In vitro studies on sex steroid-binding protein in rainbow trout (*Oncorhynchus mykiss*) hepatocytes: influence of 17β-estradiol and environmental estrogens

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Forord

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Kine Martinsen

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ABSTRACT

Environmental estrogens may modulate the endocrine system through interactions with sex steroid-binding protein (SBP), and these processes may be a novel mechanism for endocrine disruption. The endogenous hormone 17β-estradiol (E2) together with weakly estrogen compounds as di-(*n*-butyl) phthalate (DBP) and potent estrogen mimics (ethynylestradiol, EE2) were all able to induce an up-regulation of total sex-steroid binding capacity and SBP gene expression *in vitro* using a culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes. This increase was most likely due to the induction of the sex steroid-binding protein (SBP) gene and protein itself, although other non-identified proteins probably contribute to sex steroid-binding as well. The roles of non-specific binding proteins were assessed using albumin and rainbow trout vitellogenin which both showed a very low capacity for binding sex steroids. Quantification of SBP protein expression in cell media using western blot and zebrafish SBP antibodies was not possible due to low protein concentrations. Exposing hepatocytes to EE2 induced the strongest response in both total sex steroid-binding activity and SBP gene-expression followed by E2 and DBP. Exposure to endogenous sex steroids and environmental estrogens increased SBP gene-expression after 24 hours, while an increase in total sex-steroid binding capacity was seen after 48 hours of exposure, indicating an up-regulation of the SBP gene before secretion of SBP into the cell medium. A concentration-response relationship, most likely due to increased SBP secretion and gene expression was seen after 96 hours of exposure for both total sex-steroid binding capacity and SBP gene expression.

ABBREVIATIONS

[^{3}H]-E2	[2,3,6,7-^{3}H]estradiol
[^{3}H]-T	[1,2,6,7-^{3}H]testosterone
APS	ammoniumpersulphate
B_{max}	maximum specific binding
β-ME	β-Mercaptoethanol
bp	basepair
BSA	bovine serum albumin
cDNA	complementary DNA
CPM	counts per minute
DBP	di-(*n*-butyl) phthalate
DCC	dextran-coated charcoal
dH_2O	distilled water
DMSO	dimethyl sulphoxide
E2	17β-estradiol
ECL	enhanced chemiluminescence
EDC	endocrine disrupting compound
EDTA	ethylenediaminetetraacetic acid
EE2	17α-ethynylestradiol
EGTA	ethylyneglycol-bis(β-aminoethylether) N, N, N',N'- tetraaceticacid
ELISA	enzyme linked immunosorbent assay
ER	estrogen-receptor
ERE	estrogen response element
EtAc	ethyl acetate
EtBr	ethidium bromide
GH	growth hormone
HRP	horseradish peroxidase
IGF	insulin-like growth factor
K_d	equilibrium dissociation constant
kDa	kilo Dalton

L-15	Leibowitz 15 medium
Ligand	molecule that binds to a specific receptor or other target molecule
mRNA	messenger RNA
Ns	non-specific binding
OD	optical density
PCR	polymerase chain reaction
PEI	poly(ethyleneimine)solution
Phytoestrogens	estrogenic substances from plants
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
rtVtg	rainbow trout vitellogenin
SBP	sex steroid-binding protein
SDS	sodium dodecyl (lauryl) sulfate
SDS-PAGE	sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
T	testosterone
T3	triiodothyronine
T4	L-thyroxine
TBS	tris-borat electrophoresis buffer
TBST	tris-borat electrophoresis buffer with Tween 20
Temed	N, N, N',N'-tetra-methylethylenediamine
TEMG	tris-EDTA-1 thioglycerol-glycerol
Triplex III	ethylenediaminetetraacetic acid disodium salt
Tween 20	polyoxyethylene-sorbitan monolaurate
Vtg	vitellogenin
Xenoestrogens	synthetic substance that imitate or enhance the effects of estrogens

1 INTRODUCTION

Numerous chemicals are used and generated through municipal, agricultural and industrial activities. When some pesticides were found to affect wildlife during the 1950s and 1960s widespread concern about possible ecological effects started developing (Carson, 1962). A discharge into the environment may cause changes, great or small and the aquatic ecosystem is of particular concern because this is where most contaminants released into the environment are finally deposited either from direct discharge into bodies of water or from terrestrial runoff and atmospheric deposition (Pritchard, 2003).

The main threats to the aquatic environment, as it has been perceived have changed from time to time, from heavy metals, radioactive discharges and eutrophication to oil pollution. In the recent years, a number of chemicals from anthropogenic sources have shown the ability to modulate the endocrine system and the topic has emerged as a major issue in terms of both science and public policy (Clark, 2001).

1.1 Endocrine disrupters

1.1.1 Background

Large numbers and large quantities of endocrine-disrupting compounds (EDCs) of both natural and anthropogenic origin have been released into the environment since the 1940s (Colborn, *et al*., 1993; Colborn, 1996) and endocrine disruption has been postulated as the cause of a great number of adverse affects on the health of various wild species (Colborn *et al.*, 1993). Chemicals that have shown endocrine toxicity include pesticides, herbicides, fungicides, plasticizers, halogenated polyaromatic hydrocarbons and phytoestrogens (Muller *et al*., 1995; Cooper and Kavlock, 1997). These chemicals may interact with multiple targets, acting at the level of hormone synthesis, secretion, transport, site of action and metabolism (Klaasen, 2001). Exposure to endocrine disrupting chemicals has been linked to diminished fertility in birds, fish and mammals, abnormal thyroid function in birds and fish and demasculinization and feminization in

fish, gastropods and birds (Vos *et al*., 2000). Some organochlorines including dioxins stimulate the arylhydrocarbon (Ah) receptor-mediated xenobiotic metabolism and result in the metabolic disruption of steroid hormones as reported in eggshell thinning in birds of prey and uterus occlusion in seals (Ratcliffe, 1970; Helle *et al*., 1976).

1.1.2 Environmental estrogens

For the last decade there has been a large focus on EDCs that show estrogenic activity the environmental estrogens including the xenoestrogens. These chemicals are able to mimic the action of the female sex steroid, 17β-estradiol (E2) and have been claimed to have the potential to induce severe effects on reproductive performance in wildlife and humans (Colborn and Clement, 1992; Sharpe and Skakkebaek, 1993). Environmental estrogens may elicit effects through a number of pathways including direct binding and activation of the estrogen receptor (ER), by binding to other nuclear receptors which interact with an estrogen response element (ERE) and through other receptor and/or signal transduction pathways (Gillesby and Zacharewski 1998; Arukwe and Goksøyr, 2003; Kirk *et al*., 2003).

In aquatic environments, the sources of environmental estrogens are mainly human sewage and industrial activities (Atkinson *et al*., 2003; Tashiro *et al*., 2003). Numerous studies have assessed the effects of E2 and associated xenoestrogens on wild fish (Folmar *et al*., 1996; Jobling *et al*., 1998; Lee *et al*., 2000; Folmer *et al*., 2001) and all of these studies have found induction of the yolk protein vitellogenin (vtg) in exposed male fish. Although the gene that produces vtg is present in both male and female, the protein is normally only activated by estrogens in maturing females (Korsgaard *et al*., 1983). Production of large quantities of vtg has also been seen in male trout held in lagoons downstream of inputs of sewage effluent containing EDCs mostly estrogens (MAFF, 1994). Other effects such as gonadal abnormalities (Gimeno *et al*., 1998; Jobling *et al*., 1998; Miles-Richardson et al., 1999; Rodgers-Gray et al., 2001) and changes in behavior (Bayley et *al*., 1999; Bjerselius et *al*., 2001) have also been reported in male fish exposed to environmental estrogens.

In addition, these compounds may alter the levels of critical endogenous hormones or show effects on hormone secretion and transport (reviewed in van der Kraak *et al*., 1998). Environmental estrogens can effect hormone-binding proteins in the blood such as the sex steroid-binding protein (SBP) and disrupt hormone transport by increasing and decreasing the bound-to-free ratio of the hormone in plasma (Wells and Van Der Kraak, 1999).

1.2 Objectives

The objective of this thesis was to determine whether the natural estrogen 17β-estradiol and common environmental chemicals such as ethynylestradiol and di-(*n*-butyl) phthalate may modulate the production of sex steroid-binding proteins and potentially play a role in endocrine disruption in fish.

To achieve this aim, exposure studies with hepatocytes from rainbow trout *(Oncorhynchus mykiss)* were used to determine whether these chemicals were able to affect:

a. intracelluar gene expression of SBP
b. release of sex steroid-binding proteins into the cell medium
c. total sex steroid-binding capacity of proteins released into the cell medium

2 BACKGROUND

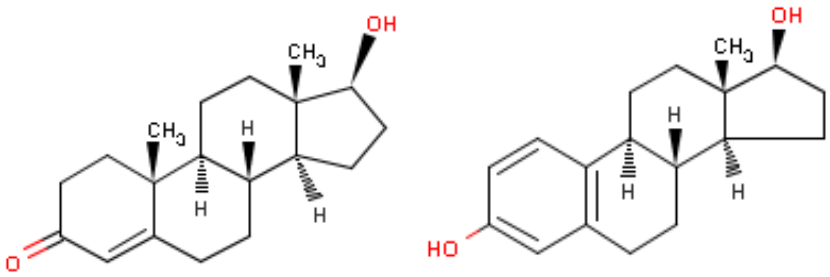
2.1 Sex steroids

Sex steroids such as androgens, estrogens and progesterone (**Figure 2-1**) move through the blood stream from their site of production (the testis and ovaries) to target tissues, where they enter cells, bind to highly specific receptor proteins in the nucleus, such as the estrogen receptor (ER) or androgen receptor, and trigger changes in gene expression and metabolism (Callard and Callard, 1987). The steroid hormones are removed from the blood by metabolism as they circulate through the liver, usually coupled to either a sulphate ion or glucornic acid, thus increasing their solubility in water and the ease with which they are excreted in the bile or urine (Evans, 1993).

Major androgens produced by testicular tissue vary between fish species and developmental stages, but include: testosterone (T), 11-ketotestosterone, and androstenedione (Fostier *et al*., 1983; Loir, 1990; Bourne 1991). Testosterone is the precursor hormone for the production of E2 in the ovarian follicle of females by the actions the enzyme aromatase (Lazier and MacKay, 1993). Estrogens play a major role in controlling reproduction in females, and to a less extent in males and are essential for cell growth and several other biological activities (Cakmak *et al*., 2006). Other female sex steroids include estrone, 17α, and 20β, 21-trihydroxy-4-pregnen-3-one (Fostier *et al*., 1987; Barry *et al*., 1993).

Figure 2-1 *Chemical structures of testosterone and 17β-estradiol*

Testosterone **17β-estradiol**



Steroid hormones are synthesized in only a few organs, as the testis and ovaries, and due to their lipophilic nature do not dissolve easily in plasma and other body fluids. For this reason, most of the steroid hormone molecules found in the blood is bound to specific and non-specific steroid carrier molecules (Sitteri *et al*., 1982).

2.2 Sex steroid-binding protein

2.2.1 Function

Most vertebrate species sex steroid hormones are carried in the bloodstream bound to specific high affinity sex steroid-binding proteins (SBP, alternative name; sex hormone-binding globulin, SHBG) and to low affinity proteins such as albumins and corticosteroid-binding proteins (Sitteri *et al*., 1982). In addition to their role as steroid carriers, SBPs protect circulating sex steroids from rapid metabolic degradation and excretion (Westphal, 1986) and play a role in regulating the amount of steroid that is available to target tissues (Pardrige, 1981; Rosner, 1990).

It has also been suggested that SBP plays an additional role in assisting in the cell uptake of sex steroids by interacting with a specific receptor on the membrane of target cells. The activity of the SBP specific receptor (R_{SBP}) appears to be regulated by occupancy of the SBP steroid-binding site (Rosner, 1990) and leads to the induction or suppression of a

nuclear steroid receptor by the G-protein cAMP-phosphokinase A pathway and the regulation of sex steroid cellular actions (Fortunati, 1999; Rosner *et al*., 1999).

2.2.2 Sex steroid-binding proteins in fish species

Sex steroid-binding protein has been identified in the plasma of several fish species and exhibits a broad capability for binding endogenous hormones. Steroid binding activities have been characterized in plasma in a number of teleosts including Atlantic cod, *Gadus morhua* (Freeman and Idler, 1971), goldfish *Carassius auratus* (Pasmanik and Callard, 1986), carp *Cyprinus carpio* (Chang and Lee, 1992), spotted seatrout *Cynoscion nebulosus* (Laidley and Thomas, 1994) and the salmonoids, rainbow trout *Oncorhynchus mykiss* (Fostier and Breton, 1975), brown trout *Salmo trutta* (Pottinger, 1988) and Atlantic salmon *Salmo salar* (Freeman and Idler, 1971).

It is well documented that the SBP binding affinity and specificity for endogenous hormones vary greatly among different vertebrates (Renoir *et al*., 1980; Westphal, 1986). This is also true for several fish species where studies on the SBP characteristics produce a varying degree in the affinity for endogenous sex steroids (Westphal 1986; Chang and Chen, 1990). This is reflected in the dissociation constants (K_d: the molar concentration of ligand at which half of the available ligand-binding sites are occupied) and B_{max} values, representing the total number of available binding sites for SBP to endogenous sex steroids in different fish species.

The dissociation constant (K_d) is used to express the affinity a protein has for a ligand (i.e. E2 or T) in which a lower K_d value corresponds to a higher affinity of ligand, that is the more tightly a protein binds a ligand, the lower the concentration of ligand required for half the binding sites to be occupied. Dissociation constant (K_d) values and B_{max} values for spotted seatrout have been reported to be 5.75 nM and 415 nM (Laidley and Thomas, 1994), while lower values for both K_d and B_{max} are seen in common carp (Kd; 1.43 nM while B_{max} values vary between male and female; 18.9 and 29.9 fmol [^{3}H]-E2/ mg protein, respectively) (Klaos *et al*., 2000). Rainbow trout has rather high binding affinity for SBP (K_d 4.7 nM) which coincides with low values for B_{max} (Foucher *et al*.,

1991). Binding affinity and total number of binding sites have also shown to vary with reproductive state in some fish species. Differences between SBP characteristics of nonreproductive and reproductive rainbow trout and black bream have been shown by higher B_{max} levels in vitellogenic trout and bream (Hobby *et al*., 2000). SBP levels in spotted seatrout are highest when plasma concentrations of E2 are peaking (Laidley and Thomas, 1997). In contrast no differences in B_{max} or K_d are found between vitellogenic and nonreproductive greenback flounder (Hobby *et al*., 2000) or female goldfish (Pasmanik and Callard, 1986), male or female carp (Chang and Chen, 1990, 1991). In addition to reproductive stage, phylogeny and other factors may determine sex steroid binding characteristics in fish (Rosner, 1991).

Despite interspecies variations in K_d and B_{max} values, SBPs relative high affinity binding of estrogens and androgens and low affinity binding of progesterone and corticosterone seems to be common (Fostier and Breton, 1975; Pasmanik and Callard, 1986; Laidley and Thomas, 1994; Tollefsen, 2002). The rank order of affinity of steroids binding to SBP in several teleost fish species is E2 and T followed by androstenodione, 11-ketotesterone and estrone, then estriol, progesterone and cortisol. (Hobby *et al.*, 2000; Øvrevik *et al*., 2001; Tollefsen, 2002, see **Table 2-1**). This is similar to that seen in the salmonids, and also to a few non-salmonoids, such as goldfish, carp and spotted seatrout (Pottinger 1986; Laidley and Thomas, 1994). It is suggested that the relative affinity of SBP for different steroids is related to the steroid structure (Hobby *et al*., 2000) and the circulating steroid concentrations with higher affinity binding in species with low circulating steroid levels (Laidley and Thomas, 1994).

Table 2-1 *Relative binding affinity (RBA) of various endogenous steroids in blood plasma for Arctic charr, rainbow trout and spotted seatrout SBPs.*

Steroid	Arctic Charr[1] RBA (%)	Rainbow trout[2] RBA (%)	Spotted seatrout[3] RBA (%)
17β-estradiol	100	100	100
Testosterone	75.83	86.6	59.9
11-Ketotestosterone	8.35	13.3	8.3
Estrone	1.52	-	70.6
Estriol	0.47	-	1.7
Progesterone	<0.01	0.22	7.5

** [1] Øvrevik et al., 2001; [2] Tollefsen, 2002; [3] Laidley and Thomas, 1994*

Moreover, numerous factors other than steroid hormones could influence SBP concentrations including growth factors, nutritional status and hormones implicated in general metabolism such as growth hormone (GH), insulin, insulin like growth factor (IGF), triiodothyronine (T3) and cortisol (Mercier-Bodard *et al*., 1989; Vermeulen, 1986; Mercier-Bodard *et al*., 1987; Adlercreutz *et al*., 1987).

2.2.3 Mechanisms regulating sex steroid-binding proteins

Sex steroid-binding protein, which is mainly produced in the liver (Foucher *et al*., 1991) have been purified and fully characterized in a small number of species including human and amphibians. Sex steroid-binding proteins in these species are dimeric proteins composed of identical protomers with varying degree of glycosylation. The two subunits dimerize to form a glycoprotein of approximately 90 kDa (Petra, 1991; Santa-Coloma *et al*., 1985).

The SBP found in various fish species is also a glycoprotein, and as seen in humans it is suspected that each sub-unit of the homodimer contains a high-affinity steroid-binding site (Grishkovskaya *et al*., 2002; Miguel Queralt *et al*., 2005). The molecular weight of SBP has been reported for a few fish species. SBP in eel *Anguilla japonica* has a size of 64 kDa (Chang *et al*., 1994), in common carp 194 kDa (Chang and Lee, 1992), in

rainbow trout 65 kDa (Fostier and Breton, 1975), Arctic charr *Salvelinus alpinus* 60 kDA (Øvrevik *et al*., 2001) and in spotted seatrout, 135-150 kDA depending on purification method (Laidley and Thomas, 1994). In sea bass *Dicentrachus labra,* SBP has a molecular mass of approximately 118 kDa (Migeul-Queralt *et al*., 2005).

The cDNA of SBP has been cloned and sequenced for many species, but public databases only contain the complete genomic sequence of a few of these including human, rat and cat (review by Munell *et al*., 2002). Partial zebrafish and fugufish SBP coding sequences are available within public databases together with rainbow trout which share a 95% gene sequence identity with the coding sequence for SBP in Atlantic salmon (Miguel-Queralt *et al*., 2005). Little is known about the structure of SBP in fish species or how expression of the SBP gene is regulated, but the expression of SBP in developing zebrafish larvae and in the gut and testis of adult suggest that SBP might display important functions in development, uptake and regulation of steroids in several organs (Miguel-Queralt *et al.*, 2004).

The exposure of E2 induces SBP production both *in vivo* and *in vitro* in rainbow trout plasma and liver cells, in spotted seatrout, and in human hepatoma cells (Foucher *et al*., 1991; Thomas and Laidley, 1994; Mercier-Bodard *et al*., 1987). Effects of testicular androgens appear to have little or no influence on SBP regulation in mature male trout, the same is seen *in vitro* with exposure of testosterone to cultures of rainbow trout liver cells (Foucher *et al*., 1992; Foucher *et al*., 1991). Other steroids such as cortisol have no effect on SBP regulation in black bream and rainbow trout (Hobby *et al*., 2000) either. Non-steroidal factors such as growth hormone regulate SBP by increasing its production in rainbow trout liver cells, while IGF appears to have an inhibitory effect on the secretion of SBP and on specific SBP mRNA (Mercier-Bodard *et al*., 1989).

In addition to this, total sex steroid-binding activity also depend on several other factors such as the age of experimental animals, the dose of hormones or compound distributed, cellular type and the use of *in vivo* or *in vitro* models (review by Munell *et al*., 2002).

Many questions arise regarding the expression and role of SBP in teleosts and other species and there is still much controversy regarding the subject.

2.3 Sex steroid-binding proteins potential role in endocrine disruption

2.3.1 Potential mechanisms of action

In addition to binding endogenous steroids, SBP can interact with several natural and synthetic xenobiotics that bind reversibly to SBP (Danzo, 1997; Dechaud *et al*., 1999; Klaos *et al*., 2000; Tollefsen 2002; Tollefsen *et al*., 2002; Tollefsen *et al*., 2006). The binding and modulation of SBP in regard to xenoestrogens could be an additional route that may be disturbed by EDCs (Danzo, 1997; Milligan *et al*., 1998). As shown in several fish species, xenoestrogens are able to displace estrogen from the SBP binding site in blood plasma, although at considerably higher concentrations than endogenous hormones (Klaos *et al*., 2000; Milligan *et al*., 1998; Tollefsen *et al*., 2002; Gale *et al*., 2004). Despite their lower affinity for SBP, it has been suggested that xenoestrogens affect endocrine activity in part by disrupting the equilibrium between free and SBP-bound sex steroids in blood plasma, or by altering the levels of SBP (Danzo, 1997; Dechaud *et al*., 1999; Hodgert *et al*., 2000; Tollefsen, 2002; Tollefsen *et al*., 2002). The binding of environmental estrogens to the high-affinity sites on SBP may potentially displace biologically active steroid and through the reversible binding that occurs, at the same time enhance specific delivery of exogenous compounds to the target sites that produce sex steroids.

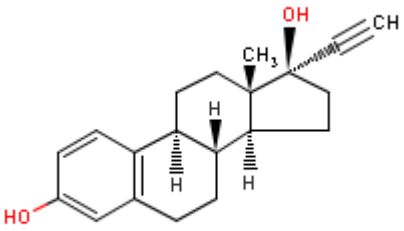
2.3.2 Compounds known to interact with and modulate SBP

Natural hormones including estrogens and testosterone occurs frequently in effluent discharge of sewage treatment plants (Desbrow *et al*., 1998; Rodgers-Gray *et al*., 2000) where it can cause endocrine disruption in fish (Purdom *et al*., 1994; Harries *et al*., 1997; Routledge *et al*., 1998). The natural hormone E2 completely displaces radio-labeled estrogen from the steroid-binding site on SBP (Klaos *et al*., 2000; Tollefsen, 2002) and increases the amount of SBP in blood plasma in humans and several wildlife species (Foucher *et al*., 1991; Lermite and Terqui, 1991; Orlando and Guillette, 2002).

Synthetically produced pharmaceuticals have together with E2 been implicated as the primary contaminants contributing to estrogenic activity in surface waters in several countries (Desbrow *et al*., 1998; Snyder *et al*., 1999). The pharmaceutical estrogen ethynylestradiol (EE2) which is used in contraceptive pills and treatments for hormone-responsive cancers is highly hormonally active and have been detected in sewage effluents (**Figure 2-2**) (Arcand-Hoy and Benson, 1998; Larsson *et al*., 1999). Ethynylestradiol binds with high affinity to the ER-receptor of most vertebrates and can induce mammary and hepatic carcinogenesis (Kloas *et al*. 2000). Ethynylestradiol is also reported to induce a dose-dependant increase in the circulating levels of SBP in Atlantic salmon blood plasma and has one of the highest affinities for SBP in rainbow trout plasma, although the binding occurs at concentrations 130 times higher than those needed for E2 (Tollefsen, 2002; Tollefsen *et al*., 2002). This binding affinity to EE2 is in general agreement with other research on both mammalian and fish SBP, although SBP in the channel catfish *Ictalurus punctatus* has shown to have a higher affinity for EE2 than E2 (Gale *et al*., 2004), thus concluding that species-specific differences among fish exist.

Figure 2-2 *Chemical structures of the synthetic estrogen ethynylestradiol*

17α-ethynylestradiol



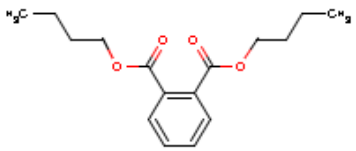
Man-made chemicals and by-products designed for uses in industry, agriculture, certain pesticides and consumer goods such as plastic additives may have estrogenic activity. An example is bisphenol A, (**Fig 2-3**) used in the production of polycarbonates which partially displaces E2 from SBP in common carp and rainbow trout (Klaos *et al*., 2000; Tollefsen

et al., in press). Estrogenic degradation products from detergents and surfactants are present in sewage effluents and are both lipophilic and persistent. Alkylphenols were first found to be estrogenic in the 1930s. (Dodds and Lawson, 1938) and display estrogenic effects in rainbow trout hepatocytes (Jobling and Sumpter, 1993). 4-nonylphenol, 4-octylphenol and 4-tert-butylphenol all have low binding affinities for SBP in several fish species including common carp, rainbow trout and catfish plasma (Klaos *et al*., 2000; Tollefsen 2002; Gale *et al*., 2004; Tollefsen *et al*,. in press).

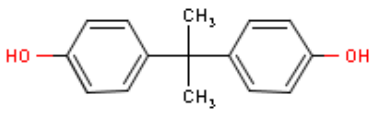
Phthalates are found in groundwater, rivers and drinking water and several of these chemicals used as plastic additives are estrogenic in tests using a mammalian cell line and an *in vitro* bioassay from rainbow trout (Jobling *et a*l., 1995). Both di-(*n*-butyl) phthalate (DBP) (**Fig 2-3**) and butyl-benzyl phthalate are able to bind to the rainbow trout estrogen receptor and initiate the transcriptional activity of the ER (Jobling *et al*., 1995). In addition to this, DBP disrupts androgen regulated male sexual differentiation (Myhlchreest *et al*., 1999). Phthalates interference with SBP is a possible answer for why these weakly acting xenoestrogens cause reproductive disturbances in developing males (Tollefsen *et al*., 2002). Like EE2, DBP induces a dose-dependent increase in the circulating levels of SBP (Tollefsen *et al*., 2002). Phthalates as diethyl phthalate and DBP also compete with E2 for the binding to SBP, but at concentrations far higher than those needed for endogenous hormones. (Tollefsen 2002, Klaos *et al*., 2004)

Figure 2-3 *Chemical structures of di-(n-butyl) phthalate and bisphenol A*

Di-(*n*-butyl) phthalate



Bisphenol A



In addition to the exposure of single compounds, fish exposed to complex industrial effluent from paper mills and offshore and land-based oil production show modifications of SBP properties including binding affinities to sex steroids and concentration of SBP in plasma which again could influence transport, clearance or actions of both E2 and T (Pryce-Hobby *et al*., 2002; Tollefsen *et al*., in press).

3 MATERIALS AND METHODS

3.1 Experimental design

Liver cells (hepatocytes) were isolated from rainbow trout and kept in culture where the cells were exposed to 17β-estradiol (E2), ethynylestradiol (EE2) and di-(*n*-butyl)-phthalate (DBP) dissolved in DMSO. The cell medium surrounding the hepatoyctes was used in analysis for total steroid binding capacity (binding assays) and in analysis of protein expression (western blot). RNA was isolated from the hepatocyte cells and used in calculation of the relative gene expression of SBP (qPCR). The role of non-specific binding proteins was also evaluated using purified rainbow trout vitellogenin (rtVtg) and albumin (BSA). Rainbow trout plasma was used in saturation analysis and in western blot with assumed heterologous anti-zebrafish SBP specific antibodies.

3.2 Chemicals

17β-estradiol (E2), 17α-ethynylestradiol (EE2), Di-(*n*-butyl) phthalate (DBP), Testosterone (T), Bovine serum albumin (BSA), Collagenese, Dimethyl sulphoxide (DMSO), Ethidium bromide (EtBr), Trizma-base, Trizma-HCl, Ethylenediaminetetraacetic acid (EDTA), *Ethylyneglycol-bis(β-aminoethylether) N, N, N',N'- tetraaceticacid* (EGTA), Heparin, Aprotinin, Glycine, Glycerol, Polyoxyethylene-sorbitan monolaurate (Tween 20), Activated carbon (charcoal), Ficoll-400, Xylene cyanol, 1-thioglycerol and Poly(ethyleneimine)solution (PEI) were all purchased from Sigma (St.Lois, Oregon, USA). Leibowitz L-15 medium, L-glutamine, penicillin, streptomycin and fungizone was supplied by Biowhittaker Inc (Walkersville, Maryland, USA). Rainbow trout vitellogenin (rtVtg) was bought from Biosense Laboratories AS (Bergen, Norway). NaCl, KCl, $MgSO_4$, Na_2HPO_4, $NaHCO_3$, $CaCl_2$, β-mercaptoethanol (β-Me), Bromphenolblue, Acetic acid, Ethylacetate, Ethylenediaminetetraacetic acid disodium salt (Triplex III) and Methanol (Me-OH) were all from Merck (Whitehouse Station, New Jersey, USA). Ethanol was bought from Arcus As (Oslo, Norway). The RNA isolation kit was from Qiagen (Hilden, Germany) while the high capacity cDNA

archive kit came from Applied Biosystems (Foster City, California, USA). TAQ SYBR® Green Supermix with ROX, Agarose, *N, N, N',N'-tetra-methylethylenediamine* (TEMED), Ammonium persulphate (APS), Acrylamide/bis solution (30%), Sodium dodecyl sulfate (SDS) and Goat anti-rabbit IgG (H+L)-HRP-conjugate were from Bio-Rad Laboratories (Hercules, California, USA). OLIGOLD SBP forward and reverse primers and β-actin forward and reverse primers were from Eurogentec (Seraing, Belgium). The radiolabelled steroids [1,2,6,7-^{3}H]testosterone ([^{3}H]-T, 95 Ci/mmol) and [2,3,6,7-^{3}H]estradiol ([^{3}H]-E2, 83 Ci/mmol) were from Amersham Biosciences (Buckinghamshire, England). Dextran T-70 was from Pharmacia Biotech (Uppsala, Sweden) and Optiphase Supermix came from Perkin Elmer (Wellesly, Massachusetts, USA). Milli-Q® water was collected from a Milli-Q® Ultrapure water purification system from Millipore (Billerica, Massachusetts, USA). Antibodies against zebrafish SBP was a generous gift from Dr. Geoffrey L. Hammond (University of British Columbia, BC, Canada).

3.3 Animals used for experiments

Rainbow trout, *Oncorhynchus mykiss* from Killi Oppdrettsanlegg (Dombås, Norway), was kept in tanks at the Department of Biology, University of Oslo (Norway) at a water temperature of 12ºC, oxygen saturation of approximately 100 % and pH 6.6. The fish were fed Ewos Transfer pellet (EWOS, Norway). The tanks received artificial illumination 12 h/day.

Zebrafish, *Danio rerio* from Akvarie fisken Eivind AS (Oslo, Norway) were kept in aquariums at the institute of Norwegian Water Research (NIVA). Water temperature was approximately 25ºC. Feeding was not necessary as the fish were only kept alive for a couple of hours.

3.4 Exposure of hepatocytes

3.4.1 Isolation of cells

Hepatocytes from rainbow trout were isolated and modified in a two step perfusion method as described by Tollefsen *et al*., (2003). Sexually undifferentiated juvenile or male fish were killed by a blow to the head and perfused *in situ* with a calcium-free solution containing NaCl (7.14 g/l), KCl (0.36 g/l), $MgSO_4$ (0.15 g/l), Na_2HPO_4 (0.4 g/l), $NaHCO_3$ (0.3 g/l), and EGTA (10 mg/l) at 12ºC at a rate of 10 ml/min for 10-15 min. The liver was perfused with the same buffer (37ºC), containing calcium ($CaCl_2$, 0.22 g/l) and collagenese (0.3 g/l) at the same rate for 10-15 minutes and transferred to a Petri dish containing ice-cold calcium free re-suspension buffer (EGTA-buffer containing 0.1% (w/v) bovine serum albumin) for dispersion. The cells were washed three times by centrifugation for (4 min, 500 rpm, 4ºC) and re-suspended in 20 ml Leibowitz 15 medium (L-15) containing L-glutamine (0.29 g/l), $NaHCO_3$ (0.38 g/l), penicillin (100,000 units/l), streptomycin (100 mg/l) and fungizone (0.25 g/l).

Cell viability and total number of cells was measured with trypan blue exclusion test and Coulter particle counter (cell size 10-20 μm) respectively. The cells were diluted in 6 well plates (Falcon, Bekton Dickinson Labware, Oxnard, CA, USA) as a monolayer culture with a density of 1.5 million cells per well and kept in an ambient environment (15ºC) for 24 hours before the onset of the exposure studies.

3.4.2 Exposure of cells

The hepatocytes were exposed to E2, EE2 and DBP by dissolving the test chemicals in DMSO and diluting the stock solution in cell culture media (L-15). Prior to hepatocyte exposure half the volume of cell medium was removed and the exposure solution added to each well. The cells were exposed for 24, 48 and 96 hours (15ºC) and samples of media and cells prepared for analysis of gene expression, protein expression and total steroid-binding activity in media.

3.4.3 Preparation of cell and media samples

The cell medium (2 ml) was removed, transferred to microtubes and stored at -80ºC. The cells were subjected to RNA isolation using an RNeasy Mini Kit (Qiagen, Hilden,

Germany) according to the protocol described in the "*RNeasy mini protocol for isolation of total RNA from animal cells*" (RNeasy Mini Handbook, Qiagen 2002). Each well was washed with 350 µl RNeasy lysis buffer (RLT; supplied by Qiagen) containing β-Mercaptoethanol (β-Me) and the homogenate was transferred to RNase-free microtubes. Samples were lysed by passing the homogenates 5 times through a 20-gaug needle (0.9 mm diameter) fitted to an RNase-free syringe and stored at -80ºC until further RNA isolation.

3.5 Determination of sex steroid-binding activity

3.5.1 Preparation of rainbow trout plasma

Blood was removed from the caudal artery using pre-cooled syringes containing 0.1% Heparin (v/v) and 1% protease (v/v) inhibitor Aprotinin (10 TIU/ml, Sigma, St.Lois, Oregon, USA). Blood samples were centrifuged at 2000 g (4ºC) for 10 min. The supernatant was carefully removed and frozen in aliquots at -80ºC.

3.5.2 Stripping of sex steroids

Stripping of plasma and cell media were performed essentially as described by Tollefsen, (2002). One volume of Dextran coated charcoal buffer (Dextran T-70 (2.5 mg/ml) and activated carbon (25 g/ml) mixed in TEMG; Trizma-HCl (1.404 g/l), Trizma-base (0.134 g/l), glycerol (100 ml/l), Triplex III (0.372 g/l) and 1-thioglycerol (1.3 g/l) was added to 9 volumes of sample. Charcoal was eliminated by centrifugation (4500 rpm, 10 min, 4 ºC). Two successive charcoal treatments of 5 and 45 min were performed to allow efficient elimination of high concentrations of endogenous or exogenous steroids.

3.5.3 Determination of sex steroid-binding activity

Saturation binding studies were conducted essentially as described by Tollefsen *et al*., (2002). In essence, 50 µl of [^{3}H]-E2 or [^{3}H]-T standards (0.6-20 nM) were incubated with (non-specific binding) and without (total binding) a three fold excess of inert steroid (T or E2 mixed in ethyl acetate (EtAc)) in 96-round bottomed polypropylene plates (Nunc, Roskilde, Denmark) together with 100 µl of diluted rainbow trout plasma (100 times in TEMG buffer) or undiluted DCC stripped cell medium. The plate was covered with

sealing tape (Nunc, Roskilde, Denmark) gently swirled and incubated in a refrigerator for a minimum of 4 hours. Separation of protein bound sex steroids were performed according to microplate based filtration method (Olsen *et al*., 1995; Gattu *et al*. 1995; Roychoudhury *et al.,* 1997).

The wells of a Millipore Multiscreen® $_{HTS}$96-well filtration plate (Millipore, Billerica, MA, USA) were coated with 200 µl of coating buffer (0.1% PEI (v/v) in dH_2O), sealed with sealing tape and incubated in a refrigerator for a minimum of 2 hours. The filtration plate was mounted on the vacuum manifold (Millipore, Billerica, MA, USA) and washed three times in 200 µl ice-cold TEMG buffer by vacuum suction (10 mm Hg''). To avoid the filter paper from running dry, the last wash was emptied by inverting the filtration plate and the underdrain was dried using a clean paper towel. 100 µl of the incubation solutions were applied to the filtration plate wells and the volume was reduced to approximately 25% by vacuum and rapidly washed 5 times in 200 µl of ice-cold TEMG buffer as described. The filters were allowed to dry with vacuum suction for one minute, the underdrain was removed and the filtration plate was dried for one hour at 50ºC.

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After drying, the bottom of the plate was sealed by a plastic liner (Perkin Elmer, Wellesly, MA, USA) and 30 µl of Optiphase Supermix scintillation cocktail (Perkin Elmer) was added to each well. The plate was incubated in the dark (2 hr) before radioactivity was determined by a MicroBeta Trilux microplate scintillation counter (Perkin Elmer, Wellesly, MA, USA) using standard tritium conditions. All incubation steps were performed on ice.

Total sex steroid binding capacities were essentially determined as described for saturation studies, although a fixed concentration of radio-labelled steroid was used. In brief 2.5 nM tracer ([^{3}H]-E2 or [^{3}H]-T) in combination with (non-specific binding) and without (total binding) a three fold excess of inert steroid were added in duplicates to a 96-well round bottomed plate and 100 µl of undiluted DCC-stripped medium were added to the wells. The sex-steroid binding activity was determined as described earlier.

3.5.4 Determination of protein concentration, specific binding and Scatchard plots

Protein content was determined by method of Bradford (1976). A protein dye solution (Bio-Rad Laboratories, Hercules, CA, USA) was diluted in distilled water and filtered (Whatman no.1). Samples were diluted in TEMG buffer, applied in triplicate to microtitier wells and a protein dye working solution was added. After incubation (5 min, 20ºC) the absorbance (590 nm) was measured and protein content determined by the use of bovine serum albumin (BSA).

Binding data (dpm) from saturation analysis is calculated as specific binding where;

Specific binding (dpm) = [Total background] – [Non-specific background]

To line data from saturation analysis, Scatchard plots were used. In this plot the X-axis is specific binding and the Y-axis is the ratio of specific binding to concentration of free radioligand (labelled bound/free). Total available binding sites (B_{max}) is the X intercept; the equilibrium dissociation constant (K_d) is the negative reciprocal of the slope.

3.5.5 Determining the role of non-specific binding proteins

The binding of estrogens and testosterone to other proteins than SBP might be of importance when characterizing total steroid-binding capacity. The effect of vitellogenin (vtg) and albumin (BSA) on the total-steroid binding capacity assay was determined to quantify the role of non-specific binding proteins.

In brief, 0.1% bovine serum albumin in TEMG, purified rainbow trout vitellogenin (Biosense Laboratories, Bergen, Norway) (4µg/ml TEMG), DCC-stripped cell medium (control cells), DCC-stripped cell medium from hepatocytes exposed to E2 (exposed cells) and rainbow trout plasma (10% v/v in TEMG) and TEMG alone were incubated with 5 and 20 nM of either [^{3}H]-E2 or [^{3}H]-T (50 µl) for a minimum of 4 hours in a refrigerator. Total sex steroid-binding activity was determined as described earlier.

3.6 Immunological detection of sex steroid-binding proteins

3.6.1 Preparation of plasma from zebrafish

Zebrafish were anesthetized in ice-water and blood-samples were taken from the fish's dorsal vein using a small pre-cooled syringe containing Heparin (5000 IU/ml, Sigma, St.Lois, MA, USA). Blood-samples from several fish were collected in a microtube and centrifuged in a micro-centrifuge (4ºC). Plasma was removed using a pipette and stored in aliquots at -80ºC.

3.6.2 SDS-PAGE

SDS polyacrylamide-gel electrophoresis (SDS-Page) was performed using a mini-PROTEAN II Electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) essentially as described by Miguel-Queralt *et al*., (2005). The glass plate/clamp assembly was set in the casting stand and 3.5 ml resolving gel (TEMED (0.5 µl/ml) and APS (10 µl/ml), dH_2O (0.4 ml/ml), Trizma-Base 1.5 M pH 8.8 (0.4 ml/ml), acryl/bis 30% solution (334 µl/ml) and SDS (10 µl/ml)) was poured between the glass plates and allowed to polymerise for 30 min with a thin layer of dH_2O on top. TEMED (0.75 µl/ml) and APS (10 µl/ml) were added to a 4% acrylamide gel stock solution (dH_2O (0.71 ml/ml, Acryl/Bis 30% solution (0.14 ml/ml), 0.5 M Tris pH 6.8 (0.125 ml/ml), SDS (10 µl/ml)) and poured directly on top of the resolving gel. After the insertion of a 10-well comb, the gel was left to polymerize for approximately one hour. The gel apparatus was fitted to an electrophoresis gasket and placed in the electrophoresis tank containing running buffer; Trizma-Base (3.02 g/l), glycine (18.8 g/l) and SDS (1.0 g/l).

Cell medium and plasma from rainbow trout and zebrafish were diluted 1:1 in boiling buffer (Tris-HCl (1.97 g), glycerol (20 ml), SDS (4 g), β-Me (4 ml) and bromophenolblue in 100 ml) and boiled (5 min, 95ºC) in microtubes.10 µl of a low molecular weight protein standard (Bio-Rad Laboratories, Hercules, CA, USA) and 20 µl of lysate (protein and boiling buffer) where added to the gel and the electrophoresis performed at 100 V on ice until the bromophenolblue marker dye reached the bottom of the plate (approximately 1 hr).

3.6.3 Western blotting

The SDS-gel, still glued to one of the glass plates was placed in transfer buffer containing Trizma-Base (3.03 g/l), glycine (14.4 g/l) and 20 % (v/v) methanol for 20 min while a PVDF membrane (Millipore, Billerica, MA, USA) was equilibrated in 100 % methanol (10 s) and washed in dH_2O (2 min). Sponges, 4 pieces of Whatmann paper and the PVDF membrane were all soaked in transfer buffer. A transfer cassette was prepared, and bubbles of air removed by rolling a glass flask over the assembly. The cassette was fitted into a blotting apparatus filled with transfer buffer and blotted at 100 V (45 min) in a cooled electrophoresis chamber. The transfer cassette was dismantled and the blot cleaned in TBST; TBS containing 0.01% Tween 20 (NaCl (8 g/l), KCl (0.2 g/l), Trizma-Base (3 g/l) and Tween 20 (500 µl/l)).

The blot was incubated overnight in blocking buffer (5% BSA (w/v) in TBST) (4 ºC), rinsed in TBST (10 min) and incubated (37ºC) with the primary antibody; rabbit anti zebrafish SBP diluted 500 times in TBST. The use of zebrafish antibodies in western blot was chosen as, rainbow trout antibodies for SBP are not available and anti-zebrafish SBP has shown to recognize SBPs in the blood of several fish species. The blot was washed 6 times (10 min) in TBST before incubation with a goat anti-rabbit IgG (H+L)-HRP-conjugate (60 min, 37ºC) (Bio-Rad Laboratories, Hercules, CA, USA). The blot was washed again in TBST and TBS (1 X 10 min) to remove any trace of Tween 20.

Developing was done by incubating the blot (5 min) in a solution containing equal amounts of stable peroxide solution and enhancer solution from a Supersignal® West Pico kit (PIERCE, Rockford, IL, USA). Inside a darkroom, the membrane was laid between two transparent films, bubbles of air were removed and the blot placed in a radiography cassette with its protein side up. An ECL™-Hyperfilm (Amersham, Buckinghamshire, UK) was placed on top, the cassette was closed and the film exposed for 1 – 30 min depending on intensity. Developing was done in an OPTIMAX film processor (Protec, Obersterfeld, Germany) and the film was visually rated.

3.7 Quantitative real-time PCR analysis of SBP gene expression

3.7.1 Isolation and purification of RNA

One volume (350 µl) of 70 % RNase- free ethanol (Arcus, Oslo, Norway) was added to the thawed homogenized lysate, mixed by pipetting, applied to an RNeasy mini column (Qiagen) in a 2 ml collection tube and centrifuged (10000 rpm, 15 s). RNeasy wash buffer (RW1 buffer) was added (700 µl) and the excess DNA was removed by centrifugation (10000 rpm, 15 s) before the column was washed with 500 µl RPE buffer (Qiagen) and centrifuged (10000 rpm for 15 s). This step was repeated with a longer centrifugation time (10000 rpm for 2 min) before drying the silica-gel membrane completely by centrifuging at full speed (1 min). The column was transferred to a new 1.5 ml RNase-free collection tube, 50 µl of RNase-free water was added and RNA was eluted by centrifugation (10000 rpm, 1 min). Samples were stored at -80ºC until analysis by Reverse Transcriptase Polymerase Chain Reaction (rtPCR)

The purity and amount of RNA in the isolated samples were determined by optical density (OD) of RNA at A_{280}, A_{260} and A_{230} in a Lambda 40 UV/V15 Spectrometer (Perkin Elmer, Wellesly, MA, USA) and the relationship A_{260}/A_{280} calculated as the total RNA concentration in each sample. Samples were diluted in RNase-free water to a final RNA concentration of 20 µg RNA/µl. All steps were performed in a RNase-free environment as possible.

3.7.2 DNA amplification

Reverse Transcriptase Polymerase Chain Reaction was performed using a High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). A master mix (see appendix) was made in a 1.5 ml microtube depending on the number of samples. The mixture was mixed with 10 µl RNA templates (20 µg RNA/ µl), transferred to PCR-tubes, run in an Eppendorf mastercycler gradient PCR (Eppendorf, Hamburg, Germany) according to specifications given in **Table 3-1**. The resulting cDNA samples were stored at -20ºC until analysis by Quantitative Realtime polymerase chain reaction (qPCR)

Table 3-1 *PCR program for cDNA synthesis.*

Temperature ºC	Time
25	10 min
37	2 hours
4	hold

3.7.3 Quantitative polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed according to method by Torsdalen (2003). A master mix containing TAQ SYBR® Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA, USA), milliQ water and optimal concentrations for OLIGOLD forward and reverse primer (Eurogentec, Seraing, Belgium, see **Table 3-2**) was made depending on the number of samples. 10 µl cDNA was added to each microtube, applied in triplicates of 25 µl to a 96 well PCR plate (Sarsted, Numbrecht, Germany) and coated with optically clear sealing tape (Sarsted, Numbrecht, Germany). Air bubbles were removed by centrifugation at 1000 rpm (1 min). The plate was run in an Absolute Quantification Assay by a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

Table 3-2 *Primers used in qPCR.*

Primer (OLIGOLD)	Sequence
β-actin -Reverse	5'-CGT-AGT-CCT-CGT-AGA-TGG-GTA-CTG-3'
β-actin 378 -Forward	5'-TAC-CAC-CGG-TAT-CGT-CAT-GGA-3'
SBP 1251 -Reverse	5'-CAG-GAT-AGC-CTT-GAT-TCA-GAC-TTC-3'
SBP 1171 -Forward	5'-GAT-CTA-GAC-CGG-GCG-GTG-TA-3'

For quantification of cDNA samples a standard curve was made by diluting a mix of cDNA samples tenfold (five samples). The housekeeping genes (β-actin) were used as an endogen control, by running samples including standard curve samples with forward and reverse β-actin primers.

The calculation of relative gene expression was done using the standard curve. Samples were normalised in relation to the endogen control (β-actin) and in relation to unexposed samples (0 hr of exposure to DMSO and test compounds):

- Ratio of SBP gene amount to amount of endogen control
- Ratio (sample)/ Ratio (control)

To avoid false positive signals by SYBR® Green binding non-specifically to double-stranded DNA sequences, the qPCR products were checked for non-specific product formation using gel analysis. Samples mixed with gel loading buffer (bromophenolblue (2.5 mg/ml), Xylene Cyanol (2.5 mg/ml) and Ficoll- 400 (0.15 g/l) and a 100 bp DNA ladder (ABgene, Surrey, UK) were all run in a 1% agarose gel containing EtBr (100 V, 20 min). The gel was completely covered in TAE buffer containing Trizma-base (4.84 g/l), concentrated acetic acid (1.142 g/l) and 0.5 M EDTA pH 8.0 (2 ml/l). DNA bands were visualised by placing the gel on a UVT-20M UV-table (Herolab, Wiesloch, Germany) and photographed by a Polaroid Gel cam (Peca Products, Beloit, WI, USA).

3.8 Graphical and statistical methods

Cells were exposed in duplicates and qPCR and total binding activity assays were performed on hepatocytes from two individual fish. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc. San Diego, USA).

4 RESULTS

4.1 Determination of sex steroid-binding activity

4.1.1 Saturation analysis of rainbow trout plasma

Saturation and Scatchard analysis were conducted to characterize both [^{3}H]-T and [^{3}H]-E2 binding to the assumed sex steroid-binding protein in diluted samples of rainbow trout plasma. Both steroids bound to a high affinity and moderate capacity binding protein in rainbow trout plasma and obtain saturation of ligand binding sites at about 10 nM (**Fig 4-1**). Scatchard transformation of these specific binding data (**Fig 4-2**) revealed a linear relationship for the binding of both testosterone (T) and estradiol (E2) to the assumed rainbow trout SBP. Scatchard analysis yielded a mean equilibrium dissociation constant (K_d) and mean total available binding sites (B_{max}) of 3.074 nM and 9616 fmol/mg protein for testosterone and for estradiol; K_d of 4.72 nM and B_{max} 8192 fmol/mg protein. The K_d value in saturation analysis using [^{3}H]-T are apparantly slightly lower than in saturation analysis with [^{3}H]-E2, while the opposite is true for B_{max} values. These are higher for [^{3}H]-T than [^{3}H]-E2.

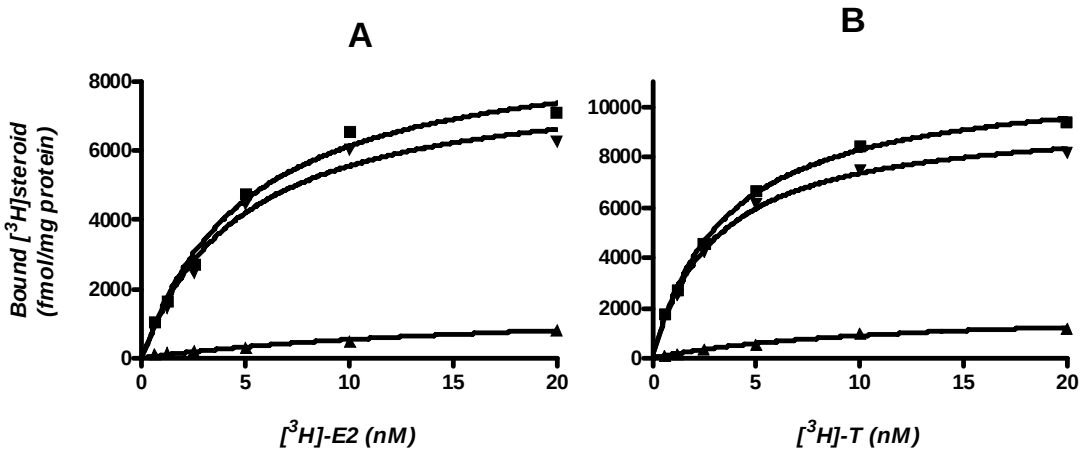


Figure 4-1 *Total(■), non-specific(▲) and specific binding (▼) of [^{3}H]estradiol (A) and [^{3}H]testosterone (B) to diluted plasma from rainbow trout (0.5 mg protein/ml).. The values represent analysis of plasma from one representative fish.*

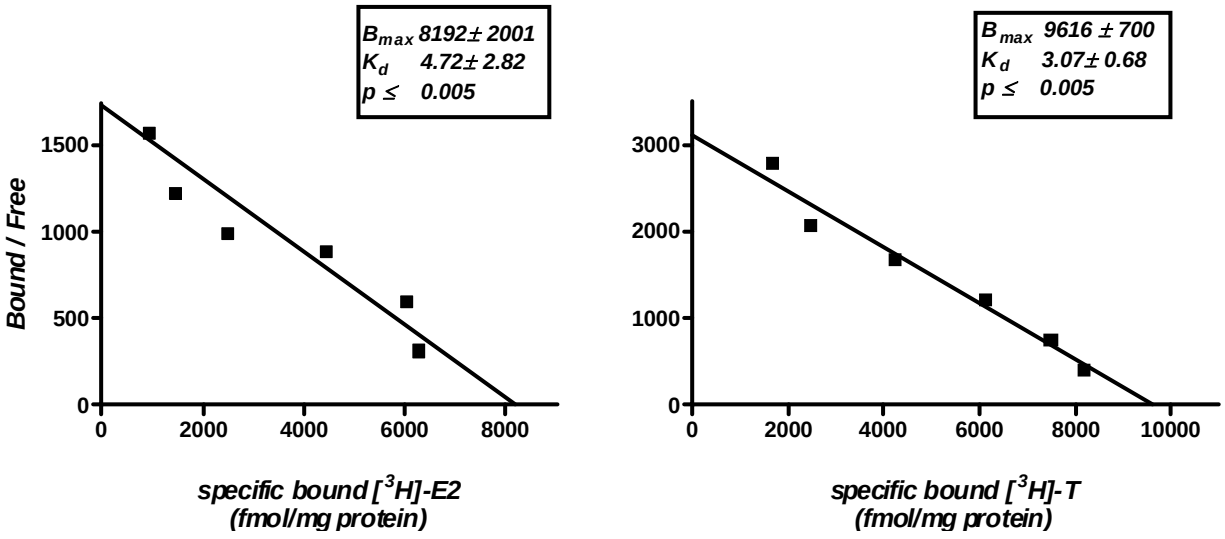


Figure 4-2 *The maximum specific binding (B_{max}) and dissociation constant (K_d) estimated by Scatchard transformation of the specific binding data from saturation analysis for [^{3}H]estradiol(left) and [^{3}H]testosterone (right) in diluted rainbow trout plasma (0.5 mg protein/ml). The values (mean ± SEM) represent analysis of plasma from one representative fish.*

4.1.2 Saturation analysis of cell medium

Cell medium from rainbow trout hepatocyte cultures exposed to 17β-estradiol for 48 hours was stripped in a 1 to10 cell medium: DCC ratio. The DCC-stripped cell medium was subjected to saturation and Scatchard analysis to determine whether cell media display similar binding characteristics as plasma for estradiol and testosterone. Saturation analysis showed a large difference between total and non-specific binding as seen for plasma, but no clear saturation of estradiol or testosterone binding sites were obtained (**Fig 4-3**). Scatchard analysis confirmed that that high affinity binding sites as seen in saturation analysis of rainbow trout plasma were not clearly identified in the medium. The K_d and B_{max} values were calculated using non-linear regression of the specific binding data. Values for K_d and B_{max} in saturation analysis using [^{3}H]-E2 were given as 29.94 ± 22 nM and 5347 ± 2773 nM and respectively. The same results were obtained in saturation analysis using [^{3}H]-T (**Fig 4-4**), where K_d values were given as 31.72 ± 20 nM and Bmax, 6122 ± 2752 nM.

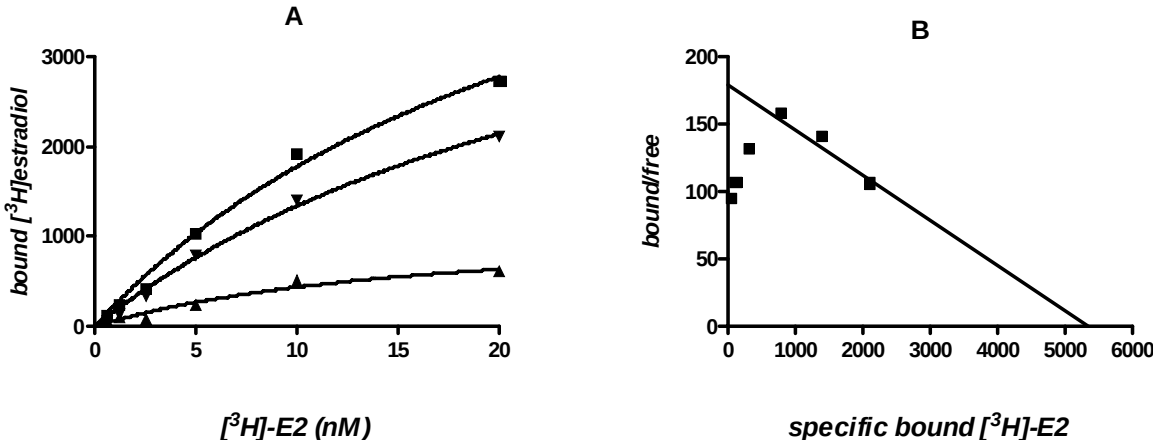


Figure 4-3 *Total(■), non-specific(▲) and specific binding (▼) of [^{3}H]estradiol in 1:10 DCC stripped cell medium (A) from a rainbow trout hepatocyte culture exposed to E2 for 48 hours. Scatchard transformation of the specific binding data (B) revealed no linear relationship. The values represent analysis of cell medium from one representative experiment.*

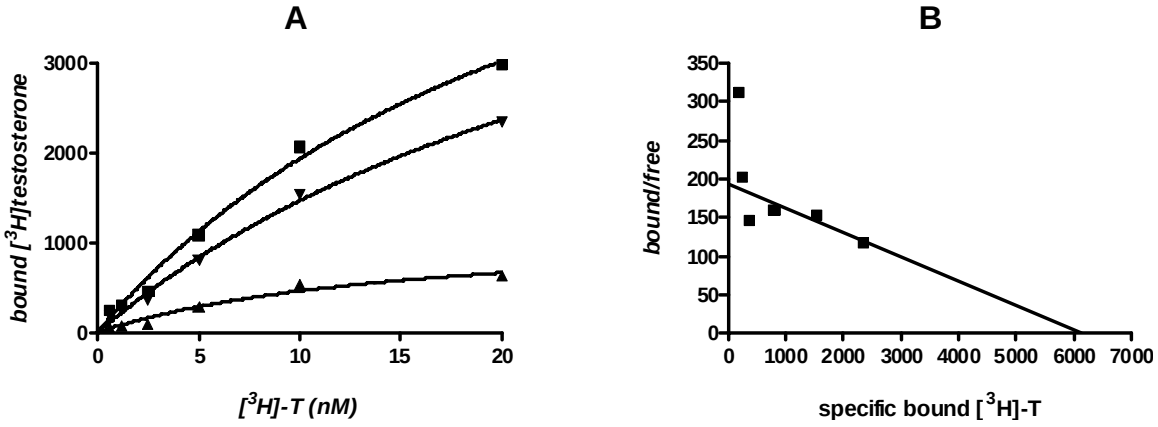


Figure 4-4 *Total(■), non-specific(▲) and specific binding (▼) of [^{3}H]testosterone in 1:10 DCC stripped cell medium (A) from a rainbow trout hepatocyte culture exposed to E2 for 48 hours. Scatchard transformation of the specific binding data (B) revealed no linear relationship. The values represent analysis of cell medium from one representative experiment.*

4.1.3 Total sex steroid-binding activity

Total binding activity in rainbow trout hepatocytes increased with both time and concentration compared to control. All chemicals showed an increase in total binding activity after 48 hours of exposure. The largest increase was seen in hepatocyte cultures exposed to EE2 (**Fig 4-5 *B and E***). Compared to solvent control, total binding activity increased 4 times. Total binding in hepatocyte cultures exposed to E2 (**Fig 4-5 *A and D***) increased with two times, while a three time increase was seen for hepatocyte cultures exposed to DBP (**Fig 4-5 *C and F***). Although the present increase is almost equal for testosterone and estradiol after 96 hours, the curves appear different at 24 and 48 hours of exposure.

A concentration-response relationship was clearly seen for EE2, E2 and DBP after 96 hours exposure. (**Fig 4-6**). Maximum induction was seen at 10^{-8} mol/l for E2 and 10^{-7} mol/l DBP (**Figure 4-6 *A and C***). A concentration-response relationship was also seen for total binding activity assays with [^{3}H]-T, displaying maximum induction at 10^{-5} mol/l for E2 and 10^{-8} mol/l for DBP. The maximum induction of EE2 (**Fig 4-6 *B and E***) is not apparent as response was still increasing at the highest exposure concentration (10^{-5} mol/l). Interestingly, total sex steroid-binding activity in [^{3}H]-E2 assays was clearly higher than control even at the lowest concentrations of EE2 and E2, suggesting that these compounds induce total binding activity responses even at lower concentrations than those tested in this work. Variation increases in the highest concentrations of EE2, which is probably due to the high concentrations of the potent estrogen affecting cell viability. A similar, but not as extreme trend is seen for DBP.

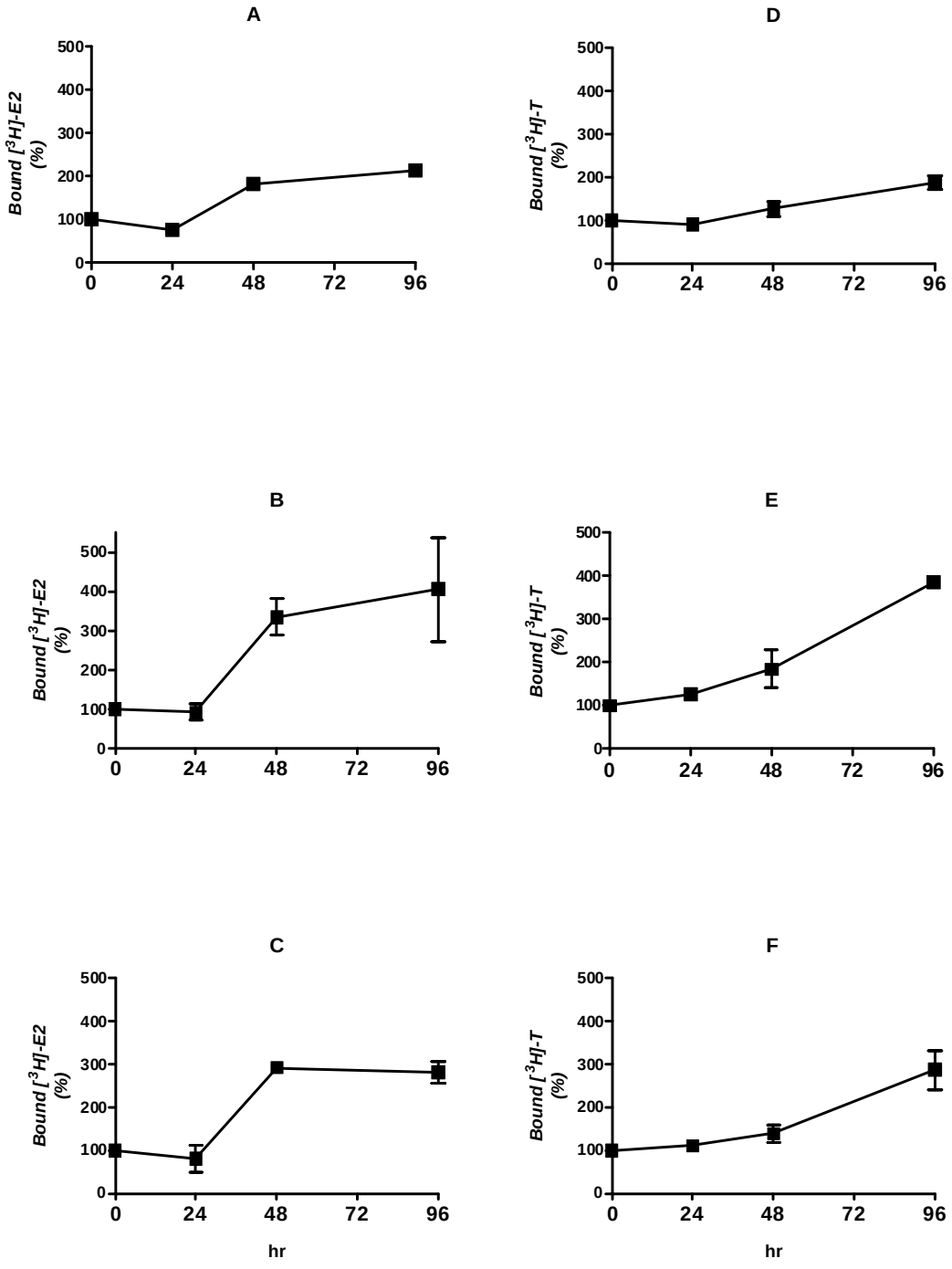


Figure 4-5 *Total binding activity to cell medium proteins from rainbow trout hepatocytes exposed to 1 µM of the test chemicals 17β-estradiol (A, D), ethynylestradiol (B,E) and di(n-butyl) phthalate (C, F) over time. Left graphs displays data for total binding capacity to [^{3}H]-E2 and the right for total binding capacity to [^{3}H]-T. The values (mean ± SEM) represent analysis of cell medium from one representative fish.*

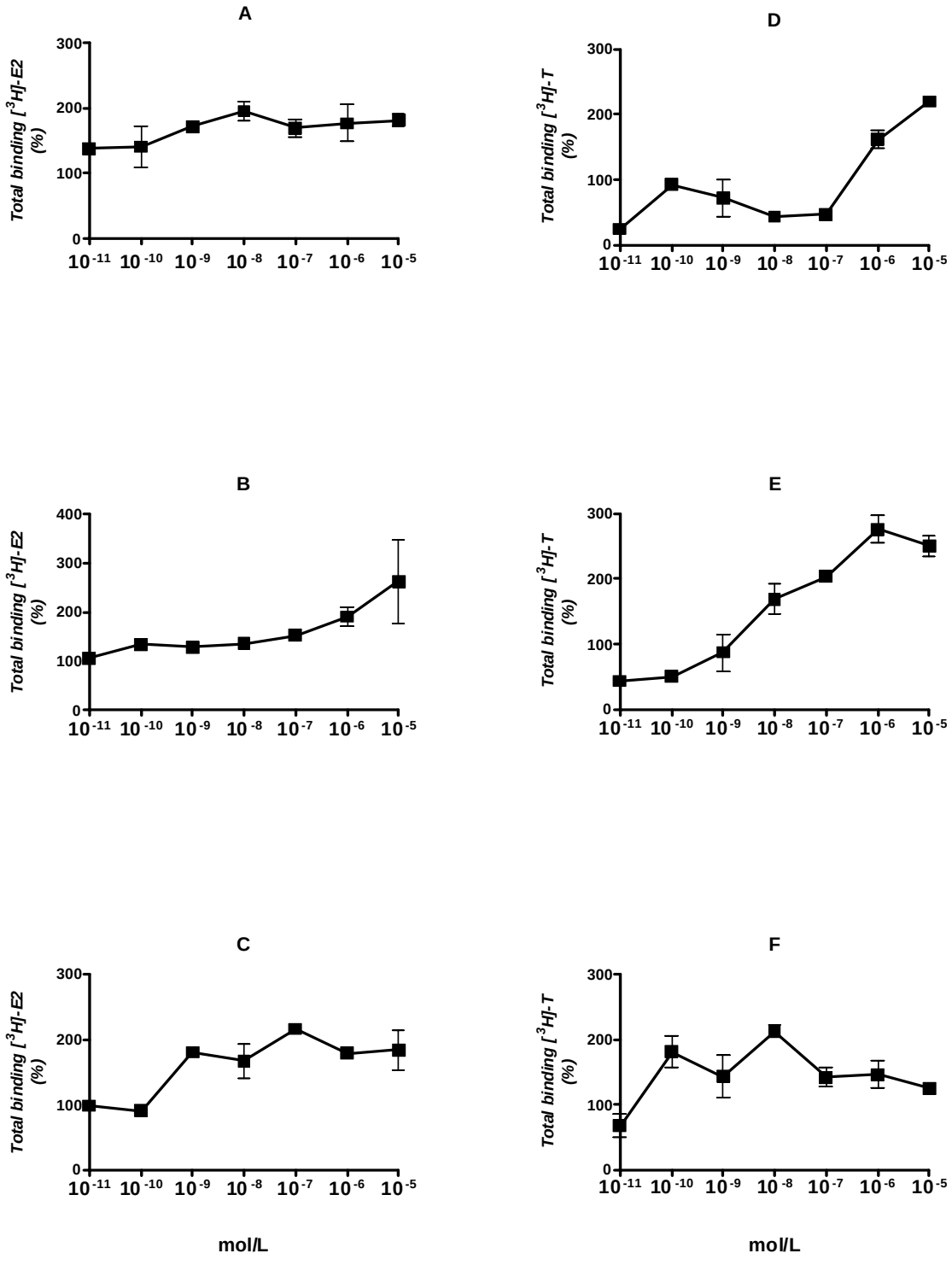


Figure 4-6 *Total binding activity to cell medium proteins from rainbow trout hepatocytes exposed to E2 (A, D), EE2 (B,E) and DBP (C, F) after 96 hours of exposure at increasing concentrations. Left graphs displays data of binding to [^{3}H]-E2 and the right for binding to [^{3}H]-T. The values (mean ± SEM) represent analysis of cell medium from one representative fish.*

4.1.4 Determining the role of non-specific binding proteins

Cell medium contains many carrier proteins other than SBP. These may be an important reason for why saturation of cell medium does not occur in the full saturation analysis. Binding to bovine serum albumin (BSA) and vitellogenin (vtg) were thus determined to asess the role of the assumed non-specific binding proteins. Compared to the contribution of the total binding activity to TEMG buffer alone (blank), binding to vtg and BSA appear to play a minor role, in total binding activity assays. Cell medium (control cells) however, increases total binding activity two times compared to the blank, whereas total binding steroid-binding activity of plasma and E2 exposed cells increase 10-20 times (**Fig 4-7**).

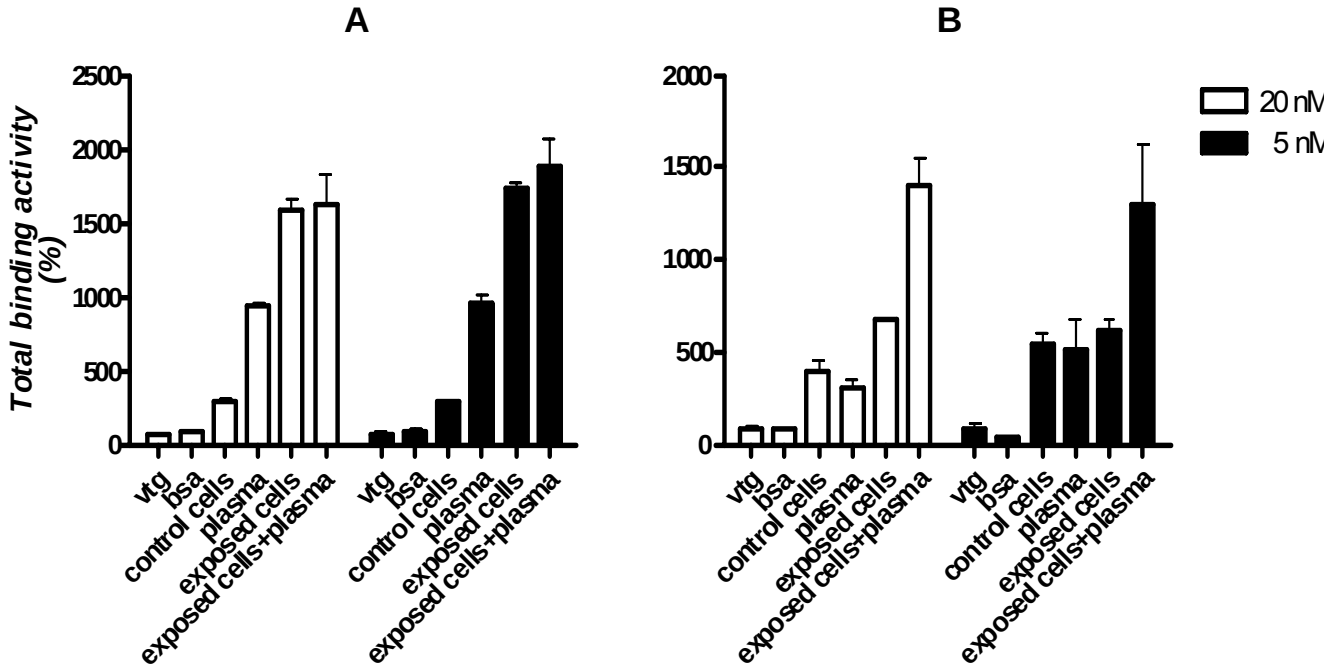
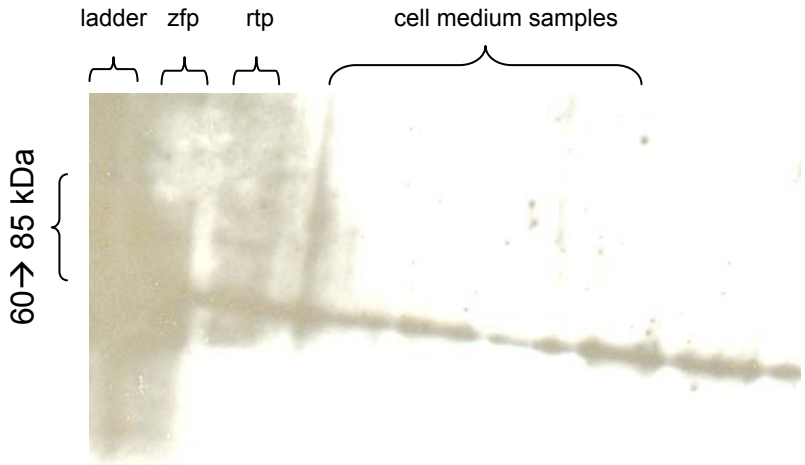


Fig 4-7 *Total binding activity (%) of [^{3}H]-E2 (A) and [^{3}H]-T (B) to 0.4 % vitellogenin in TEMG (vtg), 01 % albumin in TEMG (BSA), DCC-stripped control cell medium (control cells), DCC stripped cell medium collected from E2 exposed hepatocyte cells (exposed cells) and rainbow trout plasma. The values (mean ± SEM) show percentage increase in total binding activity compared total binding activity of TEMG buffer.*

4.2 Immunochemcial detection of sex steroid-binding proteins

Western blots (**Fig 4-8**) of fish plasma samples from rainbow trout and zebrafish probed with anti-zebrafish SBP antiserum showed weak but comparable bands indicating that the polyclonal antibodies bind to both zebrafish and rainbow trout SBP. High protein concentrations due to a low dilution of plasma samples in boiling buffer gives quit broad blurry bands Compared to the low molecular weight protein standard, the size of the protein bands appear similar to 60 to 85 kD for rainbow trout and zebrafish. The western blot confirms what appears to be the presence of a protein with similar molecular size as the assumed rtSBP in cell medium from rainbow trout hepatocytes exposed to increasing concentrations of E2 for 96 hours. The bands were not as clear as that for rainbow trout plasma, but this may be caused by the relative lower levels of proteins present in cell growth media.

Fig 4-8 *Expression of SBP in zebrafish plasma (zfp), rainbow trout plasma (rtp) and cell medium from hepatocytes exposed to E2 for 96 hours.*



4.3 Analysis of sex steroid-binding protein gene expression

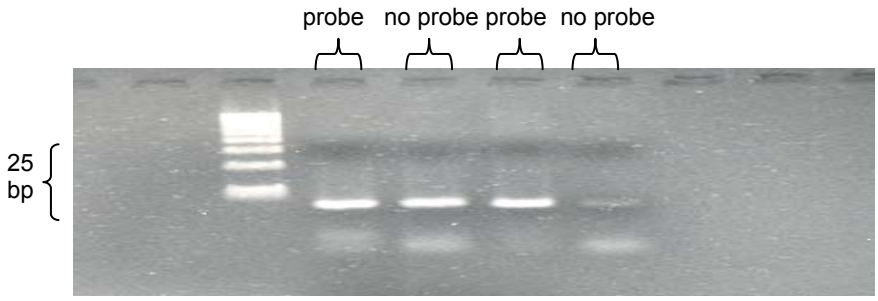
4.3.1 Assay optimalisation

Optimal concentration was tested for both forward and reverse SBP primers. This was done by testing all possible concentrations on cDNA from the same sample. The clearest qPCR product was seen at primer concentrations of 900 nM.

Standard curves for β-actin and SBP in an absolute quantification assay displayed slopes with gradients between -3.0 and –3.4 (**results not shown**). This is a good indication of the qPCR reaction being close to optimal (exponential). A selection of PCR products run in agarose gel (**Fig 4-9**) show equal sized products of approximately 25 bp. The qPCR products were therefore assumed to quantify the SBP gene.

To verify that the PCR products were indeed SBP, SBP-specific probes were used together with SBP forward and reverse primers. The SBP probes were tested, but did not produce a qPCR that corresponded to the chosen primers. As no PCR product was formed, the probes nucleotide was sequenced and the reported sequence displayed limited similarity with the rainbow trout SBP nucleotide sequence. This suggests that the SBP probes were not representing SBP, although more unlikely due to high quality gene bands in agarose gel.

Fig 4-9 *Selection of qPCR products run with and without SBP specific probes.*



4.3.2 Quantification of SBP in E2, EE2 and DBP exposed hepatocytes

The gene-expression of SBP in rainbow trout hepatocytes increased with both time (**Fig 4-10**) and concentration (**Fig 4-11**) when exposed to the test compounds. Induction of gene expression was seen after 24 hours for all compounds, but the highest increase in SBP gene expression compared to β-actin is seen in hepatocytes exposed to E2 and EE2 after 48 hours of exposure, thus coinciding with attaining maximum binding activity in the cell media. SBP gene expression was induced with a factor of almost four times for EE2, whereas E2 exposure led to three times increase in SBP gene expression. The increase in SBP gene expression agree well with the increase in total binding capacity of both EE2 and E2, reported to be four and two times, respectively. Although maximum SBP gene expression was also obtained after 48 hours of exposure to DBP, this compound was only able to induce a two times increase in SBP gene expression, slightly lower than what was observed in total binding capacity assay.

A concentration-response relationship was clearly seen for EE2 and DBP after 96 hours exposure, where maximum induction was obtained for 10^{-7} mol/l EE2 and DBP (**Fig 4-11 B *and C***). This is similar to DBP concentrations reported in total binding capacity assays. Slightly lower concentrations for EE2 maximum induction are however seen in qPCR data compared to total sex steroid-binding activity. The natural estrogen E2 did not yield a clear concentration-response relationship at the concentrations tested (**Fig 4-11 *A***). The SBP gene expression was clearly higher than SBP expression in control cells even at the lowest concentrations of DBP, EE2 and E2, thus suggesting that these compounds induce SBP gene up-regulation even at lower concentrations than those tested in this work.

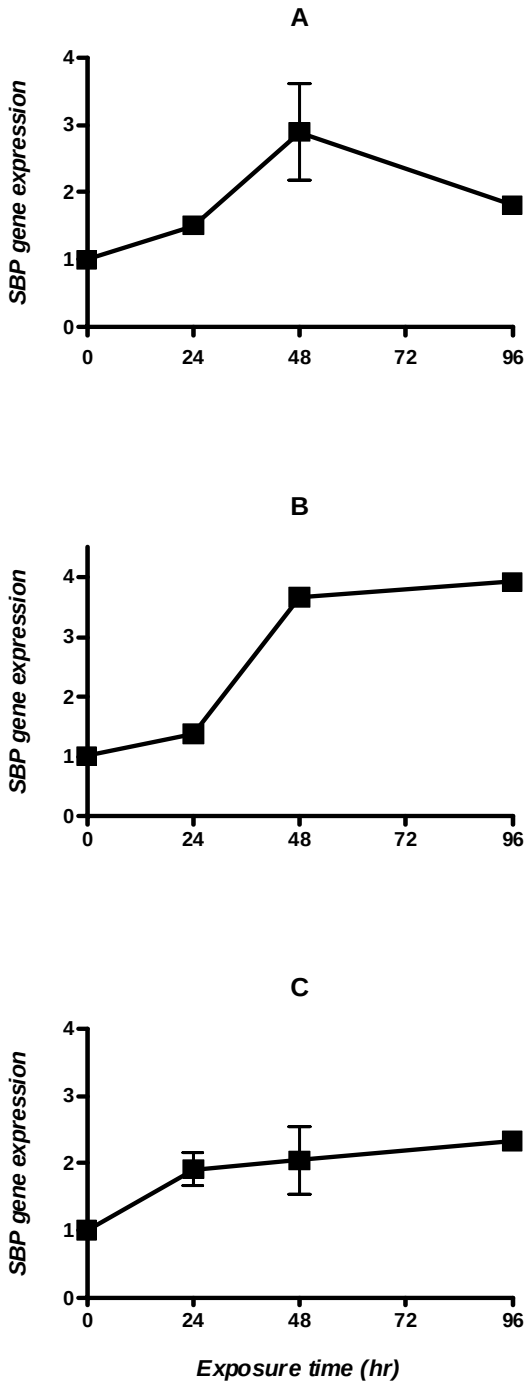


Fig 4-10 *Quantitative gene expression of SBP in rainbow trout hepatocytes exposed to 1 μM of the test chemicals E2 (A), EE2 (B) and DBP (C) over time. Results from one representative fish, are displayed as an increase in the expression of the SBP gene compared to the expression of β-actin and solvent (mean ± SEM).*

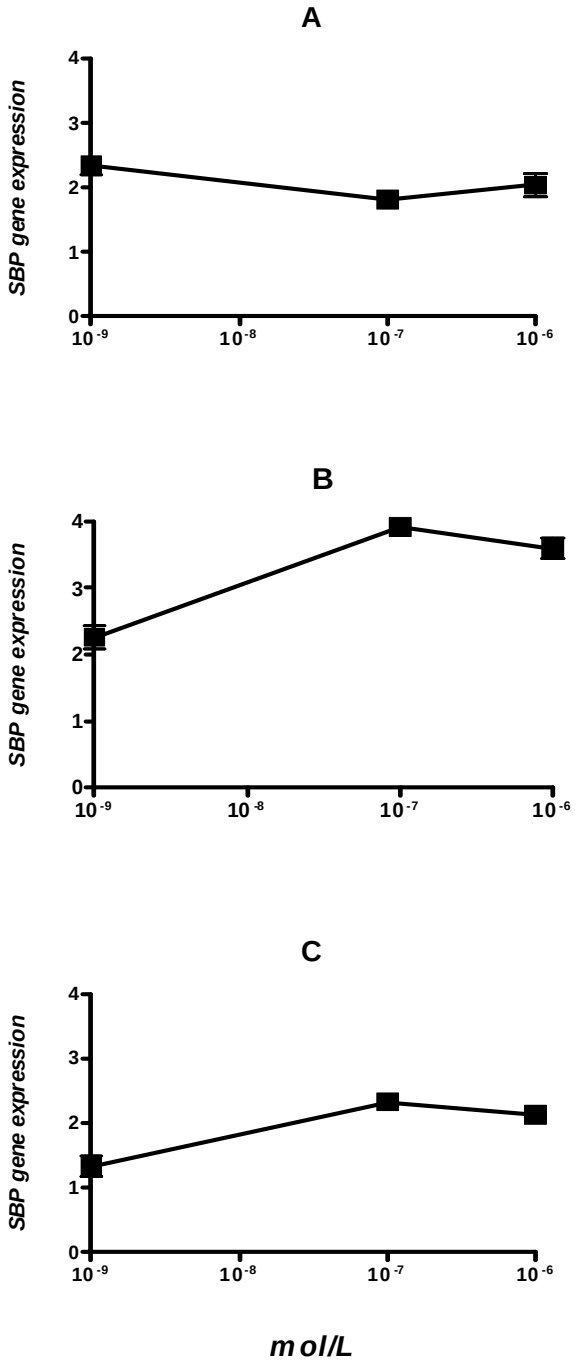


Fig 4-11 *Quantitative gene expression of SBP in rainbow trout hepatocytes exposed for 96 hours to increasing concentrations of E2 (A), EE2 (B) and DBP (C). Results from one representative fish, are displayed as an increase in the expression of the SBP gene compared to the expression of β-actin and solvent (mean ± SEM).*

5 DISCUSSION

The objective of this thesis was to determine whether common environmental chemicals may modulate the production of sex steroid-binding proteins (SBP) and potentially play a role in endocrine disruption in fish. This was done by optimising and applying methods to measure total extracellular sex steroid-binding capacity in a primary cell culture from rainbow trout hepatocytes and measure the gene expression of SBP. A small selection of environmental compounds that previously has been shown to modulate the circulating levels of SBP in fish (Tollefsen, 2002) was used to study the mechanism of action.

The metabolism and tissue availability of endocrine disrupting compounds (EDCs) may be modulated through binding to SBP and can be a mechanism for endocrine disruption in wildlife species (Crain *et al*., 1998). Low concentrations of EDCs may lead to induction of SBP and modulation of the free bio-available levels of both EDCs and sex steroid concentrations in the bloodstream (Tollefsen et al., 2002; Wells and van der Kraak, 1999). The consequence of this being that altered EDCs interact with steroid specific receptors.

SBP has been reported to bind several endocrine disrupting compounds, including bisphenol A, diethylstilbestrol and 4-nonylphenoxyacetic acid in rainbow trout plasma (Milligan *et al*., 1998). Later studies have further revealed the binding of both natural and anthropogenic EDCs from distinct chemical groups to rainbow trout plasma (Tollefsen 2002; Tollefsen *et al*. in press). Some of these compounds, including ethynylestradiol (EE2) are able to displace the endogenous sex steroids from SBP, and by doing so increase the equilibrium dissociation constant (K_d) in binding assays in a dose-dependant manner. The same has been seen following *in vivo* exposure of Atlantic salmon to EE2 and di-(*n*-butyl) phthalate (DBP), which both induce a concentration-dependant increase in plasma estradiol binding capacity (Tollefsen *et al.*, 2002).

5.1 Total sex steroid-binding capacity

In order to document a similar sex steroid-binding site in cell medium and rainbow trout plasma, radio-ligand assays were first studied on rainbow trout plasma. Saturation analysis showed that the radio-labelled testosterone and estrogen bound to SBP in a saturable and specific manner. K_d and B_{max} values were similar to those found in earlier studies (Tollefsen 2002; Øvrevik *et al*., 2001; Laidley and Thomas, 1994). K_d values for the testosterone assays were smaller than estradiol, indicating that T may have a higher affinity for the ligand. Lower K_d values in saturation analysis with T has also been seen in rainbow trout plasma and cell medium in other binding assays (Foucher *et al*.., 1990).

The SBP characteristics of hepatocyte cell medium did not show the same high-affinity binding capacity to SBP as in rainbow trout plasma. A total binding activity assay measures the total binding activity of all proteins that bind to E2 or T; one can only assume that the binding is due to an increase in SBP production. The data from saturation analysis suggest the presence of low-affinity binding sites and it was suspected that other proteins in the cell medium bound to estrogen and testosterone, the consequence of this being that the binding to E2 or T never became saturated. Earlier studies on trout hepatic cell culture media has revealed a saturable testosterone binding site where Scathcard plot analysis of saturation experiments showed a class of high affinity binding sites (K_d 4.7 nM), indicating SBP to be the same protein in plasma and cell media (Johnson *et al*., 1985).

In the present study the total sex steroid-binding capacity increased in hepatocyte cultures exposed to E2, EE2 and DBP. This is the same as has been seen for SBP levels in plasma of rainbow trout and Atlantic salmon exposed to the same estrogenic compounds (Tollefsen *et al*., 2002). Increase in total binding capacity was measured after 48 hours, but a concentration-related response was seen after 96 hours of exposure. The results do however, vary between binding assays using [^{3}H]-E2 or [^{3}H]-T. The reason for differences between the two radio-labelled ligands is uncertain, but could be due to different affinities for SBP. Unlabelled testosterone is reported to have a higher affinity for SBP in competition with other steroids to [^{3}H]-T in rainbow trout hepatocyte cultures

(Foucher et al., 1990). Another possibility is the effects of incubating filtration plates with coating buffer (0.1 % PEI) at different time-intervals. This is at the time-being unknown, and could be a possible explanation for differences between [^{3}H]-T and [^{3}H]-E2. The greater sex-steroid binding values obtained for the binding of estradiol compared to testosterone may be a result of slightly lower losses during the DCC-stripping associated with higher binding affinity for estradiol than for testosterone (Laidley and Thomas, 1994). Despite these higher values, the increase in total binding activity after 96 hours of the exposed cultures of heptocytes was approximately the same.

There has not been conducted many comparable studies on the effects of SBP in cell medium of non mammalian species. A slow increase of SBP binding capacity has been reported in rainbow trout plasma and culture media from hepatocytes after *in vivo* or *in vitro* exposure to 17β-estradiol (E2) (Foucher *et al.,* 1991). The increase in SBP production was seen together with a large production of vitellogenin (vtg) (Foucher *et al*., 1991). *In vitro* vtg induction to E2 occur earlier and to a larger degree than the induction of SBP and this suggest that low susceptibility to SBP in male hepatocytes is not the only reason for the slow SBP response to E2 (Foucher *et al*., 1991). Concentration-related responses of SBP production have been obtained during E2 stimulation from 96 hr to 144 hr in rainbow trout hepatocytes, but the increase in SBP production compared to controls was not always significant (10 and 100 nM were effective for increasing SBP) (Foucher *et al*., 1991). Such delays prior to increasing SBP production under exposure to E2 has also been shown *in vivo* in women and *in vitro* using hepatoma cell lines of human origin (Mercier-Bodard *et al*., 1987; Clair *et al*., 1985). The exposure to androgens was not tested in this particular study but, androgens have been found to increase SBP production in several studies *in vitro* and *in vivo* (Lee *et al*., 1987; Mercier-Bodard *et al*., 1987; Plymate *et al*., 1988).

In human hepatocarcinoma cell lines, the secretion of SBP increased significantly following exposure to both E2 and T (Mercier-Bodard *et al*., 1987). Incubation with EE2 also led to an increased SBP production, although this particular experiment reported no increase in SBP after exposure to testosterone (Li and Humpel, 1990). Our results suggest

that estrogens of both synthetic and natural origin appear to be important potential regulators of total sex steroid-binding capacity, potentially due to SBP from hepatocyte cultures.

Binding of sex steroids in plasma consist of high-affinity binding proteins like the SBP and low-affinity binding proteins like corticosteroids and albumin (Sitteri *et al*., 1982). It is possible that the low affinity binding sites represent a substantial part of the capacity plasma has to bind sex steroids (Westphal, 1986) (97-99 % of sex steroids in the blood are bound to carrying proteins, where of SBP accounts for 40-70 %) (Tollefsen, 2002). The presence of non-specific binding proteins can be seen when measuring the total binding of fractioned plasma samples in several fish species. Measuring total sex steroid-binding of each fraction reveals one large peak, most likely the SBP, and a smaller peak that also contributes to the total sex steroid-binding activity (Hobby *et al*., 2000, Øvrevik *et al*., 2001). These studies indicate a presence of proteins other than SBP or multiple forms of SBP that bind E2 and T.

It has previously been suggested that decreased binding affinity to SBP found in reproductive female fish may be an artefact of the increased plasma levels of vtg (Laidley and Thomas, 1997). Our studies show that vtg binding to estradiol is minor and that the same is true for vtg binding to testosterone. Other studies where vtg have been partially purified from SBP plasma samples also indicate that estradiol does not appear to bind to vtg (Hobby *et al*. 2000).

When testing the binding of albumin to radio labelled E2 and T in our studies, albumin did not appear to bind estradiol nor testosterone. Although the overall contribution of albumin was minor, the source of the albumin was from a mammal and there might be a possibility that albumin-like proteins from fish will bind testosterone and estradiol with a higher affinity. Albumin has not been found in fish, but proteins with some of the characteristics of albumin are present (Davidson *et al.*, 1989). Proteins that share these characteristics with mammal albumin have been identified in the plasma of trout. (Davidson *et al*., 1989; Maillou and Nimmo, 1993) and these albumin-like proteins may

be responsible for low-affinity, high capacity binding of E2 (Westphal, 1986). There is also a strong possibility that other binding proteins present in the medium could bind sex steroids as well, for example the corticosteroid binding globulin (Tollefsen, 2002).

Total sex steroid-binding activity to unexposed DCC-stripped cell medium was also tested. The binding activity increased two times compared to blank samples, which was higher than total sex steroid-binding activity of both albumin and vtg. This increase further suggests the presence of other binding proteins contributing to total sex steroid-binding activity, together with a baseline production of SBP secreted by the unexposed hepatocytes. The over ten time increase seen in total sex steroid-binding activity in cell cultures exposed to E2 compared to unexposed cells is most likely due to an increase in SBP secretion by the hepatocytes.

Regulation of SBP appears to show similar patterns in teleost and mammalian species. As shown in this thesis and elsewhere, exposure to sex steroids clearly increase SBP production. Growth hormone (GH) has been suggested to play an important role in SBP basal production in the liver, by increasing SBP accumulation in rainbow trout hepatocyte cells. Insulin is clearly inhibitory of SBP production in liver cells from both teleost and human origin (Foucher, 1991; Plymate *et al*., 1988). The thyroid hormones T3 and T4 did not influence SBP concentration in rainbow trout hepatic cell culture media (Foucher et al., 1991), while T4 has been shown to stimulate SBP production in hepatoma cell lines (Plymate *et al*., 1998).

5.2 Sex steroid-binding protein expression

To confirm that SBP was the main contribution to total sex steroid-binding activity and not other binding proteins, the protein expression of SBP was examined using zebrafish SBP specific antibodies in a western blot. Antibodies for SBP in fish species are only available for sea bass and zebrafish. Zebrafish antibodies were chosen as they are reported to bind to SBP from several fish species as well as human SBP (Hammond pers comm.). An increase in total-binding activity over time in cell medium from exposed

hepatocyte cultures had already been documented and the aim of this experiment was to determine whether the observed activities can be attributed SBP. Anti-zebrafish SBP was able to bind specific proteins in rainbow trout plasma and cell medium, but due to the low protein concentrations in cell medium the protein bands were very weak. This made it impossible to distinguish the intensity of the protein bands which could have given an indication of the approximate amount of SBP in each cell medium sample. The results conclude that western blot using zebrafish SBP antibodies was not a suitable method for comparing the amount of SBP protein in cell medium samples from rainbow trout hepatocyte cultures. As a consequence of this partial confirmation of SBP modulation by immonuchemical methods, gene expression studies were conducted.

5.3 Gene expression of sex-steroid binding protein

Quantitative realtime polyclonal chain reaction (qPCR) was used to quantify the amount of SBP specific genes in rainbow trout hepatocytes. In the present study, an increase in SBP gene expression was seen in rainbow trout hepatocytes after exposure to the natural estrogen 17β-estradiol and the well known endocrine disrupters EE2 and DBP over time and concentration. An up-regulation of the SBP gene was seen after 24 hours and kept increasing over time in DBP and EE2 exposed samples, while even the lowest concentrations of all test compounds tested induced SBP two times or more compared to endogen control and unexposed samples. Earlier studies with human hepatoma cells have shown that E2 has been able to stimulate SBP gene expression to a similar degree to that observed for rainbow trout hepatocytes in the present study (Mercier-Bodard *et al*., 1987). Exposure to androgens has also shown a very moderate effect on gene expression of SBP in human hepatoma cells (Mercier-Bodard *et al*., 1990; Hoop *et al*., 1990). Other studies have seen no effect of the sex steroids on SBP mRNA thus suggesting that the response to E2 may involve a different mechanism than the transcriptional activation of the estrogen receptor (Hoop *et al*, 1985).

The fact that the SBP probes were tested and did not produce a qPCR product was confusing. The sequencing of the probes reported limited similarity with the rainbow

trout SBP nucleotide sequence. There could be two possible explanations for this. Either the primers are amplifying something other than SBP, assuming that the sequencing was correct and that the Genebank clone was incorrect. Another possibility is that the sequencing of the nucleotide sequence was incorrect. Although not determined in this work, agarose gels document one distinct gene, thus suggesting that the probes utilised in some of the optimalisation steps do not represent SBP.

5.4 Relationship between synthesis and binding capacity

The increase in SBP gene expression occurs to be much faster compared to the increase in total sex steroid-binding capacity. The qPCR data from exposed hepatocytes showed an increase in SBP gene expression after 24 hours, while total binding activity increased after 48 hours exposure, indicating an up-regulation of the SBP gene before the excretion of the protein into the cell medium. More concentrations of exposure solutions were tested in total sex steroid-binding activity assays than what was measured in qPCR. The qPCR data do however show similarity with total sex steroid-binding activity, inducing SBP gene-expression in EE2, E2 and DBP exposed hepatocytes with approximately the same factor as total sex steroid-binding activity over time. Concentration-response curves appear similar for EE2 and DBP in both assays, although total sex steroid-binding activity shows maximum induction at lower concentrations. 17β-estradiol does not display a clear concentration-response curve in qPCR. The reason for this could be that an increase in total sex steroid-binding activity and gene expression is seen at lower concentrations of test compounds than what is clear from the qPCR concentration-response curve. The graph for qPCR data therefore only displays the portion of the concentration-response curve as indicated by a three-fold induction at the lowest concentrations. In both total sex steroid-binding activity and qPCR studies the potent estrogen, EE2 induced the strongest responses followed by endogenous E2 and the weakly estrogenic DBP. In conclusion, the large increase in total sex steroid-binding activity in hepatocyte cultures exposed to these compounds was most likely due to the induction of SBP since the increase in total binding coincides with SBP gene production.

5.5 The role of sex steroid-binding protein in endocrine disruption

Sex steroid-binding protein is able to bind both natural and anthropogenic estrogenic compounds. Interactions with SBP and modulation of SBP properties may represent a novel mechanism for endocrine disruption as studies on the plasma transport of sex steroids have shown. The environmental chemicals are able to interact with SBP and modulate the sex steroid-binding properties (Tollefsen, 2002), by inhibiting the binding of [^{3}H]-E2 to binding sites in plasma whereof a reduction in the affinity for E2 is seen (Martin et al., 1996; Hodgert Jury et al., 2000; Tollefsen, 2002). Although these chemicals normally exist in complex mixtures in the environment, synergistic effects between endogenous and exogenous estrogenic compounds have not been demonstrated. Their structural resemblance make the compounds behave in a similar manner when binding to SBP and therefore act in an additive fashion where all of the natural and synthetic ligands compete with each other for binding to SBP (Tollefsen *et al*. 2002).

In fish the plasma SBP resembles the estrogen receptor (ER) by binding both naturally and synthetic environmental estrogens, although much lower concentrations are often needed to induce SBP than endocrine responses directly associated with ER (Milligan *et al*., 1998; Kloas *et al*., 2000; Tollefsen 2002). The modulation of SBP by xenobiotics may influence the concentration of SBP molecules, or displace endogenous steroids from SBP binding sites. It is however still unclear if the transport of sex steroids by SBP, if the ratio of free (bio-available) to bound steroids are affected and if SBP may represent a novel mechanism for endocrine disruption or if this mechanism interacts with ER-mediated responses. Consequences of the interactions between SBP and EDC's are still unclear, but present work has shown that environmental estrogens are able to increase expression of the SBP gene and at the same time increase total sex steroid-binding capacity An increase most likely due to increased SBP production by the liver cells, highlighting an understanding of how SBP gene expression and secretion in the liver is impacted by the actions of estrogens.

5.6 Variability between hepatocyte isolations

The different isolations of trout hepatocytes showed variability in total binding activity and qPCR data. This variability could be due to the stress shown to affect sex steroid binding to SBP in black bream (Hobby *et al*., 2000) induced under cell isolations or differences in sexual maturity, genetic variation or differences in metabolic status due to age, size, reproductive stage or nutritional status. Capture and handling stress has been. In addition, the amount of SBP could be influenced by the presence of endogen and exogenous hormones and other non-hormonal factors. Variation could also be due to differences in sensitivity towards induction by estrogens (Campbell *et al.*, 1994) in the hepatocytes and/or the ability to synthesize SBP by E2 stimulation.

4.7 Proposal for future activities

It is obvious from the present data that EDCs may influence SBP mediated mechanisms, but the assessment or role in endocrine disruption is limited, and a number of issues need to be addressed.

- The exposure of rainbow trout hepatocytes cultures to the same chemicals at even lower concentrations and with more replicates is needed to properly characterize concentration-response relationships for total binding activity and qPCR assays.
- The problems with sequencing SBP specific probes and primers need to be clarified to confirm if this is of relevance for the results obtained with quantitative PCR.
- Develop enzyme linked immunosorbent assay (ELISA) to quantify the excretion of the sex steroid-binding protein in rainbow trout hepatocyte cultures based on specific antibodies towards rainbow trout or antibodies with broad species specific reactivity
- The effects of SBP production and mechanisms for regulating gene expression and protein excretion are still unclear. The use of mechanistic studies in *in vivo*

experiments and the established methods in this thesis will help reveal the role of SBP in steroid regulation, intracellular signalling and possibly in endocrine disruption

5.8 Conclusions

An increase was seen in SBP specific gene expression and total sex steroid-binding capacity in a rainbow trout hepatocyte culture exposed to the endogenous hormone 17β-estradiol, and the well known endocrine disrupters ethynylestradiol (EE2) and di-(*n*-butyl) phthalate (DBP). This increase was most likely due to the induction of the sex steroid-binding protein (SBP) gene and protein itself, although other non-identified proteins probably contribute to total sex steroid-binding activity as well. The quantification of SBP using anti zebrafish SBP antibodies was not possible due to low protein concentrations present in the cell medium. Overall, the exposure to EE2 induced the strongest response in both total sex steroid-binding activity and qPCR assays, followed by E2. As an increase in total sex steroid-binding activity over time and concentration was comparable with an in increase in SBP gene expression over time and concentration, the increase in sex-steroid binding activity measured in cell medium was most likely due to increased secretion of sex steroid-binding protein by the rainbow trout hepatocytes.

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APPENDIX

A1 Mastermixes used in qPCR and PCR

A1.1 Mastermix used in reverse trancriptase PCR

Reagents /material	µl in each sample
10X RT buffer	2
25X DNTP*	2
10X Random primer	0.8
Multiscribe RT	1
Milli Q H20	4.2
TOTAL	**10**
RNA	10

A1.2 Mastermix used in quantitative PCR

Reagents/Material	1/2X µl	3.25Xµl	Reagents/material	1/2Xµl	3.25Xµl
TAQ Sybr Green Supermix	12.500	40.625	TAQ Sybr Green Supermix	12.500	40.625
Primer reverse 20 pmol/ul (900nm)	1.125	3.65625	Primer reverse 20 pmol/ul (300nm)	0.375	1.21875
Primer forward 20pmol/ul (900nm)	1.125	3.65625	Primer forward 20pmol/ul (300nm)	0.375	1.21875
Milli Q H2O	8.000	26.8125	Milli Q H2O	9.75	31.6875
TOTAL	**23.000**	**74.75**	**TOTAL**	**23.000**	**74.75**
cDNA	2.000	6.5	cDNA	2.0000	6.5
Total	25.000	81.25	Total	25.0000	81.25