated by means of charcoal adsorption and bound moxestrol derived radioactivity was quantified to determine total and non-specific binding. Specific binding was calculated (total minus non-specific binding) and the equilibrium dissociation constant (K_d) and maximum binding site number were determined from Scatchard plots.

Plasma Vg and Estradiol Assays

Plasma Vg was quantified by a competitive enzyme linked immunosorbent assay (ELISA) as described in Chapter 2. Plasma estradiol was measured by radioimmunoassay, following ether extraction, as described by Fitzpatrick *et al.* (1986). Average plasma estradiol values were determined from duplicate assays performed with triplicate sample replication.

EROD Assay

Microsomal pellets, isolated by differential centrifugation according to the method outline in the estrogen binding assay, were obtained from liver homogenates. Microsomes were resuspended in resuspension buffer (0.1 M phosphate buffer, pH 7.25; 20% glycerol; 1 mM EDTA) and were frozen (-80°C) until use. The EROD assay was conducted at 27°C and followed the method of Prough *et al.* (1978) as modified by Williams *et al.* (1983).

Immunoblots

Duplicate microsome samples for each treatment were prepared by pooling equal portions of microsomal protein from 2 to 4 fish in 1 ml of resuspension buffer. SDS-PAGE (4% stacking; 7.5% separating) of microsomal samples utilized a Mini-Protean II (Bio-Rad, Hercules, CA) electrophoresis apparatus. Proteins were transferred (20 V, 30 min; Bio-Rad Transblot) to nitrocellulose and immunostained with rabbit anti-trout LM4b-IgG, followed by incubation with [^{125}I]Protein A (Burnette, 1981 as modified by Miranda *et al*, 1990). Radioactivity was quantified by a PhosphoImager (Molecular Dynamics Inc, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics, Inc) was used to integrate signal in identical areas in each blot lane. CYP1A protein was quantified by calibrating PhosphoImager density units to purified P450 LM4b standards (Williams and Buhler, 1984).

Northern Blots

Liver RNA was extracted and Northern blot analysis was performed as described by Buhler *et al.* (1994). Blots were sequentially hybridized with ^{32}P-labeled 2K1,7c cDNA (Buhler *et al.*, 1994), trout 1A cDNA (kindly donated by John Lech, Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI; as prepared by Heilmann *et al.*, 1988) and an 18S ribosomal RNA oligonucleotide probe (1406R, gift of Stephen Giovannoni, Department of Microbiology, Oregon State University). Membranes were quantitated by ß-emission densitometry (AMBIS Systems, San Diego, CA) at the Central Laboratory of the Center for Gene Research and Biotechnology, Oregon State University. Quantitation of the 18S ribosomal RNA internal standard showed variation between blot lanes averaged less than 15%, indicating uniform RNA loading concentrations.

Statistics

Data are presented as mean $\pm$ SE unless otherwise noted. Condition factor was calculated as: 100 x [wet weight fish (g) / (standard length (cm))^3]. Liver somatic index (%LSI) was determined by multiplying liver-to-body-weight ratio by 100. Data from the 345-HCB experiment were analyzed by two-way ANOVA. One-way ANOVA and Newman-Keuls multiple comparison tests were performed when significant ($p \leq 0.05$) estrogen or 345-HCB effects were observed. Individual means were compared using Student's t-test for the ßNF experiment.

RESULTS

Effects of 345-HCB, Estradiol and ßNF on Liver Somatic Index

Exposure to 345-HCB resulted in no mortality and did not influence the final body weights (63 ± 2 g, n=72) or condition factor (1.62 ± 0.02) of trout. Liver somatic index (%LSI) was significantly increased by 345-HCB and estradiol but there was no significant interaction between the two treatments (Figure 4.1). This indicated 345-HCB did not antagonize estradiol induced changes in %LSI. Regardless of estradiol treatment, all 345-HCB doses, except the 0.25 mg/kg dose, significantly elevated %LSI above corn oil control levels. 345-HCB produced greater changes in %LSI (200% of corn oil control at 100 mg/kg) than estradiol treatment (20% of control). Liver total and cytosolic protein, RNA and DNA concentrations were not altered by 345-HCB or estradiol exposure (data not shown). ßNF produced a subtle increase in %LSI compared to controls (0.98 ± 0.03 vs 0.79 ± 0.02, n=8).

Modulation of Vitellogenesis

Mean cytosolic ER concentrations were not significantly different between 345-HCB treated fish. Estradiol significantly elevated receptor levels (69.7 ± 4.6 fmol/mg protein or 0.92 ± 0.09 fmol/μg DNA, n=30; Figure 4.2) compared to vehicle controls (29.7 ± 2.16 fmol/mg protein, n=17). The dissociation constant (Kd) was also moderately elevated by estradiol treatment (3.1 ± 0.2 vs 1.5 ± 0.1 nM) but was still indicative of high affinity binding. In contrast, BNF significantly

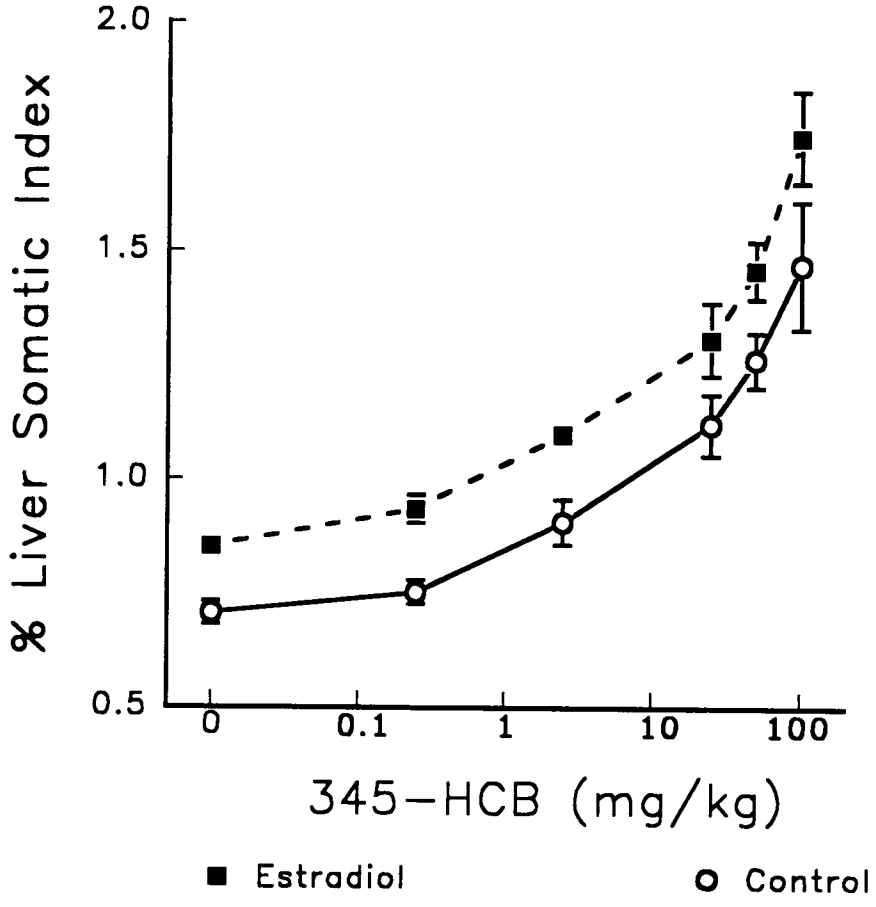

Figure 4.1. **3,4,5,3',4',5'-Hexachlorobiphenyl (345-HCB) elevated liver somatic index (%LSI) but did not antagonize estradiol-induced increases in %LSI.** Trout (≈ 46 g) were injected ip with 345-HCB (0, 0.25, 2.5, 25, 50 and 100 mg/kg, n=12/dose) and ten weeks later were killed or injected ip with 17ß-estradiol (0.1 mg/kg, n=6/dose). Estradiol-treated fish were killed 3 days after injection and liver and body weights were determined. Two-way ANOVA indicated 345-HCB and estradiol significantly affected %LSI ($p \leq 0.05$) but there was no interaction between the treatments.

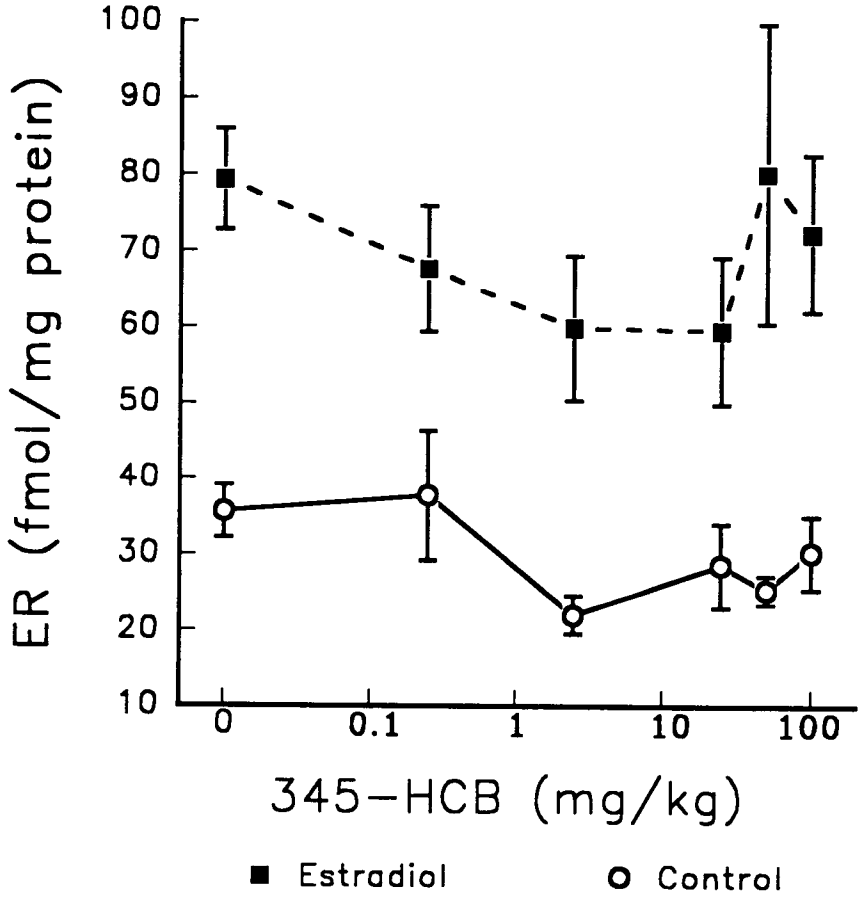

Figure 4.2 **Hepatic cytosolic estrogen receptors (ER) concentrations were significantly elevated by estradiol but were not affected by 3,4,5,3',4',5'-hexachlorobiphenyl exposure.** Trout were treated as described in Figure 4.1. Values are mean (± SE) ER concentrations for five livers in estradiol treated fish and three pools of two livers in controls. Two-way ANOVA showed estradiol significantly elevated ER content ($p \leq 0.05$) but 345-HCB had no effect.

reduced hepatic cytosolic estrogen receptor levels (22.1 ± 3.9 fmol/mg protein) compared to controls (64.7 ± 0.64 fmol/mg protein, duplicate run on 4 pooled livers) in the second experiment.

In estrogenized trout exposed to 345-HCB, Vg synthesis was induced but plasma Vg levels were not significantly different from controls (Figure 4.3). However, plasma estradiol was significantly reduced, relative to controls, at the highest 345-HCB dose (100 mg/kg; Figure 4.3).

Induction of CYP1A Enzyme Activity, Protein and mRNA Content

Two-way ANOVA indicated EROD activity was not significantly changed by estradiol, but was altered by 345-HCB (Figure 4.4). EROD activities were above control values at doses of 0.25 to 2.5 mg 345-HCB/kg. EROD activity was not induced at higher doses (25 to 100 mg 345-HCB/kg). ßNF exposure (100 mg/kg) induced EROD activities to a much greater extent than maximal induction by 345-HCB (Figure 4; 427 ± 40 vs 65 ± 25 pmol/min/mg microsomal protein).

CYP1A content was significantly elevated above control at all 345-HCB doses (Figure 4.5). Additionally, there was a subtle but significant estrogen interaction. Estradiol reduced CYP1A content in fish treated with 0.25 mg 345-HCB/kg. Induction of CYP1A content appeared to peak at doses between 2.5 and 25 mg 345-HCB/kg (329 ± 47 pmol/mg microsomal protein). ßNF induced CYP1A content with comparable potency (253 ± 23 pmol/mg microsomal protein). The catalytic efficiency of the EROD reaction was substantially higher in BNF treated trout (33.5 ± 8.2 pmol/min/ug 1A protein) compared to 345-HCB exposed fish (3.4 ± 1.0

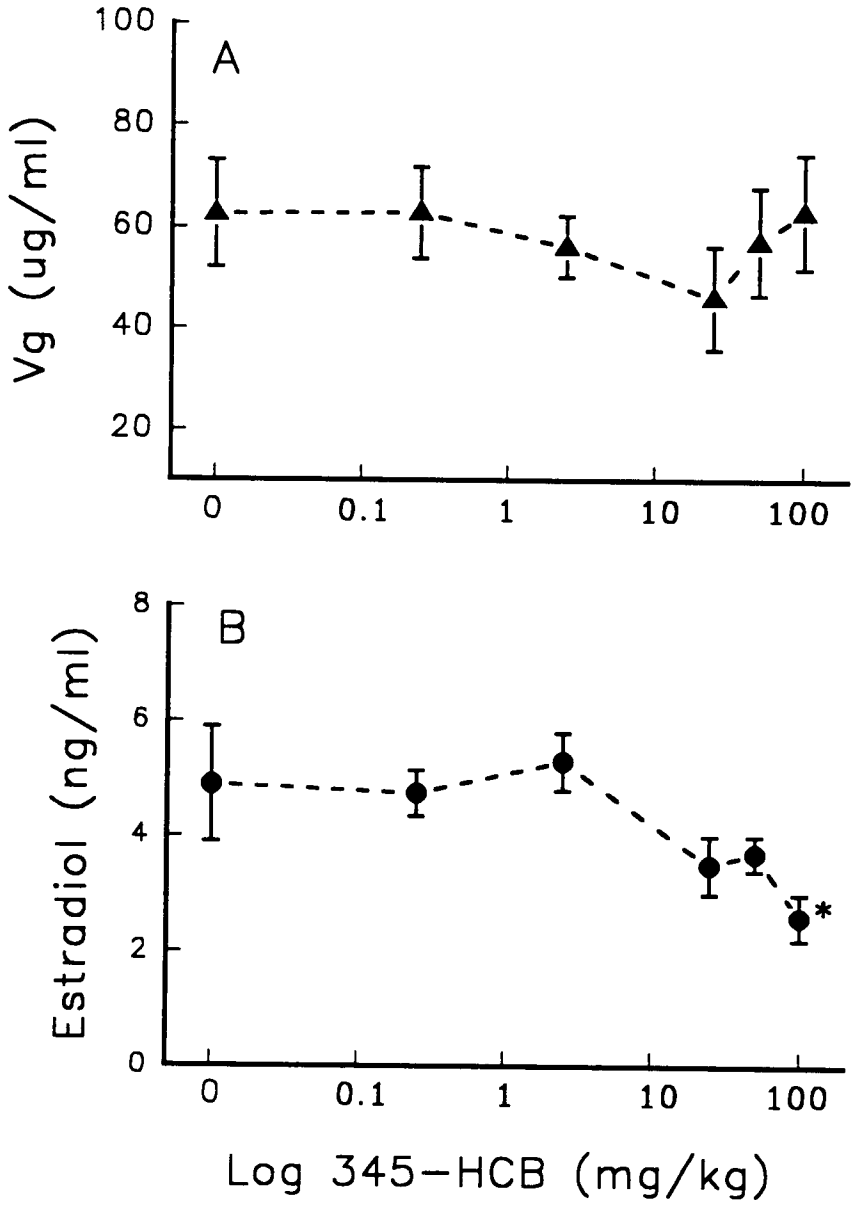

Figure 4.3. **3,4,5,3',4',5'-Hexachlorobiphenyl (345-HCB) did not antagonize estradiol-induced vitellogenin (Vg) synthesis but reduced plasma estradiol levels.** Trout were treated as described in Figure 4.1. Plasma Vg concentrations in estradiol-treated fish were not significantly altered by 345-HCB ($p \leq 0.05$; n=6/dose). Plasma estradiol levels were significantly reduced, compared to control, in estradiol-treated fish at the 100 mg/kg 345-HCB dose ($p \leq 0.05$; n=6/dose).

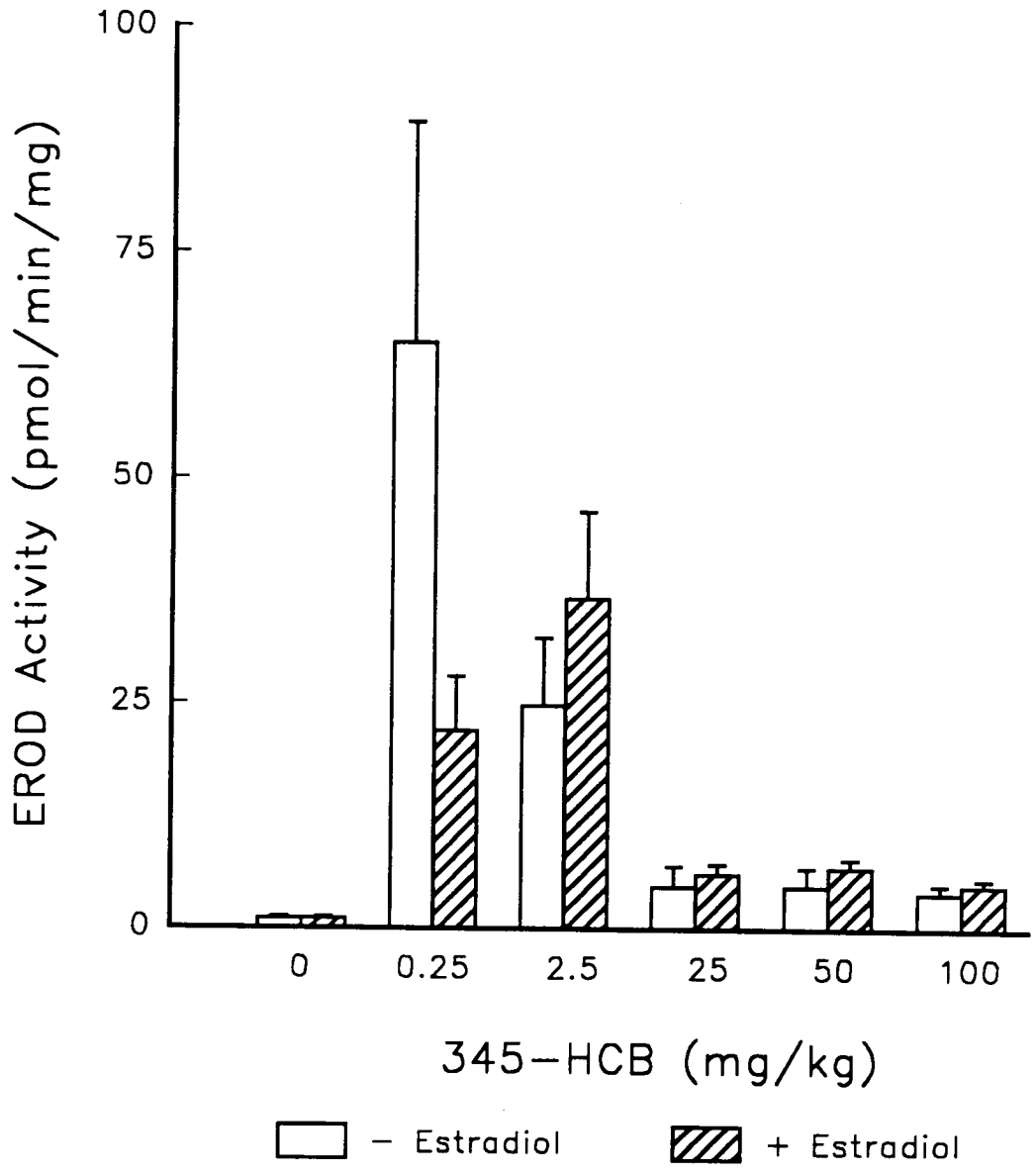

Figure 4.4. **Ethoxyresorufin-O-deethylase (EROD) activity was induced by 3,4,5,3',4'5,-hexachlorobiphenyl but was not influenced by estradiol treatment.** Trout were treated as described in Figure 4.1. Results of a two-way ANOVA indicated EROD activity was elevated by 345-HCB (0.25 - 2.5 mg/kg doses were significantly different from corn oil control; $p \leq 0.05$) but not estradiol.

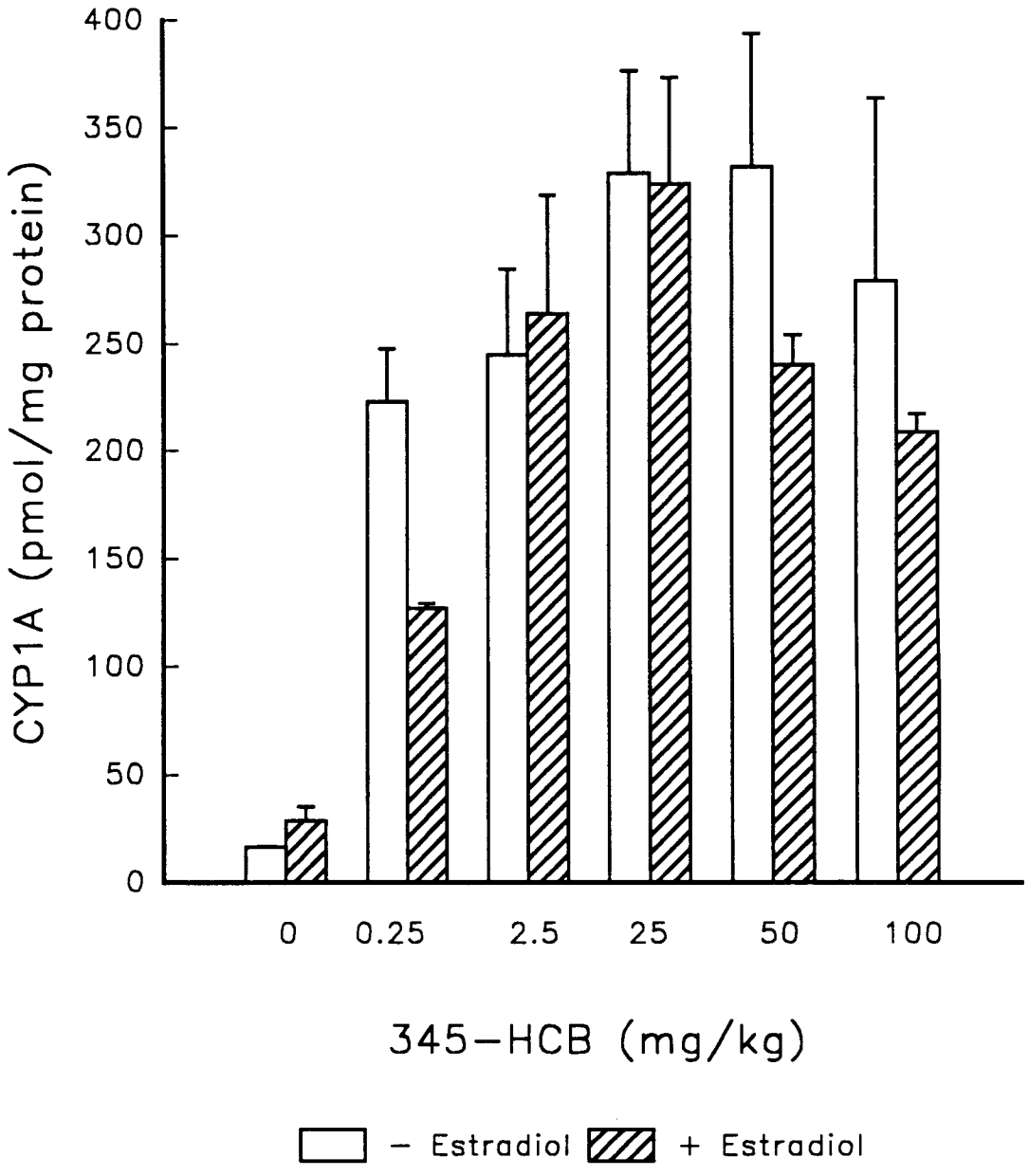

Figure 4.5. **CYP1A protein induction by 3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB).** Significant CYP1A protein induction by all 345-HCB doses, compared to controls, and a reduction in protein content by estradiol at the 0.25 mg/kg 345-HCB dose were detected by ANOVA ($p \leq 0.05$). Experiment design was as described in Figure 4.1. Values are means ($\pm$ SD) of duplicate pooled microsomes (2-4 fish/pool).

pmol/min/ug 1A protein at 0.25 mg/kg). Higher 345-HCB doses (25-100 mg/kg) further reduced mean catalytic efficiency (0.3 to 0.5 pmol/min/ug 1A protein).

345-HCB significantly induced CYP1A mRNA content, compared to controls, but there was no estradiol interaction (Figure 4.6). Similar to the CYP1A protein dose-response relationship, 345-HCB doses greater than or equal to 2.5 mg/kg significantly elevated CYP1A mRNA amount regardless of estradiol exposure. The mRNA content for the constitutive P450 isozyme, CYP2K1, was not significantly altered by 345-HCB or estradiol (Figure 4.6).

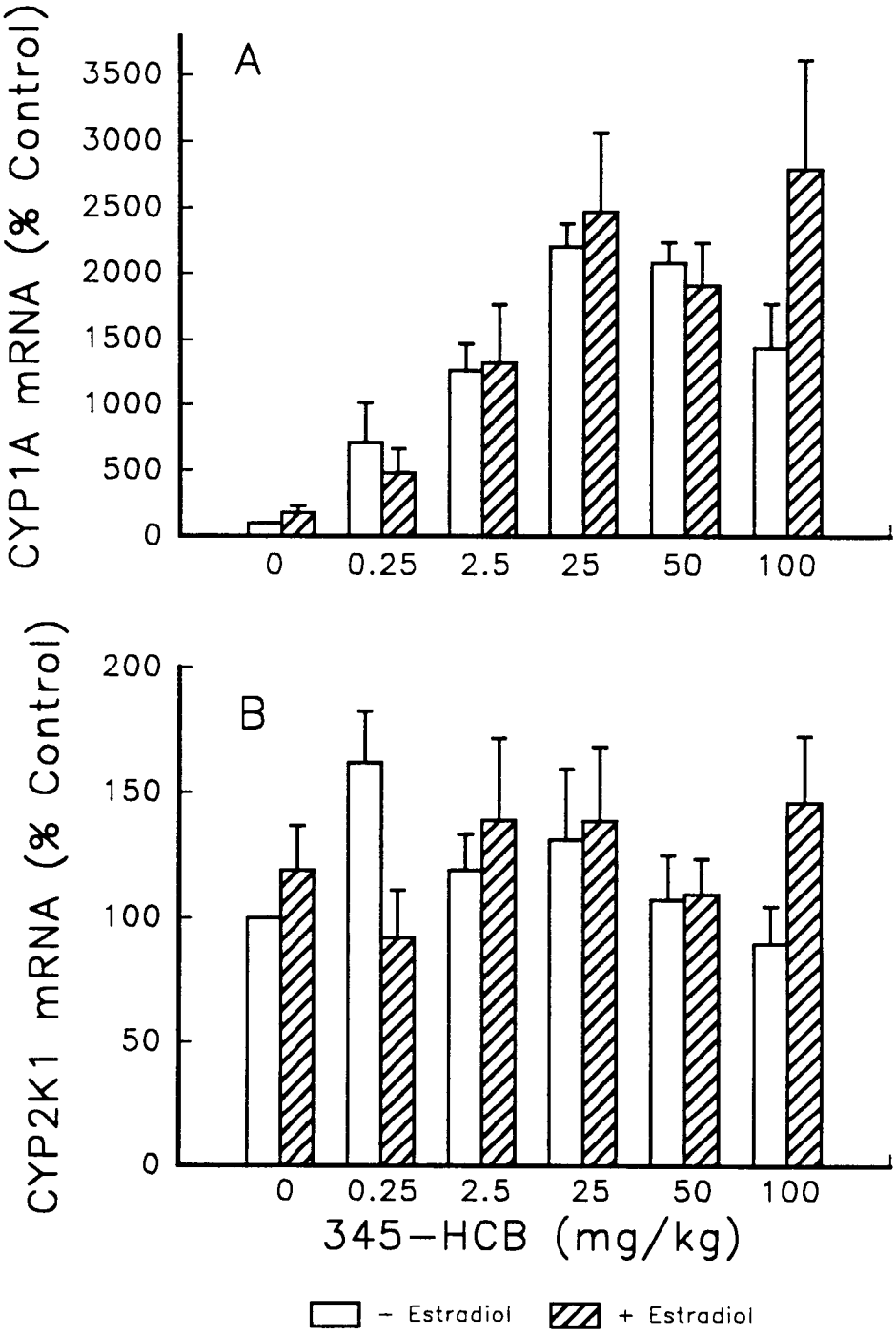

Figure 4.6. **CYP1A, but not CYP2K1, mRNA content was elevated by 3,4,5,3',4'5'-hexachlorobiphenyl without an estradiol effect.** Values, expressed as percent corn oil control, are means (± SE) of four RNA extracts of individual livers loaded onto separate gels.

DISCUSSION

The antiestrogenic effects of AhR ligands in mammals have been attributed to reduced ER number and/or suppressed estrogen-induced protein secretion (Safe *et al*, 1991). In the present study, we utilized estrogen regulated synthesis of the yolk-protein precursor, Vg, as a model to assess antiestrogenic effects of two AhR ligands (345-HCB and ßNF) in juvenile rainbow trout. Our results indicated 345-HCB (0.25 - 100 mg/kg; 0.7 - 280 μmol/kg) was not antiestrogenic in this model. Hepatic ER concentrations were not reduced in 345-HCB treated trout. Additionally, 345-HCB did not antagonize estrogen-induced increases in %LSI, hepatic ER or plasma Vg. However, a comparable dose of ßNF (100 mg/kg; 330 μmol/kg) did reduce hepatic ER to 33% of control values, suggestive of an antiestrogenic effect. Slightly higher control ER values in the ßNF experiment, compared to the 345-HCB experiment, were most likely due to use of larger juvenile fish (Pottinger, 1986) or immediate evaluation of ER activity which may have reduced effects of freezing on binding activity.

Based on the assumption that ER downregulation was AhR-mediated, these results were unexpected because ßNF has been reported to be a less potent AhR ligand than 345-HCB in mammalian studies. For example, ßNF exhibited CYP1A induction potency comparable to 3-methylcholanthrene (3MC) in rats (Boobis *et al.*, 1977), approximately 30,000-fold less potent than TCDD. ßNF was approximately 10,000-fold less potent than TCDD in a rainbow trout liver cell line (Lee *et al.*, 1993). In rats, 345-HCB was estimated to be 250-fold less potent as an inducer of

hepatic EROD activity than TCDD (Safe, 1994) but equivalent data has not been reported for trout. Due to the apparent saturability of CYP1A induction in the present study, we were unable to determine the relative potency of 345-HCB to ßNF based on the single ßNF dose.

The antiestrogenic properties of ßNF have received little attention. Duvivier *et al.* (1981) showed ßNF (60 mg/kg) reduced hepatic ER concentrations in adult ovariectomized rats to 36% of control values. We observed a similar decrease in trout hepatic ER levels following exposure to ßNF (100 mg/kg). Our results were also in agreement with Anderson *et al.* (1993) who reported reduced Vg synthesis in an *in vitro* trout hepatocyte culture system exposed to ßNF. No direct comparison of the antiestrogenic potency of ßNF to 345-HCB or TCDD in mammals was available. However, work of Chaloupka *et al.* (1992) and Krishnan and Safe (1993) indicated 3MC (EC_{50} = 0.1 to 1 μM), a ligand with CYP1A1 induction potency similar to ßNF, was less effective than 345-HCB (EC_{50} = 0.18 μM) in reducing estrogen-induced secretion of cathepsin D in MCF-7 cells. The relative antiestrogenic potency of 345-HCB to TCDD was 0.05 in the MCF-7 cell assay (Krishnan and Safe, 1993). *In vivo* rodent studies demonstrated hepatic ER concentrations were reduced by 10-80 μg TCDD/kg (0.03-0.24 μmol/kg; Lin *et al.*, 1991 and DeVito *et al.*, 1992). An estimated effective 345-HCB dose for reducing ER concentrations, based on a relative potency of 0.05 in mammals, would range from 1-2 mg/kg 345-HCB, within the range of our study. Thus, deviation from the mammalian-derived antiestrogenic

potency of 345-HCB relative to ßNF in the trout vitellogenesis model may have been attributed to factors such as unequivalent target organ (hepatic) dose for the two compounds or species/age/tissue-related differences.

The comparative disposition of ßNF and 345-HCB in fish has not been reported. However, based on solubility differences of the two compounds, similar ip injected doses of 345-HCB and ßNF may not have produced equivalent hepatic concentrations in trout which may have affected apparent antiestrogenic potencies. ßNF was absorbed more rapidly from the rat peritoneal cavity than the more non-polar compound, 3MC (Boobis *et al.*, 1977) and was also susceptible to metabolism by CYP enzymes (Vyas *et al.*, 1983). In fish, short-lived induction of CYP1A activity occurred in response to a single ip injection of ßNF but prolonged induction was reported following continuous ßNF exposure (Kloepper-Sams and Stegeman, 1989; Haasch *et al.*, 1993), indicating rapid absorption and clearance. Hexachlorobiphenyl disposition differs because of high lipophilicity, resistance to metabolism and preferential distribution to adipose tissue (World Health Organization, 1993). Additionally, work by Brown *et al.* (1993) and Vodicnik *et al.* (1981) indicated fish absorbed hexachlorobiphenyls from the ip cavity very slowly. Lake trout liver reached apparent steady state concentrations six weeks after 3,4,5,3',4'-pentachlorobiphenyl exposure when muscle plus liver retained 25% of the dose (Brown *et al.*, 1993). Vodicnik *et al.* (1981) reported trout hepatic concentrations five days after an ip injection of 2,4,5,2',4',5'-HCB were less than 1% of the injected dose. While we were unable to measure hepatic 345-HCB and ßNF

concentrations after equivalent ip injections, due to lack of sufficient tissue, it was likely target organ concentrations were higher in ßNF compared to 345-HCB exposed trout. Differences in target organ doses may have been responsible for the greater apparent efficacy of ßNF, compared to 345-HCB, to reduce hepatic ER concentrations. Further research is needed to relate target organ doses, rather than exposure levels, to antiestrogenic responses to establish *in vivo* AhR ligand potencies in mammals and fish.

Alternatively, mammalian-derived AhR ligand antiestrogenic potencies may not have been applicable to juvenile trout vitellogenesis model due to species-, reproductive status- and tissue-related differences. Evidence for trout-mammalian species differences has been presented by Clemons *et al.* (1994) who suggested trout liver cells were more sensitive to CYP1A induction by PCDD congeners than a rodent liver cell line. There have also been reports of mammalian species differences. Hruska and Olson (1989) reported rats and hamsters but not guinea pigs were responsive to TCDD-induced decreases in uterine ER levels. A second factor modifying antiestrogenicity of AhR ligands was reproductive status. DeVito *et al.* (1994) suggested immature mice were more sensitive to TCDD-induced reduction of ER levels than mature females. In mink, uterine ER dynamics in anestrous females responded differently to 345-HCB exposure than pregnant animals (Patnode and Curtis, 1994). Antiestrogenic effects have also exhibited tissue-specific responses such as disparate time course and dose-response relationships in hepatic and uterine ER dynamics following TCDD exposure in mice (DeVito *et al.*, 1992). Freyschuss *et*

al. (1994) concluded that rat liver ER was under multihormonal control (estrogen, thyroid and growth hormones) and differed substantially from uterine ER regulation. Estrogen plays a major role in fish and amphibian (*Xenopus*) hepatic ER regulation but cortisol and thyroid hormone also modulate levels (Pottinger and Pickering, 1994; Ulisse and Tata, 1994). Thus, elucidation of the mechanism of action and potency of AhR ligands in various species is complicated by several modifying factors and requires further research. Our results indicate ßNF may be a suitable compound to explore mechanisms of antiestrogenicity in fish.

Enhanced estradiol catabolism has been proposed to contribute to the antiestrogenic effects of AhR ligands (Spink *et al*, 1994). In the present study, 100 mg 345-HCB/kg significantly decreased plasma estradiol in estrogen-treated trout. However, plasma estradiol depression was not associated with antiestrogenic effects such as decreased hepatic ER or plasma Vg levels. These results were in agreement with other mammalian studies where plasma estradiol depression did not occur or appeared at relatively high TCDD exposures and was not correlated with antiestrogenicity (Peterson *et al.*, 1993). Additionally, plasma estradiol decline in trout did not parallel CYP1A protein or mRNA induction responses. Estradiol catabolism in trout likely involves more than one CYP isozyme (Miranda *et al.*, 1989). We conclude CYP1A induction was not solely responsible for the observed decline in plasma estradiol. The contribution of other AhR-responsive

biotransformation enzymes, such as UDP-glucuronyl transferases (Forlin and Haux, 1985) to enhanced estradiol catabolism and excretion by AhR ligands requires further attention.

We evaluated CYP1A induction and its modulation by estradiol 10 weeks after single ip doses of 345-HCB in trout. CYP1A enzyme activity, measured as EROD activity, was induced by low doses (0.25 - 2.5 mg/kg) but not higher doses of 345-HCB (25 - 100 mg/kg) despite elevated CYP1A protein levels. This indicated 345-HCB suppressed CYP1A catalytic activity. Similar EROD activity inhibition by 3,4,3'4'-tetrachlorobiphenyl in scup (Gooch *et al.*, 1989) and flounder (Monosson and Stegeman, 1991) and by ßNF in trout (Haasch *et al.*, 1993) has been reported. Thus, markers other than CYP1A catalytic activity (protein or mRNA content) may be required to assess CYP1A induction after high level 345-HCB exposures.

The 345-HCB dose-response relationships for CYP1A protein and mRNA content exhibited similar patterns; an estimated ED_{50} (dose that induced 50% of maximal value) below 2.5 mg/kg with maximal induction (2000-2500% of control) at doses above 2.5 mg/kg (< 7.0 μmol/kg). CYP1A protein content resulting from long-term 345-HCB exposure in our study were substantially higher than values reported for short term exposure to similar ip injected 345-HCB doses in trout (Miranda *et al.*, 1990). These results reemphasize the need to consider target organ doses in the dose-response relationship when fish are exposed to highly lipophilic compounds by ip injection.

CYP-dependent activities (Andersson and Forlin, 1992) and CYP2K1 levels (Buhler *et al.*, 1994) were lower in sexually mature female, compared to male, rainbow trout. Additionally, estradiol reduced EROD activity in juvenile rainbow trout (5 mg estradiol/kg; Vodicnik and Lech, 1983) and reduced EROD activity and P450E content in flounders (1-50 mg/kg estradiol; Snowberger-Gray *et al*, 1991). We observed a significant decline in CYP1A protein content, a subtle but insignificant decrease in EROD activity and no change in CYP1A mRNA content in estradiol (0.1 mg/kg) as compared to vehicle control treated fish after a single 345-HCB dose (0.25 mg/kg). Estradiol had no effect on CYP1A induction at higher 345-HCB doses or on CYP2K1 mRNA levels. The mechanism by which estradiol exerted CYP1A suppression in fish is not known. However, reduction of CYP1A protein but not mRNA content indicates post-transcriptional processes are targeted. Additional work is required to define the relationship of estrogen dose to the suppression of CYP1A induction by AhR ligands.

An objective of our study was to evaluate the use of estrogen-induced Vg production in juvenile rainbow trout as a biomarker of reproductive dysfunction resulting antiestrogenic AhR ligand exposure. Our results indicated 345-HCB hepatic concentrations were sufficient to increase %LSI, to decrease plasma estradiol, and to induce CYP1A enzyme activity, protein content and mRNA content. However, hepatic ER and plasma Vg concentrations in estrogen-induced juvenile trout were not reduced by 345-HCB exposure. We speculate higher hepatic 345-HCB concentrations would be required to reduce Vg production and these concentrations may not be

representative of environmental residue levels (Elskus *et al.*, 1994). Our results were consistent with Monosson *et al.* (1994) who reported 3,4,3',4'-tetrachlorobiphenyl exposure in adult white perch had no effect on plasma Vg but impaired maturation of adult females and the survival of their offspring. Collectively, these results indicate that other markers, such as maternal transport of PCBs to eggs and resulting reduced hatchability (Mac and Edsall, 1991) or embryolethality (Walker and Peterson, 1991) may more sensitively characterize PCB-related reproductive dysfunction in fish. The vitellogenesis model may be most useful in elucidating the mechanisms by which AhR ligands alter ER dynamics or estrogen regulated gene expression.

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CHAPTER 5

Summary

Although there is growing evidence that environmental estrogens and antiestrogens may impair reproduction and development in wildlife, dose-response information required to assess this environmental hazard is lacking. These manuscripts contribute needed information on the doses of estradiol, chlordecone (CD), o,p'-DDT/DDE and 3,4,5,3',4',5'-hexachlorobiphenyl (345-HCB) associated with the induction or inhibition of the estrogen regulated process, vitellogenesis, in juvenile rainbow trout. Results of the present research indicated estrogen increased a series of vitellogenesis markers [%LSI, hepatic estrogen receptor (ER), plasma lipids and plasma vitellogenin (Vg)] in a dose-responsive manner and plasma Vg was the most sensitive indicator. Our inability to detect antagonism of estrogen induced vitellogenesis by 345-HCB suggests further investigation is warranted to examine antiestrogenicity of Ah receptor ligands in fish. Preliminary research with ßnaphthoflavone indicated this compound may be an appropriate candidate for further study.

Three compounds (CD, o,p'-DDT and o,p'-DDE) with reported estrogenic activity in mammals induced vitellogenesis in juvenile trout, providing evidence of estrogenicity in fish. Whereas plasma Vg in estrogen treated (36 μmol/kg) fish reached mg/ml quantities, CD and o,p'-DDT/DDE were relatively weak inducers of

Vg synthesis, producing μg/ml concentrations of Vg in response to higher doses. *In vivo* studies showed similar CD and o,p'-DDE administered doses (CD, 187 μmol/kg; o,p'-DDE, 282 μmol/kg) and hepatic concentrations (CD, 30 μmol/kg; o,p'-DDE, ≈ 40 μmol/kg) were associated with comparable mean plasma Vg levels (CD, 1 μg/ml; o,p'-DDE, 23 μg/ml). In contrast, *in vitro* ER binding assay results indicated o,p'-DDE was weaker a estrogen agonist than CD. These results demonstrate the utility of incorporating *in vivo* studies into estrogenicity assessment because they integrate metabolic, hormonal and dispositional influences.

Additionally, the present research demonstrated large hepatic CD and DDE burdens, relative to environmental concentrations, were related to vitellogenesis induction in juvenile trout. Vitellogenesis may be induced in feral trout by xenoestrogens with similar potency when environmental concentrations and/or bioaccumulation elevate hepatic concentrations significantly (μmol/kg range). Further research is required to relate target organ doses to estrogenic responses in order to perform comparisons between species and biomarkers. The relationship of vitellogenesis induction to mature female reproductive dysfunction also requires investigation. An additional area for future evaluation is examination of the relative sensitivity of different target organs (*e.g.* gonads, brain) or developmental stages (*e.g.* larvae) in fish to estrogenic actions by organochlorines. However, our results suggest plasma Vg is a valuable biomarker to incorporate into field evaluations of fish reproductive health.

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