AN ABSTRACT OF THE THESIS OF

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David E. Williams

The disruption of the critical balance between estrogens and androgens as a result of xenobiotic exposure can have major systemic consequences. Rainbow trout were utilized to distinguish between androgenic and estrogenic mediated responses. Ovarian microsomes were used to characterize aromatase activity in the trout. Pharmaceutical aromatase inhibitors, aminoglutethimide, 4-hydroxyandrostenedione (4-OHA) and letrozole were found to be effective in this model. To determine relative estrogenicity of compounds, a liver slice assay quantifying vitellogenin (Vg) induction was developed. Bisphenol A, OH-PCB30 (4-hydroxy-2',4',6'-trichloroPCB), *o,p'* DDE and 3,3'-diindolylmethane (I33') induced Vg in slices with relative potencies ranging from 10^{-4}-10^{-6} ng/mg protein compared to the most potent estrogen tested, 17β-estradiol (E_2). The practicality of this model was demonstrated using Chinook salmon liver slices, which were less sensitive than trout, but exhibited similar Vg induction profiles. I33' was also a strong estrogen *in vivo*, possessing no antiestrogenic properties in the trout. The *in*

vivo studies and slice experiments with SKF525A suggested that estrogenicity of indole-3-carbinol (I3C), was primarily via I33' formation resulting from acid condensation in the stomach and that I33' needed to be further metabolized, possibly to a hydroxylated metabolite to attain maximum Vg induction. Elucidating direct and indirect responses of androgens was studied by feeding trout aromatizable androgens dehydroepiandrosterone (DHEA) and androstenedione and the non-aromatizable androgen, dihydrotestosterone (DHT). DHEA and androstenedione induced Vg by 40-fold while DHT decreased Vg by up to 80%. These data along with E_2 increases in the DHEA and androstenedione treated trout suggest that estrogenic responses by DHEA and androstenedione were due to their conversion to estrogens. Co-treatment with DHT and the androgen receptor antagonist, flutamide, did not reverse the Vg and E_2 decreases observed with DHT alone. In fact, flutamide alone lowered Vg and E_2 levels in a similar fashion observed with DHT, with no effect on testosterone levels observed. It appears that the regulation of estrogens and androgens overlap and that crosstalk pathways are critical in maintaining balance between these sex steroids. These studies demonstrated that rainbow trout are very useful for deciphering responses elicited by estrogens and androgens and provide a model for predicting effects in other species.

CHARACTERIZATION OF ANDROGENIC AND ESTROGENIC RESPONSES IN THE RAINBOW TROUT BY COMPARING LIVER SLICE TO WHOLE ANIMAL STUDIES

by

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CONTRIBUTION of AUTHORS

Dr. David E. Williams was instrumental in the preparation of all manuscripts (Chapters 2, 3, 4, 5 and 6) and was responsible for their submission. Dr. Williams provided advice and expertise in experimental design of the studies in this thesis. Dr. David Carlson trained me in the sampling of fish, set the groundwork for the aromatase assay (Chapter 2) and in the sampling and vitellogenin analyses performed for Chapter 5. Sirinmas Katchamart helped in the diet preparation and sampling of fish in Chapter 5. Dr. Aram Oganesian instructed me in the execution of tissue slice assays and the use of the Krumdieck slicer. Sheila Cleveland sectioned and stained tissue slices and Dr. Jan Spitzbergen and Dr. Jerry Hendricks examined them for the slice studies performed in Chapters 4, 5 and 6. Dr. Marilyn Henderson and Dr. Donald Buhler instructed me in the use of the double beam spectrophotometer for total P450 assays in Chapter 3. Dr. Buhler also graciously provided me with vitellogenin and cytochrome P450 antibodies for ELISAs (Chapter 2, 3, 4, 5, 6) and Western Blots (Chapter 5). Dustin Leibelt instructed me in the use of the HPLC for analyses performed in Chapter 5.

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and to my parents, George and Florence

CHARACTERIZATION OF ANDROGENIC AND ESTROGENIC RESPONSES IN THE RAINBOW TROUT BY COMPARING LIVER SLICE TO WHOLE ANIMAL STUDIES

CHAPTER 1

INTRODUCTION

Environmental Protection Agency (EPA) mandates, Food and Water Quality and Safe Drinking Water Acts of 1996 made it necessary to develop effective screening assays to detect endocrine disrupting chemicals. Compounds high on the testing list are those produced synthetically such as pesticides and by-products of industrial processing. There are numerous other compounds found in the diet, however, that may pose more of a risk in disturbing the hormone-balanced processes in humans and animals. It is this vast unknown that requires such a large effort by scientists today.

The exact parameters encompassing endocrine disruption is as controversial as the determination of which screening assays are to be used to detect them. Currently, the definition of an endocrine disrupter according to EPA's Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) is, "an exogenous substance that changes endocrine function and causes adverse effects at the level of the organism, its progeny and/or (sub) populations of organisms." One major stumbling block was whether or not the definition should encompass nonadverse effects to the endocrine system. In any case, the definition includes compounds that act to disrupt estrogen and androgen signal transduction pathways through agonist/antagonist mechanisms. The most common mechanism studied has

been through direct receptor binding. Scores of cell line, fusion protein, reporter gene and binding assays have been developed, primarily with the estrogen receptor to identify and quantify relative estrogenicity of compounds (Andersen *et al.*, 1999, Sonnenschein and Soto, 1998; Bolger *et al.*, 1998, Petit, *et al.*, 1997, Ren *et al.*, 1996). The chapters in this thesis address several issues of endocrine disruption.

The focus is on androgens, which classically are known as the male sex hormones, and estrogens, the class of female sex hormones. The importance of estrogens and androgens for the proper development and function of organisms of both sexes is well characterized. Estrogens and androgens play a role in all animals from sponges to primates. They have been implicated in so many physiological processes that there are probably more biological pathways affected by these hormones than not. Their standard modes of action overlap considerably and play critical roles that include sex determination, sexual development of males and females, neural and brain function and development, lipid and cholesterol homeostasis, muscle formation, bone metabolism and integrity, cell-cycle regulation, gonadal function, reproduction and cardiovascular regulation. (Grumbach and Auchus, 1999, Mendelsohn and Karas, 1999, McEwen, 1999, Rickard *et al.*, 1999, Miller and Franklin, 1999). These two classes of hormones elicit and promote several kinds of carcinogenesis such as prostate (androgens) and breast and uterine (estrogens) cancer (Kelloff *et al.*, 1998, Fujimoto *et al.*, 1998, Blamey 1997, Miller, 1997 and DeCoster *et al.*, 1996). It is the formation and promotion of tumor development and the alterations in reproductive function that are primary reasons estrogens and androgens are the major focus of endocrine disruption research today. The major biomarker for estrogen receptor binding utilized in this thesis is vitellogenin (Vg), a 170 kDa glycophospholipoprotein, egg-yolk protein precursor produced in oviparous animals made up of three components, two lipovitellins (I and II) and phosvitin (Davis, 1997, Mouchel *et al.*,

1996 and Goulas *et al.*, 1996). Vg is a very high density lipoprotein (VHDL) with a sequence similar to apolipoprotein B100, the triglyceride/ cholestrerol transporter (Davis, 1997 and Baker, 1988). Vg is synthesized in the liver in response to estrogen receptor binding (Islinger *et al.*, 1999 and Bieberstein *et al.*, 1999). Normally, production of Vg occurs in mature females that have synthesized estrogens (mainly 17β-estradiol (E_2)) in the ovary. Figure 1.1 depicts the pathway involving Vg synthesis and secretion in response to estrogen stimulation. The E_2 goes to the liver via the bloodstream, binds estrogen receptors in the liver, which then bind estrogen receptor response elements in the promoter region of the Vg gene. Vg mRNA is produced and Vg protein is synthesized in the liver, secreted into the blood and sent back to the ovary. Vg is then cleaved in the ovary and incorporated into the developing eggs. The detection of Vg in the blood plasma or liver is a well-characterized biomarker for estrogen exposure (Davail *et al.*, 1998, Sumpter and Jobling, 1995, Maitre *et al.*, 1986). The best subjects for measuring Vg induction are usually immature males, which generally have little or no basal levels of this protein. Immature males, however, still have the capacity to synthesize Vg in a similar fashion to females in response to exogenous estrogen exposure. Since males have no ovaries, the Vg will just circulate futilely through the bloodstream, which can be measured in extracted blood plasma with an enzyme linked immunosorbant assay (ELISA). When exposed to estrogen (i.e. E_2) Vg protein levels peak at about 7 –10 days post treatment and the half-life of Vg in the bloodstream is several weeks (Donohoe and Curtis, 1996, Sumpter and Jobling, 1995 and Shapiro, 1982). The evolutionary explanation for why males have retained the ability to synthesize Vg is still unknown. Since it is a lipoprotein, perhaps it serves a secondary function as a binding or carrier protein. In any case, it is a very useful biomarker for estrogen exposure that has been exploited for several years.

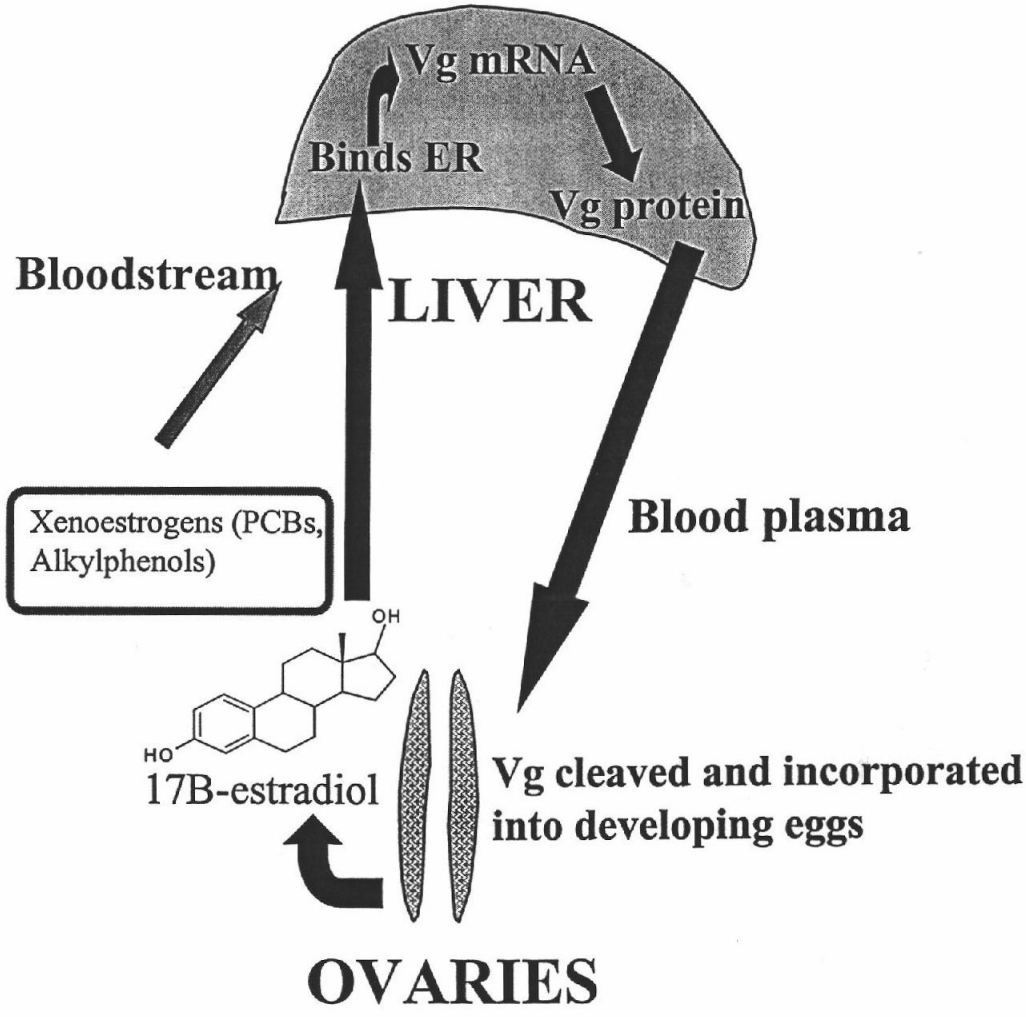

Figure 1.1: Diagram of Vg synthesis pathway in oviparous animals. This process normally occurs only in mature females. Addition of exogenous estrogens (shown in box as PCBs and alkylphenols) can induce Vg production in males.

There are many other mechanisms by which chemicals can exert a response such as through altered metabolism, absorption and clearance of the hormone, interaction (co-exposure) with other compounds. Sensitivity of exposed organism, which includes parameters such as sex, age, genotype, body size and current health status, can influence response to a given dose. Moreover, many of the previously mentioned receptor binding assays measure receptor binding only in an artificial environment. In this thesis, the effects of androgens and estrogens on sex steroid levels *in vivo* as well as metabolism via cytochrome P450 content were examined.

The first area of endocrine disruption investigated dealt with was aromatase inhibition. Aromatase is a cytochrome P450 from the CYP19 gene that is responsible for the conversion of androgens to estrogens (Zeitoun and Bulun, 1999, Njar and Brodie, 1999, Blamey, 1997 and Miller, 1997). It is found in the ovaries as well as peripheral tissues such as the brain, breast and adipose tissue (Njar and Brodie, 1999 and Kelloff *et al.*, 1998). Aromatase has been implicated in the growth of estrogen dependent cancers such as breast and uterine cancer (Zeitoun and Bulun, 1999, Ginsburg, 1999 and Feigelson and Henderson, 1996). Studies have shown that aromatase activity is very high in and around tumor tissue in the breast and endometrium (Brodie *et al.*, 1999, Blankenstein *et al.*, 1999 and Zeituon and Bulun, 1999). It is believed that the tumor tissue upregulates androgen synthesis and the aromatase gene in neighboring tissue to produce the estrogen that allows the cells to grow and replicate at a high rate (Zeituon and Bulun, 1999, Blankenstein *et al.*, 1999). This can be observed because these cancers normally affect post-menopausal women, who typically have very low estrogen levels. Blocking aromatase activity has been the target of chemotherapy for estrogen dependent cancers for several reasons. The synthesis of estrogen is the final step in the cholesterol biosynthetic pathway (see Figure 1.2), therefore, highly specific

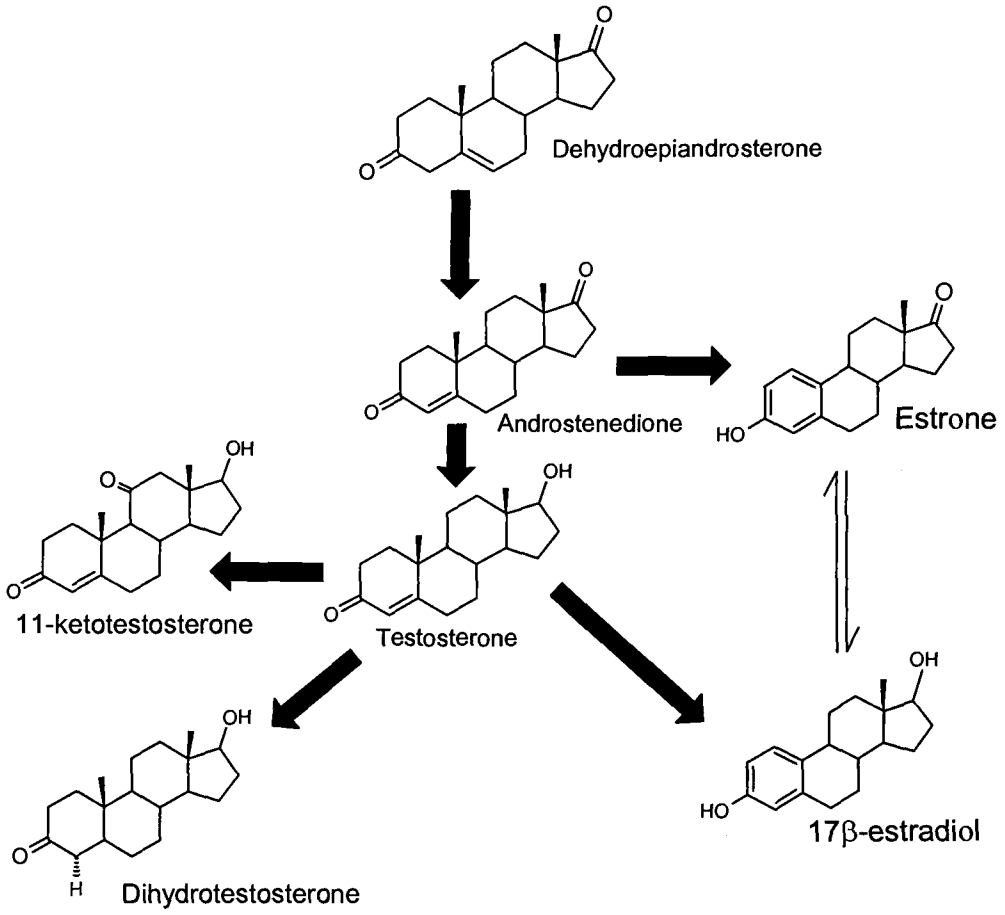

Figure 1.2: Synthesis pathway of major estrogens and androgens from DHEA. E_2 can be formed from testosterone via aromatase or estrone via a dehydrogenase reaction. DHT is the major circulating androgen in mammals while 11kT is the major circulating androgen in fish. E_2 is the major estrogen in both.

inhibitors of this enzyme should have little effect since little estrogen is produced in post-menopausal women. Classic treatment techniques such as masectomy, radiation and general chemotherapy are more destructive and have often devastating side-effects. The tumor cells depend on the estrogen to grow and proliferate whereas normal cells do not and will not be affected. Traditional hormone therapy with estrogen receptor antagonists such as tamoxifen target the site at which estrogens exert their action. Although prevention of breast cancer by tamoxifen shows promise, this kind of treatment almost always results in relapse due to the fact tumor cells can alter expression of critical genes such as aromatase, the β-form of estrogen receptor, sulfotransferases, or upregulate androgen levels, decreasing the potency of the antiestrogen (Atkinson *et al.*, 1999, Speirs *et al.*, 1999 and Purohit *et al.*, 1999). Treatment with aromatase inhibitors is designed for these patients as another type of specific hormone therapy with fewer side-effects. Successful aromatase inhibitors have been classified as either steroidal or non-steroidal. In the 1970s, the main treatment was with a non-steroidal drug called aminoglutethimide. This compound however is a broad-spectrum cytochrome P450 inhibitor with only slight preference towards the aromatase enzyme (Newton *et al.*, 1991, Halpert *et al.*, 1994). In fact, it blocked the enzyme responsible for the conversion of corticosterone to aldosterone (18-hydroxylase) effectively enough that patients taking aminoglutethimide had to be co-administered corticoid (DeCoster *et al.*, 1996). In the early 1980s, more potent and selective steroidal aromatase inhibitors such as 4-hydroxy-4-androstene-3,17-dione (4-OHA) and 6-methyleneandrosta-1,4-diene-3,17-dione were developed and marketed under the names of Formestane and Exemestane, respectively. These androstenedione analogs act as irreversible suicide inhibitors, inactivating the enzyme quite effectively (Njar and Brodie, 1999 and Evans *et al.*, 1992, Dowsett *et al.*, 1989). Disadvantages are that 4-OHA is a steroid and is not orally bioavailable so it must

be administered by intramuscular injection. More recently developed inhibitors, deemed third and fourth generation aromatase inhibitors are non-steroidal compounds usually containing imidazole or triazole moieties. These drugs are more potent and specific with less side effects than previously marketed drugs, including 4-OHA. Examples include anastrozole, letrozole, fadrozole and vorozole (Njar and Brodie, 1999, Ingle *et al.*, 1997, Kelloff *et al.*, 1996). The goal of the studies involving aromatase inhibition in rainbow trout was to characterize the aromatase enzyme in the female ovary and to determine if inhibitors of mammalian aromatase were effective on the trout enzyme. The rational was that aromatase inhibitors could be used in trout tumor studies to determine mechanisms of action of tumor promotion. Previous studies showing promotion of aflatoxin B_1 and N-methyl-N'-nitro-nitrosguanidine induced liver tumors by dehydroepiandrosterone (DHEA) suggested that the mechanism may be through estrogenic pathways (Orner *et al.*, 1998, Orner *et al.*, 1996b and Orner *et al.*, 1995). Blocking aromatase activity by an inhibitor would aid in the understanding of tumor promotion by DHEA and elucidating if the mechanism was through conversion of DHEA to 17β-estradiol. To distinguish the different responses between endogenous androgens, liver slice and whole animal studies were conducted. The use of an aromatase inhibitor would again be useful in these whole animal studies. Vg induction both through direct estrogen receptor binding by the androgen and by binding of the converted androgen to estrogens were studied. Effects mediated through the androgen receptor using the antiandrogen, flutamide, on metabolism via total cytochrome P450 content modulation, on blood steroid level alterations and tumor initiation and promotion were also examined

Androgens are intriguing endocrine disruptor candidates because they can be converted to other compounds that can elicit similar or opposite responses. Androstenedione, for example, is a weak androgen that can be converted to

testosterone, a strong androgen receptor agonist. Testosterone can then elicit an androgenic response or be converted to 5α-dihydrotestosterone (DHT) the most potent circulating androgen. Testosterone, however, can also be converted to 17β-estradiol by aromatase, which is the most potent circulating estrogen (Kelloff *et al.*, 1998). The picture is complicated by studies that demonstrate estrogenic activity of androgens in the absence of aromatase activity. Testosterone, androstenedione and DHT all have the ability to bind the estrogen receptor and induce Vg (Mori *et al.*, 1998, LeMenn *et al.*, 1980 and Hori *et al.*, 1979). In these studies, the induction was blocked by tamoxifen co-treatment, further supporting the hypothesis that androgens can directly elicit estrogenic responses. Moreover, altering metabolizing enzyme levels and activity could also modulate responses by androgens (Lazier *et al.*, 1996, Gustafsson *et al.*, 1983, Hansson *et al.*, 1980).

Rainbow trout, *Oncorhynchus mykiss*, were the animal model used in the studies of this thesis. They were utilized in a manner that was acceptable to the Oregon State University Institutional Animal Care and Use Committee. Rainbow trout have been utilized as a viable model for carcinogenesis for dozens of years (Breinholt *et al.*, 1999, Hayashi *et al.*, 1999, Bailey *et al.*, 1998, Dashwood *et al.*, 1998, Harttig *et al.*, 1996, Williams *et al.*, 1992 and Bailey *et al.*, 1987). The liver in particular has demonstrated applicability towards human risk assessment. This is due to certain similarities in morphology and enzymes of xenobiotic metabolism. In the case of hormone mediated responses, particularly by estrogens, the rainbow trout liver has shown close correspondence to other animals (Williams *et al.*, 1998, Orner *et al.*, 1998 and Orner *et al.*, 1996). The commitment of the National Institutes of Health (NIH) to the use of lower vertebrates as viable models for risk assessment has been a motivating factor for the application of the rainbow trout model. Applying aquatic models including animals such as fish, amphibians and reptiles, has been validated largely based on biochemical similarities (Matthiessen

and Sumpter, 1998, Kleinow *et al.*, 1987, Stegeman and Kloepper-Sams, 1987 and Williams *et al.*, 1992).

Rainbow trout possess certain qualities that are desirable for extrapolation in risk assessment (Bailey *et al.*, 1996, Bailey *et al.*, 1992). Trout are a sentinel species for certain end points such as Vg induction in response to estrogens and liver tumor formation in response to aflatoxin (Orner *et al.*, 1998, Hyashi *et al.*, 1999, Breinholt *et al.*, 1999). They are large enough so that tissue samples can be collected for various analyses. Their size also allows for collection of multiple samples of blood without detrimental consequences to the fish, which is critical for time-course studies. They can be housed and will reproduce in the laboratory setting. Studies can be done at all life stages including egg exposure, which allows for research to be performed with minute amounts of rare and/or expensive test chemicals. Studies can be performed with hundreds to thousands of individuals allowing for strong statistical power because the trout are easy and inexpensive to maintain. Depending on size, dozens to hundreds of fish can be kept in a single tank, which is approximately a twenty times cheaper than caring for laboratory rodents. There are drawbacks of the rainbow trout model as well. Extrapolation to humans can be difficult in some cases. Fish are evolutionarily farther from humans than rodents making extrapolation from trout to humans a more remote phylogenetically than from mice or rats, which are mammals. The physiology of trout is different as they possess organs not present in humans (gills, swim bladder, scales and fins) and visa versa (lungs) particularly in females (breast, uterus and cervix). Trout have a relatively long life history compared to other animals commonly used in laboratory studies such as rodents making reproduction and multi-generation studies more difficult and time consuming. Their application to environmental risk assessment and extrapolation to other aquatic species, however, is quite relevant. The careful use of the trout in risk assessment for carcinogenesis,

endocrine disruption and toxicity studies allows this model to have many applications.

Indole-3-carbinol (I3C) was studied extensively as an estrogen in the rainbow trout. I3C is found in cruciferous vegetables in the *Brassica* genus such as broccoli, cauliflower and brussel sprouts. I3C enters the acid environment of the stomach of humans (and rainbow trout) and undergoes condensation into numerous dimers, trimers and higher order oligomers (Wortelboer *et al.*, 1992, Bradfield and Bjeldanes, 1987 and Leete and Marion, 1953). The major products formed are a dimer, 3,3'-diindolylmethane (I33'), and a linear trimer, 2-(indol-3-ylmethyl)-3,3-diindolylmethane (LT) (Grose and Bjeldanes, 1992 and Stresser *et al.*, 1995a). Other minor products identified include, but are not limited to, indolo-[3,2-b]-carbazole (ICZ), a potent aryl hydrocarbon receptor agonist, another dimer, 1-(3-hydroxymethyl)idolyl-3-indolylmethane (HI-IM), and a cyclic trimer, 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b':7,8-b"] triindole (CT). Of these products, the properties of I33' have been investigated to the greatest degree. The rational has been that I33' possesses characteristics that make it a promising chemopreventive agent. I33' has demonstrated antiestrogenic properties through inhibition of estrogen dependent tumor cell line growth and proliferation (Chen *et al.*, 1998, Liu *et al*, 1994). It also increases the 2-OH estrone (good estrogen) to 16α-OH estrone (bad estrogen) ratio which is indicative of antiestrogenicity (Telang *et al*, 1997, Michnovicz *et al.*, 1997 and Wong *et al.*, 1997). These characteristics are the reason I3C and I33' are currently in clinical trials for prevention and treatment of estrogen dependent cancers such as breast cancer.

I33' may also be chemopreventive by other means. It has been shown to induce apoptosis and cell-cycle arrest in certain cell lines (Chang *et al.*, 1999, Cover *et al.*, 1998, Chen *et al.*, 1996, and Ge *et al.*, 1996). I3C and its condensation products are effective Phase I and Phase II metabolizing enzyme

inducers as well which can elicit a host of responses relevant to carcinogenesis (Renwick *et al.*, 1999, Takahashi *et al.*, 1995a,b, Wortelboer *et al.*, 1992 and Bradfield and Bjeldanes, 1987). The induction of Phase II enzymes such as NADPH quinone reductase and UDP-glucuronyl transferase is generally seen as beneficial and chemopreventive. The induction of Phase I enzymes by I33' such as those in the cytochrome P450 enzyme family can have beneficial or detrimental consequences depending on timing and the endpoint (Stresser *et al.*, 1994, Vang *et al.*, 1999, Bradlow *et al.*, 1991, Michnovicz and Bradlow, 1990). In the case of carcinogenesis, I3C has been shown to block tumor formation at the initiation, promotion and progression stages (Xu and Dashwood, 1999, Srivastava and Shukla, 1998, Manson *et al.*, 1998, Oganesian *et al.*, 1997c, Takahashi *et al.*, 1995b, Dashwood *et al.*, 1994, Agrawal and Kumar, 1999, Takahashi *et al.*, 1995c and Bradlow *et al.*, 1991). There have also been studies reporting I3C to enhance tumor development, demonstrating tumor promotion with this compound when administered with carcinogen (Oganesian *et al.*, 1999, Dashwood *et al*, 1991 and Bailey *et al.*, 1987). Postulated mechanisms of chemopreventive by I3C and/or its acid condensation products are scavenging of the carcinogen by I3C, blocking of carcinogen activation by Phase I enzyme inhibition, transient induction of Phase I enzymes that act to detoxify the carcinogen and decrease formation of the ultimate carcinogen and induction of conjugating enzymes to detoxify the ultimate carcinogen. Induction of the detoxifying pathway steers metabolism away from the activation pathway. This has been demonstrated in zebrafish and rainbow trout exposed to cytochrome P4501A inducers that lowered aflatoxin B_1 initiated liver tumor incidence (Troxel *et al.*, 1997, Takahashi *et al.*, 1995a, Stresser *et al.*, 1994 and Fong *et al.*, 1990). In the tumorigenic scenario, I3C may act to promote the development of tumors from cells already initiated by a carcinogen. One explanation is that I3C acts as an estrogen to promote growth and proliferation of

the initiated cells. Previous studies in the trout liver have demonstrated the effectiveness of estrogens to promote tumor development (Williams *et al.*, 1998, Orner *et al.*, 1996 and Nunez *et al.*, 1989). This mechanism of action has been observed with DHEA co-treatment, which is believed to act through conversion to E_2. To test this, the estrogenicity of I3C and at least one of its condensation products had to be established. Vg induction could be measured in a whole animal study, but since I3C is converted to numerous oligomers *in vivo*, narrowing down the product(s) responsible for the Vg induction would be extremely difficult. Development of a quick and easy *in vitro* assay to determine estrogenicity of compounds that mimics the *in vivo* system was required to solve this problem.

Tissue slices were applied to many aspects of pharmacology and toxicology for several decades. The major drawback of this model was the inability for scientists to produce dozens of slices that were equal in size and thickness. The advent of the Krumdieck and other automated slicers in the early 1980s, allowed for the extensive use of precision-cut tissue slice technology (Price *et al.*, 1998; Bach *et al.*, 1996; Parrish *et al.*, 1995; Krumdieck *et al.*, 1980). Using one of these modern tissue slicers, slices of equal size and thickness could be made rapidly without further damaging the tissue. In this thesis, a Krumdieck tissue slicer was utilized to produce 8 mm diameter x 250 μm thick liver slices. Vg induction had been observed *in vitro* in hepatocytes of various fishes such as carp and rainbow trout (Smeets *et al.*, 1999a,b, Pelissero *et al.*, 1993; Maitre *et al.*, 1986), but choosing slices as the model incurred several advantages (Guillouzo, 1998, Vickers, 1994; Beamand *et al.*, 1993). Structurally, liver slices represent the whole organ to a greater extent by preserving the cell heterogeneity of the liver. In cultured cells, the tissue is enzymatically treated with collagenase, and the desired cell type isolated (Seglan, 1975). For studies requiring more than 24 hr incubation periods, isolated cells then need to be cultured, which requires more than a week in

time. Liver slices can be cut and ready to use from a fresh liver in 2-3 hours while preserving the cell-to-cell communication that exists *in vivo*. The liver slice is basically a microcosm of the whole organ that can be treated in a controlled laboratory situation.

Liver slices have been applied effectively to metabolism and toxicity studies (Ferrero and Brendel, 1997; Ekins, 1996, Singh *et al.*, 1996). Previous studies have demonstrated the capacity of liver slices to produce a variety of proteins in response to chemical treatment. For example, induction of cytochrome P450s has been observed after treatment with inducers such as β-naphthoflavone and I3C (Drahushuk *et al.*, 1999, Renwick *et al.*, 1999, Oganesian *et al.*, 1997a, Oganesian *et al.,* 1997b, Ekins *et al.*, 1995 and Stresser *et al.*, 1995b). Enzyme inhibition/ induction has been exhibited in liver slices derived from several different species (Renwick *et al.*, 1999, Vang *et al.*, 1999, Ekins *et al.*, 1996, Larsen-Su and Williams, 1996, Takahashi *et al.*, 1995c and Stresser *et al.*, 1994). Radiolabelled ligand metabolism and fate studies have been performed using slices based on the ability of slices to metabolize compounds in a similar fashion to that observed *in vivo* (Carlile *et al.*, 1999, Ball *et al.*, 1996, Worboys *et al.*, 1996 and Price *et al.*, 1995). Vg induction has never been reported utilizing liver slice technology. Vg synthesis in the liver is in response to estrogen receptor signal transduction pathways, therefore it seems feasible that liver slices have the capacity to produce Vg. Secondly, as previously mentioned, Vg induction has been demonstrated in hepatocytes. As long as the estrogens are bioavailable to the slice, Vg should be produced in liver slices as well.

When determining the usefulness of a model for estrogenicity, aspects such as sensitivity to weak estrogens and extrapolation to other species must be addressed. The use of the Vg liver slice model was applied to another salmonid species, Chinook salmon, *Oncorhynchus tshawytscha*. The rationale was that if Vg

induction was observed in the salmon, then the applicability of this model to other species would be justified. Moreover, a comparative study would also support the rainbow trout as a sensitive species for Vg induction in response to estrogen exposure. Chinook salmon is a commercially important species that has been in decline the past several years possibly due in part to xenoestrogens released into their environment. Demonstrating adverse effects on this species by environmental contaminants possessing estrogenic properties such as *o,p'*-DDE and Bisphenol A may support established evidence of the harm these compounds induce. In regards to I3C and I33', Vg induction in the salmon would solidify the trout findings that these components can be estrogenic.

CHAPTER 2

RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, AS A MODEL FOR AROMATASE INHIBITON

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ABSTRACT

The feasibility of utilizing rainbow trout, *Oncorhynchus mykiss,* as an alternative model for studying the inhibition of aromatase (CYP 19) was investigated. The suppression of estrogen dependent tumors by aromatase inhibitors has been important in the treatment of breast cancer. Estrogens, estrogen precursors and xenoestrogens have been found to promote liver cancer in the trout model. A steroid, 4-hydroxy-4-androstene-3,17-dione (4-OHA), and non-steroids, aminoglutethimide (AG) and Letrozole (CGS 20267), all of which are known aromatase inhibitors in rats and humans, were examined *in vitro* for activity in trout ovarian microsomes. Aromatase activity was quantified as the release of ^3H_2O from the conversion of [^{3}H]-4-androstene-3,17-dione to 17ß-estradiol and estrone. Trout ovarian microsomes exhibited activity between 39 - 60 fmol mg^{-1} min^{-1} with a calculated V_{max} of 71.1 fmol mg^{-1} min^{-1} when incubated at 25^{0}C with 200 nM 4-androstene-3,17-dione (K_M = 435 nM). Significant inhibition by 4-OHA up to 80% was observed at 1.5 μM. At 2000 μM, AG decreased aromatase activity by up to 82%. Letrozole reduced aromatase activity a maximum of 90% in a dose dependent manner, but the K_i (2.3 μM) was 1000 fold higher than reported in human trials. Indole-3-carbinol and some of its derivatives, two DDE isomers, and four flavones (except α-naphthoflavone) at 1000 μM did not significantly inhibit aromatase *in vitro.* Letrozole and clotrimazole, fed to juvenile rainbow trout at doses up to 1000 ppm for 2 weeks, were not effective in suppressing dehydroepiandrosterone (DHEA) induced increases in vitellogenin and 17β-estradiol levels. These results document that trout aromatase is sensitive to inhibition *in vitro* by known inhibitors of the mammalian enzyme. The mechanism(s) for lack of inhibition *in vivo* is currently unknown and must be further investigated in order to develop a trout model for studying the role of aromatase in carcinogenesis.

INTRODUCTION

Inhibition of estrogen synthesis has become a major focus in the treatment of estrogen-dependent cancers such as breast cancer. Aromatase, the CYP19 gene product, is the enzyme responsible for the conversion of androgens to estrogens, the final step in the estrogen biosynthetic pathway [1-3]. Development of specific inhibitors of aromatase has proven critical for efficacy and safety. General cytochrome P-450 inhibitors, such as aminoglutethimide, would inhibit other steroid cytochrome P-450 biosynthetic enzymes such as those involved in glucocorticoid synthesis and lead to unwanted drug-drug interactions by inhibition of other cytochrome P-450 subfamilies [4-6]. The discovery of highly potent and specific compounds, such as 4-hydroxy-4-androstene-3,17,dione (4-OHA) and Letrozole (CGS 20267), has centered interest on developing aromatase inhibitors that could be used as treatment for breast carcinomas [7-11].

It is possible that some environmental antiestrogens may function, at least in part, through aromatase inhibition. Xenoestrogens have been postulated to play a role in reproductive dysfunction, and in diseases such as cancer, both in wildlife and in humans [12-13]. Lower vertebrate models may prove to be practical alternatives to mammalian models, provided similar mechanisms of metabolism are characterized. Studies involving reptiles have demonstrated the role aromatase plays in sex determination [14-19]. The presence and effect of naturally occurring and synthetic aromatase inhibitors are not well studied. Natural products and xenobiotics, such as some flavonoids and imidazole fungicides, have been found to inhibit aromatase in rainbow trout ovarian microsomes [20,21]. Rainbow trout have become an established model for carcinogenesis [22,23] and estrogenic pathways for hepatocarcinogenesis have been documented [24]. A crucial advantage is that *in vivo* studies involving rainbow trout allow for larger sample sizes, providing stronger statistical power at a lower cost than mammalian models.

Our goal was to characterize trout ovarian microsomal aromatase and its sensitivity to inhibitors *in vitro* and *in vivo* in order to identify the role of aromatase in estrogen dependent promotion of hepatocarcinogenesis by compounds such as dehydroepiandrosterone (DHEA) [25-27]. We determined the type of inhibition and K_i values of known inhibitors of human aromatase in trout ovarian microsomes. Several dietary and environmental chemicals were assayed as potential aromatase inhibitors. We also investigated the ability of two compounds, Letrozole, 4-4'-(1-H-1,2,4-triazol-1-yl-methylene) bis-benzonitrile, (CGS 20267) and clotrimazole (1-[o-chloro-α, α-diphenyl-benzyl] imidazole), an imidazole fungicide, to inhibit aromatase activity *in vivo* by blocking DHEA induced vitellogenesis.

MATERIALS and METHODS

Letrozole (CGS 20267) was obtained as a gift from Ciba Geigy, Switzerland. [1,2,6,7-^{3}H]-4-Androstene-3,17-dione, specific activity, 93Ci / mmol, was acquired from Amersham (Buckinghamshire, England). All other chemicals were purchased from Sigma Biochemicals (St. Louis, Mo). Materials for aromatase assay and TLC were purchased from Fisher Scientific (Santa Clara, CA). Enzyme immunoassay (EIA) kits for 17β-estradiol (E_2) and testosterone (T) were developed by Cayman Chemical (Ann Arbor, MI).

Ovaries were removed from mature, vitellogenic female rainbow trout (2-3 years old), Mt. Shasta strain, euthanized with an overdose of tricane methanesulfonate (MS 222) as approved by the Oregon State University Institutional Animal Care and Use Committee and snap frozen in liquid nitrogen. Microsomes were prepared using a modified method of Guengerich [28]. Tissue was homogenized in ice cold phosphate buffer (0.1 M potassium phosphate, 0.15 M KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) with a

Polytron homogenizer (Brinkman instruments, Westbury, NY). The homogenate was centrifuged at 600 x g for 10 min and the lipid layer was removed. The remaining supernatant was centrifuged for 25 min at 10000 x g. The microsomal fraction was obtained by centrifugation of the supernatant at 100000 x g for 95 min. The pellet was washed in 0.1 M potassium pyrrophosphate, pH 7.4, containing 1 mM EDTA, 0.1 mM butylated hydroxytoluene (BHT) and 0.1 mM PMSF. The pellet was resuspended in phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and stored at -80°C. Lyophilized monkey placental microsomes, obtained as a gift from John Resko (Dept. Physiol. and Pharmacol., Oregon Health Sciences University, Portland), were also resuspended in phosphate resuspension buffer.

Aromatase activity was determined using a variation of the method measuring the release of tritiated water from the conversion of [1,2,6,7-^{3}H]-4-androstene-3,17-dione to E_2 [29]. Each incubation mixture consisted of 1 mg protein from mature rainbow trout ovarian microsomes determined by the method of Lowry *et al.* [30], the desired inhibitor concentration, 1 μCi tritiated 4-androstene-3,17-dione, 200 nM 4-androstene-3,17-dione (androstenedione), and 2 mM NADPH. The reaction mixture was brought to a final volume of 300 μl with phosphate buffer (0.1 M Tris-acetate, 0.1 M KCl, 1.0 mM EDTA, 0.1 mM butylated hydroxytoluene (BHT), pH 7.4) and incubated at 25^{0}C for 1 hr while mixing at 100 rpm on an orbital shaker. The conditions were identical for the monkey placental microsomes except that the incubation was carried out at 37^{0}C. The reaction was stopped with 1.7 ml H_20 and 4.0 ml methylene chloride. After vortexing briefly and centrifuging for 10 min at 2000 x g, the aqueous layer was removed and extracted again with methylene chloride. The aqueous layer was stripped of remaining organics with 1% dextran-coated charcoal. The mixture was centrifuged at 10000 x g for 10 min and the aqueous layer measured for $^3H_20$

released from the 1β position of androstenedione on a Beckman LS 6500 Scintillation counter. The activity was expressed in fmol mg^{-1} min^{-1} based on negative controls containing no NADPH. One way ANOVA and F-tests were performed to determine statistical significance of aromatase inhibition compared to positive controls containing vehicle alone.

Thin layer chromatography was used to verify the conversion of [^{3}H]-androstenedione to [^{3}H]-17β-estradiol using an 85:15 dichloromethane:ether solvent system [31]. The organic fraction from the tritiated water assay was concentrated to dryness under a stream of argon gas and resuspended in 100 µl methylene chloride. Using a microsyringe, 10 µl were spotted onto KSF silica gel plates, 60 Å, 5 x 10 cm, 250 µm thick, and substrates and products visualized by fluorescence detection under short wave UV light. The E_2 and androstenedione bands were cut out of the plate and distintegrations per minute (dpm) measured to quantify the percent conversion of androstenedione to E_2.

Juvenile rainbow trout, *Oncorhynchus mykiss*, 6 per treatment group, of 50 - 100 g were allocated randomly into and maintained in 375 liter flow through tanks at 14^{0}C with a 12-hour light:dark cycle. Control fish were fed a maintenance ration (2.8 % wet wt.) of Oregon Test Diet (OTD), a casein based semipurified diet [32]. Test fish received OTD with vehicle containing either 100 ppm DHEA, a high dose of the test compound (1000 ppm Letrozole or clotrimazole), or a combination of 100 ppm DHEA and test compound for 2 weeks. The vehicles for Letrozole and clotrimazole were dichloromethane and dimethylsulfoxide (DMSO), respectively, which were added to control diets and accounted for less than 0.1% of the diet. Blood samples were drawn from the caudal artery into 3 ml Vacutainer tubes containing 45 USP units of sodium heparin. The protease inhibitors, aprotinin (50 Kallikrein Inhibitory Units (KIU) / ml blood), and EDTA (1 mM) were added to each sample to reduce vitellogenin degradation. Blood was stored on ice until

plasma was obtained by centrifugation at 2000 x g for 10 min at 4^0C. Plasma was stored at -80^0C until later analyses.

Blood plasma vitellogenin, a glycophosphoprotein normally present only in mature females, has been used as a biomarker of estrogenic activity in fish, amphibians and reptiles [33-35]. Vitellogenin concentrations were determined by a modification of a previously described ELISA method [35]. Steroid levels were determined using an EIA method for E_2 and T. Colormetric readings for both immunoassays were performed on a microtiter plate reader (Biotek EL 340, Winooski, VT) and analyzed with plate reader software (Deltasoft 3, Princeton, NJ).

RESULTS

Trout ovarian microsomes exhibited aromatase activity ranging between 39 - 60 fmol mg^{-1} min^{-1}, comparable to the 70 - 80 fmol mg^{-1} min^{-1} observed for the monkey placental microsomes. The K_M and V_{max} for trout ovarian aromatase was calculated to be 435 nM and 71.1 fmol mg^{-1} min^{-1}, respectively. Kinetic analysis revealed that the steroid analogs, 4-hydroxy-4-androstene-3,17-dione (4-OHA), and 4-acetoxy-4-androstene-3,17-dione (4-OHA acetate), displayed mixed inhibition of aromatase with estimated K_i values of about 0.2 μM. Maximum inhibition was observed at 1.5 μM at which point aromatase activity was decreased by 80 % ($p < 0.01$) (Fig. 2.1A). Inhibition of trout aromatase by 4-OHA determined by the ^3H_2O assay was similar to values obtained by thin layer chromatography of the organic fraction (Table 2.1), which measured 17β-estradiol production during the incubation. With an estimated K_i of 300 μM, aminoglutethimide had a potency that was 1000-fold less than 4-OHA, although at 2000 μM, the efficacy for inhibition was similar (82 %, $p < 0.01$ (Fig. 2.1B)). Letrozole significantly inhibited aromatase activity at doses in the micromolar range ($K_i = 2.28$ μM, $p < 0.008$),

with a maximal reduction of 90% at 100 μM (Fig 2.1C). Interestingly, Letrozole displayed noncompetitive inhibition of the rainbow trout ovarian aromatase enzyme (Fig. 2.2) with a potency that was about 1000 fold less than has been reported for the human and rodent enzyme [36,37]. Clotrimazole was found to significantly inhibit ovarian microsomal aromatase activity by up to 92% at concentrations above 10 μM (Fig 2.3).

Several dietary and environmental compounds known to have estrogenic and antiestrogenic activities, were screened for aromatase disrupting properties. Indole-3-carbinol, its acid condensation reaction mixture products [38], and purified dimer, 3,3'-diindolylmethane, had no effect on aromatase activity at concentrations up to 1000 μM (Table 2.2). Our laboratory had previously shown that 3,3'-diindolylmethane was an effective inhibitor of trout, rat and human drug-metabolizing cytochrome P450s, with K_is in the low micromolar range [39]. Neither *o,p'*- nor *p,p'*- DDE altered aromatase activity at the highest concentrations. Flavone derivatives have been shown to inhibit aromatase in human preadipocytes and ovarian and placental microsomes [40,41]. α-Naphthoflavone significantly inhibited trout ovarian aromatase at concentrations of 1000 μM, but only to a maximum reduction of 40% compared to controls (Table 2.2). Other flavones tested, including chrysin, apigenin, and kaempferol, did not inhibit aromatase at concentrations up to 1000 μM (Table 2.2).

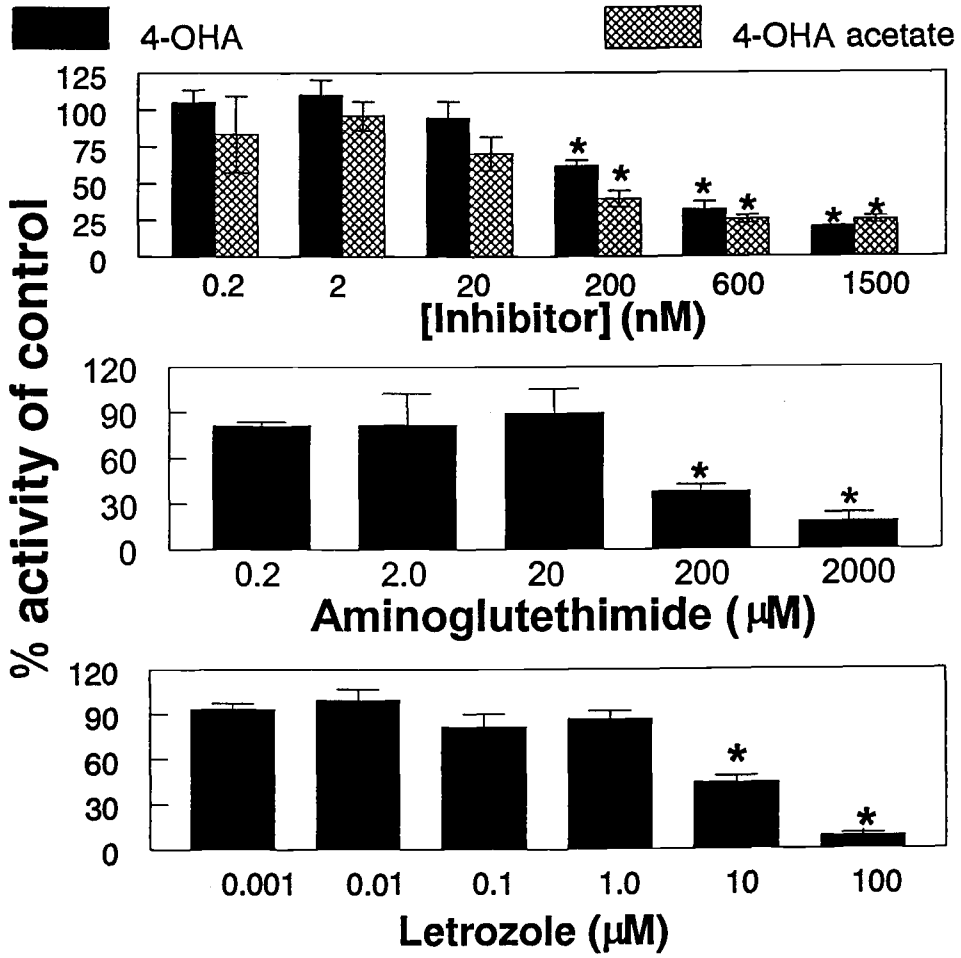

Figure 2.1: *In vitro* inhibition of trout ovarian microsomal aromatase by 4-OHA and 4-OHA acetate (A), aminoglutethimide (B), and Letrozole (C), expressed as percent activity of positive controls. Bars represent ±SE (n = 4 / group). * denotes $p < 0.01$ (ANOVA F-test).

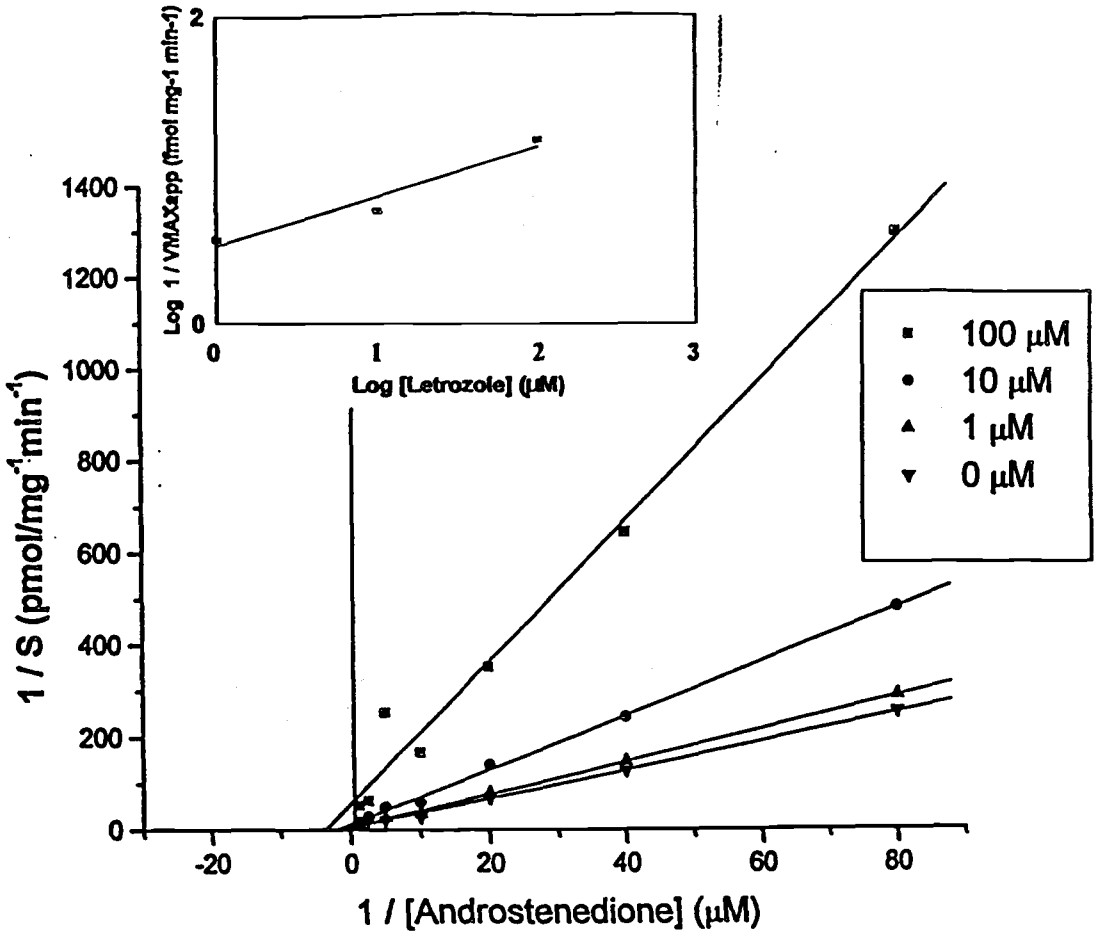

Figure 2.2: Lineweaver-Burke plots of Letrozole indicate non-competitive inhibition with androstenedione at concentrations of 12.5 - 800 nM. The K_i was calculated to be 2.3 μM from the inhibition curve (inset).

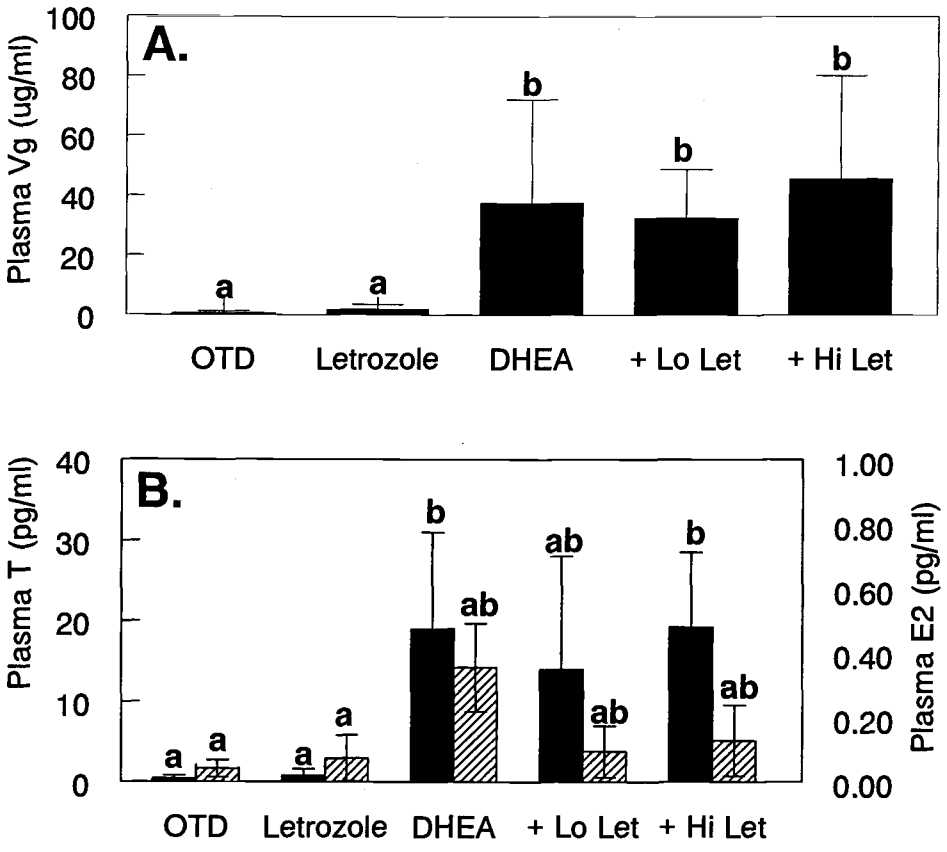

Figure 2.3: Blood plasma analyses of juvenile rainbow trout fed DHEA and/or a low dose (100 mg/kg/day) or a high dose (1000 mg/kg/day) of Letrozole **(A)** Blood serum vitellogenin levels: different letters represent significant differences between comparison groups ($p < 0.05$). **(B)** Blood serum T (solid bars) and E_2 (striped bars) concentrations: different letters represent significant differences between comparison groups ($p < 0.05$). Bars represent $\pm$SE ($n = 6$ / group).

Table 2.1. Comparison of percent inhibition of trout ovarian microsomal aromatase activity by 4-OHA measured by thin-layer chromatography (TLC) and tritiated water (^3H_2O) assay. Values are expressed as percent dpm compared to controls. For TLC analyses, bands corresponding to standards on the plate visualized by UV light were cut out, sonicated in methylene chloride, put in Ultima Gold scintillation cocktail and dpm quantified by scintillation counting.

Thin-layer chromatography			*^3H_2O Assay*	
4-OHA (nM)	**E_2 (% control)**	**SE (+/-)**	**Activity (% control)**	**SE(+/-)**
0	100.00	9.45	100.00	13.04
200	59.49*	3.83	61.90*	3.55
600	25.03*	0.48	31.84*	5.67

* Significant decreases in were observed at these concentrations of 4-OHA compared to control ($p < 0.01$, t-test).

Table 2.2. Percent inhibition of trout ovarian microsomal aromatase activity by flavones, indoles and DDEs (* α-naphthoflavone significantly inhibited aromatase at 1000 μM ($p < 0.05$, ANOVA F-test). # Induction of aromatase was observed with DDEs, but were not significant ($p > 0.05$, ANOVA F-test).

Chemical[a]	Concs (μM)	Max inihibiton (% control)
Flavones		
Kaempferol	10, 100	91.3 ± 12.8
Apigenin	10, 1000	89.5 ± 2.2
Chrysin	10, 1000	87.1 ± 22.0
α-naphthoflavone	10, 1000	62.9 ± 10.6*
Indoles		
I3C	1, 10, 100, 1000	92.6 ± 2.1
I33'	1, 10, 100, 1000	78.9 ± 3.1
I3C rxn mixture	1, 10, 100, 1000	85.9 ± 8.1
DDEs		
o,p' DDE	100, 1000	105.0 ± 14.8#
p,p' DDE	100, 1000	146.3 ± 15.7#

[a] Abbreviations: Indole-3-carbinol (I3C), 3,3'-diindolylmethane (I33')

An *in vivo* study was conducted to analyze vitellogenin suppression by Letrozole in DHEA treated juvenile trout. Our laboratory had previously documented induction of vitellogenin in trout by DHEA [42]; an estrogenic mechanism of DHEA may be responsible for its promotion of liver cancer in the trout model [25-27, 42]. In this study, we observed a 60-fold induction of vitellogenin after two weeks of feeding with 100 ppm DHEA in both males and females ($p < 0.0001$). There was no observed sex difference in either the control or the DHEA treated group. Letrozole did not significantly decrease DHEA-induced vitellogenin production at doses of 100 and 1000 ppm ($p = 0.14$) (Fig. 2.4A). E_2 and T production was increased by 8- and 34- fold ($p < 0.0002$ and $p < 0.0001$, respectively), respectively, by DHEA as determined by EIA analysis (Fig 2.4B). The levels of E_2 in DHEA treated trout co-treated with Letrozole decreased, suggesting an inhibition of aromatization. This decrease, however, was not statistically significant ($p = 0.07$).

Clotrimazole was tested for inhibiting properties *in vivo*. It was not an effective inhibitor of DHEA induced E_2 plasma levels at doses up to 1000 ppm, consequently, vitellogenin production was not inhibited significantly either (data not shown).

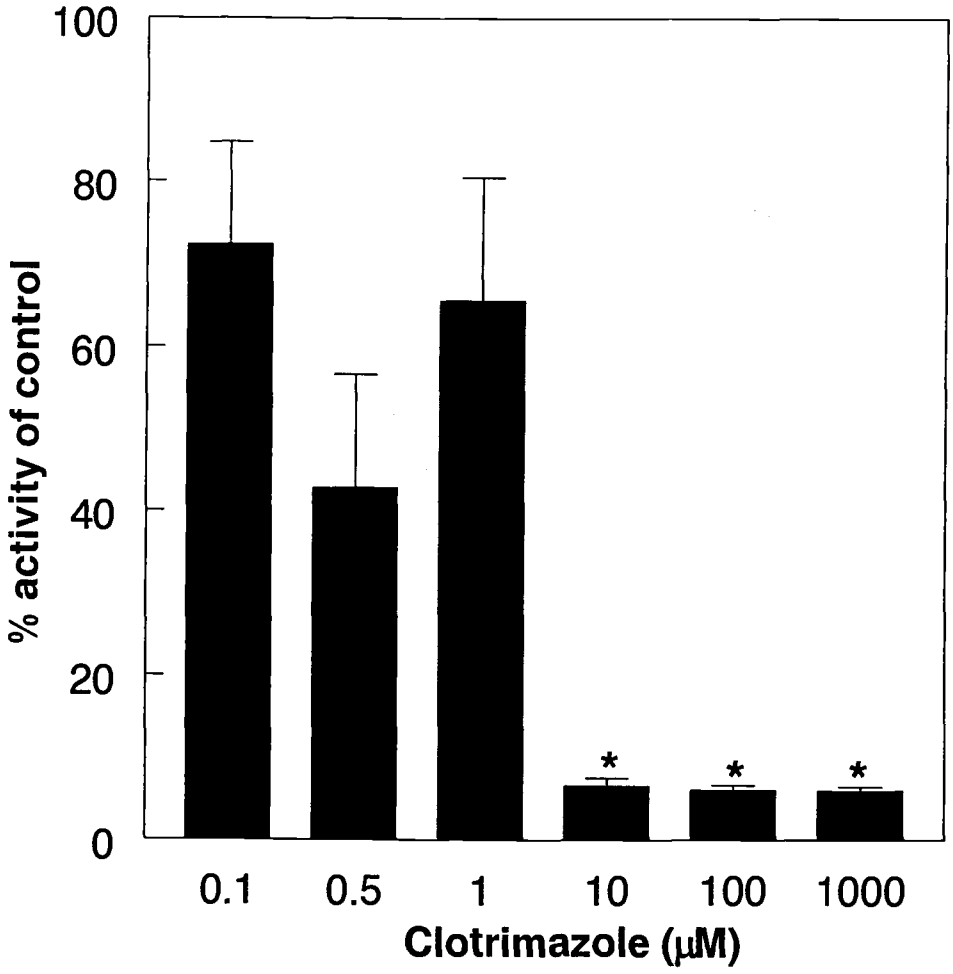

Figure 2.4: Dose response of clotrimazole *in vitro* on aromatase activity of trout ovarian microsomes. Bars represent ± SE (n = 4 / group). * denotes $p < 0.0005$ (ANOVA F-test).

DISCUSSION

Aromatase activity observed in mature female rainbow trout ovarian microsomes was comparable to that observed in mammalian estrogenic tissues such as monkey placenta. Trout ovarian microsomes proved to be a useful model for discovering effective *in vitro* aromatase inhibitors. Several compounds of varying structures were found to be effective and potent aromatase inhibitors *in vitro*. The most potent inhibitor *in vitro* was 4-OHA, a steroid analog of androstenedione, which has been demonstrated to be an irreversible inhibitor in primates [43]. Aminoglutethimide, a compound used to treat estrogen dependent breast cancer, achieved similar maximal inhibition of aromatase activity *in vitro* compared to 4-OHA, but required a 200-fold higher dose for this response. Our *in vitro* data support the concept that the rainbow trout aromatase enzyme itself is similar to other species in activity, but at least in the case of Letrozole, the mechanism and efficacy of inhibition are different.

Two compounds reported as orally bioavailable in humans, Letrozole and clotrimazole, were tested further for the possibility of inhibiting aromatase *in vivo*. Neither Letrozole nor clotrimazole blocked vitellogenin production or E_2 synthesis *in vivo*, at doses up to 1000 ppm. Based on our *in vitro* data and previous data reported in mammalian studies, these results were unexpected. This suggests that rainbow trout may exhibit a species difference compared to humans for aromatase inhibition or that there are pharmacokinetic reasons these two compounds are not reaching the target organ at levels sufficient for inhibition. E_2 levels tended to be lower with Letrozole treatment *in vivo*, suggesting inhibition of aromatizing androgens. The high variablility between individual fish, a common observation because they are not as inbred as some mammalian biological models, may have accounted for the lack of an observed significant decrease ($p = 0.07$).

There were differences in activity for Letrozole in trout aromatase compared to mammals. Bioavailability is not an issue when incubating with ovarian microsomes, and perhaps there is a difference in metabolism and absorption of these chemicals *in vivo* compared to mammals. To increase sensitivity, juvenile trout (< 18 months) were experimental subjects, because at this age they have very low circulating E_2, T, and vitellogenin levels; juvenile trout fed the proandrogen DHEA respond with elevated levels of all three parameters. As estrogens are known liver tumor promoters in trout, our goal was to evaluate the role of E_2 synthesis in DHEA carcinogenesis by aromatase inhibition *in vivo*. The dose of DHEA used was based on tumor study data and was perhaps too high for vitellogenin and steroid inhibition studies. By looking at inhibition of endogenous steroids in mature fish, confounding variables involving DHEA cotreatment would be eliminated, possibly revealing aromatase inhibiting activity. Unlike humans, rainbow trout do not have appreciable circulating levels of this androgen precursor in their blood [44,45]. There is the potential for unique metabolic pathways in rainbow trout to upregulate aromatase, counteracting any inhibitory effect, to enhance elimination, or to render the inhibitor unavailable to target organs by binding to free inhibitor or metabolizing it to an inactive form. Future studies to determine the role of gonadotropins and serum binding proteins will hopefully shed light on this issue.

Although 4-OHA was a potent *in vitro* aromatase inhibitor, this compound was not tested *in vivo* because it requires dosing via intramuscular injection and the goal of this study was to find an effective aromatase inhibitor that could be administered orally for long term tumor studies. Due to the low specificity of aminoglutethimide towards inhibiting aromatase, corticoid co-treatment would have been necessary for an *in vivo* study, thereby introducing confounding variables. This is because in mammalian models, aminoglutethimide has be

documented to suppress adrenal function, specifically 18-hydroxylase, the enzyme that converts corticosterone to aldosterone, with a higher specificity than aromatase [46].

Optimizing an established assay used to quantitate aromatase activity in mammalian microsomes, aromatase activity in rainbow trout ovaries was determined *in vitro* to be similar to mammals *in vitro*. Known mammalian inhibitors were largely successful in blocking *in vitro* E_2 synthesis in rainbow trout ovarian microsomes, although in the case of Letrozole, which was 1000 times less potent in trout compared to mammals, the activites were different. This demonstrates that the rainbow trout model is suitable for evaluating potential aromatase inhibitors *in vitro*. Caution must be used when extrapolatng from *in vitro* results to whole animal effects as shown by the ineffectiveness of Letrozole and clotrimazole to inhibit estrogen synthesis *in vivo*. The rainbow trout is still a very good candidate for modelling aromatase inhibition with respect to screening environmental chemicals. The mechanisms of endocrine modulating compounds that alter estrogen synthesis in aquatic and terrestrial animals can also be investigated using the rainbow trout. It seems likely that some estrogenic and antiestrogenic compounds act directly on the aromatase enzyme either by inhibition or induction, and this method is a quick means for the screening of such compounds. It is advantageous to discover species differences because it will allow for a better understanding of mechanisms and evolution of the aromatase enzyme. These limited *in vivo* studies do not support the use of rainbow trout as a model for human aromatase inhibition without further development, but they can be used as an environmental model and potentially for endocrine dependent cancers in the future.

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CHAPTER 3

THE NON-AROMATIZABLE ANDROGEN, DIHYDROTESTOSTERONE, INDUCES ANTIESTROGENIC RESPONSES IN THE RAINBOW TROUT

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ABSTRACT

In order to satisfy government mandates, numerous studies have been performed categorizing potential endocrine disrupting chemicals as (anti)estrogens or (anti)androgens. We report here that dihydrotestosterone (DHT), a potent, non-aromatizable androgen receptor agonist, induces antiestrogenic responses through direct and/or indirect modulation of vitellogenin (Vg), steroid hormone and total cytochrome P450 levels. DHT and two weak, aromatizable androgens, DHEA and androstenedione, (0.05 – 50 mg/kg/day) were fed to juvenile trout for 2 weeks. DHEA and androstenedione significantly increased blood plasma Vg by up to 30 and 45-fold, respectively ($p < 0.05$, t-test). 17β-Estradiol (E_2) increases were also observed with both androgens, albeit to a lower degree. DHT markedly decreased Vg and E_2 levels, suggesting that Vg and E_2 increases elicited by DHEA and androstenedione were via conversion to E_2. DHEA and androstenedione had no effect on total cytochrome P450 content, while DHT significantly decreased P450 content in a dose-dependent fashion. These results indicate that alterations in metabolism mediated by androgen receptor binding may be responsible for the Vg and E_2 decreases by DHT. In an attempt to distinguish between receptor and non-receptor androgenic mechanisms of the observed DHT effects, DHT (0, 50 or 100 mg/kg/day) and flutamide (0 - 1250 mg/kg/day), an androgen receptor antagonist, were fed to juvenile rainbow trout for 2 weeks. Flutamide alone was as effective as DHT in decreasing E_2 and Vg levels in males but did not significantly antagonize DHT induced Vg decreases in either sex ($p > 0.05$, F-test). DHT decreases in total P450 content were partially attenuated in males by flutamide co-treatment, but not females, suggesting a partial androgenic mechanism underlies P450 decreases as well as a fundamental sex difference responding to androgen receptor binding. Moreover, flutamide alone decreased P450 content by up to 30% in males and 40% in females. These effects may be mediated through direct androgen receptor

binding irrespective of whether the binding is agonistic or antagonistic. This study indicates that androgen receptor agonists/ antagonists can elicit significant antiestrogenic effects that may not necessarily be mediated through classic receptor binding mechanisms and signal transduction pathways. (Supported by ES03850 and ES07060)

INTRODUCTION

Modulation of carcinogenesis, enzyme induction/inhibition and sex determination by estrogenic compounds have become an increasingly important area of research. The influence of androgens on such responses has been studied to a much lesser extent. Dehydroepiandrosterone, (DHEA) a weak androgen, has been established as a potent liver tumor promoter in rainbow trout in the absence of peroxisome proliferation [1,2]. DHEA is also a precursor to estrogens such as 17β-estradiol (E_2), a potent promoter of carcinogenesis in many organs such as the liver and breast [3-8]. Androstenedione can be directly converted to testosterone via 17β-hydroxysteroid dehydrogenase or estrone via aromatase. E_2 is synthesized via aromatase from testosterone or estrone by the dehydrogenase enzyme [4]. Several mechanisms have been postulated for tumor promotion by estrogens such as production of catechol metabolites that can induce redox cycling and subsequent DNA damage and covalent binding, to increases in cell growth and division [3-8]. Since DHEA is converted to androstenedione, a precursor to potent androgens such as testosterone, dihydrotestosterone (DHT) and in teleosts, 11-ketotestosterone (11kT), the ability to distinguish between estrogenic and androgenic responses is critical.

Vitellogenin (Vg) is an egg-yolk protein precursor synthesized in the liver of oviparous animals such as fish, amphibians, reptiles and birds in response to estrogen receptor binding [9]. It is secreted into the blood where it is taken up by

the ovaries to be incorporated into the developing eggs [10]. Vg synthesis is a well characterized, sensitive biomarker for estrogen receptor binding in the rainbow trout liver [11]. Many studies have utilized Vg production to screen for and determine the efficacy and potency of environmental contaminants such as alkylphenols, DDEs, and PCBs (12-18). Phytochemicals and natural products that modulate Vg production have been postulated as possible promoting or chemopreventive agents in estrogen dependent cancers (19,20).

The contribution of natural and synthetic androgens to endocrine disruption has not been studied to nearly the degree of estrogens. In some cases androgens are investigated for estrogenic effects instead of pure androgenic mechanisms. In previous studies, the potent androgens, dihydrotestosterone (DHT) and 17α-methyltestosterone were shown to induce Vg in fish [21,22]. These studies, however, were carried out at extremely high doses (1000 mg/kg) and were not believed to be physiologically relevant. In contrast, another study reported that 17α-methyltestosterone inhibited Vg gene expression and serum E_2 levels in tilapia [23]. One reason for the contrasting results in the few studies that have been performed, may be the lack of a well characterized biomarker like Vg. In mammals, screening for prostate specific antigen levels in blood serum is an accepted biomarker for androgen status to indicate high risk subjects for prostate cancer. In mice, the use of 6α/15α-OH ratios has been proposed as another biomarker, which is superior to measuring circulating hormone levels, which are highly variable and make detection of alterations by xenobiotics difficult [24]. In rats, testosterone and DHT were found to lower luteinizing hormone secretion in the presence and absence of E_2 [25].

Alterations in steroid metabolizing enzymes, either through modulation of synthesis or degradation could have profound effects on estrogenic pathways. Numerous hepatic Phase I and II (detoxifying and conjugating, respectively)

enzymes metabolize estrogens and androgens [26-28]. Marked shifts in the levels and activities of xenobiotic metabolizing enzymes, as previously demonstrated [29-31], would presumably affect the type and degree of estrogenic and androgenic responses. The importance of this and other indirect mechanisms of action by androgens may affect responses that are classically regarded as estrogen mediated [32]. In one study, You *et al.* [33] found that male rats exposed to *p,p'* DDE had elevated cytochrome P4502B1 and 3A1 protein levels which resulted in an increase in the respective hydroxylated testosterone products. Administration of 17β-estradiol to rainbow trout lowered mean hepatic 6β-hydroxylase activity and total cytochrome P450 content which indicates that steroids can modulate metabolic enzyme protein levels and activity [34].

In this paper, we compared the ability of DHEA, androstenedione, and DHT, a non-aromatizable androgen, to elicit estrogenic responses, by measuring Vg induction in rainbow trout. We also quantified blood plasma E_2 in order to measure conversion rates of each androgen. In an attempt to shed light on some possible metabolic effects of androgens, we quantified total liver microsomal cytochrome P450 content for each group. Additionally, we investigated the possible mediation of Vg, E_2 and total cytochrome P450 content through the androgen receptor utilizing the antiandrogen, flutamide. The aim of these studies was to determine sensitive endpoints and indicators of androgen exposure and to distinguish them from estrogenic responses that would serve as a basis for future studies.

METHODS and MATERIALS

Juvenile Mt. Shasta rainbow trout, *Oncorhynchus mykiss*, 12-18 months old, were kept in 375 liter tanks at 14^0C under a 12:12 hr light:dark cycle and fed a maintenance ration (2.8% w/w) of Oregon test diet (OTD). To determine the

estrogenic contribution of the three androgens in this experiment, treatment groups of 12 rainbow trout were fed OTD containing DMSO (<0.2% by volume) with or without dissolved DHEA, androstenedione or DHT (0.05 – 50 mg/kg/day) for 14 days. In a second study, we focused on the relationship of the effects observed in the first study with the androgen receptor. Rainbow trout, 16 per treatment group, were administered diets of OTD containing DHT (0, 50 or 100 mg/kg) or flutamide (0, 100, 250, 750 or 1250 mg/kg) dissolved in DMSO for 14 days.

On day 15 of both studies, fish were euthanized with an overdose of tricane methanesulfonate (MS222) following protocols approved by the Oregon State University Institutional Animal Care and Use Committee. Blood was collected from the caudal vein into heparinized vials containing 1 mM EDTA and 50 KIU/ml aprotinin to slow Vg degradation. Plasma was collected by centrifugation of blood at 2000 x g for 10 min at 4^0C and stored at -80^0C. Livers were weighed, snap frozen in liquid N_2 and stored at -80^0C.

Blood plasma Vg was quantified using an ELISA described by Donohoe and Curtis [13] and Shilling and Williams [35]. Antibody used was raised in rabbits against chum salmon Vg (1:1500 dilution), which was graciously obtained by way of Donald Buhler, Oregon State University from A. Hara at Hokkaido University. Colorimetric readings for the ELISA were performed on a microtiter plate reader (Biotek EL 340, Winooski, VT) and analyzed with plate reader software (Deltasoft 3, Princeton, NJ). The linear range of a given assay was determined to be between 6.25 and 3200 ng Vg / ml of sample. Samples were diluted accordingly in phosphate buffered solution, pH 7.2, containing 1% bovine serum albumin, 0.1 % Triton X-100 and 0.1% Tween-20. Although rabbit anti-trout Vg antibody was available, we found that the anti-chum salmon antibody gave relatively similar values throughout the linear concentration range with about a third of the background, allowing for greater sensitivity (data not shown). A 100

µl aliquot of each blood plasma sample was taken for steroid analysis. These samples were extracted twice with 2 ml diethyl ether and vortex shaken for 20 sec. The tubes were placed in liquid N_2 for 8 sec and then the ether layer was decanted and collected. The extract was concentrated to dryness using a SpeedVac concentrator system (Savant, Holbrook, NY), resuspended in 1 ml of EIA buffer and stored at – 20^{0}C. 17β-Estradiol (E_2) and testosterone (T) levels were measured by EIA with reagents from Cayman Chemical (Ann Arbor, MI). Statistical analyses performed within sex groups only using t-tests for treatment effect and one-way ANOVA and F-tests comparing differences between treatment groups.

Microsomes were prepared from livers by the method of Guengrich [36]. Briefly, tissue homogenized in cold 0.1 M phosphate buffer containing 0.15 M KCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was centrifuged for 25 min at 10000 x g at 4^{0}C. The microsomal fraction was obtained by spinning the supernatant at 100000 x g for 95 min at 4^{0}C. The pellet was washed by resuspension in 0.1 M potassium pyrophosphate containing 1 mM EDTA, 0.1 mM BHT and 0.1 mM PMSF followed by a second 95 min centrifugation at 100000 x g. The final microsomal pellet was resuspended in phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF and stored at -80°C. Total liver microsomal P450 content was quantified by the CO versus CO-reduced difference spectra [37] on a Cary 300 UV-Vis spectrophotometer (Varian, Walnut Creek, CA). Values are reported as nmol P450 and normalized to protein content determined by the Lowry method using bovine serum albumin as the standard [38].

RESULTS

After two weeks, untreated female trout were found to have significantly higher levels of Vg than males, representing a fundamental sex difference in basal

Vg levels, even in immature trout ($p < 0.01$, t-test). Comparisons were performed intrasex only, due to significant sex differences in Vg levels ($p < 0.05$, one-way ANOVA and ANOVA F-test) in most of the treatment groups as well. A dose related increase in plasma Vg was observed in trout fed DHEA and androstenedione (Figure 3.1A). In trout fed DHEA, Vg levels increased in males and females with about 30-fold induction observed at 50 mg/kg/day ($p < 0.0001$) compared to corresponding control (OTD). The Vg response to androstenedione administration was similar to that of DHEA with female levels generally somewhat higher than male levels. Maximum induction at 50 mg/kg/day androstenedione was 45-fold for females and 30-fold for males compared to controls fed vehicle. In contrast, induction of Vg was not observed in DHT fed groups (Figure 3.1B). In fact, Vg significantly decreased by up to 80% in male and female trout fed DHT compared to trout fed vehicle only ($p < 0.05$, F-test).

As was the case with Vg, basal E_2 levels were twice as high in untreated females compared to untreated males ($p < 0.01$, t-test, Figure 3.2). Unfortunately, we were only able to report values for males due to loss of some female samples. Plasma E_2 levels were significantly higher in males fed 50 mg/kg DHEA or 5 and 50 mg/kg androstenedione compared to controls ($p < 0.05$, t-test). A 3-fold induction compared to OTD was observed at 50 mg/kg for DHEA and androstenedione. These increases support the hypothesis that these androgens are indeed being converted to estrogens *in vivo*. If the previously observed estrogenic effects are through direct conversion to estrogens, we would not expect increased E_2 in trout fed an androgen that cannot be aromatized, such as DHT. At 50 mg/kg DHT, we actually noticed a significant 55% reduction in male plasma E_2 (Figure 3.2, $p < 0.005$, t-test). The E_2 decrease may be the basis of the Vg decrease since the values of the two parameters corresponded closely.

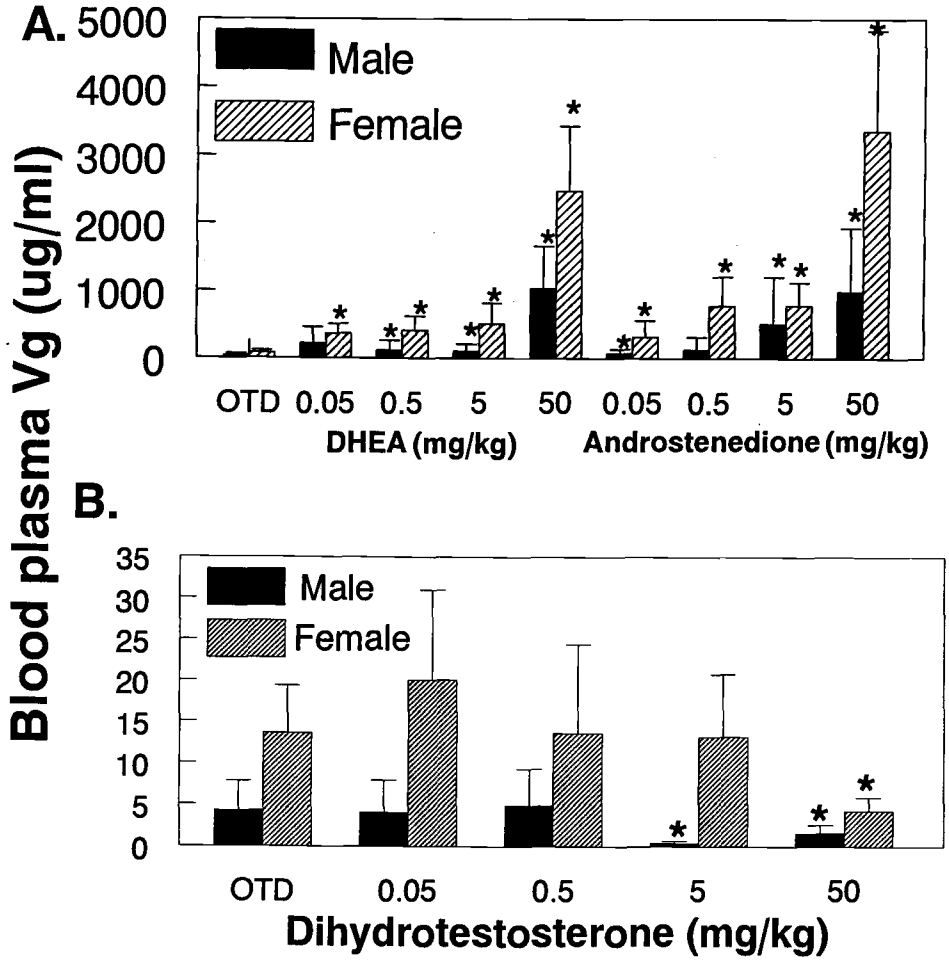

Figure 3.1: Plasma Vg for male and female trout fed 0.05 – 50 mg/kg/day (A) DHEA and androstenedione and (B) DHT for 2 weeks. Significant sex differences were observed in most groups. (*) denotes significant Vg modulation compared to controls ($p < 0.05$, t-test assuming unequal variances). Error bars represent $\pm$ SE, ($n = 4 – 8$, total $n = 12$).

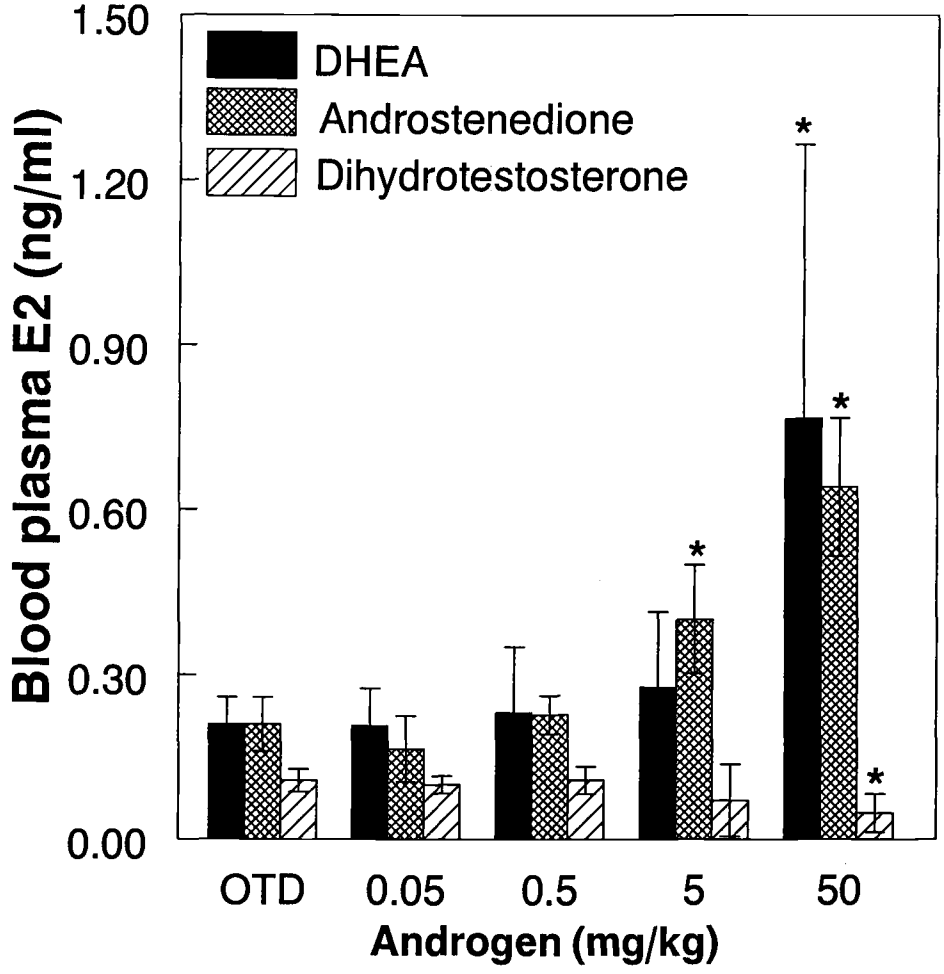

Figure 3.2: Plasma E_2 levels in male trout fed 0.05 – 50 mg/kg/day DHEA, androstenedione and DHT for 2 weeks. E_2 was not reported for female trout due to loss of samples. Significant differences in E_2 from controls are denoted with (*), ($p < 0.05$, t-test assuming unequal variances). Error bars represent $\pm$ SE, ($n = 4 – 8$).

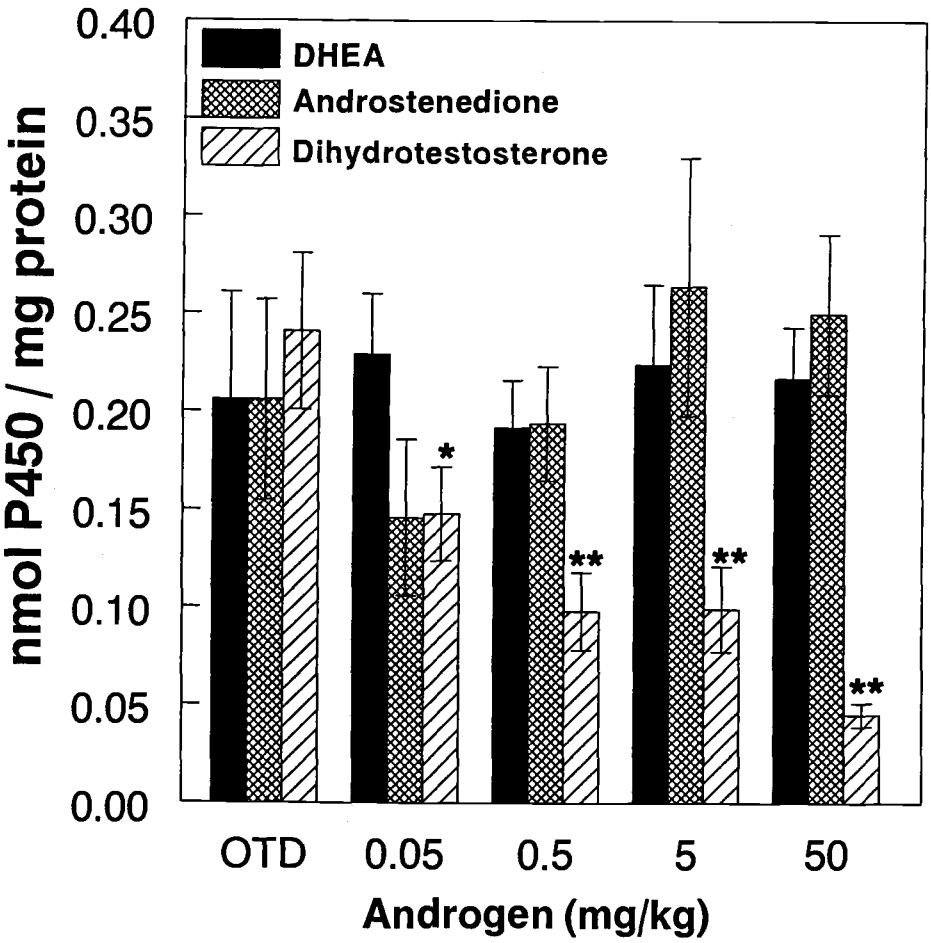

Figure 3.3: Total liver P450 content in trout fed 0.05 – 50 mg/kg/day DHEA, androstenedione or DHT for 2 weeks. Sex data are pooled since no differences were observed in any group($p > 0.05$). Error bars represent $\pm$ SE ($n = 12$ or 15, respectively). (*) and (**) denote values significantly lower than control ($p < 0.03$ and 0.0005, respectively, t-test assuming unequal variances).

To investigate possible indirect effects on Vg and E_2 levels by androgens via alterations in metabolism, total liver microsomal cytochrome P-450 content was quantified. In trout fed DHEA or androstenedione, total cytochrome P-450 content was not affected (Figure 3.3). DHT, however markedly decreased P450 content in a dose responsive fashion at all doses compared to OTD (Figure 3.3, $p < 0.03$ at 0.05 mg/kg and $p < 0.0005$ at doses above 0.05 mg/kg, t-test). At the highest dose of DHT, an 81% decrease was observed.

In the first study, the non-aromatizable androgen, DHT, exhibited antiestrogen-like characteristics in the rainbow trout. We hypothesized that some of these effects may be mediated through androgen receptor binding and subsequent down-regulation of aromatizable androgen and/or estrogen synthesis, which would explain the observed Vg and E_2 decreases. To test this hypothesis, DHT was fed at two high doses (50 and 100 mg/kg/day) to trout with flutamide, an effective mammalian androgen receptor antagonist. After 2 weeks, the antiestrogenic responses were observed in both male and female rainbow trout in the absence of flutamide. At 50 mg DHT/ kg/day, Vg levels were 80% lower than trout fed vehicle (Figure 3.4). At 100 mg DHT/kg/day, this response was not as marked, possibly due to some estrogen receptor agonism, especially in males where Vg levels were higher than controls. The decrease, however, appeared to be in response to a decline in E_2 levels which was also observed in males and females fed DHT (Figure 3.5). Flutamide did not antagonize the DHT effect, suggesting that the Vg and E_2 decreases were not due to pathways directly related to androgen receptor binding. It does seem that the androgen receptor plays some role because this receptor antagonist was as effective as DHT in decreasing Vg and E_2 levels, particularly in males. DHT increased T levels two-fold at 100 mg/kg, an effect that was partially blocked by increasing flutamide dose (Figure 3.6). Flutamide had no effect on trout fed 50 mg/kg DHT and there was high variability, making it difficult

to generate relationships between T and the Vg and E_2 decreases. Flutamide alone, however, had a profound effect on female T levels, decreasing the steroid by 90% at 1250 mg/kg ($p < 0.0005$, F-test, Fig 3.6). This decrease was not observed in males, suggesting the presence of a sex-specific antiandrogenic feedback mechanism.

We investigated a possible metabolic effect of DHT through androgen receptor binding by measuring total P450 content in liver microsomes from trout fed DHT and flutamide. Although there were no significant differences in liver somatic index between groups, DHT lowered P450 content significantly in males and females (Figure 3.7). Flutamide had a substantial impact on this effect in males, blocking P450 decreases induced by DHT in a dose-dependent manner. In females, total P450 content was similar with or without flutamide. Flutamide alone also lowered total P450 content by 30% in males and 40% in females. These results suggest a sex specific role for the androgen receptor pathway that may not be related to classic agonistic/ antagonistic mechanisms.

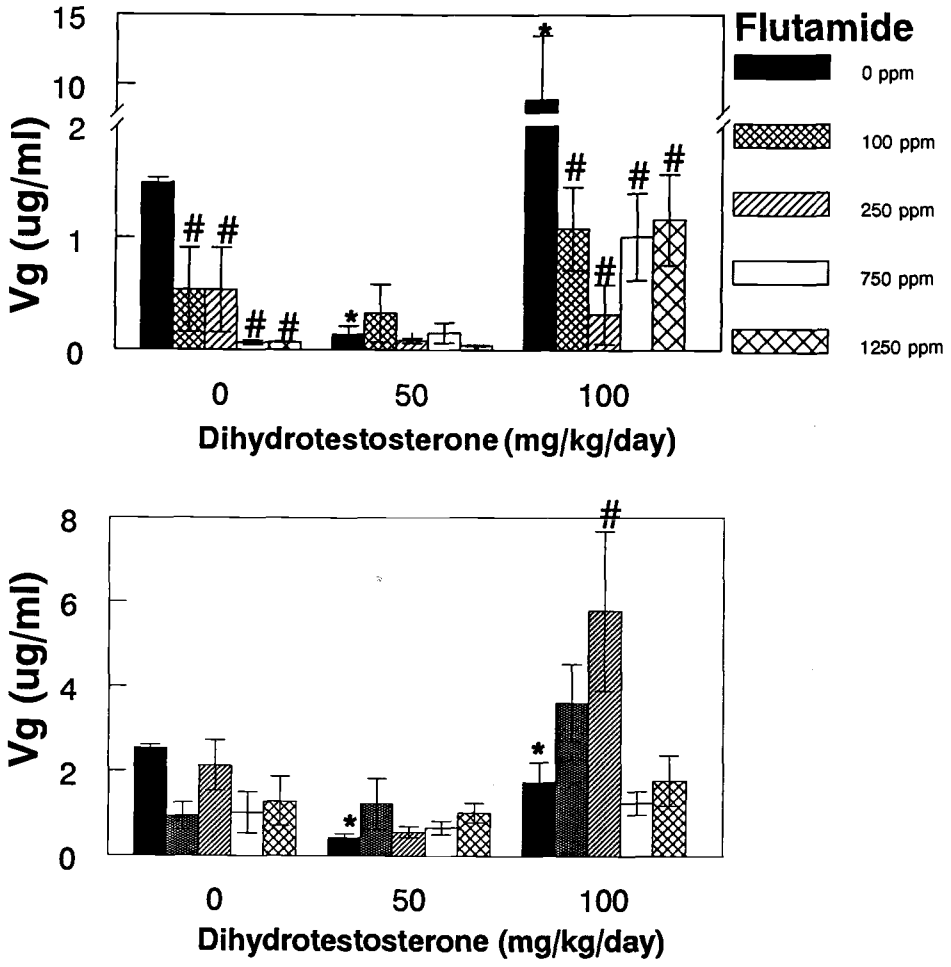

Figure 3.4: Blood plasma Vg for male (upper graph) and female (lower graph) trout fed DHT and flutamide. Error bars represent ± SE (n = 4 – 12). (*) denotes values significantly different from trout fed vehicle ($p < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only, ($p < 0.05$, ANOVA F-test).

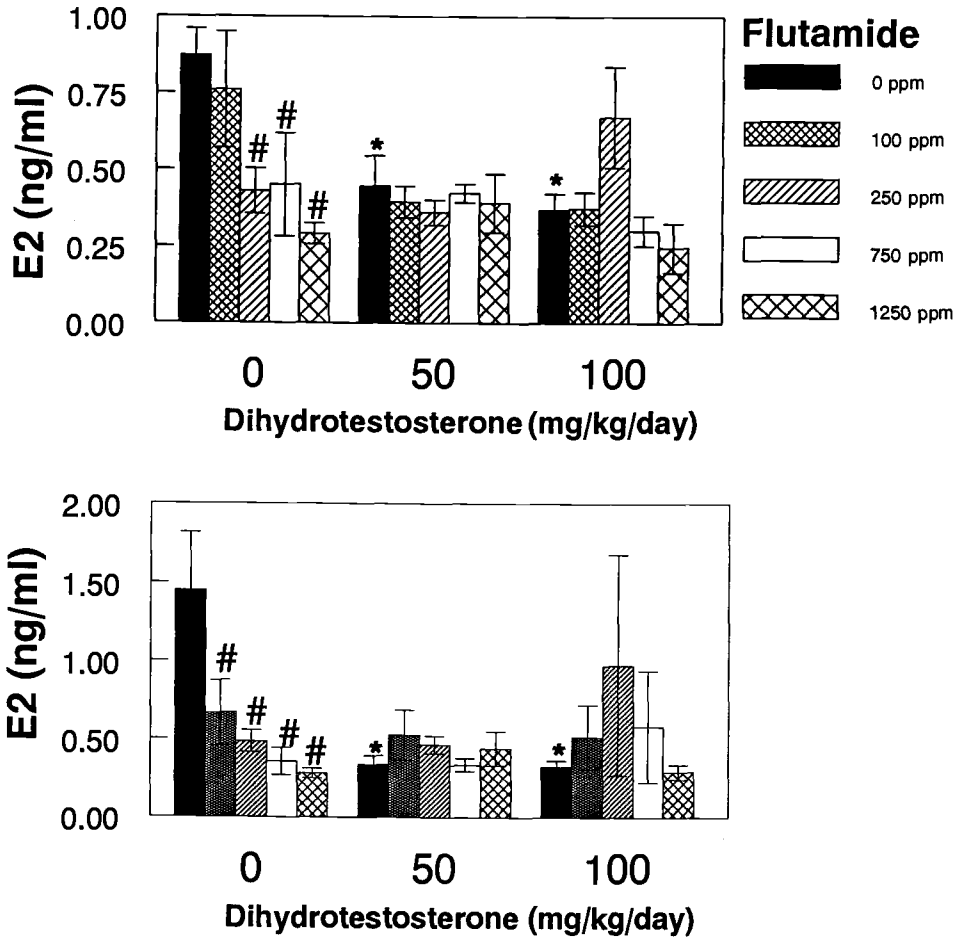

Figure 3.5: Blood plasma E_2 for male (upper graph) and female (lower graph) trout fed DHT and flutamide. Error bars represent $\pm$ SE (n = 4 – 12). (*) denotes values significantly different from trout fed vehicle ($p < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only, ($p < 0.05$, ANOVA F-test).

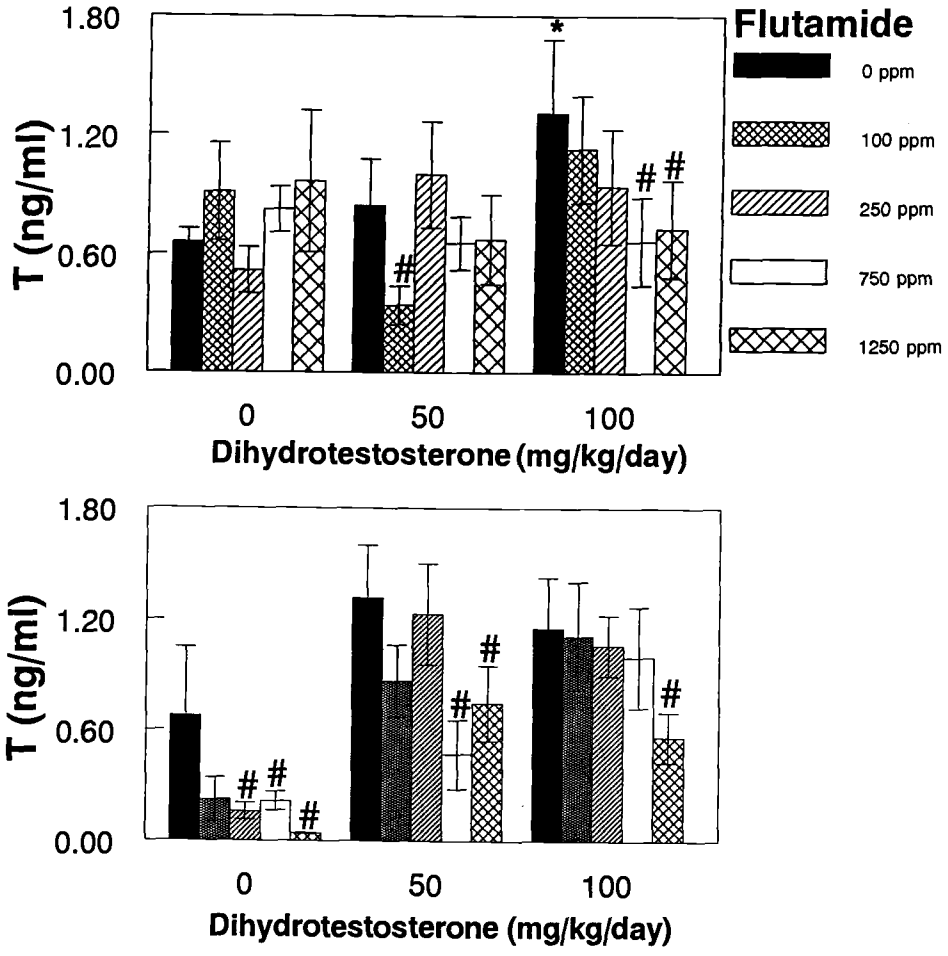

Figure 3.6: Blood plasma testosterone for male (upper graph) and female (lower graph) trout fed DHT and flutamide. Error bars represent ± SE (n = 4 – 12). (*) denotes values significantly different from trout fed vehicle ($p < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only, ($p < 0.05$, ANOVA F-test).

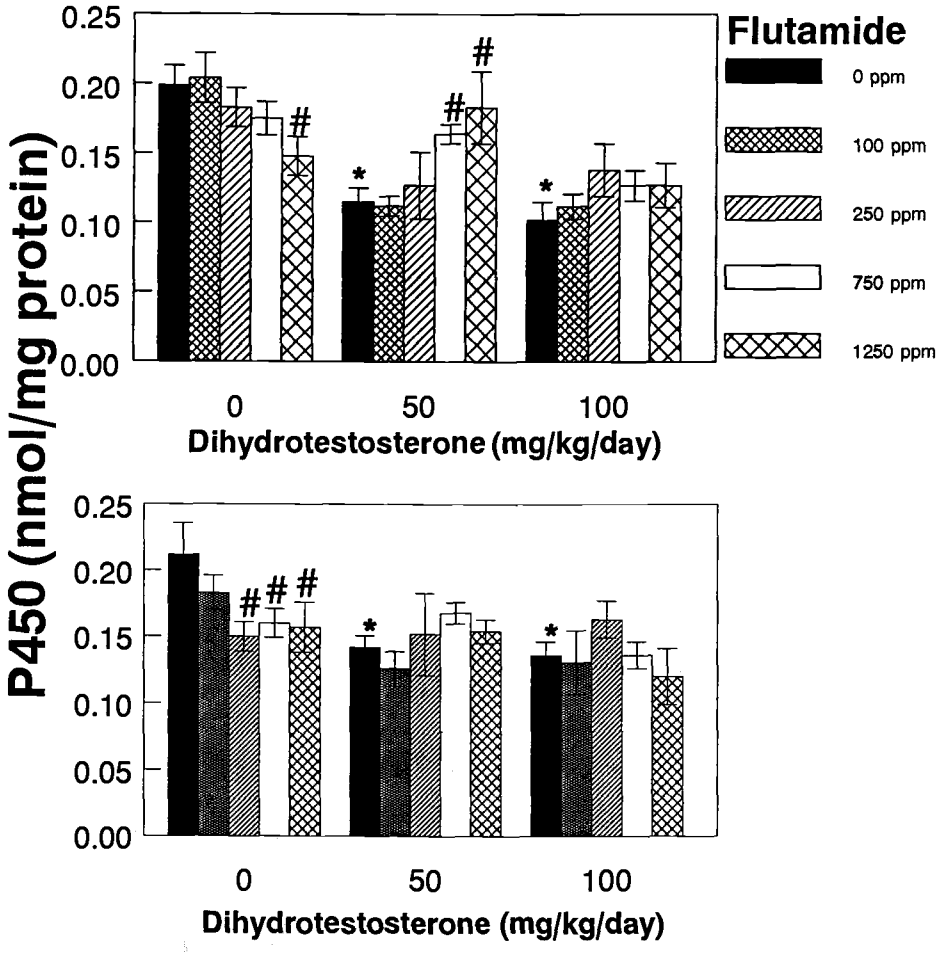

Figure 3.7: Total P450 content for male (upper graph) and female (lower graph) trout liver microsomes. Error bars represent ± SE (n = 4 – 12). (*) denotes values significantly different from trout fed vehicle ($p < 0.05$, t-test assuming unequal variances. (#) denotes significant difference from corresponding group fed DHT only, ($p < 0.05$, ANOVA F-test).

DISCUSSION

We observed striking differences in responses of the trout fed the aromatizable androgens, DHEA and androstenedione, and the structurally similar non-aromatizable androgen, dihydrotestosterone (DHT). DHEA and androstenedione significantly induced plasma Vg and E_2 levels in juvenile rainbow trout demonstrating the ability for these androgens to elicit an estrogenic response. DHT did not induce Vg or E_2 levels, suggesting induction by DHEA and androstenedione was not by direct estrogen receptor binding, but by conversion to estrogens. Support for this conclusion was observed in the DHEA and androstenedione treated fish, where E_2 levels increased in concert with the Vg increases, albeit with less sensitivity. In fact, DHT markedly lowered Vg and E_2 levels in the absence of toxicity, which was assessed grossly, by body weight, and liver somatic index. Decreases in Vg and E_2 levels in trout fed DHT suggest that either this androgen does not bind the trout estrogen receptor or it is an estrogen receptor antagonist. Receptor binding assays are underway to answer this question. There is also the possibility of estrogen down regulation by this androgen. Perhaps it is inhibiting aromatase, the enzyme that converts androgens to estrogens. Our laboratory has been unsuccessful in uncovering an effective aromatase inhibitor for trout *in vivo* to test this hypothesis. The conversion could be blocked in another way such as inhibition of luteinizing hormone (LH) secretion that would be a signal to synthesize estrogens from androgens. Turgeon and Waring [25] found that DHT blocked gonadotropin-releasing hormone induced LH secretion in female rat gonadotropes *in vitro*.

In the second study, DHT elicited antiestrogenic-like responses similar to the first study, reducing Vg levels to 20% of control levels at 50 mg/kg in both males and females. Flutamide alone, however, also decreased Vg to levels comparable to DHT. DHT and flutamide normally act as opposite entities in regard

to androgenic responses, but both of these evoked similar responses concerning Vg suppression. There may be some signal transduction pathways initiated by androgen receptor binding separate from classic androgen/ antiandrogen pathways that ultimately reduce E_2. Females may be less sensitive to E_2 decreases due to higher estrogen receptor levels and/or affinity for DHT and/or flutamide as a substrate, which could offset decreases in E_2 levels. Carlson and Williams [39] found that even in immature rainbow trout there is a sex difference in Vg production in response to E_2.

We also examined the potential for DHT to block the production of endogenous testosterone (T), thus depleting the available pool of androgens for conversion to estrogens. DHT increased T levels, an effect that potentially is an artifact of crossreactivity in the EIA, which has been estimated by the vendor to be 20%. Although flutamide significantly decreased E_2 in both sexes, T levels were only lowered in females. It is possible that decreases in E_2 are a consequence of a depleted androgen pool, induced by androgen receptor binding. Separate mechanisms exist that control E_2 and T levels in response to androgen receptor binding which may be sex-specific.

Increases in estrogen metabolism by specific cytochrome P450s provide an explanation for the E_2 decreases that ultimately would lead to suppressed Vg levels. The highly significant, dose responsive decrease in total liver P450 content by DHT does not support this hypothesis. Feedback mechanisms by DHT that decrease syntheses of estrogens or estrogen precursors, however, would decrease E_2 and Vg, a hypothesis that is supported by the DHT data. A previous study in rainbow trout, implanted with 5 mg of different androgens and E_2, found that DHT, T, and 17α-methyltestosterone had no effect on total P450 content, however, 11-ketotestosterone (11kT) acted as an inducer [40]. Decreases in hepatic P450 levels may mirror repression of extrahepatic P450s or other sex-steroid-regulated

enzymes important for E_2 and/or Vg synthesis. Perhaps the levels of aromatase in Sertoli cells, a cytochrome P450 ($P450_{arom}$) responsible for the conversion of androgens to estrogens, is also being lowered in response to DHT, resulting in an accumulation of T. Our laboratory is currently investigating the androgenic effects on specific P450 isozymes, such as aromatase. Although DHT significantly lowered P450 content in males and females, the antiandrogen flutamide attenuated this effect in males only. Total P450 levels may be under androgen control in males in a fashion that is quantitatively or qualitatively different from females.

We hope to compare the responses by trout to DHT, the major circulating androgen in mammals to 11kT, the major androgen in trout, which is also non-aromatizable. This will allow us to study a physiologically relevant androgen in trout to determine if the DHT responses are predictive of those of the major teleost androgen. This is critical since significant differences in Phase I and Phase II activities have been reported between trout and other species [41,42]

In summary, trout fed DHT have lower Vg, E_2, and total P450 content without the standard indications of toxicity. The antiandrogen, flutamide also had some antiestrogenic-like effects in the trout supporting the role of the androgen receptor in these responses. The mechanisms seem less clear due to the similar responses elicited by these different compounds. There may be some metabolic modulation associated with DHT treatment related to the antiestrogenic effects. The data presented suggest that antiestrogenic responses can result from androgenic and antiandrogenic compounds acting in a direct or indirect manner.

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CHAPTER 4

DETERMINING RELATIVE ESTROGENICITY BY QUANTIFYING VITELLOGENIN INDUCTION IN RAINBOW TROUT LIVER SLICES

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ABSTRACT

Precision cut tissue slices, by modeling the entire organ, are a valuable tool for studying protein induction or inhibition by test chemicals. This manuscript describes parameters to quantify relative estrogenicity of chemicals in rainbow trout liver slices by measuring vitellogenin (Vg) induction, a well characterized biomarker of estrogen receptor signal transduction. Hank's medium (phenol-red free) supplemented with HEPES, sodium bicarbonate and 1% bovine serum albumin was utilized. The experimental parameters were optimized using 1000 nM 17β-estradiol, a potent estrogen in rainbow trout that induces Vg production *in vivo*. The addition of trout serum and retention of the media were essential, probably to allow for the accumulation of Vg in the slices and media. Histological examination and ATP analyses indicated no toxicity in control or 17β-estradiol treated liver slices after 120 hours. Induction was 4-fold greater with 25% serum containing media compared to media with 10% serum. We observed Vg induction as great as 500-fold over controls at 96 hours in liver slices and media containing 25% serum and 1000 nM 17β-estradiol. Controls without 17β-estradiol, incubated in media with 10% or 25% serum, exhibited no detectable Vg production, indicating that the observed induction was not from the media or serum. We observed that 48 hours was required for significant Vg induction in the media and liver slices. Maximum induction in slices occurred at 96 hours whereas media Vg levels continued to increase to 120 hours, suggesting a time delay between Vg production and excretion by the liver. The feasibility of this model to detect weak environmental estrogens was determined with 0 – 250 μM *o,p'*DDE and bisphenol A. Both compounds induced Vg in this model with EC_{50} values of 10^4 and 2 x 10^5 higher than E_2, respectively. Our results indicate the importance of media, serum, and time selection for optimal Vg induction. This model allows for the

determination of relative estrogenicity of chemicals in a controlled *in vitro* system while utilizing the advantages of precision cut slice technology.

INTRODUCTION

Establishing the estrogenicity of compounds found in the environment has become a widespread concern (Sonnenschein and Soto, 1998; Bolger *et al.*, 1998). Amendments added to the Food Quality Protection and Safe Drinking Water Acts in 1996 required the screening for endocrine disrupting chemicals. The development of assays to quantify xenoestrogens is a critical aspect of this mandate by Congress. One effective biomarker for estrogen exposure is vitellogenin (Vg), an egg-yolk protein precursor produced in the liver of oviparous animals in response to estrogen receptor binding (Sumpter and Jobling, 1995). The development of simple and rapid *in vitro* methods to test for estrogenicity via Vg induction is important as a screening tool. The use of cultured hepatocytes has been shown to be effective for Vg induction (Pelissero *et al.*, 1993; Maitre *et al.*, 1986). The advent and subsequent modifications of precision-cut tissue slicing techniques and slicers that generate slices of consistent thicknesses (Price *et al.*, 1998; Bach *et al.*, 1996; Parrish *et al.*, 1995; Krumdieck *et al.*, 1980) has allowed for the application of this technology to metabolism studies (Ferrero and Brendel, 1997; Ekins, 1996). Liver slices are more representative of the dynamic organ system *in vivo* allowing for the preservation of cell heterogeneity and cell-to-cell interactions (Guillouzo, 1998) and retention of metabolic activity over a longer period of time (Vickers, 1994; Beamand *et al.*, 1993).

Rainbow trout have been shown to be sensitive to xenoestrogens and their effects on responses such as modulation of cytochromes P450 (CYPs) and carcinogenesis (Williams *et al.*, 1998). The screening of estrogenic compounds using rainbow trout liver slices offers an attractive link between cultured cell

models and *in vivo* studies. Another advantage is that in contrast to mammalian liver slices, rainbow trout slices can be maintained for several days allowing for more extensive studies of induction and inhibition (Oganesian *et al.*, 1997a;b). Slices are also faster and easier to prepare than hepatocytes because no collagenase, isolation or culturing steps are required (Seglan, 1975).

We developed optimal conditions for Vg induction utilizing precision-cut liver slice technology with 17β-estradiol as our model estrogen. Incubations were performed with rainbow trout liver slices in phenol-red free medium containing trout or fetal bovine serum and the test compound. A modified version of the well-plate shaker system described by Dogterom (1993) was utilized for incubations so that contact of the slices with media and test compounds would not be limited, a drawback of the dynamic culture system for which availability of medium to the slice cells is limiting (Drahushuk *et al.*, 1996). Liver slice and media Vg levels were quantified by an enzyme-linked immunosorbant assay (ELISA) with viability monitored by histological examination and ATP analyses. This model demonstrated the capacity to screen environmental and dietary compounds for estrogenicity in rainbow trout via Vg induction.

METHODS and MATERIALS

All glassware and tools were sterilized at 105^0C for 30 min prior to use. Unless noted otherwise, all chemicals were purchased from Sigma Chemicals (St.Louis, MO). Livers were extracted from male juvenile (< 18 month) rainbow trout, Mt. Shasta strain, which were euthanized with an overdose of tricane methanesulfonate (MS222) according to a protocol approved by the Oregon State University Institutional Animal Care and Use Committee. The livers were put in ice cold Hank's modified salts buffer which contained 10 mM HEPES and 8 mM sodium bicarbonate, pH 7.2. All media were sterilized by filtering through a 0.22

μm filter. Using a sterile stainless steel coring device, 8 mm cores were generated from the livers and then cut into precision slices of about 250 μm using a Krumdieck tissue slicer (Alabama Research and Development Corp, Munford, AL). The slices were placed randomly into 12-well Falcon plates, one per well, containing the previously described sterile Hank's media additionally supplemented with 1% bovine serum albumin and 0.1% gentamicin. Male rainbow trout serum collected by centrifugation of blood at 2000 x g for 10 min or fetal bovine serum was then added to the media at the proper concentration yielding a final volume of 1 ml. Test chemicals were added to the wells before introduction of the slice using ethanol or dimethylsulfoxide as the vehicle (0.2% of final volume). Two plates were dedicated per concentration due to the requirement of enough slices for histology, ATP, and Vg assays. The plates were covered and put into sealed plastic containers that were saturated with 95% O_2 / 5% CO_2, which was refreshed at least every 12 hours. Incubations were performed at 14^0C on an orbital shaker at 90 RPM. Media was not changed for the duration of the incubations. For time course experiments, samples were collected every 24 hr.

At t = 0, four slices randomly were fixed in 10% phosphate buffered formalin for histological analyses to estimate the percentage of viable cells. ATP production was measured using kits purchased from Sigma Chemicals which is a modification of the procedure described by Adam (1963). We chose to use ATP content as a viability parameter because it represents the energetic state of the cell, an important parameter when considering the complexity of Vg synthesis. This assay detects for an important biochemical change that occurs in the cell before the appearance of general cytotoxicity such as lactate dehydrogenase leakage. Two slices were pooled for a total sample size of six per treatment group and homogenized in 200 μl phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylflouride, pH 7.4 and stored at

-80°C for later analyses. Six samples of media were also collected and stored at -80°C.

At termination of each experiment, slices were assayed for ATP production or fixed in 10% buffered formalin for histology. Remaining slices, two per replicate, for a sample size of six, were homogenized in phosphate buffer and aliquots of media were collected for Vg assays and stored at -80°C. Media and slice homogenate Vg was quantified utilizing an ELISA previously described by Donohoe and Curtis (1996). Briefly, samples were incubated at 4°C for 24 hr in 96-well plates with antibody raised in rabbits against anti-chum salmon Vg (1:1500 dilution), which was graciously obtained by way of Donald Buhler, Oregon State University from A. Hara at Hokkaido University. The solution was transferred to plates coated with 25 ng/well rainbow trout Vg and incubated for 24 hr. Plates were incubated with biotin linked donkey-anti-rabbit IgG and with a streptavidin horseradish peroxidase conjugate (Amersham, Buckinghamshire, England) each for two hours at 37°C with four washings between each step. The colormetric reaction was performed using 0.01% 3,3',5,5'-tetramethylbenzidine and 0.01% hydrogen peroxide in 0.5 M sodium acetate, pH 6.0 for 10 min and stopped with 2 M sulfuric acid. Colormetric readings for the ELISA were performed on a microtiter plate reader (Biotek EL 340, Winooski, VT) and analyzed with plate reader software (Deltasoft 3, Princeton, NJ). The limit of detection was determined to be 6.25 ng Vg / ml sample. Intrassay or well-well and interassay or plate-plate variability for all reported values were < 10% and 15%, respectively. Those samples above these acceptable levels were reanalyzed. Liver slice Vg was reported as a function of protein concentration which was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Significant Vg induction between treatment groups with six samples of two slices each and negative controls containing vehicle alone was determined by t-tests, assuming unequal variances.

One-way ANOVA and F-tests were used to determine differences between treatment groups.

RESULTS

Vitellogenin was quantified in both liver slice homogenates and surrounding media. Liver slice homogenates and media assayed at the beginning of each experiment usually had little or no detectable levels of Vg. Time course experiments revealed that induction of Vg by 17β-estradiol was detectable after 24 hr, but not significant in liver slices until after 48 hr of exposure ($p < 0.05$, ANOVA F-test, Figure 4.1a). Media Vg levels were significantly higher than controls after 48 hr as well, but were not maximized until the 120 hr timepoint (Figure 4.1b). Peak levels of Vg in the slices and media were over 500-fold higher than Vg levels in corresponding 120 hr samples not treated with E_2. There were no significant differences observed in liver slices or surrounding media Vg levels between the 0 hr and 120 hr controls without E_2, indicating that there were no estrogenic compounds in the media or serum.

Based on the previous time course data, dose response experiments were run for 96 hr because Vg induction at this timepoint was in the area of peak response. A dose response study revealed significant induction in slices at 0.1 nM and above at 96 hr ($p < 0.05$, t-test, Figure 4.2a). Significant induction was not observed in media samples until 10 nM ($p < 0.05$, t-test, Figure 4.2b), supporting the hypothesis that measuring Vg in slices is the more sensitive endpoint. The EC_{50} for E_2 was estimated to be 1 nM. Vg levels of the homogenates (Figure 4.2a) were nearly 800 ng / mg protein which was more than 100 times the Vg of controls not treated with E_2. The low EC_{50} value and relatively wide induction window provides a broad area for quantifying Vg induction of much weaker estrogens. The

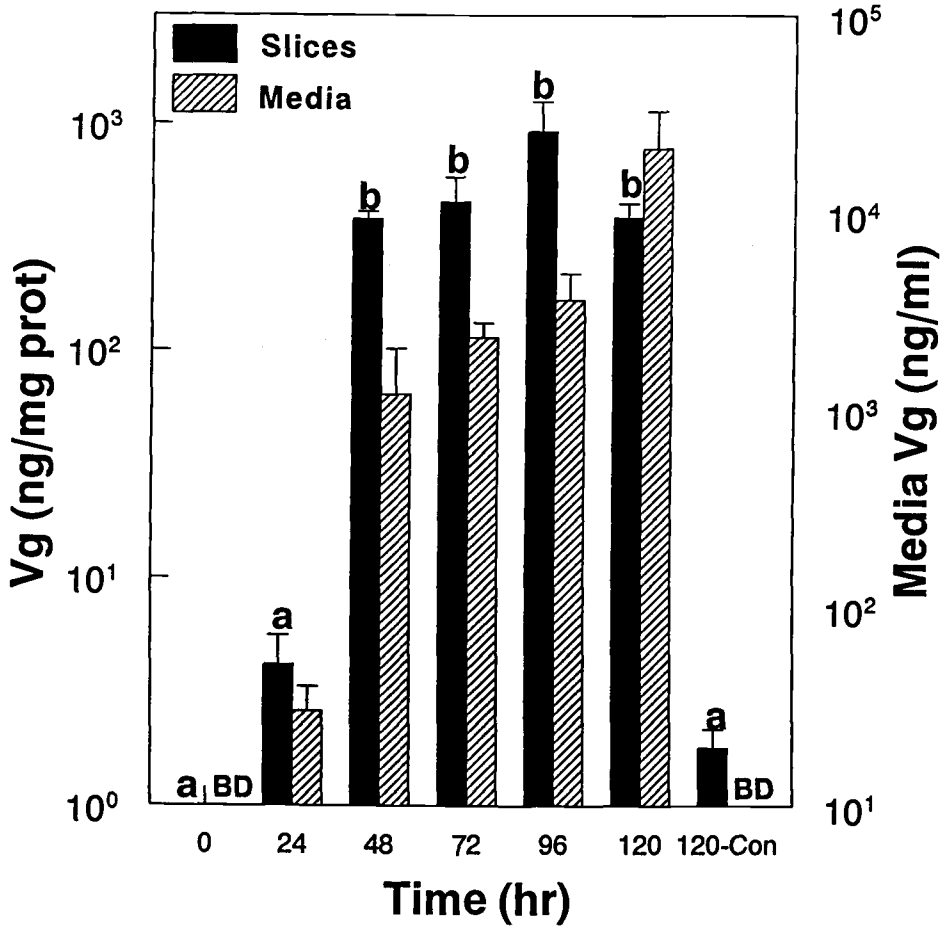

Figure 4.1: Vg levels plotted on a log scale for liver slice homogenates and media treated with 1000 nM E_2 in supplemented Hank's media and 25% serum for 120 hr. Different letters denote significant differences between groups ($p < 0.05$, ANOVA F-test). Error bars denote $\pm$ SE ($n = 3$). BD = Vg values below the detection limit.

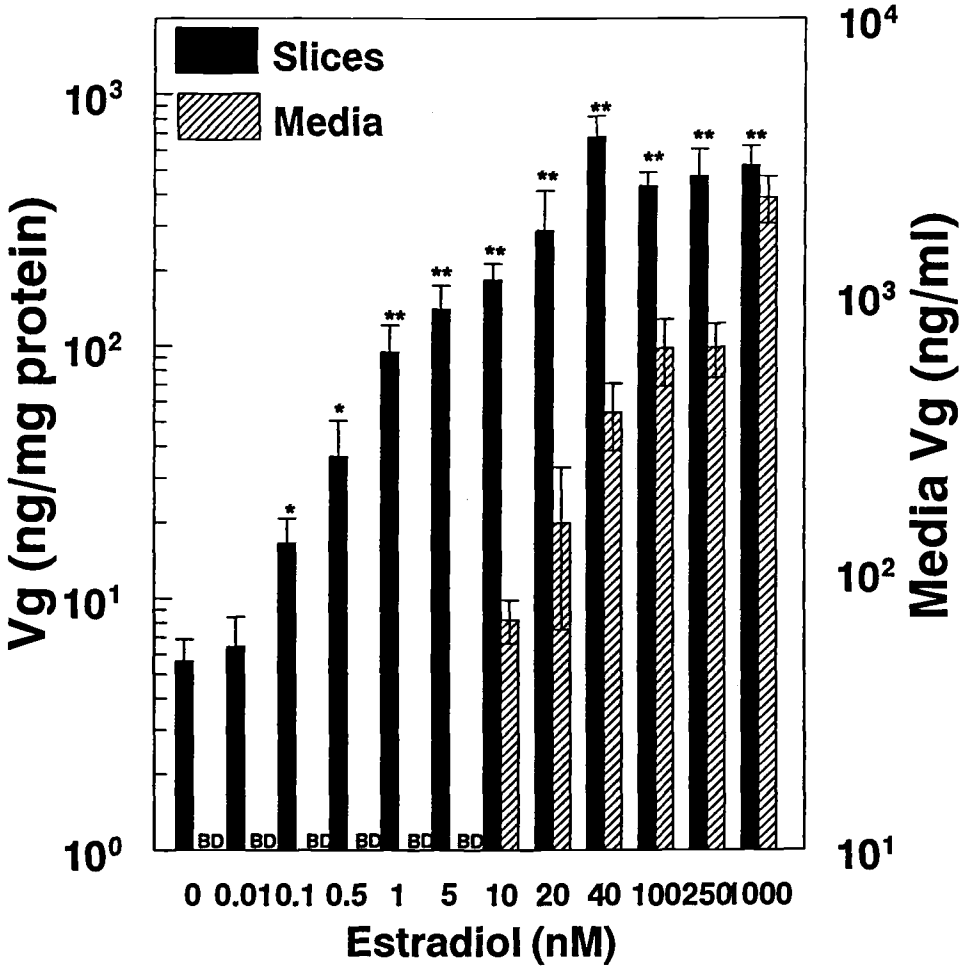

Figure 4.2: Vg levels plotted on a log scale for slices and media treated with 0 - 1000 nM E_2, for 96 hr in supplemented Hank's media containing 25% fetal bovine serum. Significant differences in Vg compared to control where $p < 0.05$ or $p < 0.001$ are denoted by (*) and (**), respectively (t-test, assuming unequal variances). Statistical comparisons were not performed for between media groups because of non-detectable Vg levels in the control group. Error bars denote $\pm$ SE ($n = 6$). BD = Vg values below the detection limit.

estrogen receptor antagonist, tamoxifen, significantly decreased E_2 mediated Vg induction in slices ($p < 0.0001$, ANOVA F-test) at 5 and 100 μM (Figure 4.3). The effect of tamoxifen as measured by media Vg levels was also observed, but could not be quantified because levels were below detection at 100 μM. Tamoxifen alone did not significantly induce Vg in slices or media ($p > 0.05$) and in fact appeared to markedly inhibit basal Vg expression.

The addition of serum proved critical for Vg induction. Liver slices treated with 1000 nM E_2 using Hank's media and supplements described above without serum yielded no significant induction. Experiments run under the same conditions using rainbow trout serum at 10 and 25% by volume demonstrated significant induction, with the 25% serum containing samples producing more Vg than slices in media of 10% serum (Figure 4.4). Substituting fetal bovine serum with rainbow trout serum did not affect Vg induction significantly. Interestingly, at 25% media volume, Vg induction in slices incubated in heat inactivated serum was only one-tenth that of slices incubated in non-heat inactivated fetal bovine serum.

No media changes were performed over the course of these experiments, making liver slice viability a concern. Histological examination and ATP assays were conducted to assure that structural or biochemical damage was not occurring and introducing confounding variables to the model system. ATP production by liver slices did not diminish over time for any of the concentrations of chemicals tested (Figure 4.5). ATP production was compared to slices at $t = 0$ and control samples run concurrent with experimental samples. In some cases, ATP production increased for experimentally treated slices. Examination of slices sectioned and stained with hematoxylin and eosin revealed no major structural injury even after 120 hr. Results of these two assays confirmed that the liver slices were viable and useful for Vg induction studies.

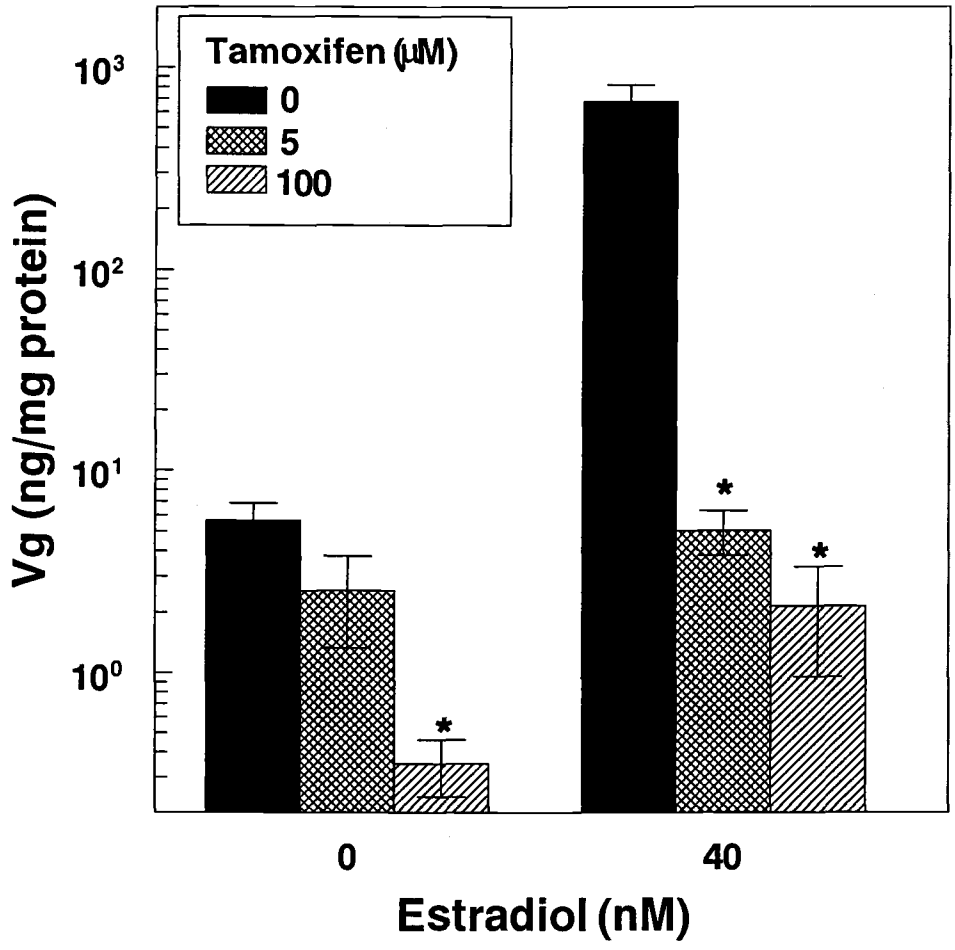

Figure 4.3: Inhibition of Vg production by tamoxifen (0, 5 and 100 μM) in the presence or absence of E_2 in slices and media incubated for 96 hr in supplemented Hank's media containing 25% fetal bovine serum. Significant inhibition by tamoxifen is denoted by (*) ($p < 0.05$, ANOVA F-test). Error bars represent $\pm$ SE ($n = 6$). Vg levels plotted on a log scale.

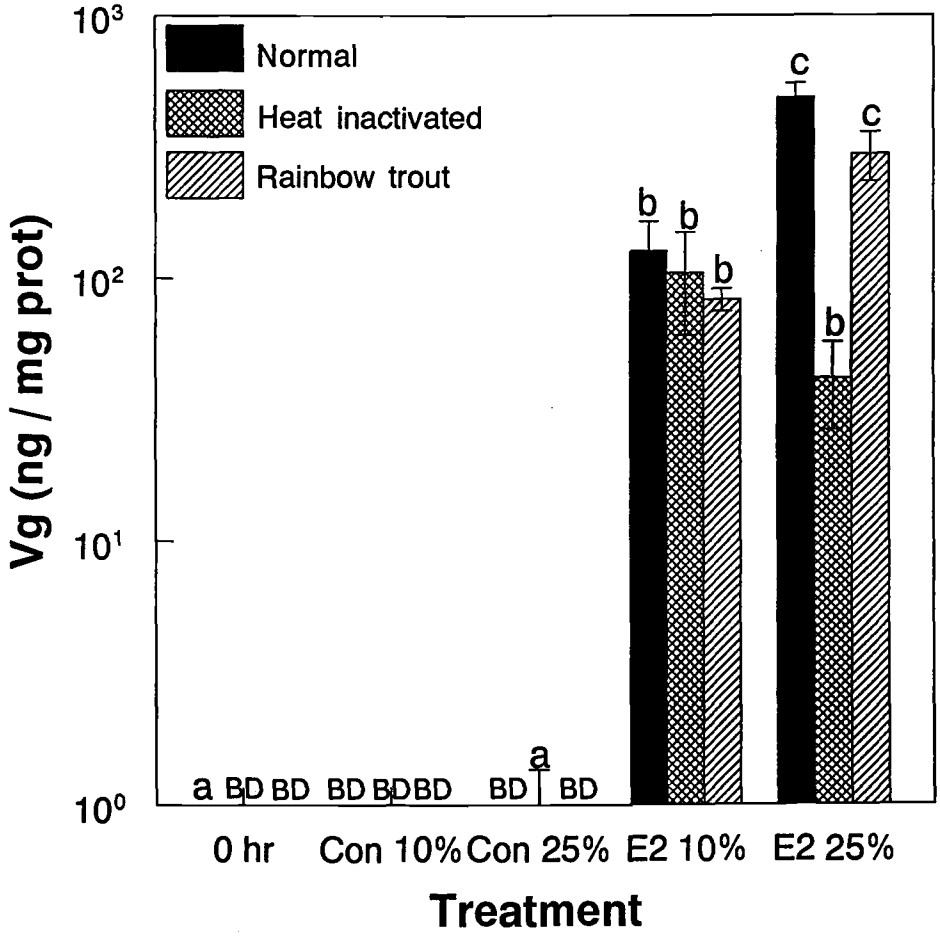

Figure 4.4: Comparison of induction of Vg in slices treated with 1000 nM E_2 for 96 hr in 10% or 25% normal or heat inactivated fetal bovine or rainbow trout serum. Different letters denote significant differences between groups ($p < 0.05$, ANOVA) and BD denotes Vg values below detection. Error bars denote $\pm$ SE ($n = 4$). Vg levels plotted on a log scale.

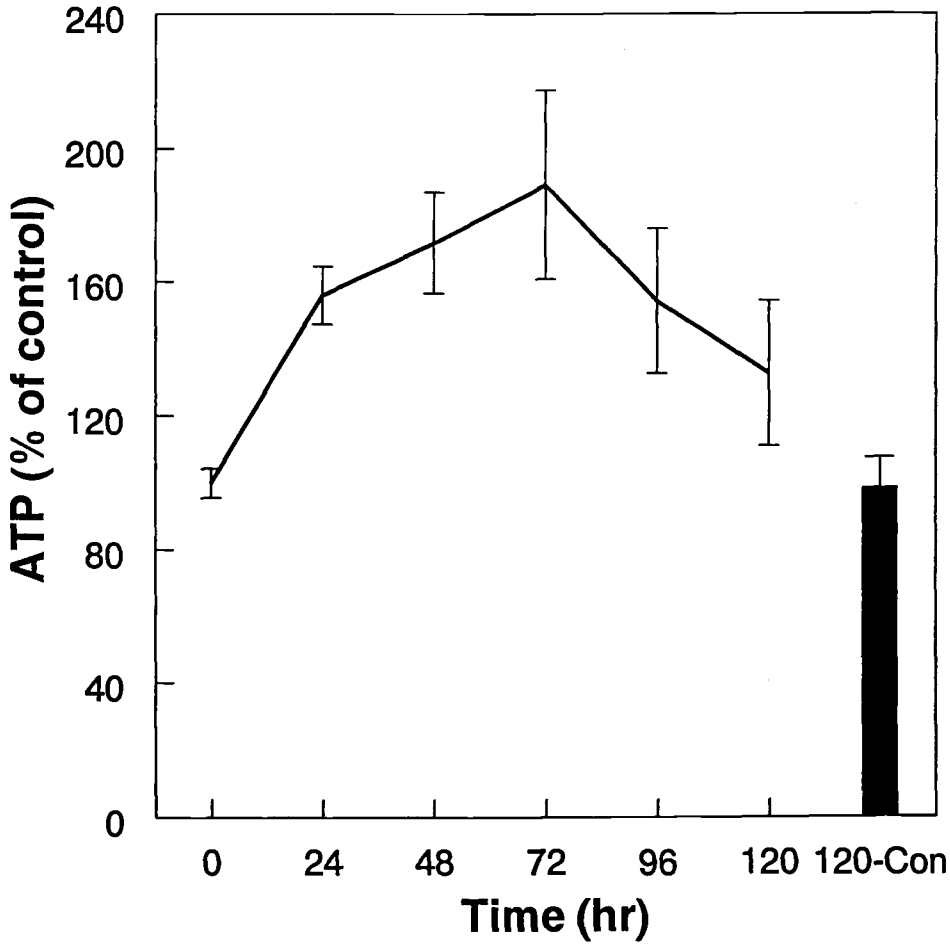

Figure 4.5: ATP levels for slices treated with 1000 nM E_2 over a 120 hr time course in supplemented Hank's media containing 25% fetal bovine serum. Values represent percent of control. Error bars denote $\pm$ SE (n = 3).

The ultimate goal of this study was to examine relative estrogenicity of compounds in relation to E_2. To validate this model, we tested three environmentally relevant weak estrogens, OH-PCB30, a hydroxylated metabolite of 2,4,5-trichloroPCB, *o,p'*DDE, a metabolite of *o,p'*DDT, a once widely used pesticide and Bisphenol A, a product formed during the production of resins and epoxies. Both compounds were found to be estrogenic by inducing Vg, which was quantified and compared to E_2, verifying the usefulness of this model (Figure 4.6). *o,p'*DDE induced Vg with an efficacy similar to E_2, albeit at a much lower potency (EC_{50} = 10 μM). Bisphenol A, an even weaker estrogen induced Vg with an efficacy of 8% and EC_{50} value 2 x 10^{-5} of E_2.

Induction of Vg in response to E_2 was compared between slices obtained from juvenile male and female trout livers. There was no observable sex difference for initial Vg levels at the start of each experiment involving male and female slices. Dose-response curves from 1 - 1000 nM E_2 resulted in similar Vg induction profiles for liver slices of male and female trout (Figure 4.7). At 1000 nM E_2 after 96 hr, Vg levels were more than 30-fold higher for males and 40-fold higher for females than the corresponding controls ($p < 0.0003$, t-test). Although at 10 nM E_2 only male slice Vg was significantly induced ($p < 0.001$, t-test assuming unequal variances), there was not any significant sex differences in the values between the other treatment groups ($p > 0.05$, ANOVA F-test).

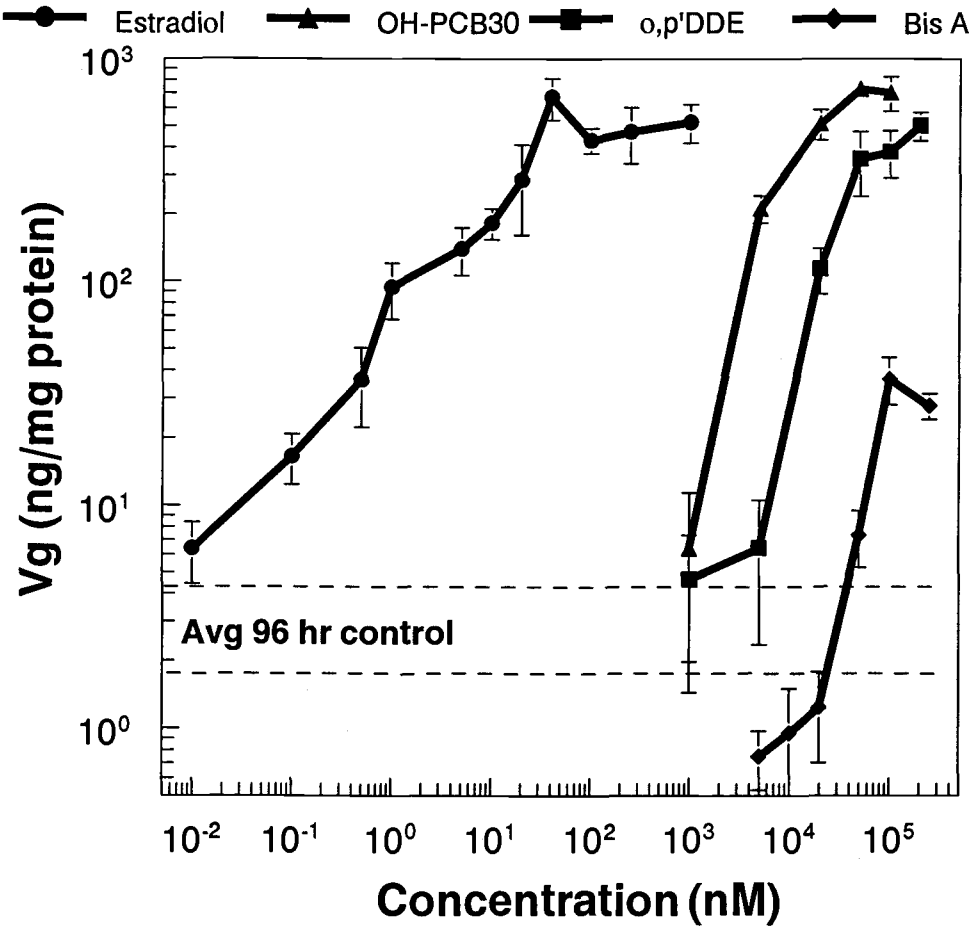

Figure 4.6: Vg induction curves plotted on a log scale for slices treated with OH-PCB30, *o,p*'DDE and Bisphenol A compared to E_2. In each experiment, slices were incubated in supplemented Hank's media containing 25% fetal bovine serum for 96 hr. Error bars represent $\pm$ SE (n = 6). EC_{50} values were calculated from data depicted in this graph.

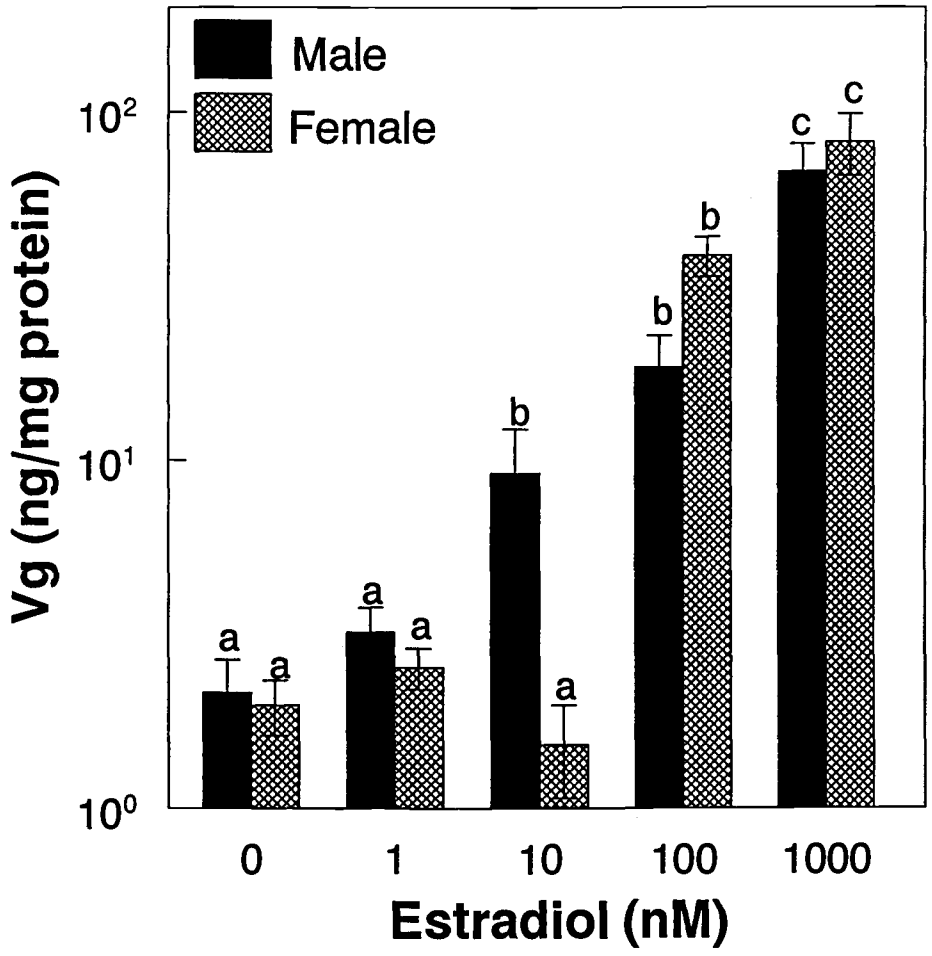

Figure 4.7: Vg induction for liver slices obtained from male or female rainbow trout exposed to 1, 10, 100 or 1000 nM E_2 for 96 hr in supplemented Hank's media containing 25% fetal bovine serum. Different letters represent significant differences between groups ($p < 0.01$, ANOVA F-test). Error bars denote $\pm$ SE (n = 6). Vg levels plotted on a log scale.

DISCUSSION

Rainbow trout liver slices demonstrated the capability to produce vitellogenin in response to estrogenic chemicals. Using 17β-estradiol as our prototypic estrogen, we developed a model system in which relative estrogenicity could be quantified via Vg induction by liver slices. Detection of 17β-estradiol-induced Vg induction in the liver slice samples was the more sensitive endpoint with significant induction observed at 0.1 nM while media samples required 10 nM (Figure 4.2). Co-treating with the antiestrogen, tamoxifen also demonstrated the superiority of quantifying slice Vg levels compared to the media levels which were too low to detect in some of the comparison groups (Figure 4.3).

Certain conditions were critical for the sensitivity and validation of this model. Only livers from juvenile male trout were used to assure extremely low to no initial Vg at commencement of each study. The addition of serum, whether it was rainbow trout or fetal bovine, was absolutely essential for Vg induction. In previous studies performed without serum, no significant Vg induction was detected even after 120 hr with 1000 nM 17β-estradiol (data not shown). We found that media containing 25% serum yielded higher Vg induction than media that was only 10% serum. To assure higher levels of consistency between experiments and reduce the number of animals required, we tried using commercially available fetal bovine serum in our media instead of rainbow trout serum that must be extracted from the fish and centrifuged before use. Similar induction was observed with the normal fetal bovine serum. Interestingly, we found fetal bovine serum heat inactivated for 30 min at 56^0C was much less effective in supporting Vg induction than non-heat inactivated serum. Perhaps the heat inactivation degrades certain proteins that are important for the Vg production and/or stability.

Instead of changing the media throughout an experiment, we allowed liver slices to incubate in media with the test chemical for the duration so that Vg could

be produced and accumulate. In previous studies performed in our laboratory and by other investigators, liver slices were placed in fresh media every 24 hr. As exhibited in Figure 4.1a, significant Vg induction was not observed until after 48 hr of treatment, which may explain why previous studies did not detect significant Vg induction. Although significant Vg induction was observed in media, it lagged behind the induction seen in liver slice homogenates, indicating a delay between Vg production by the liver slices and subsequent release into the media. Vg production and its subsequent release are potentially two separate mechanisms to consider when determining the effects that an estrogen has on increasing Vg levels. It is likely that compounds alter Vg induction *in vivo* by other mechanisms outside of classic estrogen receptor binding, but deciphering these mechanisms was beyond the scope of these studies. Moreover, measuring secretion in this model may not be critical because, like all *in vitro* systems, the incubating slice is not part of a closed system, but its own entity.

From our data comparing Vg induction in slices and media with E_2 (Figure 4.2) and tamoxifen (Figure 4.3), we determined that quantifying Vg in liver slices was the more sensitive endpoint. This is supported by the fact we observed significant Vg induction in slices $\geq$ 0.1 nM while in media, 10 nM E_2 was required for significant induction. We observed a similar difference in slice and media samples for OH-PCB30, *o,p'*DDE and Bisphenol A (data not shown). Both slice and media samples, however, were collected in every study, but for quantifications of relative estrogenicity, slice homogenate Vg data is reported (Figure 4.6).

Tamoxifen (5 μM and higher) significantly inhibited Vg induction by E_2, demonstrating that induction was dependent upon the estrogen receptor. Previous *in vivo* studies with rainbow trout have found tamoxifen to induce Vg at high doses (Carlson *et al.*, submitted). There was not significant Vg induction in slices

incubated with up to 100 μM tamoxifen alone, however, indicating that it was not eliciting a significant estrogenic response in this model.

It was critical to compare our results with those reported by investigators utilizing hepatocytes. Cultured rainbow trout hepatocytes from immature females incubated with 0.1 - 1000 nM E_2 displayed a similar induction pattern to what we report in this paper (Pelissero *et al.*, 1993). Vg induction appears to be similar among different teleost species as well. Carp hepatocytes from mature males and females exposed to E_2 for four days showed a similar induction pattern for Vg, but with an EC_{50} two orders of magnitude less sensitive than the rainbow trout liver slice model reported here (Smeets *et al.*, 1999a). Although we found Bisphenol A estrogenicity to be comparable, Smeets *et al.* (1999b) reported that *o,p*'DDE did not induce Vg in carp hepatocytes, yet we observed significant induction above 10 μM. Not surprisingly, these observations suggest possible species differences in the sensitivity to Vg induction by estrogens. It was also reported that tamoxifen blocked E_2-induced Vg production above 0.6 μM without having an estrogenic effect when administered alone (Smeets *et al.*, 1999a). Our data regarding tamoxifen inhibition of E_2-induced Vg production in the absence of estrogenic effects corresponds to this finding (Figure 4.3).

The difference of liver slices from male and female to produce Vg in response to E_2 was studied. No sex differences were observed between slices from male and female trout either at the beginning of the experiment or after exposure to 100 or 1000 nM E_2 for 96 hr. This corresponds to hepatocyte cultures from male and female rainbow trout and carp in which a sex difference in E_2 induced Vg production was not observed (Pelissero *et al.*, 1993 and Smeets *et al.*, 1999a). In fact, a significant increase was observed in males at 10 nM, but not in female slices. We are suggesting that *in vivo* sex differences reported previously by Carlson and Williams (1999) may not be due to inherent sex differences in the liver

such as estrogen receptor numbers and may involve other factors. Considering that the liver is the major tissue for metabolizing compounds, it will be important to assess the contribution of xenobiotic metabolism to Vg induction observed in liver slices. Our laboratory is currently investigating the role of metabolizing enzymes in liver slices, particularly cytochrome P450s, in the modulation of Vg induction by estrogens over the course of a 96 hr experiment.

We have demonstrated this *in vitro* model to be practical and sensitive for quantifying estrogenicity of chemicals via Vg induction. Vg induction in slices reported in this study is quite comparable to induction observed in hepatocytes. Liver slices are easier and faster to prepare than hepatocytes and provide a more realistic *in vitro* model of the whole organ owing to the inclusion of different types of cells and maintaining cell-to-cell interactions. A limitation to this model is the use of compounds at high concentrations (> 1 mM) that are relatively insoluble in media. It should be noted, however, that the weak estrogens tested in this model induced Vg; the maximal levels well below levels in which solubility became a concern. Rainbow trout liver slices are viable for several days, allowing for Vg production in response to estrogenic chemicals. Our laboratory is currently screening compounds that are known or suspected estrogens and believe this model may prove to be useful in predicting relative estrogenicities of compounds *in vivo*.

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CHAPTER 5

3,3'-DIINDOLYLMETHANE, A MAJOR CONDENSATION PRODUCT OF INDOLE-3-CARBINOL, IS AN EFFECTIVE ESTROGEN IN THE RAINBOW TROUT

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ABSTRACT

Indole-3-carbinol (I3C), a compound found in *Brassica* vegetables has been widely studied for its chemopreventive properties. I3C has been shown to block tumor initiation and promotion; however, it also acts as a tumor promoter. I3C and some of its acid condensation products, particularly 3,3'diindolylmethane (I33'), have exhibited antiestrogenic properties. We report that I33' acts as an estrogen in the rainbow trout liver *in vitro* and *in vivo* by inducing vitellogenin (Vg), a well-characterized biomarker for estrogens. Precision-cut liver slices from male rainbow trout, *Oncorhynchus mykiss*, were incubated at 14^0C for 96 hr. I3C, I33' and a mixture of I3C acid condensation products (RXN) (0-250 μM) were added to the medium with DMSO as the vehicle. I33' and RXN increased Vg levels in rainbow trout liver slices by over 300- and 20-fold, respectively, vs vehicle. The maximal I33' induction of Vg was comparable to 17β-estradiol with 2500-fold less potency. I33' and E_2 co-treatment resulted in additive Vg induction. Moreover, tamoxifen completely inhibited I33' induced Vg induction suggesting that Vg induction by I33' is entirely through the estrogen receptor. *In vivo*, juvenile male rainbow trout were fed I3C, RXN (0- 2000 mg/kg) or I33' (0-250 mg/kg) for two weeks. At 2000 mg/kg, I3C induced Vg by over 100,000-fold compared to controls, which was comparable to 5 mg/kg 17β-estradiol (the dose resulting in maximum induction). I33' was five times as potent and as efficacious as I3C, but the potency of RXN was only 5% of I3C. I33' and E_2 co-treatment resulted in additive Vg induction. The antiestrogen toremifene, however, was ineffective in reducing E_2 or I33' mediated Vg induction, suggesting that it is not a useful antiestrogen in the rainbow trout. I33' may have accounted for Vg increases observed in trout fed I3C as it is present in liver after oral dosing, at concentrations (70 μM) expected to maximally induce Vg. In trout, results *in vitro* and *in vivo* document that I33' is estrogenic, consistent with our hypothesis that I3C promotes

liver cancer in trout by estrogenic pathways. I33' apparently functions as a pro- or antiestrogen in a tissue- and/or species-specific manner.

INTRODUCTION

Within the past decade, interest has focused on the use of natural products for the treatment of a wide range of ailments. In the past, plant derived compounds such as indole-3-carbinol (I3C), a natural constituent of *Brassica* plants, such as broccoli, cauliflower and brussel sprouts, have been studied for its chemopreventive properties (Bailey and Williams, 1993, Boone *et al.*, 1990 and Hocman, 1989). I3C has been implicated in the reduction of tumor development by a number of mechanisms across varying species (Xu and Dashwood, 1999, Srivastava and Shukla, 1998, Manson *et al.*, 1998, Oganesian *et al.*, 1997b, and Dashwood *et al.*, 1994). *In vitro*, I3C has the ability to block cell cycle progression and proliferation in certain cell lines (Chang *et al.*, 1999, Cover *et al.*, 1998, Chen *et al.*, 1996, and Ge *et al.*, 1996). In whole animal experiments, it has been reported to be chemopreventive by blocking tumor development at the initiation, promotion and progression stages (Agrawal and Kumar, 1999, Xu and Dashwood, 1999, Takahashi *et al.*, 1995b and Bradlow *et al.*, 1991). In certain scenarios, however, I3C has exhibited just the opposite effects, inducing and promoting cancer formation (Oganesian *et al.*, 1999, Dashwood *et al*, 1991 Bailey *et al.*, 1987).

Distinguishing between the bioactive and inactive metabolites of I3C has been an ongoing task for researchers. I3C is an unstable compound that undergoes rapid oligomerization in the acid environment of the stomach (Wortelboer *et al.*, 1992, Bradfield and Bjeldanes, 1987 and Leete and Marion, 1953). This results in the formation of dimers, trimers, tetramers, and several other higher order oligomers (Grose and Bjeldanes, 1992). Stresser *et al.* (1995a), found that in rats,

the two major products formed in the stomach were the dimer, 3,3'-diindolylmethane (I33'), and a linear trimer, 2-(indol-3-ylmethyl)-3,3-diindolylmethane (LT). Other minor products identified include, but are not limited to, indolo-[3,2-b]-carbazole (ICZ), an aryl hydrocarbon receptor agonist, another dimer, 1-(3-hydroxymethyl)idolyl-3-indolylmethane (HI-IM), and a cyclic trimer, 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b':7,8-b"] triindole (CT). In fact, I3C cannot be found at any appreciable quantities in the liver indicating complete transformation of the parent compound in the stomach (Dashwood *et al.*, 1994 and Bjeldanes *et al.*, 1991).

The dimer, I33', has been implicated as a major chemopreventive agent, particularly because it can attain significant concentrations in the liver due to its higher stability than I3C (Takahashi *et al.*, 1995a). It has also displayed antiestrogenic properties in certain systems, making I33' a candidate for treating estrogen dependent cancers such as breast cancer (Chen *et al.*, 1998 and Telang *et al*, 1997, Liu *et al*, 1994). Limited clinical trials have been conducted to determine the efficacy of I3C and I33' supplements in treated breast cancer (Michnovicz *et al.*, 1997 and Wong *et al.*, 1997). It is critical to note that very few whole animal studies in mammals have been conducted to verify the *in vitro* findings and that the long-term effects of I33' supplementation have yet to be determined.

Rainbow trout, *Oncorhynchus mykiss*, have demonstrated to be a very useful model for human risk assessment of certain compounds in certain target organs, such as the liver (Bailey *et al.*, 1996, Bailey *et al.*, 1992). In regards to estrogen mediated carcinogenesis, the trout liver has been well characterized (Williams *et al.*, 1998, Orner *et al.*, 1996 and Nunez *et al.*, 1989). The rainbow trout model offers the advantage of providing an established biomarker for estrogen exposure, via vitellogenin (Vg) induction (Davail *et al.*, 1998, Sumpter and Jobling, 1995 and Maitre *et al.*, 1986). This endpoint is particularly powerful in the

juvenile male, which has very low Vg levels when unexposed to estrogenic compounds. For example, rainbow trout liver slices were shown to be a viable model for screening potential compounds for estrogenic properties via Vg induction (Shilling and Williams, 2000). In previous studies performed in our laboratory, the trout were utilized for *in vitro* studies (Oganesian *et al.*, 1997a, Stresser *et al.*, 1995b) as well as *in vivo* induction/inhibition and tumor experiments involving I3C (Oganesian *et al.*, 1999, Oganesian *et al.*, 1997b, Orner *et al.*, 1996, Dashwood *et al.*, 1991). Additionally, this model has been utilized to measure the modulation of metabolizing and conjugating enzyme levels and activities by I3C and some of its acid condensation products (Renwick *et al.*, 1999, Vang *et al.*, 1999, Larsen-Su and Williams, 1996, Takahashi *et al.*, 1995c and Stresser *et al.*, 1994).

In this paper, we determined the estrogenic potential of I3C, I33' and RXN by measuring Vg induction *in vitro* using liver slices. The liver slice model provides advantages over other *in vitro* systems such as cultured hepatocytes or microsomes because cell heterogeneity is retained, thus mimicking the whole organ to a greater extent (Guillouzo, 1998, Vickers, 1994 and Beamand *et al.*, 1993). The trout slices are incubated at 14^0C allowing for studies to be drawn out for more than 5 days, an excellent tool for induction and inhibition studies. These results were compared to a two-week feeding study that was performed using juvenile rainbow trout. Our model estrogen was 17β-estradiol (E_2) to which relative potencies and efficacies were normalized. We also measured blood plasma 17β-estradiol levels to rule out any indirect estrogenic effects by I3C, I33' or RXN. Additionally we performed mixture studies with I33' and E_2 as well as an inhibition study with toremifene *in vivo* and tamoxifen *in vitro*. In an attempt to determine the exact metabolite responsible for the estrogenic activity of I33' we

performed *in vitro* time course studies with I33' in liver slices using HPLC analyses and mass spectrophotometry.

METHODS and MATERIALS

All chemicals utilized for the slicing procedure were purchased from Sigma Chemical Co. (St.Louis, MO), unless noted. The 3,3'-diindolylmethane (I33'), >99% pure, was obtained from Bioresponse, LTD. (Boulder, CO). The mixture of acid condensation products (RXN), processed to mimic the oligomerization of I3C in the stomach was prepared using a modification of the method of Grose and Bjeldanes (1992). Briefly, I3C was dissolved in HPLC grade ethanol, an aliquot was removed and blown to dryness under nitrogen gas. To this, equal volumes of water and methanol were added to dissolve the residue. A 1N hydrochloric acid solution was added and the mixture vortex shaken for 1 min. The mixture was neutralized with 0.25 N ammonia and then HPLC grade acetonitrile was added to produce the desired working concentration. The two major products of this procedure are I33' and the linear trimer, (LT).

All glassware and tools were sterilized at 105^0C for 30 min prior to use. The slicing and incubations are based on the method previously optimized in our laboratory (Shilling and Williams, 2000). Briefly, livers were extracted from male juvenile (< 18 month) rainbow trout, Mt. Shasta strain, which were euthanized with an overdose of tricane methanesulfonate (MS222) according to a protocol approved by the Oregon State University Institutional Animal Care and Use Committee. Livers were kept in ice cold Hank's modified salts buffer containing 10 mM HEPES and 8 mM sodium bicarbonate, pH 7.2 and filter sterilized through a 0.22 μm filter. Cores, 8 mm in diameter, were generated from the livers. A Krumdieck tissue slicer (Alabama Research and Development Corp, Munford, AL) cut the cores into precision slices of about 250 μm. The slices were placed randomly into

12 well Falcon culture plates (Fisher Scientific, Pittsburgh, PA) containing the Hank's media additionally supplemented with bovine serum albumin (1%) and 100 µl gentamicin per 100 ml, fetal bovine serum (25%) and the test chemical dissolved in dimethylsulfoxide (0.2% of final volume). The plates were covered, put into sealed plastic containers and saturated with 95% O_2 / 5% CO_2, which was refreshed at least every 12 hours. Incubations were performed at 14^0C on an orbital shaker at 90 RPM. Media were not changed for the duration of the incubations.

At t = 0, six replicates of two pooled slices were homogenized in 200 µl phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4 and stored at -80°C for later analyses. Six samples of media were also collected and stored at -80^0C. After each experiment, slices were homogenized in phosphate buffer and aliquots of media were collected for Vg assays and stored at -80^0C.

Viability of the slices was determined structurally and energetically. At t = 0, four slices randomly were fixed in 10% phosphate buffered formalin for histological analyses to estimate the percentage of viable cells. ATP production was measured using kits purchased from Sigma Chemical Co. which is a modification of the procedure described by Adam (1963). At the end of each experiment, slices were fixed for histological examination or assayed for ATP content with the percentage of viable cells and ATP content, respectively, compared to 0 hr and experimental controls (vehicle treated slices). Slices with ATP contents and histologically viable cells > 80% of controls were deemed viable.

Juvenile Mt. Shasta rainbow trout, *Oncorhynchus mykiss*, 12-18 months old, were kept in 375 liter tanks at 14^0C under a 12:12 hr light:dark cycle and fed a maintenance ration (2.8% w/w) of Oregon test diet (OTD). In the first study, the ability of I3C, I33' and a mixture of I3C acid condensation products to induce Vg

was investigated. Dietary administration of I3C and RXN (25 – 2000 mg/kg/day), I33' (2.5 – 250 mg/kg/day), 17β-estradiol (0.05, 0.5, and 5 mg/kg/day) and toremifene, (1 – 250 mg/kg/day) was carried out for 2 weeks. All test compounds were dissolved in DMSO which constituted > 0.25% of the diet. In the second study to determine any modulation of E_2 mediated Vg induction, E_2 (0.5 or 5.0 mg/kg/day) and I33' (250 mg/kg/day) were fed to juvenile trout for 2 weeks.

On day 15 of both studies, fish were euthanized with an overdose of tricane methanesulfonate (MS222) as approved by the Oregon State University Institutional Animal Care and Use Committee and weighed. Blood was extracted from the caudal vein into vials containing 45 units sodium heparin, 1 mM EDTA and 50 KIU/ml aprotinin. Plasma was collected by centrifugation of blood at 2000 x g for 10 min at 4^0C and stored at -80^0C for later Vg and steroid quantification.

Quantification of homogenate and media Vg were based on an ELISA previously described by Donohoe and Curtis (1996) and modified by Shilling and Williams (2000). We used an antibody raised in rabbits against anti-chum salmon Vg (1:1500 dilution), which was given to us by Donald Buhler, Oregon State University, from A. Hara at Hokkaido University. Colormetric readings for the ELISA were performed on a Biotek EL 340 microtiter plate reader (Winooski, VT) and analyzed with Deltasoft 3 plate reader software (Princeton, NJ). The limit of detection was determined to be 6.25 ng Vg / ml sample with intrassay and interassay variability for all reported values < 10% and 15%, respectively. Liver slice Vg was normalized to protein concentration determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Significant Vg induction between treatment groups of six samples of two slices each and negative controls containing vehicle alone was determined by t-tests, assuming unequal variances. One way ANOVA and F-tests were used to determine differences among treatment groups.

For steroid analyses, steroids were extracted 2X from blood plasma with 2 ml diethyl ether. Tubes were frozen in liquid nitrogen and the ether collected each time. The ether was evaporated off in a SpeedVac concentrator system (Savant, Holbrook, NY). Steroids in the dried tubes were resuspended in buffer supplied by enzyme immunoassay (EIA) kits purchased from Cayman Chemical (Ann Arbor, MI), used to determine estradiol and testosterone levels, and stored at -80^0C until use. The limits of detection for these EIA assays were 10 pg/ml for estradiol and 6.0 pg/ml for testosterone.

The homogenates stored in the previously described phosphate buffer containing glycerol were diluted in loading buffer containing 4 ml H_2O, 1.6 ml 10% sodium dodecylsulfate (SDS), 1 ml 0.5 M Tris, pH 6.8, 0.8 ml glycerol, 0.4 ml β-mercaptoethanol, and 0.4 ml 0.1 % pyronin Y, to a concentration of 2 μg protein / μl, based on Lowry protein data. To a 1 mm thick, 3% acrylamide stacking gel (0.5 M Tris, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate and 0.1% TEMED) that was placed on top of an 8% polyacrylamide gel (1.5 M Tris, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.1 % TEMED), 5 μl (10 μg) of each sample in loading buffer was added. Gels were degassed under vacuum and poured immediately after the addition of TEMED. Gels were run in Hoefer SE 260 running units (Pharmacia Biotech Inc., San Francisco, CA) in buffer containing 3 g Tris base, pH 8.3, 14.4 g glycine and 1 g SDS per liter of water. Gels were transferred using a BioRad (Hercules, CA) semi-dry transfer cell at 10V for 25 min unto 0.45 μm nitrocellulose paper. Blots were dried at 65^0C for 30 min and then placed in phosphate buffer, pH 7.4 containing 2% BSA. After 1 hour, blots were placed in rabbit anti-trout CYP1A for 1 hour and then washed 4X in phosphate buffer containing 0.05% Tween-20 for 5 min. Blots were placed in goat anti-rabbit IgG for 1 hour and washed 4 more times. Blots were developed using ECL kits from Amersham (Buckinghamshire, England) and exposed to X-ray film.

Liver slice homogenate and media samples were extracted with 4 volumes ethyl acetate and vortex shaken for 1 min. The tubes were snap frozen in liquid nitrogen and the ethyl acetate layer poured into clean tubes. The aqueous fraction was extracted again. The ethyl acetate fractions were evaporated to dryness with a SpeedVac concentrator and resuspended in 250 µl acetonitrile and stored at -80^0C. Samples (50µl) were injected into a Shimadzu LC-6A (Kyoto, Japan) liquid chromatograph that had a Beckman C-18 ODS column (4.6 mm x 25 cm). The flow rate was 1 ml /min of 80% water and 20% acetonitrile from t = 0 – 30 min, 15% water, 85% acetonitrile from 30 – 45 min and 100% acetonitrile from 45 – 55 min. Absorption was measured at 280 nm with a deuterium lamp detector (Shimadzu LC-6AV). Desired peaks were verified by electrospray mass spectrophotometry.

RESULTS

I3C has been implicated in altering the metabolism profile in the liver through induction of Phase I and Phase II enzymes such as cytochrome P450s and glutathione-S-transferase, respectively. Since Vg is produced in the liver of the trout, it was critical to assess any toxicological factors that could introduce confounding variables to our studies. For liver slice viability we examined the slices histologically and found E_2, I33', I3C, RXN to be non-toxic under these conditions. Energetic analysis by ATP content quantification also indicated that the test compounds were non-toxic under these parameters. In fact, ATP content was often elevated after 96 hr in slices at the highest concentration of test compounds compared to 0 hr and 96 hr controls. Another viability parameter measured was protein content of slices, which in most cases was + 20% of control slices (Figure 5.1). *In vivo*, we observed no adverse effects to the trout as determined by gross

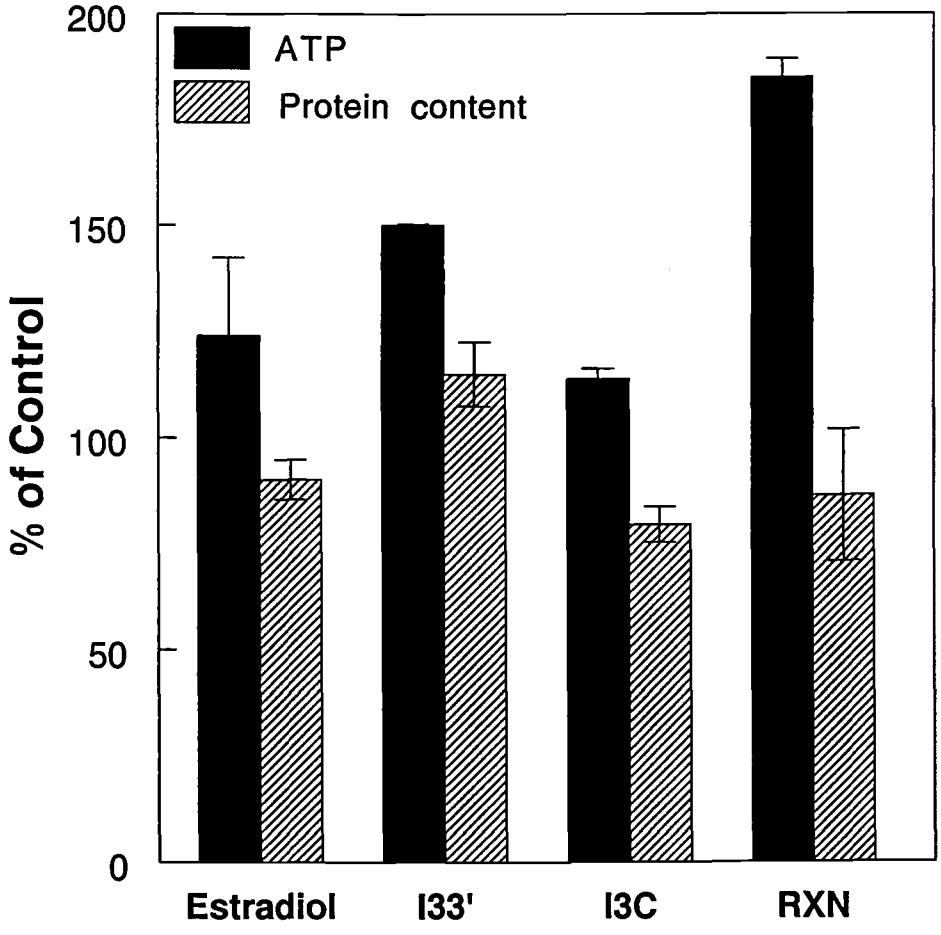

Figure 5.1: ATP content (n = 3) and protein content (n = 6) for slices incubated with E_2 (1000 nM), I33', I3C and RXN (250 μM) for 96 hr. Values are exhibited as a percentage of corresponding controls treated with vehicle only. Error bars denote ± SE.

examination and by body weight. For the I33' and E_2 co-feeding study, we measured livers as well as body weights to calculate the liver somatic index (LSI). Both compounds increased LSI values in male and female trout by about 50% compared to controls, but in combination, the E_2 and I33' effects did not appear to be additive (Table 5.1).

Table 5.1: Body weight and liver somatic index (LSI) values for male and female rainbow trout fed 0.5 or 5 mg/kg E_2 alone or with 250 mg/kg I33' for 14 days. Significant differences in LSI by E_2 and I33' alone or in combination ($p < 0.05$) are denoted with (*). SD is the standard deviation of the means (n = 4-12).

	MALE		**FEMALE**	
	Weight ± SE(g)	**LSI ± SE**	**Weight ± SE(g)**	**LSI ± SE**
Control	278 ± 61	1.55 ± 0.21	280 ± 78	1.41 ± 0.23
0.5ppm E_2	336 ± 62	1.87 ± 0.37	306 ± 73	1.99 ± 0.27*
5ppm E_2	300 ± 89	2.40 ± 0.38*	284 ± 36	2.44 ± 0.17*
250ppm I33'	278 ± 93	2.33 ± 0.36*	282 ± 84	2.08 ± 0.23*
0.5ppm E_2+I33'	249 ± 144	2.43 ± 0.41*	249 ± 97	2.29 ± 0.14*
5ppm E_2+I33'	237 ± 74	2.66 ± 0.25*	277 ± 82	2.66 ± 0.31*

Treatment in liver slices and *in vivo* established I33' as a fairly potent estrogen in the rainbow trout liver by inducing Vg. In liver slices from juvenile male rainbow trout, I33' was as effective as E_2 albeit with 2500-fold less potency in inducing Vg levels, which were increased by up to 300-fold compared to 96 hr controls (Figure 5.2). RXN, which was previously reported by Grose and Bjeldanes, (1992), to contain 5.9% I33', was about 15-fold less potent and efficacious as I33' in inducing Vg. I3C did not significantly induce Vg even at concentrations up to 200 μM. Analyses of liver slice homogenates and surrounding media by HPLC indicate the presence of I33' in samples treated with I33' and RXN with none detected in I3C samples even after 96 hr (Figure 5.3).

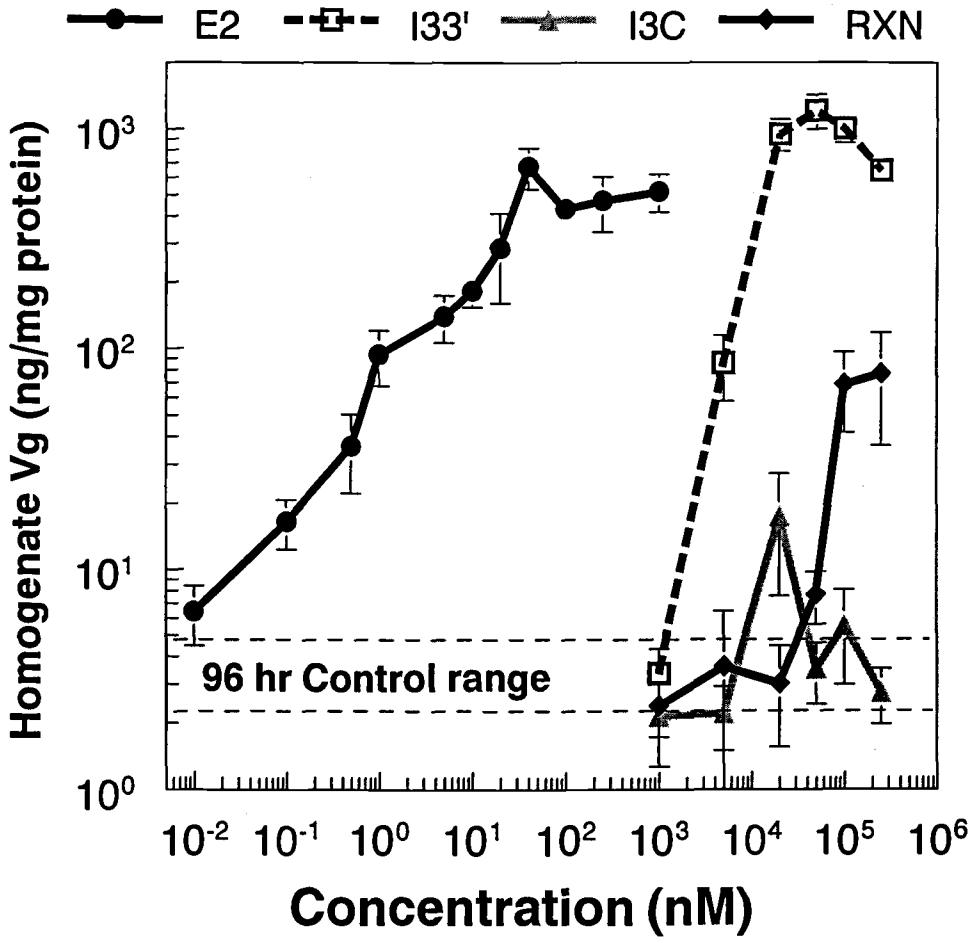

Figure 5.2: Vg levels for liver slices incubated for 96 hr in E_2 (0.01 – 1000 nM), I33', I3C, and RXN (1 – 250 μM). Significant induction was observed in slices treated with $E_2 \geq 0.1$ nM, ≥ 5 μM I33' and ≥ 50 μM RXN ($p < 0.05$, ANOVA F-test). Significant induction was observed only at the 20 μM concentration of I3C and did not represent a trend. The range of the corresponding 96 hr control slice Vg levels is drawn for reference. Error bars represent $\pm$ SE ($n = 6$).

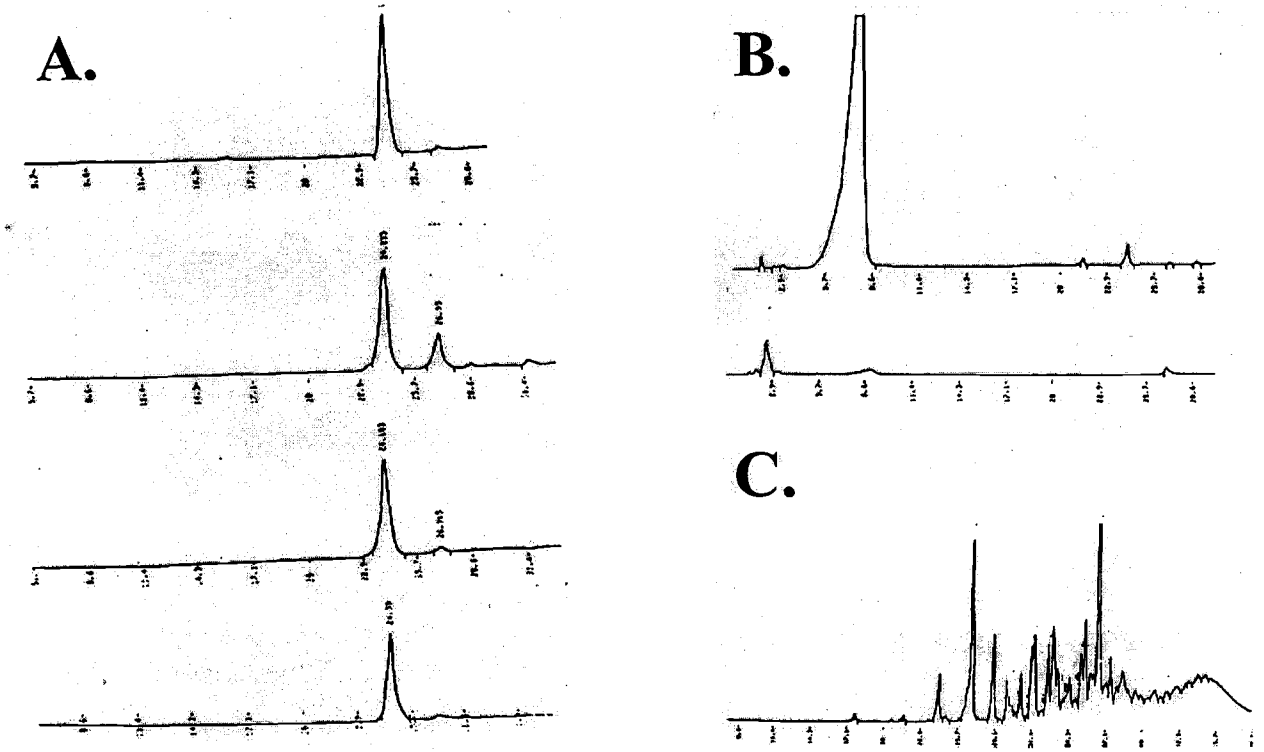

Figure 5.3: HPLC analyses for I3C oligomers in liver slices incubated with I33', RXN or I3C (50 μM). The left column is for slices treated with I33' for 6 – 48 hr with the t = 0 hr media sample shown for reference. The right column is I3C t = 0 media samples (top) and t = 96 hr slice samples (middle). No I3C oligomers were detected at any timepoint. A representative RXN slice sample (48 hr) is also shown (bottom).

To support the concept that I33' is actually binding the estrogen receptor, slices were incubated with tamoxifen. Tamoxifen alone did not induce Vg in the liver slices, a phenomenon observed *in vivo*, however, it blocked the estrogenic action of 100 μM I33' at 5μM and decreased I33' induced Vg levels to below control levels at 100 μM (Figure 5.4). When I33' was incubated with E_2, at submaximal concentrations, the effects were not different from an additive effect (Figure 5.5). We also co-incubated slices treated with I33' at a concentration near its EC_{50} value (20 μM) with a non-specific P450 inhibitor, SKF525A (5, 20 and 100 μM). SKF525A alone had no effect on Vg levels and was not toxic to the slices in the presence or absence of I33' as measured by ATP and protein content and visualized by histological examination. In the test groups, SKF525A significantly reduced I33' mediated Vg induction by up to 80% at the three concentrations tested ($p < 0.01$, F-test, Figure 5.6). I33', RXN as well as I3C induced the cytochrome P4501A protein in liver slices at a higher concentration than was needed to significantly increase Vg (Figure 5.7). This indicates that estrogenicity is more sensitive than CYP1A protein induction as a biological endpoint of I33' exposure; i.e. I33' is more potent as an estrogen receptor- than an aryl hydrocarbon (AhR) agonist.

The *in vivo* data generally support the *in vitro* findings (Figure 5.8). In the whole animal feeding model, however, I3C was an effective Vg inducer albeit with about 1000-fold less potency than E_2. In fact, at 2000 ppm, I3C induced Vg to about 20 mg/ml, extremely high levels compared with administration of 5 ppm E_2. These Vg values are normally only observed in mature, vitellogenic females. This supports the requirement for an acidic environment, such as that found in the stomach, to form the estrogenic components of I3C. I33' was the most potent acid condensation product of I3C tested for inducing Vg in the trout. It was five times

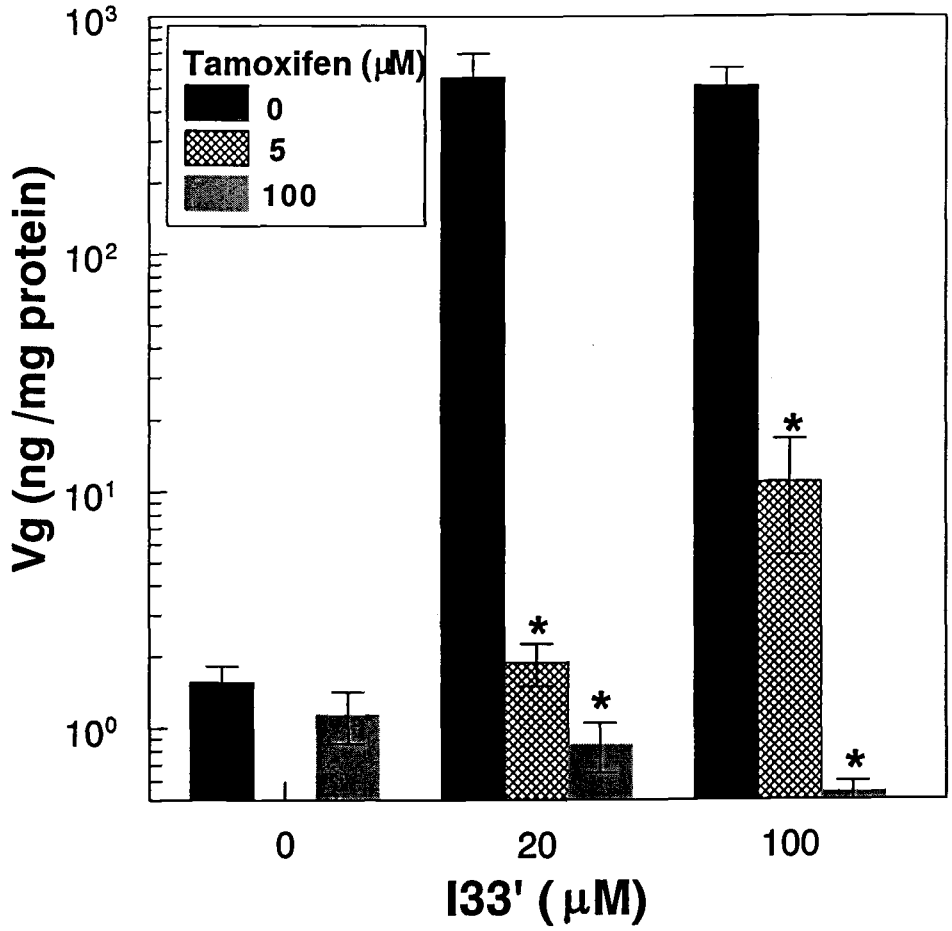

Figure 5.4: Inhibition of I33' induced Vg production by tamoxifen. Tamoxifen was not estrogenic in this model and decreased Vg with high statistical significance at 5 and 100 μM when combined with 20 or 100 μM I33', * denotes $p < 0.001$ (ANOVA F-test). Error bars denote ± SE (n = 6). ND = not determined.

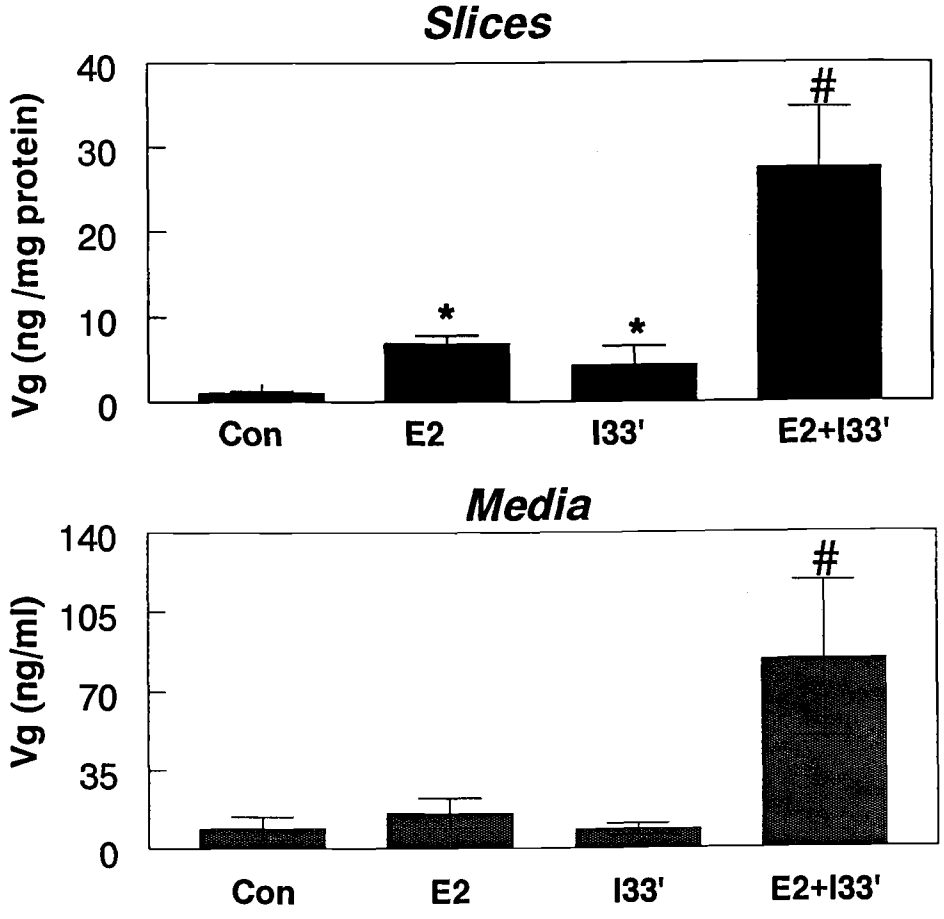

Figure 5.5: Vg levels for liver slices and surrounding media incubated for 96 hr with 5 nM E_2, 5 μM I33' and co-incubation of E_2 and I33'. Significant Vg induction compared to corresponding control ($p < 0.05$, ANOVA F-test) is denoted with (*). Significant differences by the E_2 + I33' groups compared to both of the corresponding E_2 and I33' alone groups is denoted with (#). Error bars represent ± SE (n = 6).

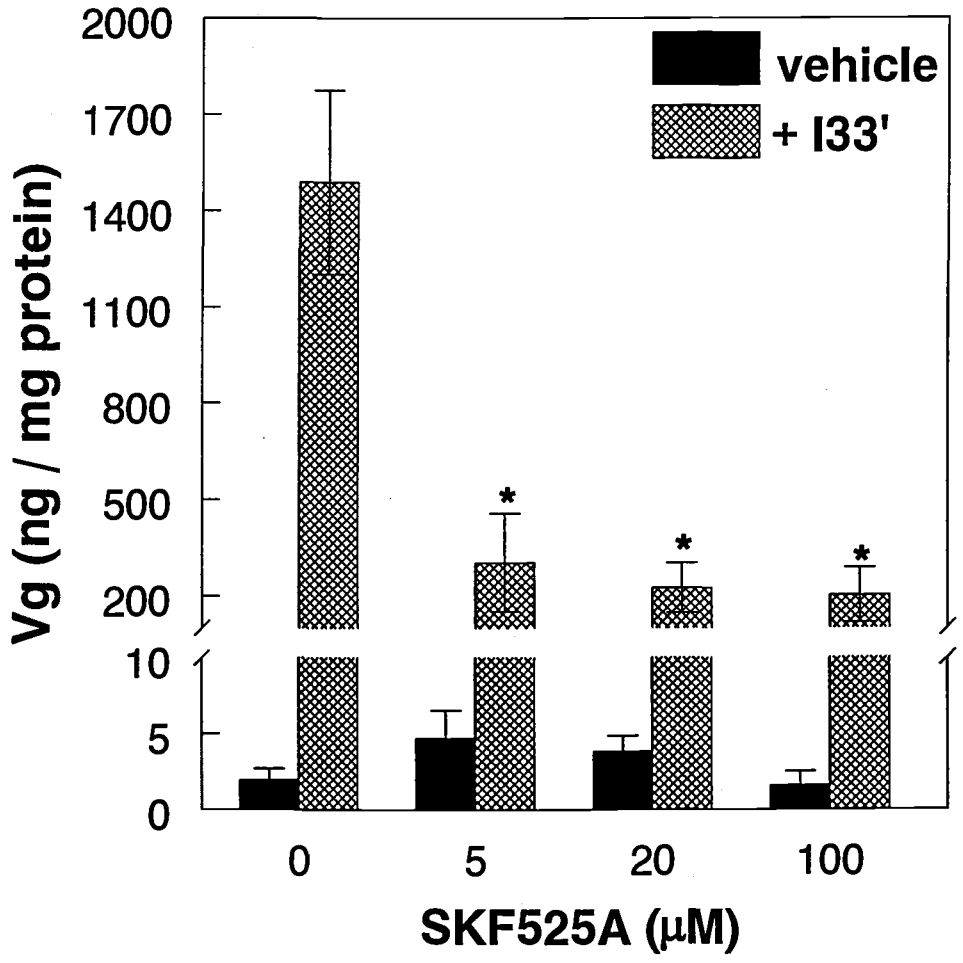

Figure 5.6: Effect of SKF525A (5, 20 and 100 μM), a broad-spectrum cytochrome P450 inhibitor, on Vg induction by 20 μM I33'. SKF525A had no significant effects alone, but reduced I33' induced Vg significantly at all concentrations tested by up to 80% ($p < 0.01$, F-test) and is denoted with (*). Error bars represent ± SE ($n = 6$).

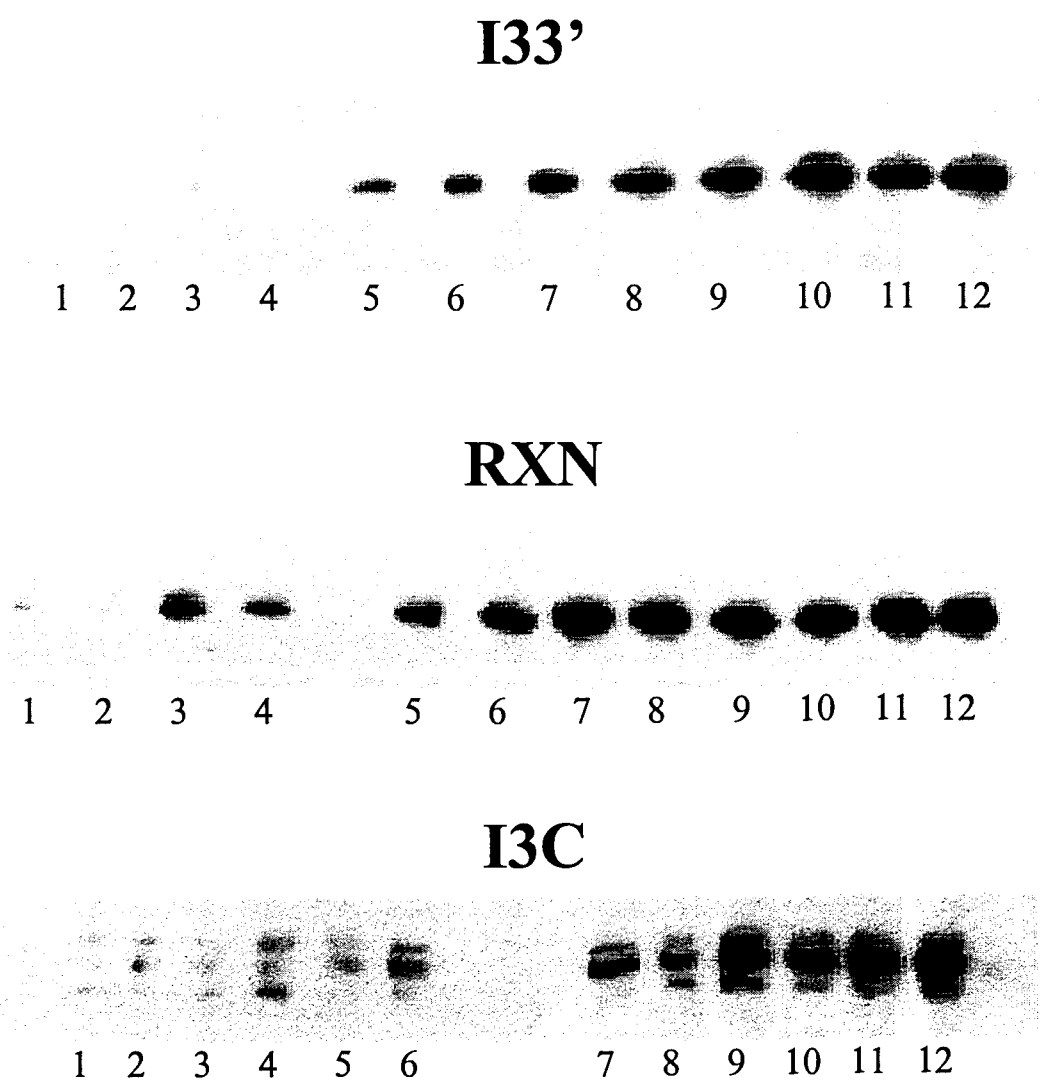

Figure 5.7: Western blots of CYP1A as fold induction vs vehicle treated slices. Liver slices were treated with I33', RXN or I3C (0 – 100 μM) for 96 hr. Lanes 1,2 - 0 μM; 3,4 - 1 μM; 5,6 - 5 μM; 7,8 - 20 μM; 9,10 - 50 μM; and 11,12 - 100 μM.

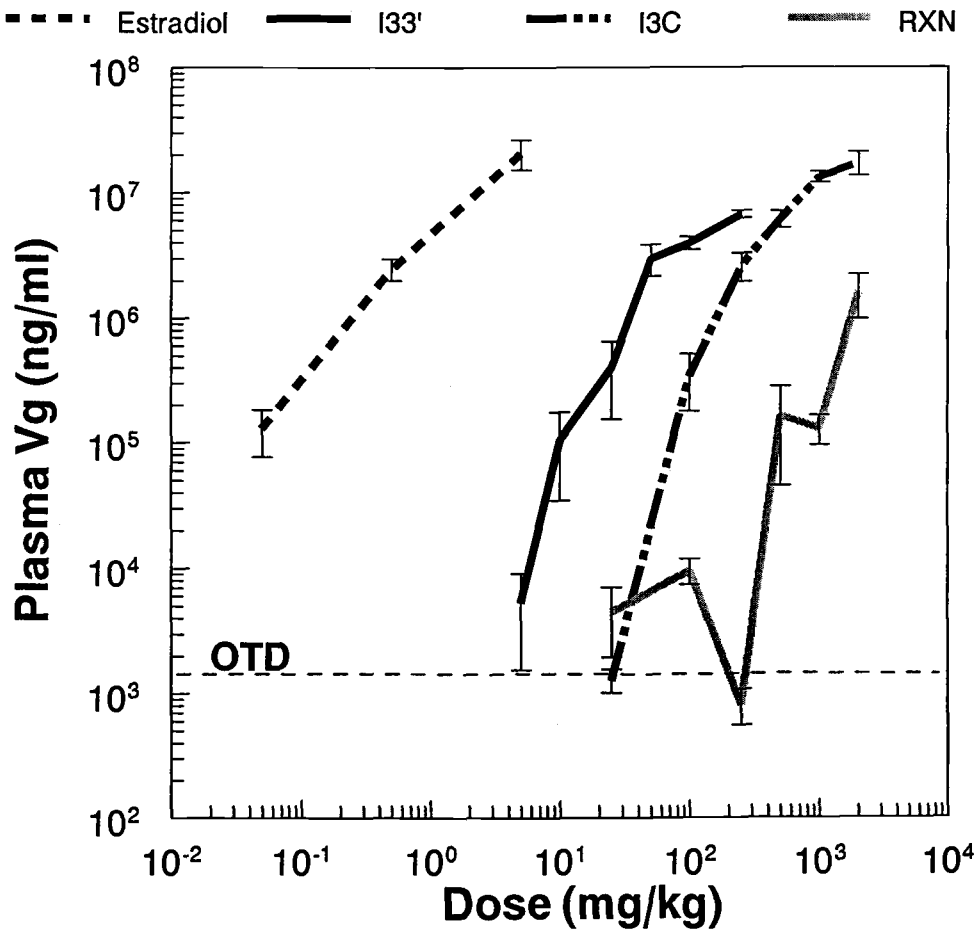

Figure 5.8: Blood plasma Vg induction in male rainbow trout fed E_2 (0.05 – 5 mg/kg), I33' (2.5 – 250 mg/kg), I3C and RXN (25 – 2000 mg/kg) for 14 days. Mean plasma Vg in trout fed OTD containing vehicle is drawn for reference. Significant Vg induction vs vehicle only trout was observed $\geq$ 0.05 ppm E_2, $\geq$ 10 ppm I33', $\geq$ 100 ppm I3C. For RXN significant induction was seen at 100 and $\geq$ 500 ppm. Error bars represent $\pm$ SE (n = 4).

as potent as I3C alone, but was not as efficacious at 250 ppm, the highest dose tested. This may be due to the fact a higher I33' dose was required to reach maximal efficacy, potential toxicity at higher doses, or the requirement of other I3C metabolites to additively maximize Vg induction. RXN was an effective Vg inducer as well, but was the least potent, perhaps due to the lower amount of estrogenic compounds present. If approximately 6% of the RXN is I33', then we would have expected RXN to be about 15-20 times less potent than I33'. Table 5.2 summarizes the *in vitro* and *in vivo* estrogenicity studies supporting this relationship *in vivo*.

Table 5.2: Comparison of relative estrogenicities for E_2, I33', I3C and RXN as measured by Vg induction in rainbow trout liver slices (*in vitro)* and feeding studies (*in vivo*). Values are based on the data for curves drawn in Figures 5.2 and 5.8. Numbers in parentheses next to EC_{50} values represent potency values relative to E_2. Maximum efficacy was calculated as the highest magnitude of induction as fold increase compared to corresponding vehicle controls. I3C did not produce an induction curve *in vitro* and is denoted as not active (NA).

	In vitro		***In vivo***	
	EC_{50} (nM)	**Max efficacy**	**EC_{50} (ppm)**	**Max efficacy**
E_2	2.0 (1)	220	0.1 (1)	1.31×10^5
I33'	5000 (4.0×10^{-4})	309	20 (5×10^{-3})	4.22×10^4
I3C	NA	NA	100 (1×10^{-3})	1.08×10^5
RXN	70000 (2.9×10^{-5})	36	500 (2×10^{-4})	1.02×10^4

We wanted to determine if the Vg induction observed *in vivo* was solely due to estrogen receptor binding or by an indirect mechanism such as upregulation of E_2 levels. Figure 5.9 depicts E_2 levels in trout fed the highest dose of each compound.

Only E_2 treatment resulted in a significant elevation in blood plasma E_2 levels compared to vehicle treated trout. Although tamoxifen was used in the liver slice experiments, we chose to use toremifene instead of tamoxifen as our antiestrogen *in vivo* because we have found tamoxifen to be an inducer of trout Vg in previous feeding studies (unpublished data). Toremifene, when fed to male trout, weakly induced Vg at doses > 10 mg/kg, but displayed no antiestrogenic properties (Figure 5.10), while having no effect on plasma E_2 levels (Figure 5.11). It also significantly added to the Vg induction seen in trout fed 5 mg/kg E_2 alone ($p < 0.05$). It is apparent by this study that toremifene is not a useful antiestrogen in the trout.

The question arose as to whether I33' could antagonize the estrogenic effects of other compounds, such as E_2. Rainbow trout were fed 250 mg/kg I33' with 0.5 or 5 mg/kg E_2 for 14 days. Both compounds administered alone displayed Vg induction profiles similar to those observed in the first study (Figure 5.12). When combined, an additive effect was observed to a maximal level of 100 mg/ml Vg which became sex non-specific. This level is believed to be the maximum for Vg levels in rainbow trout. No significant effect on plasma E_2 levels in either sex (Figure 5.13). This study is consistent with the hypothesis that I33' has no antiestrogenic properties in the rainbow trout.

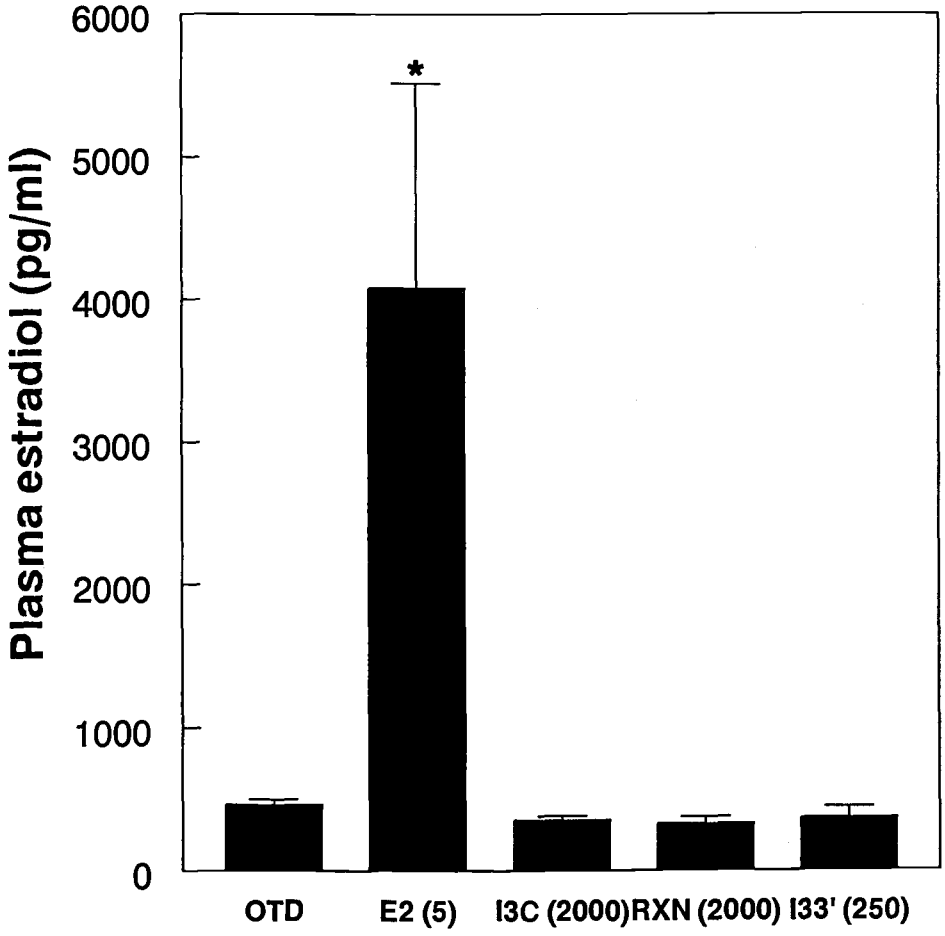

Figure 5.9: Blood plasma E_2 levels for male trout fed the highest dose of each compound (next to label in mg/kg in parentheses). Only trout fed E_2 had significantly different plasma E_2 levels compared to vehicle only trout ($p < 0.05$, F-test). Error bars represent $\pm$ SE ($n = 4$).

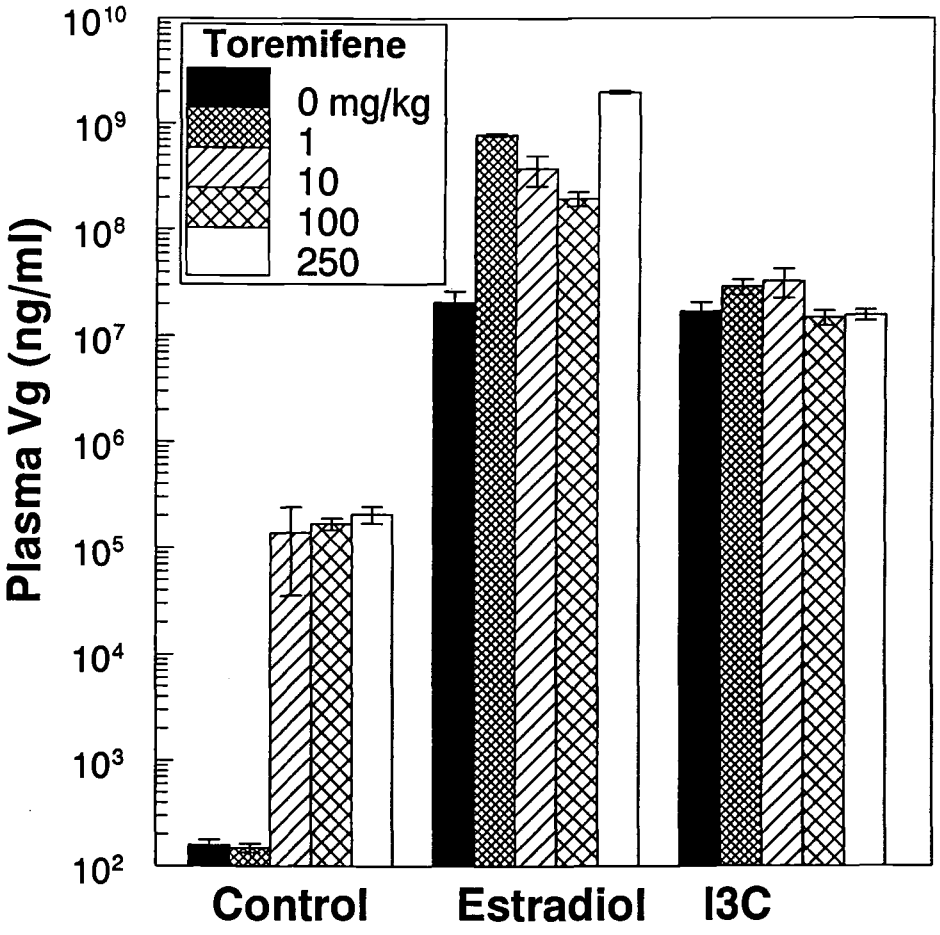

Figure 5.10: Vg levels for male trout fed 5 mg/kg E_2 or 1000 mg/kg I3C in combination with toremifene (0 – 250 mg/kg). Significant induction in the absence of toremifene is denoted with (*) and differences within groups by toremifene treatment is denoted with (#), ($p < 0.05$, F-test). Error bars represent $\pm$ SE ($n = 4$).

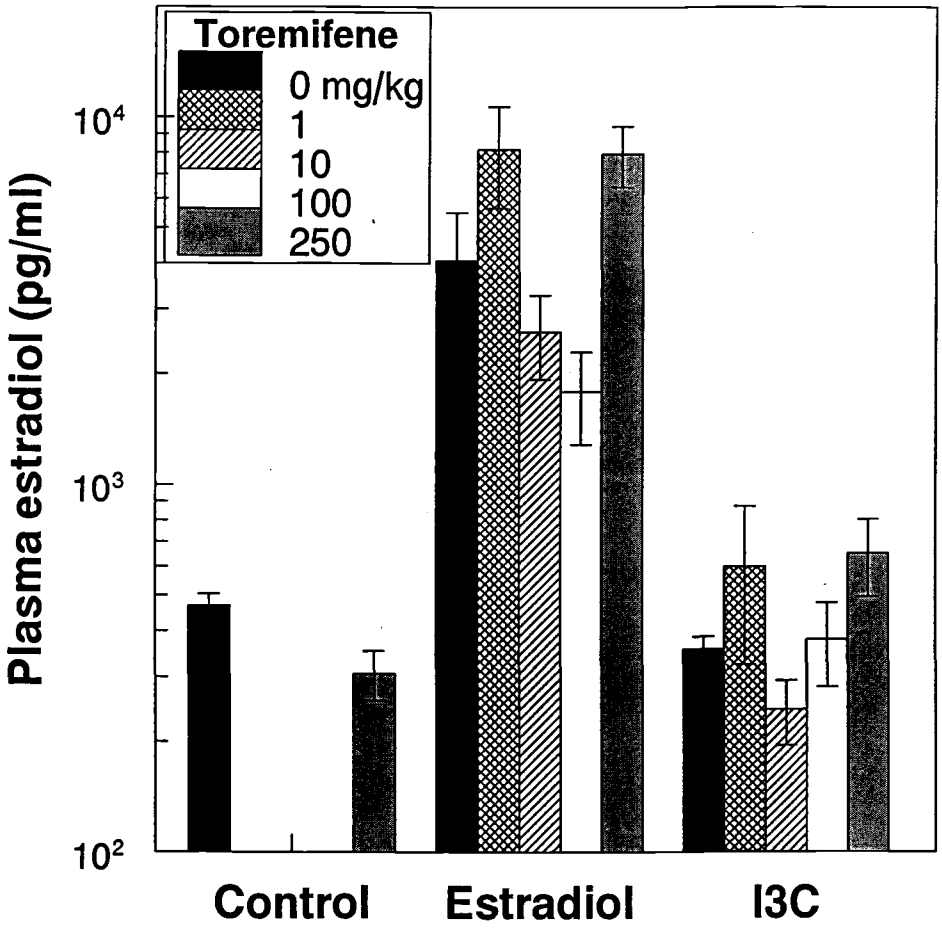

Figure 5.11: E_2 levels for male trout fed 5 mg/kg E_2 or 1000 mg/kg I3C with or without toremifene. E_2 significantly raised plasma E_2 levels ($p < 0.05$, F-test). No significant effect was observed with I3C or toremifene co-administration in any group. Error bars represent $\pm$ SE ($n = 4$).

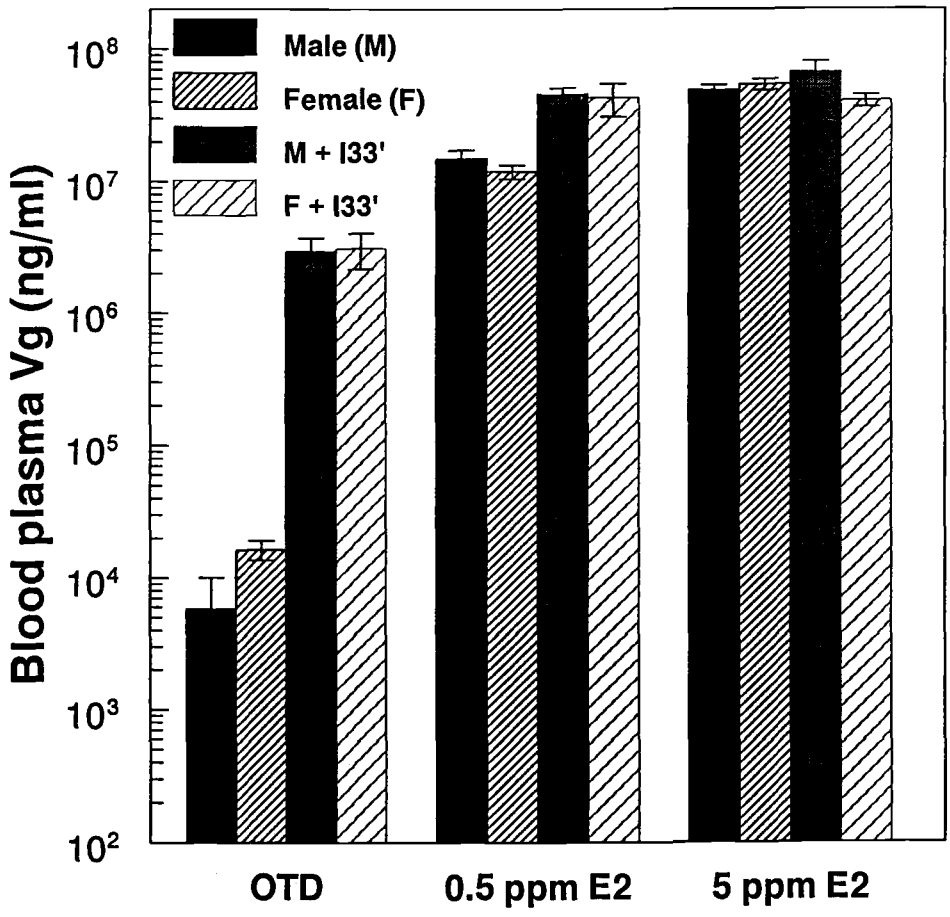

Figure 5.12: Blood plasma Vg induction for male and female trout fed E_2 (0.5 and 5 mg/kg) alone or with 250 mg/kg I33'. The estrogenic effects were additive with no significant sex differences in Vg levels. There appeared to be a saturation point for plasma Vg at 100 mg/ml. Error bars represent $\pm$ SE (n = 4 - 12).

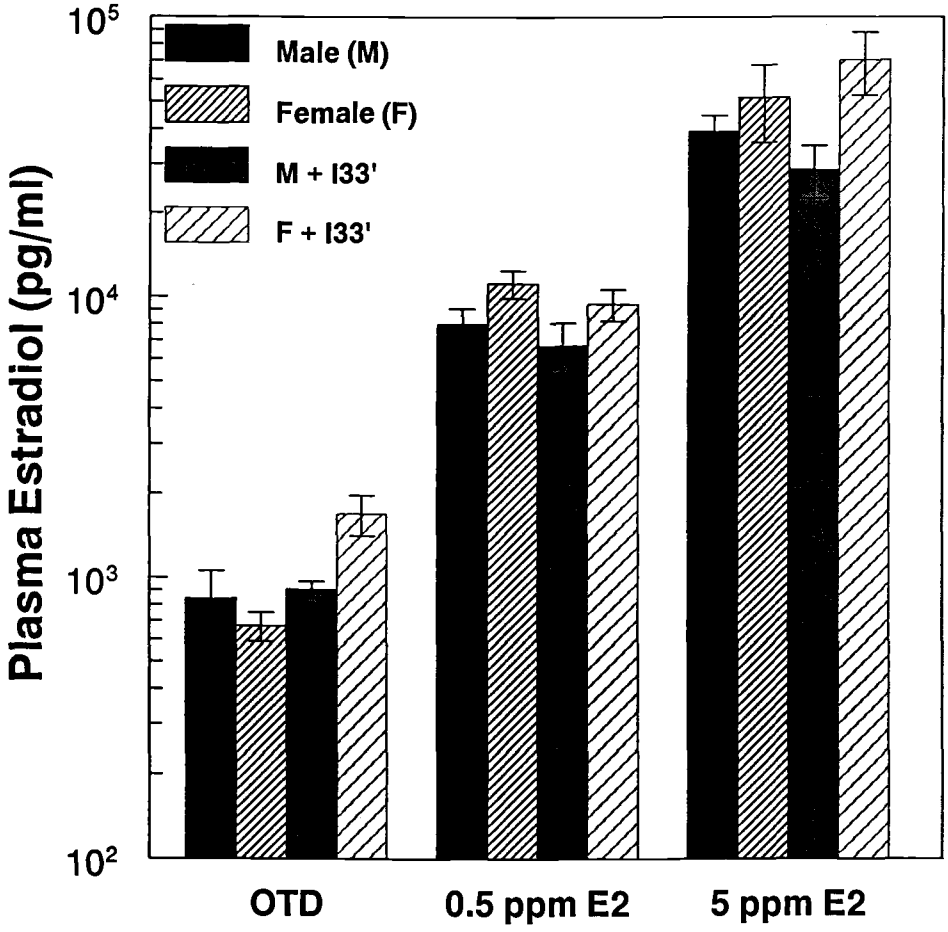

Figure 5.13: E_2 levels in male and female trout fed E_2 (0.5 and 5 mg/kg) alone or with 250 mg/kg I33'. As demonstrated previously, E_2 alone increased plasma E_2 levels significantly in both sexes. Co-treatment with I33' had no effect on modulating the E_2 increases. Error bars represent $\pm$ SE (n = 4 - 12).

DISCUSSION

In contrast to previous reports implicating I3C to be an antiestrogen, we documented substantial evidence in this paper that I3C and at least one of its acid condensation products (I33') are estrogenic in the rainbow trout. Utilizing rainbow trout liver slices from juvenile males, I33' was a fairly potent estrogen (EC_{50} = 5 μM), inducing Vg to the same maximal levels as was observed with E_2. It was approximately 4 x 10^4 fold less potent than E_2, but would be considered a relevant environmental estrogen because its EC_{50} value is lower than *o,p'*DDE (10 μM) and Bisphenol A (50 μM), well established estrogens previously tested in this model (Shilling and Williams, 2000). Based on consumption levels of cruciferous vegetables, this value may be practically relevant to humans. Much of the literature covering I3C recognize the prerequisite for the formation of condensation products as I3C has very little activity in its parent form. In the case of inducing Vg, this also seems to be true. I3C did not significantly induce Vg in the trout liver slices at concentrations up to 250 μM. RXN, however, which contains I33' as well as several other I3C oligomers, significantly induced Vg, with about one-fifth the potency and efficacy of I33' (EC_{50} = 70 μM). Tamoxifen completely eliminated the I33' induced Vg response, supporting the hypothesis that I33' was directly binding the estrogen receptor and inducing Vg production. When co-incubated, I33' and E_2 induced Vg in an additive fashion supporting the notion that I33' possesses no antiestrogenic properties in this liver slice model.

We were interested in verifying that I33' was entering the liver slices to induce Vg. Analysis by HPLC found that after 6, 24 and 48 hr incubations, liver slices treated with I33' contained significant amounts of I33'. In RXN treated slices, I33' was also present as well as many other higher order oligomers. In I3C treated slices, no I33' was present. In fact, no significant peaks, including parent I3C were detected even after a 96 hr incubation. Single or multiple hydroxylated

metabolite(s) of I33' may be the ultimate estrogen(s) inducing Vg in the rainbow trout. It is possible that a cytochrome P450 would be responsible for this transformation. Our hypothesis was that I33' mediated Vg induction would be blocked if this conversion did not take place. In liver slices treated with I33', SKF525A, a broad spectrum cytochrome P450 inhibitor, reduced Vg levels by up to 80% in the absence of toxicity. This suggests that cytochrome P450s are important for Vg induction by I33'. The two-week feeding studies in juvenile male trout fed the test compounds supported the liver slice data that conversion of I3C was critical for Vg induction. Moreover, I33' or an I33' metabolite appears to be the major, if not primary metabolite responsible for the Vg induction. When fed alone, I3C induced Vg with an EC_{50} of 100 ppm while I33' was about 5 times as potent. I3C and I33' elevated Vg to extremely high levels (> 5 mg/ml) at 2000 ppm and 250 ppm, respectively, supporting the assumption that these compounds and dietary constituents containing these compounds possess potent estrogenic potential. The induction was observed in the absence of increased E_2 levels suggesting that Vg induction by these compounds are mainly due to an estrogen receptor mediated response. Unfortunately, this hypothesis could not be tested because the antiestrogen utilized *in vivo*, toremifene, was proven to be ineffective. We did find that blood plasma E_2 levels were not affected by I33', I3C or RXN treatment at the highest doses tested, ruling out increased E_2 synthesis as an indirect mechanism for Vg induction. Numerous reports have demonstrated the ability of I3C and its condensation products to modulate hepatic metabolism, particularly inducing cytochromes P450 (P450s). P450s play an important role for metabolizing estrogens and estrogen precursors. Alterations in this homeostatic balance could increase/decrease estrogenic responses. These effects may be magnified in a population that normally is absent of estrogenic stimulation, i.e. juvenile male rainbow trout.

It is possible that I33' is estrogenic alone, but possesses antiestrogenic properties when combined with another estrogen. In the liver slice and whole animal models, administration of I33' in conjunction with E_2 resulted in an additive effect on Vg induction. There were no effects on circulating E_2 levels in the feeding studies as well. The data from these studies suggest no evidence to support that I33' possesses any estrogenic characteristics in the rainbow trout.

Combining the data from the liver slice (*in vitro*) and two-week feeding (*in vivo*) studies leads to the conclusion that I33' is an estrogen in the rainbow trout. The mechanism appears to be through direct estrogen receptor binding and subsequent Vg synthesis as demonstrated in the liver slice model. We were unable to confirm the interaction of I33' and the estrogen receptor *in vivo*, due to the ineffectiveness of toremifene, but could rule out any indirect effects on E_2 levels. In attaining extremely high levels of Vg induction comparable to that of a high dose of E_2 ($\sim 10^4 - 10^5$ fold), it is conceivable that the majority of the I33' response is from estrogen receptor binding. We are currently investigating the ability of I3C, I33' and major metabolites isolated by HPLC to bind the trout liver estrogen receptor.

Enzyme modulation is considered to be a major mechanism of chemoprevention by I3C and its condensation products (induction in mammals and inhibition in trout). We reported substantial Vg induction at a lower concentration of I33' than was required for CYP1A protein induction. This indicates that in the rainbow trout, Vg induction (estrogen receptor binding) is a more sensitive endpoint than CYP1A protein induction (AhR binding). The implication that I3C or one of its major condensation products formed in the stomach could be estrogenic is critical. We fed rainbow trout 2000 ppm I3C for two weeks and observed a near maximal response comparable to E_2. Takahashi *et al.* (1995a), reported that in trout fed 2000 ppm I3C for 1 week, I33' concentrations in the trout

liver were 70 μM, which would also elicit a maximal Vg response in liver slices. Given the role of estrogens in promotion of carcinogenesis, particularly in the liver (Williams *et al.*, 1998, Metzler *et al.*, 1990, Yager and Liehr, 1996) and extrahepatic organs such as the breast and uterus (Brodie *et al.*, 1999 and Fujimoto *et al.*, 1998), more research should be conducted to determine whether the use of I3C or I33' to treat and/or prevent estrogen dependent tumors is a practical approach.

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CHAPTER 6

VITELLOGENIN INDUCTION BY ENVIRONMENTAL ESTROGENS IN CHINOOK SALMON LIVER SLICES

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ABSTRACT

Utilization of sensitive species in screening assays for estrogenicity is critical for their effectiveness. We have previously applied rainbow trout, *Oncorhynchus mykiss*, liver slices as a model to assess estrogenicity via vitellogenin (Vg) induction. In this paper, Vg induction in Chinook salmon (*Oncorhynchus tshawytscha*) liver slices by 17β-estradiol (E_2) was quantitated using a method previously developed in our laboratory. The relative estrogenicity of several dietary and environmental compounds via Vg induction in liver slices was also quantified. Dose response experiments revealed that the liver slices produced Vg in response to estrogens as determined by ELISA. Significant induction was observed at 40 nM E_2 and above in liver slices, which is more than a hundred times higher than previously reported for rainbow trout. Maximal induction in liver slices was about 50-fold higher than controls not treated with E_2. In time course studies, significant Vg induction was detected by 72 hr after treatment with 1000 nM E_2 in liver slices. The salmon liver slices were viable for 120 hr as determined by ATP content and histological examination. Xenoestrogens, *o,p'*-DDE and OH-PCB30 and the dietary constituent, 3,3'-diindolylmethane (I33') significantly induced Vg in salmon, but with less potency than previously observed in the rainbow trout. PCB30, *o,p'*-DDE and I33' significantly induced Vg (potency - OH-PCB30 = I33' > *o,p'*-DDE) with I33' being the most efficacious. Bisphenol A, shown to induce Vg in rainbow trout had no effect in the salmon. In summary, the Chinook salmon produced Vg in response to estrogen treatment, but appear to be less sensitive than the trout. Our findings support the application of liver slices from oviparous animals across different species to quantify relative estrogenicity via Vg induction. These studies also stress the importance of comparative studies between species and support the value

of rainbow trout liver slices as a sensitive and useful *in vitro* model for quantifying potential estrogenic compounds. (Supported by grants ES03850 and ES07060).

INTRODUCTION

The synthesis and mechanisms of action of estrogens is critical throughout the life history of nearly all animals. Recent articles review several of the beneficial physiological roles of estrogens such as in regulation of development, neural function, bone integrity, hormone balance, cardiovascular health and disease, and as an antioxidant (Grumbach and Auchus, 1999, Mendelsohn and Karas, 1999, McEwen, 1999, Rickard *et al.*, 1999, Miller and Franklin, 1999). Estrogens also have properties that could be classified as negative such as the ability to induce and promote tumor formation, decrease male potency, disturb reproduction, induce oxidative damage through metabolite formation and alter metabolic enzyme activities and levels (Metzler *et al.*, 1990, Yager and Liehr, 1996, Zhu and Conney, 1998, Wibbels and Crews, 1992, Crain *et al.*, 1997, Janz *et al.*, 1997, Sonnenschein and Soto, 1998, Gray, 1998 and Colborn *et al.*, 1993). The regulation and balance of endogenous estrogens is critical for proper development and function. Disruption of this balance by exogenous exposure can be detrimental at the individual level in the case of tumor modulation, or at species level, particularly if it involves reproductive defects. The potential implications of estrogens have been one of the areas of focus for the recent Environmental Protection Agency Acts.

The mandates to test chemicals for endocrine disrupting properties require assays that can determine the ability of a chemical to mimic endogenous hormones such as 17β-estradiol (E_2). The development of simple and rapid screening models that accurately predict the estrogenic effects of chemicals in humans is critical. Investigating target endpoints in different species provides insight to mechanisms

behind the response and allows for higher confidence for extrapolation. The determination of a biomarker in sensitive or sentinel species is the basis of screening assays. One effective biomarker for estrogen exposure is vitellogenin (Vg), an egg-yolk protein precursor produced in the liver of oviparous animals in response to estrogens (Sumpter and Jobling, 1995). The classification of numerous environmental contaminants as estrogens such as certain DDTs, DDEs, PCBs, alkylphenols as well as phytoestrogens such as coumestrol and genstein has been determined based on Vg induction (Donohoe and Curtis, 1996, Mellanen *et al.*, 1996, White *et al.*, 1994, Smeets *et al.*, 1999, Andersen *et al.*, 1999, Carlson *et al.*, 2000, Petit *et al.*, 1997 and Pelissero *et al.*, 1991).

A wide array of *in vitro* screening assays have been developed for discovering and quantifying estrogenic compounds utilizing reporter gene assays, estrogen sensitive cell lines and receptor binding assays (Nimrod and Benson, 1997, White *et al.*, 1994, Mellanen *et al.*, 1996, Legler *et al.*, 1999, Andersson *et al.*, 1999, Andersen *et al.*, 1999, Ren *et al.*, 1996, Arukwe *et al.*, 1997). Cultured hepatocytes from fish have also been proven to be effective *in vitro* models for Vg induction (Smeets *et al.*, 1999a, Smeets *et al.*, 1999b, Miller *et al.* 1999, Fluoriot *et al.*, 1993, Pelissero *et al.*, 1993, Maitre *et al.*, 1986). A major disadvantage of hepatocytes as a representative model, however, is the lack of liver cell heterogeneity and cell-to-cell interactions found *in vivo*. In our laboratory, we have developed an assay utilizing precision-cut liver slices, which are more representative of the dynamic organ system *in vivo* (Shilling and Williams, 2000). In the liver slices, the structure of the different cells is kept intact, mimicking the whole organ to a greater degree than cultured cells. Moreover, slices are faster and easier to prepare than hepatocytes for this assay because no isolation or culturing steps are required (Ekins 1996, Sidelmann *et al.*, 1996, Bach *et al.*, 1996, Vickers, 1994, Beamand *et al.*, 1993, Price *et al.*, 1998).

Previously we developed optimal conditions for Vg induction in rainbow trout, *Oncorhynchus mykiss*, using precision-cut liver slices. Vg induction by E_2 as well as by environmental and dietary estrogens in Chinook salmon, *Oncorhynchus tshawytscha*, was quantified and compared to that of the rainbow trout. Incubations were performed with liver slices in phenol-red free medium containing fetal bovine serum and the test compound. A modified version of the well-plate shaker system described by Dogterom (1993) was utilized for incubations so that contact of the slices with media and test compounds would not be limited. Liver slice and media Vg levels were quantified by an enzyme-linked immunosorbant assay (ELISA) with viability monitored by histological examination and ATP analyses.

METHODS and MATERIALS

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and supplies from Fisher Scientific (Pittsburgh, PA) unless noted otherwise. Liver slicing was performed using the method previously reported (Shilling and Williams, 2000). All tools and glassware were sterilized before use at 105^0C for 30 min. Livers were extracted from male juvenile (< 18 month) Chinook salmon (100-200 g) and put in ice cold Hank's buffer containing 8 mM HEPES and 0.35 g/L sodium bicarbonate, pH 7.2. Media was filtered through a 0.22 μm membrane to assure sterilization. Using an 8 mm coring device, cores were generated from the livers and cut into precision slices of about 250 μm thickness using a Krumdieck tissue slicer (Alabama Research and Development Corp. Munford, AL). The slices were placed in 12 well culture plates containing 748 μl Hank's media supplemented with HEPES, sodium bicarbonate, 1% bovine serum albumin, and 0.1% gentamicin, pH 7.4. Fetal bovine serum (250 μl) was added to the media for a final volume of 1 ml. Test chemicals were dissolved in dimethylsulfoxide (DMSO) and added to the media in the wells before introduction of the slices

(0.2% of final volume). OH-PCB30 (4-hydroxy-2',4',6'-trichloroPCB) and *o,p'*-DDE were purchased from AccuStandard (New Haven, CT) and I33' was obtained from BioResponse, LTD (Boulder, CO). All compounds were readily soluble in the media at a concentration of 100 μM. The plates were covered and put into sealed plastic containers saturated with 95% O_2 / 5% CO_2, which was refreshed at least every 12 hours. Incubations were performed at 14^0C on an orbital shaker at 90 rpm. Media were not changed for the duration of incubations. Except for time course studies, incubations were run for 96 hr.

At t = 0, slices were fixed in 10% buffered formalin for histological analyses. Slices were then stained with eosin and hematoxylin and viewed microscopically to determine the percentage of viable cells. Other slices were assayed for ATP content that was measured using kits from Sigma Chemical Co. (St. Louis, MO) and a variation of the method of Adam (1963). Additionally, two slices were pooled together in six replicates and homogenized in 200 μl phosphate buffer containing 30% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and stored at -80°C for later analyses. Six samples of media were also collected and stored at -80^0C.

At termination of the experiment, slices were assayed for ATP production or fixed in buffered formalin for histology. Remaining slices, two per replicate, were homogenized in phosphate buffer and aliquots of media were collected for Vg assays and stored at -80^0C. Media and slice homogenate Vg was quantified utilizing an ELISA previously described by Donohoe and Curtis (1996) and based on protein concentration determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Significant Vg induction between treatment groups of six samples of two slices each and negative controls containing vehicle alone was determined by t-tests, assuming unequal variances. One way ANOVA and F-tests were used to

determine significant differences between Vg induction for Chinook salmon slices and that of rainbow trout slices reported previously (Shilling and Williams, 2000).

RESULTS

Vitellogenin (Vg) was quantified in both liver slice homogenates and surrounding media. Measuring Vg in the slice homogenates was the more sensitive endpoint than medium (data not shown) and will be reported in the following figures. Liver slice homogenates and media assayed at the beginning of each experiment usually had little or non-detectable levels of Vg.

No media changes were performed over the course of these experiments to allow for Vg accumulation, making liver slice viability a possible concern. Histological examination and ATP assays were conducted to assure that structural or biochemical damage had not occurred. ATP production in liver slices treated with 1000 nM E_2 for up to 120 hr did not significantly diminish compared to 0 hr samples (Figure 6.1) or any concentrations of other chemicals reported in this paper (not shown). In some cases, ATP production increased in treated slices. Examination of slices sectioned and stained with hematoxylin and eosin revealed no major structural injury even after 120 hr. Slices averaged $\geq$ 95% viable cells and no slice examined had less than 80% viable cells. Since maximal induction was detected by 96 hr, results of these two assays confirmed that the liver slices were viable and a valid model for Vg induction studies.

Time course experiments revealed that significant induction of Vg in Chinook salmon slices by 1000 nM 17β-estradiol (E_2) occurred after 72 hr ($p < 0.05$, t-test, Figure 6.2). In a dose response study with Chinook salmon slices, 40 nM E_2 was required for significant Vg induction after 96 hr ($p < 0.01$, t-test, Figure

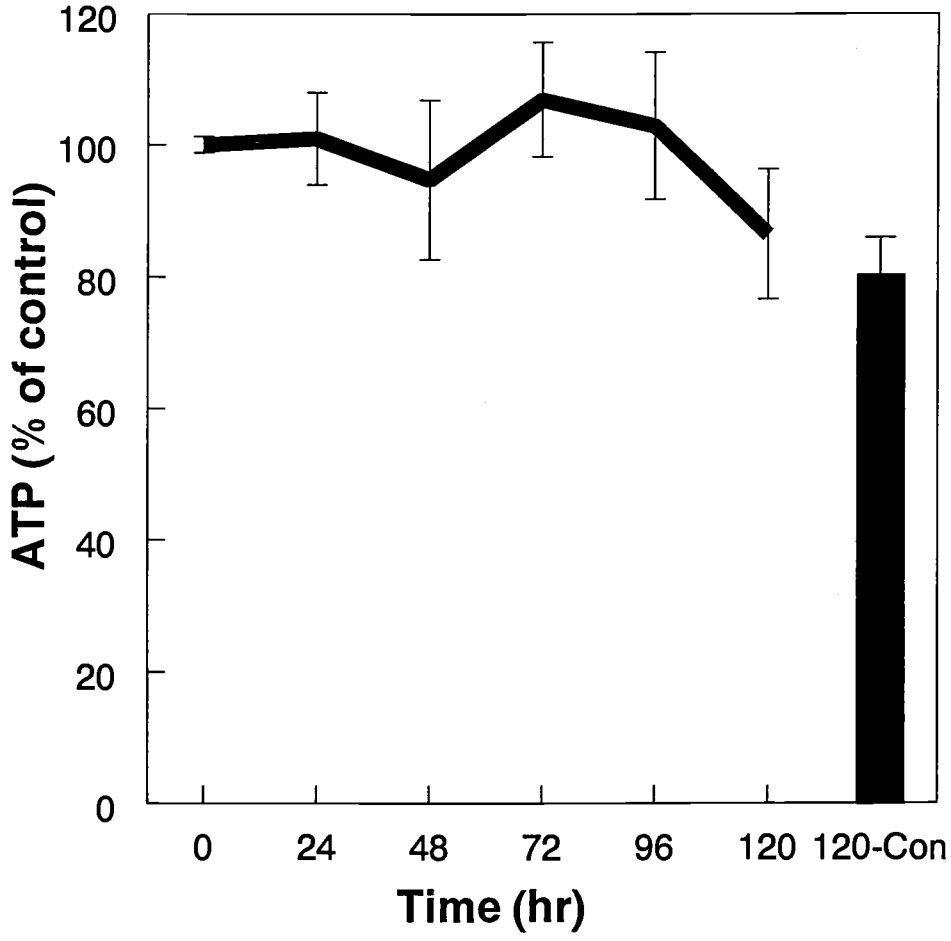

Figure 6.1: ATP levels for Chinook salmon slices treated with 1000 nM E_2 over a 120 hr time course. Values are percent of control. Error bars are $\pm$ SE (n = 3).

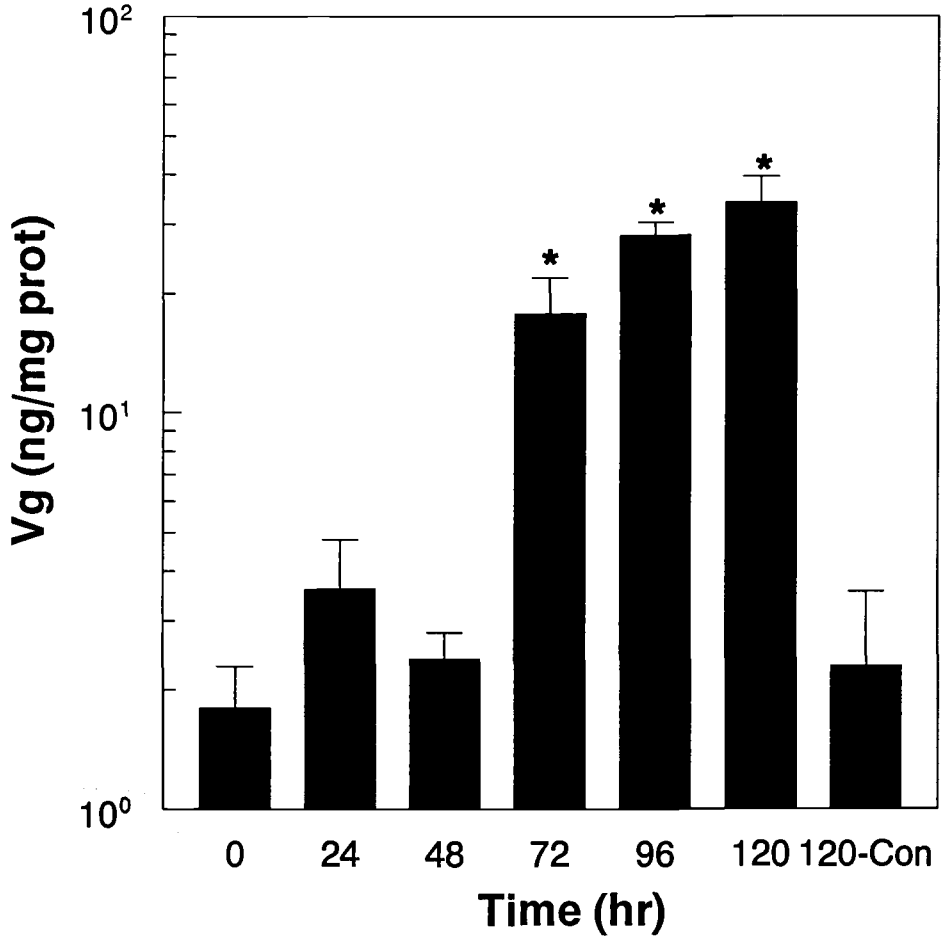

Figure 6.2: Vg levels for Chinook salmon liver slice homogenates treated with 1000 nM E_2 in Hank's media and 25% serum for 120 hr. (*) Denotes significant induction compared to 0 hr control ($p < 0.05$, t-test). Error bars denote $\pm$ SE ($n = 6$).

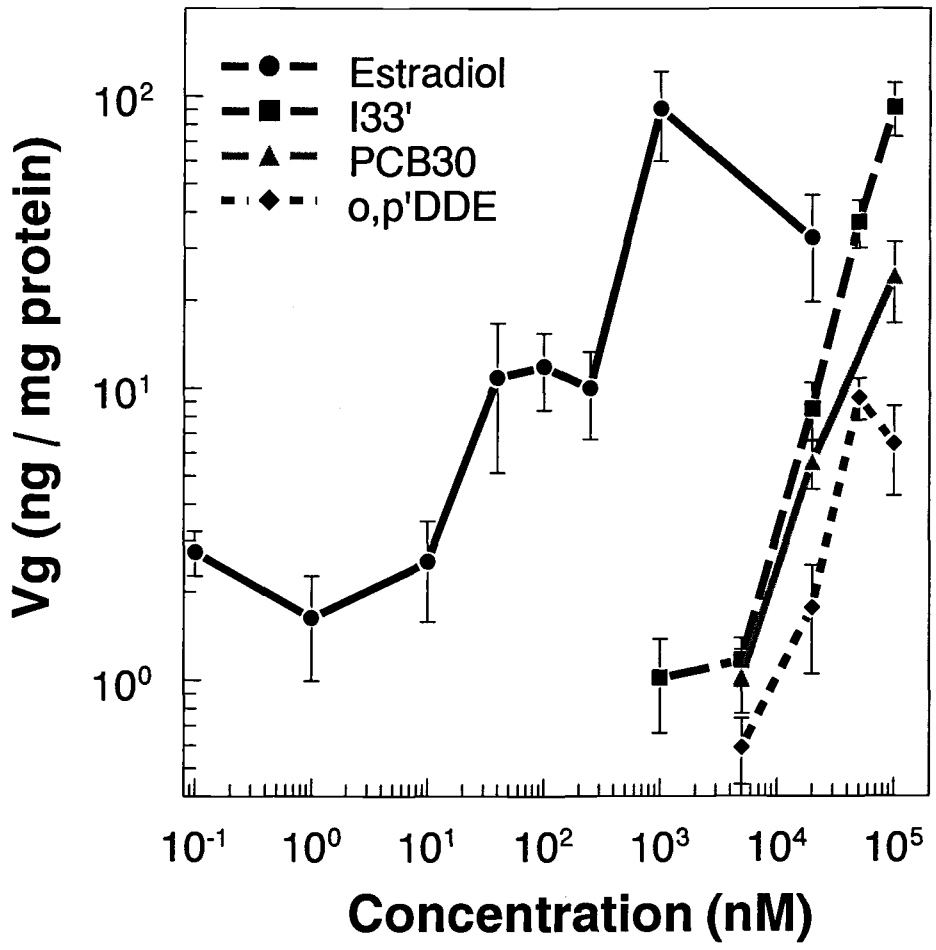

Figure 6.3: Vg levels for Chinook salmon liver slices treated with E_2 (0.1 – 20000 nM), I33' (1 - 100 μM), OH-PCB30 and *o,p'*-DDE (1 - 100 μM) for 96 hr. Vg values for concentrations ≥ 40 nM E_2, ≥ 20 μM I33', PCB30 and *o,p'*-DDE were significant ($p < 0.05$, ANOVA F-test). Bisphenol A (5 – 100 μM) did not significant induce Vg at any concentration ($p > 0.05$) and is not depicted in this figure. Error bars denote ± SE (n = 6).

6.3). Maximal induction by salmon slices depicted in Figure 6.2 was about 50-fold greater than controls not treated with E_2. Vg induction was not observed in slices incubated with vehicle, even after 120 hr, indicating that there were no estrogenic compounds in the slices or media.

Several known or suspected estrogenic compounds were screened using this model. The conditions were kept constant for all chemicals tested (supplemented Hank's media with 25% fetal bovine serum incubated at 14^0C for 96 hr). Environmental contaminants that have demonstrated estrogenic activity in rainbow trout such as *o,p'*-DDE, Bisphenol A and PCB30 were assayed at 1 – 100 μM. *O,p'*-DDE and PCB30 significantly induced Vg in this model with *o,p'*-DDE being as effective as E_2 albeit at about 2 x 10^4 higher concentration (Figure 6.3). The dietary constituent, I33', was also a fairly strong inducer of Vg in the salmon (80-fold induction, EC_{50} = 25 μM), which supports previously reported trout data. Bisphenol A, however, did not increase Vg at any concentration supportive that Chinook salmon are less sensitive to estrogenic exposure. A summary of relative estrogenicities for the compounds studied in this paper are reported in Table 6.1 with comparative trout data previously published (Shilling and Williams, 2000).

Table 6.1: EC_{50} values, relative EC_{50} values (in parentheses) and maximum Vg induction (Max efficacy) for Chinook salmon slices treated with several estrogens. Rainbow trout values are reported here for comparison (from Shilling and Williams, 2000).

	Chinook salmon		*Rainbow trout*	
	EC_{50} (μM)	**Max efficacy**	**EC_{50} (μM)**	**Max efficacy**
E_2	0.1 (1)	50	0.002 (1)	220
o,p' DDE	20 (5 x 10^{-3})	15	10 (2 x 10^{-4})	105
OH-PCB30	10 (0.01)	35	5 (4 x 10^{-4})	120
I33'	25 (4 x 10^{-3})	80	5 (4 x 10^{-4})	309
Bisphenol A	NA	NA	50 (4 x 10^{-5})	40

DISCUSSION

Chinook salmon liver slices produced vitellogenin (Vg) in response to estrogenic chemicals. Using 17β-estradiol (E_2) as our model estrogen, we used a previously developed assay in our laboratory to quantify relative estrogenicity via Vg induction in liver slices. Only livers from juvenile males were used to assure low or non-detectable background levels of Vg. The salmon slices appeared to be less sensitive to Vg induction by E_2 than rainbow trout, suggesting a quantitative difference between these salmonid species. As exhibited in Figure 6.2, significant Vg induction in the salmon liver slices was not observed until 72 hr after treatment with 1000 nM E_2. In rainbow trout liver slices, significant induction occurred after 48 h. In dose response experiments, Chinook salmon slices required a higher E_2 concentration for significant induction than did the trout slices (0.5 nM vs 40 nM) and the response was about an order of magnitude less as well. This supports our hypothesis that rainbow trout liver slices offer a very sensitive model for screening potential estrogens *in vitro*.

By observing significant Vg induction in Chinook salmon slices, we demonstrated that Vg induction can be observed in liver slices of other fish species, supporting the applicability of the slice model. Moreover, by observing Vg induction in slices of other teleosts and in the future, higher vertebrates such as amphibians, reptiles and birds, a more complete evaluation of estrogenic potentials of compounds can be determined. Decreases in fish populations due to environmental contaminants can be associated to xenoestrogen exposure by Vg induction. Exposure can be quantified by Vg induction using the liver slice model and compared to the potency and efficacy of the suspected estrogen(s) to assess the relative risk to the organism.

The environmentally relevant estrogens, *o,p'*-DDE and PCB30, both of which have demonstrated estrogenic potential in trout and several other species

(Carlson *et al*, in press, Conner *et al.*, 1997 and Safe *et al.*, 1997), induced Vg in salmon liver slices, also with lower efficacies than E_2. E_2 was the most potent Vg inducer tested in the liver slices. Induction by the xenoestrogens tested, however, was seen at concentrations well below the EPA mandate of 100 μM, supporting the application of this model for screening potential estrogenic compounds. Quantitatively, the difference in sensitivity between Chinook salmon and rainbow trout to xenoestrogens was within an order of magnitude. Bisphenol A, a very weak estrogen in rainbow trout liver slices (EC_{50} = 50 μM) did not induce Vg in Chinook salmon up to 100 μM. This qualitative difference may be due to a quantitative difference in sensitivity. Perhaps if we treated the salmon slices with a higher concentration of Bisphenol A, Vg induction would have been observed. Interestingly, the dietary component, I33', was the most effective estrogen tested in the salmon, inducing Vg to a greater extent than even E_2, albeit with about 250 fold less potency. This also supports previously reported rainbow trout studies.

We have demonstrated the liver slice model to be practical and sensitive for quantifying estrogenicity of chemicals via Vg induction in the Chinook salmon. Liver slices are easier and faster to prepare than dispersed hepatocytes and provide a more realistic *in vitro* model of the whole organ owing to the inclusion of different types of cells and maintaining cell-to-cell interactions (Guillouzo, 1998). Rainbow trout and Chinook salmon liver slices were both viable for several days, allowing for Vg production in response to E_2, however, the Chinook salmon appear to be less sensitive. Very weak estrogens such as Bisphenol A, may not be detectable as estrogens in the salmon liver slice model due to lower sensitivity in regard to Vg induction. Smeets *et al.* (1999b) reported that Bisphenol A, but not *o,p'*-DDE was estrogenic in carp hepatocytes based on Vg induction. Perhaps this was due to a difference in sensitivity. Our findings in the salmon support this

notion and indicate the importance of species comparisons when attempting to apply the most sensitive model for estrogens.

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CHAPTER 7

SUMMARY and CONCLUSIONS

Rainbow trout were very responsive to estrogen and androgen treatment both *in vitro* and *in vivo*. The development of a liver slice assay to quantify Vg induction in order to assess estrogenicity was critical for many of the studies performed. It was found that I33', a major acid condensation product of I3C, was a strong estrogen both in liver slices and whole animal studies with an efficacy similar to E_2. Using the *in vitro* ovarian microsome aromatase model that was developed, I33', I3C and RXN had no effect on this enzyme. *In vivo* studies revealed that Vg induction by these compounds was observed in the absence of elevated blood E_2. Additionally, tamoxifen blocked Vg induction by I33' in liver slices suggesting that this compound was a direct estrogen receptor agonist. These reports are in sharp contrast to the current dogma that stipulates I33' is an antiestrogen. The issue of species and, perhaps, tissue specificity is raised based on these findings. Ongoing clinical trials have targeted the suppression and prevention of estrogen dependent cancers such as breast and uterine cancer. It is feasible that I33' is estrogenic in the liver, but antiestrogenic in other tissues. Unfortunately, the limitation of the rainbow trout is demonstrated by this scenario as accurate extrapolation to these tissues would not be justified. Nevertheless, estrogens play a role in tumor promotion in the liver and the strong evidence that I33' is estrogenic in this tissue raises the possibility of unwanted side effects by this compound.

Comparison between liver slice and *in vivo* studies revealed that I3C possessed no estrogenic activity in the parent form. Examining the relative levels of Vg induction between I3C, I33' and RXN also suggested that I33' was the major condensation product responsible for the estrogenic response. Studies with SKF525A suggest that the ultimate estrogen may be a $(OH)_n$ metabolite of I33'. The presence of the parent compound was observed by HPLC in the liver slices and surrounding media, but mass spectrophotometry analyses was unable to identify the active metabolite.

Endogenous androgens, androstenedione and testosterone, elicited estrogenic responses *in vivo* via Vg induction. The hypothesis that this was due to conversion to estrogens by aromatization was supported by observed increases in blood E_2 levels in trout fed these androgens. It was demonstrated using the *in vitro* aromatase model that androstenedione is converted effectively to E_2. The whole animal studies established that even male trout are capable of aromatization of androgens. DHT, a non-aromatizable androgen, did not result in Vg or E_2 increases further supporting this hypothesis. In fact DHT significantly lowered Vg and E_2 in males and females, suggesting a negative or antiestrogenic effect by this androgen. The androgen receptor antagonist, flutamide, did not reverse the DHT effects and in fact when administered alone, flutamide lowered Vg and E_2 in a similar manner to DHT. It appears that there is crosstalk between androgen and estrogen receptor signal transduction pathways. This is not surprising since androgens and estrogens are often required together to elicit a full response. Examples of this are seen in brain and neural development and function and sex differentiation. Additionally, utilization of the liver slice model, revealed that androstenedione, testosterone and DHT were all weak inducers of Vg (Figure 7.1). Reversal of the induction by tamoxifen suggested that these compounds act as weak estrogen receptor agonists (Figure 7.2). The mechanisms by which levels of and modes of actions by these

androgens and estrogens are regulated, however, appear to be far-reaching and complex as discovered in the *in vivo* studies.

When developing and establishing a useful model, in this case, for estrogenicity, comparative studies verify and determine its effectiveness. Chinook salmon, a closely related species to the rainbow trout, was chosen for liver slice experiments to compare relative estrogenicity of some environmental estrogens. Vg induction was observed in salmon slices in response to estrogen treatment albeit with less sensitivity than the trout. These data supported the choice of rainbow trout as a sentinel species for Vg induction and demonstrated the estrogenicity of the test compounds between species. Rainbow trout appear to be an effective species for measuring estrogenicity via Vg induction. The ease and precision of the liver slices allows for the screening of potential estrogenic compounds with confidence. Given the numerous advantages of liver slices and that they are viable tissue, more complete metabolism/toxicity studies in addition to just quantifying Vg induction can be performed which is the aim of any good model. The rainbow trout liver slice model would be an excellent screening tool for use by EDSTAC.

Rainbow trout are responsive to androgen and estrogens and represent a sensitive species for the study of responses mediated by these sex steroids. Although the argument against extrapolation for use in clinical trials is valid, the trout are an excellent model for studying estrogen and androgen effects in regards to endocrine disruption. Rainbow trout offer a well-characterized model for studying the effects of environmental contaminants on aquatic and terrestrial species. Careful extrapolation can allow the use of trout for human risk assessment studies, especially regarding the trout liver, which is structurally and biochemically similar to humans. The studies with androgens and estrogens demonstrated the complexity of responses in the rainbow trout. Utilizing the liver slice and *in vivo* estrogenicity models, direct estrogenic and androgenic responses

via receptor mediated pathways were studied. As demonstrated with I33' and I3C, these models allowed for the investigation of indirect mechanisms of action by sex steroids, which may play a more important role than classic receptor mediated pathways. By looking at alterations by these compounds via endpoints such as Vg levels, aromatase inhibition/induction, cytochrome P450 induction/inhibition, circulating steroid levels, some of the responses to and mechanisms of sex steroid signal transduction are better understood.

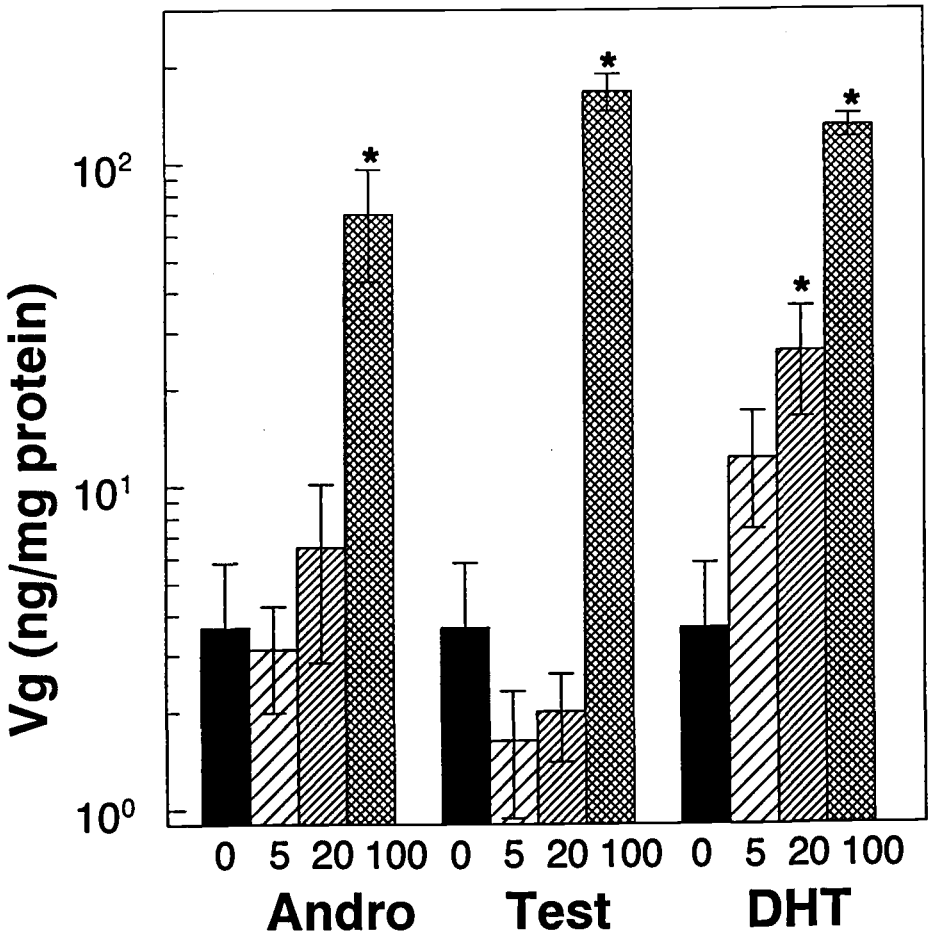

Figure 7.1: Vg induction in rainbow trout liver slices treated with 0, 5, 20 and 100 μM of androstenedione (Andro), testosterone (Test) and dihydrotestosterone (DHT). (*) denotes significant Vg induction compared to corresponding control ($p < 0.05$). Error bars represent $\pm$ SE ($n = 6$).

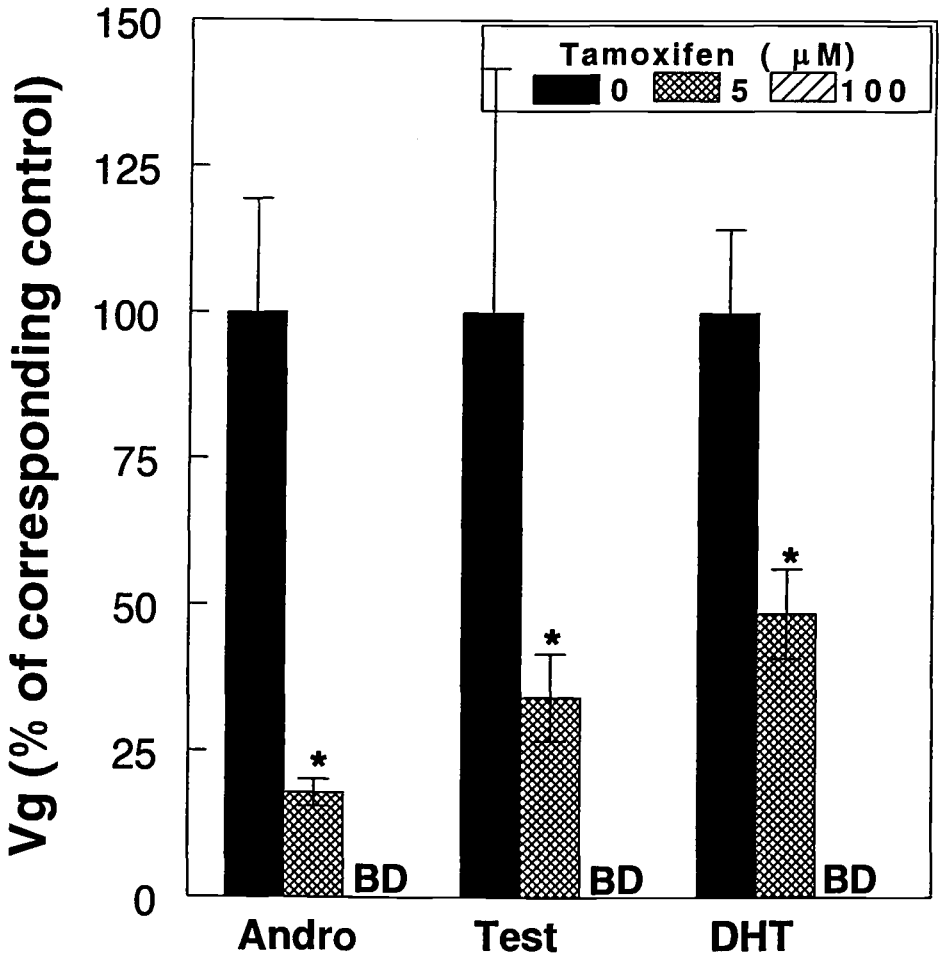

Figure 7.2: Inhibition of androgen induced (100 µM) Vg by tamoxifen in rainbow trout liver slices. (*) denotes significant inhibition of Vg induction by 100 µM of corresponding androgen by tamoxifen ($p < 0.05$, F-test). Error bars represent $\pm$ SE ($n = 6$). BD represents values below the detection limit for Vg.

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