AN ABSTRACT OF THE THESIS OF

David B. Carlson for the degree of Doctor of Philosophy in Toxicology presented on July 20, 1998. Title: Sex and Life Stage Sensitivity of Rainbow Trout to Xenoestrogens.

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Abstract approved: _____

David E. Williams

Numerous natural and anthropogenic chemicals interact with endocrine systems of animals. The most widely studied of these endocrine active chemicals (EACs) are estrogen receptor agonists and antagonists. Because of the many important roles of estrogens in animals, xenoestrogens have the potential to impact environmental health. It has been proposed that xenoestrogen contaminants are responsible for recent increases in estrogen dependent human diseases and sexual and developmental abnormalities in wildlife. Aquatic species are particularly susceptible to persistent EACs that accumulate in sediments and biomagnify along trophic levels. Rainbow trout, *Oncorhynchus mykiss*, commonly used in biomedical research and as a sentinel species, was chosen as a model for studying mechanisms of xenoestrogen activity. The aims of this research were to assess the estrogenic activity of individual persistent, organic contaminants and simple mixtures *in vivo*. Emphasis was placed on determining the potential for xenoestrogens to alter sexual development or to induce sexually dimorphic biochemical responses. Gonadal abnormalities in trout exposed as embryos to the xenoestrogen *o,p'*-DDE showed that xenobiotics can affect trout sexual development. However, the absence of endocrine

disruption by low doses of *o,p'*-DDE, by the xenoestrogens chlordecone and octylphenol, or by the anti-androgen *p,p'*-DDE, suggested that lethality is likely to precede endocrine disruption in highly exposed, feral salmonid populations. Sexually distinct responses in immature trout were documented with respect to vitellogenin induction (2 to 4 fold higher in females) and cytochrome P450 expression. Sex differences occurred only when doses of estrogens or xenoestrogens were below levels that cause maximal estrogenic responses. Evidence suggests that estrogen regulation may be fundamentally different in immature males and females, which may have implications for natural populations exposed to xenoestrogens. Vitellogenin induction and P450 modulation were responsive to mixtures of estrogens and xenoestrogens in a manner suggestive of additive activation of estrogen receptors. Cytochrome P450 dependent induction of lauric acid hydroxylation was observed for the first time in trout, in response to tamoxifen and mixtures of tamoxifen and 17β-estradiol. The estrogenic activity of tamoxifen and 4-hydroxy-2',4',6'-trichlorobiphenyl were greater *in vivo* than what was predicted by *in vitro* studies, which emphasized the need for mechanism based investigations of xenoestrogens in whole organisms.

Sex and Life Stage Sensitivity of Rainbow Trout to Xenoestrogens

by

David B. Carlson

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented July 20, 1998
Commencement June 1999

Doctor of Philosophy thesis of David B. Carlson presented on July 20, 1998

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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David B. Carlson, Author

Acknowledgments

I'm happy to take this opportunity to thank the many people who, through their direct involvement, encouragement, inspiration, or emotional support, helped me achieve this ambitious goal. First and foremost, I would like to thank my parents, Lucinda and Barton Carlson, whose love, support, and trust have been the true foundation for my life and this undertaking. My brother Ken, my sister Kari, the Bedford Carlson's, and the rest of my family also provided unlimited love and encouragement. And, my family has always believed in education as the key to a happy and successful life, which brings me back to the task at hand...

I thank my major professor, Dr. David Williams, for welcoming me into his lab in midstream and for providing guidance and support. Dr. Williams and his golfing buddy, Dr. Larry Curtis, were instrumental in convincing me that working with trout at O.S.U. was the perfect way to pursue environmental and biomedical research. Dr. Martin Fitzpatrick provided a link to the wonderful world of fish endocrinology and wound up being an invaluable mentor. My other committee members, Dr. Donald Buhler, Dr. Henry Schaup, Dr. Frank Moore, and Dr. Jeff Stone provided a broad base of knowledge, expertise, and support, which contributed greatly to my graduate studies. Without the encouragement and inspiration of great teachers throughout my life, my love for learning would have withered and died. I hope that one day I can inspire students the way that Dr. Denis Maika, Dr. Celia Bonaventura, and Dr. Joe Bonaventura inspired me.

My friends in Corvallis made the rainy winters a little more bearable. Thanks to the Kerkvliet lab for helping out with rent; Carl, Kirsten, and Adam for biking, volleyball,

and being there; Susan for making Corvallis feel like home (sometimes); Roxanne and Jeff

and the many others. And, of course, my two best friends, Mancha and Lisa, for cold

noses, wet kisses, warm hearts, smiles, laughter, and adventure!

Contribution of Authors

Dr. David Williams provided laboratory and financial support and was involved in the design and interpretation of data in all experiments. Dr. Lawrence Curtis provided laboratory facilities and was involved in the design and analysis of experiments in Chapter 2. RIAs in Chapter 2 were performed in the laboratory of Dr. Carl Schreck, Oregon Cooperative Fisheries Research Unit at O.S.U., under the guidance of Dr. Martin Fitzpatrick. Many folks at the Food Toxicology and Nutrition Laboratory provided assistance with aquaculture, diet preparations, and histology, including (but not limited to) Dr. Jerry Hendricks, Dr. Jan Spitzbergen, Dan Arbogast, Greg Gonnerman, Dwayne King, Chance MacDonald, Sheila Cleveland, John Kelly, Eric Johnson, and Lara Martini. Drs. Steven Arnold and John McLachlan of Tulane University provided chemicals and were involved in the design of experiments in Chapter 3. The P450 assays in Chapter 4 were performed by Dr. Cristobal Miranda in the laboratory of Dr. Donald Buhler. Both Drs. Miranda and Buhler were involved in the design and interpretation of results in Chapter 4.

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Chapter 1

Introduction

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Background

Endocrine toxicology has received much attention in the latter half of this century. A series of events in the early part of this decade, including hypotheses linking human breast cancer (1-4) and decreasing sperm counts (5,6) to toxic chemicals, coupled with disturbing fish and wildlife data (7-12), have created renewed awareness to a historical problem. Since the 1950s, scientists have known that certain xenobiotics interact with hormone systems in animals (13). The majority of both historical and modern research on endocrine modulating chemicals has focused on steroid hormones, particularly estrogens. Silent Spring (14), by Rachael Carson, struck a chord with the American public by implicating pesticides and organochlorine chemicals such as DDT, as the cause of abnormalities and declining populations in birds and wildlife. Studies in the scientific literature had shown that DDT and other chemicals were estrogenic in animals (15-22), but those findings had not gained the attention of the public as a whole. While the cause of population declines in predatory birds due to eggshell thinning was eventually linked to DDTs and metabolites (DDEs), the mechanism involved changes in enzyme function and sequestering Ca^{2+} for shell formation (23,24). In subsequent years, research in endocrine toxicology became sufficiently broad that a common link between many studies was lost. Two conferences on "Estrogens in the Environment", in 1979 and 1985 (25,26), provided a forum for discussion of estrogen modulating chemicals, but it wasn't until 1991 that scientists from a variety of fields were brought together at the "Wingspread Conference" (27) that the breadth of related endocrine toxicology research was realized. Public interest was piqued as the popular press published stories on the issue that has been commonly

called "endocrine disruption". Stories with titles such as "Hormone Hell" (*28*) and "The Gender Benders" (*29*) undoubtedly caught the attention of the United States Congress, which passed provisions contained in the Safe Drinking Water Act (1996) and the Food Quality and Protection Act (1996) that require the Environmental Protection Agency (U.S. EPA) to identify screening tools for testing estrogenic activity of new chemicals this year, and to start chemical screening in 1999. Public television recently aired a program of Frontline (2 June, 1998) dedicated to the endocrine disruption issue and the consequences of congressional testing mandates. It is fair to say that "endocrine disruption" is an issue of toxicological interest that has attracted the attention of the American public.

The descriptive term "endocrine disruption" has been coined to describe all chemical interactions with the hormone systems of animals. Strong evidence exists that populations of animals, including trout in the United Kingdom (*7,8*) and alligators in Florida (*9,10*), have been affected by chemicals in heavily contaminated areas. Humans have suffered ill effects from the pharmacological estrogen diethylstilbestrol (DES), ingested by pregnant women in the 1950s and 1960s to prevent spontaneous miscarriage, resulting in sexual abnormalities and unusual hormonally dependent cancers in people exposed *in utero* (*30,31*). In such cases, normal function of the endocrine system has been disrupted and the term "endocrine disruption" is an accurate description of the consequences. However, the ability of chemicals to interact with components of endocrine systems does not prove that "disruption" of normal physiological activity has occurred. Better terms for chemicals that interact with hormone systems are "endocrine active chemicals" (EACs) or "endocrine modulating chemicals" (EMCs), neither of which have gained widespread use. For lack of an accurate, widely used term, the most general

description of "endocrine active chemicals", or EACs, will be used within this body of work.

Aquatic populations are particularly susceptible to EACs. EACs are released into aquatic systems in bleached kraft mill effluent (BKME) from paper mills and in treated sewage effluent (32,33). Other chemicals accumulate in sediments, which are slowly released into water and bioaccumulate and bioconcentrate in animals. Evidence from field studies suggest that various populations of aquatic vertebrates have been affected by human derived EACs. Predatory bird populations exposed to EACs, described by Rachel Carson, have rebounded but exposure to widespread, low level contamination persists and the topic has recently been reviewed (24). Feminization of feral fish in the United Kingdom is thought to be caused by contaminants in sewage outfall, including alkylphenols and pharmacological estrogens (7,8,34). Male alligators in heavily contaminated Lake Apopka, Florida, have small penises thought to be caused by the demasculinizing effects of the DDT metabolite p,p'-DDE (9,10). Reviews from the Wingspread Conference discussed the role of organochlorines in sexual abnormalities observed in Great Lakes salmon and marine mammals (11,12), as well as the unknown causes of masculinization of fish in the Southeastern United States (35). Sufficient evidence suggests that populations in heavily contaminated areas have been affected by EACs, but less research exists to implicate residual environmental contamination in endocrine disruption.

Recent interest in endocrine toxicology surged after reports implicating EACs in human health abnormalities. Selected populations are exposed to high levels of EACs in chemical manufacturing, pesticide application and farming, point-source pollution, and

diets high in contaminated foods (*36-40*). Breast cancer rates have increased in the past 50 years and a preliminary epidemiological study reported a correlation between EACs and cancer incidence (*2*). Results in several subsequent studies have been mixed, but the most comprehensive data have failed to establish a link between organochlorine EACs and breast cancer (*3,4,37,41*). Worldwide declines in male sperm counts were described and attributed to EACs in a 1993 report (*5*). Several studies showed that sperm counts have declined in certain geographical regions, but not globally (*6,42,43*). A role for estrogens in the male reproductive system has been discovered (*44*), which may be a target of EACs; however, no causes of declining sperm counts or other male reproductive system anomalies have been proven (*37,45,46*).

There is evidence that humans are sensitive to exposure to exogenous estrogens. *In utero* exposure to diethylstilbestrol (DES) has been linked to rare vaginal cancers in women and sex organ alterations in men (*30,31*). A major difference between DES and other EACs is that DES, a potent, pharmacological estrogen, was taken intentionally in high doses. The general human population is exposed to very low levels of persistent EACs, although certain lipophilic EACs accumulate in animal and human tissues. Developmental exposure to EACs *in utero* and through breast milk has been documented in infants (*39,47*), some of whom are thought to have suffered permanent detrimental effects on learning and cognitive function (*48,49*). Research continues to investigate the role of EACs in human health, but at the present time, it is difficult to make any conclusions about adverse health effects of environmental EACs (*37,46,50*).

Interest in EACs is due, in part, to the large numbers of chemicals and chemical classes that interact with endocrine systems. Xenoestrogens have received the most

attention and study. No clear structure-activity relationships have been determined for chemicals that interact with estrogen receptors, but hydroxyl groups and aromatic rings seem to be important (Figure 1.1) (51,52). Early work with organochlorines such as DDTs and chlordecone established their estrogenic activity *in vivo* (13,15,16,18-20). Interaction with estrogen receptors *in vitro* has recently been used to classify xenoestrogens (51,53-57). Chemical classes with estrogen modulating activity include natural products (e.g. phytoestrogens: genistein; coumestrol; β-sitosterol), industrial chemicals (PCBs, surfactants, phthalates), pesticides and pollutants (DDT, chlordecone, methoxychlor, dioxins, BKME, sewage effluent) and pharmaceuticals (DES, contraceptive estrogens). Exposure to these chemicals is likely to occur simultaneously, so it is advantageous to understand the activity of simple and complex mixtures, in addition to individual chemical effects. Many of these chemicals are lipophilic and resistant to degradation by design, which contributes to their bioaccumulation and persistence.

Persistence is an important issue, because humans are exposed to larger amounts of dietary phytoestrogens than any other EACs (50). Phytoestrogens undoubtedly evolved as defense mechanisms in plants to disrupt reproduction in pest species. Phase I and Phase II enzymes quickly metabolize xenoestrogens from plants, preventing accumulation and prolonged exposure (50,58). Chemicals such as organochlorines (OCs) and alkylphenol ethoxylates (APEs), on the contrary, are difficult to metabolize and persist in animal lipid stores. Partly because of their role in declining bird populations and endocrine modulating potential, the production and use of OCs has ceased in most industrialized nations. However, OCs are still widely used in developing countries, which has resulted in global atmospheric transport and ubiquitous contamination in Arctic

Figure 1.1- Structures of estrogenic chemicals. (1) 17β-estradiol (natural); (2) tamoxifen (pharmacological); (3) diethylstilbestrol (DES, pharmacological); (4) 4-hydroxy-2',4',6'-trichlorobiphenyl (industrial contaminant); (5) nonylphenol (alkylphenolic contaminant); (6) *o,p'*-DDT (pesticide contaminant); (7) 4-hydroxy-2',3',4',5'-tetrachlorobiphenyl (industrial contaminant); (8) genistein (natural phytoestrogen); (9) chlordecone (pesticide contaminant)
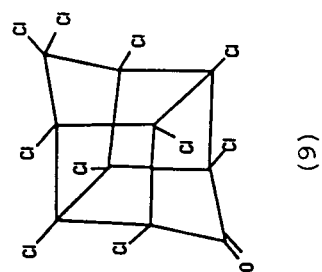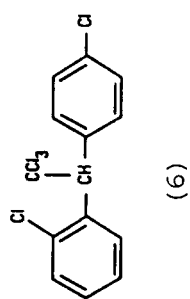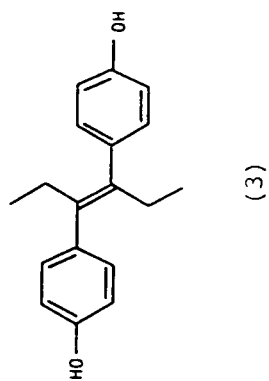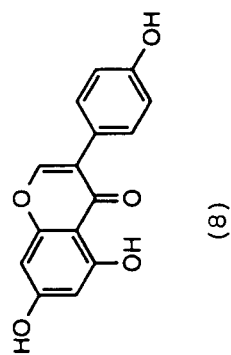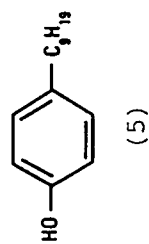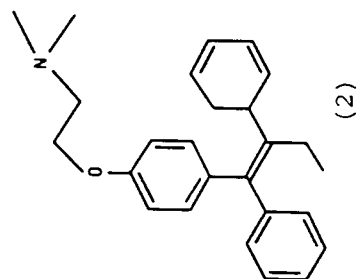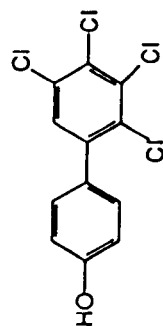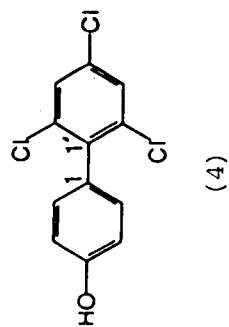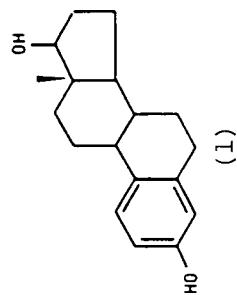
Figure 1.1

regions and flora and fauna, including marine vertebrates and humans (*12,59,60*). APEs

are heavily used in sewage treatment works, which also contain pharmacological

estrogens, contributing to ongoing pollution (*33,34,61*).

In vitro vs. *in vivo*

A majority of recent research in endocrine toxicology has followed the general

trend in biology towards *in vitro* and molecular studies. Many chemicals have been

classified as steroid hormone mimics or antagonists, based on their interaction with

receptors *in vitro*. While reductionist approaches in mechanism based studies have

provided a more accurate description of chemical activity than the historical bioassay

approach, endocrine systems are exquisitely complex in animals (see *62,63*). Perhaps the

best approach is to combine mechanism based *in vitro* analyses with *in vivo* studies.

Common approaches for *in vitro* EAC characterization include: receptor binding

studies (*51,53,56,57,64,65*); stable and transient transfections of hormonally responsive

genes (or promoter regions), in combination with reporter genes such as luciferase (*54-

56,65-70*); and studies using immortal cell lines and cultures that are responsive to natural

estrogens (*55,56,65,66,69*). Some advantages of *in vitro* systems include the ability to

screen large numbers of chemicals quickly, to estimate ligand affinity for hormone

receptors, and to gauge the strength of ligand-receptor-DNA interactions. A major

disadvantage of the *in vitro* approach is the potential lack of regulatory elements (both

positive and negative) present in intact organisms, including cell and tissue specific

transcription factors, coactivators and repressors. Physiological factors are also involved

in hormone homeostasis, which may be altered by xenobiotics. The activity of EACs will

depend, in part, on absorption and metabolism during exposure, partioning between

aqueous and lipid compartments, and the effective concentration of chemical at target

sites. Indeed, endocrine responses in whole organisms cannot necessarily be considered to consist of the sum of their parts.

Various examples in the literature illustrate *in vivo* responses that were different from *in vitro* predictions. Welshons and colleagues observed that the estrogenicity of octylphenol was greater in mice than predicted, presumably due to interactions with serum binding proteins (*71*), and that prostates were enlarged in mice exposed *in utero* to low, but not high, doses of estrogen and DES (*72*). Studies with rainbow trout also showed up to 10 fold higher *in vivo* estrogenicity of octylphenol than predicted, possibly due to bioaccumulation (*34,73*). Metabolism is necessary for many drugs and xenobiotics to be bioactivated, which has been shown to be important for the *in vivo* estrogenicity of the pesticide methoxychlor (*74*). Tamoxifen, designed to be an inhibitor of estrogen dependent breast tumors, was found to be an estrogen agonist in other tissues *in vivo*, indicating the importance of tissue specific transcription factors in estrogen receptor activation (*75*). An analysis of the existing research with EACs led participants in a U.S. EPA sponsored workshop to conclude that *in vivo* characterization of EACs should be a high priority (*76*).

In addition to receptor interactions, a simplified look at steroid hormone regulation reveals multiple sites of xenobiotic action, encompassing all endpoints influenced by endogenous steroids. Synthesis of steroids in endocrine glands and tissues is under the direct control of gonadotropin hormones, which are regulated by the hypothalomo-pituitary axis (*62*). Steroids are involved in feedback loops that signal gonadotropin release, thereby influencing their own metabolism. Proteins and lipoproteins in the blood bind to steroids, facilitating transport throughout the circulatory system and regulate

movement across cell membranes (71). Cytochrome P450 enzymes are likely sites of modulation by EACs because they are directly involved in steroid metabolism (77), they are involved in Phase I metabolism and bioactivation of xenobiotics (78,79), and they are susceptible to induction, downregulation, and inhibition by xenobiotics (79-82). Interference with receptor-DNA complexes is possible, not only through direct interactions with receptors, but by modifying binding to response elements and transactivation domains of other proteins (83,84) or through cross-talk with other signaling pathways (85). Regulation of cell growth, including interactions with growth factors and proliferative activity in tumor cells (86), may be a target of EACs. Alternatively, EACs could modulate endogenous estrogen function in positive ways by enhancing bone growth or activity at other non-target tissues (86,87).

Physiological contributions and susceptibility of animals to subtle endocrine modulating effects have not been widely explored. Steroid hormones are involved in sexual determination and differentiation in most animals. Lipophilic chemicals are often designed to cross the blood-brain barrier (e.g. the neurotoxic pesticides DDT and chlordecone) and they easily cross through the placenta to expose developing embryos. Evidence from animal models and humans have demonstrated that alterations in sex steroid levels during development can have profound effects including feminization, masculinization, and sex changes (31,88-92). Many chemicals are also more toxic to early life stages of animals, including endocrine systems (93,94). Sex steroid expression and activity are different in males and females, therefore, chemicals that interact with steroids may have sex specific consequences. Depending on the stage of sexual development,

toxicity may occur with respect to growth and development immediately, or effects may be delayed until sexual maturity is reached.

Hormone metabolizing enzymes and estrogen responsive genes are common targets of xenobiotics. Early studies with DDT showed that enzyme systems involved in hormone regulation could be altered, which contributed to endocrine toxicity (95,96). Recent evidence has built upon earlier work, showing that treatment with exogenous estrogens or xenoestrogens alters cytochrome P450 expression and activity (97-100) and estrogen-dependent proteins such as vitellogenin and lactoferrin have proven useful as biomarkers of estrogen exposure (101-103).

A recurring concern in environmental research is the activity of complex chemical mixtures in vivo. Environmental health agencies such as the U.S. EPA regulate chemicals, and implement toxicity testing and health guidelines, based on the assumption that activity of mixtures is additive. The most common example of this is the use of toxic equivalency factors (TEFs) to assess the risk of exposure to aryl hydrocarbon receptor (AhR) agonists based on the sum of chemical affinity for AhR, compared to dioxin (104,105). Mechanistic laboratory studies usually focus on single chemicals or simple mixtures, but evidence exists in vitro and in vivo that suggests that synergistic interactions of estrogenic chemicals are possible (85,106). Functional synergy could occur between transcription factors (106) or through mechanisms that have not been documented, such as cross-talk between signaling pathways, conformational changes in gene promoter regions, or through multiple binding sites on receptors. Interactions between different receptor subtypes (107) or with retinoic acid receptors (108) could lead to complex transcriptional regulation of

estrogen receptors. Investigations of the toxicity of mixtures will continue because of the continuous exposure of humans and wildlife to low levels of complex chemical mixtures.

Rainbow trout model

Research in aquatic toxicology varies greatly, from environmental and regulatory, to biomedical and basic research. Rainbow trout, *Oncorhynchus mykiss*, are desirable animal models for a variety of reasons. Trout are predatory fish, with a fairly high fat content, which makes them susceptible to bioaccumulation and bioconcentration of lipophilic contaminants (*109*). Wild trout populations have been used as indicator species of contamination (*94,109,110*) and fisheries biologists have studied salmonids because of their economic importance and use in aquaculture. Rainbow trout have been widely used in biomedical research, particularly as a model for carcinogenesis (*111*) and in the characterization of xenobiotic metabolizing enzymes (*82*). Extensive knowledge of trout biology, including steroid hormone systems, make them excellent models for endocrine toxicity research.

Early life stages, particularly embryos and sac-fry, are more sensitive to xenobiotics than older fish (*93,112*) and endocrine development can be permanently altered by early exposures. Sex determination in trout is thought to be governed by sex chromosomes (*113*), but sexual development can be manipulated by exogenous hormone treatments (*90,114*). Sexual differentiation occurs between hatching and yolk-sac absorption (*114,115*), throughout which time EACs could cause irreversible changes in sexual development. Examples of xenobiotic induced changes in sexual differentiation exist, including: sex inversion in all female gynogenetic lizards (*116*) and in turtles (*117*), caused by aromatase (CYP 19) inhibition; sex reversal in reptiles with temperature-dependent sex determination by OH-PCBs (*85*); and abnormalities in trout gonads due to

developmental PCB exposure (*118*). *In utero* exposure in humans and laboratory animals

to steroid hormones or analogs can alter fetal sexual development in the absence of

maternal toxicity, and problems may only be evident after sexual maturity (*31*).

The modulation of estrogen receptors, steroid metabolizing enzymes, blood

transport proteins, hypothalamo-pituitary feedback loops, and estrogen-dependent gene

products have been studied in trout with respect to natural hormone homeostasis. All of

those endpoints are targets of xenoestrogens, either directly, or indirectly by altering

endogenous hormone function. A single trout estrogen receptor has been sequenced and

cloned (*119-121*), though multiple mRNAs exist (*120,122*). Two estrogen receptors have

been characterized in mammals (*107*), which suggests that other trout ERs may exist

(*123*). ER is autoinduced in trout, resulting in upregulation of receptor number by

estrogens (*64,124,125*), ultimately saturating the response (*125*). Mature female brown

trout were shown to have higher ER expression than mature males (*126*), but rainbow

trout express ER and ER responsive genes early in development at levels comparable to

adult males (*122*). Sex differences in juvenile fish have not been explored. Species

differences appear to exist in teleost ER sensitivity to xenoestrogens (*53,127*), but little

work has been done to characterize fish responses to pharmacological antiestrogens or

partial agonists. Studies showed P450 sex differences in mature fish (*128*), which were

supported by later evidence that estrogen exposure caused female fish to exhibit lower

P450 levels (*99,100*). Xenobiotic induced changes in steroid transport proteins or

gonadotropins have not been studied extensively. EACs have been shown to modify

thyroid hormone status in animals, but specific work in fish is lacking (*129-131*).

Induction of the egg yolk protein precursor vitellogenin (Vg), which is dependent on

estrogen receptor binding (*132*), has been used as a biomarker of xenoestrogen exposure in trout (*34,64,102*). The promotion of liver cancer in trout by estrogens has also been documented (*133*), which is an additional potential endpoint of estrogenic xenobiotics.

Present studies

This thesis research was designed to investigate effects of xenoestrogens in rainbow trout, particularly whether endocrine function could be transiently or permanently altered by *in vivo* exposure. Because *in vitro* systems are necessarily more simplistic than intact organisms, mechanistic models do not always accurately predict results in animals. Specific questions of interest were: (1) can xenoestrogens alter normal sexual development?; (2) are early life stages exquisitely sensitive to EACs?; (3) are chemicals that are classified as xenoestrogens based on *in vitro* data also estrogenic *in vivo*?; (4) are estrogenic responses of simple chemical mixtures equivalent to the sum of individual chemicals?; and, (5) do sexually immature males and females respond similarly to xenoestrogens?

Exogenously administered steroid hormones can alter sexual differentiation in salmonid fishes (*90,114,134*), which suggests that EACs may alter trout sexual development. Chapter 2 contains information about rainbow trout that were exposed as embryos to persistent, lipophilic chemicals known to interact with steroid hormone receptors. Treatments were designed to model maternal transfer of chemicals to eggs, which results in continuous exposure to EACs throughout sexual development. Multiple physiological and biochemical endpoints were studied, including gonadal morphology, histology, and *in vitro* steroid production. Fish were also reared to maturity to examine the effect of *in ovo* exposure on sexual maturation and reproductive potential.

The estrogenicity of PCBs and OH-PCB metabolites is discussed in Chapter 3. PCBs have been classified as estrogen agonists and antagonists based on their ability to

bind directly to estrogen receptors and because of interactions associated with Ah receptor binding. Evidence from other species, including turtles and mice, demonstrated the estrogenicity of various OH-PCBs *in vivo (51,66)*. An enzyme-linked immunosorbent assay (ELISA) for vitellogenin, modified from an existing method (*64*), was used to gauge estrogenicity. Responses of fish to single chemicals and mixtures of natural estrogens and OH-PCBs were compared. Immature fish at different stages of sexual maturation were also compared in order to investigate sex and life stage sensitivity to chemical mixtures.

Estrogens and steroid hormones have complex physiological functions. While changes in gene transcription by estrogens are mediated through receptor-DNA interactions, regulation of steroid metabolizing enzymes may be receptor dependent or independent. Expression and activity of cytochrome P450 enzymes involved in steroid and xenobiotic metabolism can be modulated by natural estrogens (*99,100*). Studies described in Chapter 4 investigated the mechanism of P450 downregulation by 17β-estradiol. Tamoxifen, a partial estrogen receptor agonist and pharmaceutical agent in humans, was used as a model xenoestrogen in combination with 17β-estradiol to investigate the role of estrogen receptors in P450 regulation. Little is known about the activity of tamoxifen in trout and studies were designed to determine the potential of a tissue-specific estrogen to induce vitellogenin or alter P450s. Most EACs are weakly estrogenic, like tamoxifen, and may alter normal endocrine function by disrupting the physiological role of natural estrogens.

Prior to sexual maturation, rainbow trout and other salmonids are difficult to sex without using invasive techniques. A prevailing assumption is that sexes are physiologically similar until maturation, when circulating sex steroid levels rise as gonads

become fully functional. Consequently, when trout are used as biomedical models, results from juveniles are often pooled and analyzed together. Patiño and Schreck, however, showed evidence of sexually dimorphic steroid production in immature salmon (135). Exposure to xenoestrogens and other EACs may have different consequences in fish of different sexes, because estrogens and androgens have different physiological roles. In chapter 5, the sex-specific vitellogenic responses of immature fish to estrogens are compared.

Ultimately, scientists must assess the risk of EACs to the general health of animal and human populations. The final chapter of this thesis provides a brief discussion of the significance of results presented here, as well as recommendations for future research. Observations in rainbow trout may be useful in uncovering mechanisms of xenoestrogen induced endocrine toxicity and will provide a basis for species comparisons and insight into potential human health effects.

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Chapter 2

Salmonid Sexual Development Is Not Consistently Altered By Embryonic Exposure To Xenoestrogens

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Abstract

Sexual development in fish is sensitive to exogenous hormone manipulation and salmonids have been used extensively as environmental sentinels and models for biomedical research. Maternal transfer of contaminants was simulated by microinjection of rainbow trout (*Oncorhynchus mykiss*) and chinook salmon (*Oncorhynchus tshawytscha*) embryos. Fish were reared for six months, sexed, and gonads removed for histology and measurement of *in vitro* steroid production. Analysis of fat samples showed that dichlorodiphenylethylene (DDE) levels, *o,p'*-DDE and *p,p'*-DDE isomers, were elevated six months after treatment. A preliminary study showed an increased ratio of males:females after treatment with 80 mg/kg and 160 mg/kg of the xenoestrogen *o,p'*-DDE. One fish treated with 160 mg/kg *o,p'*-DDE had gonads with cells typical of both males and females. A follow-up study, using more fish and excluding the highly toxic 160 mg/kg *o,p'*-DDE dose, showed no effect on sex ratio or gonadal histology. Embryonic exposure of monosex male trout, monosex female trout, and mixed sex salmon to *o,p'*-DDE, *p,p'*-DDE, mixtures of DDE isomers, and octylphenol failed to alter sexual development. No treatment dependent changes in *in vitro* gonadal steroid production were seen in any experiments. Trout exposed *in ovo*, that were reared to maturity, spawned successfully. These results suggest that lethality due to the xenoestrogens *o,p'*-DDE, chlordecone, and octylphenol, and the anti-androgen *p,p'*-DDE, are likely to precede subtle changes in sexual development. However, trout appeared to be sensitive to endocrine disruption, so we cannot dismiss the threat of heavily contaminated sites or complex mixtures to normal sexual development of salmonids or other aquatic organisms.

Introduction

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

Laboratory and field data have implicated OCs in impaired reproductive success and abnormal sexual development in fish and wildlife species. DDT and metabolites have caused egg shell thinning and endocrine and reproductive toxicity in wild birds, altering population structure (*5*). Sexual abnormalities reported in Florida alligators are thought to be caused by the demasculininizing effects of DDT metabolites, including p,p'-DDE (*6,7*). Reviews have discussed the role of OC exposure in reproductive and sexual abnormalities in Great Lakes salmon (*8*) and marine mammals (*2*). Feminization of trout in United Kingdom rivers has been observed and is likely due to contaminants in sewage outfalls, including alkylphenols and pharmacological estrogens (*9,10*). Laboratory studies with

organochlorines and alkylphenols, including o,p'-DDE, chlordecone, and octylphenol, have

documented the estrogenicity of these chemicals (4,11-13).

Because hormones are involved in the etiology of various human cancers, EACs

have been predicted to increase cancer risks. Selected human populations are exposed to

high levels of OCs from chemical manufacturing, heavy pesticide use, point-source

pollution, and diets high in contaminated fish. Correlations have been suggested between

xenoestrogens and breast cancer (14) and declining sperm counts (15), however, causal

links with adverse human health effects have not been established (reviewed in 16,17). In

utero exposure to DES, a potent non-steroidal pharmacological estrogen, has been linked

to rare vaginal cancers in women and sex organ alterations in men (18,19).

Developmental exposure of EACs to humans in utero and from breast milk has been

documented (20) and implicated in permanent cognitive dysfunction (21).

Rainbow trout, Oncorhynchus mykiss, have been studied extensively in fisheries,

environmental, biomedical, and endocrine research. Lipophilic OCs and alkylphenols are

resistant to metabolism, resulting in bioaccumulation in fish and subsequent human

exposure from fish consumption. Female fish transfer persistent chemicals to eggs,

effectively clearing contaminant burdens but exposing developing embryos to EACs

(22,23). These chemicals may alter sexual development of embryos, which has been

documented in laboratory studies with other chemicals in fish and reptiles (24-26). There

is a great deal of sexual plasticity in teleost fish, but sex steroid hormones appear to

control sexual characteristics in all species (27,28). Sexual differentiation likely occurs

between hatch and yolk-sac absorption in rainbow trout (24,29,30). Complete and partial

feminization of trout have been achieved by various estrogen treatments to eggs and fry

(*30,31*) and androgens can be used to create functional all male populations of salmonids (*24,32*). Hormone receptor expression, endocrine feedback loops, and steroid metabolizing enzymes can be altered by chemical exposure (*11,33-35*). Although physiological consequences may differ, biochemical and molecular functions of steroid receptors in fish seem to be homologous to humans (*36-38*).

In this study, we examined the effects of the xenoestrogens *o,p'*-DDE, chlordecone and octylphenol, and the anti-androgen *p,p'*-DDE, on various endpoints of sexual development in rainbow trout. Differences in species sensitivity to the xenoestrogen *o,p'*-DDE were investigated by comparing sexual development of chinook salmon, *Oncorhynchus tshawytscha*, to rainbow trout. Eggs containing eyed embryos were microinjected with chemicals to ensure accurate dosing. Developing fish were continuously exposed to contaminants throughout sexual development, modeling exposure to maternally transferred chemicals. Sex ratios and gonadal morphology and histology were examined after six months. Immature gonads were removed and incubated *in vitro* to determine steroid synthesizing potential of exposed fish. Fish were reared to sexual maturity and fertility and egg viability assessed. Experiments were conducted with mixed sex populations, as well as single sex populations of males and females.

Materials and Methods

Chemicals

Two persistent metabolites of 1,1-dichlorodiphenyltrichloroethane (DDT), *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 obtained from AccuStandard (New Haven, CT). 4-*tert*-Octylphenol (OP; 97% pure) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Chlordecone (CD; 99.5% pure) was purchased from ChemService (West Chester, PA). Ring-labeled [^{14}C]*p,p'*-DDE (> 95% pure; 12.7 μCi μmol^{-1}), [^{14}C]CD (96% pure; 6.1 μCi μmol^{-1}), and salmon pituitary powder were from Sigma Chemical Co. (St. Louis, MO). A mixture of *p,p'*-DDE and *o,p'*-DDE (5.4:1) was prepared based on the ratio of technical grade DDT isomers and residual environmental levels (*39,40*). All chemicals were dissolved in menhaden oil for microinjection of embryos. Sterile RPMI-1640 culture medium (without glutamine) and gentamicin sulfate (50 mg/ml) were purchased from Whitakker Bioproducts (Baltimore, MD).

Embryo microinjections

Mt. Shasta strain rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from the Marine/Freshwater Biomedical Sciences Center aquaculture facility at Oregon State University. Chinook salmon (*Oncorhynchus tshawytscha*) were obtained from the Fish Genetics and Performance Laboratory at Smith Farm, Oregon State University. Fish were maintained and euthanized with the approval of the Institutional Animal Care and Use

Committee (IACUC) at Oregon State University. Trout were maintained in tanks with continuously running well water, 12-14°C, on a 12 h light:dark photoperiod, except for EXP 1 and the maturation stage of EXP 2, which were subjected to the natural photoperiod. Eyed embryos (21 days post fertilization (DPF)), averaging 110 mg/egg, were microinjected with 1 μl menhaden oil containing chemical (or vehicle only) directly into the yolk, using a Hamilton MicroLab 900 automated syringe pump fitted with a 31 g needle. Microinjections have been used successfully to administer accurate carcinogen doses (41). Preliminary range finding experiments showed that the DDE and chlordecone doses employed approximated LOAEL of six month old fish injected *ip*, and OP doses were below levels lethal to embryos (unpublished data). A total of five microinjection experiments were performed as follows: Experiment 1 (EXP 1)- mixed sex rainbow trout population, 100 eggs per treatment, in duplicate, at 40, 80, or 160 mg/kg *o,p'*-DDE or 7.5, 15, or 30 mg/kg chlordecone; Experiment 2 (EXP 2)- mixed sex rainbow trout population, 150 eggs per treatment, in duplicate, at 10, 40, or 80 mg/kg *o,p'*-DDE, 10, 40, or 80 mg/kg *p,p'*-DDE, or 10, 40, or 80 mg/kg DDE mix; Experiment 3 (EXP 3)- monosex male trout, 100 eggs per treatment at 1, 40, or 80 mg/kg *o,p'*-DDE or 65 eggs per treatment at 0.01, 0.1, or 1 mg/kg OP; Experiment 4 (EXP 4)- monosex female trout, 50 eggs per treatment, in duplicate, at 1, 10, 40, or 80 mg/kg *o,p'*-DDE or 0.01, 0.1, or 1 mg/kg OP; Experiment 5 (EXP 5)- chinook salmon, 85 - 100 eggs per treatment, in duplicate, 3 μl injections at 1, 13.33, 40, or 80 mg/kg *o,p'*-DDE (except 40 mg/kg and 13.33 mg/kg (1 μl injection) were without replicate treatments). Mortality was monitored over time and fish were reared for approximately 6 months, until gonads were large enough to easily sex and remove. Mortality differences were analyzed by ANOVA after hatch and time of first

feeding after yolk absorption, two critical windows of fish development. Sex ratios were compared by Chi-square analysis.

Gonad incubations and histology

After approximately six months fish were anesthetized in a 50 mg/L solution of tricainemethanesulfonate (MS222). Fish weights were recorded and sex was determined grossly by examination of gonad morphology. Gonads were removed and placed in 24 well culture plates containing ice cold medium (RPMI-1640). Fish were then killed by severing the spinal chord. *In vitro* gonadal steroid production was measured using a modification of the method of Fitzpatrick et al. *(30)*. Gonads were washed twice in 1 ml RPMI-1640 containing 0.1% gentamicin for 1 h. Steroid hormone production was induced by addition of 12.5 µg salmon pituitary powder into 1.25 ml medium. Gonads were incubated for 24 h at 4°C in sealed chambers saturated with a 95% O_2:5% CO_2 gas mixture. Media was removed and stored at -80°C until measurement of sex steroids by radioimmunoassay *(30)*. Preliminary experiments were performed to optimize sex steroid production and it was found that addition of 10 µg salmon pituitary extract per ml medium stimulated maximal steroid production by gonads and steroid levels increased linearly up to 24 h. No sex steroids were detected in media alone or when gonads were incubated in the absence of pituitary powder. Histology of gonads confirmed that tissues were viable after 24 h incubations. Steroid production was compared between treatments by ANOVA and the nonparametric Kruskal-Wallis Test (StatView v4.5, Abacus Concepts, Berkeley, CA).

At least ten pairs of gonads from each treatment were fixed in 10% buffered formalin for histological examination. After fixation, tissues were embedded in paraffin and sectioned into 5 μm slices. Sections (3-6 per gonad pair)were stained in haematoxylin and eosin and examined under light microscopy for sex specific gonad structures and germ cells.

Residue analysis and $t_{1/2}$

Persistence of DDE isomers and chlordecone were estimated in fish using radiochemicals. Embryos (21 DPF) were injected with 10 or 80 mg/kg [^{14}C]*p,p'*-DDE or 7.5 or 15 mg/kg [^{14}C]chlordecone. Eggs were maintained in an aerated, static water bath for 20 d, with subsamples removed and snap frozen in liquid N_2 at time 0, 1 d, 7 d, and 20 d. Embryos or fry (hatch occurred at 25 DPF) were solubilized in 1 ml soluene and shaken overnight in a 37°C water bath. Solutions were decolorized with 30% H_2O_2, cooled and radioactivity measured by liquid scintillation counting (Beckman LS6500), employing automatic quench and chemiluminescence correction. Estimations of residue $t_{1/2}$ were determined by least squares regression (Microsoft Excel, v7.0).

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

Sexual maturation

A subset of mixed sex trout from EXP 2 were reared for 2.5 years, in order to monitor sexual maturation. Fish were maintained under a natural photoperiod for the final six months prior to the winter spawning season. Fish were then anesthetized in MS222 and eggs removed from ripe females. Total egg weight was compared to total fish weight to determine the gonadal somatic index (GSI) for individual females. Total egg weight was compared to individual egg weight (average weight of 30-50 eggs) which was used to estimate fecundity, or total eggs released. Eggs from each female were fertilized with a mixture of sperm from at least three stock (control) males. Sperm from experimental males was used to fertilize eggs from two separate control females. From each cross, 100 eggs were removed immediately after fertilization and reared in incubation cups (PVC pipe with fine mesh bottoms) with continuously running water. Fertilization success was determined after 24 h, timing of hatch and hatch success were recorded, and egg mortalities were recorded until one week post-hatch. Gonads of fish that did not spawn were examined grossly to determine if fish were maturing normally.

Results

Early life stages of salmonids were more sensitive to o,p'-DDE and chlordecone than older fish. Mortality (96 h) of two month old fish injected *i.p.* with o,p'-DDE (160 mg/kg highest dose) was 5% and with chlordecone (7.5 - 30 mg/kg doses) ranged from 10 - 35% (data not shown). Mortality of mixed sex trout injected as embryos, with equivalent doses of o,p'-DDE was as high as 49% above oil injected controls (Table 2.1). Chlordecone (EXP 1) mortality ranged from 20 -45 % above oil injected controls (p < 0.05), which increased in a dose-dependent manner. Mortality in o,p'-DDE treated salmon (EXP 5) was no different than oil injected controls (Table 2.1). In monosex trout populations, mortality in treatment groups did not differ from oil injected controls, however, injection itself increased mortality in males (Table 2.1). Octylphenol exposure did not affect survival in a dose-dependent manner in monosex male or female trout (Table 2.1). While mortality was lower in monosex female treatments compared to monosex males (Table 2.1), these data are confounded by the fact that fish were genetically manipulated during fertilization. Approximately 50% of monosex males and 80 - 90% of monosex females died prior to chemical treatment, resulting in varying degrees of selection for hearty fish.

Xenoestrogen treatment in EXP 1 resulted in increased ratios of male:female at all doses. Results were consistent between duplicate treatments and final analyses were performed on pooled data from tanks containing replicates. There were significantly more males than females in 80 and 160 mg/kg o,p'-DDE treated fish (Figure 2.1). Trends from chlordecone treatments suggested increased male:female (1.9, 1.25, and 2.25 at 7.5, 15,

Table 2.1- Mortality of salmonid fry treated as embryos with endocrine active chemicals

Embryo treatment	Mortality (%)									
	EXP 1 (Dimorphic)		EXP 2 (Dimorphic)		EXP 3 (Males)		EXP 4 (Females)		EXP 5 (Salmon)	
	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]	Hatch[a]	1st Feed[b]
Uninjected	17[c]	19[c]	17[c]	19[c]	8	12	1	11	23	36
Oil injected	3	22	18	45	56	56	5	20	26	46
o,p'-DDE (mg/kg)										
1	--	--	--	--	42	44	3	13	27	57
10	--	--	17	58*	--	--	8	22	--	--
40	14*	29*	17	54*	55	62	8	28	10	23
80	10*	38*	32	75**	47	47	3	29	30	50
160	13*	71**	--	--	--	--	--	--	--	--
chlordecone (mg/kg)										
7.5	27*	42*	--	--	--	--	--	--	--	--
15	15*	51*	--	--	--	--	--	--	--	--
30	22*	67**	--	--	--	--	--	--	--	--
p,p-DDE (mg/kg)										
10	--	--	12	48	--	--	--	--	--	--
40	--	--	11	44	--	--	--	--	--	--
80	--	--	13	48	--	--	--	--	--	--
DDE mix (mg/kg)										
10	--	--	15	74	--	--	--	--	--	--
40	--	--	13	51	--	--	--	--	--	--
80	--	--	8	55	--	--	--	--	--	--
octylphenol (mg/kg)										
0.01	--	--	--	--	57	62	4	14	--	--
0.1	--	--	--	--	68	69	5	8	--	--
1	--	--	--	--	25	29	3	6	--	--

[a]Mortality measured approximately 7 d post-hatch
[b]Mortality measured approximately 14 d after fish were fed for the first time
[c]Estimated mortality in stock fish, based on observations from a single spawn; corresponds well with historic levels of mortality in this partially inbred stock population (personal observation)
*Higher mortality than oil injected controls of the same experiment (p < 0.05)
**Higher mortality than lower dose treatments and oil injected controls (p < 0.05)
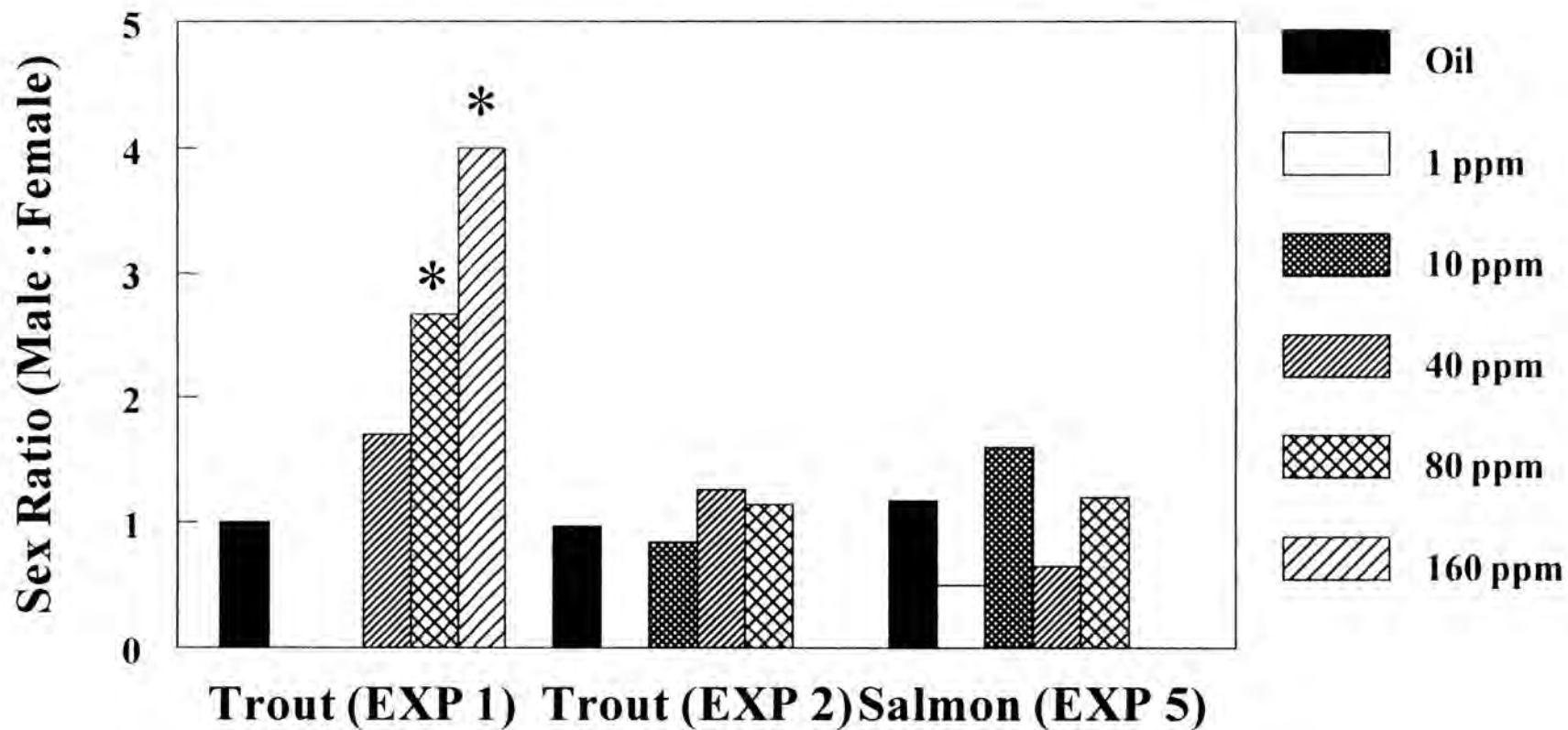
Figure 2.1- Ratio of males:females in response to embryonic *o,p*'-DDE exposure in rainbow trout, EXP 1. * denotes p < 0.05 in 80 mg/kg (n=33) and 160 mg/kg (n=15) treatments.

30 mg/kg, respectively), but the sex ratios were not statistically different than the control ratio of 1:1. Similar experiments using rainbow trout (EXP 2) and chinook salmon (EXP 5) failed to replicate the changes in sex ratio seen with o,p'-DDE in EXP 1. Neither p,p'-DDE, DDE mixtures, or octylphenol treatments altered sex ratios in trout or salmon. Monosex populations of rainbow trout were also treated with o,p'-DDE and octylphenol and no sex reversal was observed. All fish in EXP 3 were male and all fish in EXP 4 were female, regardless of treatment.

One pair of unusual gonads was seen from a fish treated with 160 mg/kg o,p'-DDE in EXP 1. These gonads were phenotypically female when examined grossly, but under light microscopy distinct male and female germ cells were apparent within each gonad (Figure 2.2). Germ cells were not mixed throughout the gonad, rather two distinct portions of the gonad exist, each containing cells indicative of male or female gonads. All other gonads were normal, in all experiments, containing either male or female organization and cells. Histological examination confirmed that gross observations of sexual phenotype were correct for trout. However, gross determination of salmon sex often resulted in incorrectly scoring males as females. Consequently, salmon sex ratios reflect only information obtained histologically.

DDEs and chlordecone were successfully administered to eggs and persisted throughout the period of sexual differentiation in developing fish. The elimination of radiolabeled [^{14}C]chlordecone and [^{14}C]p,p'-DDE from eggs provided estimates of CD $t_{1/2}$ of 19 - 29 d (7.5 and 15 mg/kg doses) and p,p'-DDE $t_{1/2}$ of 347 - 408 d (10 and 80 mg/kg doses). DDE residues were also measured in fat samples taken from fish 283 d after injection in EXP 2. Mean residues were 0.033 and 0.15 ppm in 10 and 40 mg/kg o,p'-

Figure 2.2 - Gonad of six month old morphologic female rainbow trout treated with 160 mg/kg *o,p'*-DDE *in ovo* (EXP 1). *(Top)* Gonad appears to have distinct female and male portions, with no apparent mixing of germ cells except around the transition boundary shown here. Closed arrow represents tear in gonad, probably not related to treatment. Open arrows show characteristic female gonad structure. Large arrowhead indicates characteristic male gonad structure. Small arrowheads show lamellae structures characteristic of female gonads. (8 x magnification). *(Bottom)* High power view of transition point between female and male gonad structure. Small arrowhead shows the distinct membrane characteristic of female lamellae. This appears to be a final lamellar structure, rather than a tear in the gonad, as characterized by the intact membrane. Number 1 shows a typical female germ cell at six months of age. Number 2 shows the typical cluster arrangement of male germ cells at this stage of development. This is the only portion of the gonad where male and female germ cells can be seen together. (132 x magnification).
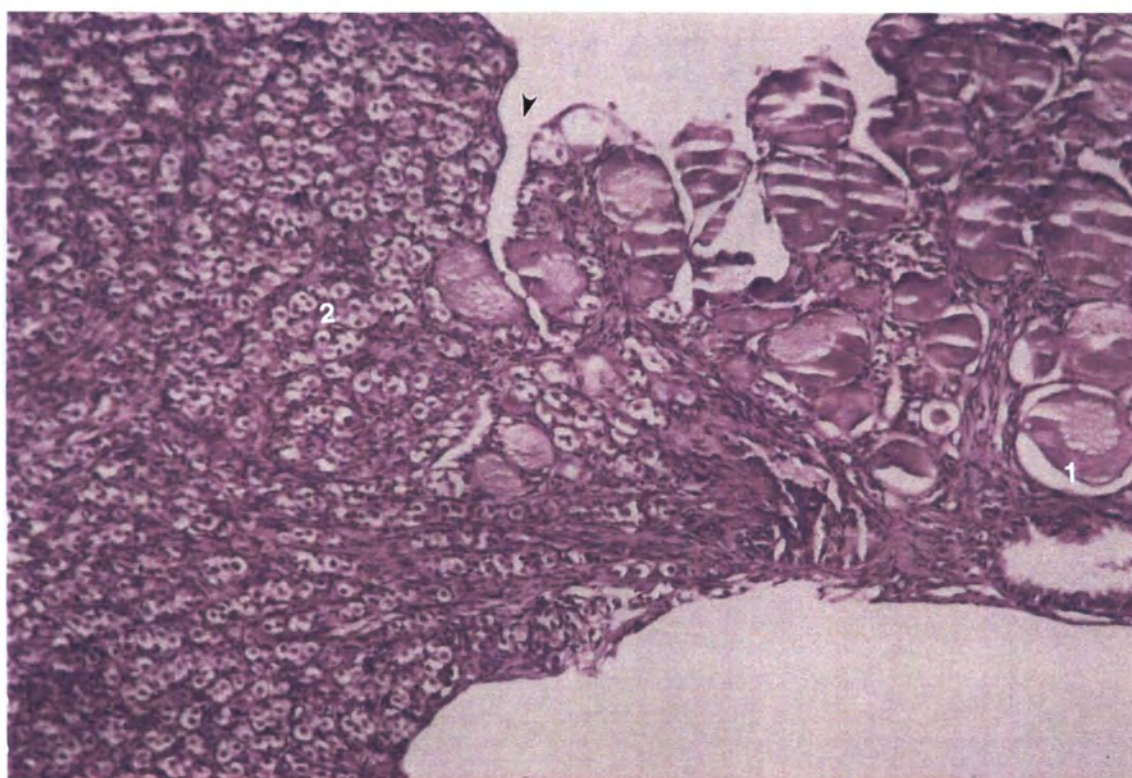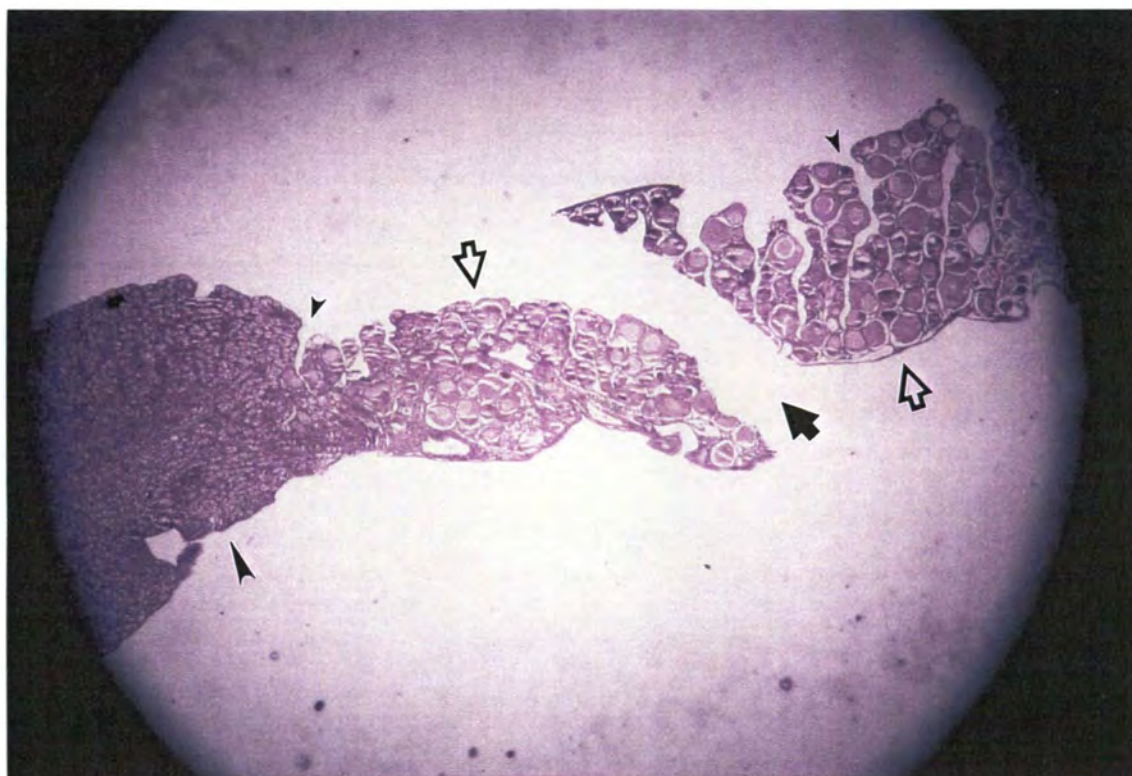
Figure 2.2

Table 2.2 -DDE residues in fish approximately nine months after embryo exposures[a]

Embryo treatment		DDE Residue Analysis (% original dose)[b]	
Chemical	Dose (mg/kg)	o,p'-DDE (µg/g)	p,p'-DDE (µg/g)
Oil (vehicle)	--	BDL[c]	BDL
o,p'-DDE	10	6.5 ± 0.1	BDL
	40	3.8 ± 1.1	< 2.6[d]
p,p'-DDE	10	BDL	78.3 ± 15.0
	40	< 2.5[d]	58 ± 6.8
	80	< 1.5[d]	57 ± 14.5
DDE mix[e]	10	44.2 ± 4.1	109 ± 11.9
	40	16.6 ± 5.3	56.9 ± 2.7
	80	7.6 ± 2.0	58.8 ± 4.7

[a] Residue analysis was determined by GC-ECD after extraction from fat samples taken 283 d after egg injections.

[b] Values represent mean ± SEM of six fish, with the exception of 10 mg/kg o,p'-DDE (n=2) and 40 mg/kg p,p'-DDE (n=5). Percent of original dose recovered was estimated based on 6.98% total body weight as fat in rainbow trout (*42*).

[c] BDL = below detection limit (0.02 and 0.01 µg/g fat for o,p'- and p,p'-DDE, respectively)

[d] Isomer was detected in some samples

[e] DDE mix was 5.4:1 p,p'-DDE:o,p'-DDE

DDE treatments and 0.402, 1.4, and 2.7 ppm in 10, 40, and 80 mg/kg *p,p'*-DDE

treatments. Table 2.2 shows that both *o,p'*-DDE and *p,p'*-DDE were present in treated

fish, but not in oil injected fish. Detection limits were 0.02 and 0.01 µg/g fat for *o,p'*-DDE

and *p,p'*-DDE, respectively. Total body burden of DDE isomers was estimated based on

the fact that approximately 6.98% of total body weight is fat in juvenile rainbow trout

(*42*). Residues of *p,p'*-DDE predicted by $t_{1/2}$ after 283 d were 61.3% and 72.1%, for low

and high doses, which were similar to the observed levels of 78.3% and 57% of the

original dose (Table 2.2). We found that *p,p'*-DDE was more persistent in fish than *o,p'*-

DDE, which, coupled with greater amounts of *p,p'*- isomers produced in technical

mixtures, likely contributes to higher environmental levels of *p,p'*-DDE.

Treatment with these known and putative xenoestrogens did not alter *in vitro*

steroid production by gonads in a dose-dependent manner in any experiments. However,

gonadal steroid production proved to be sensitive to embryonic manipulation, as

evidenced by decreased androgen production (testosterone and 11-ketotestosterone) by

gonads from male fish injected with chemicals or vehicle in EXP 3, compared to

uninjected controls ($p < 0.05$; data not shown).

Subsets of fish from EXP 2 and EXP 3 were reared for 2-2½ years until normal

sexual maturation would occur. At least ten fish from each treatment were examined

grossly and all fish had normal gonads. Ripe males and females from EXP 2 spawned

successfully with control fish. Fertilization success was virtually 100% and most eggs

survived until well developed embryos were visible within eggs (14 DPF). Between 14

DPF and 21 DPF embryo development was monitored by observing the size and intensity

of eye pigments, shortly after which hatch occurred (24-25 DPF in 13°C water).

Mortality increased between 14-21 DPF, and hatch was delayed 1-3 d, in various eggs

spawned from xenoestrogen exposed mothers (personal observation). Survival of progeny

from males was greater than females, though survival did not correlate with *in ovo*

toxicant exposure (Table 2.3). No treatment dependent differences in female GSI or egg

production were observed. Crosses of *in ovo* exposed males and females also developed

and hatched successfully (data not shown). While these data do not represent a

comprehensive study of reproductive effects on population dynamics, they show that

treated fish were maturing normally.

Table 2.3 - Reproductive performance in trout exposed as embryos to DDE isomers[a]

Embryo treatment (P$_1$)		F$_1$ Survival (%)[b]	
Chemical	Dose (mg/kg)	P$_1$ Male (n)	P$_1$ Female (n)
Untreated	--	88 (2)	44[c] (2)
Oil (vehicle)	--	76 (1)	ND[d]
o,p'-DDE	10	72 (1)	71 (1)
	40	85 (2)	ND
	80	82 (2)	33 (1)
p,p'-DDE	10	82 (2)	35 (3)
	40	77 (1)	24[c] (2)
	80	69 (1)	47 (1)
DDE mix[e]	10	87 (2)	54 (1)
	40	94 (1)	10 (2)
	80	78 (2)	ND

[a] Trout injected with chemicals as embryos were reared to maturity. Treated fish were crossed with untreated, stock fish and embryo survival was monitored. Gonads were examined grossly and were normal in all fish.
[b] Survival is represented as mean of offspring that survived until one week after hatch. Small samples prevented meaningful standard error calculations
[c] Denotes eggs from one female failed to hatch after successful fertilization
[d] ND - not determined
[e] DDE mix was 5.4:1 p,p'-DDE:o,p'-DDE

Discussion

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

Microinjection of eggs with vehicle alone had two unintended consequences.
Injection of eggs decreased time to hatch in both trout and salmon (personal observation),
which may increase background mortality rates. Oil injected control mortality in our
experiments were not higher than historical levels or comparable uninjected controls
except in EXP 2 and EXP 3. In those experiments, increases in mortality may have been
due to injections altering development or simply to genetic variations in brood fish (EXP
2) or genetically manipulated offspring (EXP 3). Injection with oil or chemicals also
caused a decrease in androgen production by males in EXP 3. Male gonads incubated six
months after exposure produced approximately 50% less testosterone and 11-
ketotestosterone than uninjected fish. No differences were seen between treatment groups
or oil injected controls, suggesting an association with injection itself. Thin layer
chromatography on menhaden oil revealed few components other than triacylglycerides
and appeared identical in composition to a canola oil control. Changes in steroid

production could be a result of an unidentified contaminant or steroid in the oil vehicle, altered development due to injection, or a stress response due to egg manipulation. Topical application of toxicants in DMSO resulted in approximately 30% incorporation into eggs (unpublished data) and while absorption was lower than injection, this may be a less stressful dosing technique (*43*).

There are no obvious explanations why sex ratios were affected in EXP 1 but not in other experiments. Fish came from different spawns and light cycle (natural vs. 12 h light:dark) and water temperature (12 - 14°C range) may have varied slightly, but those conditions varied between unaffected treatments (EXP 2-4) as well. Mortality rates may have been higher in females than males in EXP 1, but similar results should have been noted in EXP 2. Unfortunately, attempts to compare mortality in all male (EXP 3) and all female (EXP 4) treatments were confounded by high background mortality rates (approx. 50% in males, 80-90% in females) prior to experimental treatment, due to genetic manipulation of fish.

Histological observation of male and female germ cells in one of two fish examined from 160 mg/kg *o,p'*-DDE exposure in EXP 1 is compelling evidence that trout are susceptible to xenoestrogen treatment. Observations of unusual gonads in rainbow trout exposed to OH-PCBs (*44*) and 17β-estradiol (*30*) support our assumption that gonadal abnormalities were due to chemical treatment. We examined gonads from hundreds of fish and while morphological variability was common, we never observed other gonads with both male and female germ cells, suggesting that our observations were not due to random genetic variation. Because 160 mg/kg *o,p'*-DDE treatment was acutely toxic to embryos and is unlikely to be observed in natural waters, we did not repeat studies using

that dose. It is possible that a threshold for gonadal abnormalities is above all other doses used in our experiments.

It should be considered that injected chemicals were sequestered away from target tissues or somehow metabolized and cleared from fish. We did not include estrogen treatments as positive controls because trout embryos have the ability to metabolize and excrete exogenously administered estrogens within 24 h (45), rendering short term estrogen exposure ineffective at feminizing trout. Evidence from radiochemical studies and residue analyses confirmed that DDEs and chlordecone were present in fish throughout the period of sexual differentiation. No residue analysis of octylphenol was performed, but its lipophilic nature suggests a long half-life in fish (46). Additionally, injection occurred 21 days after fertilization, subsequent to organogenesis and the most sensitive stages of embryo development. Chemicals could potentially alter sexual development prior to this life stage, but sexual differentiation in salmonids is not complete prior to exposure periods tested (24,29,30).

Normal gonadal steroid production *in vitro* suggested that normal sexual development was occurring in immature fish. Fitzpatrick et al. (30) observed decreased *in vitro* steroid production in juvenile trout fed 17β-estradiol and methyltestosterone, suggesting that abnormal development of gonads in our studies would have produced anomalous steroid profiles. Sex steroid production in fish increases during maturation and, similar to humans, exposure to hormones during development can result in sexual abnormalities that are not apparent until maturation (24). Fish appeared to mature normally and no sexual or reproductive abnormalities were observed in mature males or

females. Gametes from a subset of males and females from EXP 2 crossed with either control fish or other treated fish resulted in viable offspring. Genetic variability plays a large role in reproductive success, as evidenced by the failure of all crosses with one of two female stock fish. Consequently, quantitative measurements of egg viability in treated females were impossible. Nevertheless, the majority of evidence suggests that all fish treated as embryos were maturing normally.

Significant abnormalities in sexual development were only seen in rainbow trout in one experiment with o,p'-DDE, an estrogenic metabolite of DDT. Embryonic exposure of trout to p,p'-DDE, DDE mixtures, chlordecone, and octylphenol failed to alter any of the parameters of sexual development tested. A single experiment with o,p'-DDE in chinook salmon, a species sensitive to complete feminization, also failed to cause developmental abnormalities. Our conclusions are consistent with a recent report that p,p'-DDE failed to alter sex differentiation in a marine turtle (47). With the exception of heavily contaminated sites (21, 48), environmental residues are unlikely to approach levels that would expose fish to doses used in this study. Such contamination, if present, would probably alter fish populations due to lethality prior to exerting more subtle endocrine disrupting effects. While salmonid fishes are advantageous models for biomedical research, long life spans and low sensitivity to environment levels of EACs point to aquarium fish as better models for multigenerational endocrine toxicity studies. We cannot dismiss the potential hazards of complex mixtures or variations due to species differences, but we can conclude that average environmental levels of the chemicals tested are unlikely to have profound effects on endocrine development in salmonids.

Acknowledgments

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Chapter 3

4-Hydroxy-2',4',6'-Trichlorobiphenyl and 4-Hydroxy-2',3',4',5'-Tetrachlorobiphenyl

Are Estrogenic in Rainbow Trout

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Abstract

Many natural and synthetic xenobiotics are known to interact with endocrine systems of animals. Various hydroxylated metabolites of persistent polychlorinated biphenyl contaminants (OH-PCBs) have been shown to have agonist or antagonist interactions with estrogen receptors. In this study, 4-hydroxy-2',4',6'-trichlorobiphenyl (OH-PCB 30) and 4-hydroxy-2',3',4',5'-tetrachlorobiphenyl (OH-PCB 61), and the natural estrogens 17β-estradiol (E_2) and estrone (E_1), were incorporated into diet and fed to juvenile rainbow trout. The production of vitellogenin (Vg), an egg yolk protein precursor in oviparous animals, was used as a marker of hepatic estrogen receptor binding. All compounds induced plasma Vg in a dose-dependent manner, with maximal levels of approximately 5 mg Vg/ml plasma induced by E_2, E_1, and OH-PCB 30. Maximum plasma Vg of 48 μg/ml in the highest dose (50 mg/kg) of OH-PCB 61 was approximately 100-fold lower than natural estrogens and OH-PCB 30. At doses that induced sub-maximal Vg, E_1 was 2 to 3-fold less potent, and OH-PCB potencies were up to 500 fold less potent, than E_2. Predictions from previous receptor binding studies underestimated the maximum estrogenic response of OH-PCB 30 in trout, which was achieved with a dose 10 times higher than E_2. Differences in plasma Vg induction by OH-PCB 30 and OH-PCB 61 support *in vitro* predictions that the degree and position of chlorination are important for estrogen receptor activation. Cotreatment with mixtures of E_2 and E_1 or OH-PCB 30 and OH-PCB 61 resulted in Vg levels predicted by an additive mechanism of hepatic estrogen receptor binding. Age of fish did not appear to influence

responsiveness to estrogenic mixtures. Sex differences in Vg synthesis were apparent at weakly estrogenic doses, but were not evident at maximal Vg inducing doses.

Introduction

Polychlorinated biphenyls (PCB) are persistent organic pollutants which have contributed to historical declines of fish and wildlife populations. While the manufacture and use of PCBs in the United States was banned two decades ago, residual contamination continues, particularly in the Great Lakes region (*1,2*). PCBs bioaccumulate in animals and residues are prevalent in humans, fish, and wildlife (*3-7*). PCBs have been shown to alter mixed function oxidase systems (*8*), potentially affecting steroid metabolism (*9*). Reproductive, thyroid and immune dysfunction from PCB exposure have also been observed (*1,10-12*), which are thought to be due to receptor binding of PCBs as structural analogs of dioxin, thyroxin, and estrogens (*13,14*). PCBs have been implicated in learning and memory deficits in children exposed *in utero* and through contaminated breast milk (*15*), but adverse health effects in adults have not been demonstrated conclusively (*16*). Highly chlorinated PCBs tend to be the most persistent congeners in the environment, but OH-PCB metabolites have been shown to persist in animals and the bioavailability of lower chlorinated PCBs may be greater than highly chlorinated congeners (*3,8*).

Hydroxylated metabolites of PCBs (OH-PCBs) are able to interact with estrogen receptors (ER) *in vitro*, which can result in either estrogenic or antiestrogenic activity (*17-19*). The estrogenicity of OH-PCBs has been established *in vivo* as well, resulting in alterations in sex differentiation in turtles (*20*) and increased uterine weight in mice (*21*). Unusual results seen with mixtures of OH-PCBs and other xenoestrogens *in vivo* have suggested that chemicals may act synergistically to induce estrogenic responses (*20,22*).

A recent report by Ramamoorthy et al. (21), however, documented additive estrogenic responses of mixtures of OH-PCBs *in vitro* and in mice.

The estrogenic activity of OH-PCBs *in vivo*, particularly mixtures, have not been well characterized. The studies described here were designed to determine whether two OH-PCBs are estrogenic in rainbow trout exposed *in vivo*. 4-Hydroxy-2',4',6'-trichloro-biphenyl (OH-PCB 30) and 4-hydroxy-2',3',4',5'-tetrachlorobiphenyl (OH-PCB 61) are estrogenic *in vitro* (17,21) and in turtles (20), but their activity in fish has not been examined. Induction of vitellogenin (Vg), a phospholipoglycoprotein egg yolk precursor produced in oviparous animals, has been used as a marker of hepatic estrogen receptor binding in fish (22-24). Vg is not present in appreciable amounts in immature fish, so induction by estrogenic chemicals is easily detected. We fed juvenile rainbow trout, *Oncorhynchus mykiss*, varying doses of the natural estrogens 17β-estradiol (E$_2$) and estrone (E$_1$) and compared their ability to induce vitellogenin synthesis with that of various PCBs, OH-PCB 30 and OH-PCB 61. Trout were also fed mixtures of estrogens and OH-PCBs to determine whether Vg levels increased in a manner suggestive of additive ER binding.

Materials and Methods

Chemicals

The following PCBs and OH-PCBs, 100% pure, were purchased from AccuStandard (New Haven, CT): 4-hydroxy-2',4',6'-trichlorobiphenyl (OH-PCB 30); 4-hydroxy-2',3',4',5'-tetrachlorobiphenyl (OH-PCB 61); 2,4,6-trichlorobiphenyl (PCB 30); 2,3,4,5-tetrachlorobiphenyl (PCB 61); 2,4,4',6-tetrachlorobiphenyl (PCB 75); and, 2,3,4,4',5-pentachlorobiphenyl (PCB 114). The natural estrogens 17β-estradiol (E_2) (minimum 98% pure) and estrone (E_1) (>99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade or better and purchased from Sigma unless noted.

Experimental Design

Immature rainbow trout were reared and treated at the Marine/Freshwater Biomedical Sciences Center at Oregon State University, with IACUC approval. Fish were maintained in 3' diameter tanks with continuously running, 12-14°C well water, with a density of eight fish per tank. Estrogens, OH-PCBs and PCBs were dissolved in DMSO (0.1% v/w in diet) and mixed into a semi-purified trout diet, OTD (*25*). Trout were fed a growth ration of 4% diet per day (w/w) for 7 d and euthanized on day 8, unless noted, in four separate exposure periods. **Experiment 1 (EXP 1):** An initial experiment to determine doses of natural estrogens and OH-PCBs that induce vitellogenin in a mixed sex population of trout. Fish were sexually immature, approximately 12 months old. Eight fish per treatment were fed 0.05, 0.5, 5, 12.5 mg/kg E_2 or E_1, or 0.01, 0.1, 1, 10, 25, 50

mg/kg OH-PCB 30 or OH-PCB 61. **Experiment 2 (EXP 2):** PCB congeners, lacking a hydroxyl group, were tested for estrogenic potential *in vivo*. Eighteen month old juveniles of both sexes (n=6), were fed for 5 d and killed on day 7, after fasting for one day. Diets contained 50 mg/kg of PCB 30, PCB 61, PCB 75, or PCB 114. Plasma was assayed for Vg. **Experiment 3 (EXP 3):** The estrogenicity of mixtures of natural estrogens and OH-PCBs was explored. Genetically male trout (n=5) were produced by fertilizing stock eggs from a single female with sperm containing only Y chromosomes (milt provided by Gary Thorgaard, Washington State University). Approximately 2 year old fish were fed mixtures designed to include a dose of chemical near the threshold for Vg induction, determined from EXP 1, with an additional chemical increasing in dose from low to average Vg inducing capability. The natural estrogens E_2 and E_1, which occur physiologically as mixtures, were fed in diet containing 0.1 mg/kg E_2 plus 0, 0.05, 0.5, 2, or 5 mg/kg E_1. E_1 was also fed alone at 0.5 mg/kg. Mixtures of OH-PCBs included 10 mg/kg OH-PCB 30 plus 0, 1, or 10 mg/kg OH-PCB 61 and constant 10 mg/kg OH-PCB 61 plus 0, 0.1, or 1 mg/kg OH-PCB 30. **Experiment 4 (EXP 4):** In order to determine whether the age of fish is important in the activity of estrogenic mixtures, selected mixtures were fed to 6 month old trout fry (3 - 10 g). Twelve fish per treatment were fed diet containing DMSO (0.1%), 0.1 mg/kg E_2, 0.5 mg/kg E_1, 10 mg/kg OH-PCB 30, 10 mg/kg OH-PCB 61, 0.1 mg/kg E_2 + 0.5 mg/kg E_1 or 10 mg/kg OH-PCB 30 + 10 mg/kg OH-PCB 61. Liver Vg was determined, rather than blood, because of small circulating blood volume. Sex ratios varied between treatments, so only livers from four males per treatment were analyzed. Individual liver Vg levels were adjusted to total liver protein content (*26*).

Vitellogenin analysis

Vitellogenin concentrations were determined in blood for EXP 1, EXP 2, and EXP 3, and liver in EXP 4. Fish were collected and deeply anesthetized in tricainemethanesulfonate (MS222 50 mg/L), blood was drawn from the caudal vein in heparinized syringes or vacutainers, and fish were killed by severing the spinal cord. A mixture of aprotinin (150 kallikrein inhibiting units) and EDTA (1mM in 0.02% Tris, pH 8) was added to whole blood to prevent proteolysis of Vg. Blood was kept on ice until centrifugation at 3000 g for 10 min. Livers were removed (EXP 4 only), snap frozen in liquid nitrogen and held at -80°C until homogenization in 4 volumes (v/w) of buffer (0.1 M tris-acetate, 0.1 M KCl, 1 mM EDTA, 0.1 mM butylated hydroxytoluene (BHT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4). A competitive enzyme-linked immunosorbent assay (ELISA) for Vg was modified from Donohoe and Curtis (*23*) and described elsewhere (*27*). Purified rainbow trout vitellogenin and IgG raised against chum salmon Vg were obtained from D.R. Buhler, Oregon State University (antibody originally isolated by A. Hara, Hokkaido University). Cross reactivity of anti-salmon IgG was confirmed by visualization of proteins separated by SDS/PAGE and analyzed by western blotting. There was no evidence of non-specific antibody binding to either microtiter plates or proteins. Intraassay variability was less than 10% and interassay variability less than 15%. Vg levels are presented as mean ± SEM for each treatment group. Mean Vg levels were compared statistically by ANOVA, followed by Fisher's PLSD between treatments and Student's t-tests between sexes within individual treatments.

Results

The hydroxylated PCBs investigated proved to be estrogenic in rainbow trout *in vivo*. One week of dietary exposure to natural estrogens or OH-PCBs was sufficient to induce vitellogenin production in immature trout. Plasma Vg increased in a dose-dependent manner with increasing doses of natural estrogens or OH-PCBs (Fig. 3.1). Vg reached a maximum level of approximately 5 mg/ml in plasma in E_2, E_1, and OH-PCB 30 treated fish. Increasing the dose of estrogens beyond 5 mg/kg did not increase plasma Vg (Fig. 3.1). The maximum plasma Vg induced by OH-PCB 61 was 48 ± 16 µg/ml, which was approximately 100 fold lower than E_2, E_1, or OH-PCB 30. Baseline plasma Vg from male and female pools was 1.2 ± 0.8 µg/ml, with a detection limit of approximately 62 - 125 ng/ml (depending on individual ELISA standard curves).

Male fish consistently produced less Vg than females, when fed weakly estrogenic doses. Statistical analyses were complicated by unequal sex ratios in many treatments, but males in 0.05 mg/kg E_2, 0.1 mg/kg E_1 and 25 mg/kg OH-PCB 61 doses had significantly lower plasma Vg than females ($p<0.05$). At doses of chemicals that induced plasma Vg to mg/ml levels, which were approaching the maximal observed Vg of 5 mg/ml, males and females produced equivalent amounts of protein. Female controls usually had basal Vg concentrations above detection limits, while Vg was undetectable in males in the absence of estrogenic treatments.

PCBs lacking hydroxylation in the *para* position did not induce Vg production. Neither PCBs lacking substitution in the *para* position (PCBs 30 and 61) nor

Figure 3.1- Plasma vitellogenin (Vg) in twelve month old mixed sex trout after dietary exposure to estrogens and OH-PCBs. Values represent mean ± SEM (n=5-8). *indicates significantly greater than control diets (1.2 ± .8 μg Vg/ml); p < 0.05. (a) E_2 (solid bars) and E_1(open bars). (b) OH-PCB 30 (solid bars) and OH-PCB 61 (open bars). n.b. females produced more Vg than males at sub-maximal Vg inducing doses, so sex ratio influenced statistical analyses.
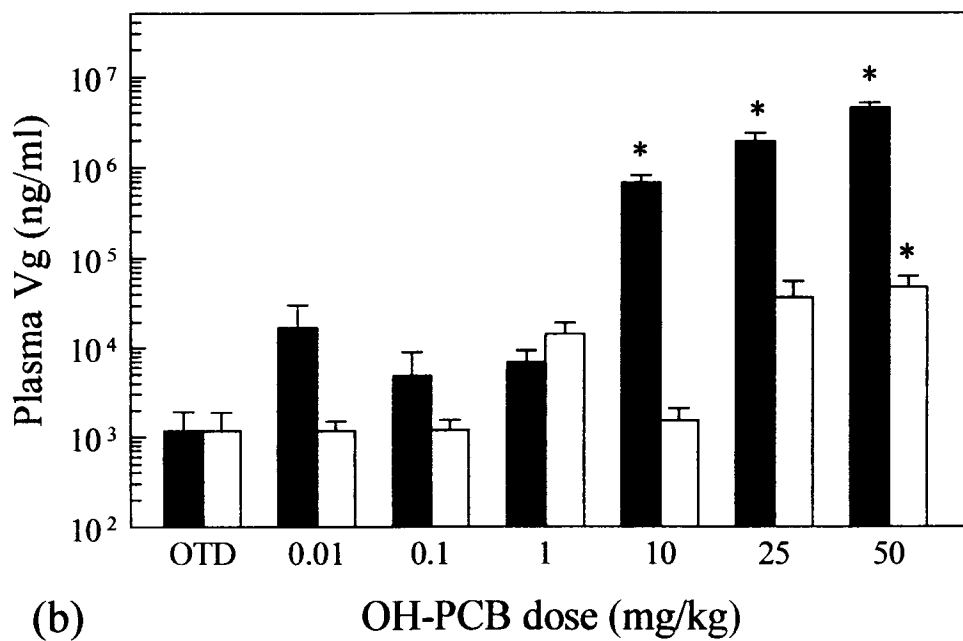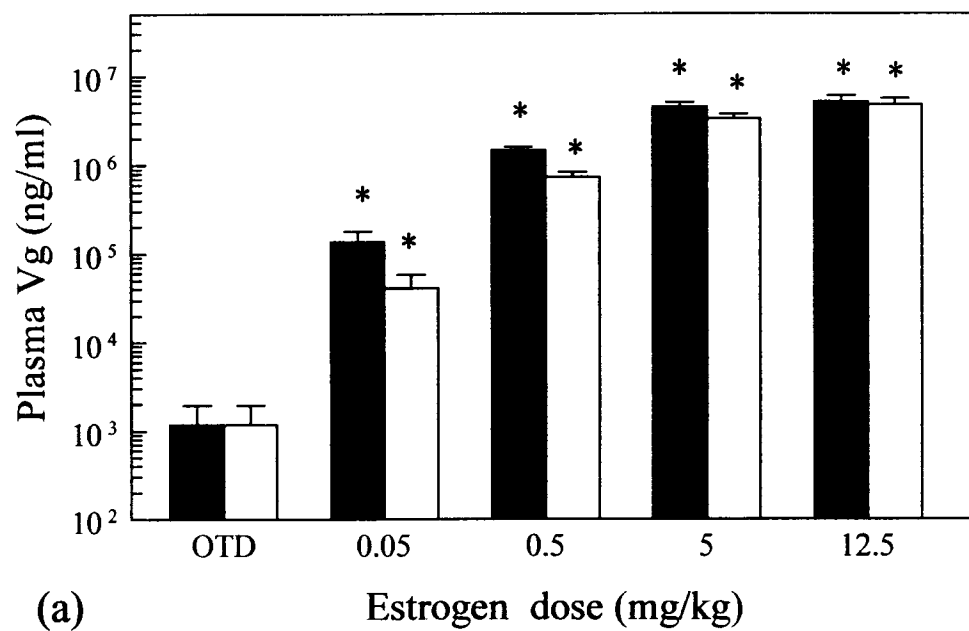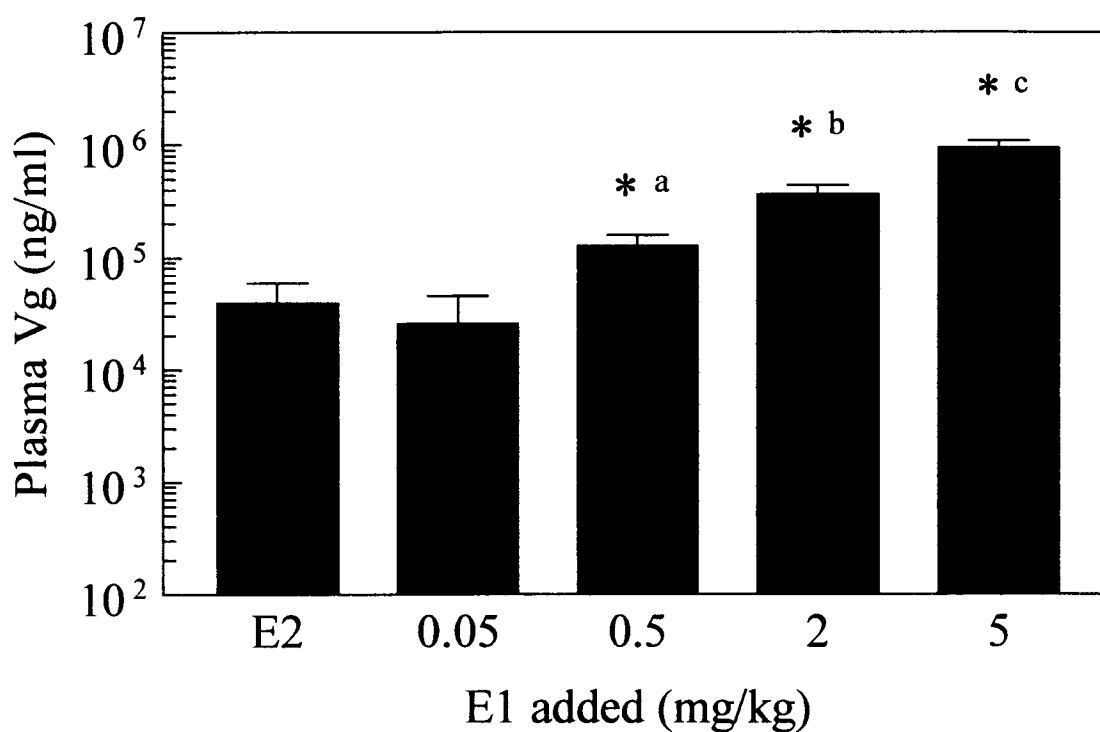
Figure 3.1

containing a Cl substitution (PCBs 75 and 114) in place of the OH group induced Vg (Table 3.1). The data suggest that non-hydroxylated PCBs were unable to activate estrogen receptors.

Fish from a population of genetic males were responsive to Vg induction by mixtures of estrogens and OH-PCBs (Figs. 3.2 and 3.3). Vg was not detected in plasma of untreated fish (detection limit of 62 ng/ml). Mixtures of chemicals induced Vg production in a manner suggestive of additive estrogen receptor activation. Doses of E_2 and OH-PCB 30 that were kept constant in respective mixtures induced Vg to 40 ± 20 and 57 ± 28 µg/ml, which were approximately 100 fold lower than saturated levels seen in EXP 1. The 10 mg/kg OH-PCB 61 dose appeared to be below the threshold for Vg induction in all male fish because only the OH-PCB 61 + 10 mg/kg OH-PCB 30 treatment induced Vg (Fig. 3.3). Unfortunately, the diet containing only OH-PCB 61 was ruined during preparation so there were no fish fed OH-PCB 61 alone. When Vg levels from males exposed to mixtures were compared to those in males from comparable mixed sex treatments (EXP 1), genetically manipulated males from EXP 3 consistently produced less Vg.

Observations in six month old fry were similar to those in older juveniles. Mixtures of estrogens and OH-PCBs induced liver Vg in males, while Vg was not detected in untreated fish. Small livers and proportionally small homogenization volumes required the dilution of samples 100 fold before assaying, resulting in a detection limit of 1.25 µg/ml liver homogenate. Vg was measurable in all treatments except controls, 0.1 mg/kg E_2, and 10 mg/kg OH-PCB 61. After Vg was adjusted to total liver protein content, mixtures were compared to individual chemical treatments and Vg levels could be

explained by classical, additive activation of estrogen receptors (Fig. 3.4). One puzzling

observation was that Vg in fish fed a mixture of E_2 and E_1 was significantly lower than fish

fed E_1 alone ($p<0.05$).

Figure 3.2- Vitellogenin induction in two year old male trout by mixtures of natural estrogens. Values are mean ± SEM from all male trout (n=5). Varying doses of E_1 were added to a constant dose of 0.1 mg/kg E_2. Vg was detected in only two of 10 control fish. * indicates significantly greater than E_2 diet alone (p<0.05). [a,b,c] indicate significantly different from each other (p<0.05).

Figure 3.3- Plasma vitellogenin induction in two year old male trout in response to mixtures of OH-PCBs. Values are mean ± SEM from all male trout (n=5). Vg was detected in only two of 10 control fish. (a) Constant dose of 10 mg/kg OH-PCB 30 with varying OH-PCB 61 doses. (b) Constant dose of 10 mg/kg OH-PCB 61 with varying OH-PCB 30 doses. [a]Value for 10 mg/kg OH-PCB 61 alone represents male fish from EXP1; no 10 mg/kg OH-PCB 61 data in all male fish was available, due to a problem with diet preparation.

(a)

(b)
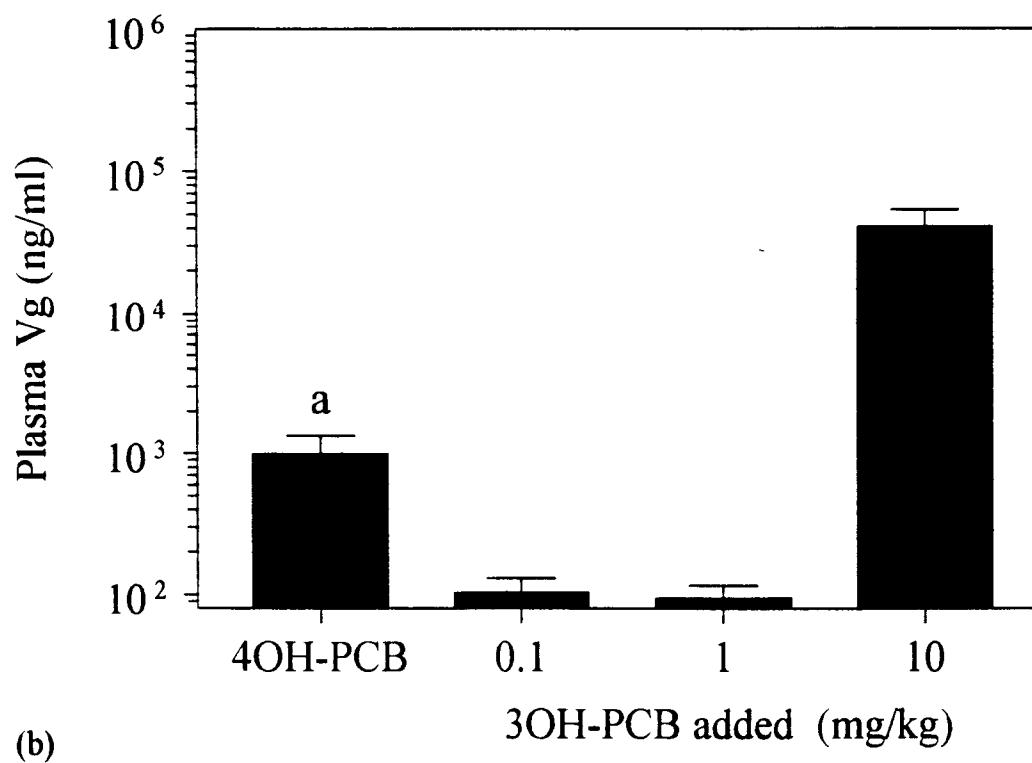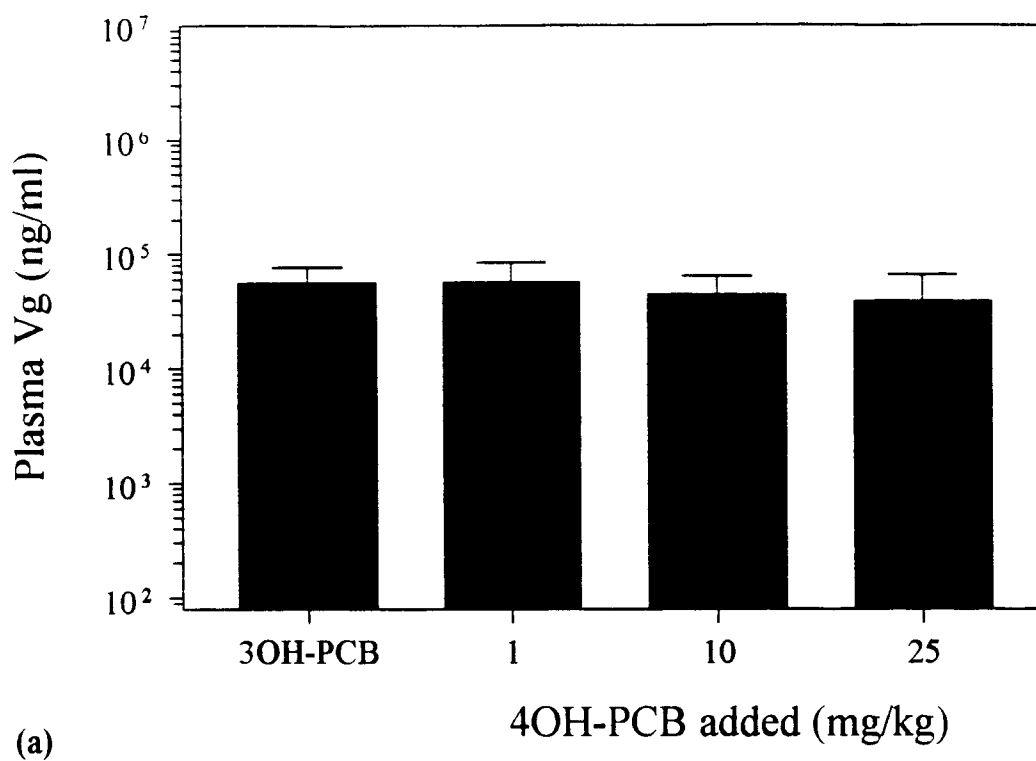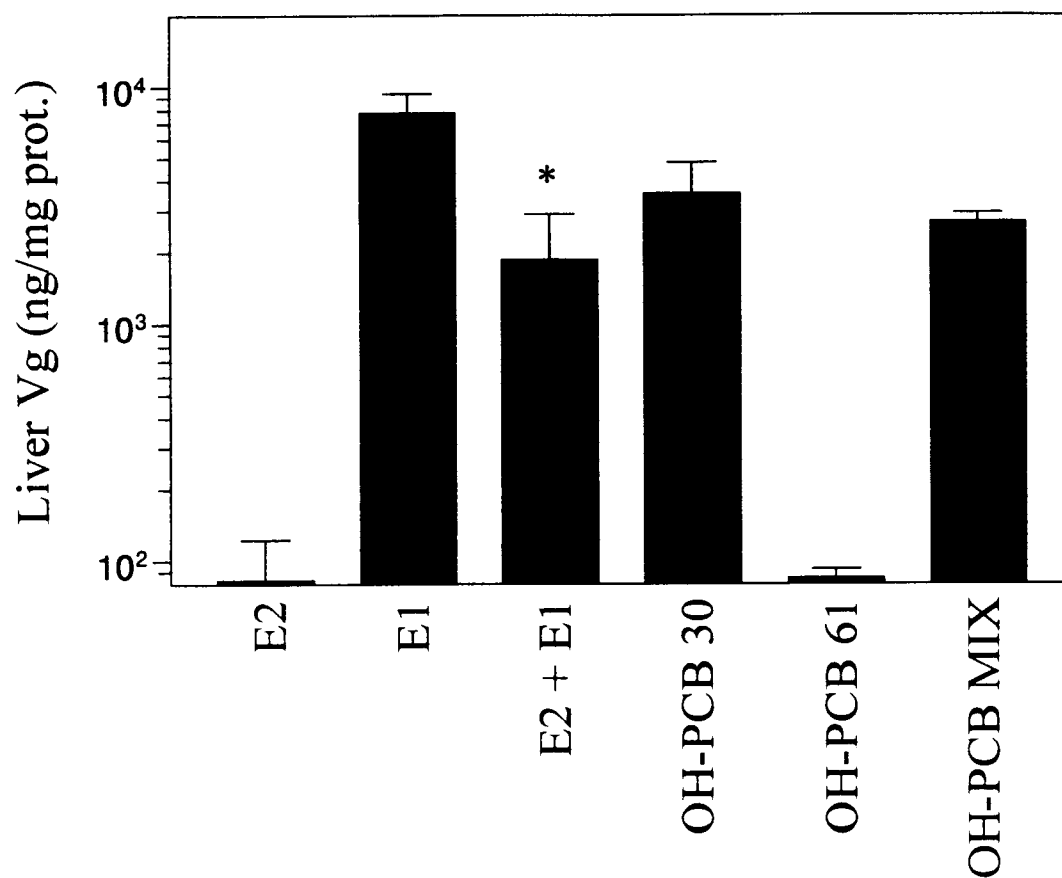
Figure 3.3

Figure 3.4- Liver vitellogenin in six month old fish fed mixtures of estrogens and OH-PCBs. Values are mean ± SEM from male trout (n=4). Vg was not detected in control fish. Doses were 0.1 mg/kg E_2, 0.5 mg/kg E_1, 10 mg/kg OH-PCB 30, and 10 mg/kg OH-PCB 61, or simple mixtures of E_2 and E_1 or OH-PCBs. *significantly lower than E_1 alone (p<0.05).

Table 3.1- Maximum estrogenicity of PCBs *in vivo*, relative to natural estrogens

	Max. Vg (mg/ml)[a]	Dose (mg/kg)[b]	Estrogenicity[c]
E_2	4.5	5	1
E_1	4.7	5	1
OH-PCB 30	4.5	50	0.1
OH-PCB 61	0.048	50	0.001
PCB 30	--[d]	50	0
PCB 61	--	50	0
PCB 75	--	50	0
PCB 114	--	50	0

[a]Maximum plasma vitellogenin (Vg) induced (i.e. "efficacy")
[b]Dose necessary for maximum Vg (i.e. "potency")
[c]Relative maximum estrogenicity compared to E_2 and E_1, determined as: efficacy^{-1} x potency^{-1} (n.b. relative estrogenicity varies for sub-maximal doses)
[d]PCBs lacking OH moiety did not induce Vg in males or females

Discussion

Dietary exposure to natural estrogens and OH-PCBs resulted in dose-dependent induction of plasma vitellogenin in immature trout. Fish were responsive to Vg induction by both OH-PCB 30 and OH-PCB 61, with the di-*ortho* substituted OH-PCB 30 having higher potency and 100-fold higher maximum Vg than the mono-*ortho* congener. Korach et al. (*17*) examined the ability of various OH-PCBs to bind to mouse uterine estrogen receptors *in vitro* and found that *ortho* substitution on the nonphenolic ring conferred the greatest ER binding. The ER binding activity of OH-PCB 61 was approximately 100-fold lower than E_2 in mice (*17*), which was similar to the difference in maximal Vg induction seen here. Surprisingly, maximal Vg induction by OH-PCB 30 was equivalent to the natural estrogens E_2 and E_1. Estrogenic potency of OH-PCB 30 was lower than E_2, with maximal Vg induced at 50 mg/kg, compared to 5 mg/kg for E_2. A 10 mg/kg dose of OH-PCB 30 resulted in plasma Vg of 680 µg/ml, which was 7 fold lower than corresponding E_2 and E_1 doses. The threshold for Vg induction was between 1-10 mg/kg OH-PCB 30, which was 20-200 times higher than 0.05 mg/kg E_2 or E_1 doses that significantly induced Vg. Receptor binding studies showed that ER affinity of OH-PCB 30 was approximately 40-fold lower than E_2 (*17*), which probably contributed to the lower potency of OH-PCB 30. The threshold for Vg induction for OH-PCB 61 was between 10-25 mg/kg.

It was interesting to find that lower Vg inducing potencies did not prevent E_1 and OH-PCB 30 from inducing Vg to the maximum seen with E_2. Both E_2 and E_1 induced Vg at all doses tested, but E_1 was 2-3 times less potent than E_2 at doses that induced sub-maximal Vg levels. The potency differences between E_2 and E_1 were similar to the 2-5

fold difference in potency in trout exposed to estrogens in water (28). E_2 is the major circulating hormone in fish (29) and fish may be more sensitive to E_2 than other natural estrogens, such as E_1. While *in vitro* predictions for ER binding by OH-PCBs correlated well with the potency differences we observed, maximal Vg induction *in vivo* did not appear to be simply a function of receptor affinity. Evidence from studies with the xenoestrogen octylphenol in mice and trout were similar to these data, showing that *in vivo* estrogenicity was higher than what was predicted from *in vitro* studies (28,30). Estrogenicity of E_1 and OH-PCBs *in vivo* was estimated based on dose, relative to E_2, that resulted in maximum plasma Vg (Table 3.1). The relative estrogenicity of OH-PCB 30 was thus estimated to be 10% of the natural estrogens. For OH-PCB 61, which did not induce Vg to maximal levels, the dose relative to E_2 was divided by 100, resulting in an estimate of 1000 fold lower *in vivo* estrogenicity (Table 3.1). The maximum Vg inducing treatment of OH-PCB 61 contained six males and only one female, so maximum induction by female fish may be higher than the data shown.

Estrogen receptors in trout liver are autoinduced by estrogen or xenoestrogen exposure (23,31), which probably contributed to the equivalent maximum Vg levels seen in OH-PCB 30 and estrogen treated fish. While we did not directly measure estrogen receptor expression, Vg production is thought to be mediated solely through estrogen receptor mediated transcription (24). Donohoe and Curtis (23) also showed that ER binding sites, as well as Vg, increased in immature rainbow trout injected *ip* with the xenoestrogens *o,p'*-DDT, *o,p'*-DDE, and chlordecone. The existence of a maximum level of plasma Vg observed in these experiments suggests that hepatic estrogen receptors were saturated, which is consistent with other studies in trout *in vitro* (31) and *in vivo* (28). We

have tested a variety of chemicals for *in vivo* estrogenicity and each chemical was found to have a particular maximum capacity for Vg induction, which were not all equal to natural estrogens (*32* and unpublished data). The fact that OH-PCB 30 and E_1 had lower potency but equivalent maximum Vg induction compared to E_2 suggests that the number of available estrogen receptors, in conjunction with chemical ER affinity, played a role in the regulation of estrogenic chemicals *in vivo*. PCBs and OH-PCBs bioaccumulate in fish tissues, which has been proposed to increase *in vivo* estrogenicity of octylphenol in trout through prolonged ER interactions (*28*).

Vitellogenin was not induced by PCBs lacking an OH moiety, which suggests that PCBs were not readily metabolized in trout and that the native PCBs tested do not bind to estrogen receptors. The phenol rings in OH-PCBs are thought to bind to the active site of ER (*17*), and our results confirm that hydroxylation is necessary to confer receptor activation. Chlorination on the nonphenolic rings of the four PCBs tested were identical to the OH-PCBs tested, with structural differences involving only the phenol ring. PCBs 30 and 61 simply lacked hydroxylation in the *para* position of the "phenol" ring, while PCBs 75 and 114 contained a Cl substitution in the *para* position. Lack of Vg induction by PCBs showed that *ortho* chlorination on the nonphenolic ring was not sufficient to activate liver ER, supporting the prediction that phenol rings mediate ER binding.

Additive estrogen receptor binding is a possible explanation of plasma Vg levels observed after dietary exposure to mixtures of natural estrogens and OH-PCBs. The constant doses of E_2, OH-PCB 30, and OH-PCB 61 were chosen to be below maximal, and close to threshold, Vg inducing doses. Increasing amounts of E_1 mixed with constant E_2 resulted in a dose-dependent increase in Vg, above the levels induced by E_2 alone. The

addition of low potency doses of OH-PCB 61 to constant OH-PCB 30 did not affect Vg

levels and only the 10 mg/kg OH-PCB 30 dose mixed with constant PCB 61 induced Vg

above background. Doses with low estrogenic potency were chosen for mixture studies,

allowing for large increases in Vg before plasma Vg levels were saturated. If chemical

mixtures enhanced liver ER binding in a synergistic manner, we should have observed

higher plasma Vg levels.

Sexes responded differently to weakly estrogenic doses, evidenced by higher

plasma Vg induced in females than males. Small initial sample sizes prevented robust

statistical power, but differences were significant in three doses. Differences between

sexes have been supported by subsequent work in our lab (27). In order to reduce

variability, we used fish from a population of all male trout, produced by crossing normal

females with sperm containing only Y chromosomes, for experiments involving mixtures

(EXP 3). Plasma Vg was consistently lower than levels in male fish from the initial study

(EXP 1), so we were unable to directly compare Vg levels in similarly treated fish from

the different studies. Trout from our facilities are partially inbred (Shasta strain) but

vitellogenin levels varied up to 100 fold between individual fish of the same sex, which

was consistent with other observations in salmonids (28,33). Trout from the genetic male

population were approximately one year older than stock fish (EXP 1), but age does not

seem to influence Vg production in male fish (27). It is not clear whether estrogen

responsiveness was inherently different in genetically manipulated fish or if Vg differences

were simply due to natural variation. Nevertheless, there was no evidence of synergism

between the natural or xenoestrogen mixtures tested in all male trout.

Many fish are sensitive to hormone treatment during a specific window of development. Sexual differentiation of rainbow trout and other salmonids can be manipulated by hormone treatment, resulting in partial or complete sex inversion (*34-36*). We hypothesized that estrogen receptor regulation may be different in trout during the period of greatest sensitivity to steroid hormones. To test this hypothesis, we treated six month old fish with simple mixtures of OH-PCBs and natural estrogens. Sexual differentiation had likely occurred by six months, but trout cannot be easily sexed before that time. Liver vitellogenin levels were estimated and results were similar to those in older fish. E_2, E_1, and OH-PCB 30 induced Vg at the doses tested, while data from chemical mixtures suggested additive (or competitive) binding of estrogen receptors. The only unusual result was that treatment with a mixture of E_2 and E_1 resulted in significantly lower liver Vg than E_1 alone (E_2 dose was below the response threshold). One explanation for this observation could be competition for ER binding sites. In older fish, E_2 was 2-3 times more potent than E_1, while the dose of E_1 in this study was 5 times greater than E_2. E_2 may have bound to a percentage of available receptors without inducing Vg gene transcription, but there is no evidence in the literature or within our data to suggest this was the case. We did not perform a comprehensive investigation of the effects of estrogen mixtures during trout sexual development, a period which may be most sensitive to xenoestrogens, but our results were consistent with simple models of estrogen receptor interaction with mixtures of ligands.

One week of dietary exposure to natural estrogens and OH-PCBs was sufficient to induce Vg in immature rainbow trout. We did not determine the residence time of Vg in plasma, but it would be interesting to see if Vg clearance time is similar in estrogen and

OH-PCB treated fish. OH-PCBs may persist in trout after short term exposures, which could result in prolonged ER stimulation and elevated plasma Vg levels compared to treatment with estrogens, which are readily metabolized. Our data in fish confirm earlier reports of the estrogenicity of OH-PCB 30 and OH-PCB 61 in other animals. These results suggest that fish may be sensitive to other estrogenic metabolites of PCBs and that persistent xenoestrogens may have higher relative potency *in vivo* than predicted from *in vitro* studies. There was no evidence to suggest synergism of either natural estrogens or OH-PCBs with respect to estrogen receptor dependent vitellogenin induction.

Acknowledgments

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Chapter 4

Tamoxifen Antagonizes Changes in Cytochromes P450 and Vitellogenin Mediated

by 17β-Estradiol in Rainbow Trout

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Abstract

Many environmental chemicals have been found to interact with endocrine systems of animals. The expression and activity of enzymes active in steroid and xenobiotic metabolism, such as cytochromes P450 (CYP), are potential endpoints for endocrine disruption. Natural estrogens are known to decrease CYP expression in fish, but it is not known whether estrogen receptors are involved. Tamoxifen, a tissue-specific estrogen receptor agonist, 17β-estradiol (E_2), and mixtures of the two were fed to immature rainbow trout, *Oncorhynchus mykiss*, in order to investigate mechanisms of endocrine modulation. Vitellogenin (Vg) induction was used as a biomarker of estrogen receptor activation. E_2 induced plasma vitellogenin to 1.4, 5.6, and 7 mg/ml (0.5, 2.5, and 7.5 mg/kg in diet, respectively), while concomitantly reducing total liver CYP, in a dose dependent manner, to 37% that of control fish. When fed tamoxifen alone (1, 10, and 100 mg/kg), vitellogenin in plasma ranged from 190 - 650 μg/ml, while total liver CYP did not change. Tamoxifen cotreatment with 2.5 mg/kg E_2 blocked the downregulation of CYP and reduced plasma vitellogenin to levels comparable to fish fed tamoxifen alone. Tamoxifen was unable to completely block Vg induction or CYP downregulation at the highest (7.5 mg/kg) E_2 dose. Sex differences were observed, as females were more susceptible to CYP modulation, and more sensitive to Vg induction, than males. Lauric acid hydroxylation (LA-OH), used to estimate CYP activity, was not affected by low doses of E_2 or tamoxifen, but 100 mg/kg tamoxifen and mixtures of 2.5 mg/kg E_2 plus 100 or 250 mg/kg tamoxifen significantly increased activity. Our results show that

tamoxifen antagonized E_2 mediated effects in trout. The data suggest that tamoxifen interacted with the trout estrogen receptor as a partial agonist and that the downregulation of liver CYP by E_2 is mediated, in part, by estrogen receptors.

Introduction

Endocrine systems of animals are sensitive to modulation by xenobiotics. Wildlife populations, particularly aquatic species, have been affected by endocrine active chemicals (EACs) (*1,2*) and numerous chemicals have been identified that interact with hormone receptors. A variety of studies have suggested that human health may be impaired by EACs, including increased rates of cancer and reproductive abnormalities; however, the data are inconclusive (*3*). Much of the recent work on EACs has focused on chemical interactions with estrogen receptors (ER) (*4,5*), but research is needed to understand the complexity of responses *in vivo* (*6*). The effects of EACs on hormone metabolizing enzymes, including CYP, have not been studied extensively.

Cytochrome P450 proteins are involved in Phase I metabolism of xenobiotics, as well as synthesis and metabolism of endogenous steroids (*7*). Steroid hormone production and function at target tissues are regulated by feedback loops involving the hypothalamo-pituitary axis, which may be altered by xenobiotic-induced changes in CYP-mediated steroid metabolism (*8,9*). The involvement of CYP in sexual development has been documented, particularly the inhibition of aromatase (CYP19) (*10*), which is a potential site of toxicant impact. Determining the regulation and consequences of CYP modulation in fish will help to assess the risk of EAC exposure in natural aquatic populations. Insight into the evolution, structure and function of human CYP has been gained from the characterization of various rainbow trout CYP (*11*).

Tamoxifen (TAM) is a pharmacological agent used in the treatment of estrogen-dependent breast cancer and it is being proposed as a chemopreventive agent. Originally

designed to be antiestrogenic, the estrogenic activity of tamoxifen is regulated by transcription factors present in target tissues, not solely by interaction with estrogen receptors (*12*). Tamoxifen blocks estrogen-mediated growth in breast tissue but enhances estrogen receptor-mediated responses in the uterus and endometrium. This example illustrates the difficulty of predicting the *in vivo* activity of estrogens or xenoestrogens based only on their affinity for estrogen receptors. There are no reports of the estrogenicity of tamoxifen in trout or its effect on fish CYP. Tamoxifen has the potential for use as an antiestrogen in trout and as a model chemical for the study of tissue specific, or weakly agonistic, xenoestrogens.

Reports of lower CYP levels in adult female fish, compared to males, suggested that natural estrogens were involved in suppressing CYP (*13*). Previous studies with the natural estrogen 17β-estradiol (E$_2$) have demonstrated that trout CYP are susceptible to downregulation by estrogens (*14,15*), but the mechanisms of modulation are unknown. A variety of xenobiotics inhibit CYP in trout, often interacting directly with the enzymes (*16*). Here, we describe studies designed to determine if CYP modulation by E$_2$ in trout is antagonized by tamoxifen, which would suggest involvement of the estrogen receptor in CYP downregulation. The estrogenicity of tamoxifen and effects on liver CYP were also investigated. Vitellogenin (Vg), a phospholipoglycoprotein produced in response to estrogen receptor binding (*17*), was used as a biomarker of liver estrogen receptor activation (*18,19*).

Methods

Chemicals

Tamoxifen (TAM; [z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene;

minimum 99% pure) and 17β-estradiol (E_2) (minimum 98% pure) were obtained from

Sigma Chemical Co. (St. Louis). [^{14}C]-Lauric acid, biotinylated donkey anti-rabbit IgG

and streptavidin linked horseradish peroxidase were purchased from Amersham (Arlington

Heights, IL). All other chemicals were of reagent grade or better.

Trout exposure

Immature rainbow trout, 16 month old males and females, were reared at the core

facility of the Marine/Freshwater Biomedical Sciences Center at Oregon State University.

Fish from each treatment were maintained, with IACUC approval, in 3' diameter tanks

supplied with continuously running, 12°C well water. Trout were fed a maintenance

ration of 2.8% (w/w) Oregon Test Diet (OTD) (*20*) containing E_2, tamoxifen, or mixtures

of E_2 and TAM. In the first experiment, eighteen fish per treatment were fed the following

doses for four weeks: OTD; 0.5, 2.5, or 7.5 mg/kg E_2; 1, 10, or 100 mg/kg tamoxifen; or,

2.5 mg/kg E_2 mixed with 1, 10, 100, 250, or 500 mg/kg tamoxifen. After 1, 2, and 4

weeks, six fish from each treatment were deeply anesthetized in tricainemethanesulfonate

(MS222), blood was collected in heparinized syringes, livers were removed and snap

frozen in liquid nitrogen, and fish were killed by severing the spinal cord. Blood was kept

on ice (maximum 6 h) until centrifugation at 3000 g for 10 min to isolate plasma. Livers

and plasma were frozen at -80°C until use. In an additional experiment, twelve fish per

treatment were fed OTD alone or containing 7.5 mg/kg E_2, 250 mg/kg tamoxifen or 7.5 mg/kg E_2 + 250 mg/kg tamoxifen, for one week. Livers and blood were collected as described above.

Liver microsomes and LSI

The liver somatic index (LSI) was determined for each fish and represents the percentage of total body weight contributed by the liver. Microsomes were prepared from individual livers as previously described (*21*). Microsomes were washed and stored at -80°C after resuspension in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol. Total cytochrome P450 content was estimated from the CO-difference spectrum (*22*). Protein content was determined using the method of Lowry et al. (*23*).

Enzyme activity

The activity of lauric acid hydroxylase (LA-OH) in liver microsomes was estimated *in vitro* using the method described by Giera and van Lier (*24*). LA-OH activity has been characterized as a marker for trout CYP 2K1 (*25,26*). Incubation mixtures contained 0.2-0.4 mg protein, 100 μM [^{14}C]-lauric acid, 1 mM NADPH, 1 mM $MgCl_2$ and 5 mM KCl, in 50 mM Tris-HCl, pH 7.4. Reactions were run for 60 min at 30°C, throughout which time hydroxylation rates were linear.

Vitellogenin ELISA

A competitive enzyme-linked immunosorbent assay (ELISA) for Vg, modified

from Donohoe and Curtis (*19*) and described elsewhere (*27*), was used to determine

plasma vitellogenin levels. Plasma aliquots were thawed a single time and dilutions that

fell within the linear portion of the standard curve were used to estimate Vg. Rainbow

trout vitellogenin was purified for use as standards (*28*) and detected by cross-reactivity

with IgG raised against chum salmon Vg (provided by A. Hara, Hokkaido University).

Western blots confirmed that anti-salmon IgG recognized rainbow trout Vg. There was

no evidence of non-specific antibody binding to either microtiter plates or proteins.

Data analysis

Experiments were designed with the intention of gathering data from individual

fish and comparing the means from all fish in each treatment. However, vitellogenin levels

suggested possible sex differences, which was unexpected in immature fish. Because of

unequal sex ratios in some treatment groups, only livers from two males and two females

were analyzed for CYP endpoints in low dose treatments Final analyses include

vitellogenin data from all fish and CYP data from four fish per treatment, unless noted.

ANOVA and Student's t-test were used to compare results in different treatment groups

(Microsoft Excel v7.0 and StatView v4.5, Abacus Concepts, Berkeley). Covariance of

fish weights were compared to Vg levels by ANCOVA. Vitellogenin standards were set

to a four parameter model using DeltaSoft 3 software (Biometallics Inc., Princeton, NJ) in

conjunction with a Bio-Tek EL340 plate reader (Winooski, VT).

Results

Dietary exposure to both 17β-estradiol and tamoxifen resulted in vitellogenin

induction in a dose-dependent manner (Fig. 4.1). Plasma Vg levels in E_2 exposed fish

were consistently higher than tamoxifen throughout four weeks of feeding. After one

week of feeding, Vg reached 45% and 71% of TAM and E_2 levels achieved, respectively,

after four weeks of exposure (data not shown). Maximum plasma Vg induced by TAM

was 654 μg/ml, which was 11 fold lower than the maximum of 7 mg/ml induced by E_2.

Tamoxifen was much less potent than E_2 as well, with maximal induction at 100 mg/kg

significantly less than the lowest E_2 dose of 0.5 mg/kg ($p < 0.05$). Males fed 1 mg/kg TAM

seemed to have lower plasma Vg than females, but the lack of statistically significant

differences ($p > 0.05$) may have been due to small sample size. Fish fed OTD alone

averaged 148 ng Vg/ml plasma, which was slightly above the detection limit of the assay

(62.5 ng/ml). Mean fish weights varied between some treatment groups, but no

correlation between weight and Vg levels was observed (linear regression and ANCOVA

analyses). Induction of vitellogenin suggested that tamoxifen acted as a partial estrogen

receptor agonist in rainbow trout liver.

When fed varying doses of tamoxifen in combination with 2.5 mg/kg E_2, Vg

decreased compared to fish fed E_2 alone (Fig. 4.2). Tamoxifen was also able to block E_2-

dependent increased LSI and decreased total liver CYP (Table 4.1). E_2 feeding caused a

dose-dependent decrease in total liver CYP. Tamoxifen alone had few effects on liver

parameters. The LSI of 10 mg/kg TAM treated fish was significantly increased, but higher

and lower doses of tamoxifen had no effect on LSI. Total CYP levels were unchanged in
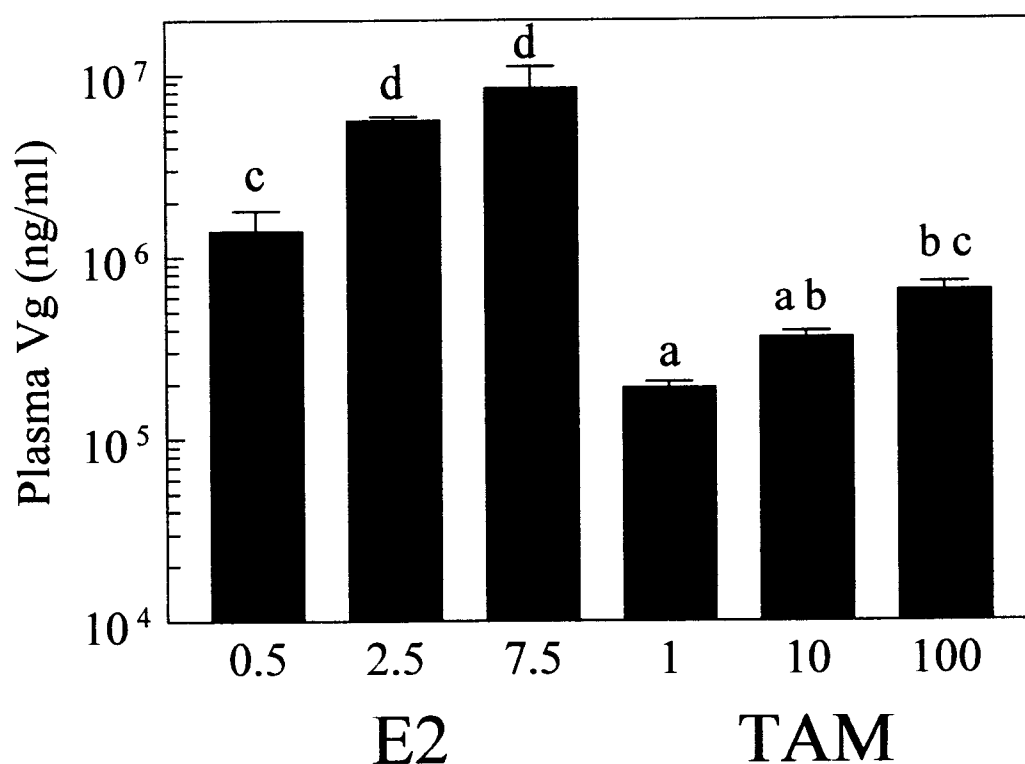
Figure 4.1- Plasma vitellogenin induction by 17β-Estradiol (E_2) and tamoxifen (TAM).
Error bars represent SEM of the mean (n=6). Background Vg in controls was 148 ng/ml.
[a,b,c,d] different letters indicate significantly different Vg (p<0.05).
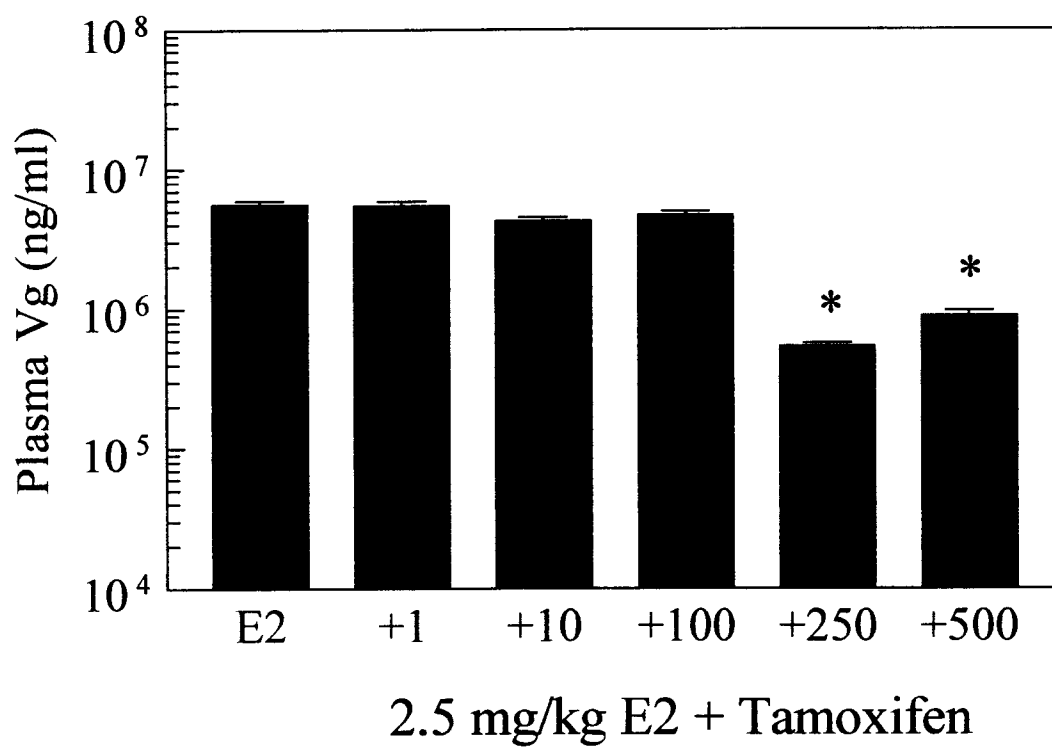
Figure 4.2- Plasma vitellogenin in trout fed mixtures of 17β-estradiol (E_2) and tamoxifen (TAM). Error bars represent SEM of the mean (n=6). * significantly lower than E_2 (p<0.01).

Table 4.1 - Modulation of liver CYP by 17β-estradiol and tamoxifen[a]

Treatment	LSI[b]	Total CYP (nmol/mg prot.)	LA-OH (nmol/mg prot./min)
OTD	0.70 ± .07	0.189 ± 0.03	0.095 ± 0.03
0.5 mg/kg E_2	1.24 ± 0.10*	0.168 ± 0.01	0.135 ± 0.04
2.5 mg/kg E_2	0.86 ± 0.10	0.129 ± 0.01	0.087 ± 0.01
7.5 mg/kg E_2	1.67 ± 0.16*	0.070 ± 0.01*	0.084 ± 0.03
1 mg/kg TAM	0.92 ± 0.02	0.187 ± 0.02	0.125 ± 0.01
10 mg/kg TAM	1.0 ± 0.10*	0.199 ± 0.01	0.121 ± 0.03
100 mg/kg TAM	0.74 ± 0.05	0.182 ± 0.04	0.181 ± 0.04[c]
2.5 mg/kg E_2 + 1 mg/kg TAM	1.18 ± 0.12*	0.124 ± 0.02	0.140 ± 0.02
2.5 mg/kg E_2 + 10 mg/kg TAM	1.34 ± 0.14*	0.142 ± 0.02	0.116 ± 0.05
2.5 mg/kg E_2 + 100 mg/kg TAM	0.94 ± 0.04	0.181 ± 0.03	0.253 ± 0.04*
2.5 mg/kg E_2 + 250 mg/kg TAM	0.92 ± 0.05	0.180 ± 0.03	0.165 ± 0.02*
2.5 mg/kg E_2 + 500 mg/kg TAM	0.69 ± 0.05	0.201 ± 0.05	0.132 ± 0.02

[a]Four week dietary exposure, values are means ± SEM (LSI n=8, CYP and LA-OH n=3 or 4)
[b]LSI=liver somatic index; percent of body weight contributed by liver
[c]Significantly greater than OTD controls when expressed as turnover (nmol/min/nmol CYP; p<0.05)
*Signifcantly different than OTD controls (p<0.05)

all tamoxifen treatments. Interestingly, lauric acid hydroxylase activity increased in fish fed 100 mg/kg tamoxifen for four weeks (Table 4.1). One fish in that treatment had low LA-OH activity, which was due to low total CYP content. The mean increase in LA-OH activity was not significant (p = .08) unless LA-OH activity was expressed as turnover rather than specific activity (p < .05). All other trends were identical whether LA-OH activity was reported as turnover or specific activity. Combinations of E_2 and tamoxifen induced LA-OH activity up to 2.7 fold higher than corresponding tamoxifen, E_2, or control fish (Table 4.1).

Results from fish fed high doses of E_2 and tamoxifen for one week were slightly different than those from lower dose treatments. Exposure to 7.5 mg/kg E_2 induced plasma vitellogenin to levels 63 fold higher than 250 mg/kg tamoxifen. Unlike lower E_2 dose combinations, tamoxifen could not fully counter the induction of Vg by 7.5 mg/kg E_2 (Fig. 4.3). Treatment with a mixture of 250 mg/kg TAM and 7.5 mg/kg E_2 resulted in plasma Vg levels significantly lower than E_2 alone, but also significantly higher than tamoxifen alone (Fig. 4.3). Males fed 250 mg/kg TAM for one week had plasma Vg levels of 20 ± 3 µg/ml, which were significantly less than female levels of 106 ± 20 µg/ml (p=.001). Males and females produced equivalent amounts of Vg when fed 7.5 mg/kg E_2 for one week.

Trends in liver CYP from high dose treatments were similar to Vg results. E_2 treatment caused a significant reduction in total CYP which, contrary to lower dose mixtures, was not antagonized by tamoxifen (Table 4.2). Unlike the four week treatments with lower tamoxifen doses, 250 mg/kg TAM in diet resulted in decreased total liver CYP (Table 4.2). When male fish were compared, there were no statistically significant
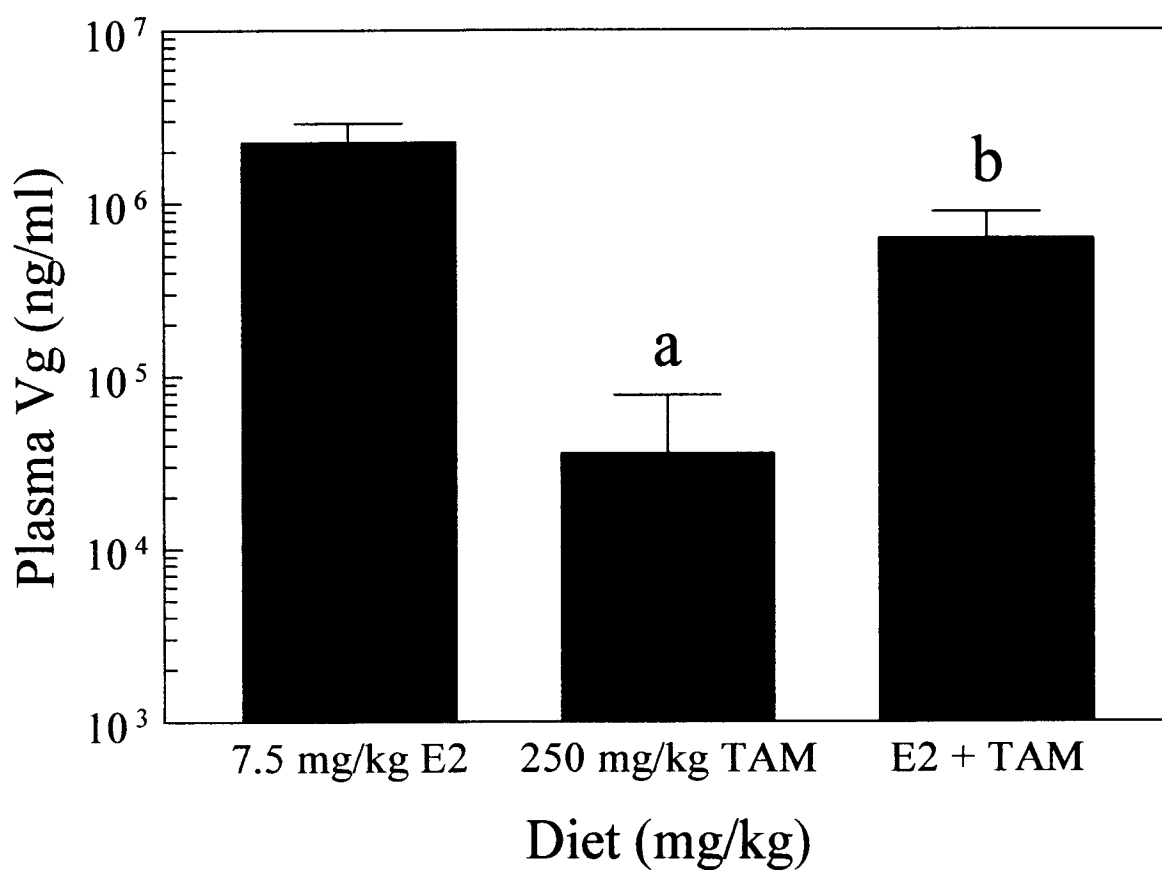
Figure 4.3- Plasma vitellogenin in trout fed high doses of 17β-estradiol (E_2), tamoxifen (TAM), or E_2 + TAM. Error bars represent SEM of the mean (n=12). [a]significantly lower than E_2 or mixture ($p<0.05$). [b]significantly lower than E_2 ($p<0.01$).

Table 4.2 - Modulation of liver CYP by high doses of 17β-estradiol and tamoxifen[a]

Treatment	LSI[b]	Total CYP (nmol/mg prot)		LA-OH (nmol/min/mg prot)
		Males	Females	
OTD	1.12 ± 0.05	0.251 ± 0.03	0.319 ± 0.02	0.293 ± 0.02
7.5 mg/kg E$_2$	1.22 ± 0.04	0.184 ± 0.03	0.211 ± 0.01*	0.143 ± 0.01*
250 mg/kg TAM	1.09 ± 0.05	0.198 ± 0.03	0.130 ± 0.01*[c]	0.074 ± 0.01*
7.5 mg/kg E$_2$ + 250 mg/kg TAM	1.15 ± 0.03	0.213 ± 0.02	0.205 ± 0.02*	0.120 ± 0.02*

[a]One week dietary exposure (n=8), values are means ± SEM. Sex differences were only evident for total CYP.
[b]LSI=liver somatic index
[c]Female CYP lower than males (p=0.03)
*significantly different than OTD controls (p<0.01)

reductions in CYP content, although levels in E_2 fed male fish seemed lower than controls (p=.07). Females were more susceptible to CYP downregulation, with significant reduction in all high dose treatments (Table 4.2). Effects in males and females appeared to be different in lower dose treatments, as well, but only two males and two females from each treatment were assayed for CYP so statistical comparisons of sexes were impossible. Lauric acid hydroxylation decreased at all high dose treatments (Table 4.2), but activity in males and females was similar.

LSI did not change after only one week of feeding, contrary to a greater than two fold increase in fish fed 7.5 mg/kg E_2 for four weeks. Liver hypertrophy is likely to be due to changes associated with Vg production (*19*), but increased liver size was not required to increase plasma Vg levels in one week, high dose exposures. Total CYP content also decreased in the absence of changes in LSI (Table 4.2).

Discussion

Tamoxifen appears to be a partial estrogen receptor agonist in rainbow trout liver. We did not determine directly whether tamoxifen interacts with trout estrogen receptors, but Flouriot et al. (29) showed that 4-OH-tamoxifen prevented ER mediated gene transcription in rainbow trout hepatocyte cultures. Vitellogenin production is thought to be mediated solely by estrogen receptor binding (17), suggesting that tamoxifen binds to hepatic ER to induce the Vg production seen *in vivo*. Nimrod and Benson (30) reported tamoxifen binding to catfish ER, but Vg was not induced *in vivo* (31). Our findings that tamoxifen had lower potency and efficacy than 17β-estradiol, with respect to Vg induction, are consistent with mammalian models that suggest tamoxifen is a partial estrogen receptor agonist (12).

Decreases in liver CYP by E_2, which were consistent with earlier studies (14,15), appear to be regulated by ER binding. At a dose of 2.5 mg/kg E_2, tamoxifen was able to suppress E_2 induction of vitellogenin, resulting in Vg levels comparable to fish fed tamoxifen alone. Cotreatment with 100 fold excess tamoxifen also prevented the increase in LSI and decrease in total CYP expression seen with E_2 alone. At a higher E_2 dose of 7.5 mg/kg, tamoxifen doses 33 times in excess of E_2 (by weight) reduced the estrogenic responses in liver, without completely restoring parameters to levels when fed tamoxifen alone. The affinity of tamoxifen for trout liver ER may, therefore, be close to 100-fold less than E_2, which would mean a greater excess of tamoxifen was needed to fully counteract the 7.5 mg/kg dose of E_2. A lower threshold for Vg induction than CYP modulation was observed, as evidenced by plasma Vg present in low dose tamoxifen and

0.5 mg/kg E_2 treated fish in the absence of changes in liver CYP content or activity. Estrogen receptor activation itself is therefore not sufficient to downregulate CYP, rather a threshold must be reached or an additional mechanism may be involved.

In the low dose study, E_2 alone had no effect on lauric acid hydroxylation but in combination with 100 and 250 mg/kg tamoxifen, activity was significantly increased compared to controls. When expressed as turnover (nmol LA-OH/min/nmol CYP), 100 mg/kg tamoxifen alone also significantly increased LA-OH. Estrogen receptor activation at those tamoxifen doses, predicted from Vg induction, was significantly less compared to E_2 alone. It does not appear that induction of LA-OH activity was due to high doses of tamoxifen alone, because 500 mg/kg TAM in combination with 2.5 mg/kg E_2 was not different from controls or E_2 alone. Additionally, 250 mg/kg TAM alone significantly decreased LA-OH activity in the one week feeding study. High doses of tamoxifen may inhibit CYP, specifically or non-specifically, by covalent binding of metabolites or reactive intermediates, which has been observed in human and rat liver microsomes (*32*). High dose tamoxifen treatment decreased total liver CYP in females, while Vg induction to sub-maximum levels suggested only partial ER activation. The high dose of 7.5 mg/kg E_2 decreased total CYP in both studies, while LA-OH activity was decreased after one week but not four. The different effects of high doses of tamoxifen and E_2, compared to lower dose mixtures and different time points, are puzzling. If LA-OH induction was mediated through the estrogen receptor in a classical manner, we would expect induction by E_2 alone. We are currently investigating the specific CYP involved in the differential induction and inhibition of LA-OH by different tamoxifen doses and mixtures, which may

help to explain the mechanisms. Nevertheless, the increased LA-OH activity seen with 100 mg/kg tamoxifen and mixtures is the first evidence of induction in trout.

Estrogen metabolism may play a role in receptor mediated responses. Depending on the position of hydroxyl groups, hydroxyestrone metabolites of 17β-estradiol can be highly (16α-hydroxy) or weakly (2α-hydroxy) estrogenic (33). The 2α-hydroxylation of 17β-estradiol is catalyzed primarily by CYP1A2 in humans (34) and by CYP2K1 (LMC2) in rainbow trout (35). In contrast, 16α-hydroxylation of 17β-estradiol is mediated by CYP2C11 in rat liver (36). In trout, the catalyst for 16α-hydroxylation of 17β-estradiol has not been identified. Tamoxifen, or xenoestrogens, may alter CYP profiles resulting in changes in the ratio of potent:weak estrogen metabolites (37). Different doses of E_2 and tamoxifen may alter CYP profiles such that more estrogenic metabolites are present after high dose treatments. Some changes in CYP profiles seen here may be estrogen receptor independent, particularly high doses of tamoxifen, but observations with sub-saturating E_2 doses suggest receptor involvement.

Estrogen receptors are autoinduced in trout and saturation of receptors (29) has been observed. Studies in our laboratory have shown that 7.5 mg/kg E_2 results in saturated plasma Vg levels, while 2.5 mg/kg was slightly below the saturation threshold (unpublished data). Those results were consistent with the present data, in which fish fed 2.5 mg/kg E_2 seemed to have lower plasma Vg than 7.5 mg/kg treatments, but differences were not statistically significant. The inability of tamoxifen to suppress the effects of E_2 at high doses may be due to different ER regulation at saturated levels. Similar to trout, studies in catfish showed that tamoxifen alone did not induce Vg but 100 fold excess tamoxifen only partially blocked Vg induction at highly estrogenic *ip* doses (31). Sex

differences in Vg induction in immature trout have recently been described for sub-saturating E_2 doses (27), which were also seen here at the 250 mg/kg TAM dose, but not at the high E_2 dose (with or without tamoxifen cotreatment). Sex differences were also observed with respect to CYP levels in high dose exposures of E_2 and tamoxifen, where total CYP content was significantly decreased only in females. The different responses of males and females may be due to differences in receptor number, however, receptor numbers were autoinduced to comparable levels in male and female salmon *in vitro* (38) and males and females produced equivalent amounts of Vg in this study at high estrogen doses. Our results suggest that there may be fundamental differences in estrogen receptor regulation between sexes, which is supported by paradoxical effects of tamoxifen on sexual development of alligators (39). Alternative mechanisms could also explain our observations, such as stabilization of receptor-ligand complexes or mRNAs at higher E_2 doses (40). A single estrogen receptor cDNA, with multiple mRNAs, has been identified in trout (41), but multiple ERs have been identified in mammals, suggesting the possibility of an additional trout receptor (42). A thorough investigation of estrogen receptor function in trout is needed to determine the mechanisms responsible for the differences in CYP content and Vg induction seen with low and high dose estrogen treatment.

Most xenoestrogens interact weakly with estrogen receptors *in vitro* (5,30) and we have observed that xenoestrogen potency and efficacy are generally lower than E_2 *in vivo* (unpublished data). In this study, receptor mediated downregulation of CYP by E_2 occurred only at doses approaching or exceeding levels sufficient to saturate plasma Vg, but nominal environmental levels of xenoestrogens are unlikely to be as potent. There are likely to be species differences in response to xenoestrogens and partial estrogen agonists,

as evidenced by different responses to tamoxifen and xenoestrogens in catfish (*30,31*)

compared to trout and other species. Ongoing investigations of the modulation of specific

trout CYP isoforms and lauric acid hydroxylation sites by E_2 and tamoxifen should help to

clarify estrogenic mechanisms.

Acknowledgments

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Chapter 5

Sex Specific Vitellogenin Production in Immature Rainbow Trout

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Abstract

Immature female rainbow trout, *Oncorhynchus mykiss*, ranging in age from six to eighteen months, consistently produced 3-4 fold higher amounts of vitellogenin than similarly aged males. The sex difference in vitellogenin induction is apparent after one week of dietary exposure to 0.05 mg/kg 17β-estradiol. Our results document the importance of considering the sex of juvenile fish when using vitellogenin production as a marker of xenoestrogen exposure.

Introduction

Many xenobiotics interact with hormone systems of animals, potentially leading to a phenomenon commonly called "endocrine disruption". Much attention has focused on steroid hormone systems and corresponding receptor proteins, particularly estrogens [1,2]. Chemicals are routinely classified as hormone agonists or antagonists based on their interaction with receptor proteins *in vitro*, but, Nagel et al. [3] showed that *in vitro* receptor binding did not accurately predict estrogenic potency in mice. Kavlock et al. [4] also reported the need for *in vivo* research to determine the mechanisms of endocrine modulating chemicals.

Vitellogenin (Vg), a phospholipoglycoprotein, is an egg yolk protein precursor produced in oviparous animals in response to estrogen receptor binding [5]. Mature females produce vitellogenin due to a rise in circulating estrogens, however, the presence of vitellogenin in liver and plasma of juvenile and male fish has been used as a biomarker of estrogen and xenoestrogen exposure [6,7]. Elevated plasma vitellogenin levels have been found in wild male fish in the United Kingdom, which are thought to be due to contaminants and pharmacological estrogens released in waste water effluent [8]. Understanding the role of exogenously administered estrogens will help to determine the effects of xenoestrogens in fish.

Previous studies have reported vitellogenin protein and mRNA levels in xenoestrogen-exposed juvenile rainbow trout, as pooled samples of males and females [7,9]. Immature salmonids lack secondary sex characteristics and they have low levels of circulating sex steroids, which have contributed to a common assumption that sexes are

physiologically similar until sexual maturation. Patiño and Schreck [10], however, have described sex differences in plasma sex steroids in immature coho salmon. Studies in our laboratory suggested that immature male and female trout responded differently to xenoestrogens, as evidenced by vitellogenin production [11]. Here, we describe work with the natural estrogen 17β-estradiol that demonstrates a sex difference in juvenile rainbow trout vitellogenin production.

Materials and Methods

Dietary 17β-estradiol exposure

Juvenile rainbow trout (*Oncorhynchus mykiss*), Shasta strain, were reared at the Marine/Freshwater Biomedical Sciences Center aquaculture facility at Oregon State University, as approved by the IACUC at O.S.U. Thirty fish per treatment, ages three to eighteen months, were separated by age and maintained in 3' diameter tanks supplied continuously with 13°C well water. Fish were fed a growth ration of 5.6% diet per day (w/w) for seven days. 17β-Estradiol (E_2) (Sigma, St. Louis, MO) was dissolved in 95% EtOH and added to Oregon Test Diet (OTD), a semi-purified trout diet [12]. Final dietary concentration of E_2 was either 0, 0.05, or 2.5 mg/kg, containing 0.025% EtOH (v/w). Doses were chosen to include diets known to induce sub-maximal and maximal Vg production [11]. On day eight, fish were anesthetized in tricainemethanesulfonate (MS222) (50 mg/L) and blood was drawn into 3 ml vacutainers containing sodium heparin. Livers were removed, sex was determined grossly by gonad morphology and fish were killed by severing the spinal chord. A mixture of EDTA (1 mM) and aprotinin (50-150 kallikrein units/ml) was added to whole blood to inhibit protealytic cleavage of Vg. Blood was immediately placed on ice and maintained at 4°C until plasma was isolated after centrifugation for 10 min at 3000 g (within 4 h after sampling). Plasma was stored in aliquots at -80°C until use. Total liver protein was determined by the method of Lowry et al. [13].

Vitellogenin ELISA

Vitellogenin was measured using a competitive enzyme-linked immunosorbent assay (ELISA), modified from Donohoe and Curtis [7]. Purified rainbow trout vitellogenin (D.R. Buhler, Oregon State University) was used to coat 96 well microtiter plates (25 ng Vg/well) and for standard solutions (0-1600 ng Vg/ml). Vitellogenin was identified by polyclonal IgG antibodies (427 ng/ml) raised in rabbits against chum salmon Vg (A. Hara, Hokkaido University). Vg standards were fit to a four parameter model using DeltaSoft 3 software (Biometallics Inc., Princeton, NJ) in conjunction with a Bio-Tek EL340 plate reader (Winooski, VT). There was no observed non-specific binding of antibody to plates or other plasma proteins, as verified by western blotting. Samples from individual treatments were always measured within a single assay, however, we occasionally had to repeat analyses with additional plasma dilutions because of variability between fish. Intraassay variability was <10% and interassay variability <15%. ANOVA and Student's t-test analyses were performed with Microsoft Excel (v7.0) and StatView (Abacus Concepts, Berkeley, CA).

Results

Liver Somatic Index

Individual fish and liver weights were recorded and compared within, and between, treatments. Liver somatic index (LSI) was calculated for each fish and represents the percentage of total body weight contributed by the liver. LSI increased over basal levels in six month old fish fed 0.05 mg/kg E_2, from 1.2 ± 0.2 to 1.9 ± 0.3 in females ($p<0.001$) and from 1.2 ± 0.1 to 1.7 ± 0.3 in males ($p<0.001$). High dose E_2 exposure of 2.5 mg/kg resulted in higher LSI in twelve month old fish (females 1.5 ± 0.2, males 1.5 ± 0.4) compared to age-matched fish fed 0.05 mg/kg (females 1.3 ± 0.1, males 1.2 ± 0.2) ($p<0.01$). Neither individual fish weight nor LSI differed between sexes in any treatment groups.

Plasma and liver vitellogenin

One week dietary exposure to 0.05 or 2.5 mg/kg E_2 resulted in the production of vitellogenin in all fish, ranging in age from 3 to 18 months. Vg was present in plasma of unexposed 12 and 18 month old females (0.556-58.1 µg Vg/ml range) but not in males (detection limit was 62.5 ng/ml at 1:10 plasma dilution). In fish aged six to eighteen months, females produced more Vg than males after feeding 0.05 mg/kg E_2. Vg averaged 70.8 ± 12.3 ng/mg protein in livers of three month old fish, however, comparisons between sexes were not made because gonads were too small to identify the sex grossly. In six month old fish, Vg was 3.7x higher in female livers than in males after adjusting for total liver protein content (Fig. 5.1). Vg was 4.2x higher in plasma of twelve month old
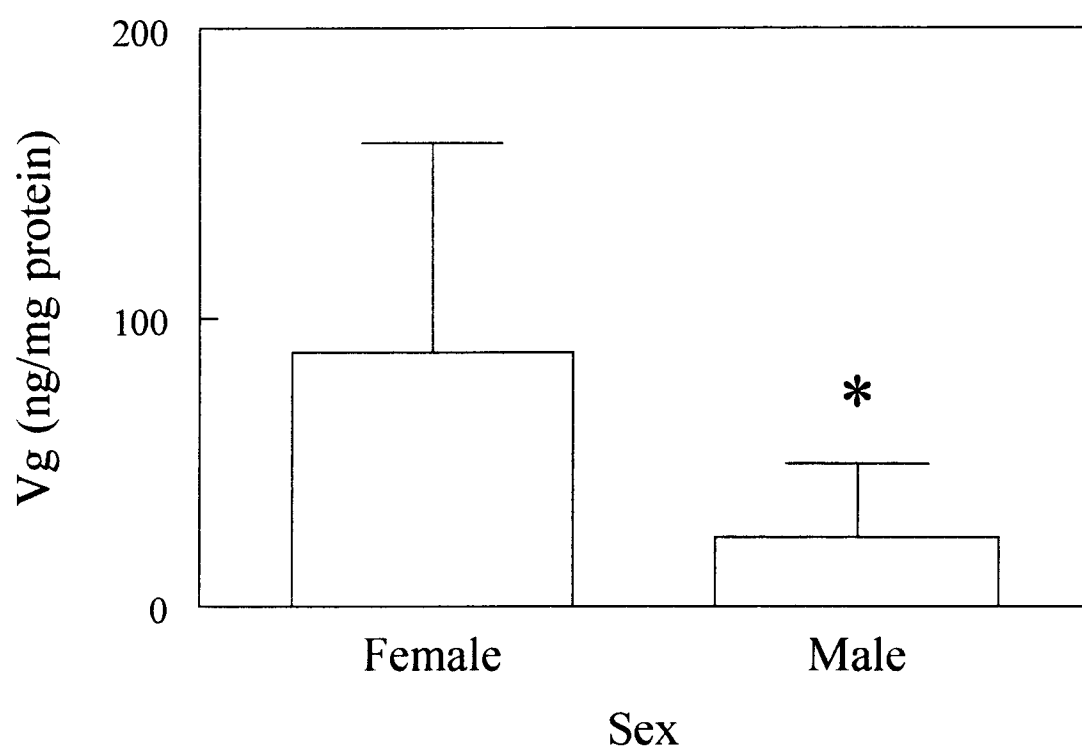
Figure 5.1- Liver vitellogenin in six month old trout fed 0.05 mg/kg 17β-estradiol for 7 d. * indicates significantly lower vitellogenin production in males (p < 0.005).

females and 3.3x higher in plasma of eighteen month old females than similarly aged males (Fig. 5.2). When fed 2.5 mg/kg E_2, twelve month old males and females produced similar amounts of Vg (4.84 ± 1.08 and 4.37 ± 1.03 mg Vg/ml plasma, respectively; p>0.05). There were no differences in the amount of plasma Vg in sex-matched twelve and eighteen month old fish fed 0.05 mg/kg E_2 (p>0.05). The mean levels of plasma Vg in twelve month old fish fed 2.5 mg/kg E_2 were significantly higher than both twelve and eighteen month old fish fed 0.05 mg/kg E_2 (p<0.0001). Trends in liver Vg were similar to plasma in twelve month old fish, with female Vg higher than males at low, but not high, E_2 exposure (data not shown).
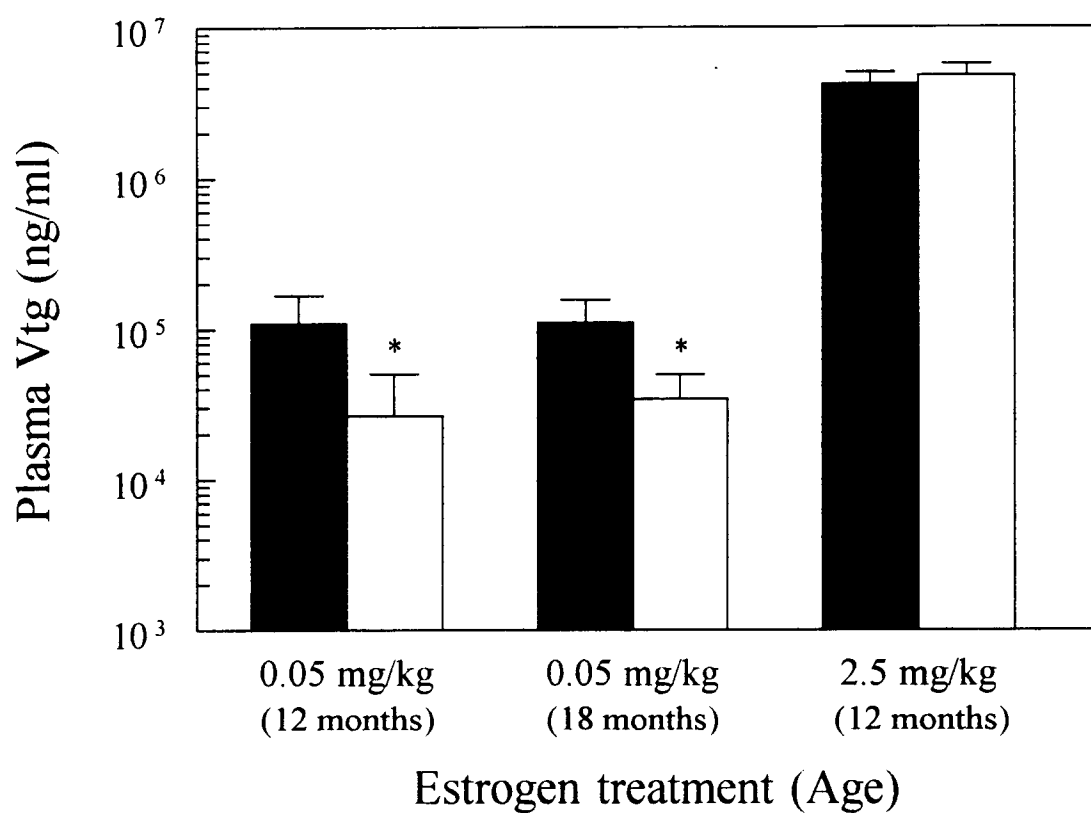
Figure 5.2 - Plasma vitellogenin in twelve and eighteen month old trout fed 17β-estradiol for 7 d. Solid bars represent females, open bars represent males. * indicates significantly lower vitellogenin production in males (p < 0.001).

Discussion

Vitellogenin production by oviparous animals has proven to be a useful biomarker of exposure to estrogenic chemicals [6,7]. Results from the current study document a sex difference in estrogen induced vitellogenin in immature trout, evident only if fish are fed a dose of E_2 (0.05 mg/kg) that induces sub-maximum Vg production (26.3 - 110.5 µg Vg/ml plasma in males and females, respectively). These observations are consistent with results from a previous study in our lab, in which tamoxifen blocked E_2-mediated Vg production and liver cytochrome P450 downregulation only at sub-maximal E_2 doses [11]. Flouriot et al. [14] showed that trout estrogen receptor mRNA and Vg mRNA transcription are saturable by estrogens, and we have previously observed saturable plasma Vg for estrogenic chemicals with varying potency and efficacy (unpublished data).

It is possible that previous studies failed to observe sexually dimorphic vitellogenin production in immature trout because of highly estrogenic doses or long exposure times. Juvenile rainbow trout can only be sexed accurately by examination of gonads and fish may not have been sexed in earlier studies. Basal and induced vitellogenin levels varied greatly (up to 10-100 fold) between same-sex individuals, which was consistent with reported variability in fish of comparable age, sex, and reproductive status [15]. Such high variability would make it difficult to see statistically significant sex differences in Vg induction with small sample sizes [7].

We are currently trying to determine the mechanism that promotes greater vitellogenin synthesis in immature females. Sex differences may be due to different estrogen receptor regulation, as age, sex, and seasonal differences in estrogen receptor

number, affinity, and regulation have been documented in trout [16,17]. Ultimately, when exposed to high E_2 doses, immature males produced equivalent amounts of Vg as females (Fig. 5.2), which could be explained by autoinduction of estrogen receptors to equivalent, saturated levels [16,14]. Alternatively, studies have shown that rainbow trout previously exposed to estrogens produce greater amounts of Vg and Vg mRNA [18,19], during subsequent estrogen exposure. Imprinting by estrogens during development, or priming by low levels of circulating estrogens, may predispose female trout to respond differently, than males to later estrogen exposure.

Because immature fish have been used successfully to characterize estrogenic chemicals *in vivo*, these models are candidates for use in screening estrogenic chemicals, as mandated by the Congress of United States in the Food Quality and Protection Act (1996) and the Safe Drinking Water Act (1996). While the mechanisms of sexually distinctive vitellogenin production are currently unknown, immature fish will continue to be useful models for *in vivo* xenoestrogen characterizations if sex is considered in analyses.

Acknowledgments

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Chapter 6

Conclusions

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Summary

The data presented in this thesis confirm that rainbow trout are sensitive to xenoestrogens and useful for mechanistic studies of endocrine modulation. As expected, early life stages of trout were more sensitive to xenobiotics than juveniles, evidenced by high lethality in embryos and fry. The xenoestrogens investigated, however, were unable to produce consistent changes in sexual differentiation seen with natural estrogens. Nominal environmental DDE contamination is unlikely to induce changes in rainbow trout sexual development. Other persistent chemicals may alter sexual development in trout (1), and species sensitivity may differ to xenoestrogen exposure during sexual differentiation (2). The high doses of o,p'-DDE and chlordecone injected into embryos were above levels that most natural populations would be exposed to and chemicals may interact with receptors at lower environmental levels (3). It has been suggested that low doses of xenoestrogens may be more potent *in vivo* than high doses (4), but there is no evidence in fish to support that theory. Embryos may be more sensitive to continuous aqueous exposure than to microinjections (2), because injection directly into yolk may result in chemicals associating with lipids and being sequestered away from developing sex organs. With respect to exposure to maternally transferred chemicals, acute toxicity of the chemicals investigated in this thesis is likely to precede subtler endocrine effects in natural populations.

Exposure to mixtures of xenoestrogens was similar to what would be expected from additive, or competitive, binding to estrogen receptors. Vitellogenin induction and P450 modulation were sensitive biomarkers for investigating chemical mixtures. At lower

relative estrogen doses, tamoxifen was able to block the effects of 17β-estradiol on Vg and liver P450 in trout. Mixtures of natural estrogens and OH-PCBs also resulted in Vg induction suggested by additive receptor binding. Estrogenic biomarkers were not, however, sensitive to unlimited chemical exposure. Responses were saturated during short term *in vivo* exposures, which supports earlier findings that estrogen receptor binding is saturable and dependent on finite receptor numbers (*5,6*). There was a high degree of variability in the responses of individual fish to xenoestrogens, but maximal Vg induction was evident. One result which could not be explained by classical ER binding was the induction of lauric acid hydroxylase by mixtures of tamoxifen with sub-saturating levels of estrogen. Tamoxifen alone did not appear to be solely responsible for LA-OH induction because the responses to E_2 and tamoxifen mixtures were greater than with tamoxifen alone and treatment with the highest dose of tamoxifen alone decreased enzyme activity.

The importance of estrogenic doses was documented by different responses in fish treated with saturating and sub-saturating amounts of estrogens. Environmental contaminants are unlikely to saturate estrogenic responses because of weak affinity for estrogen receptors (*3,7-9*). Responses seen with low doses of natural estrogens are probably more applicable to xenoestrogen effects than high dose effects. High doses of estrogens induced Vg to maximal levels, at which point sex differences in the vitellogenic response of immature fish disappeared. Tamoxifen was also unable to fully counteract the effects of high doses of estrogens, which may be due to toxic effects of estrogens (*10*) or to non receptor-mediated mechanisms. Another interesting finding was the ability of 4-OH-2',4',6'-trichlorobiphenyl to induce Vg to saturated levels *in vivo*. Although receptor

affinity of OH-PCB 30 is predicted to be about 40 fold lower than estradiol (*11*), the maximal Vg response was similar to natural estrogens, presumably because of maximal receptor activation. There may be different mechanisms involved in the activity and toxicity of estrogenic chemicals, dependent on the extent and strength of receptor binding and corresponding unbound chemicals.

Sex differences in estrogen dependent responses were documented in immature trout, which were contrary to the common assumption that immature salmonids are physiologically similar until sexual maturity. Vg induction and P450 downregulation occurred in both males and females, but the magnitude of response to estrogens was different. As noted above, the sexually dimorphic Vg response was observed only at doses of xeno- and natural estrogens that induced Vg to less than saturated levels. A sexually dimorphic downregulation of hepatic P450s was significant with high dose treatment of 17β-estradiol and tamoxifen. Similar trends were seen with lower dose estradiol treatments, but small sample sizes prevented statistical analyses. Sexing immature trout is time consuming and invasive, but the results presented in this thesis document the importance of sex specific responses to xenoestrogens.

Future Directions

Endocrine toxicology has received a tremendous amount of attention recently from lawmakers and the public. Clear, causal relationships of adverse effects of EACs on human health are lacking and documented toxicity in natural fish and wildlife populations has been limited to heavily contaminated areas. The potential certainly exists for contaminants to affect endocrine systems, but scientists should be cautious about emphasizing isolated occurrences, in highly polluted environments. It may be wise to concentrate resources and effort on the consequences of low level, ubiquitous contamination. While high dose exposures facilitate mechanism-based studies, experiments with low, environmentally relevant doses should follow. Work from vom Saal's lab showing that low doses of xenoestrogens act differently than high doses *in vivo* (*4*) suggest that low level environmental contamination may be important. The establishment of thresholds for EAC effects in different species will provide important information for regulating chemicals.

There is an important distinction between chemical *exposure* and *adverse effects* in an organism. For example, the *effects* of elevated vitellogenin in fish have not been addressed, though evidence in the field and the laboratory have shown xenoestrogens to induce Vg. Some questions to consider about xenoestrogen induced vitellogenin include: Are male fish adversely affected by high levels of circulating vitellogenin?; How long is Vg present in males and non-reproductive females?; Does xenobiotic induced vitellogenin induction in female fish alter normal reproductive function?; Is there a physiological role for Vg in male fish?; Do normally silent genes, such as male Vg genes, or male sex

determining genes in unisexual female lizards, serve a common evolutionary function? Mechanisms of endocrine modulation must be determined before complex environmental effects can be understood. Eventually, population and community structure should be addressed, in the context of ecoregions and habitat, which ultimately reflect overall environmental health.

Many interesting questions and avenues of research could be pursued concerning the mechanisms of sexually dimorphic responses of immature trout to xenoestrogens. It is not surprising that males responded differently than females to estrogens, because steroid expression is sexually dimorphic in mature fish (*12*). Directly measuring estrogen receptor expression and activity would determine whether receptor-dependent biomarkers described in this thesis accurately predicted receptor responses. It would be interesting to learn whether immature males simply have lower ER numbers than females, or whether gene regulation was controlled by other factors which are inherently different between sexes. Research in our lab is currently investigating the persistence of Vg in male fish, as well as the implications of prior estrogen exposure on the magnitude of Vg induction. Preliminary results have shown that sex differences are not due to physiological priming of females by natural estrogens, which suggests that estrogen regulation in immature males and females is fundamentally different (unpublished data). Molecular tools could be used to determine the earliest time when sexually dimorphic responses occur, which could help clarify the mechanisms of sexual differentiation in trout. Investigating the different responses of fish to high and low estrogen doses may provide interesting information about receptor-mediated gene regulation.

There were many interesting observations in this thesis, and in related work, that were not pursued but that may have implications for toxicant effects in trout. Microinjected eggs hatched earlier than uninjected embryos, which may have been due to physical damage to the chorion. Limited evidence with salmon suggested that toxicant doses may also decrease the time to hatch, but those data were confounded by injection stress to eggs. It could be that decreased time to hatch is a general stress response, unrelated to injection trauma, that may be important for fish exposed to low levels of toxicants. Hatching of underdeveloped embryos may increase mortality rates; higher mortality was noted in injected salmon that hatched earliest (personal observation). Decreased *in vitro* androgen production in male trout injected as embryos may have been due to a general stress response or to developmental changes caused by earlier hatching. Endogenous steroid production at maturity was not measured in affected males, but males from a mixed sex population reproduced successfully.

Two other projects may provide useful tools for endocrine toxicology research. Attempts to identify a pure antiestrogen in trout were unsuccessful; if one is identified, it would assist investigations of tumor promotion in trout by estrogens and xenoestrogens. By blocking estrogen receptor-mediated responses, the contribution of estrogenic effects to toxicity and carcinogenesis could be separated from other mechanisms. Identification of trout aromatase inhibitors would be useful for blocking the conversion of androgens to estrogens, which may be important in the hepatocarcinogenic activity of the androgen dehydroepiandrosterone (DHEA). Additionally, aromatase may be involved in trout sex determination or differentiation, which has been shown in reptiles (*13, 14*) and suggested in salmon (*15*). Identification of environmental contaminants that inhibit aromatase may

provide interesting information about sex differentiation, endocrine modulation, and additional risks of EACs.

Human society and the chemicals we produce affect the world around us. The science of toxicology (and science in general!) is undergoing an exciting period of discovery. We have amazing molecular biology tools and a substantial knowledge of biology and chemistry at our disposal. We are beginning to understand the mechanisms by which chemicals cause non-lethal physiological perturbations. Endocrine active chemicals may prove to pose little risk to humans, but there are well documented cases of adverse effects in wildlife. Research proceeds slowly, however, and we must decide whether to err on the side of caution or economic interests as we implement policy regarding new chemicals that we know little about.

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