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AN INVESTIGATION ON DIFFERENT MECHANISMS OF ENDOCRINE DISRUPTION IN FISH AND MOLLUSCS

Ph.D. Thesis

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"*We have to realise that the various forms of life do not construct a pyramid on top of which exists the human species. All the forms of life, construct perhaps a circle, each ring of which is connected with every other. We have to comprehend that the environment does not exist "out there" but "in here". When we poison the air, the water or the soil, we poison our own selves. Like all the other organisms, we too, form part of a big biological circle, from which nothing can detach us*."

John Sind, New South Wales, Australia
quoted from "Environment and consciousness in ancient Greece" by
Aimilios Bourantinos

"*What doesn't kill you, doesn't necessarily make you stronger*"

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ACRONYMS LIST

11-KT 11-ketotestosterone
AD androstenedione
AEAT acyl-CoA:estradiol acyltransferase
AOX acyl-CoA oxidase
AR androgen receptor
ATAT acyl-CoA:testosterone acyltransferase
BCF bioconcentration factor
CAR constitutive androstane receptor
CAT catalase
CNS central nervous system
CPs chlorinated Paraffins
DBD DNA binding domain
DDT dichlorodiphenyl trichloroethane
DES diethylstilbestrol
DHA dihydroandrostenedione
DHT dihydrotestosterone
DPHA dihydroepiandrosterone
E2 17beta-estradiol
E3 estriol
EcR ecdysone receptor
EDCs endocrine disrupting chemicals
EE2 ethynylestradiol
ER estrogen receptor
FA fatty acid
FXR farnesoid X receptor
GEM gemfibrozil
GnRH gonadotropin releasing hormone
GnRHR gonadotropin releasing hormone receptor
GR glucocorticoid receptor
GSH glutathione
GST glutathione-*S*-transferase
HDL high density lipoprotein
HPG axis hypothalamic-pituitary-gonadal axis
HPI axis hypothalamus–pituitary–interrenal axis
HRE hormone response elements
HSD hydroxysteroid dehydrogenase
HUFA highly unsaturated fatty acid
IMO International Maritime Organisation
Kd sediment-water partition coefficient
Kow octanol-water partition coefficient
LBD ligand binding domain
LDL low density lipoprotein
LXR liver X receptor
MAPK mitogen-activated protein kinase
MUFA monounsaturated fatty acids
NOEC no observed effect concentration
NR nuclear receptor
P450arom Cytochrome P450 aromatase
PBDEs polybrominated dephenylethers
PCBs polychlorinated biphenyls
PCDDs polychlorinated dibenzodioxins
PCDFs polychlorinated dibenzofurans
PPAR peroxisome proliferator-activated receptor
PPs peroxisome proliferators
PR progestin receptor
PUFA polyunsaturated fatty acids
PXR pregnane X receptor
RA retinoic acid
RAR retinoic acid receptor

RXR retinoid X receptor

SFA saturated fatty acids

StAR protein steroidogenic acute regulatory protein

STP sewage treatment plants

TBT tributyltin

ThR thyroid hormone receptor

TPT triphenyltin

UDPGTs uridine diphosphate glucuronosyltransferases

VLDL very low density lipoprotein

VDR vitamin D receptor

Vtg vitellogenin

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***ANEX I* ..... I**

Chapter 1. General Introduction

1. GENERAL INTRODUCTION

1.1 Endocrine disruption- background information and definition

1.1.1 Background information

In the last few decades it has been demonstrated that environmental contaminants may interfere with the endocrine system of humans and wildlife leading to adverse effects particularly in relation to reproduction and development of secondary sexual characteristics. The potential of environmental chemicals to exhibit hormone activity, even though their structure does not resemble those of known hormones, is not a recent discovery. In the 1930s, during the process of synthesising compounds with estrogenic activity based on the phenanthrene nucleus of the steroidal estrogens, it became apparent that other compounds that lack this estrogen nucleus, among them bisphenol A and alkylphenolic compounds, exhibited estrogenic activity as well (Dodds and Lawson, 1936; 1938). In the following years, scientific evidence revealed the estrogenic activities of phytoestrogens like isoflavones (Bradbury and White, 1954; Batterham et al., 1965) and common pesticides such as methoxychlor (Tullner, 1961) and DDT (Burlington and Lindeman, 1950). However, it was not until the early 1990s, after a series of field and laboratory documentations confirming the potential of specific contaminants to interfere with the endocrine system, that endocrine disruption received attention and became a concern of the scientific community and governments (Colborn et al., 1996).

There are numerous studies that have reported adverse health effects in human and wildlife following exposure to compounds that were later characterised as endocrine disruptors. Table 1-1 gives some examples of cases of endocrine disruption observed in human and wildlife across the world. Thus, exposure to xenobiotics with a potential to interfere with the endocrine system may result in diminished fertility and reproduction, altered sex differentiation, changes in behaviour, abnormal growth, altered immune function, neurological impairment, altered hormonal levels and deformed organ histology among other effects.

Table 1-1. Examples of endocrine disruption in humans and wildlife.

Organism	Chemical(s) under suspicion	Route of exposure	Observed effects
Humans[1,2]	PCBs and PCDFs	Consumption of contaminated cooking oil; breast milk of exposed mothers	"Oil-disease": peripheral neuropathy, chloracne and hyperpigmentation; children of the exposed mothers exhibited developmental delays and neurological dysfunction
Humans[3]	DES	Oral administration. Prenatal and transplacental exposure from mothers and grandmothers	Adolescent daughters: benign reproductive track problems, reproductive organ malformations and dysfunction, poor pregnancy outcomes, and immune system disorders. Adolescent sons: structural, functional and cellular abnormalities, hypospadias, micro-phallus, retained testes and increased genital-urinary inflammation; increased susceptibility for tumours.
Alligators[4]	DDT, DDE, DDD, dicofol, sulphuric acid	Environmental; pesticide manufacture spill;	Male reproductive and developmental abnormalities, including small penis size and suppressed plasma testosterone levels
Birds[5,6,7,8,9,10,11]	DDE and PCBs	Environmental	Egg-shell thinning, disturbed breeding and mating performance, nest construction failure

Seals[12]	DDT, DDE, PCBs and PCDDs	Environmental	Females experience damaged uterine wall, metabolic disorders, immunosuppression and hormonal imbalance
Turtles[13]	DDT, DDE, PCBs and PCDDs	Environmental	Alterations of external sexually dimorphic morphology and sex hormones
Fish[14,15,16]	Xenoestrogens (Alkylphenols, EE2)	Sewage treatment work and paper mill effluents in rivers	Skewed sex ratios, vitellogenin production and decreased testosterone levels in male fish
Marine and freshwater gastropods[17,18,19]	Organotins (TBT and TPT)	Environmental; antifouling paints and herbicides	Superimposition of secondary male sexual characteristics in females (imposex), altered hormone levels and disturbed breeding.

[1]Masuda et al. (1978), [2]Yu et al. (1991), [3]Herbst and Bern (1981), [4]Guillette et al. (1994), [5]Peakall et al. (1983), [6]Olsen et al. (1993), [7]Hartley et al. (1995), [8]Green (1998), [9]Newton et al. (1979), [10]Fry (1995), [11]Bosveld and van den Berg (2002), [12]Olsson et al. (1992), [13]de Solla et al. (1998), [14]Andersson et al. (1988), [15]Munkittrick et al. (1991), [16]Jobling and Sumpter (1993), [17]Bryan et al. (1986), [18]Horiguchi et al. (1995), [19]Matthiessen and Gibbs (1998)

For most of the cases mentioned in Table 1-1 the observed effects on the endocrine system became evident after a significant exposure to substances that were later confirmed by laboratory studies to be endocrine disruptors. However, there are several cases where signs of endocrine disruption and other developmental effects are evident demonstrating that populations may be at risk but the link between effect and exposure to contaminants remains unclear. For example, scientific data suggests that during the past 50 years there has been a significant decline in sperm quality (Carlsen et al., 1992; Swan et al., 2000) and plasma testosterone levels in men from industrialised countries (Travison et al., 2007). Likewise, in wildlife, female polar bear offspring show pseudohermaphroditism (Wiig et al., 1998; Carmichael et al., 2005) and panthers in Florida (*Felix concolor coryi*) show defects on the reproductive and immune systems (Facemire et al., 1995). It has been proposed that exogenous chemicals with endocrine disrupting potential may be the causative agents of the observed effects but the link has not been established.

Endocrine disruption is a particularly complicated issue because in order to identify an early “dysfunction” of the endocrine system a thorough understanding of its “normal” function is necessary. However, endocrinology is a continually advancing field and new insights on the function and role of the components that synthesize the endocrine system will continue to be made. Even in rodents -the animal model studied most extensively- there is continuously innovative information on the discovery of new factors with important role in the function of the endocrine system. Thus, with the discovery and cloning of mammalian hormone receptors (hormone-binding proteins), a whole new line of investigation began on what is called today nuclear receptor superfamily to elucidate the molecular basis of this model. To date, several new nuclear receptors have been cloned and for some of them their ligand-activators have not been identified yet and therefore their functional role remains unclear. Some of these receptors are common throughout not just mammals but lower vertebrates and invertebrate species as well. Since there are still gaps on the endocrinology of rodents and mammals, one can imagine the insufficient information on the function of the endocrine system of other less studied animals such as fish and invertebrates. Fish and invertebrates are subject to anthropogenic contamination and potential endocrine disrupting chemicals since the aquatic environment is the receiving end of several manufacture, municipal, industrial and house-holding facilities’ effluents. Therefore, the

lack of knowledge of the endocrinology of animals exposed to endocrine disruptors implies that populations may be at risk and we are unaware of it.

1.1.2 Definition

For the purpose of the present thesis, the following definition on endocrine disruptors has been adapted: “An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub)populations” (WHO, 2002).

The changes in the endocrine function could arise through interference of the substance with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour.

The endocrine system has a fundamental role in the regulation of endogenous metabolic processes in an organism. It coordinates the function between different organs through hormones that are released in the blood stream from specific types of cells within endocrine glands. Neurological, behavioural, nutritional and reproductive processes are regulated by the endocrine system, as are growth, organ functions and response to all forms of stress. These endocrine control mechanisms have been widely conserved among animal phyla. Disorders in any of these mechanisms with consequent alterations in hormone secretion will result in dysfunction, affecting different organs, and are often debilitating or life-threatening. Therefore, the threat posed from environmental chemicals with endocrine activity is potentially serious (WHO, 2002).

1.2 General characteristics of endocrine disrupting chemicals (EDCs)

1.2.1 Endocrine disruptors and effects

To date, there is a wide range of compounds, naturally occurring or xenobiotics, that have been recognised as EDCs through laboratory and field studies. Some of these are: (a) pesticides such as DDT, methoxyclor, dicofol, lindane, TBT and TPT; (b) industrial products and by-products such as PCDDs (dioxins), PCBs, polybrominated

diphenyl ethers (PBDEs), halogenated phenols and benzenes, polychlorinated naphthalenes, alkylphenols, phthalates and bisphenol A; and (c) pharmaceutical compounds such as ethynylestradiol (EE2), diethylstilbestrol (DES) and tamoxifen. Table 1-2 lists some of those chemicals together with a summary of their known effects on the endocrine system of animals.

Reproductive effects include any effects on the endocrine system that may lead to reproductive impairment, such as low fertilisation success and reduced breeding. Developmental effects incorporate all types of development alterations, such as deformed organ and secondary sexual characteristics growth. Behavioural effects include changes in mating behaviour as well as altered activity performance, such as eating and sleeping. Effects on steroidogenesis are all kind of alterations that are caused during the synthesis of steroid hormones, in any of the steroidogenic tissues, leading to altered hormonal levels. Estrogenic and androgenic effects refer to the effects that are naturally regulated by endogenous estrogens and androgens, respectively. Compounds that cause such effects are either estrogen/androgen agonists, i.e. they bind to the equivalent receptors and activate them in place of their natural hormone ligands or they induce the synthesis of one of these hormones. The term anti-estrogenic and anti-androgenic refers to compounds that antagonise with the natural estrogens and androgens respectively; i.e. they bind to the receptor without activating it and block the action of the endogenous hormones. Finally, thyroid dysfunction includes any effects related to thyroid function, from thyroid hormone synthesis and metabolism, to bone and tissue growth.

1.2.2 Common characteristics

EDCs are a wide range of compounds, which have little in common structurally or in terms of their chemical properties. Additionally, their mechanism of action among different species and their potential targets within the endocrine system (steroid hormones, thyroid, peptide hormones etc) are highly diverse. Nevertheless, EDCs exhibit some common characteristics that are summarised in Table 1-3.

Table 1-2. Compounds with endocrine disrupting effects.

Effects / Compounds	Reproductive	Developmental	Behavioural	Interference with Steroidogenesis	Estrogenic	Anti-estrogenic	Androgenic	Anti-androgenic	Thyroid dysfunction
Pesticides									
o,p'- DDT[1,2,3,5,6]	■	■	■	■	■				
o,p'- DDE[5,7]				■	■				
p,p'- DDE[4,7,29]	■	■	■	■				■	
Methoxyclor[1,3,13]			■			■			■
Dicofol[9,10]				■	■				
Atrazine[11,12]	■	■		■					
Lindane[14,15,16]	■	■		■	■				■
Dieldrin[8,17]	■			■	■			■	
Vinclozolin[4,8,29]	■	■	■					■	
Endosulfan[8,13]				■	■			■	■
Fenarimol[9,10]				■	■				
TBT and TPT[18,19,40]	■	■	■	■			■		
Pentachlorophenol[13,14]	■	■							■
Halogenated industrial Chemicals									
PCDD and PCDF[13,24,25,26]	■	■	■	■		■			■
PCBs[13,20,21,22,25]	■	■	■	■		■			■
Polybrominated diphenylethers (PBDEs)[13,27]	■	■		■					■
Chlorinated paraffins (CPs)[36]		■	■						■
Other Industrial Chemicals									
Alkylphenols[6,13,23,30,31,34]	■	■	■	■	■			■	■
Phthalates[13,33,34]	■	■		■		■		■	■
BisphenolA[13,23,35]	■	■		■	■			■	■
Pharmaceuticals									
Ethynylestradiol[28,29]	■	■	■	■	■				
Diethylstilbestrol[3,32]	■	■	■		■				
Tamoxifen[37]	■	■		■		■			
Fibrates[38,39]		■		■					

[1]Welch et al. (1969); [2]Robison and Stancel (1982); [3]Palanza et al. (1999); [4]Kelce et al. (1997); [5]Forster et al. (1975); [6]Mills et al. (2001); [7]Wojtowicz et al. (2007); [8]Andersen et al. (2002); [9]Vinggaard et al. (1999); [10]Vinggaard et al. (2000); [11]Cooper et al. (2000); [12]Spano et al. (2004); [13]Boas et al. (2006); [14]Rawlings et al. (1998); [15]Singh and Canario (2004); [15]Pages et al. (2002); [17]Jorgenson (2001); [18]Iguchi et al. (2007); [19]Matthiessen and Gibbs (1998); [20]Kuriyama and Chahoud (2004); [21]Halbrook and Arenal (2003); [22]Sanders et al. (1977); [23]Bonefeld-Jorgensen et al. (2007); [24]Wolf et al. (1999); [25]Krishnan and Safe (1993); [26]Heiden et al. (2006); [27]Talsness et al. (2008); [28]Scholz and Gutzeit (2000); [29]Bayley et al. (2002); [30]Bayley et al. (1999); [31]Hossaini et al. (2003); [32]Marselos and Tomatis (1993); [33]Mylchreest et al. (1999); [34]Mlynarcikova et al. (2007); [35]Hirose et al. (2000); [36]Cooley et al. (2001); [37]Gao et al. (2002); [38]Sausen et al. (1995); [39]Xu et al. (2002); [40]McAllister and Kime (2003).

Table 1-3. Common characteristics across EDCs

Common characteristics across EDCs
▪ Animals are more sensitive to EDCs exposure during early development when the endocrine system is still under formation. Exposure during such periods (embryogenesis, lactation, puberty) may result to permanent alterations and in some cases these alterations become obvious later in adult life.
▪ There are particular periods when adult organisms are sensitive to these chemicals (e.g. reproduction period, spawning season). Outside these windows of sensitivity, EDCs may have no effect.
▪ Exposure of adult organisms to EDCs may result to different effects or no effects compared to the juveniles, due to endocrine system homeostasis that may compensate the changes caused by the exposure.
▪ The endocrine system is pleiotropic, thus more than one alteration may be caused from exposure and in some cases the effect may be seen in a different part of the endocrine system from the one expected.
▪ Since the endocrine system is different between males and females, endocrine disruption effects may be gender specific.
▪ Due to inter-species variation of the endocrine system, EDCs give rise to different endocrine disruption effects across phyla.

All animals, from humans to invertebrates, are particularly sensitive to EDC exposure during their very early developmental period when gonadal sexual differentiation is still in process. The gonadal sex will determine the phenotypic sex, which involves the genital tract, secondary sex characteristics and neural structures mediating behaviour such as sexual interest and appetite (Hall and Wachtel, 1980). Differentiation towards male or female gonadal and body phenotype requires the inductive actions of gonadal androgens or estrogens, respectively. Therefore, exogenous administration of sex steroids or exposure to EDCs with hormonal action during this critical period may induce permanent gonadal and/or phenotypic sex reversal.

Adults may be sensitive to EDC exposure during specific periods (e.g. reproduction) and the effects may be observed in both adults and its progeny. However, outside these windows of sensitivity there may be no effects as hormone homeostasis may compensate the alterations. In general, the endocrine system is a homeostatic system that works to maintain a stable internal environment. An alteration in the concentration of an endogenous metabolite may cause endocrine cells to

respond by releasing a substance such as a hormone which will then act on other cells to increase the output of the metabolite (positive feedback) or release it from the body's reservoirs or promote its loss from the body (negative feedback) (Hadley, 1992). Although homeostasis may compensate an alteration in the endocrine system, it may also cause a different effect on another part of the endocrine system.

The various parts of the endocrine system work simultaneously and their functions often overlap. For example, the circulating levels of thyroid hormones during pubertal period can affect terminal differentiation of various tissues (e.g. neurons, muscle cells) including conversion of immature sertoli cells (cells in the testis that support spermatogenesis) to mature cells. Thus, abnormal supply of thyroid hormones may trigger alterations in the number of sertoli cells and consequently alter testis size and number of sperm produced leading to reproductive impairment (Hadley, 1992). Therefore alterations in thyroid hormones by EDCs would lead to effects in reproduction as well.

Finally, the effects of EDCs may vary between species and between males and females. Inter-species differences on the endocrine system may give rise to a different effect, or no effect depending on the species. Furthermore, fundamental differences in the structure of the endocrine system between males and females may result in a higher susceptibility of one of the sexes to a specific EDC.

Thus, EDCs have some common characteristics in terms of their effects demonstrating that their mechanism of action is particularly ambiguous compared to other toxic chemicals.

1.3 Laboratory experiments and identifying dose-response relationships in endocrine disruption research

Valuable information that significantly contributes to the awareness of the harmful effects of EDCs derives from laboratory studies using experimental animals. Good practice laboratory testing is important for field extrapolation since it can prove or disprove a causative relationship between levels of an environmental pollutant and a biological effect. There is extensive scientific literature on the effects of suspicious endocrine disrupting chemicals on animals, both vertebrates and invertebrates, under in-vivo and in-vitro studies, confirming the biological mode of action of these

compounds. For example, laboratory studies have demonstrated that *p,p'*-DDE, an active metabolite of *p,p'*-DDT promotes avian egg-shell thinning through inhibition of the Ca^{2+} ATPase activity, which is involved in the transport of calcium across the eggshell gland mucosa (Lundholm, 1987; Blus et al., 1997). Additionally, in-vivo and in-vitro experiments using the *o,p'*-DDT and *o,p'*-DDE isomers reveal their estrogenic action in rats (Forster et al., 1975; Robison and Stancel, 1982) and fish (Donohoe and Curtis, 1996; Mills et al., 2001) due to competitive binding to the nuclear estrogen receptor, whereas the *p,p'*-DDE metabolite shows anti-androgenic activity in rats (Kelce et al., 1997; Ashby et al., 2002) and fish (Mills et al., 2001; Bayley et al., 2002) by binding to the androgen receptor and inhibiting its activation. The estrogenic potency of DDT isomers and metabolites could explain partly the estrogenic effects observed in the alligators of Lake Apopka in Florida.

Traditional toxicological studies, which still form the base of environmental risk assessment, have as a primary objective to establish a dose-response relationship, which means to quantify the amount of a chemical, to which an organism is exposed to, that gives rise to a consequent harmful, "toxic" effect. Usually, environmental toxicological experiments focus on exposure of adult individuals to high pharmacological doses of environmental contaminants that result in mutagenesis, cancer or death as some unequivocal indications of contaminant effect. However, when dealing with EDCs it becomes difficult to establish such dose-response relationships. This is mainly because EDCs often mimic or antagonise the actions of endogenous hormones that are already present at physiological functional concentrations and thus already create a response. Additionally, as it has been mentioned, the "response" may be delayed following exposure to low concentrations of EDCs during critical periods of the endocrine system development or may be compensated by adult organisms and not lead to toxicologically significant alterations. Thus when dealing with EDCs it becomes complicated to quantify the "dose" that produces a hazardous "response" compared to other compounds that do not act directly on the endocrine system.

A successful monitoring of endocrine disrupting chemicals incorporates the use of specific biological responses as end-points that demonstrate departure from the normal health status (Walker et al., 2001). These responses may include changes at biochemical, physiological, histological, morphological and behavioural level. Biochemical responses at individual (organ or organelle) level following exposure to potential EDCs during sensitive periods of an organism's life-cycle give specific

information on the origin of the response and may be used as an “early warning” that the organism is experiencing endocrine disruption. Thus, with the use of such specific biochemical or molecular responses it becomes possible to demonstrate a dose-response relationship and evaluate the posing risk of environmental concentrations of EDCs upon exposed organisms.

When dealing with EDCs in order to identify the response and relate it to a dysfunction of the endocrine system that may lead to toxicity and disease, it is necessary to first have a thorough understanding of the fundamentals of the endocrine system, and afterwards, to identify the mechanism of action of these compounds. Next section reports the basics of the endocrine system, endogenous hormonal synthesis and biotransformation of endogenous compounds and xenobiotics.

1.4 Endocrinology overview

Endocrinology is a subdiscipline of physiology and is specifically concerned with the chemical messengers “hormones”, which are synthesised and secreted by cells of endocrine glands and tissues. There are various glands in the body excreting hormones (hypothalamus, pituitary gland, thyroid, ovary, placenta, testes, thymus gland, pancreas, kidney, heart) and new glands are still being found distributed in other tissues such as the gastrointestinal tract and liver (Downes and Liddle, 2008), the central nervous system (CNS), peripheral nerves (Michalik and Wahli, 1999; Nikolenko and Krasnov, 2007) and adipose tissue (Prins, 2002). In general, the endocrine system functions through a series of cascade mechanisms, triggered by an external or internal stimuli, that involve various hormone secretion intermediate steps from different cells, to finally give rise to a coordinated physiological response (Hadley, 1992). These coordinated physiological processes are driven by positive and negative feedback mechanisms to maintain homeostasis and avoid extreme alterations in hormone levels and responses that might have otherwise detrimental metabolic effects.

1.4.1 Hormonal action

A simplified example of such cascade of mechanisms of hormonal action is during the male sexual maturation across the hypothalamic-pituitary-gonadal (HPG) axis (Figure

1-1). After genetic sex determination, genes located on the Y chromosome send a signal for sexual maturation through neurotransmitters to the hypothalamus in the brain. The hypothalamus in response secretes a peptide hormone, the gonadotropin-releasing hormone (GnRH) from the neurons (Halász, 1985). GnRH is then transported by the hypothalamic hypophyseal blood vessels to the anterior pituitary where it binds to the GnRH receptor (GnRHR) and stimulates the synthesis and secretion of gonadotropins, which in turn travel to the testis, where they stimulate the secretion of the steroid hormone testosterone from the testis Leydig cells (Dörner, 1978). The testosterone hormone is responsible for differentiation and development of the male urogenital system characteristics and sexual maturation occurs. Testosterone will then circulate back to the pituitary and hypothalamus to reduce the production of gonadotropins and GnRH (negative feedback). It is also responsible for sexual differentiation of the brain (Hadley, 1992). Afterwards, the testes become quiescent until puberty when they are activated by pituitary gonadotropins.

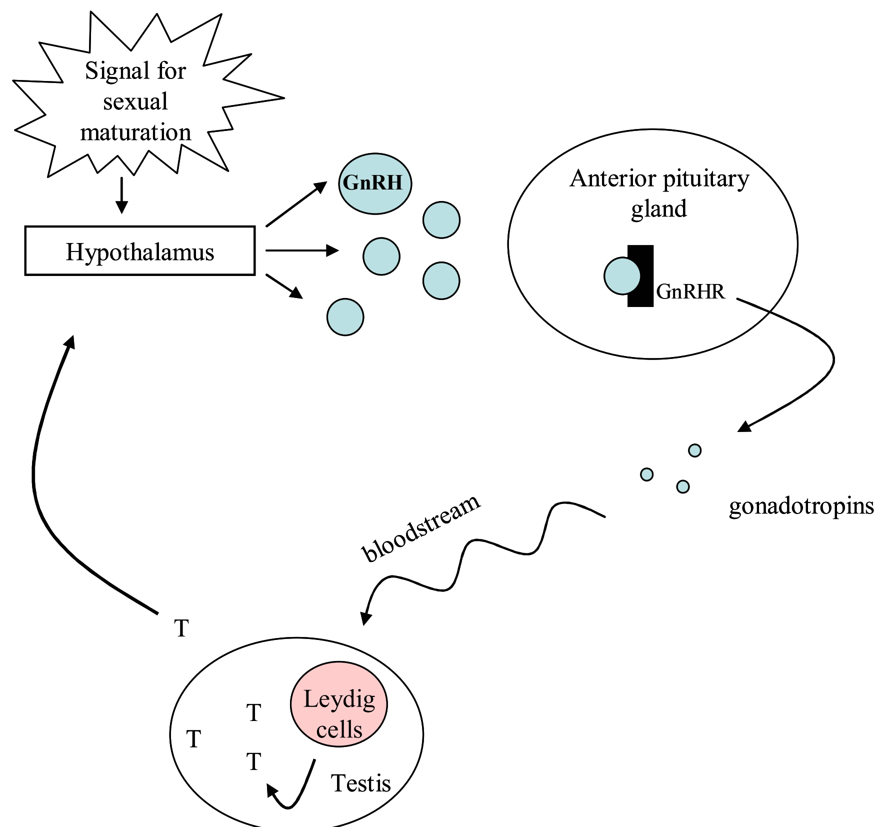


Figure 1-1. Hormonal action during the male sexual maturation across the hypothalamic-pituitary-gonadal (HPG) axis.

The genomic action of hormones is mediated by their binding to specific hormone receptors expressed in the target tissues. Such interactions lead to an increase in the production of intracellular second messengers, which are more directly responsible for the activation of the cell and the regulation of physiological processes (Nikolenko and Krasnov, 2007). In the following sections the nuclear receptor superfamily and the action of hormones through genomic and non-genomic mechanisms is discussed separately in order to underline the fundamental function of the endocrine system.

1.4.2 Nuclear Receptors

Research on the mode of action of hormones, mainly steroid hormones, brought evidence on their interaction with the nuclear receptors (NRs). These receptors are intracellular proteins that act as transcription factors and regulate the expression of target genes in target tissues to affect physiological processes (Mangelsdorf et al., 1995). In 1980s, glucucorticoid (Hollenberg et al., 1985) and estrogen receptors (Green et al., 1986) were cloned, followed shortly by the cloning of thyroid hormone (Sap et al., 1986) and retinoic acid receptors (Petkovich et al., 1987). They were all found to exhibit great sequence similarity despite their chemically distinct ligand activators. In the next years receptors for all classes of hormones were identified as well as the orphan receptors, whose ligand, target genes and physiological functions were initially unknown. Therefore, it became apparent that more NR-like genes exist than what it was previously suspected and that their action is not necessarily triggered upon hormone ligand binding but other lipophilic molecules able to pass across the plasma membrane could be ligand activators as well. Today these receptors are known as the nuclear receptor superfamily.

The overall structure of NRs is given in Figure 1-2. The characteristic structural feature of them is that they possess two conserved domains: a DNA-binding domain (DBD) and the ligand-binding domain (LBD) (Germain et al., 2003). With few exceptions these proteins have an NH_2 terminal region that contains a ligand-independent transcriptional activation function (AF-1). Following that region there is the central DBD which targets the receptor to specific DNA sequences typically known as hormone response elements (HRE). It is composed of two highly conserved zinc fingers that set the NRs away from other DNA sequences. Afterwards, there is a hinge

region that permits protein flexibility to allow for simultaneous receptor dimerisation and DNA binding. Toward the COOH-terminal region there are the LBD and a dimerisation region which allows molecules of different receptors to form dimers, thereby substantially expanding the range of the potential DNA targets and regulatory functions. At the end of this region lies a transcriptional activation function that is ligand specific (AF-2) (Mangelsdorf et al., 1995; Kimbrel and McDonnell, 2003; Chambon, 2005; Nikolenko and Krasnov, 2007).

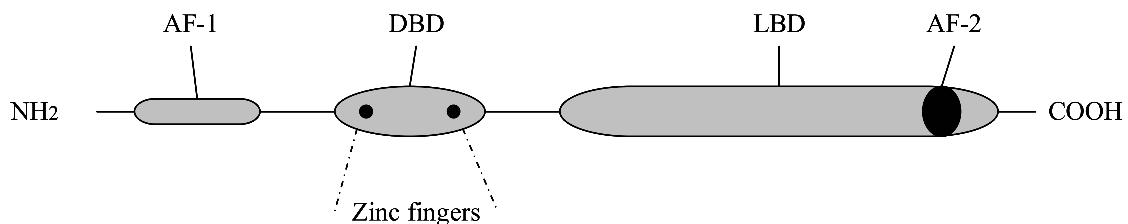


Figure 1-2. General structure of steroid hormone nuclear receptors.

NRs are located in the cytoplasm of the cell or in the nucleus. When a lipophilic ligand (steroids, thyroid hormones, retinoic acid, vitamin D, fatty acids) passes through the plasma or nuclear membrane it binds to the NR (Figure 1-3). After binding to the receptor's LBD, the hormone-receptor complex undergoes conformational change, followed by a separation of the receptor from cytoplasmic chaperone proteins (such as heat shock protein 90-Hsp90), which maintain it inactive, and by exposure of nuclear sequences of the receptor's DBD (Kimbrel and McDonnell, 2003; Nikolenko and Krasnov, 2007). This allows nuclear translocation and binding to hormone responsive elements (HRE) in the promoter region of a target gene by the DBD to regulate the transcription of that gene (Lefstin and Yamamoto, 1998) and activate or repress mRNA and protein synthesis through co-regulators bound to the LBD (Moras and Gronemeyer, 1998).

NRs have a functional flexibility and could act as activators or repressors due to the recruitment of a diverse group of supplementary factors, the co-regulators (McKenna and O'Malley, 2002). These co-regulators give flexibility to the LBD of NRs and allow it to accommodate ligands with different structures promoting specific conformational changes which will determine whether the ligand-receptor complex will act as agonists or antagonists (Kimbrel and McDonnell, 2003). Thus, in an "agonist conformation", co-activators bind to AF-2 and activate transcription. On the

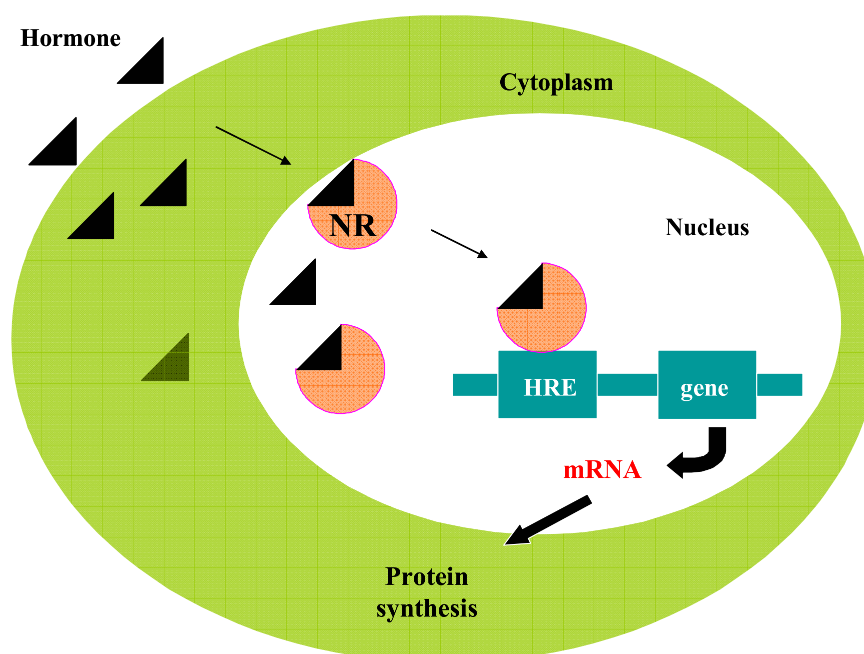


Figure 1-3. A simplified diagram of hormone action through nuclear receptors in mammalian cells.

contrary, when a ligand causes a conformational change where the co-activator pocket is hidden, transcriptional co-activation is blocked.

NRs can be classified according to their function and ligand availability. The first class of receptors comprises the classic nuclear hormone receptors such as progestin (PR), estrogen (ER), androgen (AR), glucocorticoid (GR), and mineralocorticoid receptors. This group of receptors are inactive in the absence of the ligand. The hormone-ligands are synthesised endogenously from endocrine sources that are regulated by the negative feedback control of the hypothalamic-pituitary-axis (Halász, 1985). They bind to the equivalent receptor with high affinity and specificity and thereafter the ligand-receptor complex binds to the HRE as a homodimer (Figure 1-3) (Nikolenko and Krasnov, 2007).

The second group of receptors includes nuclear receptors known as “adopted” orphan nuclear receptors that function as heterodimers with the retinoid X receptor (RXR). The term “adopted” indicates that ligands for the “orphan receptors” have been identified. The ligands of this group of receptors are dietary lipids and therefore their concentrations are not regulated by simple negative feedback mechanisms. The ligand molecules bind to the equivalent receptors with high specificity but low affinity. Thus, alterations in the physiological concentrations of the ligands occur by dietary intake. Members of this group include receptors for fatty acids and eicosanoids (PPARs)

(Michalik and Wahli, 1999), for oxysterols (LXRs) and bile acids (FXR) (Downes and Liddle, 2008), as well as for xenobiotics (PXR/SXR, CAR) (Timsit and Negishi, 2007). These receptors function as lipid sensors; ligand binding to these receptors activates a feed-forward metabolic cascade through the transcription of genes involved in lipid metabolism, storage, transport and elimination to maintain lipid homeostasis (Chawla et al., 2001).

Apart from the adopted orphan receptors there are some other receptors that form heterodimers with RXR but do not fall into the two categories mentioned above. Thus, the third class of receptors includes the thyroid hormone (ThR), retinoic acid (RAR), vitamin D (VDR) and ecdysone (EcR) receptors. The ligands of these receptors derive from dietary sources and external sources but their function is regulated by the endocrine system. For example, vitamin D and thyroid hormones require exogenous elements for their synthesis (sunlight and iodine respectively) but their action such as calcium absorption and bone growth is under strict endocrine control (Hadley, 1992).

Nuclear transcription can also occur through activation of membrane-bound receptors (Nordeen et al., 1994). Peptides, catecholamines or growth factors may bound to membrane receptors and affect cellular function by modulation of intracellular second messengers. Such changes on second messengers modulate steroid-induced transcription by an intracellular cross-talk. Furthermore, intracellular cross-talk may occur in the absence of a steroid ligand. Scientific evidence confirms that epidermal growth factor activates ERα by a signalling through the MAPK pathway (Bunone et al., 1996).

1.4.3 Non-genomic action of steroid hormones

It is now accepted that the action of steroid hormones is not exclusively through nuclear receptors and therefore does not always involve DNA binding and RNA synthesis (Falkenstein et al., 2000). These non-transcriptional effects can be indicated as: (i) actions that are too rapid to be compatible with RNA and protein synthesis (response to hormone action takes place within second to minutes); (ii) actions that occur in the presence of inhibitors of RNA or protein synthesis; (iii) actions that can be reproduced by using steroid hormones coupled to cell membrane-impermeable molecules; (iv) actions that steroid hormones induce in cells with highly compacted chromatin, in which RNA and protein synthesis are absent (such as spermatozoa); and

(v) actions that are elicited by steroid hormones via binding to receptors containing mutations which make them incapable of activating transcriptional processes (Simoncini and Genazzani, 2003). These rapid non-genomic actions are likely to be transmitted via specific membrane receptors.

1.4.4 Aquatic vertebrate and invertebrate endocrinology

The use of hormone cascades to regulate physiological processes is a strategy that has been conserved during evolution. These cascades provide a communication link between the nervous system and the endocrine system in all animal phyla. Thus, the role of the hormones to stimulate or inhibit the activity of target cells directly or indirectly through modulation of the actions of other chemical messengers is a common strategy across animals.

The NR superfamily, like other gene families, is the product of evolution and diversification of the genome within and among species. NRs have been identified across all animal phyla belonging to metazoans showing various degrees of conservation in their domains (Escriva et al., 1997). This clearly demonstrates that the receptors evolved prior the divergence of vertebrates and invertebrates. Phylogenetic trees model studies show that orphan receptors are ancient since they exist in anthropods and vertebrates (Laudet, 1997). For many of the orphan receptors it is not clear if all these receptors indeed have a ligand to be identified or if they are ligand-independent and therefore act in a constitutive manner. Thus, ancestral receptors could have been ligand independent, whose ligand-binding specificity evolved to produce the modern receptors (Escriva et al., 1997; Laudet, 1997).

Gene sequences diverge independently after duplication and therefore some NRs evolved after the separation of protostomes (anthropods, molluscs, nematodes, etc.) and deuterostomes (chordates and echinoderms). Thus, although some receptor homologues have been found across protostomes and deuterostomes (e.g. RXR), others have appeared later in evolution and are found only in vertebrates (e.g. RAR, PPARs, ThR) (Thornton et al., 2003).

Steroid receptors have also been identified in some invertebrate phyla although their role in reproduction remains unclear (Köhler et al., 2007). For example, estrogen receptor-like proteins have been identified in various invertebrate molluscs such as *Aplysia californica* (Thornton et al., 2003), *Mytilus edulis* (Stefano et al., 2003) and

Ilyanassa obsoleta (Sternberg et al., 2008) but since they do not bind to estrogens, their function is unknown. However, scientific data show that ER in molluscs is highly expressed during reproduction indicating that it may have a role in reproduction that is ligand independent (Castro et al., 2007b; Sternberg et al., 2008).

Teleost fish, being further "up" in the phylogenetic tree of evolution, appear to posses all the nuclear receptor types found in mammals. Furthermore, due to an apparent whole-genome duplication that caused the expansion of a large number of gene families in the teleost lineage, followed by the retention of some duplicates and the loss of others, teleost fish have more NRs than most mammals do (Thornton, 2003). For example, several teleost species have been found to have two androgen receptors (Sperry and Thomas, 1999), whereas mammals have only one. Similarly, cloning of PPARs from teleost fish has revealed a higher diversity of these receptors across different fish species, than what is seen in mammals. Therefore, even though mammals have three PPARs, namely PPARα, PPARβ and PPARγ, some fish species have more than one of each of PPARs. Thus, torafugu pufferfish has two types of PPARα (Kondo et al., 2007), salmon has four types of PPARβ (Leaver et al., 2007) and zebrafish has two types of PPARα and two of PPARβ (Robinson-Rechavi et al., 2001).

1.5 Steroidogenesis

Steroidogenesis is the process through which specialised cells in specific tissues synthesise steroid hormones such as glucocorticoids, mineralocorticoids, progestins and sex hormones (Sanderson and Van den Berg, 2003). The major sites of steroidogenesis are the gonads (ovaries and testes), adrenal gland and placenta, although other organs such as the brain, liver and adipose tissue can play a significant role (Ramsby, 1997). Steroidogenic cells do not store significant quantities of steroid hormones (Miller, 2002) and therefore, when steroids are needed they are principally synthesised *de novo*. Figure 1-4 shows the structures of some of the steroids that belong to these steroid hormone groups. It is common to refer to each steroid group in terms of the number of carbons they contain. Hence, the precursor of all steroids, cholesterol, is a C27 molecule, aldosterone and cortisol are C21 steroids, whereas

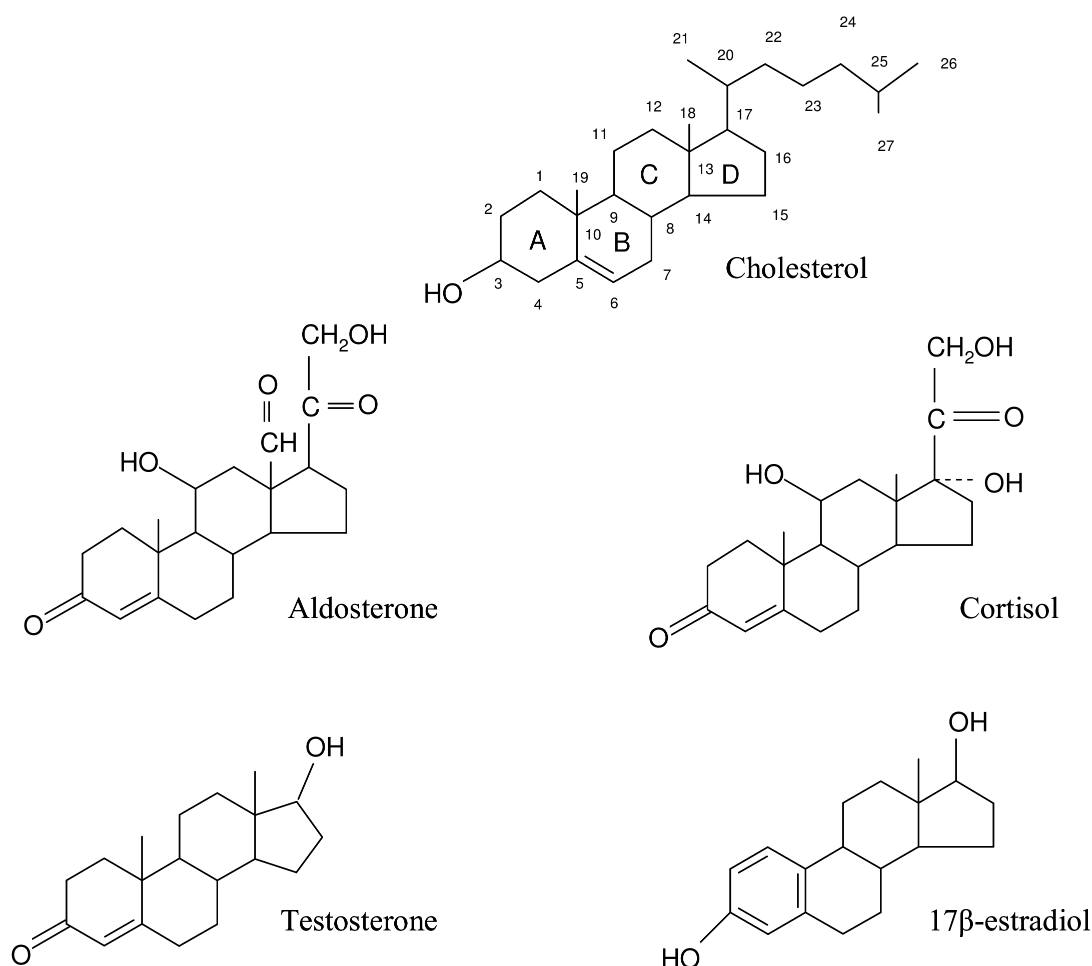


Figure 1-4. Examples of steroids and their precursor cholesterol. Aldosterone (mineralocorticoid), cortisol (glucocorticoid), testosterone (androgen) and estradiol (estrogen).

androgens are C19 and estrogens C18 steroids. Each group of hormones has a different function. Adrenal glucorticoids regulate the carbohydrate metabolism and manage stress; adrenal mineralocorticoids regulate salt balance and maintain blood pressure; ovarian and placental progestogens and estrogens regulate reproductive function and secondary characteristics in females, and testicular androgens regulate fertility and secondary sex characteristics in males (Stocco, 2001).

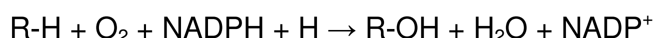
1.5.1 The enzymes that participate in steroidogenesis

Steroidogenesis involves the action of a battery of enzymes predominately in mitochondria and endoplasmic reticulum (microsomes). These enzymes fall into three

major classes of proteins: the Cytochrome P450 heme-containing mixed function oxidases (MFO), the hydroxysteroid dehydrogenases (HSDs) and the reductases.

In the present work, the nomenclature used for the Cytochrome P450 enzymes and their genes follows predominately the one proposed by Nelson et al. (1993), in which the root CYP is used to denote human Cytochrome P450 followed by Arabic numerals denoting the family (e.g. CYP19 for 19-hydroxylase), followed by a letter designating the isoforms (e.g. CYP19A and CYP19B for the two isoforms of the protein).

The P450 enzymes involved in steroidogenesis are membrane-bound proteins associated with either mitochondrial membranes (CYP11A, CYP11B) or the endoplasmic reticulum (CYP17, CYP19 and CYP21). In general, the name of Cytochrome P450 enzymes derives from the characteristic that when combined in-vitro with exogenous CO they absorb maximum light at 450 nm (Hall, 1986). In the biosynthesis of steroid hormones, these enzymes catalyse the hydroxylation and cleavage of the steroid substrate, in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor for the reduction of molecular oxygen (Ghayee and Auchus, 2007). The overall reaction is the following:



$$R\text{-}H + O_2 + NADPH + H \rightarrow R\text{-}OH + H_2O + NADP^+$$

In this reaction Cytochrome P450 activates molecular oxygen and one oxygen atom is being introduced to the substrate R-H to form a hydroxyl group and the other is reduced to H_2O. The system through which the electrons are transferred from the NADPH to the substrate depends on the location of the Cytochrome P450s. In the case of mitochondrial Cytochrome, the transfer of electrons requires two electron carriers, namely a flavoprotein and an iron-sulfur protein. Microsomal cytochromes require a single flavoprotein (Hall, 1986; Payne and Hales, 2004).

The hydroxysteroid dehydrogenases belong to the protein family named short-chain alcohol dehydrogenase reductases. They are involved in the oxidation and reduction of steroid hormones requiring NAD^+/$NADP^+$ as acceptors and their reduced forms as donors of reducing equivalents (Miller, 1988). One of the major differences between the Cytochrome P450 enzymes and the hydroxysteroid dehydrogenases is that each of the P450 enzymes is a product of a single gene, whereas there are

several isoforms of HSDs and each one of them derives from a different gene (Payne and Hales, 2004).

1.5.2 Description of the steroidogenic pathway

Although steroid hormones have diverse functions, they are all synthesised by biosynthetic pathways that are identical in the initial stages, with cholesterol being the first precursor (Miller, 2007). Thus, in all steroidogenic tissues the initial step in steroidogenesis is the conversion of cholesterol to the first C21 steroid pregnenolone, through the catalytic action of the Cytochrome P450 side-chain cleavage enzyme (P450*scc* or CYP11A) located in the inner mitochondrial membrane of the steroidogenic cells (Payne and Hales, 2004). (Figure 1-5).

In general, cholesterol is transferred to the target tissues by low and high density lipoproteins, from which it gets liberated by the action of lipases. It is also esterified with fatty acids and stored in lipid droplets as fatty acid esters from where it can get liberated by the action of esterases (Chang et al., 2006). Once in free

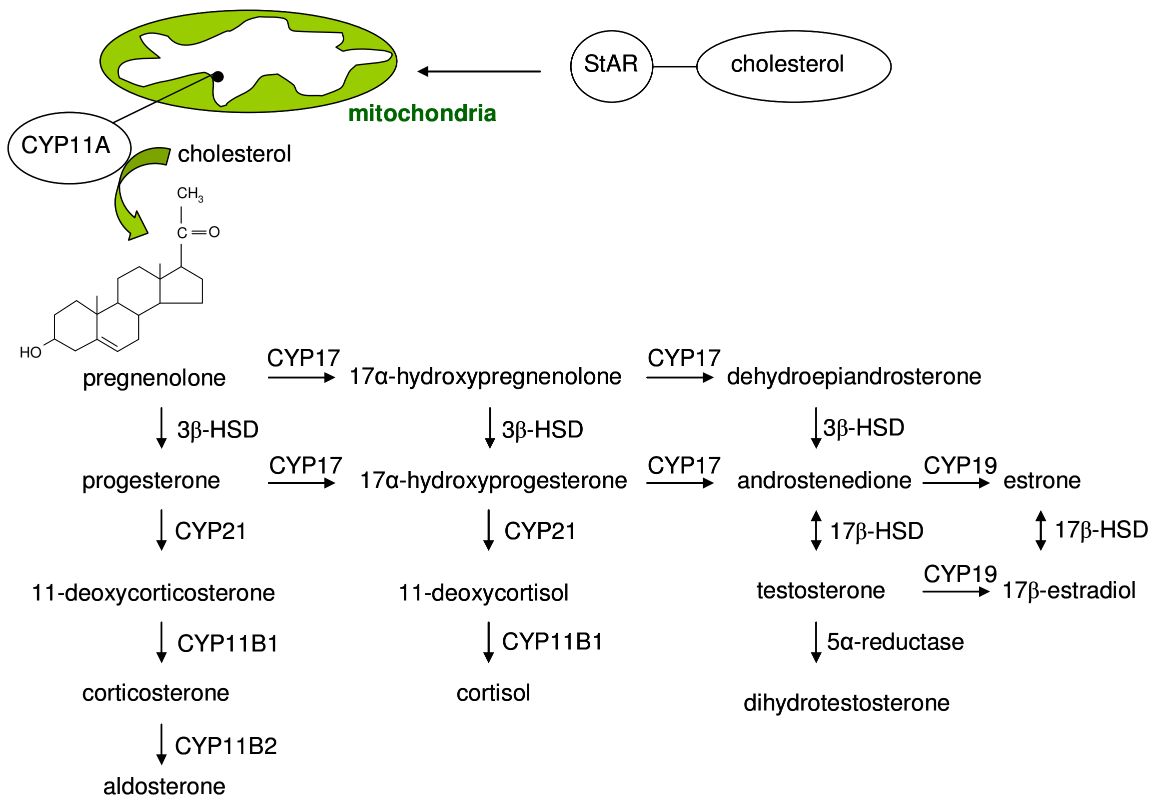


Figure 1-5. General scheme of steroidogenesis.

form, for steroidogenesis to initiate, lipophilic cholesterol must get transported first to the outer mitochondria and then across the mitochondrial cell membrane to reach the inner membrane where CYP11A is located. This is achieved by binding to proteins that decrease its lipophilicity and thus facilitate its transport across the aqueous interior of the membranes. The protein that transfers cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane is the steroidogenic acute regulatory (StAR) protein (Clark and Stocco, 1996; Stocco, 2001; Miller, 2007; Roostaee et al., 2008). The proteins that carry cholesterol to the outer mitochondrial membrane from elsewhere in the cell (e.g. lipid droplets) share similar structure with the StAR protein but their identification is still under research (Miller, 2007).

The StAR protein has received its name following the observation that it is synthesised upon stimulation for acute steroid production (Clark and Stocco, 1996). Overall, the steroid hormone biosynthesis is regulated mainly by the pituitary trophic hormones and can be acute or chronic (Ramsby, 1997). The acute regulation occurs within seconds to minutes and involves the binding of cholesterol to StAR protein for its transfer to the inner mitochondrial membrane where CYP11A is located to initiate rapid production of steroids. The chronic regulation of steroidogenesis occurs within hours to weeks and involves the induction of the transcription of the genes that encode the steroidogenic enzymes and thus their synthesis resulting in an optimal long-term steroid production (Black et al., 1993; Clark et al., 1995). In terms of the acute regulation of steroidogenesis, the production of steroids is determined by the availability of cholesterol in the inner mitochondrial membrane mediated by the StAR protein. In a study using mouse Leydig tumour cells it was established that StAR expression and steroidogenesis increased in response to trophic hormone stimulation indicating the key role of StAR in acute induction of steroidogenesis (Clark et al., 1995). Thus, it is evident that the rate limiting step in steroidogenesis following acute stimulation is the synthesis of StAR and its role in transporting cholesterol within the mitochondria.

Cholesterol side chain cleavage (CYP11A or P450*scc*). The net steroidogenic capacity of a cell is determined by the expression of the CYP11A enzyme which converts cholesterol to pregnenolone (Figure 1-5) and is expressed in all steroidogenic tissues. This CYP11A catalysed conversion is achieved by a 3-step reaction: 20α-

hydroxylation, 22-hydroxylation and scission of 20,22 carbon bond (Black et al., 1993; Ramsby, 1997; Miller, 1998).

17α-Hydroxylase (CYP17) is bound to the mitochondrial membrane of adrenal gland and gonad cells and is essential for the synthesis of glucucorticoids and sex steroids but not for mineralocorticoids, since they can be synthesised in the absence of this enzyme (Chung et al., 1987; Ramsby, 1997; Miller, 1998). It catalyses two reactions: the 17α-hydroxylation of the C21 steroids, pregnenolone and progesterone, followed by the side chain cleavage at carbon 17,20 to produce the C19 steroids dehydroepiandrosterone (DHEA) and androstenedione, respectively (Figure 1-5). In these two-step reactions 17α-hydroxypregnenolone and 17α-hydroxyprogesterone are formed as intermediates, respectively.

3β-Hydroxysteroid dehydrogenase (3β-HSD) enzymes are membrane bound enzymes that are found in both mitochondrial and microsomal membranes of the main steroidogenic tissues, namely adrenals, ovaries, testis and placenta and their enzymatic activity is essential for the initial step in the production of all classes of active steroid hormones (Pelletier et al., 1992). There are different isoforms of 3β-HSDs and their expression is tissue specific involving separate mechanisms of regulation (Mason et al., 1997). They catalyse the transformation of all 5-ene-3β-hydroxysteroids (Δ^5 steroids) into 4-ene-3-ketosteroids (Δ^4 steroids). Therefore, pregnenolone, 17α-hydroxypregnenolone and DHEA are converted to progesterone, 17α-hydroxyprogesterone and androstenedione, respectively (Ramsby, 1997; Payne and Hales, 2004; Figure 1-5).

21-Hydroxylase (CYP21) plays a crucial role in the formation of the mineralocorticoids and the corticoids and is expressed exclusively in the endoplasmic reticulum of adrenal tissue cells. It catalyses the hydroxylation of the carbon 21 of progesterone and 17α-hydroxyprogesterone to yield deoxycorticosterone and 11-deoxycortisol, respectively (Ramsby, 1997; Payne and Hales, 2004; Figure 1-5).

11β-Hydroxylase (CYP11B). The final step in cortisol and aldosterone biosynthesis is catalysed by the two isoforms of the enzyme CYP11B (CYP11B1 and CYP11B2) which are located in the inner mitochondrial membrane of adrenal tissue cells. CYP11B1

catalyses the 11β-hydroxylation of 11-deoxycorticosterone and 11-deoxycortisol to yield corticosterone and cortisol, respectively. CYP11B2 catalyses the conversion of corticosterone to aldosterone (Ramsby, 1997; Payne and Hales, 2004; Figure 1-5). In fish, CYP11B has also been shown to catalyse the synthesis of 11-oxygenated androgens (e.g. 11-ketotestosterone) in the gonads, which have been shown to be potent steroids (Socorro et al., 2007).

17β-Hydroxysteroid dehydrogenase (17β-HSD) enzymes are found in steroidogenic cells, either as membrane bound or soluble, and they catalyse the final step in the synthesis of gonadal steroid hormones, i.e. the conversion of inactive 17-keto steroids into their active 17β-hydroxy forms. Thus, inactive androstenedione and estrone are hydroxylated at carbon 17 and converted into active testosterone and estradiol, respectively and *vice versa* (Figure 1-5). 17β-HSDs enzymes are not involved in the synthesis of the adrenal steroids mineralocorticoids and glucurocorticoids (Ramsby, 1997; Payne and Hales, 2004). 17β-HSD isoforms have little homology to each other (Payne and Hales, 2004) and the expression and catalysing activity of each isoenzyme is tissue specific, catalysing either reactive or oxidative reactions (Labrie et al., 2000).

5α-Reductase enzymes are membrane-bound microsomal proteins that have an important role in androgen formation as they catalyse the conversion of testosterone to the potent androgen 5α-dihydrotestosterone (Ramsby, 1997; Wilson, 2001; Figure 1-5). 5α-Reductases reduce all Δ^4-3-ketosteroids (including progesterone, androstenedione and corticosterone) but with different kinetics and specificity according to the steroidogenic tissue (Normington and Russell, 1992; Poletti et al., 1998). The action of these enzymes is fundamental since type II 5α-reductase deficiency in mammals produces a syndrome of male pseudohermaphroditism characterised by ambiguous external genitalia and decreased levels of plasma dihydrotestosterone (Imperato-McGinley et al., 1974).

Cytochrome P450 aromatase (CYP19 or P450arom) is located in the endoplasmic reticulum mainly of gonad and brain cells (Simpson et al., 1994; Lephart, 1996; Bulun et al., 2003) and has a pivotal role in estrogen biosynthesis as it catalyses the conversion of the C19 Δ^4-3-ketoandrogens androstenedione and testosterone, to the C18 phenolic-A-ring estrogens estrone and 17β-estradiol, respectively (Hall, 1986;

Ramsby, 1997; Payne and Hales, 2004; Figure 1-5). It is the unsaturation of the A ring of the androgens that permits its conversion to a phenolic ring; therefore, steroids such as dihydrotestosterone cannot be aromatised by this enzyme (Simpson et al., 1994). Overall, experimental data from aromatase knockout mice demonstrate that in the lack of the enzyme, animals are unable to synthesise endogenous estrogens (Jones and Simpson, 2000). Additionally, CYP19 has a fundamental role in the brain as estrogen biosynthesis mediated by CYP19 during the development of the CNS, influences sexual differentiation of neural structures and modulates neuroendocrine and reproductive functions as well as sexual behaviour (Lephart, 1996). In humans, rats and mice CYP19 is encoded by a single gene (Simpson et al., 1994) and its transcripts vary across tissues due to the action of tissue specific promoters (Ramsby, 1997). However, this is not a common feature across all vertebrates. In teleost fish for example, there are two CYP19 isoforms expressed from separate gene loci and they are differentially expressed in the brain and gonads. CYP19B is predominately expressed in the brain whereas CYP19A is predominately expressed in gonads (Callard et al., 2001).

1.6 Biotransformation enzymes: Phase I and II metabolism.

The purpose of metabolism is to maintain cell homeostasis, i.e. a stable intracellular environment and can be divided in anabolism or catabolism. The first is the synthesis of larger molecules from smaller parent compounds (input energy from a source such as ATP is required) and the second is the break-down of larger parent compounds to smaller ones (energy in the form of ATP is produced). Metabolism is catalysed by a battery of enzymes and since a new compound with different chemical structure is formed, the process is called biotransformation (Yu, 2004).

Whether a compound will pass the cellular membranes and reach the sites of enzymatic metabolic activity will depend on its degree of lipophilicity and polarity. Lipophilic compounds can traverse biological membranes and therefore are subject to metabolism, whereas polar compounds are water soluble and will get excreted from the organism. Both endogenous and exogenous substances (xenobiotics such as drugs, environmental chemicals and pollutants) are subject to biotransformation by cellular enzymes within the organism. In the case of endogenous lipophilic compounds (e.g.

hormones, bile and fatty acids, postagladins and retinoids) metabolism serves in their processing and recycling to achieve homeostasis. Steroid hormones, for example, reach the target sites or become inactive, not only through steroidogenesis and transport but also through well-coordinated metabolism. Deactivation of steroid hormones is achieved through biotransformation to more polar or non-polar compounds that may be excreted from the organisms or retained in lipoidal tissues, respectively (Waxman, 1988).

The enzymatic metabolism takes place in two stages (Figure 1-6). The first stage (phase I metabolism) involves the introduction of small polar groups on to the parent compound and normally leads to the production of metabolites which contain hydroxyl groups. The hydroxyl group serves as the site for the second stage of the metabolism (phase II metabolism) where normally large polar groups are added to the compound through conjugation (Caldwell, 1998). These two phases lead to a progressive increase in water solubility, moving from a lipophilic compound to a more polar metabolite and then to an even more polar conjugate. Most conjugates are water soluble anions and thus are readily excreted through the bile and/or urine (Walker et al., 2001).

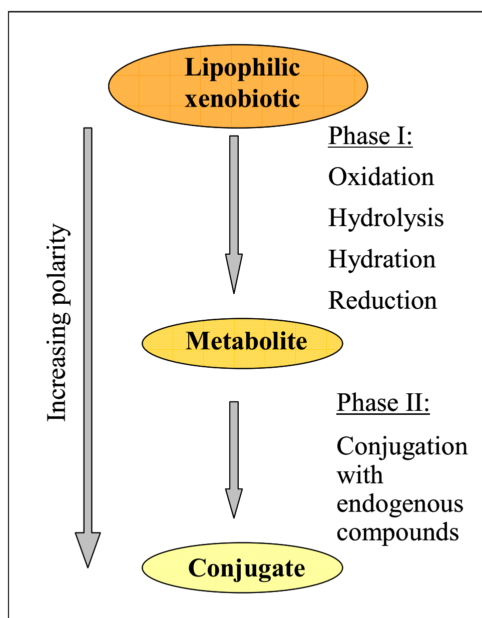


Figure 1-6. Phase I and II enzymatic metabolism of lipophilic xenobiotics.

Table 1-4 illustrates examples of the phase I enzymatic reactions which may involve oxidation, reduction or hydrolysis and the phase II enzymatic reactions which include conjugation with acetate, inorganic sulphate, glucuronic acid, fatty acids, methyl

groups or amino acids. The structural changes resulting from phase I reactions are often small, but in some cases they may enhance the reactivity of the compound instead of decreasing it producing more biologically active metabolites than their parent compounds, with desirable (drug activation) or harmful (toxicity) biological activity (Tredger and Stoll, 2002; van den Berg et al., 2003; Guengerich, 2008). Therefore, a stable inactive compound may be converted to a highly reactive one capable to interfere with intracellular molecules resulting in cell dysfunction and toxicity. By contrast, the physiochemical changes occurring as a consequence of the majority of conjugation reactions during phase II metabolism, lead to a loss of specific tissue interactions and favour excretion, resulting in inactivation or detoxification (Caldwell, 1998). Phase II reactions are not restricted exclusively to increasing the solubility of the parent compound but solubility may be decreased by conjugation of the compound with a non-polar molecule such as a fatty acid and may thus be retained in biological fat deposits and lipoidal matrices (Hochberg, 1998).

Table 1-4. Enzymes involved in phase I and II metabolism of lipophilic xenobiotics and endogenous compounds.

Enzymatic catalysis	Mechanism	Catalysing enzymes
Phase I metabolism		
Oxidation	Introduces hydroxyl, epoxide and ketone groups	Alcohol and aldehyde dehydrogenases
	Shortens alkyl side chains	Amine oxidases
	Converts alcohols to aldehydes and acids	Cytochromes P450 (MFO)
Reduction	Introduces hydrogens into ketones and nitro groups	Nitro- and azo- reductases
Hydrolysis	Break down esters to alcohols and acids	Esterases
Phase II metabolism		
Acetylation	Adds acetate to polar sites	Acetyltransferases
Amino acid conjugation	Adds amino acids to polar sites	Glutathione transferases
Glucuronidation	Adds sugars to polar sites	Glucuronyl transferases
Methylation	Adds methyl groups to polar sites	Methyltransferases
Sulfation	Adds inorganic sulfate to polar sites	Sulfotransferases

Biotransformation occurs in several tissues and organs such as the liver, kidney, intestine and lung. The liver carries out the majority of chemical reactions because it hosts a large amount of non-specific enzymes capable of catalysing the metabolism of a wide range of compounds (Caldwell, 1998). The enzymes responsible for the phase I metabolism are located in the smooth endoplasmic reticulum of the cells, whereas the phase II enzymes are located in the cytoplasm and in some cases in the endoplasmic reticulum.

1.6.1 Phase I enzymes

The most important enzyme system of phase I metabolism is the Cytochrome P450 system, and the enzymes involved are known as Cytochrome P450 mixed function oxidases (MFOs) (Yu, 2004). The properties of the Cytochrome P450 enzymes have been discussed earlier (section 1.5.1) so they are not mentioned here. Three Cytochrome P450 gene families (those being CYP1, CYP2, CYP3 and CYP4) appear to be responsible for the majority of xenobiotic metabolism in the liver (Wrighton et al., 1996; Anzenbacher and Anzenbacherova, 2001; Tredger and Stoll, 2002). Below, the enzymes of interest for the present work are discussed separately with reference to both mammals and fish.

CYP1A . The mammalian CYP1A isoenzymes are expressed mainly in the liver and the extrahepatic tissues (Nelson et al., 1996) and at least two CYP1A isoenzymes are expressed in the liver of fish (Buhler and Wang-Buhler, 1998). The particular interest toward the CYP1A subfamily comes from the field of chemical carcinogenesis and the biotransformation of chemical compounds to potent cancer inducers by the catalytic activity of these enzymes (Dipple et al., 1985; Guengerich and Shimada, 1998; Wogan et al., 2004). Such compounds include polycyclic aromatic hydrocarbons (PAHs), PCBs, dioxins, *β*-naphthoflavone (BNF) and some pesticides. The CYP1A isoenzymes present in the liver are also responsible for the metabolism of a wide range of pharmaceuticals including acetaminophen, caffeine and fluvoxamine (Anzenbacher and Anzenbacherova, 2001). The induction of CYP1A subfamily expression is regulated by a heterodimer composed by the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (Arnt). AhR is located in the cytoplasm and upon ligand binding, the ligand:AhR complex undergoes a conformational change

exposing the nuclear localisation sequences (NLSs). The complex is then translocated to the nucleus forming a heterodimer with the Arnt protein already present in the nucleus. The heterodimer has high DNA binding affinity and binds to specific DNA recognition site, AhR response elements (AhRE) or dioxin responsive elements (DRE), near the promoter region of the CYP1A genes. This leads to chromatin and nucleosome disruption, increased promoter accessibility, increased CYP1A mRNA transcription and protein synthesis, and consequently increased CYP1A catalytic activity (Denison et al., 2002; Fujii-Kuriyama and Mimura, 2005; Kawajiri and Fujii-Kuriyama, 2007). The most studied classes of AhR synthetic ligands are the halogenated aromatic hydrocarbons (HAHs) such as dioxins and PCBs, and the PAHs. Overall, HAHs bind to AhR with higher affinity than PAHs and the dioxin TCDD is one of the most potent AhR activators and CYP1A inducers (Denison et al., 2002). Apart from the synthetic ligands, natural compounds deriving from diet such as flavonoids, carotinoids and phenolics and endogenous substances such as arachidonic acid (AA) have also been found to activate AhR (Denison et al., 2002).

CYP3A metabolising enzymes are expressed mainly in the liver, intestine, lungs and placenta of mammals (Nelson et al., 1996) and a CYP3A-like protein with high similarity toward the mammalian CYP3A genes has been identified in the liver of fish as well (Buhler and Wang-Buhler, 1998). In mammals, CYP3A4 is the most abundant Cytochrome P450 in liver, accounting approximately for 30% of total liver Cytochrome P450 content. This enzyme has an important role in endogenous processes, such as steroid catabolism as well as in environmental contaminant metabolism and it participates in the oxidation of approximately 60% of all clinically used drugs making it the most important enzyme in drug metabolism (Lehmann et al., 1998; Anzenbacher and Anzenbacherova, 2001; Plant and Gibson, 2003). CYP3A4 has broad substrate specificity and catalyses the oxidation of many large and structurally complex molecules (Kadlubar and Guengerich, 1992) and a variety of compounds ranging from steroids to antibiotics induce CYP3A4 hepatic gene expression in-vivo (Anzenbacher and Anzenbacherova, 2001; Scott and Halpert, 2005). The molecular mechanism driving the transcriptional activation of CYP3A is complicated with many hormone receptors such as pregnane X receptor (PXR) and CAR participating in the process (Plant and Gibson, 2003; You, 2004). These two receptors must form a heterodimer with the RXR before binding to the response elements in the promoter region of the

Cyp3a gene (Jones et al., 2000). They both exhibit the ability to bind to multiple endogenous ligands (e.g. progesterone, pregnane, corticosterone) and a variety of xenobiotics (e.g. antibiotics, anti-depressants, fungicides); and receptor specificity is species dependent (Jones et al., 2000; Timsit and Negishi, 2007). A correlation between hepatic PXR and CYP3A mRNA expression has been observed in salmon following exposure to the xenoestrogen 4-nonylphenol (Meucci and Arukwe, 2006) and to the DDT metabolite DDE (Mortensen and Arukwe, 2006) and in zebrafish following exposure to the synthetic steroid pregnenolone (Bresolin et al., 2006). Therefore, the CYP3A gene and its regulation by PXR appear to be a general feature that has been conserved during the evolution of vertebrates (Jones et al., 2000).

1.6.2 Phase II enzymes

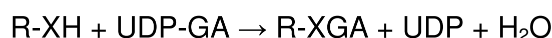
The enzymes that the present work focuses and participate in phase II metabolism are uridine diphosphate glucuronosyltransferases (UDPGTs), glutathione-*S*-transferases (GSTs) and the fatty acid acyl-CoA: steroid acyltransferases.

Uridine diphosphate glucuronosyltransferases (UDPGTs). Glucuronidation is a major conjugation pathway in the metabolism and excretion of endogenous compounds (e.g. steroids) and xenobiotics, a detoxification mechanism common to many diverse living organisms, from bacteria to humans (Jakoby, 1988; Tephly, 1990; Miners and Mackenzie, 1991; Mackenzie et al., 1992). The system has received a lot of scientific attention primarily for its role in detoxification of drugs. Indeed, more than 35% of the drugs metabolised by phase II enzymes are subject to glucuronidation (Evans and Relling, 1999). The other roles of glucuronidation is the protection against environmental contaminants such as toxic dietary components and environmental carcinogens (Nagar and Remmel, 2006) and to maintain homeostasis of various endogenous molecules within the organism, including bilirubin, steroid and thyroid hormones, fatty and biliary acids (You, 2004).

The glucuronidation reaction is catalysed by a group of enzymes called uridine diphosphate glucuronosyltransferases (UGTs). These enzymes are membrane bound proteins and they are the only phase II enzymes located exclusively in the endoplasmic reticulum (Zamek-Gliszczynski et al., 2006). They are primarily expressed in the liver

but other organs such as kidney, intestine, brain and skin also show low levels of UGT activity.

UGTs catalyse the transfer of the glucuronic acid (GA) moiety from UDP-glucuronic acid to the functional group (e.g. carboxyl-, hydroxyl-, amino-, sulfur-) of the target substrate (aglycon). The general reaction is:



$$\text{R-XH} + \text{UDP-GA} \rightarrow \text{R-XGA} + \text{UDP} + H_2O$$

Where R-XH determines the glucuronide formed (Jakoby, 1988; Guillemette, 2003; Nagar and Remmel, 2006). The carboxylic group from the GA promotes excretion by increasing the aqueous solubility of the glucuronide, which is otherwise highly hydrophobic. The glucuronide is recognised by the biliary and renal organic anion transport systems, which enable excretion into the urine or bile (Guillemette, 2003).

Although glucuronidation leads to an overall de-activation of the substrate there are some cases where the formed conjugate is retained or its biological action increases, as in the case of morphine-6-glucuronide formation which has a more pronounced analgesic effect than morphine itself (Mulder, 1992) or the hypolipogenic drug gemfibrozil which is converted to gemfibrozil 1-*O*-β-glucuronide, a potent CYP2C8 inhibitor causing drug-drug interactions (Ogilvie et al., 2006).

Glutathione-*S*-transferases (GSTs). The GST system is a cellular mechanism with the main role to protect the organism against cytotoxic and genotoxic stress. GST enzmes are found in almost all species from plants and bacteria to animals. They catalyse the conjugation of potent electrophilic compounds to the tripeptide glutathione (γ-glutamyl cysteinyl glycine) (GSH), producing less reactive chemical species (Schultz et al., 1997). In addition, a number of endogenous substances with cytotoxic potential (e.g. lipid peroxides) or steroid hormones and prostaglandins are also metabolised by GSTs to more polar compounds. The actual reaction takes place spontaneously under physiological conditions of pH and temperature, but the rate is greatly increased by the presence of GSTs. In vertebrates they are expressed mainly in cytosolic fractions but they are also found in mitochondrial and microsomal membranes (Zamek-Gliszczynski et al., 2006). They are usually up-regulated after exposure to carcinogens and GST isoenzyme null mice exposed to carcinogens show enhanced tumorgenesis

demonstrating the important role of these enzymes in cancer protection (Henderson et al., 1998).

Acyl coenzyme A (acyl-CoA) : steroid acyltransferases catalyse the conversion of steroids to an inactive non-polar form, which is retained in the lipoidal tissues of the organism instead of getting eliminated. More specifically, they catalyse the conjugation of steroids with the fatty acid moiety from acyl-CoAs forming a steroid fatty acid ester (Hochberg, 1991) and it has been suggested that their expression in rodents is controlled by the PPARα (Xu et al., 2001b). All of the families of steroid hormones (glucocorticoids, mineralocorticoids and sex steroids) can be esterified with fatty acids and steroid fatty acid esters occur in the hydrophobic, lipid fractions of several tissues, such as fat deposits but also in the brain, ovaries (and ovarian follicular fluid), placenta, uterus and mammary (Roy and Bélanger, 1989; Larner et al., 1992; Hochberg, 1998). Blood circulation of steroidal fatty acid esters takes place exclusively through binding to lipoprotein particles (LDL and HDL) in the plasma, however plasma concentrations are generally very low (Miilunpohja et al., 2006).

Esterification of steroids has received a great deal of attention particularly after the discovery that fatty acid estrogen esters have increased estrogenic potency compared with the parent compounds and could act as long-lived steroids (Hochberg, 1998). Studies on mammals, particularly on humans, rats and calves have shown that estradiol (E2) and estriol (E3) are converted to their fatty acid esters derivatives. Estradiol and estriol fatty acid esters do not bind to the estrogen receptor themselves but require enzymatic hydrolysis by esterases to liberate the bioactive hormone and exert their action. In the case of E2-17β-fatty acyl esters, injections of immature rodents with E2-17β-stereate, activated the hypothalamic-pituitary-ovarian axis, advanced puberty (Larner et al., 1985) and showed a maximal uterotrophic response (Paris et al., 2001), whereas multiple 17β-estradiol injections were needed to obtain such results. Similar results of long-acting estrogenic action were obtained by injecting female ovariectomised rats with E2-17β-oleate and –palmitate (Vazquez-Alcantara et al., 1989). Interestingly, a more profound estrogenic response was observed when female ovariectomised rats were injected with E3-17β-stereate, despite the fact that E3 is a weak estrogen compared to estradiol (Zielinski et al., 1991). Similar to estrogen fatty acid esters, endogenous testosterone fatty acid esters have an increased potency and duration of action when compared to testosterone (Borg et al., 1995).

Estradiol is esterified naturally on the hydroxyl group of C-17 to give E2-17β-fatty acyl ester (Figure 1-8 A) but not on the hydroxyl group of C-3 (Schatz and Hochberg, 1981; Roy and Bélanger, 1989; Larner et al., 1992). Similarly, estriol is esterified on the hydroxyl groups of C-17 and C-16 (Figure 1-8 B and C) but not on the hydroxyl group of C-3 (Pahuja et al., 1991). It has been suggested that this fatty acid esterification of the D-ring of sex steroids protects the steroid nucleus from metabolism resulting in a long-lived hormonal stimulation (Larner et al., 1985; Hochberg, 1991).

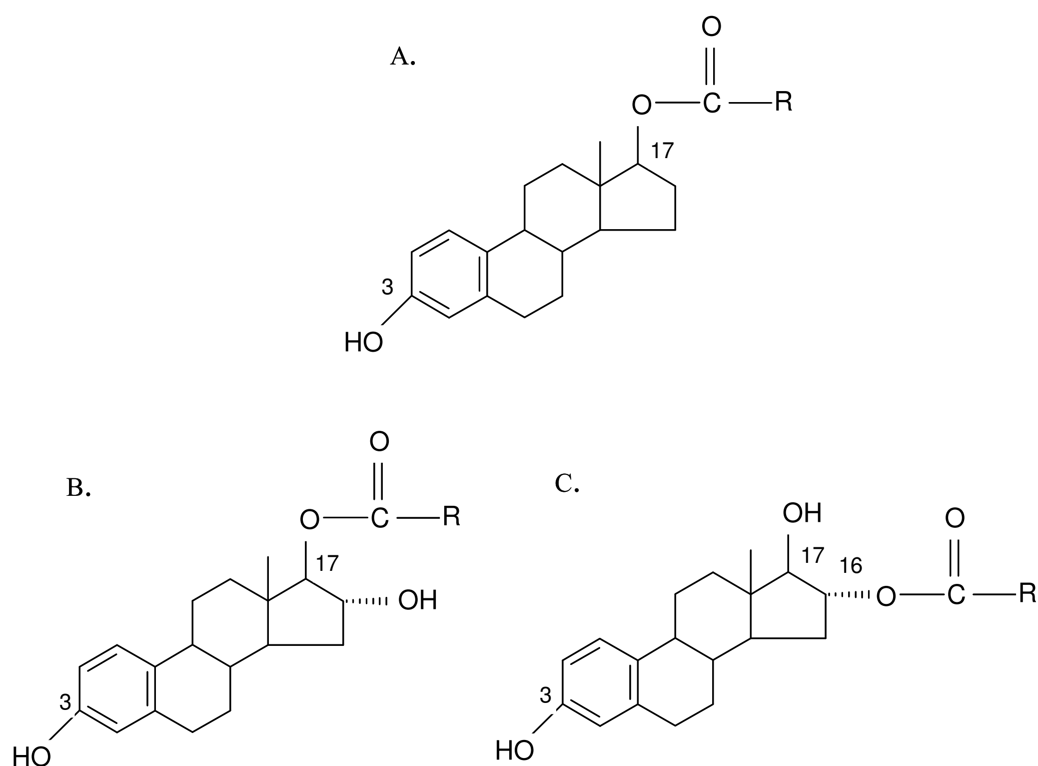


Figure 1-7. Naturally occurring esterified estrogens. A: Estradiol-17β-fatty acid ester; B: Estriol-17β-fatty acid ester; C: Estriol-16-fatty acid ester.

Scientific evidence suggests that acyl-CoA: steroid acyltransferases are not steroid specific. Incubation of estradiol with long chain fatty acyl-CoAs with human mammary cancer cells resulted in increased synthesis of estradiol-17β-monoacyl esters but addition of the C19-steroids testosterone, DHEA and 5α-androstenediol competitively inhibited the esterification of estradiol (Martyn et al., 1987). Furthermore, testosterone was found to be both substrate and inhibitor of the esterification of estradiol (Lee and Adams, 1987). It appears that the same enzyme esterifies estradiol

and testosterone indicating that acyl-CoA:acyltransferases have wide substrate specificity.

1.7 Case studies: Pharmaceuticals and Organotins

1.7.1 Pharmaceuticals, a threat for fish populations

Pharmaceuticals have an important role in modern human and veterinary medicine and due to their extensive manufacture and widespread use, significant amounts are present in the aquatic environment. They are usually resistant to biodegradation as metabolic stability is necessary for their pharmacological action and certain pharmaceuticals or their metabolites are highly soluble in water for excretion from the body. When those characteristics are combined, their removal during wastewater treatment is limited (Fent, 2006) and as a consequence, these compounds enter the aquatic environment resulting in chronic exposure of aquatic biota. The main sources of pharmaceuticals in the environment are effluents from hospitals and domestic sewage treatment plants (Halling-Sørensen et al., 1998) as well as effluents from drug manufactures (Larsson et al., 2007) and leachates from landfill sites (Bound and Voulvoulis, 2005). Worldwide studies have reported the presence of pharmaceuticals in soil, sediments, sewage treatment plant's effluents and surface, ground and drinking waters, as well as in aquatic organisms (Stumpf et al., 1999; Koplin et al., 2002; Andreozzi et al., 2003; Calamari et al., 2003; Thomas and Hilton, 2004; Brown et al., 2007; Gros, 2007).

Pharmaceuticals are excreted from humans and animals in the form of conjugates, which are inactive. However, in sewage treatment plants (STP) such conjugates are cleaved releasing the active compound (Ternes et al., 1999). Environmental contamination by pharmaceuticals is of great concern as pharmaceuticals are made to be biologically active, pass through biological membranes and are often exquisitely potent. Their biological activity is usually not limited towards one target, and side effects do occur (Li, 2004). Pharmaceuticals present in the environment may have similar modes of action and side effects on the exposed aquatic organisms as they have in mammals since target receptors and biomolecules are similar and mostly conserved during evolution (Thornton, 2003).

However, due to interspecies differences, prolonged exposure and susceptibility of aquatic organisms to chemicals, the secondary effects of pharmaceuticals that are considered irrelevant to humans may potentially play a major role in non-mammalian species. Fish are non-target organisms that are exposed to pharmaceuticals and therefore they may experience adverse health effects provoked from these compounds.

1.7.1.1 Ethynylestradiol (EE2)

A striking example of adverse effects provoked by pharmaceuticals is the feminisation of male fish in rivers that receive municipal effluents. Chemical water analysis has revealed that municipal effluents contained among other compounds the pharmaceutical ethynylestradiol (EE2), which is the synthetic estrogen used in birth control pills, at extremely low but biologically active concentrations. Concentrations of EE2 reported in STP effluents from different countries across the world are given in Table 1-5. In some cases concentrations up to 42 and 831 ng/L have been reported in Canada (Ternes et al., 1999) and USA (Koplin et al., 2002), respectively, although common concentrations are within a few ng/L or lower. Even in such low levels, EE2 is bioactive upon fish. Exposure of adult fathead minnow over their life cycle to EE_2 showed that the NOEC values for embryo hatching success and larval survival were ≥1 ng/L (Länge et al., 2001).

Table 1-5. Concentration of EE2 in effluents from sewage treatment plants.

Country	EE2 concentration (ng/L)	Reference
Sweden	<2.0 – 5.0 (4.5)	Larsson et al. (1999)
Finland	LOD – 1.1	Johnson et al.(2005)
Switzerland	<0.8 – 2.8	Johnson et al.(2005)
England	<0.2 – 7.0	Desbrow et al. (1998)
Italy	<0.31 – 1.7 (0.45)	Baronti et al.(2000)
Germany	LOD – 15.0 (1.0)	Ternes et al. (1999)
Canada	LOD – 42.0 (9.0)	Ternes et al. (1999)
USA	LOD – 831.0 (73.0)	Koplin et al. (2002)

Concentration range is given, values in parenthesis are the means, when available; LOD: Limit of detection

Table 1-6 shows the developmental and reproductive effect concentrations of EE2 in various fish species during different phases of their life cycle. An effect concentration as low as 0.1 ng/L of EE2 has been reported for vitellogenin (Vtg) induction in male adult rainbow trout (Purdom et al., 1994). Vtg is an egg-yolk protein precursor produced in the liver of female fish in preparation for reproduction. E2 stimulates Vtg synthesis and secretion into the bloodstream for transport to the developing oocytes to form the yolk. Because production is extremely low in males and immature females, Vtg induction is a highly specific biomarker for exposure to estrogenic substances (Sumpter and Jobling, 1995). Interestingly, in male fathead minnows, EE2 is 25-30 times more potent than estradiol in inducing Vtg synthesis (Brian et al., 2005). It has been demonstrated that EE2 binds to the estrogen receptor with high affinity. In fact, in fathead minnow the relative binding affinity of EE2 compared to E2 binding was found 166% (Denny et al., 2005) confirming that EE2 is a more potent estrogen than E2 in this fish species. However, in zebrafish EE2 was an equally potent ER agonist as E2 using a yeast estrogen receptor assay (Segner et al., 2003). This demonstrates that other factors, such as bioavailability of EE2 or endogenous metabolism may play a role in the estrogenic potency of EE2.

EE2 has lower water solubility (4.8 mg/L) and higher K_{ow} (4.15) than E2 (13 mg/L and 3.9, respectively) suggesting a lower rate of degradation in the environment and higher bioavailability (Ying et al., 2002). Furthermore, the administration of EE2 to humans as a contraceptive is due to its prolonged estrogenic action compared to the natural estrogens. This has been attributed partly to a lower glucuronidation of EE2 and excretion, compared to the natural estrogens. Injections of humans with E2 resulted in a 90% decrease in plasma levels after 30 min (Longcope and Williams, 1974), whereas in the case of EE2 such a decrease was evident after 7 hours (Warren and Fotherby, 1973). In the case of E2, a major metabolic pathway for excretion is its conversion to estrone by the catalytic action of 17β-HSD, followed by 16α-hydroxylation to produce 16α-hydroxyestrone, which is then reduced to produce estriol (Bolt, 1979). All estrogens can be conjugated at the phenolic hydroxyl group (C-3) of the A ring, with either glucuronic acid or sulfonate. However, in the case of estradiol and estriol, conjugation with glucuronic acid takes place at the D ring as well. The ethynyl group at C17 of EE2 (Figure 1-8) protects the compound from 17β-HSD and 16α-hydroxylase catalysis, which means that no estrone- or estriol-analogs of EE2 are formed to serve

Table 1-6. Summary of effect concentration of different fish species exposed to EE2.

Species	Exposure period[a]	Response criterion	EE2 effect concentration (ng/L)	NOEC (ng/L)	Reference
Fathead minnow	0-28 dph	Adverse larva growth	≥ 0.2		Länge et al., (2001)
	0-56 dph	Reduced juvenile growth and bulk weight	≥ 4		
	0-172 dph	Lack of sexual differentiation and testicular tissue	≥ 4	≤ 1	
	0-301dph	Vitellogenin induction	≥ 4		
		Reduced female weight	≥ 1		
		Reduced egg number per female	≥ 1		
		Lack of sexual differentiation	≥ 4		
	Male adults 2-week exposure	Vitellogenin induction	≥ 0.5 (EC_{50} = 1 ng/L)	< 0.5	Brian et al., (2005)
Rainbow trout	Male adults 3-week exposure	Vitellogenin induction	≥ 0.1	< 0.1	Purdom et al., (1994)
Zebrafish	0-75 dph	Vitellogenin induction	≥ 3		Segner et al., (2003)
		Reduced egg number per female	≥ 3		
	0- adult stage	Reduced fertilisation success	≥ 3		
		Vitellogenin induction	≥ 1.7		
		Reduced juvenile growth	≥ 1.7	< 3	
		Lack of sexual differentiation and testicular tissue	≥ 3		
		Reduced egg number per female	≥ 1.7		
		Reduced fertilisation success	≥ 1.7		

[a]dph: days post hatch

as substrates for glucuronidation and thus excretion of EE2 is lower than E2 (Bolt, 1979). Since glucuronidation is also a major metabolic pathway for steroid de-activation and excretion in fish (Clarke et al., 1991), the increased estrogenic potency of EE2 observed in fish could be attributed to a lower rate of glucuronidation compared to natural estrogens as well.

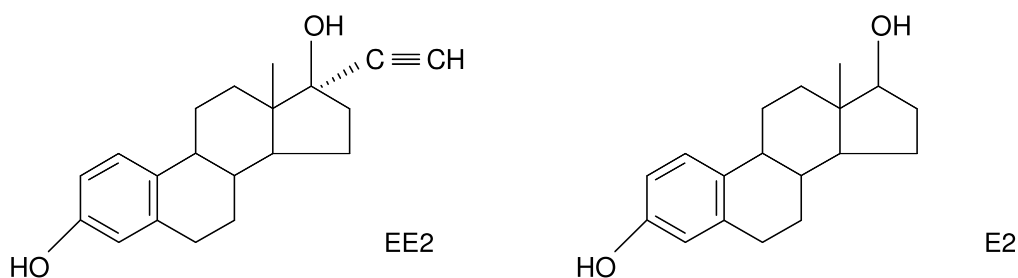


Figure 1-8. Molecular formulas of ethynylestradiol (EE2) and 17β-estradiol (E2).

Most of the studies on fish have focused on the interaction of EE2 with the ER and on the related effects on reproduction. However, there is limited data on the interference of EE2 with other steroidogenic pathways.

1.7.1.2 Gemfibrozil

Gemfibrozil (GEM) belongs to the fibrate drugs and is used in human medicine as hypolipidemic agent, as it decreases serum triglycerides and very low density lipoproteins (VLDL) and increases high density lipoproteins (HDL) (Spencer and Barradell, 1996). As most of the drugs, it is metabolised in the liver and approximately 70% is excreted through the urine, mostly as glucuronide conjugate (Zimetbaum et al., 1991). GEM has been detected in surface waters at concentrations of a few ng/L (Koplin et al., 2002; Gagné et al., 2006) but concentrations in the range of 1-5 µg/L have also been reported in sewage treatment effluents from European countries (Andreozzi et al., 2003) and Brazil (Stumpf et al., 1999). Data show that GEM has a half life of 15 hours in river surface waters due to photodegradation (Lin and Reinhard, 2005), a K_{ow} value of 4.77 (Gros et al., 2007) and a bioconcentration factor of 113 has been reported in goldfish (Mimeault et al., 2005).

GEM and other fibrates, are classified as peroxisome proliferators (PPs) as they increase to a great extent the number and size of hepatic peroxisomes, a phenomenon called peroxisome proliferation, leading to hepatomegaly and hepatocarcinogenesis in rodents (Rao and Reddy, 1991; Sausen et al., 1995). Peroxisomes are single membrane bound organelles present in all eukaryotic cells that have a fundamental role in lipid metabolism. In particular, they are responsible for the β-oxidation of long and very long fatty acids, in a process releasing hydrogen peroxide. Peroxisome

proliferation is usually accompanied by induction of the enzymes involved in the peroxisomal β-oxidation pathway, such as acyl-CoA oxidase and to a lesser extend catalase, the enzyme which converts hydrogen peroxide to oxygen (Lazarow and Duve, 1976).

Induction of the peroxisomal fatty acid β-oxidation pathway enzymes in mammals occurs through transcriptional activation of PPARs (Reddy and Hashimoto, 2001). So far, three types of these receptors have been identified: PPARα, PPARβ and PPARγ. In PPARα gene knockout mice PPs fail to induce the pleiotropic responses in the liver, including induction of the peroxisomal β-oxidation pathway enzymes, hepatomegaly and liver carcinogenesis, demonstrating the critical role of this receptor in peroxisome proliferation in this species (Lee et al., 1995). It has also been reported that PPs induce the fatty acid ω-hydroxylation in the endoplasmic reticulum catalysed by lauric acid ω-hydroxylase, a member of the CYP4A subfamily in mammals, which genes are also encoded by PPARα (Reddy and Hashimoto, 2001). Hence, exposure of male rats to PPs for 13 weeks resulted in dramatic increases in CYP4A protein expression (Fan et al., 2004). Although PPARs received their name because they are activated by PPs it is now well documented that endogenous compounds such as polyunsaturated fatty acids and eicosanoids are also ligands of these receptors (Wilson and Wahli, 1997). Therefore interaction of PPs with these receptors will interfere with other lipid signalling pathways controlled by PPARs. Thus, administration of rats with GEM or clofibrate resulted in increased esterification of E2 with fatty acids in the liver, a reaction catalysed by acyl-CoA: estradiol acyltrasferase (AEAT) (Xu et al., 2001a), which is also controlled by PPARα (Xu et al., 2001b).

Following the cloning of PPARs from various fish species (Ruyter et al., 1997; Leaver et al., 2005; Kondo et al., 2007) questions arise whether fish exposed to peroxisome proliferators such as fibrates are experiencing peroxisome proliferation leading to disturbed lipid metabolism. There is limited data on the effects of fibrates and GEM on fish. Table 1-7 lists some of the documented effects of fibrates on various fish species. As it can be seen, fibrates are potent inducers of acyl-CoA oxidase (AOX) and catalase activity in some fish as it has been observed in rodents. Whether the observed induction of the particular enzyme activities leads to adverse health effects remains unknown. Furthermore, recently it was reported that 2-week waterborne exposure to GEM resulted in decreased testosterone levels in the plasma of goldfish (Mimeault et al., 2005) at a concentration of as low as 1.5 µg/L. This suggests that GEM present in

the environment may interfere with the hormone metabolism of fish and may potentially lead to adverse reproductive and developmental effects.

Table 1-7. Reported effects of fibrates on different fish species following in-vivo exposures.

Fish species	Compound	Exposure	Effect	Effect concentration	Reference
Rainbow trout *Onchorynchus mykiss*	Gemfibrozil	2-weeks; daily intraperitoneal injection exposure	AOX induction$^{\alpha}$	≥ 46 mg/kg	Scarano et al. (1994)
Rainbow trout *Salmo gairdneri*	Ciprofibrate	3-weeks; intraperitoneal injection exposure every second day	AOX induction Catalase induction	≥ 35 mg/kg ≥ 15 mg/kg	Yang et al. (1990)
Fathead minnow *Pimephales promelas*	Clofibric acid	3-weeks; waterborne exposure	Sperm count reduction Non-viable sperm count induction	≥ 10 µg/L ≥ 1 µg/L	Runnalls et al. (2007)
Goldfish *Carassius auratus*	Gemfibrozil	96h; waterborne exposure	Reduction of plasma testosterone and StAR mRNA levels	≥ 1,500 µg/L	Mimeault et al. (2005)
		2-week; waterborne exposure	Reduction of plasma testosterone levels Alterations in StAR mRNA levels	≥ 1.5 µg/L No effect	
	Gemfibrozil	2- and 4-week; waterborne exposure	Inhibition of PPARβ mRNA levels Induction of catalase and glutathione peroxidise	≥ 1,500 µg/L ≥ 1.5 µg/L	Mimeault et al. (2006)

$^{\alpha}$expressed as mg/min/g of liver

1.7.2 Organotin compounds

1.7.2.1 Sources of environmental contamination and signs of toxicity

With the discovery of the biocidal properties of organotin compounds in the 1950s, their application use together with their production and consumption increased considerably. In particular, TBT gained widespread application as an effective antifouling paint biocide in the mid 1960s and its popularity quickly rose as its cost-effectiveness became widely recognised by paint users. Antifouling paints are used to prevent the attachment of organisms such as seaweeds, barnacles, tubeworms and sea squirts to the hulls of ships and other submerged structures. Marine fouling on ships is a constant problem encountered in the shipping industry as growth of marine biota on ship hulls increases the drag on heavily fouled ships and leads to a loss of fuel efficiency. Estimates suggest that a ship with a six month accumulation of fouling can use up to 40% more fuel just to maintain a normal cruising speed (Starbird and Sisson, 1973). The incorporation of TBT into polymers and co-polymers has resulted in the development of paints that keep ship hulls foul-free for up to 4 years, which is the maximum efficacy ever achieved with antifouling paints (Hall et al., 1987). Triphenyltin compounds have also been used as a co-toxicant with TBT in some antifouling paints, although their major employment is in agriculture as a fungicide in crop protection.

Since TBT is an extremely long-action biocide against a wide variety of marine organisms, it is not surprising that leaching of TBT from the hulls of the boats has affected non-target populations. The first negative environmental impact of TBT became evident in the 1970s when deformation and reproductive failure were observed in an important commercial stock of oysters (*Crassostrea gigas*) in Arcachon Bay, France, causing the oyster industry to collapse (Alzieu, 1991). Within the same decade, it was reported that female gastropods of *Nucella lapillus* near Plymouth harbour in England (Blaber, 1970) and of *Nassarius obsoletus* on the Connecticut coast of the United States (Smith, 1971), had developed male sexual characteristics, namely a penis and a vas deferens; a phenomenon called imposex (Smith, 1971), also known as pseudohermaphroditism (Jenner, 1979). Severity of these effects increased with proximity to yacht marinas, and TBT came under suspicion. It is now demonstrated that gastropods are probably the most susceptible species to TBT exposure, and in some cases an exposure concentration as low as 1 ng Sn/L is enough to cause oviduct

blockage, inhibit breeding and consequently lead to population extinction (Bryan et al., 1986; Gibbs et al., 1988).

Due to the continuous evidence on the harmful effects of TBT on marine organisms at such low concentrations, France, UK and the U.S. were the first to proceed to regulations on the use of TBT-based antifouling paints in 1980s. In 1990, the International Maritime Organisation (IMO) adopted a resolution which prohibited the use of organotin antifouling paints in vessels of less than 25m for all the countries-members. However, since TBT environmental levels continued to be over the NOEC levels, in 1999 a new resolution was adopted by IMO calling for the global prohibition on the application of organotin-based antifouling paints on all ships by January 2008 (IMO, 2005).

1.7.2.2 Physicochemical properties and environmental behaviour

The structures of the two organotin compounds of interest of the present thesis, namely TBT and TPT, are shown in Figure 1-9. In aqueous environment, TBT and TPT are in equilibrium with Cl^- and OH^- anions, depending on the pH and temperature (Fent et al., 2006).

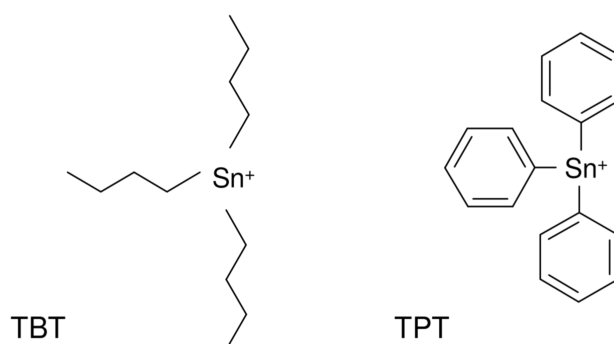
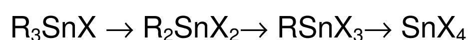


Figure 1-9. Structure of tributyltin (TBT) and triphenyltin (TPT) cations.

TBT and TPT in the water will degrade by environmental influences such as ultraviolet irradiation, biological and chemical cleavage, according to the following scheme:



$$R_3SnX \rightarrow R_2SnX_2 \rightarrow RSnX_3 \rightarrow SnX_4$$

where R is the butyl- or phenyl- moiety and X the anion (Blunded and Evans, 1990). Toxicity of trialkylated organotins decreases with the removal of the alkyl-moiety,

therefore environmental degradation results in less toxic compounds (Fent et al., 2006). Under laboratory conditions the fastest route of degradation of organotins appears to be photolysis by UV light (Table 1-8). However, penetration of sunlight in the field is restricted by the particular mater present in the water and sunlight does not reach the deeper layers; therefore this degradation pathway is not particularly important in the environment. The major breakdown pathway of organotins is through biological degradation by bacteria, microalgae and other aquatic organisms and it is faster in the water column than in sediments (Table 1-8). In some cases, biological degradation may be delayed by factors such as toxic TBT and TPT concentrations that result in elimination of the biodegrading organisms and by limiting conditions of sunlight, temperature or nutrients.

TBT and TPT entering the aquatic environment will either get adsorbed into suspended particles and pass from the water column into sediments, or stay in the water column from where they can get accumulated directly by aquatic organisms. The fate of these compounds into the environment is strongly depended to their physiochemical properties.

Table 1-8. Physiochemical properties of TBT and TPT.

Property	TBT	TPT	Reference
Degradation in water (half life)	6-126 days		Fent et al. (2006)
Degradation in sediments (half life)	0.9-5.2 years	>years	Adelman et al. (1990)
Photodegradation in seawater (half life)	0.5 – 90 days	93-115 days	Fent et al. (2006)
Solubility in seawater	1-10 mg/L	1.2-8 mg/L	Bock (1981)
K_d (L/kg; harbour and marine sediments)	(1.1-49) x 10^4	(5.4-23) x 10^4	Berg et al. (2001)
$logK_{ow}$ (pH 5.8-7.8)	3.2-3.8 (TBTCl)	3.1-3.5 (TPTCl)	Fent et al. (2006)
BCF *Pagrus major* (L/kg ww; fish; whole body)	3.2 x 10^3	3.1 x 10^3	Yamada and Takayanagi (1992)
BCF (*Nucella lapillus*; whole tissue)	100,000	-	Bryan et al. (1993)
BCF (*Mytilus edulis*; whole tissue)	1.1 x 10^4	4.3 x 10^4	Guolon and Yong (1995); Suzuki et al. (1998)

Some of the physiochemical properties of TBT and TPT according to the literature, are shown in Table 1-8. It has been documented that about 5% of the total TBT in seawater will get adsorbed into suspended particles (Seligman et al., 1989), whereas in the case of TPT 63-87% has been found preferentially associated with particulates (Tolosa et al., 1992). Since degradation of these compounds in sediments is slower than in the water column, the sediment-water partition coefficients (K_d) are moderately high, resulting in accumulation of these compounds in the sediments. The K_{ow} and water solubility values demonstrate that both TBT and TPT are hydrophobic and therefore will accumulate not only in organic matter and sediments but in aquatic organisms as well. Accumulation of these compounds on sediments and their slow biodegradation is of special concern as they become bioavailable to deposit feeders or in the case of harbour sediments, dredging will release these compounds into the water column. Bioaccumulation in aquatic organisms is also of concern, since these compounds are lipophilic and will pass through the biological membranes to reach targets of toxicity.

1.7.2.3 Mode of action of TBT and TPT

To date, imposex has been detected in more than 150 gastropod species worldwide (Fioroni et al., 1991; Horiguchi et al., 1997; Matthiessen et al., 1999) and the occurrence of the phenomenon has been correlated to TBT and TPT concentrations in their tissues (Spooner et al., 1991; Horiguchi et al., 1997; Morcillo and Porte, 1999; Birchenough et al., 2002; Barroso et al., 2005), as well as in waters and sediments (Axiak et al., 2000; Ten Hallers-Tjabbesa et al., 2003). Because the first visible stages of imposex appear in such low concentrations of TBT and/or TPT and the syndrome is irreversible, its appearance has been used internationally in monitoring programmes as an indicator of TBT and/or TPT pollution (Horiguchi et al., 1997; Oehlmann et al., 1998; Vishwakiran and Anil, 1999; Ten Hallers-Tjabbesa et al., 2003; Garaventa et al., 2006; Vasconcelos et al., 2006).

Although the link between imposex and exposure to TBT and/or TPT has been established (Bryan et al., 1986; Horiguchi, 2006; Santos et al., 2006), the exact mechanism through which this phenomenon develops remains to be elucidated. Table 1-9 lists the five documented possible mechanisms of action of TBT in female gastropods, obtained through laboratory in-vivo studies.

Table 1-9. Proposed mechanisms of action of TBT in female gastropods.

Mechanism of action	Reference
Increased testosterone levels due to inhibition of aromatase activity.	Spooner et al. (1991); Bettin et al. (1996)
Accumulation of testosterone due to the inhibition of its excretion as polar phase II metabolites (sulphate and glucose conjugates).	Ronis and Mason (1996)
Inhibition of the esterification of testosterone to fatty acid esters resulting in elevated levels of free testosterone.	Gooding et al. (2003); Janer et al. (2006)
Alterations in the secretion of neurohormones (e.g. the peptide hormone APGWamide) that contribute to sexual differentiation in gastropods.	Oberdörster and McClellan-Green (2002)
Binding of TBT and TPT to the retinoid X receptor (RXR) and the involvement of this receptor in imposex development.	Nishikawa et al. (2004)

Since the action of TBT consists in the imposition of male sexual characteristics on female gastropods, TBT is recognised as an “androgenic” compound. Interestingly, the androgenic action of TBT is not restricted to gastropod molluscs. It has been documented that TBT has an androgenic action in fish as well, since 70-day exposure of *Danio rerio* to 0.1 ng/L showed a male biased population which produced a high incidence of sperm lacking flagella (McAllister and Kime, 2003).

The first three theories listed in Table 1-9 assume that increased levels of testosterone will induce imposex. Although there is no evidence supporting the presence of an androgen receptor in gastropods that testosterone will bind to (Thornton, 2003; Sternberg et al., 2008a), the above assumption derives from scientific data demonstrating that testosterone is also a potent imposex inducer in female gastropods (Spooner et al., 1991; Bettin et al., 1996). However, a recent study showed that 2-month exposure of females of *N. lapillus* to TBT resulted in 100% imposex induction whereas exposure to testosterone induced imposex only in 30% of the females (Castro et al., 2007a). Furthermore, imposex-induced female gastropods do not always experience elevated levels of testosterone (Janer et al., 2006) suggesting that induction of testosterone levels may be a secondary effect of TBT, which is probably species depended.

It has been suggested that in the gastropod *Ilyanassa obsoleta*, the peptide hormone APGWamide is the Penis Morphogenic Factor (PMF) which induces sexual differentiation in molluscs (Oberdörster et al., 2005). Since the endocrine system of molluscs appears to depend more on neuropeptides and less on steroid hormones, the observation that APGWamide hormone and TBT induced imposex to the same extent (Oberdörster and McClellan-Green, 2002) brought questions on whether APGWamide is responsible for imposex induction following an external stimuli such as TBT exposure. However, injections of female gastropods of *N. lapillus* and *Bolinus brandaris* with APGWamide did not result in imposex development indicating that imposex induction by this peptide may be species-specific (Santos et al., 2006; Castro et al., 2007a).

The last proposed theory in Table 1-9 suggests that the action of TBT and/or TPT maybe through their interaction with the RXR receptor (Nakanishi, 2007). RXR homologues have been cloned from the gastropods *Thais clavigera* (Nishikawa et al., 2004) and *N. lapillus* (Castro et al., 2007a), as well as from the freshwater snail *Biomphalaria glabrata* (Bouton et al., 2005), all showing a high similarity with the vertebrate RXR, i.e. the ligand binding domain was highly similar (80-90%) to the LBD of the vertebrate RXR. In all three species, 9-*cis*-RA, one of the proposed natural vertebrate ligands for RXR, bind to RXR with high affinity suggesting that retinoid signalling pathways may exist in these species as well. Interestingly, injections of the gastropods *T. clavigera* and *N. lapillus* with 1 µg/g 9-*cis*-RA for 1 month resulted in induction of imposex including both an increase of penis length and vas deferens, similar to the one produced by TBT and/or TBT in these species (Nishikawa et al., 2004; Castro et al., 2007a). These data strongly suggest that induction of imposex in gastropod mollusc may be through interaction of organotins with the RXR receptor.

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Chapter 2. Objectives

2. OBJECTIVES

The overall aim of this thesis is to investigate targets of EDCs, at the molecular and subcellular level, on the endocrine system of fish and gastropod molluscs that could be used in the future as specific endpoints for assessing endocrine disruption.

In order to fulfil this aim two general objectives are being addressed:

- To investigate the effects of the pharmaceuticals ethynylestradiol and gemfibrozil, and the trialkyltin TBT on the endocrine system of juvenile fish.

- To examine the metabolism of androgens in different gastropod species and the alterations caused by exposure to TBT and/or TPT, including sex steroid levels and effects on lipids and fatty acid homeostasis.

These two general objectives have been subdivided in the following specific objectives that are addressed in Chapters 3-8:

Chapter 3 investigates whether short-term exposure of juvenile salmon to environmentally relevant concentrations of EE2 and TBT (singly or in combination) modulates brain aromatase isoform genes and activity and whether these effects will parallel direct ER-mediated responses such as brain ERα gene expression and liver Vtg content. Additionally, Chapter 4 investigates whether exposure to EE2 will also alter brain and interrenal key and rate limiting steroidogenic pathways in juvenile salmon by measuring StAR, P450*scc* and CYP11B mRNA levels.

Chapter 5 examines whether exposure of juvenile eels to the human lipid regulator gemfibrozil will interfere with key enzymes involved in phase I (CYP1A, CYP2K-, CYP2M- and CYP3A-like), phase II metabolism (glucuronyltransferase, glutathione-S-transferase, esterification of testosterone) and peroxisome proliferation induction and whether it will alter plasmatic levels of estradiol and testosterone.

Chapter 6 analyses the metabolism of AD in two gastropod species and assesses the in-vitro effect of the organotin compounds TBT and TPT.

Chapters 7 and 8 investigate whether short-term exposure of the ramshorn snail *Marisa cornuarietis* to environmentally relevant concentrations of TPT will modulate endogenous levels of free and esterified steroids and the metabolism of the androgen precursor androstenedione, and whether it will alter lipid content and fatty acid homeostasis, as well as the activity of the enzyme acyl-CoA oxidase (AOX), the first and rate limiting enzyme of peroxisomal β-oxidation of fatty acids.

*Chapter 3. Ethynylestradiol and Tributyltin modulate brain aromatase gene expression and activity in juvenile salmon (*Salmo salar*) following short-term in-vivo exposure*

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Key Words:
neurosteroidogenesis; aromatase genes; pharmaceutical; antifouling agent; endocrine disruption; fish.

Abstract

In the work presented in this chapter, the effects of two environmental endocrine disruptors, the synthetic pharmaceutical estrogen (ethynylestradiol, EE2) and antifoulant (tributyltin, TBT) representing two different modes of action on the endocrine system, were studied on brain steroidogenic pathway of juvenile Atlantic salmon (*Salmo salar*). Neurosteroidogenesis was studied using brain aromatase gene isoforms and enzyme activity, in parallel with typical xenoestrogen responses, such as brain estrogen receptor (ERα) and plasma vitellogenin (Vtg) levels. Fish were exposed to nominal waterborne EE2 (5 and 50 ng/L) and TBT (50 and 250 ng/L) concentrations dissolved in dimethyl sulfoxide (DMSO), singly and in combination. Gene expressions were quantified using real-time PCR with gene-specific primers, aromatase activity was analyzed using the tritiated water-release assay, and plasma Vtg was analysed using competitive ELISA. Data show that EE2 induced a concentration-specific modulation of P450aromA, P450aromB, and aromatase activity in addition to ERα and plasma Vtg levels in juvenile salmon at day 3 post-exposure. TBT exposure caused both the elevation and inhibition of P450aromA, P450aromB, and aromatase activity levels, depending on concentration, at day 7 post-exposure. TBT elevated and inhibited ERα and plasma Vtg and also antagonized EE2-induced expression of the studied variables at day 7 post-exposure. Interestingly, the carrier vehicle DMSO modulated the receptor-mediated and non–receptor-mediated estrogenic responses at day 7 post-exposure, compared to day 3. In general, these findings suggest that the exposed animals are experiencing impaired steroidogenesis and modulations of receptor-mediated endocrine responses. Given the integral role of neurosteroids in homeostatic process, growth, metabolism, reproduction, and development of central nervous system and function, these effects may have serious impact on this endocrine pathway and potentially affect organism's reproductive performance and health. In conclusion, the regulation of steroidogenesis is a fundamental mechanism involved in the biosynthesis of important biological compounds, irrespective of organ; therefore, the search for the molecular targets of xenoestrogens, given singly and also in combination, in these pathways will increase our understanding of organismal endocrine disruption and potential consequences.

3.1 Introduction

Human and wildlife data indicate that the reproductive system, including its associated endocrine and neural controls, can be very susceptible to alterations by occupational or environmental exposures to a variety of chemical and physical agents (Arukwe and Goksøyr, 1998, 2003; Colborn et al., 1993). Chemical compounds known to mimic the effects of endogenous estrogens in laboratory and field studies include synthetic steroids such as those used in contraceptive pills (Nash et al., 2004), many pesticides (Donohoe and Curtis, 1996), phytoestrogens (Pelissero et al., 1991), alkylphenol polyethoxylates (Kwak et al., 2001; Meucci and Arukwe, 2005), and antifouling agents (McAllister and Kime, 2003). Ethynylestradiol (EE2) is a pharmaceutical used in birth control pills and a potent endocrine modulator known to be present in the aquatic environment at biologically active concentrations (Nash et al., 2004). In sewage treatment work effluents, steroidal estrogens are believed to be responsible for, partly, the feminised responses in some wild fish species (Desbrow et al., 1998; Jobling et al., 2002). The concentration of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/L (Desbrow et al., 1998; Larsson et al., 1999), and concentrations of up to 50 ng/L have been reported by Aherne and Briggs (1989) in aquatic environments. In the United States, a survey of 139 streams showed that several rivers had concentrations > 5 ng/L with an extreme EE2 concentration up to 273 ng/L reported at some riverine sites (Kolpin et al., 2002). Despite the lower EE2 concentrations in surface waters compared to natural steroidal estrogens, its estrogenic potency in fish in vivo studies is 10- to 50-fold higher than that of 17β-estradiol (E2) and estrone (E1) (Segner et al., 2003) most likely due to its longer half-life and tendency to bioconcentrate (650- and 10,000-fold in whole-body tissues and bile, respectively) (Lange et al., 2001; Larsson et al., 1999).

Tributyltin (TBT) is an organotin compound used primarily as a biocide in antifouling paints for ships, boats, and fishing nets (Horiguchi 1994). The use of TBT as antifouling paint has been banned for small boats and fishing nets in most countries as it was shown to be toxic to aquatic life and as an endocrine disrupting chemical that causes severe reproductive effects in aquatic organisms (Horiguchi 1994). TBT was shown to exert masculinising effect in zebra fish (*Danio rerio*) (McAllister and Kime, 2003) and induce imposex that was first described for dogwhelk, *Nucella lapillus* (Babler, 1970), and later reported in the female whelks (*Buccinum undatum L.*) from

the North Sea (Borghi and Porte, 2002). Other effects of TBT include the inhibition of aromatase activity in fish (McAllister and Kime, 2003). The mechanisms by which TBT induces the modulation of the endocrine systems are not well understood, although several possible mechanisms have been proposed. These include the regulation of gonadotropin concentrations and receptor binding, regulation or modification of transcription factors, and regulation of transcription/translation or direct inhibition of the enzymes involved in steroid hormone biosynthesis (Ohno et al., 2005; Yamazaki et al., 2005). All these mechanisms are typical of non–receptor-mediated antiestrogenicity.

Studies on endocrine disruption, particularly with regard to steroidogenesis, have focused mainly on gonadal reproductive steroids, and little is known about the effects and mechanisms of xenoestrogens on reproductive neurosteroids. In vertebrates, the cytochrome P450 aromatase (CYP19) is a crucial steroidogenic enzyme catalyzing the final step in the conversion of androgens to estrogens (Callard et al., 2001; Kishida and Callard, 2001). In teleost there are two structurally distinct CYP19 isoforms, namely, P450aromA and P450aromB. The P450aromA is predominantly expressed in the ovary and plays important roles in sex differentiation and oocyte growth, while P450aromB is expressed in neural tissues such as brain and retina and is speculated to be involved in the development of the central nervous system and sex behaviours (Callard et al., 2001; Kishida and Callard, 2001). Research on endocrine toxicology has mainly focused on estrogenicity that involves direct estrogen receptor (ER)–mediated effects. Therefore, the present study was designed to investigate the effects of EE2 and TBT, singly and in combination, on neurosteroidogenic pathway using the aromatase activity and gene isoforms as model end points, in addition to direct ER-mediated end points such as the egg yolk protein (vitellogenin, Vtg) and brain ERα gene expressions. The ERα isoform was chosen as opposed to ERβ because recent data demonstrates that its expression in the brain showed a direct relationship with plasma Vtg levels in salmon after exposure to a xenoestrogen (Meucci and Arukwe, 2006). The hypothesis of this chapter is that exposure of juvenile salmon to environmentally relevant EE2 and TBT concentrations, singly and in combination, will modulate brain aromatase isoform genes and activity in a concentration dependent and time-dependent manner and that these effects will parallel direct ER-mediated responses and represent a new and quantitative measure of effect biomarker and response toxicity on the endocrine system.

3.2 Material and Methods

Chemicals.

EE2, tributyltin chloride, ^{3}H-androstenedione, bovine serum albumin (BSA), and o-phenylenediamine dihydrochloride (OPD) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Trizol reagent and oligo(dT)$_{18}$ primer were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA). DNA ladder, RNase-free DNase, RevertAid First Strand cDNA Synthesis Kit (FERMENTAS GMBH, Germany), and deoxynucleotide triphosphates were purchased from Stratagene (La Jolla, CA). SYBR Green REAL-TIME PCR Master mix was purchased from BioRad Laboratories (Hercules, CA). Microtiter plates (MaxiSorp) were purchased from Nunc (Roskilde, Denmark). Polyclonal Arctic charr Vtg antibody was purchased from Biosense Laboratories, (Bergen, Norway). All other chemicals were of the highest commercially available grade.

Fish and experiment.

Immature Atlantic salmon (*Salmo salar*, mean weight and length 10 ± 2.5 g and 9 ± 2 cm, respectively) were obtained from Lundamo Hatcheries (Trondheim, Norway) and kept in 70-L tanks at 7 ± 0.5 ºC and for a 14:10-h photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal-holding facilities. The experiment was performed after a 24-h acclimation period.

This work was designed to test two hypotheses in parallel under similar experimental conditions. Firstly, that EE2, in addition to inducing classical ER-mediated responses, will modulate the expression of brain P450arom isoform genes and enzyme activity after short-term (3-days) waterborne exposure at different concentrations. For this experiment, three groups of 12 fish were exposed once to waterborne EE2 at 5 and 50 ng/L. Secondly, that TBT, in addition to affecting EE2- induced classical ER-mediated responses, will antagonize EE2-induced modulation of brain P450arom isoform genes and enzyme activity and that this antagonistic effect will depend on TBT and EE2 concentrations. For this experiment, six groups of 12 fish were exposed once for 7-days to TBT at 50 and 250 ng/L singly and in combinations with EE2 at 5 and 50 ng/L. In addition, an EE2-positive control group and one group serving as solvent

carrier control were exposed to the carrier vehicle dimethyl sulfoxide (DMSO; 7.5 ppb or 0.00075%). The final concentration of DMSO was similar in all exposure groups. The EE2 and TBT concentrations were chosen because they represent environmentally relevant concentrations.

During the experimental period, fish were starved. For both experiments, replicate sampling of six fish each per exposure group was sacrificed for gene expression and aromatase activity. Blanks were collected at the start of the experiments by sampling brain tissue of six individuals each for gene expression and aromatase activity analysis. Samples were collected from each exposure group after the fish were anesthetized with benzocaine (5 mg/l), and blood was collected before sacrifice. After sacrifice, the brain was excised and weighed and then processed as explained below. Blood was taken from the caudal vein using heparinised syringes, centrifuged immediately at 5000 × g for 5 min and stored at -80 ºC until analyzed.

RNA purification and cDNA synthesis.

Total RNA was purified from brain tissues homogenized in Trizol reagent according to the manufacturer's protocol. Total cDNA for the real-time PCRs was generated from 0.2 µg DNase-treated total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (BioRad).

Primer optimization, cloning, and sequencing.

The PCR primers for the amplification of 96– to 216–base pairs gene-specific PCR products were designed from the conserved regions of the studied genes. The primer sequences, their amplicon size, and the optimal annealing temperatures are shown in Table 3-1. Prior to PCRs, all primer pairs were used in titration reactions in order to determine the optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon size in all reactions. PCR products from the genes to be investigated were cloned into pCRII vector in INV αF´ *Escherichia coli* (Invitrogen). Each plasmid was sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the

Table 3-1. Primer pair sequences used for real time-PCR

Target gene		Primer sequence*	Amplicon size (nucleotides)	Annealing temperature (ºC)
P450aromB	Forward	CTGACCCCTCTGGACACG	97	60
	Reverse	TCTCGTTGAGAGGCACCC		
P450aromA	Forward	CTGACCCCTCTGGACACG	96	55
	Reverse	TCTCGTTGAGAGGCACCC		
ERα	Forward	GCTCCTGCTGCTGCTCTC	216	55
	Reverse	CCCTATGCTGGAGCCTGT		

*Sequences are given in the 5´ - 3´order

Department of Biology, NTNU, Norway. Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

Quantitative (real-time) PCR

Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed as described by Arukwe (2005), using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Each 25 µL DNA amplification reaction contained 12.5 µL of iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 1 µL of cDNA and 400 nM of each forward and reverse primers. The 3-step real-time PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (30 sec), 55-60°C depending on target gene (see Table 3-1; 30 min), and 72 °C (30 sec). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into nanogram of DNA using standard plots of Ct versus log ng DNA standards. Standard plots for each target sequence were generated using known amounts of plasmid containing the amplicon of interest. Target cDNA amplification were averaged and expressed as ng/ml RNA. The use of the standard curves was based on equal amplification efficiency between target gene and plasmid containing gene of interest (Arukwe 2005).

Quantitative ELISA analysis of Vtg.

A quantitative Vtg ELISA was performed using a polyclonal Arctic charr Vtg antibody as described previously by Meucci and Arukwe (2005). Purified Vtg protein was used to

coat the plates and for preparation of the standard curve. Briefly, purified salmon Vtg was serially diluted to obtain standard concentrations between 3 and 1000 ng/mL. Standards and diluted samples were incubated for 1 h at 37 ºC with an equal volume of the primary antibody (diluted 1:5000). Triplicate aliquots of standards and samples (200 µL) were added to 96-well microtiter plates previously coated with Vtg (100 ng/mL overnight at 4 ºC) and incubated for 1 h at 37 ºC. The plates were washed with tween-phosphate buffered saline (TPBS) and a 1:2000 dilution of goat anti-rabbit peroxidase-conjugated secondary antibody (BioRad, Hercules, CA) was added and incubated for 1 h at 37 ºC. Levels of Vtg in samples were measured colorimetrically at 492 nm using o-phenylenediamine dihydrochloride (OPD) as substrate with an Ultra microplate reader Elx 808 from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA). Vtg ELISA Absorbance values (expressed as optical density, OD) were converted to the proportion of antibody bound (B) expressed as a percentage in the zero standard by the following equation: B (%) = (OD-NSB/ OD0-NSB)*100 (where OD is the absorbance of a given sample or standard, OD_0 is the absorbance of the zero standard and NSB is the non-specific binding absorbance value). Binding percentage values were logit transformed as shown below: Logit B=Log10(B/1- B) and plotted against log dose to achieve a linear transformation of standard and plasma dilution curves. In evaluating the detection limit of the ELISA assay, the minimum amount of Vtg that produced a response significantly different from OD_0 was 2 ng/mL with 90% binding. The range of the standard curve was between 2 and 500 ng/mL, with 50% of binding around 35 ng/mL. ELISA values for Vtg obtained from control and exposed fish are expressed as mean ± SEM.

Aromatase activity assay.

Aromatase activity was determined by the tritiated water release from the C-1 carbon atom of labelled androstenedione ((1,2,6,7-^{3}H)-androst-4-ene-3, 17-dione) during its conversion to estradiol (Thompson and Siiteri, 1974) and was assayed according to standard protocol as described by Ankley et al (2002) with some modifications. Briefly, brain tissue from individual fish (20–40 mg) was homogenised in 100 µl of 10 mM phosphate buffer (containing 100 mM EDTA, 1 mM dithiothreitol at pH 7.4) per mg of tissue and centrifuged at 10,000 × g for 20 min at 4 ºC. A 50 µL volume of the supernatant was incubated in a water bath at 25 ºC for 1h together with 4 nM of ^{3}H-

androstenedione and 1 mM NADPH in a total volume of 200 µL. The samples were assayed in duplicates with a blank containing buffer instead of sample for each set. The reaction was stopped by adding 300 µL of diethyl ether to the reaction tubes whilst on ice. Tubes were then transferred to –80 ºC for 10 min to freeze the lower aqueous phase. Thereafter, the ether phase containing the steroids was discarded. The solution was then mixed thoroughly with 2:1 (v/v) of 0.25% dextran-coated (2.5%) charcoal and centrifuged at 2500 × g for 30min at 4 ºC to remove residual aromatics. A 500 µL volume of the supernatant was added in a scintillation vial containing 5 mL of scintillation cocktail and ^{3}H was measured as disintegrations per min (dpm) using a liquid scintillation counter. Aromatase activity was then expressed as fmol enzyme activity per hour per mg of total protein content. Total protein concentration was measured using the Bradford assay (Bradford 1976) with bovine serum albumin (BSA) as standard.

Statistical analysis.

Comparison of different concentrations of EE2- or TBT-treated groups (singly and in combination) and control groups was performed using Dunnett's method. Statistical differences among treatment groups were tested using ANOVA and the Tukey-Kramer method. For all the tests the level of significance was set at $p < 0.05$, unless otherwise stated.

3.3 Results

Modulation of P450arom isoform genes and aromatase activity.

Quantitative RT-PCR analyses revealed that juvenile salmon relatively expressed both P450aromA and P450aromB genes in the brain of control and exposed individuals. The P450aromB mRNA expression was induced by EE2 in an apparent concentration-dependent manner after 3 days of exposure (Figure 3-1A). Exposure to 50 ng EE2/L caused a 6.2-fold significant increase of brain P450aromB gene expression, compared to control (Figure 3-1A). Brain P450aromB gene expression was significantly decreased by 0.5-fold 7-days post-exposure to 250 ng TBT/L compared to the corresponding day-7 control group (Figure 3-1B). The effect of EE2 after 7-days of

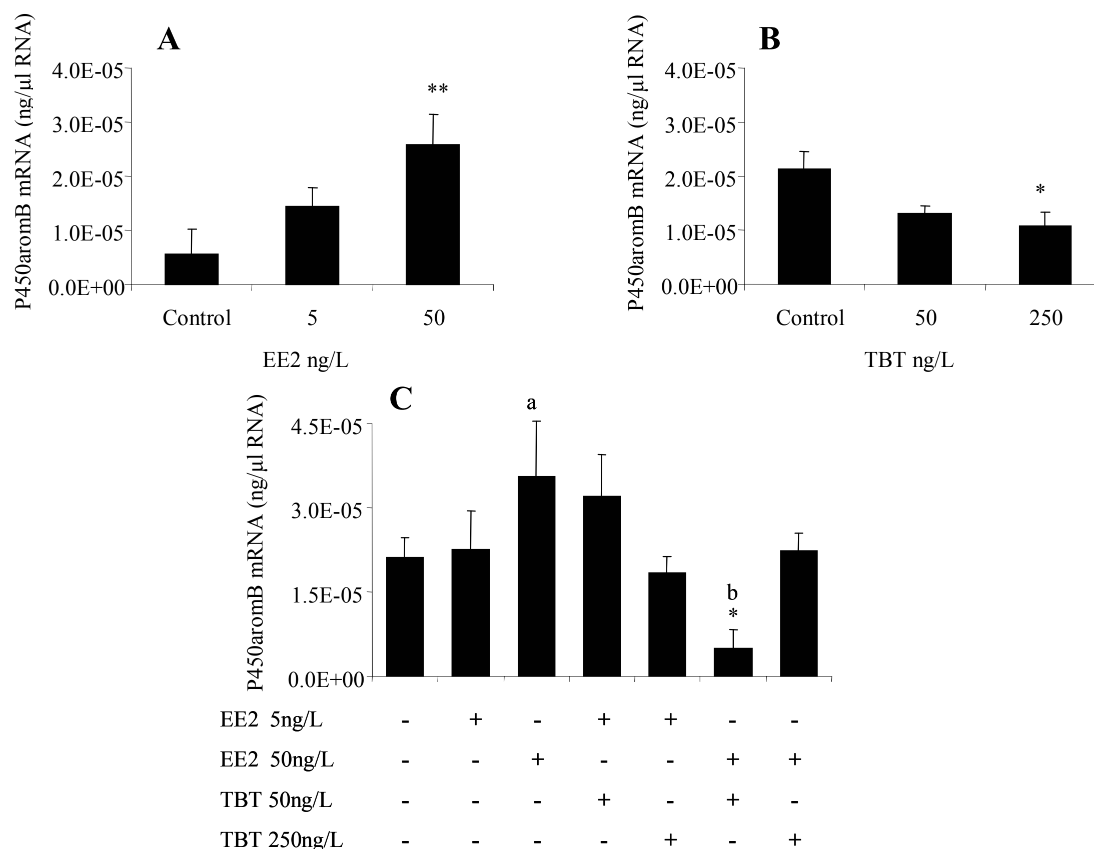

Figure 3-1. Transcriptional changes of brain P450aromB mRNA levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The P450aromB mRNA expression after 3 days of exposure to 5 and 50 ng EE2/L. (B) The P450aromB mRNA expression after 7 days of exposure to 50 and 250 ng TBT/L. (C) The P450aromB mRNA expression after 7 days of exposure to 5 and 50 ng EE2/L singly and in combination with 50 and 250 ng TBT/L. All values represent the mean (n = 6) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analysed using ANOVA.

exposure on the transcription abundance of the brain P450aromB gene, although elevated, was not significant in any of the exposures (Figure 3-1C). However, P450aromB gene expression showed a 1.7-fold increase in 50 ng EE2/L–exposed group compared to carrier control (Figure 3-1C). When EE2 and TBT were given in combination, TBT antagonized, depending on concentration, the effect of EE2 on P450aromB expression (Figure 3-1C). When compared to the 50 ng EE2/L group alone, combined exposure with 50 ng TBT/L significantly inhibited brain P450aromB gene expression (Figure 3-1C).

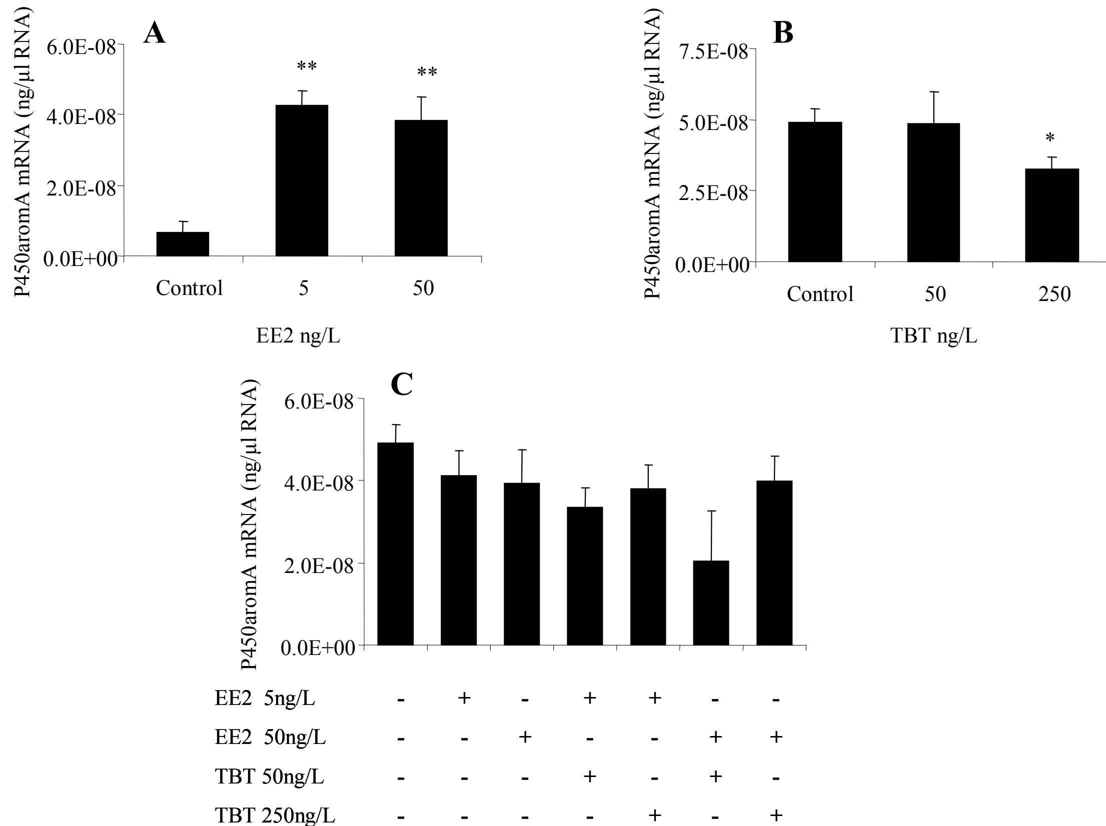

Figure 3-2. Transcriptional changes of brain P450aromA mRNA levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The P450aromA mRNA expression after 3 days of exposure to 5 and 50 ng EE2/L. (B) The P450aromA mRNA expression after 7 days of exposure to 50 and 250 ng TBT/L. (C) The P450aromA mRNA expression after 7 days of exposure to 5 and 50 ng EE2/L, singly and in combination with 50 and 250 ng TBT/L. All values represent the mean (n = 6) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control, analysed using ANOVA.

The gene expression of brain P450aromA was significantly induced 4- and 3.6-fold, respectively, after 3 days of exposure to 5 and 50 ng EE2/L, compared to the control (Figure 3-2A). In the second experiment, while exposure to 50 ng TBT/L did not have any effect, 250 ng TBT/L resulted in a 0.4-fold significant decrease in brain P450aromA gene expression after 7 days of exposure, compared to the control group (Figure 3-2B). Overall, combined EE2 and TBT exposures did not cause any significant changes in brain P450aromA gene expression (Figure 3-2C). The abundance of P450aromB was generally higher than P450aromA in the brain of controls and exposed animals.

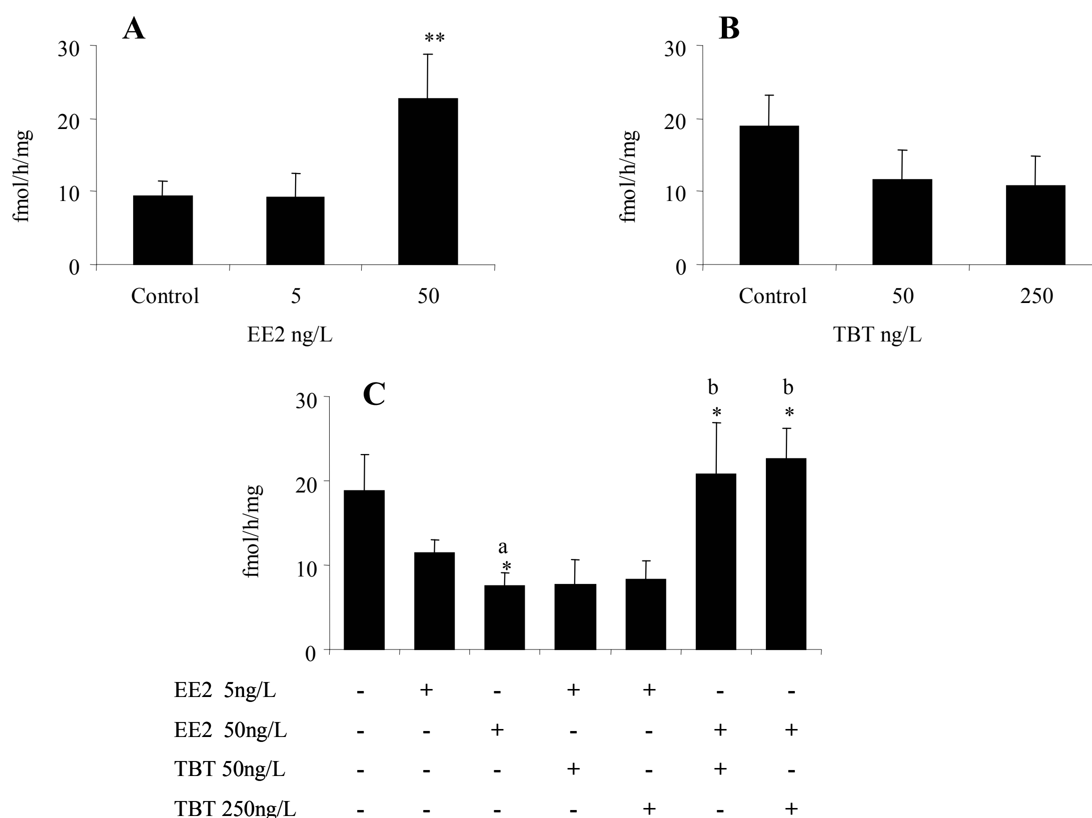

Figure 3-3. Modulation of brain aromatase activity levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) Aromatase activity levels after 3 days of exposure to 5 and 50 ng EE2/L. (B) Aromatase activity levels after 7 days of exposure to 50 and 250 ng TBT/L. (C) Aromatase activity levels after 7 days of exposure to 5 and 50 ng EE2/L, singly and in combination with 50 and 250 ng TBT/L. All values represent the mean (n = 6) ± SEM quantified using real-time PCR with specific primer pairs. $*p < 0.05$, $**p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analysed using ANOVA.

Exposure of salmon to 50 ng EE2/L for 3-days resulted in a 2.4-fold significant increase of brain aromatase activity, compared to control (Figure 3-3A). Similar to the P450arom isoform gene expression, aromatase activity was apparently inhibited after 7 days of exposure to TBT concentrations (Figure. 3-3B). Exposure to EE2 caused an apparent concentration-dependent decrease in aromatase activity at day 7 post-exposure (Figure 3-3C). In particular, 50 ng EE2/L significantly decreased aromatase activity 0.6-fold at day 7 post-exposure, compared with the control (Figure 3-3C). Co-exposure of fish to TBT concentrations with 5 ng EE2/L sustained the EE2-inhibited aromatase activity (Figure 3-3C). Interestingly, combined exposure of TBT

concentrations with 50 ng EE2/L significantly increased brain aromatase activity, compared to the 50 ng EE2/L–exposed group alone (Figure 3-3C).

Modulation of ERα gene expression

Exposure of fish to 5 and 50 ng EE2/L for 3 days caused, 8.5- and 3.5-fold significant induction of ERα mRNA expression, respectively, compared to the control (Figure 3-4A). 7-days exposure to TBT during the second experiment increased ERα mRNA levels by 1.6- and 1.7-fold at 50 and 250 ng TBT/L, exposures respectively, however not being statistically significant (Figure 3-4B). After 7-days of EE2 exposure, ERα

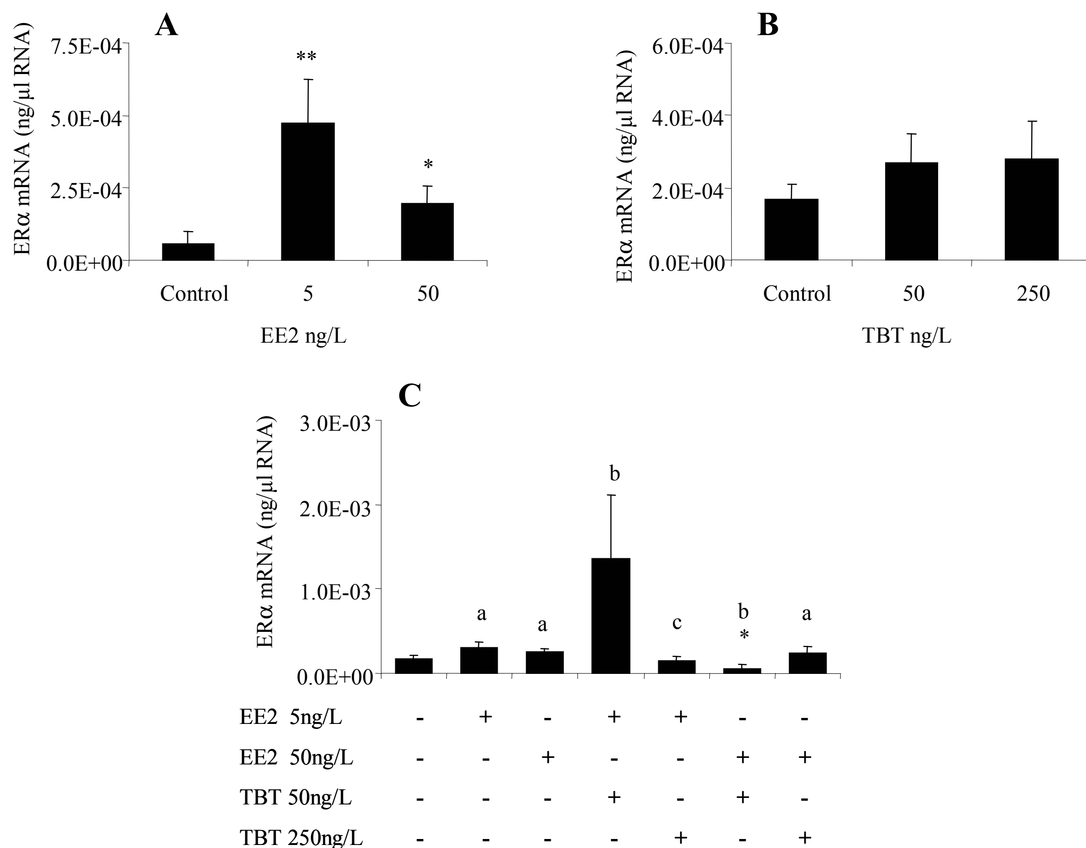

Figure 3-4. Transcriptional changes of brain ERα mRNA levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The ERα mRNA expression after 3 days of exposure to 5 and 50 ng EE2/L. (B) The ERα mRNA expression after 7 days of exposure to 50 and 250 ng TBT/L. (C) The ERα mRNA expression after 7 days of exposure to 5 and 50 ng EE2/L, singly and in combination with 50 and 250 ng TBT/L. All values represent the mean (n = 6) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analysed using ANOVA.

gene expression was still induced 1.8- and 1.5-fold in the 5 and 50 ng EE2/L exposure groups, respectively, but this induction was not significant (Figure 3-4C). Combined exposure of fish to 5 ng EE2/L and 50 ng TBT/L resulted in a 8.1-fold increase in ERα gene expression, compared to the 5 ng EE2/L exposure group alone (Figure 3-4C). When 5 ng EE2/L was given in combination with 250 ng TBT/L, the 5 ng EE2/L–induced ERα gene expression was inhibited (Figure 3-4C). Co-exposure of 50 ng EE2/L with 50 ng TBT/L significantly inhibited the EE2-induced ERα gene expression, reaching a value equal to 34% of the control (Figure 3-4C).

Modulation of plasma Vtg levels

Exposure of fish to EE2 for 3 days caused the increase of plasma Vtg levels in a concentration-dependent manner with a 2.2-fold significant increase at 50 ng EE2/L, compared to the control (Figure 3-5A). After 7 days, while TBT concentrations did not significantly affect plasma Vtg levels (Figure 3-5B), exposure to 50 ng EE2/L resulted in a significant decrease of Vtg, compared to the control (Figure 3-5C). Combined exposure of fish to 5 ng EE2/L and TBT did not significantly alter plasma Vtg levels, while coexposure of 50 ng EE2/L with 50 or 250 ng TBT/L significantly restored the 50 ng EE2-inhibited Vtg levels to almost control levels at day 7 (Figure 3-5C). Overall, it is important to note that the carrier vehicle (DMSO) used in the present study caused significant elevations of P450aromB (Figure 3-1A vs. 3-1C), P450aromA (Figure 3-2A vs. 3-2C), aromatase activity (Figure 3-3A vs. 3-3C), ERa (Figure 3-4A vs. 3-4C), and plasma Vtg (Figure 3-5A vs. 3-5C) levels after 7 days (see C-figures) of exposure compared to 3 days of (see A-figures) exposure.

3.4 Discussion

The present study has investigated the effects of EE2 and TBT (given singly and also in combination), representing two different modes of action on the endocrine system, on salmon brain P450arom gene isoforms and aromatase enzyme activity levels, in parallel with typical xenoestrogen responses (ERα and Vtg). The data of this chapter, based on nominal exposure concentrations, show that EE2 induced a concentration-specific modulation of the P450arom isoforms and activity, ERα gene, and plasma Vtg levels in juvenile salmon. TBT caused variable effects, depending on

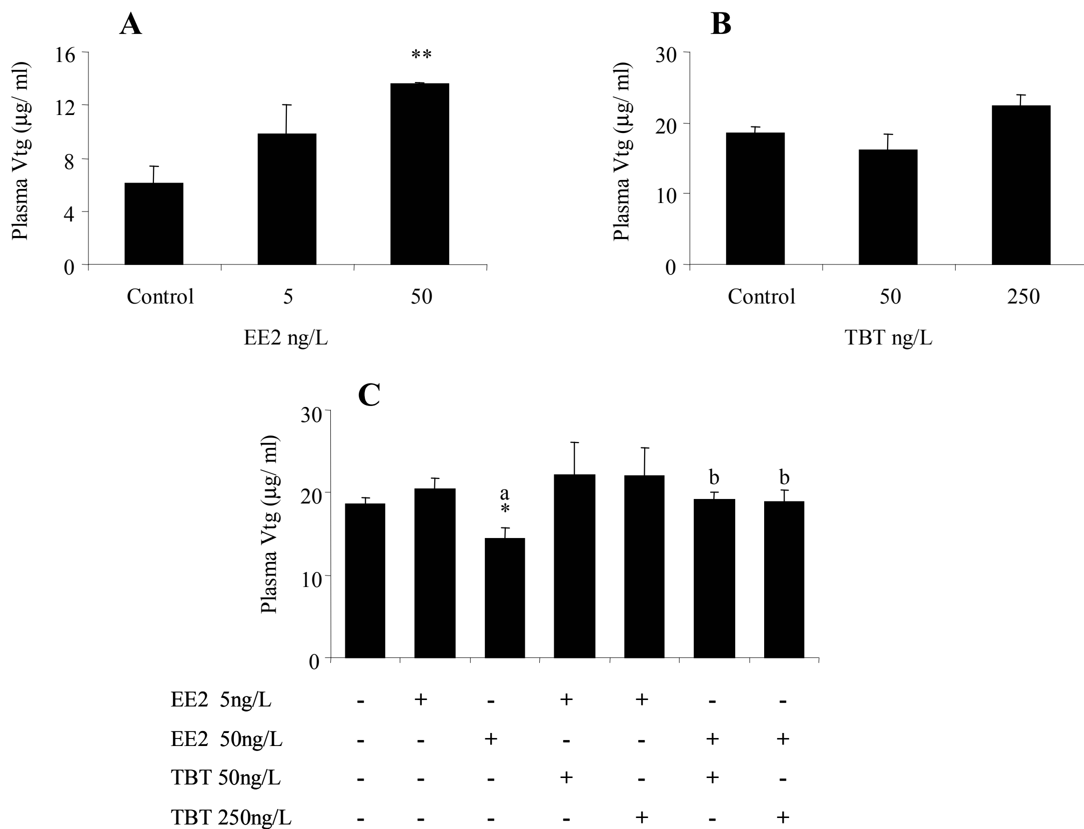

Figure 3-5. Modulation of plasma Vtg levels in juvenile Atlantic salmon exposed to waterborne EE2 and TBT. (A) The Vtg levels after 3 days of exposure to 5 and 50 ng EE2/L. (B) The Vtg levels after 7 days of exposure to 50 and 250 ng TBT/L. (C) The Vtg levels after 7 days of exposure to 5 and 50 ng EE2/L, singly and in combination with 50 and 250 ng TBT/L. All values represent the mean (n = 6) ± SEM quantified using real-time PCR with specific primer pairs. *$p < 0.05$, **$p < 0.01$ compared to solvent control. Different letters denote combined exposure groups that are significantly different ($p < 0.05$), analysed using ANOVA.

concentration, on P450arom isoforms and activity levels, ERα gene, and plasma Vtg and also antagonized EE2-induced expression levels of the studied variables.

Modulation of P450arom gene and enzyme activity

This work shows that the brain P450arom genes were differentially affected by the pharmaceutical endocrine disruptor, EE2, in juvenile salmon with P450arom isoforms showing a unique expression pattern that was dependent on concentration and time of exposure. In parallel, the brain aromatase activity was significantly increased after 3 days of exposure to EE2. Recently, several studies have shown that P450aromB mRNA levels were upregulated after steroid hormone exposure (Gelinas et al., 1998;

Lee et al., 2000; Tsai et al., 2000). In the brain of adult sea bass (*Dicentrarchus labrax*), it was shown that aromatase activity was significantly higher than that in the gonads (Gonzalez and Piferrer, 2002, 2003). In view of these studies and the unique and comparable expression pattern between brain P450aromB mRNA and activity observed in the present study, it is suggested that the brain aromatase activities reported in the present study might be due to the P450aromB isoform as opposed to P450aromA.

The finding that P450aromB has higher abundance than P450aromA is in accordance with previous studies demonstrating the expression of P450aromB in the brain with high levels of mRNA (Meucci and Arukwe, in press). The P450aromA is highly expressed in ovary with relatively low mRNA expression and enzyme activity (Callard et al., 2001). In goldfish, these two P450arom genes are nonequivalent in their tissue-specific expression, indicating distinct promoters and regulatory mechanisms (Callard et al., 2001). In accordance with the present study, brain P450arom expressions showed a differential expression pattern in neural and nonneural tissues but revealed a degree of overlap (Callard et al., 2001). Elsewhere, Kazeto et al. (2004) reported that P450aromA mRNA levels in whole-body samples of zebra fish juveniles did not show any alteration after a short-term exposure to environmental concentrations of EE2, except at high (100 nM) concentration, while significant EE2 concentration dependent inductions in P450aromB mRNA levels were observed. Similarly, continuous exposure of zebra fish fry to 170 nM of EE2 for a period of up to 10 days post-fertilization, significantly elevated the expression of P450aromB gene in whole-body tissue, and the expression of the P450aromA gene was not affected by the exposure (Trant et al., 2001).

Although the overlap between brain P450arom genes has been suggested as a result of an evolutionary remnant, it could be speculated that these differences are a result of the P450aromA function in the brain, in addition to an E2 negative feedback control mechanism. These speculations are supported by the fact that the teleost brain P450aromA isoform lacks the consensus estrogen response element (ERE) in the promoter region (Kazeto et al., 2001; Tchoudakova et al., 2001). TBT exposure inhibited both P450arom isoforms, but only the EE2-induced brain P450aromB was significantly antagonised by combined TBT exposure. These findings suggest that P450aromB could be a candidate and a sensitive exposure gene biomarker for environmentally relevant estrogenic compounds. An autoregulative loop for

P450aromB expression has been described for adult goldfish (Callard et al., 2001; Pasmanik et al., 1988) and in zebra fish embryos (Kishida and Callard, 2001; Kishida et al., 2001). The identification of EREs in the promoter region of the P450aromB gene of the zebra fish (Kazeto et al., 2001; Tchoudakova et al., 2001) points to a direct estrogen responsiveness of the P450aromB gene, and our study suggests that environmental estrogens, including synthetic estrogens such as EE2, affect the P450aromB in a similar way as the natural estrogen. The masculinising effects of TBT are well documented in invertebrates, especially in gastropods where TBT at a concentration as low as 1–2 ng/L causes the imposition of male sex organs including a penis and vas deferens (Bryan et al., 1986; Depledge and Billinghurst, 1999; Gibbs and Bryan, 1994). It has been suggested that the mechanism of TBT-induced imposex is due to the inhibition of aromatase functions, resulting in subsequent accumulation of testosterone that could otherwise be metabolised (Matthiessen and Gibbs, 1998; Spooner et al., 1991). In fish, relatively low levels (11.2 ng/L) of TBT exposure for 3 weeks decreased sperm counts in adult guppies (*Poecilia reticulata*) by 40–75% (Haubruge et al., 2000), and administration of 1 µg of TBT/g bodyweight to male Japanese medaka significantly decreased fertilisation success and suppressed sexual behavior (Nakayama et al., 2004). Juvenile zebra fish exposed to TBT concentrations for up to 70 days posthatch showed a male-biased population and production of sperm lacking flagella (McAllister and Kime, 2003). In the study by McAllister and Kime (2003), the lowest observable effect concentration (LOEC) found was below 1 ng/L. Several field studies have demonstrated a correlation between TBT concentration in water and incidence of imposex with determined LOEC of 5 ng/l. An autoradiographic study demonstrated that a substantial amount of labelled TBT accumulated in the nervous tissue of the dogwhelk, thus supporting the hypothesis that imposex is caused through TBT action on some neurohormones (Feral and LeGal, 1983). In a recent study by Yamazaki et al. (2005), it was shown that TBT at concentrations higher than 100nM suppressed all steroid biosynthesis in bovine adrenal cells, and this suppression was closely correlated to the decrease in steroidogenic acute regulatory (StAR) protein. In our study, exposure of juvenile salmon to TBT for 7 days resulted in the inhibition of brain P450arom isoform genes expression and enzyme activity and is in agreement with the theory that TBT is an aromatase inhibitor, in addition to other effects involving different mechanisms of action. Nevertheless, the TBT-induced activation of aromatase expression observed in the present study is novel and needs to

be investigated in detail, and the mechanism involved should be studied in a differently designed study. These studies are currently underway in our laboratory.

Modulation of ERα gene and plasma Vtg levels

This study has also demonstrated that EE2 induced ERα gene expression in the brain and plasma Vtg levels. The molecular basis for Vtg gene and protein expression shows that the Vtg gene activations are receptor-mediated responses that are ligand structure–dependent interactions with ER, probably involving all isoforms, in addition to other coactivators. Maintenance of Vtg synthesis and concentration in oviparous species, including fish, is achieved through activation of ERs by E2 (Specker and Sullivan, 1994). Therefore, the induction of Vtg and Zr-protein synthesis in response to E2 and their mimics has been described in several fish species (Arukwe et al., 2001; Flouriot et al., 1997; Yadetie et al., 1999). Although TBT inhibited aromatase gene expression and activity in the brain, other effects such as induction of ERα gene expression and plasma Vtg levels show that the mode of action for TBT effect is not restricted to aromatase inhibition as earlier anticipated. When adult fathead minnow (*Pimephales promelas*) were exposed to the aromatase inhibitor fadrozole for 21 days at concentrations up to 50 ng/L, aromatase activity was significantly inhibited in both males and females (Ankley et al., 2002), and this inhibition was accompanied by a significant decrease in E2 and Vtg levels in the plasma.

Relevant to the present findings, the aromatisable xenoandrogen, 17α-methyltestosterone (MT) is found to increase Vtg levels in adult fathead minnow (Hornung et al., 2004; Pawlowski et al., 2004). It was suggested that MT was possibly converted into estrogens due to the action of aromatase (Pawlowski et al., 2004; Simpson et al., 1994). The production of Vtg in goldfish by high doses of MT, ethynyltestosterone, and methylandrostenediol has also been reported, but non-aromatisable androgens did not have the same effect (Hori et al., 1979), suggesting a possible role of aromatase activity. In support of the same theory, Zerulla et al. (2002) found that estrogenic effects, such as Vtg induction, caused by MT in juvenile fathead minnow could be blocked through coadministration of an inhibitor of CYP19 aromatase to the fish. However, Hornung et al. (2004) did not find any methylestradiol in the plasma of fish, despite the fact that Vtg levels were induced. These findings and the

present study suggest a complicated mode of action for putative xenoandrogens and androgens in fish systems.

The induction of steroidogenic enzymes is highly tissue specific and cell-type specific and is controlled by different promoters and second messenger pathways. These pathways provide various targets for interaction with xenobiotics. Given the important roles of aromatase in sexual differentiation, development, reproduction, and behaviour, particularly in the gonads and the brain (Callardet al., 2001), interferences with the catalytic activity or expression of aromatase may be expected to result in disruptions of endocrine-regulated processes, such as sperm production and maturation, development of puberty, masculinisation and feminisation of (sexual) behaviour, and the inhibition or stimulation of estrogen-dependent development. Furthermore, estrogens produced from increased aromatase activity have a number of physiological functions that are dependent on the tissue and development stages of the organism. For example, estrogens are involved in the sex-dependent behavior in the brain and control bone development, lipid metabolism, and distribution in peripheral tissue. Therefore, EE2 and TBT effects, singly and also in combination, on the brain P450arom gene expressions and subsequent activity may result in altered estrogen synthesis affecting multiple tissues.

A critical observation in the present study is the fact the carrier vehicle DMSO modulated receptor-mediated and non–receptor mediated estrogenic responses at day 7 post-exposure, compared to day 3. The use of DMSO as carrier solvent as opposed to ethanol was preferred in the present work because of the evidence that alcohol may activate P450arom activity and promote the conversion of testosterone to estradiol in mature female tilapia (*Oreochromis niloticus*) and their ovaries (Kim et al., 2003). At the time of the study, there were no published data reporting possible estrogenic effects of DMSO on fish in-vivo studies. However, in our experiment, exposure of juvenile salmon to DMSO for 7 days resulted in the up-regulation of aromatase isoform genes, enzyme activity, ERα gene, and plasma Vtg, compared to the 3-day exposure levels. Kazeto et al. (2003) exposed juvenile zebra fish to EE2 and nonylphenol dissolved in DMSO (0.1% v/v) for 3 days and found that EE2 and nonylphenol induced P450aromB gene expression in a dose- dependent manner. Elsewhere, Alberti et al. (2005) exposed adult zebra fish to E2 and nonylphenol dissolved in DMSO (0.02% v/v) for 11 days and found a strong Vtg gene expression in the liver of male fish at high E2 (500 ng/L) and nonylphenol (250 µg/L) concentrations. For female fish exposed to E2 and

nonylphenol, increasing concentrations of the compounds led to a consistent decrease in aromatase gene expression in the gonads (Alberti et al., 2005). In view of these studies, the present study, and the recent review by Hutchinsonet al. (2006), it is clear that DMSO has the potential of modulating the endocrine system in addition to other effects. Therefore, the use of DMSO as carrier vehicle in both in-vitro and in-vivo fish endocrine disruption studies should be revaluated.

In summary, studies on endocrine disruption, particularly with regard to steroidogenesis, have focused mainly on reproductive steroids, but very little is known about the effects and mechanisms of xenoestrogens on neurosteroids. Recently, it was described that the brain is not only a target but also a steroid-producing area and that steroid concentration in plasma and brain fluctuate independently (Sierra, 2004). The effect of endocrine disruptors on steroidogenic pathways might have a more serious consequence for the organism than end points like egg yolk and eggshell protein inductions (Arukwe and Goksøyr, 2003). The relevance of these findings in terms of physiological, endocrine, reproductive, and ecotoxicological consequences will depend on factors such as bioavailability, biotransformation, and environmental concentration of EE2 and TBT, in addition to their interaction with other xenoestrogens. In this regard, it should be noted that the data in this work are based on nominal EE2 and TBT concentrations, and that the concentrations used might not necessarily represent the concentration of EE2 and TBT available in the experimental tanks. Regardless, in view of the present study, the fact that the concentration of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/L (Desbrow et al., 1998; Larsson et al., 1999) and concentrations of up to 50 ng/L have been reported (Aherne and Briggs, 1989), and also the U.S. study survey of 139 streams showing that several rivers had concentrations > 5 ng/L with an extreme EE2 concentration up to 273 ng/L reported at some riverine sites (Kolpin et al., 2002), pharmaceuticals in the environment represent serious health concern both to humans and wildlife.

Acknowledgments

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*Chapter 4. Ethynylestradiol modulates brain and interrenal StAR protein, P450scc, and CYP11β mRNA levels in Atlantic salmon (*salmo salar*) following short-term in-vivo exposure.*

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Abstract

Pharmaceuticals are ubiquitous pollutants in the aquatic environment, where their potential effects on non-target species like fish has only recently become subject of systematic investigations. Recently, it was shown that the documented xenoestrogen nonylphenol produced variations in brain steroidogenic acute regulatory (StAR) protein, cytochrome P-450-mediated cholesterol side-chain cleavage (P450*scc*), and cytochrome P-45011β hydroxylase (CYP11β) gene transcripts of exposed juvenile salmon (Arukwe, 2005). In the study described in this chapter, experiments were undertaken to examine the effect of the synthetic pharmaceutical endocrine disruptor ethynylestradiol (EE2), given in water at 5 or 50 ng/L and sampled at day 0 (control), 3, and 7 after exposure, on these key and rate-limiting brain and interrenal steroidogenic pathways of juvenile salmon using quantitative (real-time) polymerase chain reaction (qPCR). The data of this work, which is based on nominal exposure concentrations, show that brain and head kidney StAR and P450*scc* expression were modulated by EE2 in a time- and concentration-specific manner. While the StAR protein and P450*scc* showed EE2 concentration-dependent transcriptional increases in the brain and head kidney 3-days after exposure, no significant effect was observed at day 7. The EE2-induced effects at day 7 were underscored because the carrier solvent (dimethyl sulfoxide, DMSO) produced significant induction of the StAR protein and P450*scc* in both the brain and head kidney at day 7 compared to day 3 post-exposure. CYP11β transcript was detected in the brain and head kidney, where the expression patterns were modulated by EE2 in a concentration-and time-specific manner. In the brain, DMSO produced significant changes in the CYP11β gene expression at day 7 compared to day 3 post-exposure. These changes in the levels of StAR, P450*scc*, and CYP11β mRNA levels in important steroidogenic organs suggest that the experimental animals are experiencing a time-dependent impaired steroidogenesis. Thus, the StAR protein, P450*scc*, and CYP11β might represent sensitive diagnostic tools for short-term and acute exposure to endocrine disrupting chemicals. In view of the present study and high concentrations of EE2 reported in effluents and surface waters from Europe and the United States, pharmaceuticals in the environment represent potentially more serious health concern both to humans and wildlife than earlier anticipated.

4.1 Introduction

Human and wildlife data indicate that the reproductive system, including its associated endocrine and neural controls, may be susceptible to alterations by occupational or environmental exposures to a variety of chemical and physical agents (Singleton & Khan, 2003; DeRosa et al., 1998). Ethynylestradiol (EE2) is a pharmaceutical and potent endocrine modulator known to be present in the aquatic environment at biologically active concentrations (Rotchell & Ostrander, 2003; Nash et al., 2004). In sewage treatment work (STW) effluents, steroidal estrogens are believed to be, at least in part, responsible for the feminised responses in some wild fish species in reports from the United Kingdom (Jobling et al., 2002). The concentrations of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/L (Larsson et al., 1999), and concentrations of up to 50 ng/L have been reported (Aherne & Briggs, 1989). In the United States, a survey of 139 streams showed that several rivers had concentrations >5 ng/L with an extreme EE2 concentration up to 273 ng/L reported at some river sites (Kolpin et al., 2002). Despite the lower EE2 concentrations in surface waters compared to natural steroidal estrogens, its estrogenic potency in fish in-vivo studies is 10- to 50-fold higher than that of E2 and E1 (Segner et al., 2003), most likely due to its longer half-life and tendency to bioconcentrate (650- and 10,000-fold in whole-body tissues and bile, respectively) (Larsson et al., 1999). Steroidogenesis is the process by which specialized cells in specific tissues, such as the brain and kidney, synthesise steroid hormones. It is generally believed that the rate-limiting step in acute steroid production is the movement of cholesterol across the mitochondrial membrane by the steroidogenic acute regulatory (StAR) protein, with subsequent conversion to pregnenolone by cytochrome P-450-mediated side-chain cleavage enzyme (P450*scc*) (Sierra 2004; Geslin and Auperin 2004). Regardless of steroidogenic organ or tissue, the StAR protein and P450*scc* are rapidly synthesized in response to acute tropic hormone stimulation. Neurosteroids are produced in the brain and function in (1) stimulating and inhibiting GABAergic responses (Majewska, 1992), (2) modulating the response of Purkinje cells to excitatory amino acids (Smith 1991), and (3) controlling memory (Flood et al., 1992). Although the StAR and P450*scc* are the main proteins involved in the early steroidogenic pathway, other proteins such as 17α-hydroxylase, 3β-hydroxysteroid dehydrogenase (3β-HSD), and 21- and 11 β-hydroxylases (CYP11β) (Miller, 1988) are also key enzymes in steroidogenesis.

The CYP11β is an important and multifunctional steroidogenic enzyme that catalyzes the final step in the conversion of deoxycorticosterone and 11-deoxycortisol to corticosterone and cortisol, respectively, in steroidogenic tissues (Kime. 1993). In addition, CYP11β acts in the biosynthesis by male teleosts of the potent androgen 11-ketotestosterone (11-KT), which appears to control many aspects of spermatogenesis (Kusakabe et al., 2002). Thus, steroid hormones have diverse functions but are synthesized by biosynthetic pathways that are identical in the initial stages. Cortisol is the main corticosteroid produced by steroidogenic cells in response to pituitary secretion. The adrenocorticotropic hormone (ACTH) is the major secretory regulator, and ACTH is in turn regulated by corticosteroid releasing factor from the hypothalamus (Mommsen et al., 1999). These entire processes are under the control of negative feedback, involving the glucocorticoid receptor in the brain (Bernier & Peter, 2001), making the entire hypothalamus-pituitary-interrenal axis (HPI axis) susceptible to chemical impact (Aluru et al., 2005).

Studies on endocrine disruption, particularly with regard to steroidogenesis, focused mainly on reproductive steroids and receptor-mediated effects, but little is known about the effects and mechanisms of endocrine modulators on neural and interrenal steroid mediated effects. Recently, it was shown that nonylphenol, a documented xenoestrogen, produced a time- and dose-dependent modulation of brain StAR, P450*scc*, and CYP11β-hydroxylase (Arukwe, 2005). Research on endocrine toxicology has mainly focused on estrogenicity that involves direct estrogen receptor mediated effects. In addition to estrogenicity, there is more to endocrine disruption that may be subject to chemical disruption with equally or more severe consequences for organismal health. Furthermore, in the environment, chemical interactions may have profound consequences, since organisms are likely to be exposed to complex chemical mixtures of environmental pollutants. These complex chemical interactions have only recently become the focus of systematic investigation.

Therefore, the present study was undertaken to investigate the effect of the pharmaceutical endocrine disruptor, EE2 on brain and interrenal key and rate-limiting steroidogenic pathways using quantitative polymerase chain reduction (PCR). The hypothesis is that exposure of salmon to EE2 may induce differential time- and concentration-dependent transcriptional changes in the StAR, P450scc, and CYP11β gene expression patterns and that the differences in the profile of these key steroidogenic gene responses, including over expression or lowered expression in

exposed individuals and tissues, may be novel and prognostic of chemical susceptibility and adverse health, physiological, and reproductive effects.

4.2 Materials and Methods

Chemicals

17α-Ethynylestradiol was purchased from Sigma Aldrich Co. (St. Louis, MO). Trizol reagent for ribonucleic acid (RNA) purification and the TA cloning kit were purchased from Invitrogen Corporation (Carlsbad, CA). IScript cDNA synthesis kit and iTAQ SYBR Green Supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA) and GeneRuler 100 base pairs (bp) deoxynucleic acid (DNA) ladder and deoxynucleotide triphosphates (dNTPs) were purchased from Fermentas GmbH (Germany).

Fish and exposure

Immature Atlantic salmon (mean weight and length 10 ± 2.5 g and 9 ± 2 cm, respectively) were obtained from Lundamo hatcheries (Trondheim, Norway) and kept and exposed in 70-L aquariums at 7 ± 0.5 ºC and for a 14:10-h photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal holding facilities. In order to test the hypothesis that EE2 produces a concentration- and time-dependent modulation in the expression of brain and interrenal steroidogenic protein and enzyme genes after waterborne exposure, three groups of fish (18 individuals per group) were exposed to waterborne EE2 at concentrations of 5 or 50 ng/L and 1 group serving as solvent control was exposed to the carrier vehicle dimethyl sulfoxide (DMSO: 7.5 ppb). Fish were exposed once under static (without water replacement) and aerated environmental conditions. EE2 concentrations were chosen because they represent environmentally relevant concentrations (see introduction). Six individuals for each exposure were sacrificed in replicate at sampling day 0 (control or blank) and at day 3 and day 7 after exposure. Samples were collected after the fish were anesthetized with benzocaine (5 mg/L) and sacrificed. After sacrifice, the brain and head (anterior) kidney were excised and weighed.

Mitochondrial preparation and immunoblotting of StAR and P450scc proteins

Cells were fractionated using a mitochondrial isolation kit from Sigma-Aldrich according to the manufacturer's protocol. This kit features specially formulated extraction reagents and optimised protocols for the preparation of mitochondrial fractions from soft tissues such as liver or brain. Briefly, samples were washed twice with 2 volumes of extraction buffer A and a small portion (approximately 20–50 mg) was cut and weighed in an eppendorf tube. Tissue was homogenized in 10 volumes of extraction buffer A containing 2 mg/ml albumin using a Teflon homogeniser with 5–10 times up and down strokes on ice. The homogenate was transferred to a 2-ml Eppendorf tube and centrifuged at 600 × g for 5 min. Thereafter, the supernatant was transferred into a new tube and centrifuged for 10 min at 11,000 × g. The resulting supernatant was removed and the pellet was resuspended in 10 volumes of extraction buffer A and stored in storage buffer at –80 ºC until analysis. Total mitochondrial protein concentration was determined using the method of Bradford (1976).

In Western blotting, proteins were separated using 4% stacking and 9% separating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) before blotting as described by Towbin et al. (1979). The StAR and P450*scc* proteins were detected after reaction with the primary antibodies against synthetic peptides for the respective protein (diluted 1:3000; Arukwe et al., personal communication) and incubated overnight at room temperature. Peroxidase-conjugated goat anti-rabbit horse-radish (GAR-HRP, Bio-Rad) diluted 1:3000 was added and incubated for 1 h at room temperature. Protein bands were visualized using SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL).

Total RNA isolation and quantitative (Real-Time) polymerase chain reaction

Brain and head kidney samples obtained from individual exposures were homogenised in Trizol reagent and purified according to the manufacturer's protocol. Total RNA was DNase treated and the concentrations were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington DE). Quantitative (real-time) PCR and primer design for evaluating gene expression profiles was performed as previously described (Arukwe, 2005). The primer pair sequences and their amplicon size are as shown in Table 4-1. Total cDNA amounts for the real-time PCR reactions

were generated from 500 ng total RNA in 20 µL reaction volume from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad).

The real-time PCR assays are validated using all primer pairs in titration reactions in order to determine optimal primer pair concentrations and real-time PCR were run using reverse transcription (RT) reactions without enzyme and control RNA (Arukwe, 2005). The primer pair concentrations used for each 25 µL real-time PCR reaction were 200 pmol each of the forward and reverse primer for StAR, P450*scc*, and CYP11β. Each 25 µL DNA amplification reaction contained 12.5 µL of 2 × SYBR Green mix (Bio-Rad), 0.75 µL of 1 mM ROX (reference dye), and 1 µL of cDNA. The real-time PCR program included an enzyme activation step at 95°C (10 min) and 40 cycles of 95°C (30 s), 55 °C for CYP11β and 60°C for StAR and P450*scc* (1 min), and 72°C (30 s). Control samples lacking cDNA template or Taq DNA polymerase were included to determine the specificity of target cDNA amplification. Cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The criterion for using the standard curve was based on equal amplification efficiency (usually >90%) with unknown samples and was usually checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest as described previously by Arukwe (2005). Data obtained from triplicate runs for target cDNA amplification were averaged and expressed as nanograms per microliter of initial total RNA used for reverse transcription (cDNA) reaction.

Table 4-1. Primer pair sequences used for real time RT-PCR.

Target Gene	*	Primer sequence	Amplicon size (nucleotides)	Annealing Temperature (°C)
StAR	Forward	AGGATGGATGGACCACTGAG	163	60
	Reverse	GTCTCCCATCTGCTCCATGT		
P450*scc*	Forward	TGGAGTCCTGCTCAAGAATG	141	60
	Reverse	TTATGTACTCGGGCCACAAA		
CYP11β	Forward	AGGAGGTGGTAGTGGGGG	119	55
	Reverse	CCCCAGCCATGAGTTCAG		

*Sequences are given in the 5´ - 3´order

Statistical analyses

Standard errors were calculated using JMP statistic software V3.01 (SAS Institute, Cary, NC). Statistical differences among treatment groups were tested using analysis of variance (ANOVA) after log-transformation for variance homogeneity. For all the tests the level of significance was set at $p < 0.05$, unless otherwise stated.

4.3 Results

Changes in the StAR protein and P450scc mRNA levels

In the brain, the transcriptional levels of StAR protein were induced by exposure to EE2 in a concentration-dependent manner at day 3 post-exposure, compared to solvent control (Figure 4-1A). Exposure to 5 and 50 ng EE2/L produced a significant 5- and 11-fold increase in brain StAR protein mRNA levels (Figure 4-1A). At day 7, EE2 exposure did not affect the brain transcriptional levels of the StAR protein (Figure 4-1A). Interestingly, the carrier solvent control (DMSO) produced significant elevation of brain StAR at day 7 compared to day 3 (Figure 4-1A). In the head kidney, EE2 exposure induced a significant 1.5- and 1.4-fold respective increase in StAR protein mRNA expression at 5 and 50 ng/L at day 3 post-exposure, compared to solvent control (Figure 4-1B). EE2 exposure did not cause transcriptional changes in head kidney StAR mRNA expression at day 7 post-exposure (Figure 4-1B).

For P450*scc*, exposure to 5 and 50 ng EE2/L produced a significant 3.6- and 2.4-fold increase in brain P450scc mRNA levels, respectively, after 3 days, compared to solvent control (Figure 4-2A). While EE2 at 50 ng/L did not significantly affect brain P450*scc* mRNA levels at day 7 post-exposure, 5 ng EE2/L produced a 2-fold significant increase in brain P450*scc* at day 7 post-exposure, compared to solvent control (Figure 4-2A). In the head kidney, EE2 exposure induced a respective significant 3-fold increase in P450*scc* mRNA expression at 5 and 50 ng/L, at day 3 post-exposure, compared to solvent control (Figure 4-2B). EE2 exposure did not affect transcriptional changes in head kidney P450*scc* mRNA expression at day 7 post-exposure, compared to solvent control (Figure 4-2B). Similar to the StAR protein, the carrier solvent (DMSO)

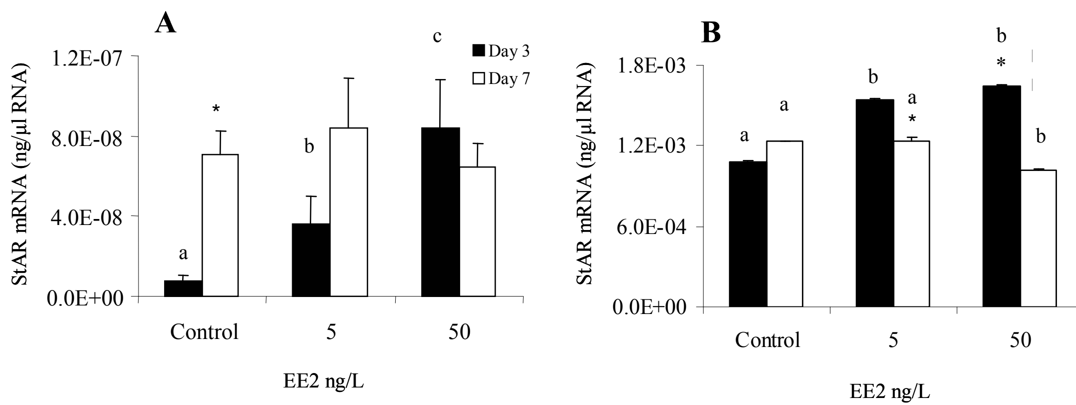

Figure 4-1. Figure 1. Changes in levels of (A) brain and (B) head kidney StAR protein mRNA in juvenile Atlantic salmon exposed to waterborne ethynylestradiol (EE2) and sampled at different time intervals after exposure. The StAR protein mRNA expression was analysed using quantitative (real-time) polymerase chain reaction (PCR) with specific primer pairs. All values represent the mean (n = 6) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$) and asterisk denotes significant different between day 7 and day 3 for corresponding exposure group ($p < 0.05$), analysed using ANOVA.

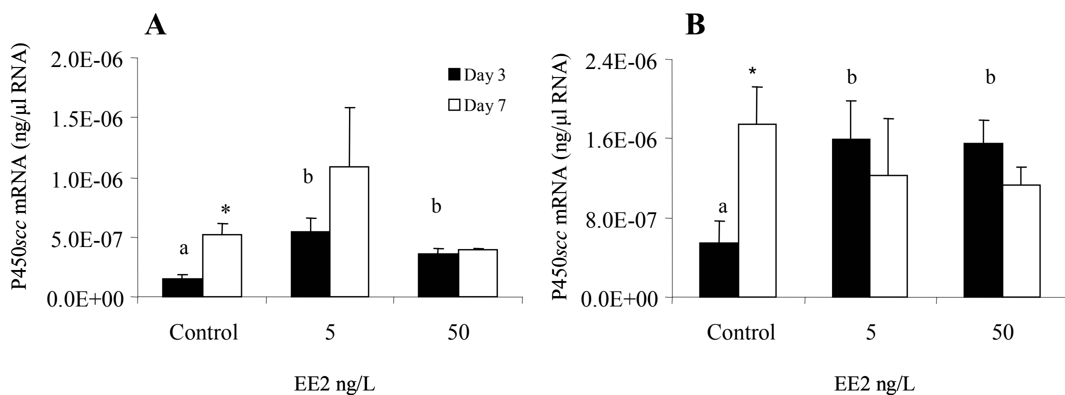

Figure 4-2. Changes in levels of (A) brain and (B) head kidney P450*scc* mRNA in juvenile Atlantic salmon exposed to waterborne ethynylestradiol (EE2) and sampled at different time intervals after exposure. The P450*scc* mRNA expression was analysed using quantitative (real-time) polymerase chain reaction (PCR) with specific primer pairs. All values represent the mean (n = 6) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$) and asterisk denotes significant different between day 7 and day 3 for corresponding exposure group ($p < 0.05$), analysed using ANOVA.

produced significant elevation of brain and head kidney P450*scc* mRNA at d 7 compared to 3 (Figure 4-2, A and B, respectively).

Immunochemical analysis, using Western blot with polyclonal antibodies against synthetic peptides for StAR and P450*scc* proteins, was used in this study to detect these proteins in randomly selected samples from control and EE2-treated juvenile salmon at d 3 post-exposure. Both antibodies recognised a protein band of 30 and 52 kD for the StAR and P450*scc*, respectively (Figure 4-3).

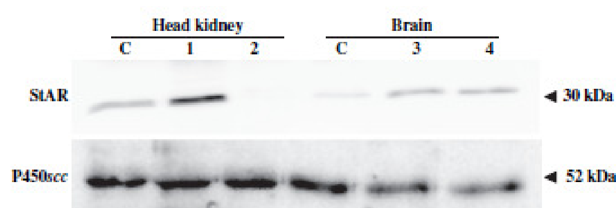

Figure 4-3. Immunoblot analysis of StAR (upper panel) and P450*scc* proteins (lower panel) in pure and crude mitochondrial fractions (respectively) in head kidney and brain of juvenile Atlantic salmon exposed to waterborne ethynylestradiol (EE2) and sampled at day 3 after exposure. Lanes C = control; Lanes 1 and 2 = head kidney tissue samples exposed to 5 and 50 ng/L EE2, respectively; Lanes 3 and 4 = brain tissue samples exposed to 5 and 50 ng/L EE2, respectively. StAR and P450*scc* proteins were probed with primary polyclonal rabbit anti-synthetic peptide antibodies. Goat anti-rabbit horseradish peroxidase (GAR-HRP) was used as secondary antibody. A total of 10 µg protein sample was applied per well, and each lane represents a randomly selected head kidney or brain tissue sample from a representative individual fish.

Changes in CYP11β mRNA levels

Changes in CYP11β mRNA Levels In the brain, the expression of CYP11β mRNA was significantly induced four- and threefold after exposure to 5 ng and 50 ng EE2/L, respectively, compared to solvent control, at day 3 post-exposure (Figure 4-4A). At day 7 post-exposure, EE2 exposure did not produce significant changes in CYP11β mRNA levels compared to solvent control (Figure 4-4A). In head kidney, EE2 did not produce significant changes (although elevated at 50 ng/L) in CYP11β mRNA levels after 3 days of exposure (Figure 4-4B).

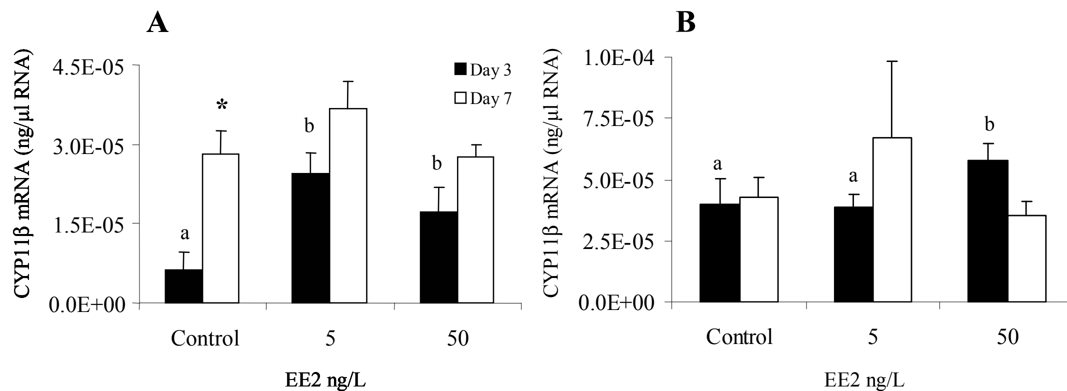

Figure 4-4. Changes in levels of (A) brain and(B) head kidney CYP11β mRNA in juvenile Atlantic salmon exposed to waterborne ethynylestradiol (EE2) and sampled at different time intervals after exposure. The CYP11β mRNA expression was analysed using quantitative (real-time) polymerase chain reaction (PCR) with specific primer pairs. All values represent the mean (n = 6) ± standard error of the mean (SEM). Different letters denote exposure groups that are significantly different ($p < 0.05$) and asterisk denotes significant different between day 7 and day 3 for corresponding exposure group ($p < 0.05$), analysed using ANOVA.

4.4 Discussion

In a recent report, experiments demonstrated the variations in three key steroidogenic proteins (StAR, P450*scc*, and CYP11β) gene transcripts in the brain of xenoestrogen (nonylphenol)-exposed juvenile salmon and suggested that these findings represent a novel aspect of neuroendocrine effects of nonylphenol in fish not previously demonstrated (Arukwe, 2005). The present study investigated the effects of EE2 on key steroidogenic genes (StAR, P450*scc*, and CYP11β) in the brain and head kidney of juvenile salmon. The present results, which are based on nominal exposure concentrations, show that EE2 produced a time- and concentration-dependent transcriptional regulation of the StAR protein, P450*scc*, and CYP11β gene expressions in the salmon brain and head kidney tissues. Thus, data showed that the synthetic pharmaceutical endocrine disruptor and ubiquitous environmental pollutant also induces variations in key and rate-limiting neuro- and interrenal steroidogenic pathways.

Changes in StAR protein and P450scc enzyme gene expressions

The present chapter shows that brain and head kidney StAR protein and P450*scc* expression were modulated by EE2 in a concentration-specific manner. The CYP11β transcript was also detected in the brain and head kidney, where the expression patterns were modulated by EE2 in an apparent concentration-specific manner at day 3 after exposure. The unique changes in the levels of StAR protein and P450*scc* (also CYP11β) mRNA levels reported in two different steroidogenic organs in the present study suggest that the experimental animals are experiencing impaired steroidogenesis. In teleosts, the endocrine response to stress is controlled by hypothalamus–pituitary–interrenal (HPI) axis (Wendelaar Bonga, 1997), leading to an increase in glucocorticoids (cortisol) that are produced in the interrenal cells of the head kidney. The linkage between the effects demonstrated in the present study lies in the fact that cortisol, like all steroids (including 11-oxygenated androgens), is generated from the precursor cholesterol (Jiang et al., 1998), and the first step in the cholesterol–steroid hormone pathway is the conversion of cholesterol to pregnenolone by P450*scc*, whose biological substrate (cholesterol) is supplied by the StAR protein. The transient increase in the StAR and P450*scc* mRNA and protein levels in response to trophic hormone stimulation provides evidence of acute steroid regulation in steroidogenic tissues (Stocco, 2000). In the present study, protein levels for the StAR and P450*scc* showed apparently a corresponding increase with the respective mRNA transcripts at day 3 post-exposure. Although the underlying mechanism for the present findings has not been characterised, it is possible that EE2 may be affecting the entire negative feedback control of steroid hormone synthesis that involve the glucocorticoid receptor in the brain (Bernier & Peter, 2001), making the entire hypothalamus-pituitary-interrenal axis (HPI axis) susceptible to chemical impact (Aluru et al., 2005).

Ethynylestradiol is a pharmaceutical endocrine disruptor known to (1) induce receptor-mediated endocrine responses, such as vitellogenin (Vtg) (Van den Belt et al., 2003), (2) produce reproductive failure (Nash et al., 2004), and (3) decrease fertility (Schultz et al., 2003) in fish species. The present findings on the effects of EE2 on key endocrine responses are novel and in accordance with previous studies demonstrating the sensitivity of the StAR protein and P450scc to pollutant mediated impairments of steroidogenesis (Fukuzawa et al., 2003, 2004; Walsh et al., 2000a). In the absence of serum hormone levels not analysed due to very low plasma samples, there are aspects

of the present study that can be compared with other studies where impaired steroidogenesis was reported in fish species after treatment with estrogenic substances. For example, it was shown that exposure of male fathead minnow to methoxychlor produced significant reduction of plasma 11-ketotestosterone (11-KT) concentrations (Ankley et al., 2001). Elsewhere, Loomis and Thomas (2000) investigated the effects of xenoestrogens on testicular androgens production using an in-vitro assay with testicular tissues from Atlantic croaker (Micropogonias undulates), and reported that several of the xenoestrogen chemicals induced concentration dependent decreases in 11-KT production. Given the important role of StAR protein and P450*scc* (also CYP11β) in the steroidogenic pathways, they may prove to be an effective molecular and cellular target for and useful biomarkers to evaluate endocrine system function in wildlife species, especially in acute and short-term exposure scenarios. Specifically, a disruption of StAR protein and P450*scc* expression may represent the first step in the sequence of related event cascades underlying xenoestrogen-induced toxicity and transmittable disturbances at the whole-organism level. Several adverse effects, including decreased fertility in wildlife species and mammals and decreased hatching success in fish, birds, and reptiles, were purportedly attributed to environmental contaminant exposure (Fairbrother et al., 2004; Rotchell & Ostrander, 2003; Arukwe & Goksøyr, 1998; Colborn et al., 1993). Some of these studies reported abnormal steroid hormone levels particular in alligators, indicating the possibility that these toxicants may block steroid hormone synthesis (Guillette et al., 1994; Orlando et al., 2002).

It was reported that two pesticides, the organochlorine insecticide lindane and the organophosphate insecticide dimethoate, which lower serum testosterone levels in animals, block steroid hormone biosynthesis in Leydig cells by reducing StAR protein expression (Walsh et al., 2000b). In another study, the modulation of interrenal steroidogenesis corresponded with lower mRNA abundance of the StAR protein and P450*scc* enzyme, but not CYP11β expression levels (Aluru et al., 2005). These findings and the present study raise the possibility that other environmental estrogens may also inhibit steroidogenesis by targeting StAR protein expression and/or other steroidogenic enzymes. In the present study, the StAR protein, P450*scc*, and CYP11β showed differential time-dependent sensitivity toward DMSO and EE2 exposure. The StAR protein and P450*scc* mediate the rate-limiting step in brain and interrenal steroidogenesis as well as other organs, and when compared to other steroidogenic

enzymes may be particularly susceptible to modulation by environmental endocrine disrupter (e.g., EE2) with negative consequences for neurosteroids, for a number of reasons. First, neurosteroidal biosynthesis may play an important role in central nervous system (CNS) development (Compagnone & Mellon, 2000) and the brain contains high concentrations of P450 and other enzymes normally utilised in steroidogenesis but the brain can also bioactivate toxicants (Volz et al., 2005), which was also demonstrated recently (Arukwe, 2005). Second, the brain is unique in its *de novo* neurosteroids synthesis, especially within glia (Compagnone & Mellon, 2000), although the relative roles of locally produced neuroactive steroids and those converted from circulating precursors remain to be defined.

Changes in CYP11β enzyme gene expression

The CYP11β is a multifunctional enzyme in the steroid hormone biosynthetic pathways. In steroidogenic tissues, pregnenolone is subjected to isomerisations and hydroxylations by several steroidogenic enzymes to produce 11-deoxycortisol that is converted to cortisol by CYP11β. CYP11β also catalyses the synthesis of 11-oxygenated androgens synthesis in testicular tissue (Rebers et al., 2000). Among the 11-oxygenated androgens, 11-KT is the most potent androgen in teleost fish (Kusakabe et al., 2002), where it controls testicular growth and differentiation. The present study shows that EE2 modulated the expression of brain and head kidney CYP11β mRNA levels. This is in accordance with the study of Yokota et al. (2005) where complete inhibition of CYP11β transcript was observed in the gonad of medaka (*Oryzias latipes*) after exposure to the xenoestrogen 4-terta-nonylphenol using RT-PCR analysis. The modulation of brain and interrenal CYP11β expression in the present study is also comparable with other studies reporting chemically induced impairment of adrenocortical responses in fish species. Impaired cortisol responses were observed in tilapia fed PCB congener 126 (Quabius et al., 1997), and in-vitro studies using rainbow trout adrenocortical steroidogenic cells showed that the pesticides atrazine, mancozeb, diazinon, and endosulfan inhibited cortisol secretion in response to ACTH or dibutyryl cAMP challenge (Aluru et al., 2005; Bisson & Hontela, 2002). These responses may have fatal consequences for animals inhabiting a stressful environment.

Effects of Dimethyl sulfoxide

A critical observation in the present work is the fact the carrier vehicle (DMSO) modulated the StAR protein, P450*scc*, and CYP11β mRNA levels at day 7 post-exposure. The 3-day EE2 exposures typically showed concentration-related increases in brain and head kidney StAR, P450*scc*, and CYP11β transcripts, but generally no significant effect was observed after 7 days of exposure. Similar effect was observed in the study presented in Chapter 3 with different estrogenic responses (induction of brain ERα mRNA and Vtg plasmatic levels) at day 7 post-exposure, compared to day 3 (Lyssimachou et al., 2006). It is unlikely that environmental stress in the exposure tanks might have contributed to the effect of DMSO observed in the present study since the experimental animals were closely monitored during the study period and showed apparent good condition. Given that modulation of brain steroidogenic enzyme genes was observed using ethanol as a carrier vehicle (Arukwe, 2005) and in accordance with the present study, it is possible that brain steroidogenic pathways might be exceptionally susceptible to organic solvents and should be studied in more detail. Despite the effect of DMSO, the time-dependent differences in the StAR and P450*scc* mRNA levels observed in the present might be explained by the physiological role of this protein and enzyme in regulating acute trophic steroid hormone levels. The use of DMSO as carrier solvent as opposed to ethanol was preferred in the present study, because of evidence that alcohol may activate steroidogenic enzymes (Arukwe, 2005) and promote the conversion of testosterone to estradiol in mature female tilapia (*Oreochromis niloticus*) and their ovaries (Kim et al., 2003).

Data recently showed that DMSO produced significant time-dependent upregulation of hepatic estrogen receptor isoforms in primary culture of salmon hepatocytes (Mortensen & Arukwe, 2006). At the time of the study there was no published data reporting possible estrogenic effects of DMSO on fish in-vivo studies. Kazeto et al. (2003) exposed juvenile zebrafish to EE2 and nonylphenol dissolved in DMSO (0.1% v/v) for 3 days and found that EE2 and nonylphenol induced P450aromB gene expression in a concentration-dependent manner. Elsewhere, Alberti et al. (2005) exposed adult zebrafish to 17β-estradiol and nonylphenol dissolved in DMSO (0.02% v/v) for 11 days and found a strong Vtg gene expression in the liver of male fish at high E2 (500 ng/L) and nonylphenol (250μg/L) concentrations. For female fish exposed to E2 and nonylphenol, increasing concentrations of the compounds led to a consistent

decrease in aromatase gene expression in the gonads (Alberti et al., 2005). In view of these studies, in the present study it is clear that DMSO has the potential of modulating the endocrine system in addition to other effects. The acute and chronic effects of carrier solvents in aquatic organisms were critically reviewed recently by Hutchinson and co-workers (2006). Therefore, the use of DMSO as carrier vehicle in both in vitro and in vivo fish endocrine disruption studies should be re-evaluated, particularly in the brain.

4.5 Conclusions

Research on endocrine toxicology has mainly focused on direct estrogen receptor-mediated estrogenicity. For a comprehensive understanding of endocrine disruption and from a regulatory point of view, research on chemical effects on the endocrine process should definitely extend beyond investigation on the androgen to estrogen conversion by aromatase (CYP19) and include, but not be limited to, key regulatory endpoints such as the role of the StAR protein, P450*scc*, and other cytochrome P-450 and dehydrogenase enzymes (Harvey & Everett, 2003; Ohno et al., 2002). Despite the integral role(s) in developmental and reproductive processes little is known about the effects and mechanisms of xenoestrogens on neural and interrenal steroids. It has been shown that the brain is not only a target but also a steroid-producing organ, and that steroid concentrations in plasma and brain fluctuate independently (Sierra, 2004). Research and current testing strategies to assess the endocrine-disrupting properties of chemicals have omitted the examination of the neural and interrenal (adrenal) gland and do not adequately cover the process of steroidogenesis. The effect of endocrine disruptors on steroidogenic pathways might have a more serious consequence for the organism than endpoints like egg-yolk and eggshell protein inductions (Arukwe & Goksøyr, 2003). In view of the present study and that the concentrations of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/L (Larsson et al., 1999) and that concentrations of up to 50 ng/L were found (Aherne & Briggs, 1989), and also that a U.S. study survey of 139 streams showed that several rivers had concentrations >5 ng/L with an extreme EE2 concentration up to 273 ng/ L reported at some river sites (Kolpin et al., 2002), pharmaceuticals in the environment represent serious health concern to both humans and wildlife.

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*Chapter 5. Gemfibrozil modulates Cytochrome P450 and peroxisome proliferation inducible enzymes in the liver of Eels (*Anguilla anguilla*) following in-vivo exposure*

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Abstract

Gemfibrozil (GEM) the human lipid regulator has been shown to induce peroxisome proliferation in rodents leading to hepatocarcinogenesis. Since GEM is found at biological active concentrations in the aquatic environment the present study was constructed to examine the effects of this drug on the metabolism of eels (*Anguilla anguilla*) representing a non-target species of exposure. Thus, eels were injected to different GEM concentrations and were sampled 24-h and 96-h post-injection. The interference of GEM with CYP catalytic enzymes involved in the metabolism of endogenous compounds, xenobiotics and fatty acid hydroxylation (CYP1A, CYP3A-, CYP2K- and CYP2M-like), phase II catalytic enzymes (glucuronyltransferase, glutathione-*S*-transferase and acyl-CoA: steroid acyltransferase), peroxisome proliferation inducible enzymes (liver peroxisomal acyl-CoA oxidase and catalase) and plasmatic levels of 17β-estradiol and testosterone was evaluated. GEM was shown to inhibit CYP1A, CYP3A- and CYP2K-like catalytic activities 24-h post-injection but 96-h post-injection only CYP1A was significantly altered, which was induced in fish from the high exposure group (200 µg GEM/g). On the contrary GEM had little effect on the phase II enzymes examined, causing an inhibition in UGT activity only 96-h post-injection in fish that received 200 µg GEM/g. 96-h post-injection AOX activity, the enzyme responsible for the β-oxidation of long and very long fatty acids, was significantly induced and so did catalase but to a minor extent. Therefore, GEM appears to induce typical peroxisome proliferation enzymatic activities however to a much lesser degree than what it has been observed in rodents. Nevertheless, GEM being a human hypolipidemic drug appears to induce peroxisomal oxidation of fatty acids and inhibit CYP catalysing activities in eels following in-vivo exposure and therefore the significance of these changes and their consequences in the health of fish should be further studied.

5.1 Introduction

GEM belongs to the group of fibrate drugs and is used in human medicine as a hypolipidemic agent, which decreases serum triglycerides and very low density lipoproteins (VLDL) and increases high density lipoproteins (HDL) (Spencer and Barradell, 1996). It is metabolised in the liver and approximately 70% is excreted through the urine, mostly as a glucuronide conjugate (Zimetbaum et al., 1991). Glucuronides are principally inactive, but they get cleaved during sewage treatment to release the active compound (Ternes et al., 1999). Thus, GEM has been detected in surface waters at concentrations of a few ng/L (Koplin et al., 2002; Gagné et al., 2006), but concentrations up to 1-5 µg/L have been reported in sewage treatment effluents from various European countries and Brazil (Stumpf et al., 1999; Andreozzi et al., 2003). GEM has an estimated half life of 15-h in river surface waters and has a K_{ow} value of 4.77 (Lin and Reinhard, 2005). Fish exposed to 1.5 µg/L for 14 days showed a bioconcentration factor in plasma of 113, resulting in plasma concentrations up to 190 µg/L (Mimeault et al., 2005).

The mechanism of action of GEM and other fibrates is mediated in part through activation of the peroxisome proliferator-activated receptor α (PPARα), which induces the transcription of genes encoding for proteins that control lipoprotein metabolism (Rader and Haffner, 1999; Krey et al., 2007). Fibrates are recognised to be peroxisome proliferators, as upon administration to rodents, they increase the size and number of hepatic peroxisomes, as well as the activity of catalase, a typical peroxisomal enzyme (McGuire et al., 1992). Long-term exposure to synthetic peroxisome proliferators leads to the development of hepatocellular carcinoma in rats and mice but not in humans and other mammals (Reddy, 2004). Peroxisome proliferation is linked to the induction of the transcription of genes involved in peroxisomal β-oxidation of fatty acids (Reddy and Hashimoto, 2001). Thus, exposure of male rats to GEM increased hepatic peroxisomal fatty acyl-CoA oxidase (AOX) activity, the rate limiting enzyme of the β-oxidation of fatty acids (Sausen et al., 1995). Additionally, fibrates, including GEM have been shown to induce esterification of sex-steroid hormones and ω-hydroxylation of fatty acids in rodents through the induction of acyl-CoA: steroid acyltransferases and CYP4A, respectively (Xu et al., 2001, 2002; Fan et al., 2004). Exposure of rainbow trout to ciprofibrate and to GEM induced hepatic peroxisomal AOX activity (Yang et al., 1990; Scarano et al., 1994), suggesting that fibrates act as peroxisome proliferators in

fish as well. Nevertheless, data regarding the effects of GEM on fish is scarce. It has been reported that waterborne exposure to GEM decreased plasma testosterone levels in goldfish and induced oxidative stress (Mimeault et al., 2005; 2006).

Oxidation and conjugation reactions catalysed by cytochrome P450 monoxygenases and glycol- and sulfotransferases are among the most important metabolic systems involved in human drug metabolism. Similarly, those reactions play a key role in the metabolism of endogenous and xenobiotic compounds in both human and fish (Williams et al., 1998). Thus, in rainbow trout for example, CYP proteins belonging to CYP1A (Williams and Buhler, 1984; Heilmann et al., 1988) , CYP2K-like(Buhler et al., 1994), CYP2M-like(Yang et al., 1998; Thibaut et al., 2002) and CYP3A (Lee et al., 1997) subfamilies have been identified and sequenced predominately from liver samples. Additionally, phase II enzymes including UDP-glucuronosyltransferases (UPDGTs) have been identified in various fish species(Martin-Skilton et al., 2006; Linderoth et al., 2007).

Pharmaceuticals undergo a series of toxicological tests before they are manufactured and distributed in the market. Therefore, there is acute toxicity data on laboratory aquatic organisms such as algae, zooplankton, *Daphnia magna* (Halling-Sørensen et al., 1998), but studies focussing on chronic toxicity using sublethal concentrations of pharmaceuticals are scarce (Ferrari et al., 2004). In-vitro studies are useful tools to identify the modes of action of pharmaceuticals in non-target organisms and then to proceed to in-vivo studies. Following this approach, we previously incubated carp liver subcellular fractions with various fibrate, anti-inflammatory and anti-depressive drugs in-vitro and evaluated their interaction with CYP catalysed pathways (CYP1A, CYP3A-, CYP2K-, and CYP2M-like) and phase II activities (UDP-glucuronosyltransferases and sulfotransferases) which are involved in both xenobiotic and endogenous metabolism in fish (Thibaut et al., 2006). GEM acted as an inhibitor of all the measured catalytic activities.

Therefore the study of the present chapter was designed to further investigate the effects of GEM on eels (*Anguilla anguilla*) metabolism after single injections of different GEM concentrations, and sampling 24h and 96h post-injection. The interference of GEM with the enzymes involved in phase I (CYP1A, CYP2K-, CYP2M- and CYP3A-like), phase II metabolism (glucuronyltransferase, glutathione-S-transferase, esterification of testosterone) and peroxisome proliferation (liver

peroxisomal AOX activity and catalase) was evaluated together with the plasmatic levels of 17β-estradiol and testosterone.

5.2 Materials and Methods

Chemicals.

[4-^{14}C]-testosterone and [1-^{14}C]-lauric acid were purchased from Amersham Biosciences (Buckinghamshire, England). Gemfibrozil, *p*- nitrophenol, UDPGA, 1-Cl-2,4-dinitrobenzene, H_2O_2 (30%), NADPH and palmitoyl-CoA were obtained from Sigma (Steinheim, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin was purchased from Cypex (Dundee, Scotland, UK). Radioimmunoassay (RIA) kits for testosterone and 17β-estradiol were obtained from Radim (Rome, Italy). 2,7- Dichlorodihydrofluorescein (H_2DCF) diacetate was purchased from Molecular Probes (Paisley, UK). All solvents and reagents were of analytical grade.

Experimental design

Immature eels (*Anguilla anguilla*; mean weight and length 81 ± 8 g and 35 ± 2 cm, respectively) were obtained from the Centre of Aquaculture, IRTA (Tarragona, Spain) and were kept in 100L (×10) tanks at 18 ± 0.2 ºC.

In order to examine the effects of GEM exposure on the enzymatic metabolism of the eels, two experiments were carried out with different exposure times and doses. For each experiment, 8 fish were randomly selected and assigned to each of the five treatment groups. Fish were anaesthetised using fenoxiacetic solution and a final volume of 2 µl/g of experimental solutions was injected into the peritoneal cavity of the fish. GEM was dissolved in sunflower oil and was administered just once at the beginning of each experiment. The first experiment was carried out for a 96-h period and fish from the different treatment groups received injections of 2, 20 and 200 µg GEM/g. The second experiment was carried out for a 24-h period and fish from the different groups received 0.1, 1 and 10 µg GEM/g. Solvent control groups from each experiment received sunflower oil injections of 2 µl/g. Fish from the control group were anaesthetised and manipulated as the other fish but they were not injected. During the experiment fish were starved.

After the corresponding period fish were anaesthetised and their weight and length was recorded before decapitation for blood collection. Blood samples were collected in eppendorf tubes containing heparin, centrifuged at 5,000 × g for 5 min at 4 ºC, and plasma was removed and stored at -80 ºC until analysis for sex steroids. Liver samples were excised, frozen in liquid nitrogen and maintained at -80 ºC until analyses.

Preparation of liver subcellular fractions.

After weighting, a small portion of the liver was cut and processed for AOX activity as described below and the rest of the liver was processed for the preparation of cytosolic and microsomal fractions. Livers were homogenised in 1:4 (w/v) of cold 100 mM potassium phosphate buffer pH 7.4, containing 100mM KCl, 1mM EDTA, 1mM dithiothreitol, 0.1 mM phenantroline and 0.1 mg/ml trypsine inhibitor. Microsomal and cytosolic fractions were prepared by differential centrifugation and ultracentrifugation as described in Thibaut and Porte, (2004). Protein content was measured according to Lowry et al. (1951), using bovine serum albumin as standard.

Cytochrome P450 catalysed pathways.

7-Ethoxyresorufin *O*-deethylase (EROD) activity was assayed as described by Thibaut et al. (2006). Briefly, 100 µg of liver microsomal proteins were incubated with 3.7 µM 7-ethoxyresorufin and 225 µM NADPH in 100 mM potassium phosphate buffer pH 7.4 (final volume 250 µL) at 30 ºC for 10 min. Reaction was stopped by the addition of 400 µL of acetonitrile and after centrifugation (2000 × g; 10 min) an aliquot of the supernatant (200 µL) was transferred to a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelengths pars of 537/583 nm using a microplate reader (Varioskan, Thermo Electron Corporation). Quantification was made by calibration with 7-hydroxyresorufin. Duplicates were done for each sample. The blank contained microsomal proteins and NADPH, and the reaction was blocked by addition of acetonitrile before incubation. BFC-*O*-debenzyloxylase (BFCOD) activity was analysed as described by Thibaut et al. (2006). Briefly, 25 µg of liver microsomal protein were incubated with 200 µM 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 µM of NADPH in 100 mM potassium phosphate buffer pH 7.4 (final volume 250 µL) at 30 ºC

for 10 min. The reaction was stopped by addition of 0.5 M Tris-base/acetonitrile (20:80, v/v), and after centrifugation (2000 - g; 10 min) an aliquot of the supernatant (200 µL) was transferred to a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelengths pars of 409/530 nm, using a microplate reader (Varioskan, Thermo Electron Corporation). Quantification was made using a calibration curve of 7-hydroxy-4-(trifluoromethyl)-coumarin standard. Duplicates were done for each sample. The blank contained microsomal proteins, NADPH and BCF, and the reaction was blocked by addition of 0.5 M Tris-base/acetonitrile (20:80, v/v) before incubation. Laurate hydroxylase activities were analysed according to a procedure adapted from Thibaut et al., (2002). Briefly, 100 µg of liver microsomal proteins were incubated with 50 µM [1-^{14}C]-lauric acid (LA) and 1 mM NADPH in 50 mM Tris-HCl buffer pH 7.4 (final volume 250 µL) at 30 ºC for 30 min. The reaction was stopped by adding 250 µL of methanol and after centrifugation (2000 × g; 10 min) 250 µL of the supernatant were analysed by reverse-phase-HPLC as described in Thibaut et al., (2002). Chromatographic peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo-scint 3 (Packard Bioscience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were identified on the basis of retention time and quantified by integrating the area under the radioactive peaks.

*Phase II activities***.**

UDP- glucuronosyltransferase (UGT) activity was measured using *p*- nitrophenol (*p*-NP) as a model substrate as described in Clark et al. (1992). Briefly, 250 µg of liver microsomal proteins (pre-treated with 0.2 % Triton X-100 and left for 15 min on ice) were incubated with 3.15 mM UDPGA and 80 µM of *p*-NP in 50 mM Tris-HCl buffer pH 7.4 containing 10 mM $MgCl_2$ (final volume 260 µL) at 30 ºC for 30 min. The reaction was stopped by adding 0.2 M of cold tricloroacetic acid, the solution was centrifuged (2000 × g; 15 min), then 1200 µL of the supernatant was alkilinised with 10 M KOH, centrifuged again (2000 × g; 15 min) and 1ml of the supernatant was transferred to a glass quartz cuvette. Decrease in absorbance of *p*-NP was measured spectrophotometrically at 405 nm. The samples were assessed in duplicates with a blank containing an equal amount of microsomal proteins but stopped before incubation for each set. Glutathione-S-transferase (GST) activity was determined

according to the method of Habig et al. (1974). Briefly, the assay mixture consisted of 100 mM phosphate buffer pH 7.4, 1 mM reduced glutathione, 1 mM of 1-Cl-2,4-dinitrobenzene (CDNB) and an adequate dilution of the liver cytosolic fraction. The change in absorbance was recorded at 340 nm and GST activity was calculated as CDNB conjugate formed/min/mg of cytosolic protein using the molar extinction coefficient $\varepsilon_{340} = 9.6\ mM^{-1}\ cm^{-1}$. Acyl-CoA: testosterone acyltransferase (ATAT) activity was measured using testosterone and palmitoyl-CoA as substrates as it has been described by Janer et al. (2004) following some modifications. Microsomal proteins (250 µg) were incubated in 0.1 M sodium acetate buffer pH 6.0 with 5 µM [^{14}C]testosterone (150,000 dpm), 0.1 mM palmitoyl-CoA and 5mM $MgCl_2$ in a final volume of 250 µL. The reaction was initiated by the addition of palmitoyl-CoA and samples were incubated for 30 min at 30 ºC. The reaction was stopped by adding 1 mL of ethyl acetate and then extracted (×3). The ethyl acetate fraction was evaporated to dryness, the dry residue was redissolved in methanol and injected into the HPLC system that consisted of a Perkin-Elmer Binary 250 LC pump system equipped with a 250 mm × 4 mm LiChrospher 100 RP-18 (5 µm) reversed-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 µm). Separation of testosterone and its palmitoylester was performed at 1.2 mL/min with a mobile phase composed of (A) 56% water containing 0.1% acetic acid (pH 3.0), 13% acetonitrile and 31% methanol and (B) 100% methanol. The run consisted of 9 min of isocratic 100% A, 6min of a linear gradient from 100% A to 100% B and 25 min of isocratic 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radio flow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks and identified by comparison of the retention times with authentic standards.

Peroxisome proliferation enzymatic activities.

Fatty acyl-CoA (palmitoyl-CoA) oxidase (AOX) activity was assayed by the determination of H_2O_2 production coupled to the oxidation of leuco-DCF in a reaction catalysed by exogenous peroxidase. The method was modified after Small et al., (1985). A portion of the liver of each fish (0.1 ± 0.01 g) was dissected and

homogenised in 0.5 mL of TVBE buffer pH 7.6, containing 1 mM sodium bicarbonate, 0.1 M EDTA, 0.1% ethanol and 0.01% Triton X-100. After homogenisation, samples were centrifuged at 500 $\times$ *g* for 15 minutes and the supernatant containing the peroxisomes was assayed for FAO activity. 50 μL of the enriched peroxisomal fraction were pre-incubated in the dark with 0.05 mM leuco DCF (prepared every 5 days by hydrolysing 2.66 mM H_2DCF diacetate in 1:9 v/v, dimethylformamide: NaOH (0.01 M) and stored at -20°C), 0.07 mg horseradish peroxidase, 40 mM sodium azide, 0.02% Triton X-100, 15 μM FAD in 100 mM Tris-HCl buffer pH 8.5 (total volume 1 mL) at 25 ºC for 3 min. Pre-incubation is necessary as some impurities in the peroxidase may cause a small amount of oxidation of the leuco-DCF (Köchli and von Wartburg, 1978). After this time, the slow rate of auto-oxidation of the dye was determined by measuring spectrophotometrically the absorption at λ= 502 nm for 2 min. The reaction was then started with the addition of 30 μM of palmitoyl-CoA and after 15 sec incubation in the dark, the enzymatic reaction rate was determined for 2 min. Rates were corrected by subtracting the blank and calculations were further processed by using the DCF molar extinction coefficient of 91,000 M^{-1} as obtained by Kochli and von Wartburg (1978) from the peroxidase-catalysed oxidation of leuco-DCF. Catalase (CAT) activity was determined in liver cytosolic fractions as described by Livingstone et al. (1992). Briefly, the assay mixture contained 100 mM phosphate buffer pH 7.0, 50 mM H_2O_2 and an adequate dilution of the liver cytosolic fraction in a final volume of 3 mL. The change in absorbance was recorded at 240 nm spectrophotometrically and CAT activity was calculated in terms of the H_2O_2 consumed/minute/mg of cytosolic protein using the molar extinction coefficient ε_{240} = 40 M^{-1} cm^{-1}.

Plasma steroid analysis.

Testosterone (T) and estradiol (E_2) levels in plasma were assayed by radioimmunoassay using ^{125}I-RIA kit. Plasma samples from the fish (300-400 μL) were extracted with diethyl ether ($\times$3), the extracts were dried under a nitrogen stream and resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine. Detection limits were 50 pg/ml for T and 10 pg/ml for E_2.

Calculations and statistics.

Fulton's condition factor (K) was calculated according to the equation K = (Body Weight $\times$ Length $^{-3}$) $\times$ 100. The Hepato Somatic Index (HIS) was calculated as Liver Weight / Body Weight $\times$ 100. Results are mean values ± S.E.M. Statistical significance was assessed by one-way ANOVA (Dunnett´s test for differences from solvent control).

5.3 Results

Morphological parameters.

Morphometric data of control and exposed fish from both experiments is given in Table 1. In both experiments GEM exposure had no effect on H.S.I and condition factor. At the 96-h experiment, GEM caused a significant decrease (6.6%) in body weight of fish injected with 200 µg GEM/g, which is 4-times the weight loss detected in the control group.

Table 5-1. Morphological data of the eels at the end of the two experiments.

	GEM µg/g	Body Weight (g)	Body Length (cm)	% Weight Loss	H.S.I.	Condition Factor
24-h	Control	77 ± 2	34.7 ± 0.5	1.2 ± 0.3	1.10 ± 0.05	1.84 ± 0.07
	0.1	81 ± 3	34.2 ± 0.3	0.7 ± 0.6	1.12 ± 0.05	2.02 ± 0.05
	1	78 ± 3	34.5 ± 0.4	1.3 ± 0.3	1.06 ± 0.03	1.89 ± 0.04
	10	83 ± 4	34.8 ± 0.5	0.6 ± 0.1	1.15 ± 0.08	1.96 ± 0.07
96-h	Control	82 ± 3	36.0 ± 0.5	1.7 ± 0.5	1.12 ± 0.06	1.78 ± 0.06
	2	81 ± 5	34.6 ± 1.4	1.2 ± 0.2	1.08 ± 0.03	2.01 ± 0.17
	20	80 ± 2	34.8 ± 0.4	2.4 ± 0.4	1.10 ± 0.06	1.90 ± 0.08
	200	75 ± 3	33.9 ± 0.7*	6.6 ± 0.5*	1.15 ± 0.06	1.93 ± 0.08

Values are expressed as mean ± S.E.M. Body weight and length values provided were taken at the end of the experiments. * indicates significant differences from the control group (n = 8; p < 0.05).

CYP-catalytic activities.

The interaction of GEM with CYP1A was assessed by measuring EROD activity in liver microsomal fractions. 24-h post-injection, fish that received 1 and 10 µg GEM/g

showed a 46 and 42% inhibition of EROD activity respectively when compared to the control (Figure 5-1). 96-h post-injection only fish in the high dose group (200 µg GEM/g) showed a significant inhibition (33%) of EROD activity (Figure 5-1). The effect of GEM on CYP3A-like catalytic activity was examined by measuring BFCOD activity. 24-h post-injection, CYP3A-like activity was inhibited significantly in all the fish groups (0.1, 1 and 10 µg GEM/g) by 37, 51 and 40% respectively (Figure 5-1). 96-h post-injection CYP3A-like activity showed a tendency towards inhibition (20-33%) but it was not statistically significant. Regarding laurate hydroxylase activities, the HPLC metabolic profile obtained for LA in eel liver microsomes showed the presence of three metabolites that could be identified as ω-, (ω-1)- and (ω-2)-hydroxy-LA by comparison of their retention times with authentic standards. In trout, the CYP2M1 form (LMC1) catalyzes the ω-hydroxylation of LA, while the CYP2K1 form (LMC2) catalyzes

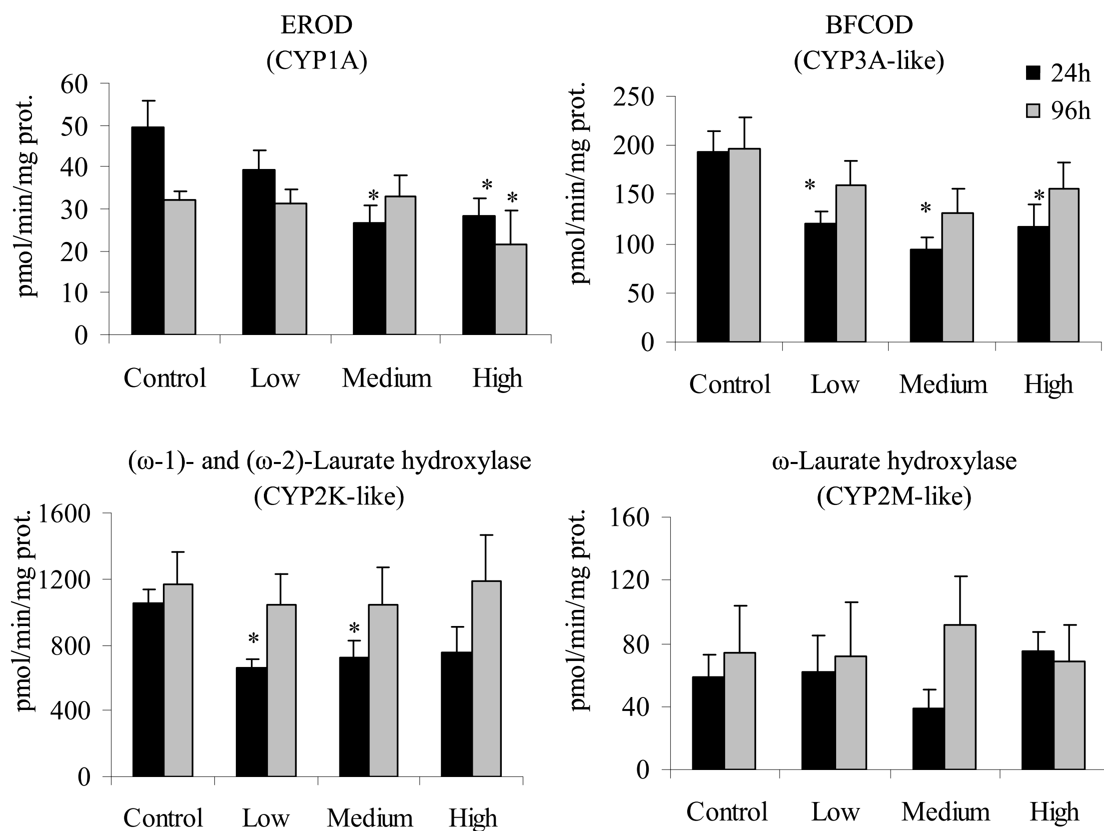

Figure 5-1. The effect of gemfibrozil on different cytochrome P450 related activities in liver microsomal fractions of eels 24-h and 96-h post-injection (for 24-h, Low, Medium, and High, correspond to 0.1, 1 and 10 µg GEM/g respectively; for 96-h, Low, Medium, and High correspond to 2, 20, and 200 µg GEM/g respectively). Values are mean ± S.E.M., n= 8. * indicates significant differences from the control for p< 0.05.

the formation of (ω-1)- and (ω-2)-hydroxylates (Buhler and Wang-Buhler, 1998). Since CYP2M- and CYP2K-like enzymes with these same regio-specific hydroxylation properties exist in other fish species such as salmon and carp (Thibaut et al., 2002; Thibaut et al., 2006), eel subterminal and terminal hydroxylations were attributed to CYP2K- and CYP2M-like, respectively. Thus, the effects of GEM were assessed on CYP2K- (sum of (ω-1)- and (ω-2)-hydroxy-LA) and CYP2M-like (ω-hydroxy-LA) related activities (Figure 5-1). 24-h post-injection of 0.1 and 1 µg/g of GEM, CYP2K-like activity decreased by 37 and 31% respectively (Figure 5-1). An apparent and weaker decrease of CYP2K-like activity was observed in fish injected with 10 µg/g of GEM (28 %). CYP2M-like activity was not affected by GEM exposure. 96-h post-injection of higher doses of GEM (2, 20 and 200 µg/g), no significant changes in LA hydroxylation were observed.

Phase II activities.

24-h post-injection no significant alterations in UGT activity in GEM exposed fish were observed. 96-h post-injection, fish that received the highest dose of GEM (200 µg/g) showed a 32% inhibition compared to the control group (Figure 5-2). In the case of GST activity, GEM exposure did not cause any significant alterations in either of the experiments (Figure 5-2). ATAT activity measured in liver microsomal fractions incubated with ^{14}C-testosterone and palmitoyl-CoA was within the range of 1.7-7.7 pmol/min/mg of microsomal protein in both experiments and GEM exposure did not cause a significant alteration. However, 96-h post-injection a 40% induction in ATAT activity was observed in organisms injected with 2 µg/g GEM exposure, nevertheless this increase was not statistically significant (p= 0.10).

Peroxisome proliferation enzymatic activities

The effect of GEM on the peroxisomal β-oxidation of fatty acids of the eels was measured in peroxisomal enriched liver fractions using a spectrophotometric assay based on the H_2O_2-depended oxidation of leuco-DCF catalysed by exogenous peroxidase. 96-h post-injection, GEM caused an induction of AOX activity and fish that received 20 and 200 µg GEM/g, the AOX activity was induced significantly by 22 and 30% respectively (Figure 5-3). However, no significant alteration of AOX activity was

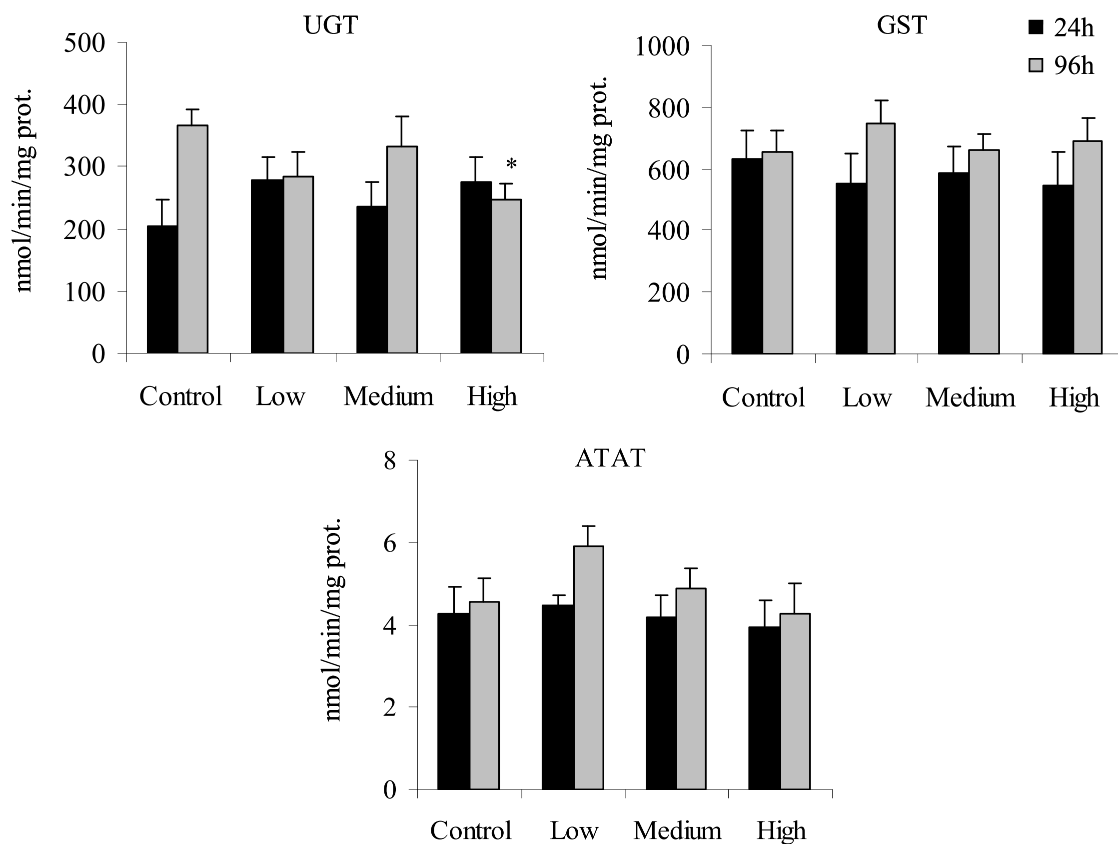

Figure 5-2. The effect of gemfibrozil on the phase II metabolism-enzymes, UDP-glucuronosyltransferase (UGT), glutathione-*S*-transferase (GST) and Acyl-CoA: testosterone acyltransferase (ATAT) activity in eels 24-h and 96-h post-injection (for 24-h, Low, Medium, and High, correspond to 0.1, 1 and 10 µg GEM/g respectively; for 96-h, Low, Medium, and High correspond to 2, 20, and 200 µg GEM/g respectively). Values are mean ± S.E.M., n= 8. * indicates significant differences from the control for $p< 0.05$.

observed 24-h post-injection, despite of a tendency towards higher activities in fish that received 1 and 10 µg GEM/g. Similarly, CAT activity was induced 96-h post-injection in fish that received 2 and 200 µg GEM/g but only at the low dose (2 µg GEM/g) the induction (39%) was statistically significant (Figure 5-3). No significant alterations were observed on CAT activity 24-h post-injection.

Plasma steroids

Plasma T (25-981 pg/ml) was 10-fold higher than plasma E_2 (2.8-80.4 pg/ml) and E_2 levels were below the detection limit (10 pg/ml) in some of the fish. 24-h post-injection, E_2 levels in fish that received 1 and 10 µg GEM/g were reduced by 50% but this

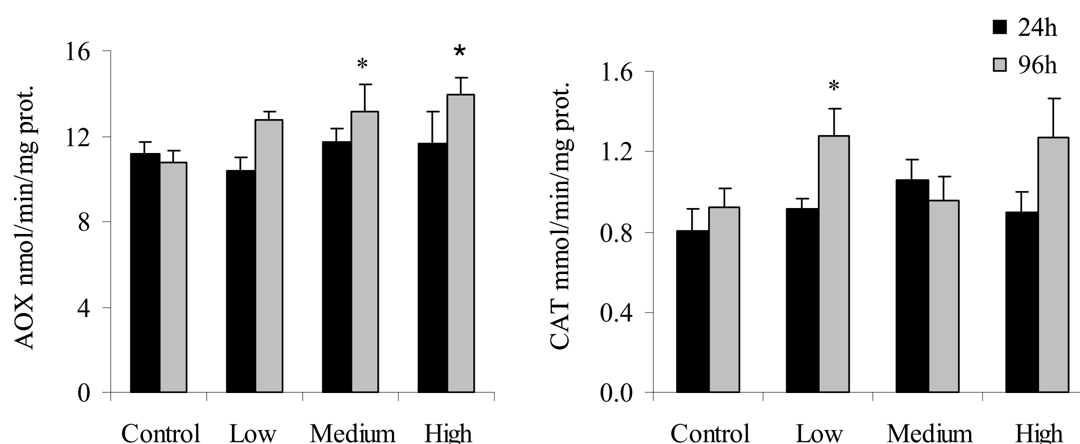

Figure 5-3. The effect of gemfibrozil on the peroxisome proliferation induced enzymes, fatty acyl-CoA oxidase (AOX) and catalase (CAT) activity, in eels 24-h and 96-h post-injection (for 24-h, Low, Medium, and High, correspond to 0.1, 1 and 10 µg GEM/g respectively; for 96-h, Low, Medium, and High correspond to 2, 20, and 200 µg GEM/g respectively). Values are mean ± S.E.M., n= 8. * indicates significant differences from the control for $p< 0.05$.

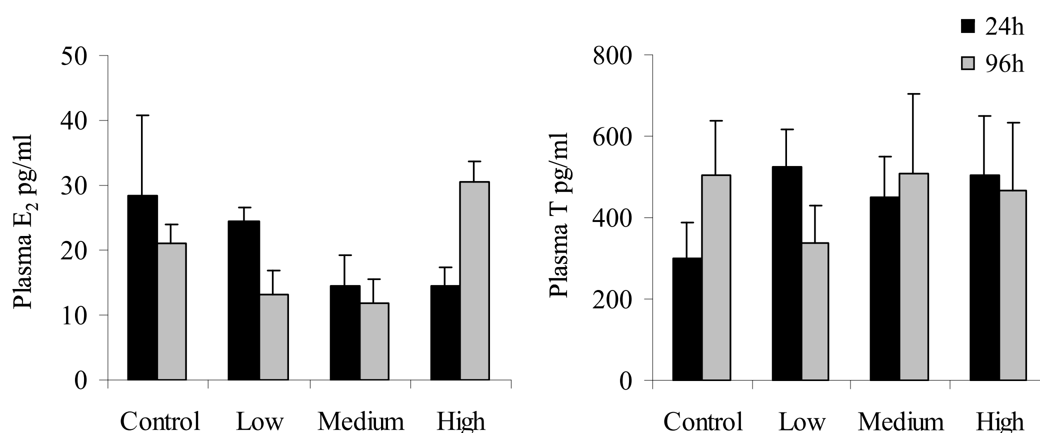

Figure 5-4. The effect of gemfibrozil on the circulating plasma estradiol (E2) and testosterone (T) in eels 24-h and 96-h post-injection (for 24-h, Low, Medium, and High, correspond to 0.1, 1 and 10 µg GEM/g respectively; for 96-h, Low, Medium, and High correspond to 2, 20, and 200 µg GEM/g respectively). Values are mean ± S.E.M., n= 8.

reduction was not statistically significant ($p = 0.11$) due to the high variability. T levels showed a tendency to increase (51-76 %) across all the GEM exposures (0.1-10 µg/g) but again differences in respect to the control group were not statistically significant ($p = 0.08$-0.22) (Figure 5-4). 96-h post-injection, 2 and 20 µg/g GEM resulted in

decreased E_2 levels (37 and 44% respectively) that was not statistically significant (p= 0.12 and 0.08 respectively). T levels did not show any alteration 96-h post-injection.

5.4 Discussion

CYP-Catalytic activities

CYP1-, CYP3- and CYP2- families play a key role in the hydroxylation of endogenous (hormones, fatty acids) and exogenous (xenobiotics) compounds, increasing their solubility and converting them to substrates of phase II enzymes, to finally become excreted from the organism. Two CYP1A isoforms have been cloned from the European eel (Anguilla anguilla), sharing 77% amino acid sequence identity (Mahata et al., 2003). As with mammals, CYP1A activity in eels is induced following in-vivo exposure to PCBs, dioxins and PAHs (benzo[a]pyrene), in both liver and extrahepatic tissues (Van der Oost et al., 1996; Schlezinger and Stegeman, 2000; Gorbi and Regoli, 2004). CYP3A-like enzymes have been cloned from various fish, among them rainbow trout, salmon and sea bass (Buhler and Wang-Buhler, 1998; Vaccaro et al., 2007; Matsuo et al., 2008) and in eels from contaminated sites an induction of CYP3A-like protein has been observed (Van der Oost et al., 1996). Unlike CYP1A and CYP3A-like, the isoenzymes CYP2K- and CYP2M-like have only been identified in few fish species until now, among them rainbow trout, zebrafish and salmon (Buhler and Wang-Buhler, 1998; Wang-Buhler et al., 2005; Matsuo et al., 2008).

24-h post-injection, GEM had a significant inhibitory effect on CYP3A-like catalysed activity at all doses tested; a significant inhibitory effect on CYP1A catalysed activity in eels that received 1 and 10 µg/Kg and a significant inhibitory effect on CYP2K-like catalysed activity in eels that received 1 and 10 µg/Kg (Figure 5-1). Interestingly, no significant effect was observed on CYP activities 96-h post-injection, with the exception of CYP1A catalysed activity, which was inhibited in fish that received 200 µg/Kg. These data are in agreement with previous results from in-vitro studies on fish where incubation of carp liver microsomes with 1mM GEM resulted in significant inhibition (50%) of CYP1A, CYP3A-like and CYP2K-like activities (Thibaut et al., 2006). There is limited data on the in-vivo effects of GEM on cytochrome P450 system. In contrast to the present study, Mimeault et al. (2006) exposed goldfish to waterborne GEM (1.5 and 1,500 µg/L) for 14 and 28-days, with a 76-h water and GEM renewal,

and found no effects on hepatic CYP1A expression. It is possible that the authors did not detect an effect due to the 72-h interval dosing, within which time fish could have cleared out GEM and recovered CYP1A mRNA levels. Since CYP3A- and CYP2K-like isoenzymes present similarities with mammalian members of the CYP3A and CYP2 families, observations on the effects of GEM on these mammalian enzymes are relevant to the present study. Therefore, mice exposed orally to 8,000 ppm of GEM for 13 weeks showed increased 6β-testosterone hydroxylase activity, principally catalysed by CYP3A1 and CYP3A2, and 16β-testosterone hydroxylase activity, catalysed by CYP2B1 but decreased 2α- and 16α-testosterone hydroxylase activities, principally catalysed by CYP2C11 (Fan et al., 2004). In rodents, hydroxylation of long chain fatty acids at ω- and ω-1 carbon terminal is regulated by enzymes of the CYP4A subfamily (Simpson, 1997). The CYP4A enzymes are induced in mammals following treatment with fibrates, including GEM (Fan et al., 2004; Reddy and Hashimoto, 2001). In the present study, the enzymatic activity of CYP2K- and CYP2M-like enzymes, responsible for the ω-1 and ω- hydroxylation of lauric acid respectively, was either inhibited or remained unaltered following exposure to GEM (Figure 1). Thus it becomes evident that the effect of GEM on cytochrome P450 enzymatic activities is species, dose and period of exposure-dependent.

This work shows that GEM is a potent inhibitor of the hepatic cytochrome P450 enzymatic activities examined but only 24-h post-injection. 96-h post-injection, eels appear capable of recovering the enzymatic activity. However, in the environment where fish may become exposed in periodic intervals through the effluents of sewage treatment plants or pharmaceutical manufacture installations, GEM may interfere with CYP1A, CYP3A-, CYP2K- and CYP2M-like enzymatic activities and thus pose the fish at risk. Therefore, it should be further investigated whether continuous exposure to low dose of GEM would have a permanent effect on the cytochrome P450 system.

Phase II metabolism enzymes

No statistically significant alterations were observed in fish at 24-h post-injection on the activity of the phase II enzymes, whereas 96-h post-injection microsomal UGT activity at 200 μg/kg dose exposure was significantly inhibited. In humans, up to 50% of an oral dose has been recovered in urine as gemfibrozil 1-O-β-acylglucuronide, indicating that glucuronidation is a major pathway in the biotransformation of GEM (Knauf et al., 1990;

Sallustio and Fairchild, 1995). In the present study, chemical analysis of the bile indicated that GEM had been conjugated with glucuronic acid; an average of 10.9 and 2.3 % was excreted at 24- and 96-h post-exposure (data not shown). These results on UGT activity are in agreement with the in-vitro effect of GEM in carp liver microsomal fractions, where 1mM of GEM caused 78% inhibition (Thibaut et al., 2006). In terms of GST activity, our results contrast the ones of another in-vivo study, where 14 and 28-day exposure of goldfish to 1.5 µg GEM/L resulted in ~15-20% induction of GST activity (Mimeault et al., 2006). It is possible that in the present study GST activity was not affected due to the short length of the experiment (24- and 96-h) and hence our results suggest that GST is not a primary target of GEM action.

Studies on rodents show that GEM and clofibrate induce liver microsomal esterification of steroid hormones such as estradiol, testosterone, dehydroepiandrosterone (DHEA), pregnenolone and corticosterone by acyl-CoA:steroid acyltransferase (Xu et al., 2001, 2002). In the present experiment acyl-CoA:testosterone acyltransferase (ATAT) was altered by GEM 96-h post-injection only in fish from low dose exposure (2 µg GEM/g) showing a 30% induction, however not being statistically significant. To our knowledge, this is the first study reporting effects of fibrates on esterification of steroids in fish. Exposure of female rats to 100-400 mg/kg clofibrate or 50-300 mg/kg GEM for 5 days increased the rate of estradiol esterification 5- to 9-fold, which is a much higher response than the one observed in the present study. Similarly, esterification of estradiol, and testosterone, with oleyl-CoA in rodents following 4 weeks administration of 0.6% clofibrate was induced by 14- and 17- fold, respectively (Xu et al., 2002), showing that the response is time dependent.

Peroxisome proliferation inducible enzymes.

It has been reported that GEM causes peroxisome proliferation and hepatocarcinogenesis in rodents; thus the present work aimed to measure AOX and CAT activity, two inducible enzymes during peroxisome proliferation (Lazarow and Duve, 1976). 96-h post-injection to GEM AOC activity was significantly induced in liver peroxisomal fractions being significant at 20 and 200 µg/g exposures (22 and 33% respectively; figure 3) whereas CAT activity although induced, was statistically significant only at 2 µg/g exposure (39% increase). The increases seen in AOX and CAT activity in this experiment are in accordance with another in-vivo experiment on

rainbow trout following exposure to a different fibrate, the ciprofibrate. Hence, after 3 weeks exposure, a 27 and 78% increase in AOX activity and a 83 and 236% in CAT activity was observed in rainbow trout peroxisomal fractions that received 25 and 35 µg/g ciprofibrate injections, respectively (Yang et al., 1990). Similarly, intraperitoral exposure of rainbow trout to GEM (46-152 µg/g/day) for 2 weeks resulted in 26-37% increase in AOX activity expressed as activity per gram of liver (Scarano et al., 1994). The observed induction in AOX activity of eels in the present study is of a much lower magnitude than what it has been observed in rodents. Therefore, in a 42-day study with rats, dietary concentrations of 0.9-2.0% GEM induced AOX activity in liver peroxisomal fractions by 16- to 18-fold, approximately 15 times more than the observed induction in this experiment (Sausen et al., 1995). Thus, it becomes evident that the peroxisome proliferation in ells caused by GEM is considerably lower than that in rodents and therefore, quantitative morphometric analysis of peroxisomes is suggested as a complementary measurement for peroxisome proliferation. Nevertheless, the induction of AOX activity indicates that GEM, being a hypolipogemic drug for humans, induces peroxisomal β-oxidation of fatty acids in the liver of fish as well and consequently stimulates lipolysis; thus the implications of aquatic contamination by this drug should be considered.

Steroid hormones

A recent study showed that goldfish exposed to waterborn GEM concentrations of 1.5 and 1,500 µg/L for 14 days exhibited concentration-depended decreased plasma testosterone levels by 49 and 72%, respectively (Mimeault et al., 2005). In the present study 24-h and 96-h of GEM exposure did not decrease plasma testosterone levels. On the contrary, a tendency for increased T and decreased E2 plasma levels was observed 24-h post-exposure. Data on the effect of fibrates on sex steroid levels are inconclusive. Thus, exposure of fathead minnow to waterborne clofibric acid (fibrate metabolite) concentrations of 10 µg/L and 1mg/L for 21 days resulted in reduced spermatogenesis in males but testosterone levels were only marginally reduced not being statistically significant (Runnalls et al., 2007). Exposure of juvenile zebrafish to 0.01-1 µM clofibrate during the period of 17 to 20 days post-fertilisation had a tendency to decrease CYP19A2 mRNA levels, which gene encodes the P450 aromatase, the enzyme responsible for the conversion of androgens to estrogens. Nevertheless, GEM

does not appear to regulate sex steroid plasma levels in eels (following short term exposure).

Peroxisome proliferation leading to hepatocarcinogenesis has been observed in rodents but not in humans or other mammals such as hamsters and rhesus monkeys (Bentley et al., 1993). The enzymes that have been reported to get induced by peroxisome proliferators (AOX, CAT, AEAT/ATAT and CYP4A) are regulated principally by PPARα a as in PPARα null mice such effects do not occur (Reddy and Hashimoto, 2001; Xu et al., 2001b). Rodents have a very high concentration of hepatic PPARα compared to humans and other mammals, which could explain their high susceptibility to peroxisome proliferators (Palmer et al., 1998). To our knowledge there is no data on hepatic PPARα expression in eels. However, exposure of another fish species (goldfish) to GEM did not alter hepatic PPARα expression suggesting that GEM may not regulate that gene in this species. Furthermore, unlike mice where highest PPARα expression is in the liver, goldfish showed low and equal distribution of PPARα across the kidney, intestine, liver and spleen, and PPARα and PPARγ were equally expressed in the liver (Mimeault et al., 2006). Thus it is possible that the low response on PPARα-regulated enzymes in eels following exposure to GEM is either due to the low concentration of hepatic PPARα in this species or due to regulation of these enzymes by different receptors. Indeed, in-vitro exposure of salmon hepatocytes to the fibrates clofibric acid or bezafibrate, resulted in a 1.7-fold increase in PPARγ and AOX activity suggesting that salmon PPARγ may have a regulatory role in the peroxisomal β-oxidation pathway of fatty acids (Ruyter et al., 1997). Furthermore, cloning of PPARα from torafugu puffer fish, plaice, sea bream and grey mullet showed a longer additional sequence in the LBD compared to rodent PPARα ((Leaver et al., 2005; Raingeard et al., 2006; Kondo et al., 2007). It is possible that these additional sequences in the LBD may influence ligand-binding. Hence, typical mammalian peroxisome proliferators may bind weakly or not at all to PPARα of some fish (Kondo et al., 2007), which could also explain the low observed response of PPARα regulated enzymes to GEM in eels of the present work.

To summarise, the present study shows that short term in-vivo exposure to GEM results in inhibition of the activity of different cytochrome P450 and UGT isoforms in eels indicating the interference of this compound with the metabolism of xenobiotics and endogenous compounds in fish. Furthermore, this study shows that GEM induces typical peroxisome proliferation enzymes in fish as it has been observed in rodents but

to a lesser extent. The significance of these peroxisome proliferation responses in the endocrine system of fish must be studied further and confirm whether such alterations lead to adverse health effects.

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Chapter 6. A study on the metabolism of androstenedione in two gastropod species and its modulation by tributyltin and triphenyltin following in-vitro exposures

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Abstract

A comparative study was performed to assess the metabolism of the androgen precursor androstenedione (AD) in two gastropod species from the Muricidae family: *Bolinus brandaris* and *Hexaplex trunculus*. AD was mainly converted to 5α-dihydrotestosterone by microsomal fractions isolated from *Bolinus brandaris*, whereas it was primarily metabolized to testosterone by *Hexaplex trunculus*. Sex differences in the metabolism of AD were only detected in *Bolinus brandaris* and attributed to higher 5α-reductase activity in males. Thereafter, the effect of the organotin compounds, tributyltin (TBT) and triphenyltin (TPT), on the metabolism of AD was investigated. A significant interference was only detected in females, and differences between the modes of action of the two compounds were observed: TPT was a strong inhibitor of 5α-reductase activity in *B. brandaris* at a concentration as low as 100 nM whereas only TBT (10 μM) altered the metabolism of AD in *H. trunculus* by increasing the activity 17β-hydroxysteroid dehydrogenase (17β-HSD). Thus, this work shows that the metabolism of the androgen precursor AD strongly differs among gastropod species, both in terms of activity and metabolic profile, and further demonstrates the ability of TBT and TPT to interfere with key enzymatic pathways involved in androgen synthesis.

6.1 Introduction

A recent study demonstrated that in contrast to vertebrates and other invertebrates, which metabolize androstenedione (AD) principally to testosterone (T) through the catalytic action of 17β-hydroxysteroid dehydrogenase (17β-HSD) (Mindnich et al., 2004), microsomal fractions isolated from digestive gland/gonad complex of the freshwater ramshorn snail *Marisa cornuarietis*, metabolised AD mainly to 5α-dihydroandrostenedione (DHA) in females, and to 5α-dihydrotestosterone (DHT) and testosterone (T) in males (Janer et al., 2005a). This is the only sexual dimorphic pathway detected so far in a gastropod species, since neither hydroxylation nor sulfation of T showed any clear sexual dimorphism in *M. cornuarietis* (Janer et al., 2005b) and only transient differences were found in the conjugation rate of T with fatty acid moieties (Janer et al., 2006a).

Endogenous vertebrate-like androgens (e.g. T, androsterone) and their precursors such as AD and dehydroepiandrosterone (DHEA) have been identified in invertebrates, including gastropod molluscs (Lafont and Mathieu, 2007; Janer and Porte, 2007). Nevertheless, their functional role remains unclear since a vertebrate-like AR has not yet been identified in invertebrates, and scientific evidence suggests that AR evolved after the emergence of jawless fish (Thornton, 2003; Sternberg et al., 2008). However, a number of studies show that vertebrate-like steroids may function as hormones in gastropods as well. Thus, exposure of the apple snail *Pomacea canaliculata* and the marine gastropods *Nucella lapillus* and *Nassarius reticulatus* to testosterone resulted in imposex development (Spooner et al., 1991; Bettin et al., 1996; Takeda, 2000). On the other hand, injections of the gastropods *Deroceras reticulates* and *Limax flavus* with estradiol and estrone stimulated egg-laying, while testosterone failed to have this effect (Takeda, 1979). In males of *Ilyanassa obsoleta*, temporal changes in testosterone levels were consistent with a positive role in reproductive tract recrudescence suggesting the involvement of this hormone in reproductive maturation (Sternberg et al., 2008). In addition, the vertebrate androgens 11-ketotestosterone (11-KT) and DHEA were potent inducers of spermatogenesis in the male slug *Ariolimax californicus* (Gottfried and Dorfman, 1970a).

Since vertebrate-like androgens appear to have a role in the development of secondary sexual characteristics and reproduction in gastropods, environmental contaminants interfering with endogenous androgen synthesis/metabolism may cause

significant alterations to exposed individuals and populations. The induction of imposex in female gastropods (superimposition of male secondary characteristics on females) following exposure to organotin compounds is a well documented issue (Matthienssen and Gibbs, 1998). Imposex induction has been attributed to: (i) elevated testosterone levels and inhibition of P450 aromatase activity (Spooner et al., 1991; Bettin et al., 1996); (ii) accumulation of testosterone due to the inhibition of its excretion as polar metabolites, such as sulfate and glucose conjugates (Ronis and Mason, 1996); and (iii) inhibition of the esterification of testosterone to fatty acid esters resulting in elevated levels of free testosterone (Gooding et al., 2003; Janer et al., 2006a), among other mechanisms (Oehlmann et al., 2007).

So far, little attention has been given to the interference of organotin compounds with the synthesis of androgens as a potential mechanism leading to alterations in endogenous steroid levels. In a previous work, we observed that in-vitro incubation of microsomal fractions isolated from the digestive gland/gonad complex of *M. cornuarietis* in the presence of the organotin compounds (TBT or triphenyltin –TPT-) resulted in a inhibition of the metabolism of AD, particularly the formation of DHA and DHT (Janer et al., 2006b). However, in vivo experiments showed no significant alteration in the metabolism of AD in males, but a marginal (TBT) and a significant (TPT) inhibition of the formation of DHA in females exposed for 150 days to concentrations that had significantly induced the development of imposex. Thus, the data suggest that the metabolism of the androgen precursor AD, may be targeted by organotin compounds in the gastropod *M. cornuarietis*.

Following the above observations, the present study was designed to further investigate the metabolism of AD in other gastropod species and to see whether previous data obtained for *M. cornuarietis* is species specific or on the contrary can be generalized to other species. For this purpose, AD metabolism was assessed in microsomal fractions isolated from the digestive gland/gonad complex of two common Mediterranean species: *Bolinus brandaris* and *Hexaplex trunculus* (Mollusca: Gastropoda: Muricidae). The in-vitro effect of the organotin compounds TBT and TPT on the metabolism of AD were additionally examined. *B. brandaris* is a subtidal muricid that lives on muddy and soft substrates from few to 50 m depth; it feeds on bivalves and other gastropods and reproduces in late spring-early summer (Ramón and Amor, 2002). *H. trunculus* lives within 1-100m depth range on rocky and muddy substrates; it is carnivorous but also saprophagous, and it reproduces in May-June. Both species

have shown the development of imposex across the Mediterranean region and have been frequently used in organotin monitoring studies (Solé et al., 1998; Ramón y Amor, 2001; Chiavarini et al., 2003; Gómez-Ariza et al., 2006; Garaventa et al., 2007).

6.2 Materials and Methods

Chemicals

Tributyltin chloride (TBT) was obtained from Sigma (Steinheim, Germany) and triphenyltin chloride (TPT) was purchased from Merck (Darmstadt, Germany). [1α-^{3}H]-androstenedione (15–30 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). NADPH was obtained from Sigma (Steinheim, Germany). All solvents and reagents were of analytical grade, except TBT (96%) and TPT (95%).

Animals

Specimens of *Bolinus brandaris* and *Hexaplex trunculus* were collected in June 2006 from Sant Carles de la Ràpita (Catalonia, NW Mediterranean). Animals were transferred to the animal-holding facilities (ICM, Barcelona) and kept in running sea water for one day before examination. The shell was measured to the nearest 0.1 mm using a vernier caliper. In *B. brandaris*, the length without siphonal canal (LWS) was chosen instead of total length due to the frequent breaks of the long and narrow siphonal canal. The shell was cracked with a vice and the animals were analyzed under a stereo microscope. Sex was determined by presence or absence of the capsule gland and genital pore and the state of maturity by looking at gonad colour (yellow-orange in mature males and pink in mature females). Approximately 30 specimens of *B. brandaris* and 20 of *H. trunculus* were analyzed. Female imposex stages were defined according to the vas deferens sequence (VDS) index, i.e. the mean score of the various stages of vas deferens development in females (Fioroni et al, 1991). After recording the body weight, the digestive gland/gonad complex was dissected, placed in liquid nitrogen and kept at -80ºC until biochemical analysis.

Subcellular fractioning

Digestive gland/gonad complex of *B. brandaris* and *H. trunculus* were individually homogenized in ice-cold 100 mM potassium phosphate buffer pH 7.4, containing 100 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline and 0.1 mM trypsin inhibitor. Homogenates were first centrifuged at 500-*g* for 20 min and the supernatant was further centrifuged at 12,000-*g* for 45 min. After centrifugation at 100,000-*g* for 90 min, the obtained pellet was resuspended in homogenization buffer and centrifuged again at 100,000-*g* for 30 min. The obtained pellet, termed microsomal fraction, was resuspended in microsomal buffer, consisting of 100 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, and 0.1 mM trypsin inhibitor, and 20% w/w glycerol. Microsomes were stored at -80°C until assays were performed. Protein concentrations were determined by the method of Lowry et al. (1951) by using bovine serum albumin as a standard.

Androstenedione metabolism

Androstenedione metabolism was assessed as indicated in Janer et al. (2005a). Briefly, microsomal proteins (200 µg) were incubated in a total volume of 250 µl, containing 100 mM potassium phosphate buffer pH 7.4, containing 1.0 mM EDTA, 0.2 µM [^{3}H]-androstenedione (150,000 dpm), and 1.0 mM NADPH. The reaction was initiated by the addition of NADPH, and incubated in constant shaking at 30°C for 5, 30 and 60 min for *B. brandaris* and 30 min for *H. trunculus*. Incubations were stopped with acetonitrile (250 µl) and after centrifugation (1500-*g*, 5 min), 200 µl of the supernatant were injected onto the RP-HPLC column. Separation of androstenedione metabolites was performed on a Perkin-Elmer Binary LC pump 250 system equipped with a 250 mm × 4 mm LiChrospher 100 RP-18 (5 µm) reversed-phase column (Merck, Darmstadt Germany) protected by a guard column LiChrospher 100 RP-18 (5 µm). The mobile phase pumped at 1 mL/min was composed of (A) 75% water and 25% acetonitrile and (B) 25% water and 75% acetonitrile. The run consisted on a linear gradient from 100% A to 100% B (0–30 min), followed by isocratic mode 100% B (5 min), linear gradient from 100% B to 100% A (5 min), and isocratic mode 100% A (5 min). Radioactive metabolite peaks were monitored by online radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany)

using Flo-Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks. Identification of the metabolites was based on the comparison of their retention times to those obtained from commercially available standards monitored at 254 nm using a UV-detector (Knauer LC-photometer).

In-vitro effect of organotin compounds on the metabolism of androstenedione

To evaluate the in-vitro effect of TBT and TPT on androstenedione metabolism, microsomes (200 µg of protein) were pre-incubated at 25 ºC with different concentrations of TBT or TPT: 0, 0.1, 1, and 10 µM. Each compound, dissolved in ethanol, was added into the test tubes, evaporated and re-dissolved in assay buffer, microsomal proteins (200 µg) added and pre-incubated for 15 minutes (*B. brandaris*) or 5 minutes (*H. trunculus*). Thereafter, the metabolism of AD was assessed in the presence of NADPH for a period of 5 min (*B. brandaris*) or 30 min (*H. trunculus*) as described in section 2.4.

Data analysis

Results are mean values ± S.E.M. Statistical significance was assessed by one-way ANOVA (Dunnett´s test for differences from control) and a Student's *t*-test when comparing males and females. *P* values lower than 0.05 were considered statistically significant.

6.3 Results

The main characteristics of the specimens analysed are given in Table 6-1. The occurrence of imposex in females of *B. brandaris* was of 100%; all females had developed a penis and VDSI was scored as 4 (formation of penis with duct and vas deferens that reaches the vulva but overpasses it without blocking) which is the last, still fertile, stage of imposex development. Females of *H. trunculus* revealed lower imposex intensity than those of *B. brandaris*: 2 out of 10 females had no imposex, 3 were classified as imposex stage 1B (formation of a short distal vas deferens section behind the right ocular tentacle), 1 was at stage 2A (development of a penis with duct),

Table 6-1. Measurement of imposex in *Bolinus brandaris* and *Hexaplex trunculus* collected from the Ebro Delta. Values mean ± S.D. Number of individuals in brackets

		n	Shell length (cm)	Body weight (g)	% Imposex	Imposex stage
Bolinus	♂	11	3.14 ± 2.0	8.6 ± 1.5		
brandaris	♀	10	3.21 ± 1.2	8.2 ± 1.2	100	4 (10)
Hexaplex	♂	9	4.75 ± 3.1	17.9 ± 4.8		
trunculus	♀	10	47.2 ± 2.3	15.4 ± 1.6	80	0 (2), 1B (3), 2A (1), 3A (2), 4 (2)

2 had reached stage 3A (formation of short distal vas deferens together with a penis with duct) and 2 were at stage 4.

The metabolism of AD by microsomal fractions isolated from the digestive gland/gonad complex of *B. brandaris* led to the formation of a major metabolite that was identified as 5α-dihydroandrostenedione (DHA) (Table 6-2). After 5 min incubation of AD in the presence of NADPH, only DHA was formed in males at a rate of 1162 ± 154 pmol/h/mg protein. Similarly, females formed DHA (893 ± 28 pmol/h/mg protein) and a minor amount of testosterone (2 out of 10 females) (Table 6-2). If microsomal fractions were incubated for 30 min, AD was totally metabolized, and DHA was further metabolized to 5α-dihydrotestosterone (DHT) in both sexes; the formation of testosterone was only detected in females. The formation of DHA and consequently the activity of 5α-reductase were significantly higher in males than in females (Table 6-2; Figure 6-1A).

In contrast, microsomal fractions isolated from the digestive gland/gonad complex of *H. trunculus* metabolized AD to T, a 17β-hydrosteroid dehydrogenase catalysed reaction (Table 6-3). No significant differences between males and females were observed, although it is worth mentioning that the formation rate of T was 2-fold higher in the two females that had not developed imposex in comparison to the other females. Interestingly, the metabolism of androstenedione was much more active in *B. brandaris* than in *H. trunculus*. Following 30 min incubation, 90% of AD was metabolized by *B. brandaris*, whereas only 9% of AD was metabolized by *H. trunculus* (Tables 6-2 & 6-3).

Table 6-2. Metabolism of androstenedione in microsomal fractions isolated from the digestive gland/gonad complex of males and females of *Bolinus brandaris*.

	T	DHT	DHA	% AD metabolised
5 min incubation				
Males	n.d.	n.d.	1161.9 ± 153.9	41.8 ± 7.7
			(767.3 – 1693.0)	(24.1 – 68.6)
Females	40.7 ± 17.6	n.d.	893.3 ± 27.8	30.1 ± 6.8
	(n.d.- 76.0)		(804.5 – 949.5)	(29.1 – 32.2)
30 min incubation				
Males	n.d.	35.5 ± 11.1	463.9 ± 16.1	97.8 ± 0.4
		(6.5 - 55.7)	(427.7 – 503.3)	(96.6-98.6)
Females	9.6 ± 2.2*	45.7 ± 27.6	240.2 ± 26.0*	82.2 ± 11.2
	(4.0 -14.0)	(8.7 – 127.0)	(191.7 – 312.7)	(47.9 - 96.8)

Values are mean ± SEM (n = 4-6) expressed as pmol/h/mg microsomal protein; ranges of values are given in brackets. *significant differences between males and females ($p < 0.05$).

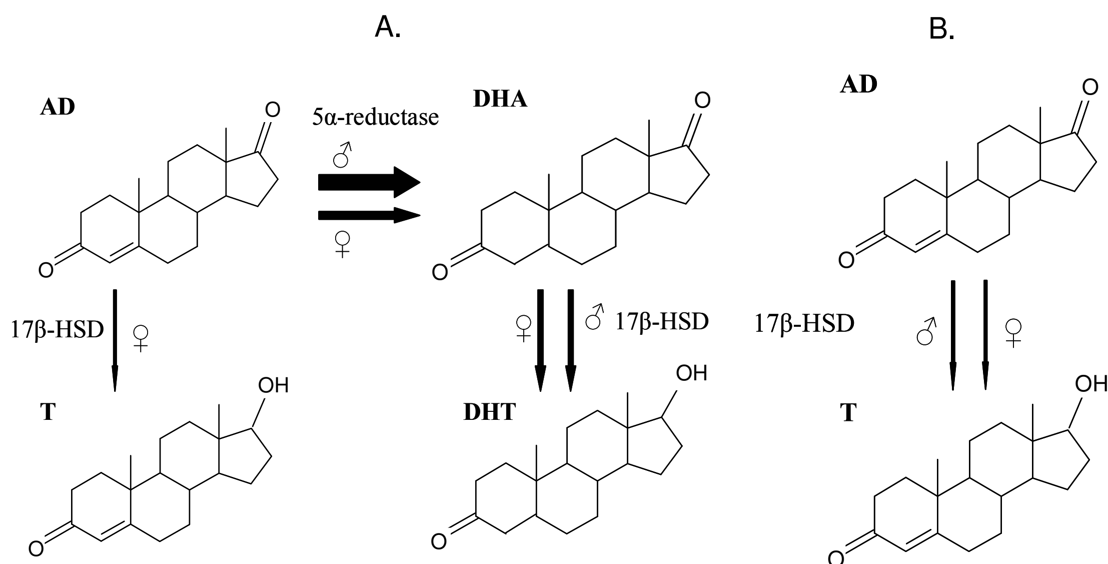

Figure 6-1. Metabolism of the androgen precursor androstenedione in microsomal fractions isolated from the digestive gland/gonad complex of (A) *Bolinus brandaris* and (B) *Hexaplex trunculus*. Width of arrows indicates the rate of conversion of the metabolite.

Table 6-3. Metabolism of androstenedione in microsomal fractions isolated from the digestive gland/gonad complex of males and females of *Hexaplex trunculus*

	T (pmol/h/mg microsomal protein)	**% AD metabolised**
Males	20.9 ± 2.6	7.9 ± 1.0
	(12.1-26.7)	(4.6-10.1)
Females	22.7 ± 5.4	8.5 ± 2.1
	(11.8-39.5)	(4.4-14.9)

Values are mean ± SEM (*n* = 5); ranges are given in brackets.

The interaction of TBT and TPT with the metabolism of AD was investigated by pre-incubating microsomal fractions of *B. brandaris* and *H. trunculus* with different concentrations of the organotin compounds (0.1, 1 and 10 µM), followed by incubation with AD in the presence of NADPH. Pre-exposed microsomes of *B. brandaris* were incubated with AD for 5 min in order to assess the interference of TBT and TPT with the formation rate of DHA. TPT significantly inhibited the formation of DHA (40 – 56%) in females of *B. brandaris* at the three concentrations tested (0.1, 1, and 10 µM) whereas no significant effect was observed in males (Figure 6-2). TBT was a less potent inhibitor of DHA formation in females causing a significant inhibition (18%) only at the high dose tested (10 µM). Similarly, the amount of DHA formed in males was not altered by TBT.

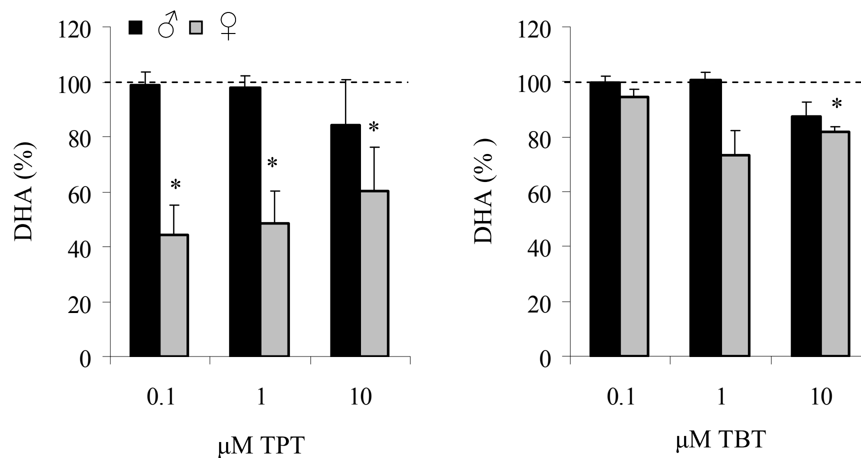

Figure 6-2. In-vitro effect of different concentrations of TBT and TPT (0.1-10 µM) on the metabolism of the androgen precursor androstenedione by microsomal fractions isolated from the digestive gland/gonad complex of *Bolinus brandaris*. Effects on the formation of 5α-dehydroandrostenedione (DHA) are shown. Values are mean ± SEM (n=4) expressed as % of control. * indicates significant differences from the control group (*p*< 0.05).

Regarding *H. trunculus*, TPT had no significant effect on the formation rate of testosterone in either males or females. On the contrary, TBT slightly enhanced the formation rate of testosterone in females at the concentrations tested, but this increase was statistically significant only at the highest concentration tested (10 μM) (Figure 6-3).

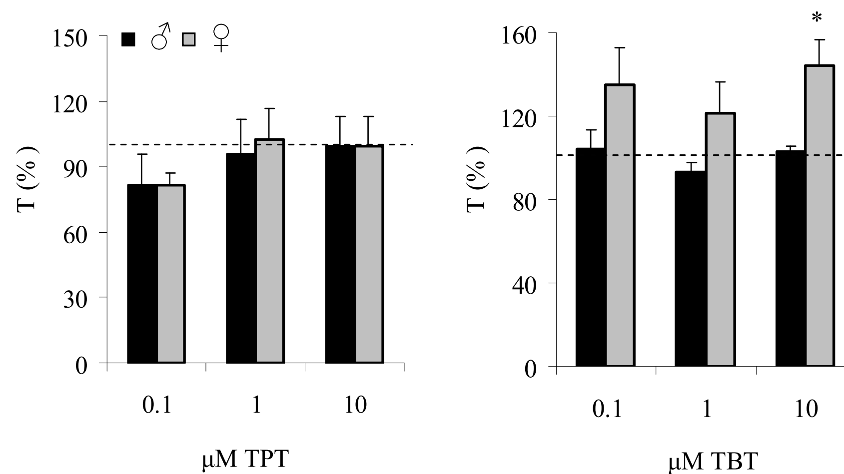

Figure 6-3. In-vitro effect of different concentrations of TBT and TPT (0.1-10 μM) on the metabolism of the androgen precursor androstenedione by microsomal fractions isolated from the digestive gland/gonad complex of *Hexaplex trunculus*. Effects on the formation of testosterone (T) are shown. Values are mean ± SEM (n=4) expressed as % of control. * indicates significant differences from the control group (p< 0.05).

6.4 Discussion

The study of the present chapter shows the presence of vertebrate-type enzymes involved in androgen synthesis in two gastropod species, namely *Bolinus brandaris* and *Hexaplex trunculus* and demonstrates significant differences between the two species. Thus, *B. brandaris* metabolised AD mainly to DHA –a 5α-reductase catalysed pathway- whereas *H. trunculus* metabolised AD to form T -a 17β-HSD catalyzed pathway- (Figure 6-1). Indeed, it has been reported that 5α-reductase plays a more important role than 17β-HSDs in the metabolism of androstenedione in molluscs (Janer and Porte, 2007): e.g. the metabolism of AD to DHA was detected in the gastropod *Marisa cornuarietis* and in other molluscs, such as the mussel *Mytilus galloprovinciallis* and the slug *Ariolimax californicus* (Gottfried and Dorfman, 1970b, Janer et al., 2005c). Surprisingly, 5α-reduced metabolites of AD were not detected in *H. trunculus*, and 17β-

HSD was the main metabolizing enzyme (Table 6-3). 17β-HSDs play a key role in vertebrates where they catalyse the last steps of steroid synthesis, leading to the formation of 17β-hydroxylated steroids, but they have also been described in other invertebrates including molluscs (Baker 2001; Janer and Porte, 2007). Nonetheless, substrate specificity of 17β-HSDs is higher for DHA than for AD in the gastropod *M. cornuarietis*, and this contrasts with the high affinity of vertebrates 17β-HSDs for AD.

Additionally, the metabolism of AD was much more active in *B. brandaris* (82 to 98% of AD metabolized after 30 min incubation) than in *H. trunculus* (approximately 8% of AD metabolized after 1 h incubation) under our assay conditions. Actually, the metabolic activity reported for *H. trunculus* is comparable to the one observed in *M. cornuarietis* (11-13% after 3 h incubation; Janer et al. 2006b); this contrasts with the high variability reported for the mussel *M. galloprovinciallis* (16-80% of AD metabolized by digestive gland microsomal fractions after 1 h incubation; Lavado and Porte, unpublished observations). Thus, the metabolism of the androgen precursor AD strongly differs among gastropod species and across molluscs both in terms of activity and metabolic profile, confirming the wide diversity of this invertebrate phylum. Nonetheless, despite of the observed differences in androgen synthesis, endogenous testosterone levels do not significantly differ between species. Thus, T levels ranged from 0.7 to 1.2 ng/g w.w. in the digestive gland/gonad complex of *B. brandaris* and from 0.13 to 2.3 ng/g w.w. in *M. cornuarietis* (Morcillo and Porte, 1999; Lyssimachou et al., 2008). Higher variability on endogenous T levels has been reported for the marine mussel *M. edulis* and *M. galloprovincialis* depending on the analyzed tissues (from 0.06 to 3.3 ng/g) (Janer et al., 2006a; Lavado et al., 2006a).

Gender differences in the metabolism of androstenedione were only detected in *B. brandaris*; the formation of DHA, a 5α-reductase-catalyzed pathway, occurred at a higher rate in males than in females, while the formation of T was only detected in females. No significant differences between males and females were observed for the formation of DHT (Table 6-2). The higher 5α-reductase activity observed in males, together with the observation that T formation was only detected in females contrasts with previous data for *M. cornuarietis* that metabolised AD in a sexually dimorphic manner, but the formation of DHT and T occurred at a much higher rate in males than in females, indicating a higher 17β-HSD activity in the former. Also, affinity constants (*K*m) and specific activities for 5α-reductase did not differ between male and female of *M. cornuarietis* (Janer et al., 2006b).

In-vitro exposure to TBT or TPT indicated the higher susceptibility of females than males to AD metabolism alteration in both species. TPT (0.1–10 μM) decreased the rate of formation of DHA in females of *B. brandaris*, a 5α-reductase catalyzed pathway, but had no significant effect in males (Figure 6-2). Likewise, TBT (10 μM) significantly inhibited 5α-reductase, although it was a less effective inhibitor than TPT. The susceptibility of 5α-reductase to inhibition by organotin compounds has already been reported in several gastropod species (Ronis and Mason, 1996; Janer et al., 2006b), but the higher sensitivity of females in comparison to males has not been previously documented. Contrasting results were obtained for *H. trunculus*, where TPT did not alter the in-vitro metabolism of AD, and TBT (10 μM) increased the rate of formation of T in females but not in males (Figure 6-3). In-vitro activation of steroid-metabolizing enzymes has been reported previously (Korzekwa et al., 1998), however, the mechanism by which TBT enhances 17β-HSD activity is unknown. These enzymes convert inactive 17-keto-steroids into their active 17βhydroxy-forms or vice versa, and although some studies have indicated no effect of TBT on the metabolism of AD to testosterone when tested at concentrations up to 100 μM (Thibaut and Porte, 2004), others reported an 50% inhibition in the conversion of testosterone to AD by digestive gland microsomal fractions of the clam *Ruditapes decussata* (Morcillo et al., 1998).

The present results regarding the higher susceptibility of females than males to AD metabolism alteration by TBT and/or TPT are to some extent supported by the higher sensitivity of females to organotin exposure. Indeed, injections of *Nucella lapillus* with TBT (1 µg/g body weight) for 2 months induced the development of imposex in females and increased the penis length of males; however, the effect in females was more severe as penis growth reached about 1.2 mm whereas in males, penis increased only by approximately 0.4 mm (Castro et al., 2007). Furthermore, exposure of the abalone *Haliotis gigantean* to 100 ng/L of TBT or TPT caused significant spermatogenesis in ovaries of exposed females, whereas no significant histological changes were observed in the testis of exposed males (Horiguchi et al., 2002). In-vivo exposure of *M. cornuarietis* to TBT for 100 days and to TPT for 7 days resulted in significant alterations in esterified testosterone and estradiol levels in females whereas no effects were observed in males (Janer et al., 2006a; Lyssimachou et al., 2008). Moreover, 4-week exposure of *P. lividus* to TPT significantly induced 5α-reductase and 3β-HSD activity in females, but in males, the effect was only observed at the high exposure dose (225 ng TPT/L) (Lavado et al., 2006b).

Overall, this chapter demonstrates significant differences in the metabolism of the androgen precursor AD in the two gastropod species investigated, *B. brandaris* and *H. trunculus*. AD was mainly metabolized to DHA in the former, whereas T was formed by the latter. The study also describes the sexual-dimorphic metabolism of androstenedione in *B. brandaris* but not in *H. trunculus*. The interference of TBT and TPT with the metabolism of AD was predominately detected in females, and differences between the mode of action of the two compounds were observed; TPT was a strong inhibitor of 5α-reductase activity in *B. brandaris* at a concentration as low as 100 nM whereas only TBT appeared to affect the AD metabolism pathway in *H. trunculus* by increasing the activity of 17β-HSD.

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Chapter 7. Effects of TPT on free and esterified steroid levels and androstenedione metabolism in the ramshorn snail Marisa cornuarietis *following 7-days in-vivo exposure*

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Keywords: esterified steroids, testosterone, estradiol, gastropod, aromatase, 17β-hydroxysteroid dehydrogenases, 5α-reductases

Abstract

Long-term exposure to organotin compounds has shown alterations on endogenous steroid levels in gastropods together with the development of imposex. However, information regarding short-term effects of these compounds on the endocrine system of gastropods is lacking. The work presented in this chapter aimed at investigating those responses in the ramshorn snail *Marisa cornuarietis* by looking at both, endogenous levels of free and esterified steroids and the metabolism of the androgen precursor androstenedione by digestive gland/gonad microsomal fractions. One-week exposure to the organotin compound triphenyltin (TPT) led to a significant increase in esterified testosterone (60-85%) and a decrease in esterified estradiol (16-53%) in females, but had no effect on males. The observed alterations in esterified steroids were not directly related to changes in P450 aromatase activity that remained unchanged in exposed females. The enzymes involved in the metabolism of the androgen precursor androstenedione, namely 17β-hydroxysteroid dehydrogenases and 5α-reductases, were not significantly altered by TPT exposure, suggesting that such enzymes are not primary targets of TPT in *M. cornuarietis*. Additional studies are needed to fully understand the significance of the observed alterations in females and their potential relationship with the development of imposex.

7.1 Introduction

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been used across the world in antifouling paints for ships and fishing nets as well as fungicides, and their release to the aquatic environment has resulted in widespread pollution. They are known to have harmful effects in vertebrates and invertebrates, being the induction of imposex in meso- and neo-gastropods one of the most studied effects (Fent, 1996). Imposex is defined as an irreversible syndrome that consists of the imposition of male-type genital organs, such as penis and vas deferens, in females (Smith, 1971; Bryan et al. 1986) and it is typically induced at very low concentrations of TBT, TPT or both (Matthiessen and Gibbs, 1998). Overall, more than 150 species of gastropods worldwide are affected by imposex which is thought to be one of the clearest evidences of endocrine disruption in wildlife (Horiguchi et al., 1997).

Although the link between imposex induction and exposure to TBT or TPT has been well established, the actual mechanisms through which this phenomenon occurs remain unclear. Hence, imposex induction has been attributed to: (i) elevated testosterone levels and inhibition of P450 aromatase activity (Spooner et al., 1991; Bettin et al., 1996); (ii) accumulation of testosterone due to the inhibition of its excretion as polar metabolites, such as sulfate and glucose conjugates (Ronis and Mason, 1996); (iii) inhibition of the esterification of testosterone to fatty acid esters resulting in elevated levels of free testosterone (Gooding et al., 2003; Janer et al., 2006a); (iv) alterations in the secretion of neurohormones (e.g. the peptide hormone APGWamide) that contribute to sexual differentiation in gastropods (Oberdörster and McClellan-Green, 2002); (v) binding of TPT and TBT to the retinoid X receptor (RXR) and the involvement of this receptor in imposex development (Nishikawa et al., 2004).

The first three theories imply the involvement of vertebrate-like steroids in the induction of imposex. Steroid hormones, such as testosterone and estradiol, have been detected in gastropod species (Bettin et al., 1996; Morcillo and Porte, 1999a; Janer et al., 2006a), but their physiological role is not yet fully understood. Studies have shown that exposure of the apple snail *Pomacea canaliculata* and the dogwhelk *Nucella lapillus* to testosterone resulted in imposex development (Spooner et al., 1991; Takeda, 2000). In addition, injection of estrone and estradiol to the gastropods *Deroceras reticulatus* and *Limax flavus* stimulated egg-laying, whereas testosterone failed to have this effect (Takeda, 1979). Thus, steroid hormones appear to have a role in

reproduction and development of secondary sexual characteristics in gastropods; although, it is still unclear whether gastropods have functional steroid hormone receptors (Horiguchi, 2006).

In vertebrate and some invertebrate species, steroids can be conjugated with fatty acids to form apolar esters that are retained in the lipoidal matrices of the body (Borg et al., 1995). Those steroid esters do not bind to receptors but can be hydrolyzed by esterases to liberate the active steroids when needed (Hochberg, 1998). Interestingly, gastropods appear to metabolise significant amounts of testosterone and 17β-estradiol to their fatty acid ester derivatives (Gooding and LeBlanc, 2001; Janer et al., 2005a). Gooding and LeBlanc (2004) reported that at the beginning and at the end of the egg-laying season, testosterone existed predominately in the free form in wild specimens of *Ilyanassa obsoleta*, whereas at other times the majority of testosterone existed as fatty acid esters. Additionally, endogenous levels of esterified steroids are altered by exposure to certain pollutants. Thus, exposure to TBT led to a decrease in esterified testosterone (60–85%) in females of *M. cornuarietis* that had developed imposex, but had no significant effect on free testosterone levels (Janer et al., 2006a). Gooding et al. (2003) found that imposex induced *I. obsoleta* showed a decrease in esterified testosterone with increasing exposure to TBT. Thus, both studies suggest that TBT may inhibit the esterification of testosterone and consequently increase the percentage of endogenous testosterone available in the free form.

Additionally, regarding androgen synthesis in *Marisa cornuarietis*, it was recently documented that androstenedione was mainly converted to testosterone (T) and 5α-dihydrotestosterone (DHT) by digestive gland/gonad complex microsomal fractions isolated from males, whereas it was primarily metabolized to 5α-dihydroandrostenedione (DHA) by females (Janer et al., 2006b). This sexual dimorphic metabolism of androstenedione was further investigated and attributed to a higher 17β-hydroxysteroid dehydrogenase (17β-HSD) activity in males than in females. Thereafter, the hypothesis was tested on whether the sexual dimorphic metabolism of androstenedione was altered in this species by exposure to TBT and TPT, and exposed females would have a ‘masculinised’ metabolism of androstenedione. The in-vitro metabolism of androstenedione, particularly the formation of DHA and DHT, was inhibited by both compounds; however, in-vivo experiments showed no significant alteration in the metabolism of androstenedione in males, but a significant inhibition of

the formation of DHA in females exposed to 250 and 500 ng/L TPT for 150 days (Janer et al., 2006b).

Most of the above-indicated studies are chronic exposure experiments and alterations on endogenous steroid levels, and steroid metabolising enzymes have been observed to occur after long-term exposure to organotin compounds. However, it should be considered that the endocrine system has complex feedback signalling pathways that act as homeostatic mechanisms, therefore, an alteration observed in a specific endpoint might not be due to a direct interaction of the xenobiotic on that level, but the result of indirect regulatory mechanisms. Thus, this study aims to investigate short-term responses to organotin exposure in the ramshorn snail *Marisa cornuarietis* by looking at (a) endogenous levels of free and esterified steroids, and (b) microsomal metabolism of androstenedione, an androgen precursor that might regulate levels of testosterone and other androgenic metabolites within the organism. To this end, adult specimens were exposed to three different concentrations of TPT (30, 125 and 500 ng/L) for 7 days. TPT was selected for the study because it is reported to be a potent inducer of imposex in *M. cornuarietis* (Schulte-Oehlmann et al., 2000) and a potential human endocrine disruptor (Golub and Doherty, 2005).

7.2 Materials and Methods

Chemicals

Triphenyltin chloride (TPT) was purchased from Merck (Darmstadt, Germany). [1β-^{3}H]-androstenedione (15–30 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA). Unlabeled steroids were obtained from Sigma (Steinheim, Germany). Radioimmunoassay (RIA) kits for testosterone and 17β-estradiol were obtained from Radim (Rome, Italy). NADPH was obtained from Sigma (Steinheim, Germany). All solvents and reagents were of analytical grade.

Animals

Ramshorn snails, *M. cornuarietis* (Mollusca: Prosobranchia: Ampullariidae), came from a laboratory breeding stock which was derived from a stock at Aquazoo Düsseldorf (Germany) in 1991 with regular cross-breeding of wild-caught animals from Florida

(USA) to avoid inbreeding. The breeding stock was kept in a flow-through system with fully reconstituted water under constant conditions regarding temperature and light/dark cycle (12:12 h). Water parameters (pH, temperature, conductivity, nitrite, oxygen concentration and saturation) were measured twice a week per tank. Parameters of the fully reconstituted influx water were pH 7.5, 850 µS/cm, <1 mg NO_2/L and >95% O_2 saturation.

Exposure experiment

For the exposure experiments, two replicate groups of 10 sexually mature male and female snails each were exposed to three nominal concentrations of TPT (30, 125, and 500 ng as Sn/L) for 7 days (June 2005) in fully reconstituted water at 24±1 ºC. TPT was added in absolute ethanol, the concentration of ethanol in water being 0.001% in all experimental groups, including solvent control (SC). Test concentrations were selected based on results from earlier studies in *M. cornuarietis* (Janer et al., 2006a) and on reported values of TPT in the aquatic environment. The exposure experiment was performed in 60 L glass aquaria with an Eihem filter system and additional aeration under 12-h light/12-h dark cycles. The exposure system was designed as semi-static renewal with addition of the test substance every 24 h (weekend 48 h) and 50% exchange of the water twice a week. Water parameters (pH, conductivity, temperature, nitrite, O_2 concentration and saturation) were measured twice a week per tank. Animals were fed daily with TetraMin® (Tetra, Melle, Germany) *ad libitum*. Exposed organisms were cooled in ice and the digestive gland/gonad complex was dissected, deep-frozen in liquid nitrogen, and stored at −80 ºC for determination of steroid levels and enzymatic activities.

Sex hormone analysis

Tissue levels of testosterone (T) and estradiol (E2) were analysed in the digestive gland/gonad complex of *M. cornuarietis* as described in Janer et al. (2005b). Briefly, individual tissue samples (0.4–0.7 g. wet weight) were homogenised in ethanol and frozen overnight at −80 ºC. Homogenates were then extracted three times with ethyl acetate. The organic extract was separated into two aliquots for determination of free and total steroids.

For the determination of free steroids, the ethyl acetate aliquot was evaporated under nitrogen and the solid re-dissolved in 80 % methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was re-dissolved in Milli-Q water and passed through a C18 cartridge, which was washed with Milli-Q water (8 mL), dried and then connected to a NH2 cartridge (Waters, Sep-Pack® Plus). The C18-NH2 system was then washed with hexane, and the steroids were eluted with dichloromethane:methanol (7:3). This fraction was collected and evaporated to dryness under nitrogen. For the determination of total steroids (free and esterified), the ethyl acetate aliquot was evaporated under nitrogen and processed as described by Gooding et al. (2003). The dry residue was resuspended in methanol containing 1% KOH and incubated at 45°C for 3 h. After the saponification step, Milli-Q water was added, and the sample was extracted three times with dichloromethane. The efficiency of the extraction and delipidation was 74 ± 3 % for testosterone and 80 ± 3% for estradiol (Morcillo and Porte, 1999a). The recovery of the purification step was in the range of 95-97% for both testosterone and estradiol (Janer et al., 2005b).

Dry extracts of free and total steroids were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine and assayed for estradiol and testosterone concentrations using commercial ^{125}I RIA kits (Radim, Rome, Italy). Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection of the method were 20 pg/g for E_2 and 100 pg/g for T.

Subcellular fractioning

Digestive gland/gonad complex of *M. cornuarietis* individuals was homogenised in ice-cold 100 mM potassium phosphate buffer pH 7.4, containing 100 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline and 0.1 mM trypsin inhibitor. Homogenates were first centrifuged at 500 × *g* for 20 min and the supernatant was further centrifuged at 12,000 × *g* for 45 min. After centrifugation at 100,000 × *g* for 90 min, the obtained pellet was resuspended in homogenization buffer and centrifuged again at 100,000 × *g* for 30 min. The obtained pellet, termed microsomal fraction, was resuspended in microsomal buffer, consisting of 100 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM phenanthroline, 0.1 mM

trypsin inhibitor and 20% (w/w) glycerol. Microsomes were stored at -80 °C until assays were performed. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Androstenedione metabolism

Androstenedione metabolism was assessed as indicated in Janer et al. (2005c). Briefly, microsomal proteins (200 µg) were incubated in 100 mM potassium phosphate buffer pH 7.4, containing 1.0 mM EDTA, 0.2 µM [^{3}H]-androstenedione (150,000 dpm), and 1.0 mM NADPH. The reaction was initiated by the addition of NADPH, and incubated in constant shaking at 30 °C for 60 min. Incubations were stopped with acetonitrile and after centrifugation (1500 × *g*, 5 min), 200 µl of the supernatant was injected onto the RP-HPLC column. Separation of androstenedione metabolites was performed on a Perkin-Elmer Binary LC pump 250 system equipped with a 250 mm × 4 mm LiChrospher 100 RP-18 (5 µm) reversed-phase column (Merck, Darmstadt Germany) protected by a guard column LiChrospher 100 RP-18 (5 µm). The mobile phase pumped at 1 mL/min was composed of (A) 75% water and 25% acetonitrile and (B) 25% water and 75% acetonitrile. The run consisted on a linear gradient from 100% A to 100% B (0–30 min), followed by isocratic mode 100% B (5 min), linear gradient from 100% B to 100% A (5 min), and isocratic mode 100% A (5 min). Radioactive metabolite peaks were monitored by online radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo-Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks. Identification of the metabolites was based on the comparison of their retention times to those obtained from commercially available standards and monitored at 254 nm using a UV-detector (Knauer LC-photometer) and further analysed by gas chromatography-mass spectrometry (GC-MS).

P-450 aromatase activity was assessed as described in Morcillo et al. (1999b), with some modifications. The assay is based on the release of tritiated water from the C-1 carbon atom of labelled androstenedione ([^{3}H]1β-androst-4-ene-3,17-dione) during its conversion to estrone by the enzyme P450 aromatase. Microsomal proteins (250 µg) were incubated at 25 ºC for 3 hours in the presence of 100 mM Tris-HCl buffer pH 7.6, 10 µM of androstenedione (1 µCi) and 200 µM NADPH in a final volume of 0.5 ml.

The samples were assayed in duplicates with a blank containing buffer instead of sample for each set. The reaction was stopped by adding 0.5 ml of cold buffer to the reaction tubes whilst on ice, and the aqueous phase was washed immediately with dichloromethane (3x) to remove steroids and other organic compounds. The solution was then mixed thoroughly with 2.5% (w/v) activated charcoal containing 0.25% dextran in Milli-Q water and centrifuged at 1500 $\times$ *g* for 1 h to remove residual steroids. Two aliquots of the supernatant were counted for ^{3}H in a liquid scintillation counter (tri-Carb 2100TR, Packard). The lowest activity detected by the method was 90 fmol/h/mg of protein.

Statistical analysis

Statistical significance was assessed by using one-way ANOVA (Dunnett's test for differences from control) and a Student *t*-test when comparing males and females. *P* values lower than 0.05 were considered statistically significant.

7.3 Results

Endogenous levels of steroids

Steroid levels were determined with and without a mild saponification step in the digestive gland/gonad complex of *M. cornuarietis*. In the absence of the saponification step, unconjugated/free steroids were measured. Endogenous levels of free testosterone and estradiol showed high variability between individuals and were within the same range of concentration for both sexes. Exposure to TPT for one week did not alter significantly the levels of free steroids in either males or females (Figure 7-1).

After saponification of the samples, the ester bonds of steroid metabolites were cleaved and total steroids (including both esterified and free steroids) were determined. Esterified/conjugated steroids were calculated by subtraction of the free steroids from the total steroid content. Both testosterone and estradiol were found predominately in their esterified form: esterified testosterone was 97.0 ± 1.3% of total testosterone in males and 91.9 ± 2.9% in females; esterified estradiol constituted 99.4 ± 0.3% of total estradiol in males and 98.0 ± 0.9% in females. Esterified estradiol content was much higher (~ 7-fold) than esterified testosterone, and males had higher concentration

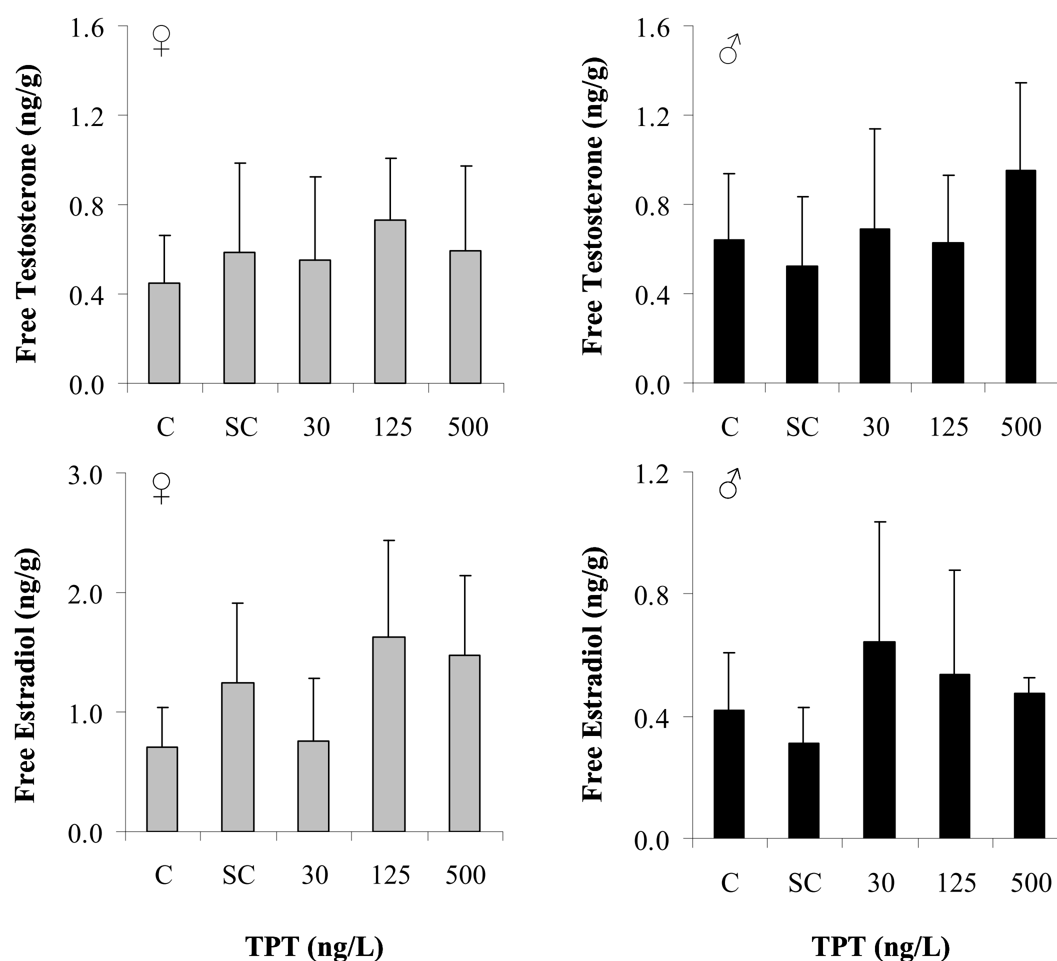

Figure 7-1. Free testosterone and estradiol levels determined in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT (ng as Sn/L) for 7-days. Values are mean ± SEM (n = 4).

of esterified steroids than females (Figure 7-2). After one-week exposure to TPT, levels of esterified steroids were significantly altered in exposed females that showed increased levels of esterified testosterone (1.9-fold increase at 30 and 125 ng TPT/L) and decreased levels of esterified estradiol (3.4-fold decrease at 500 ng TPT/L) (Figure 7-2). Interestingly, no alteration on esterified steroids was observed in TPT-exposed males (Figure 7-2).

Androgen metabolism

The metabolism of androstenedione (AD) by microsomal fractions isolated from the digestive gland/gonad complex of *M. cornuarietis* was found to be sexually dimorphic,

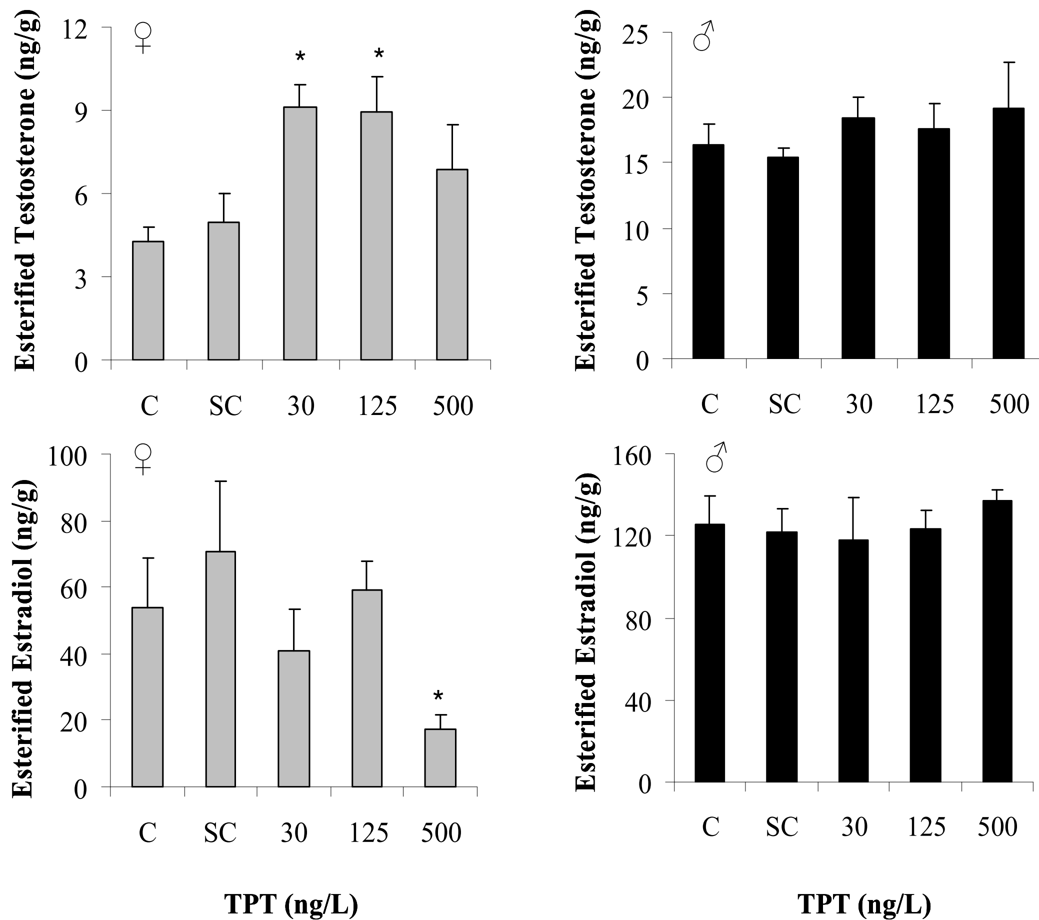

Figure 7-2. Esterified testosterone and estradiol levels determined in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT (ng as Sn/L) for 7-days. Values are mean ± SEM (n=4). *Significant differences respect to control ($p < 0.05$).

Table 7-1. Androstenedione (AD) metabolism in microsomal fractions isolated from the digestive gland/gonad complex of males and females of *Marisa cornuarietis*.

	%AD metabolised	**T**	**DHT**	**DHA**	**(DHT+T)/DHA**
Males	5.5±0.5	4.9±0.5	8.4±1.0	1.3±0.2	15.8±3.1
	(1.9-11)	(1.8-10.7)	(0.7-15.2)	(n.d.-3.2)	(4.9-49.3)
Females	2.8±0.2*	0.6±0.1*	1.2±0.2*	5.8±0.5*	0.4±0.05*
	(0.9-4.9)	(n.d.-2.1)	(n.d.-2.7)	(1.1-10.8)	(0.1-1.2)

Values are the mean ± SEM (n = 20 males; n = 25 females) expressed in pmol/h/mg of microsomal protein. The range of values is shown in parenthesis.*Significant differences between males and females (Student's t-test, $p<0.01$)

as previously reported for this species (Janer et al., 2005c). AD was metabolised to dihydrotestosterone (DHT) and testosterone (T) in males, and to dihydroandrostenedione (DHA) in females (Table 7-1). The ratio DHT + T (17β-reduced metabolites) to DHA (5α-reduced metabolite) was higher in males (15.8 ± 3.1) than in females (0.40 ± 0.05), an indication of comparatively high 17β-HSD activity in males and high 5α-reductase activity in females. Overall, the metabolism of AD in *M. cornuarietis* was rather low, as only 2.8 to 5.5% of the AD was metabolised under the assay conditions (Table 7-1). After one-week exposure to TPT, no significant alteration in the metabolism of AD was detected despite a tendency towards increased formation of T and DHT from AD in TPT-exposed males (125-500 ng/L) (Figure 7-3).

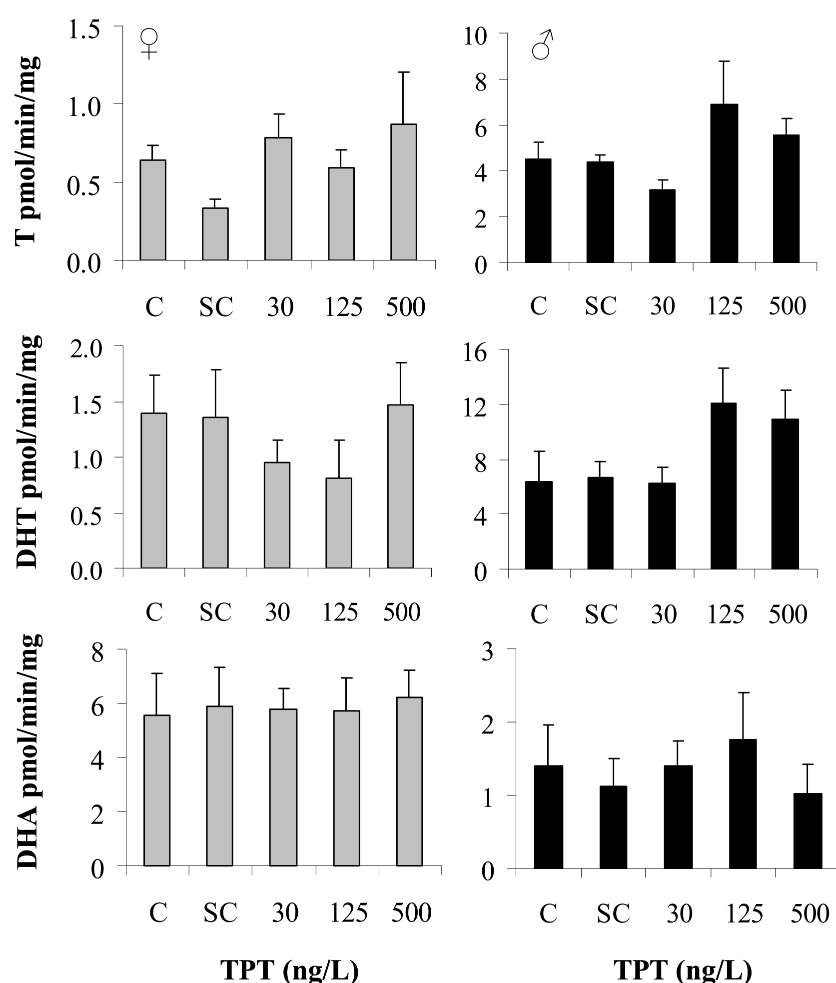

Figure 7-3. Rate of formation of T, DHT and DHA by microsomal fractions isolated from the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT for 7-days. Values are mean ± SEM (n = 5).

P450 aromatase activity was additionally determined in microsomal fractions isolated from the digestive gland/gonad complex of females. After one-week exposure to TPT, no significant alteration of P450 aromatase activity was observed in exposed females (139-627 fmol/min/mg of protein) when compared to controls (202-547 fmol/min/mg of protein), despite of a tendency to elevated P450 aromatase in females exposed to 500 ng TPT/L (Figure 7-4).

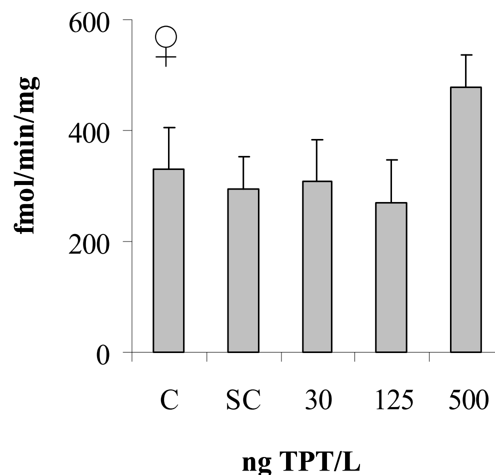

Figure 7-4. P450 aromatase activity in microsomal fractions isolated from the digestive gland/gonad complex of females of *Marisa cornuarietis* exposed to TPT for 7-days. Values are mean ± SEM (n = 4).

7.4 Discussion

Most of the estradiol and testosterone determined in the digestive gland/gonad complex of *M. cornuarietis* were in the esterified form, confirming previous results by Gooding and LeBlanc (2004) and Janer et al. (2006a). Short-term exposure to TPT did not significantly alter free steroid levels, but lead to a significant increase of esterified testosterone and a decrease of esterified estradiol at the highest concentration tested (500 ng/L TPT). Interestingly, these changes were observed only in exposed females, whereas no significant differences among control and exposed males were observed.

The significance of the observed changes in esterified steroids in females is unknown. Fatty acid esterification increases the lipophilicity of the steroid molecule and presumably facilitates its storage in lipoidal matrices while reducing its bioactivity, bioavailability and susceptibility to elimination processes (Borg et al., 1995). However,

research in rodents has revealed that steroid esters are more potent than free forms and that esterification does not deactivate the biological effect of the steroids but on the contrary, it prolongs their action (Hochberg, 1998). Thus, a single injection of immature rodents with esterified estradiol activated the hypothalamic-pituitary-ovarian axis and advanced puberty, a response that can only be mimicked by multiple injections of estradiol (Hochberg, 1991). Similarly, testosterone fatty acid esters have increased potency and duration compared to free testosterone in the brain of rodents (Hochberg, 1991; Borg et al., 1995). The prolonged action of esterified steroids has been attributed to the esterification of the D-ring, which dramatically protects the steroid nucleus from metabolism and consequently prolongs their biological life (Larner et al., 1985). These steroidal fatty acid esters can be converted back to their free steroid form through enzymatic hydrolysis by esterases. Thus, short-term exposure to TPT leads to an accumulation of testosterone as fatty acid esters in exposed females that may act as a continuous testosterone reservoir from which testosterone can be converted back to its active free form by esterases. Furthermore, the decrease in esterified estradiol observed at the 500 ng TPT/L exposure indicates a decrease of the estradiol reservoir in exposed females which could be understood as a ‘masculinising’ effect of TPT in exposed females.

The ‘masculinising’ effect of TPT was still evident in females after 50-days exposure to 500 ng/L TPT (Janer and Porte, unpublished results). Thus, levels of esterified estradiol decreased from 4.82 ± 0.98 ng/g in control females to 1.82 ± 0.35 ng/g in those exposed to 500 ng/L TPT, while esterified testosterone increased from 3.77 ± 0.98 ng/g in control females to 5.65 ± 3.10 ng/g in those exposed to 500 ng/L TPT. After 150 days of exposure, only a significant decrease in esterified estradiol was observed in 500 ng/L TPT-exposed females (3.20 ± 1.07 ng/g) when compared to controls (6.49 ± 1.49 ng/g).

These findings contrast with previous data on TBT long-term studies. Gooding et al. (2003) reported an increase in free testosterone and a concomitant decrease in esterified testosterone in the gastropod *I. obsoleta* exposed to 10 ng/L TBT for 3 months. Also, 100-day exposure of *M. cornuarietis* to 30, 125 and 500 ng/L TBT lead to a significant decrease in esterified testosterone in females (Janer et al., 2006a). Thus, although both TBT and TPT induce imposex in neogastropod species, they show different mechanisms of action in terms of alteration of endogenous steroid levels. In-vitro studies have demonstrated that 100 µM TBT had a rather low inhibitory effect

(<20%) on the activity palmitoyl coenzyme A:testosterone acyltransferase, the enzyme responsible for the fatty acid esterification of testosterone, while TPT failed to inhibit the enzyme (Janer et al., 2005c). Sex steroid acyltransferases have not yet been fully characterized, and it is uncertain whether they are specific for a single sex steroid or if they can conjugate different sex steroids. So far, available evidence indicates that a single enzyme conjugates different sex steroids and that it can do so with different fatty acid moieties (Janer et al., 2004, 2006a). Thus, the mechanisms through which TPT increases the esterification of testosterone and decreases the esterification of estradiol remain to be investigated. Factors, such as the activity of esterases or the availability of cofactors (i.e., fatty acids and acyl-CoA), might regulate the equilibrium between free and esterified steroids, and might have been affected by TPT-exposure.

In an attempt to identify other potential targets of TPT, we investigated the microsomal metabolism of AD by the digestive gland/gonad complex of control and exposed individuals. The metabolism of AD in *M. cornuarietis* was rather low in comparison to other invertebrate species (Janer et al., 2005c). Unexposed males metabolized 11% of AD in the incubation system, whereas only 5% was metabolised by females. AD metabolism was sexually dimorphic: males converted AD mainly to T and DHT, whereas females metabolised AD to DHA. However, the metabolism of AD was not significantly altered by TPT exposure. Similarly, the metabolism of AD was unchanged after 50 days exposure of *M. cornuarietis* to different concentrations of TPT, despite the fact that females from the high exposure group (500 ng TPT/L) had developed imposex (Janer et al., 2006b). Only after 150 days exposure to TPT, a decrease of the formation of DHA and a decrease in the % of AD metabolised was observed in exposed females. However, an indirect toxic effect caused by TPT at the highest dose tested cannot be discarded. Thus, both short and long-term exposure studies indicated that 5α-reductase and 17β-HSD, the enzymes involved in AD metabolism in *M. cornuarietis*, are not substantially affected by TPT exposure.

Regarding the activity of P450 aromatase, no significant differences were observed between control and exposed females, an indication that P450 aromatase might not be a primary target of TPT in *M. cornuarietis*. Additionally, the observed increase in esterified testosterone with the concomitant decrease in esterified estradiol observed in exposed females was not directly related to an inhibition of P450 aromatase.

An increasing number of studies suggest that the action of organotin compounds in gastropods may be mediated through the action of nuclear receptors (Nishikawa, 2006). Both, TBT and TPT, were high affinity ligands of the retinoid X receptor in the marine gastropod *Thais clavigera* and injections of the snail with 9-*cis* retinoic acid, the vitamin A derivative, which has been shown to be one of the endogenous vertebrate RXR ligands (Chawla et al., 2001), resulted in the development of imposex (Nishikawa et al., 2004). Vertebrate RXRs form heterodimers with a number of other nuclear receptors, among them PPARs (Szanto et al., 2004). It has been reported that TBT and TPT activate the RXR:PPARγ heterodimer in humans and this heterodimer is involved in lipid regulation (Kanayama et al., 2005). Although PPARs have emerged later in evolution and have not been identified in invertebrates yet (Thornton et al., 2003), the hypothesis of a regulation of lipid homeostasis by organotin compounds through activation of RXR is a challenging one and will possibly help to understand the observed changes in terms of esterified steroids. Hence, exposure of *M. cornuarietis* to 500 ng TBT/L for 100 days induced lipid and fatty acid accumulation in imposex induced females (Janer et al., 2007); changes in the bioavailability of fatty acids could affect the synthesis of steroid esters and consequently their accumulation in the tissue from where they act as a “steroid reservoir”.

In conclusion, this study shows that short-term exposure of *M. cornuarietis* to environmentally relevant doses of TPT did not alter levels of free testosterone and estradiol in the digestive gland/gonad complex of exposed individuals, but it significantly increased testosterone fatty acid esters and decreased estradiol fatty acid esters in exposed females, being the effect not detected in males. The enzymes involved in the metabolism of the androgen precursor androstenedione (specifically 17β-HSD, 5α-reductase and P-450 aromatase) were not significantly altered by TPT exposure, suggesting that such enzymes are not primary targets of TPT in *M. cornuarietis*. Other mechanisms, such as alterations in fatty acids bioavailability, changes in the activity of steroid esterases and probably the binding of TPT to nuclear receptors, might have lead to the observed changes in esterified steroids in exposed females. Additional studies are needed to fully understand the lack of sensitivity of exposed males as well as the significance of the observed alterations in esterified steroids in females and their potential relationship with the development of imposex.

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Chapter 8. TPT modulates fatty acid profile, metabolism and lipid content in females of Marisa cornuarietis *following 7-days in-vivo exposure*

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Abstract

Molluscs are sensitive species to the toxic effects of organotin compounds, particularly to masculinisation. Both tributyltin (TBT) and triphenyltin (TPT) have been recently shown to bind to mollusc retinoid X receptor (RXR). If RXR is involved in lipid homeostasis, exposure to TPT would have an immediate effect on endogenous lipids. To test this hypothesis, the ramshorn snail *Marisa cornuarietis* was exposed to environmentally relevant concentrations of TPT (30, 125, 500 ng/L as Sn) in a semi-static water regime for 7-days. Percentage of lipids and total fatty acid content decreased significantly in TPT-exposed females while the activity of peroxisomal acyl-CoA oxidase, involved in fatty acid catabolism, increased. In addition, fatty acid profiles (carbon chain length and unsaturation degree) were significantly altered in exposed females but not in males. This work highlights the ability of TPT to disrupt lipid metabolism in *M. cornuarietis* at environmentally realistic concentrations and the higher susceptibility of females in comparison to males.

8.1 Introduction

Since the late 1960s, organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT), have been extensively used across the world as biocides in antifouling paints, applied on ship hulls and fishing nets, and as fungicides in agricultural crops. Despite their gradual removal from the market and their prohibition of use, their release into the environment combined with their low solubility in water and high octanol-water partition coefficient has resulted in worldwide contamination of the aquatic environment (Fent, 1996). Both TBT and TPT are potent endocrine disruptors; abnormalities in the endocrine system related to TBT and TPT exposure have been observed in vertebrates (Iguchi et al., 2007; Kanayama et al., 2005; McAllister and Kime, 2003) and invertebrates (Alzieu, 2000; Oehlmann et al., 2007), with gastropods and oysters being among the most susceptible organisms. A concentration of 1 ng/L TBT is enough for the induction of imposex (superimposition of male secondary sexual characteristics, including a penis and vas deferens) in females of the gastropod *Nucella lapillus* (Bryan et al., 1986; Spooner et al., 1991).

Imposex has been reported in over 150 species of gastropods worldwide (Horiguchi et al., 1997), including *Marisa cornuarietis* following exposure to TBT and TPT (Schulte-Oehlmann et al., 1995, 2000). Although the link between imposex in female gastropods and exposure to TBT or TPT has been established, the exact mechanism through which this phenomenon occurs remains unclear. Scientific data demonstrate that imposex induced females experience elevated levels of free testosterone and this has been attributed among others to an inhibition of aromatase activity (Bettin et al., 1996), inhibition of the esterification of testosterone (LeBlanc et al., 2005) or alterations in the excretion of neurohormones that contribute to sexual differentiation in gastropods (Oberdörster and McClellan-Green, 2002).

Recent studies indicate that TBT and TPT may act through interaction with nuclear receptors (Nakanishi, 2007). Different ligand binding assays show that both TBT and TPT bind to the human Retinoid X receptor (hRXR) with high affinity, similar to that of 9-*cis* retinoic acid (9-*cis* RA), a proposed natural ligand of RXR (Nishikawa et al., 2004). RXR homologues have been cloned from the gastropods *Thais clavigera* (Nishikawa et al., 2004) and *N. lapillus* (Castro et al., 2007), as well as from the freshwater snail *Biomphalaria glabrata* (Bouton et al., 2005); all of them showing high similarity with vertebrate RXR. In all three species, 9-*cis* RA was a high affinity ligand,

suggesting that retinoid signalling pathways may exist in these species. Moreover, injections of *T. clavigera* and *N. lapillus* with 1 μg/g 9-*cis* RA induced imposex, leading to an increase in penis length and vas deferens similar to that induced by TBT and/or TPT in these species (Castro et al., 2007; Nishikawa et al., 2004).

In mammals, RXR forms heterodimers with orphan nuclear receptors (whose endogenous ligand is unknown: peroxisome proliferator-activated receptor (PPAR), liver X (LXR), farnesoid X (FXR), and pregnane X (PXR) receptor, as well as with the retinoic acid, thyroid hormone and vitamin D receptors (Szanto et al., 2004). These orphan receptors are lipid sensors as they get activated by lipid molecules and therefore play an important role in lipid homeostasis, whereas the later regulate the endocrine system and resemble more closely the action of steroid hormone receptors (Chawla et al., 2001). The RXR heterodimer is activated by ligands of either receptors and subsequently binds to the corresponding response elements in the promoter region of the target genes to modulate their transcription (Michalik et al., 2006). Knocking out RXR in mice disturbed lipid metabolism functions controlled by PPARα, PPARγ, LXRα, PXR and FXR (Szanto et al., 2004) showing the importance of this receptor in lipid homeostasis. Interestingly, TBT and TPT activate both RXR and PPARγ human receptors (Kanayama et al., 2005) and exposure of mice and the amphibian *Xenopus laevis* to TBT and RXR/PPARγ ligands stimulated lipid accumulation and ectopic adipocyte formation, respectively (Grün et al., 2006).

Additionally, activation of RXR and/or PPARs has been linked to alterations in the steroidogenic pathway: modulation of STAR protein expression (Seto-Young et al., 2007) and P450aromatase activity (Mu et al., 2000; Saitoh et al., 2001) in human granulosa cells. Thus, alterations in steroid hormone levels observed after exposure to TBT and TPT may also be a consequence of the interaction of the compounds with RXR rather than a direct interaction at the enzyme level (Nishikawa, 2006). Although receptors such as PPARs appear to have emerged later in the evolution of the nuclear receptor family (Thornton, 2003) and up to date they have not been identified in invertebrates, a lipid regulation mechanism possibly mediated by RXR in gastropods cannot be excluded. Indeed, females of the freshwater ramshorn snail *Marisa cornuarietis* exposed to different concentrations of TBT for 100 days showed increased percentage of lipids and total fatty acid content as well as significant alterations in the fatty acid profile (Janer et al., 2007).

Following the above observations, the study of this chapter hypothesises that being RXR involved in lipid homeostasis in gastropods as it is in mammals and vertebrates, exposure to TPT -a RXR agonist- will have an immediate effect on lipid homeostasis. More specifically, this study aims to investigate changes on lipid content and fatty acid profiles in *M. cornuarietis* after short term exposure to environmentally relevant concentrations of TPT. Additionally, the effect of TPT on the activity of acyl-CoA oxidase (AOX), the first and rate limiting enzyme of β-oxidation is examined. Peroxisomal AOX catalyzes the β-oxidation of very long (C>20) and long chain (C14-C18) fatty acids and its gene transcription is regulated by PPARα in mammals and vertebrates (Reddy and Hashimoto, 2001).

8.2 Materials and Methods

Chemicals

Triphenyltin chloride (TPT) was purchased from Merck (Darmstadt, Germany). Palmitoyl-CoA and NADPH were obtained from Sigma (Steinheim, Germany); 2,7-dichlorodihydrofluorescein (H_2DCF) diacetate was from Molecular Probes (Paisley, UK). All solvents and reagents were of analytical grade.

Animals

Ramshorn snails, *Marisa cornuarietis* (Mollusca: Prosobranchia: Ampullariidae), came from a laboratory breeding stock which was derived from a stock at Aquazoo Düsseldorf (Germany) in 1991 with regular cross-breeding of wild-caught animals from Florida (USA) to avoid inbreeding. The breeding stock was kept in a flow-through system with fully reconstituted water under constant conditions regarding temperature and light dark cycle (12:12 h). Water parameters (pH, temperature, conductivity, nitrite, oxygen concentration and saturation) were measured twice a week per tank. Parameters of the fully reconstituted influx water were pH 7.5, 850 µS/cm, <1 mg NO_2/L and >95% O_2 saturation.

Exposure experiment

For the exposure experiments, two replicate groups of 17 sexually mature snails each were exposed to three nominal concentrations of TPT (30, 125, and 500 ng as Sn/L) for 7-days (June 2005) in fully reconstituted water at 24±1 ºC. TPT was added in absolute ethanol, the concentration of ethanol in water being 0.001% in all experimental groups, including solvent control (SC). Test concentrations were selected based on results from earlier studies in *M. cornuarietis* (Janer et al., 2006) and on reported values of TPT in the aquatic environment (Becker-van Slooten and Tarradellas, 1995). The exposure experiment was performed in 40 L glass aquaria fitted with an Eheim filter system and additional aeration under 12-h light/12-h dark cycles. The exposure system was designed as semi-static renewal with addition of the test substance every 24 hours (weekend 48 hours) and 50% exchange of the water twice a week. Water parameters (pH, conductivity, temperature, nitrite, O_2 concentration and saturation) were measured twice a week before the water was changed. Animals were fed daily with TetraMin® (Tetra, Melle, Germany) *ad libitum*. Exposed organisms were cooled in ice, the digestive gland/gonad complex was dissected, deep-frozen in liquid nitrogen, and stored at –80 ºC for determination of steroid levels and enzymatic activities.

Peroxisomal fatty acyl-CoA oxidase activity

Acyl-CoA (palmitoyl-CoA) oxidase was assayed by the determination of H_2O_2 production, coupled to the oxidation of leuco-DCF in a reaction catalysed by exogenous peroxidase. The method was modified after Small et al. (1985). The digestive gland/gonad complex (0.3-0.7 g) from each *M. cornuarietis* individual was homogenised in TVBE buffer pH 7.6 (4 ml buffer/g of tissue), containing 1 mM sodium bicarbonate, 0.1 M EDTA, 0.1% ethanol and 0.01% Triton X-100. After homogenisation, samples were centrifuged at 500 x *g* for 15 min and the supernatant containing the peroxisomes was assayed for acyl-CoA oxidase activity. The reaction was carried out at 25 ºC in a final volume of 1 ml. The reaction mixture contained 0.05 mM leuco DCF (prepared weekly by hydrolysing 2.66 mM H_2DCF diacetate in 1:9 v/v, dimethylformamide: NaOH (0.01 M) and stored at -20 ºC), 0.07 mg horseradish peroxidase, 40 mM sodium azide, 0.01% Triton X-100, 10 mM potassium phosphate

buffer pH 7.4 and sample. This mixture was pre-incubated in the dark for 3 min, as some impurities in the peroxidase cause a small amount of oxidation of the leuco-DCF (Köchli and von Wartburg, 1978). After this time, the slow rate of auto-oxidation of the dye was determined by measuring spectrophotometrically the absorption at λ = 502 nm for 2 min. The reaction was then started by the addition of 30 μM of palmitoyl-CoA, and after 15 sec of incubation in the dark, the enzymatic reaction rate was determined for 2 min. Rates were corrected by subtracting the blank (before the addition of palmitoyl-CoA) and calculations were made by using a DCF molar extinction coefficient of 91,000 M^{-1} as obtained by Köchli and von Wartburg (1978) from the peroxidase-catalysed oxidation of leuco-DCF. Protein concentrations were determined by the method of Lowry et al. (1951) by using bovine serum albumin as a standard.

Fatty acid analysis

The digestive gland/gonad complex of *M. cornuarietis* individuals (0.3-0.7 g) were lyophilised and processed for lipid and fatty acid analysis. Lipids were extracted from the lyophilised samples by homogenisation in 2 ml ice-cold chloroform/methanol (2:1 v/v) plus 0.01% (w/v) butylated hydroxytoluene (BHT) as an antioxidant, following a modification of the method of Folch et al. (1957). After homogenisation, 0.25 ml of 0.88% KCl was added to the homogenates and the solution was mixed. After phase separation, the chloroform layer was removed, filtered and the solvent evaporated by flushing with nitrogen. The solid residue was then weighted to determine the total lipid levels, and afterwards redissolved in chloroform/methanol (2:1, v/v) with 0.01 % BHT, flushed with nitrogen and stored at -20 ºC in a screw cap vial. Lipid aliquots were transmethylated overnight (Christie, 1982) after addition of a known amount of nonadecanoic acid (19:0) as internal standard (Sigma). Fatty acid methyl esters (FAME) were extracted with hexane/diethyl ether (1:1, v/v), and purified by thin layer chromatography (silica gel G60, Merck) using hexane/diethyl ether/acetic acid (85:15:1.5, v/v/v) as solvent system. FAME were analysed with a Fissons 8000 gas chromatograph equipped with a fused silica 30 x 0.25mm open tubular column (Tracer, TR-WAX, film thickness: 0.25 μm), and a cold on-column injection system, using helium as carrier, and a 50-220 ºC thermal gradient. Peaks were recorded and integrated in a personal computer using Azur software (Datalys, France), and identified

by comparison with a well characterised sardine oil named Marinol (Fishing Industry Research Institute, Rosebank South Africa).

Statistical analysis

Results are mean values ± SEM. Differences between control groups (control and solvent control) were assessed with Student's *t*-test and they were not statistically significant (p<0.05). Thereafter, exposure groups were compared with the average of the control and solvent control groups by using one way ANOVA with Dunnett's post-hoc test.

8.3 Results

Peroxisomal acyl-CoA oxidase activity

AOX activity was determined in peroxisomal enriched fractions obtained from the digestive gland/gonad complex of *M. cornuarietis* individuals. After one week exposure to TPT, the activity AOX was significantly increased in females exposed to 30 and 500 ng TPT-Sn/L, resulting in 1.3- and 1.4-fold increase, respectively (Figure 8-1). AOX activity was also significantly increased (1.4-fold) in males exposed to 30 ng TPT-Sn/L, but no further differences were observed at higher TPT concentrations (Figure 8-1).

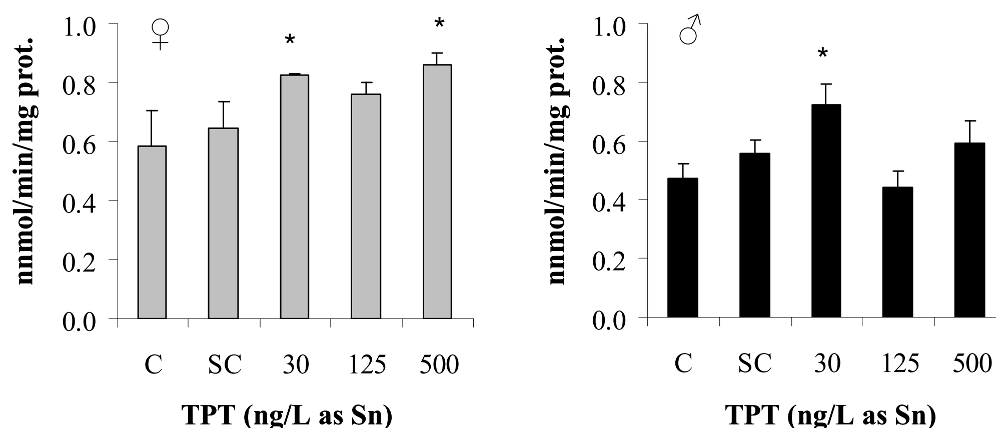

Figure 8-1. AOX activity in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT for 7-days. Values are the mean ± SEM (n=4). *Significant differences respect to control (p<0.05).

Fatty acid profile and lipid content

A detailed description of the fatty acid composition of control and exposed males and females of *M. cornuarietis* is given in Table 8-1. At least 33 fatty acids with carbon atoms from 14 to 24 were detected in the digestive gland/gonad complex of both males and females. Unsaturated fatty acids were the major group, accounting for 54% and 61% of total fatty acids in control males and females, respectively. Within this group, monounsaturated fatty acids (MUFA) constituted 28-30% of the total fatty acids and polyunsaturated fatty acids (PUFA) 26 to 31%. The most abundant unsaturated fatty acids were linoleic (18:2n-6) and oleic (18:1n-9) acids. Highly unsaturated fatty acids (3 or more saturations) of 20 or more atoms of carbon (HUFA) represented only about 10% of total fatty acids. The major forms were in decreasing order, arachidonic (20:4n-6), docosahexaenoic (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3). Saturated fatty acids represented 33% of the total fatty acids in males and 30% in females, and among them palmitic (16:0) and stearic (18:0) were the most abundant.

One-week exposure to TPT caused a shift in the fatty acid profile in the digestive gland/gonad complex of *M. cornuarietis* with alterations being more evident in females (Table 8-1). Thus, a ~10% decrease of MUFA (% FAME) and a ~20% increase of HUFA was observed in females exposed to 125 and 500 ng TPT-Sn/L; the increase in HUFA was associated to a relative increase of the n-6 HUFA group. To further understand the effect of TPT exposure on individual fatty acids, those were expressed as mg/g dry tissue. Interestingly, one week exposure to TPT resulted in a decrease in fatty acids, both in terms of chain length and saturation degree, and this decrease was mainly detected in females (Tables 8-2 & 8-3). Almost all exposed females revealed a decrease in fatty acid content in terms of chain length (Table 8-2). C18 fatty acids that accounted for 50% of total fatty acids in the control groups, showed a 40% decrease in females exposed to 30 and 500 ng TPT-Sn/L, whereas no significant alteration was observed in exposed males. In terms of unsaturation degree, saturated (ΣC:0), mono-unsaturated (ΣC:1) and di-unsaturated (ΣC:2) fatty acids, which account for 32, 34 and 23% of total fatty acids in the control groups, decreased in TPT-exposed females (30-40%) (Table 8-3). Only the tetra-unsaturated fatty acids (ΣC:4) were not significantly altered by TPT-exposure in females; within this group, arachidonic acid (20:4n-6) was the most abundant (97%).

Table 8-1. Fatty acid profile in the digestive gland/ gonad complex of *Marisa cornuarietis* following 7-days exposure to TPT.

	TPT (ng/L as Sn)									
	Males					Females				
Fatty acid	Control	Solvent	30	125	500	Control	Solvent	30	125	500
14:0	2.72±0.25	2.84±0.19	2.97±0.28	2.63±0.22	2.37±0.49	1.10±0.07	1.13±0.08	1.55±0.66	1.17±0.06	1.06±0.05
15:0	0.36±0.03	0.37±0.02	0.37±0.02	0.43±0.04	0.41±0.05	0.39±0.01	0.37±0.04	0.38±0.05	0.38±0.02	0.31±0.01*
16:0	18.9±0.92	19.6±0.78	18.9±0.67	20.0±1.08	19.6±1.80	17.1±0.19	16.1±0.24	17.1±1.65	16.3±0.20	15.7±0.33
16:1n-9	0.36±0.01	0.40±0.03	0.35±0.03	0.30±0.05	0.46±0.05	0.42±0.02	0.43±0.02	0.48±0.03	0.44±0.01	0.40±0.01
16:1n-7	1.14±0.05	1.24±0.19	1.17±0.04	1.33±0.08	1.32±0.18	1.24±0.04	1.30±0.10	1.00±0.17	1.22±0.12	1.12±0.09
16:2	0.21±0.01	0.19±0.03	0.20±0.01	0.21±0.01	0.21±0.01	0.23±0.02	0.19±0.01	0.20±0.01	0.19±0.01	0.20±0.01
17:0	1.04±0.04	1.03±0.06	0.94±0.05	1.05±0.06	1.23±0.08	1.15±0.02	1.03±0.03	1.23±0.03**	1.08±0.02	1.11±0.03
16:3	0.19±0.04	0.21±0.06	0.15±0.04	0.24±0.05	0.13±0.02	0.13±0.02	0.21±0.02	0.18±0.05	0.12±0.01*	0.11±0.00*
18:0	8.35±0.37	8.72±0.32	8.25±0.39	8.40±0.45	8.76±0.55	7.96±0.15	6.97±0.33	8.12±0.57	7.32±0.41	7.43±0.24
18:1n-11	0.18±0.01	0.14±0.01	0.16±0.02	0.20±0.05	0.19±0.04	0.21±0.03	0.11±0.01	0.15±0.03	0.17±0.04	0.13±0.01
18:1n-9	13.7±0.51	14.0±1.61	13.9±0.36	16.4±0.92	15.9±2.06	16.9±0.69	16.0±0.47	13.5±1.34	14.5±0.55*	14.4±0.32**
18:1n-7	1.98±0.10	1.93±0.11	2.01±0.15	1.81±0.09	1.90±0.20	1.53±0.09	1.47±0.09	1.36±0.23	1.37±0.06	1.34±0.05
18:2n-6	13.1±1.62	13.4±1.60	14.4±1.18	13.4±2.42	11.8±3.10	17.3±1.13	19.3±0.57	14.8±2.26	18.1±0.48	17.7±0.64
18:3n-6	0.13±0.01	0.12±0.003	0.16±0.01	0.10±0.01	0.11±0.02	0.09±0.00	0.10±0.00	0.10±0.00	0.09±0.00	0.09±0.00
18:3n-3	0.44±0.17	0.59±0.14	0.66±0.08	0.61±0.17	0.49±0.17	0.80±0.09	0.96±0.04	0.60±0.14	0.88±0.06	0.78±0.08
18:4n-3	0.08±0.01	0.08±0.01	0.06±0.01	0.09±0.02	0.08±0.02	0.11±0.02	0.12±0.01	0.11±0.00	0.12±0.01	0.11±0.01
20:0	1.14±0.07	1.04±0.06	1.08±0.07	1.25±0.13	1.17±0.19	1.19±0.06	0.87±0.05	0.74±0.08	0.88±0.13	0.85±0.03
20:1n-9	4.76±0.19	4.38±0.21	4.25±0.09	3.99±0.61	5.07±0.34	4.62±0.25	4.16±0.15	4.72±0.33	4.54±0.25	4.35±0.23
20:1n-7	2.79±0.11	2.68±0.25	2.73±0.08	2.73±0.35	3.15±0.36	3.21±0.17	3.03±0.10	2.58±0.25	2.82±0.05*	2.86±0.11
20:2n-6	1.04±0.12	0.96±0.04	0.97±0.05	0.95±0.16	0.93±0.18	1.18±0.09	1.29±0.09	1.29±0.20	1.19±0.10	1.30±0.02
20:3n-6	0.63±0.04	0.53±0.07	0.67±0.05	0.50±0.05	0.49±0.11	0.39±0.01	0.45±0.03	0.46±0.01	0.49±0.04	0.31±0.07
20:4n-6	3.61±0.2	3.59±0.55	3.35±0.16	2.96±0.35	3.65±0.82	3.52±0.24	4.00±0.31	5.66±1.33	4.97±0.34*	4.92±0.33*

20:4n-3	0.05±0.01	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.01	0.06±0.00	0.07±0.00	0.07±0.01	0.06±0.005	0.05±0.002
20:5n-3	1.30±0.13	1.27±0.24	1.28±0.18	1.26±0.27	1.22±0.40	1.75±0.25	1.96±0.04	1.92±0.40	1.99±0.07	2.21±0.05
22:0	0.75±0.05	0.73±0.04	0.69±0.06	0.83±0.08	0.74±0.12	0.81±0.03	0.65±0.03	0.54±0.04	0.64±0.07	0.64±0.03*
22:1n-11	2.91±0.09	2.65±0.36	2.94±0.13	2.98±0.32	2.72±0.22	2.85±0.27	2.81±0.09	2.23±0.41	2.44±0.18	2.74±0.22
22:1n-9	0.09±0.01	0.09±0.02	0.15±0.06	0.09±0.01	0.16±0.08	0.08±0.01	0.08±0.01	0.09±0.004	0.07±0.01	0.08±0.005
22:1n-7	0.05±0.00	0.06±0.01	0.06±0.00	0.06±0.00	0.05±0.01	0.06±0.004	0.05±0.0	0.07±0.007	0.05±0.003	0.05±0.0
22:2n-6	1.02±0.04	0.99±0.08	0.96±0.02	0.84±0.09	1.09±0.21	1.11±0.08	1.27±0.11	1.68±0.33	1.46±0.13	1.50±0.13
22:5n-6 /22:3n-3	1.33±0.09	1.28±0.19	1.48±0.24	0.97±0.19	0.85±0.32	0.09±0.02	0.12±0.01	0.46±0.3	0.10±0.01	0.10±0.01
22:5n-3	0.34±0.06	0.32±0.06	0.35±0.06	0.33±0.08	0.64±0.28	0.43±0.04	0.48±0.01	0.41±0.08	0.46±0.01	0.49±0.01
22:6n-3	2.93±0.39	2.64±0.50	3.23±0.39	2.48±0.44	1.96±0.44	2.18±0.32	2.43±0.08	1.82±0.25	2.26±0.09	2.23±0.13
24:1n-9	0.24±0.02	0.25±0.03	0.22±0.02	0.28±0.01	0.25±0.02	0.24±0.02	0.23±0.01	0.18±0.03	0.22±0.01	0.23±0.01
SFA	33.3±1.57	34.3±1.27	33.2±1.31	34.6±1.87	34.0±3.18	29.7±0.45	27.1±0.57	29.6±3.00	27.7±0.66	27.1±0.53
MUFA	28.2±0.58	27.8±2.53	27.9±0.62	30.2±1.91	31.1±3.14	31.3±1.50	29.6±0.60	26.3±2.14	27.8±0.74*	27.7±0.49*
PUFA	26.4±2.27	26.2±2.86	28.0±1.78	24.9±3.81	23.5±5.46	29.3±1.98	32.9±0.29	29.6±3.81	32.4±0.62	32.1±0.43
n-3	5.11±0.69	4.90±0.96	5.60±0.69	4.81±0.96	4.25±1.19	5.29±0.71	6.02±0.10	4.84±0.81	5.76±0.20	5.87±0.26
n-6	20.9±1.68	20.9±1.99	22.0±1.08	19.7±2.87	18.9±4.31	23.6±1.31	26.5±0.24	24.3±3.10	26.4±0.48	25.9±0.22
HUFA	10.18±0.46	9.66±1.49	8.77±1.72	8.54±1.10	8.68±1.93	8.35±0.82	9.50±0.25	10.65±1.50	10.32±0.31*	10.31±0.28*
HUFA n-3	4.61±0.56	4.26±0.82	4.88±0.61	4.12±0.78	3.70±1.01	4.39±0.60	4.94±0.06	4.19±0.66	4.77±0.14	4.98±0.17
HUFA n-6	7.62±0.18	7.34±0.78	7.43±0.39	6.21±0.68	6.99±1.33	6.25±0.32	7.12±0.46	9.44±1.63	8.20±0.52*	8.13±0.55*
Total FAME (mg/g d.w.)	59.4±8.96	52.1±3.34	62.2±6.50	64.2±4.17	41.7±3.20*	56.4±1.63	61.5±5.68	41.2±4.53*	49.1±0.83*	40.1±2.15**
Total lipids (% of d.w.)	15.9±0.91	15.8±0.52	17.7±0.89	17.5±0.99	14.7±1.44	14.5±0.56	14.1±0.80	12.7±1.52	13.6±0.69	11.6±0.16**

Data are expressed as % of total fatty acid methyl esters (mean±SEM; n=4). * and **significant differences respect to controls ($p<0.05$ and $p<0.01$ respectively).

Table 8-2. Levels of fatty acids grouped by chain length in the digestive gland/gonad complex of *Marisa cornuarietis* following 7-days exposure to TPT.

	TPT (ng/L as Sn)				
	C	SC	30	125	500
Females					
Σ C14	0.62±0.04	0.70±0.10	0.70±0.36	0.58±0.03	0.42±0.03**
Σ C15	0.22±0.01	0.65±0.08	0.28±0.07	0.19±0.01*	0.12±0.01*
Σ C16	10.77±0.39	11.20±1.02	8.01±1.55	8.95±0.10*	7.06±0.43**
Σ C17	0.65±0.03	0.63±0.05	0.50±0.05	0.53±0.02	0.45±0.03*
Σ C18	25.3±0.80	27.7±2.80	16.1±2.20**	20.9±0.37*	16.8±0.76**
Σ C20	8.96±0.24	9.73±0.94	6.94±0.24**	8.31±0.32	6.78±0.53**
Σ C22	4.03±0.21	4.83±0.45	2.96±0.43*	3.67±0.07*	3.13±0.09**
Σ C24	0.13±0.02	0.14±0.02	0.08±0.02*	0.11±0.01*	0.09±0.00**
Males					
Σ C14	1.56±0.11	1.46±0.03	1.80±0.04*	1.67±0.07	1.00±0.21
Σ C15	0.21±0.03	0.38±0.08	0.52±0.10	0.27±0.02	0.17±0.03
Σ C16	12.15±1.38	11.22±0.73	12.79±1.02	14.16±1.05	9.15±1.43
Σ C17	0.61±0.07	0.53±0.03	0.58±0.05	0.67±0.05	0.51±0.06
Σ C18	22.9±4.48	20.5±2.06	24.8±3.25	26.5±2.50	16.3±1.20
Σ C20	9.08±1.31	7.51±0.43	8.93±0.99	8.72±0.34	6.40±0.44
Σ C22	5.70±1.18	4.56±0.37	6.12±0.70	5.53±0.61	3.36±0.48
Σ C24	0.14±0.02	0.13±0.02	0.13±0.02	0.18±0.02	0.10±0.02

Values are expressed as mg/g of dry weight (mean ± SEM; *n*=4). Significant differences respect to controls indicated by *p<0.05 and **p<0.01.

Table 8-3. Levels of fatty acids grouped by unsaturation degree in the digestive gland/gonad complex of *Marisa cornuarietis* following 7-days exposure to TPT.

	TPT (ng/L as Sn)				
	C	SC	30	125	500
Females					
Σ C:0	16.6±0.68	16.6±1.32	12.5±2.51	13.7±0.19**	10.9±0.63**
Σ C:1	17.7±1.34	18.3±1.97	11.1±1.96*	13.7±0.33*	11.1±0.40**
Σ C:2	10.9±0.58	13.6±1.43	7.3±1.12**	10.3±0.28	8.29±0.41**
Σ C:3	0.79±0.04	1.06±0.12	0.55±0.07**	0.77±0.04	0.52±0.04**
Σ C:4	2.06±0.09	2.56±0.25	2.19±0.23	2.53±0.20	2.05±0.22
Σ C:5	1.25±0.13	1.57±0.12	1.08±0.09*	1.25±0.04	1.12±0.07*
Σ C:6	1.22±0.16	1.50±0.17	0.75±0.13**	1.11±0.03	0.89±0.05**
Males					
Σ C:0	19.4±2.14	17.8±0.90	20.4±1.42	22.2±1.53	14.3±2.17
Σ C:1	16.8±2.83	14.6±1.89	17.4±2.06	19.4±1.87	13.2±2.23
Σ C:2	9.49±2.30	8.19±1.15	10.48±1.73	9.96±2.06	5.65±1.28
Σ C:3	0.86±0.22	0.76±0.08	1.03±0.14	0.94±0.17	0.50±0.09
Σ C:4	2.18±0.27	1.90±0.29	2.13±0.24	1.97±0.20	1.52±0.33
Σ C:5	1.79±0.34	1.49±0.27	1.93±0.25	1.63±0.23	1.05±0.29
Σ C:6	1.84±0.54	1.38±0.30	2.04±0.37	1.60±0.34	0.80±0.19*

Values are expressed as mg/g of dry weight (mean ± SEM; *n*=4). Significant differences respect to controls indicated by *p<0.05 and **p<0.01.

Overall, one week exposure to 30, 125 and 500 ng TPT/L resulted in a drop in the total fatty acid levels (FAME) in females equal to 33, 20 and 35% (Figure 8-2). TPT caused a significant decrease (20%) in total fatty acid levels in males as well but only

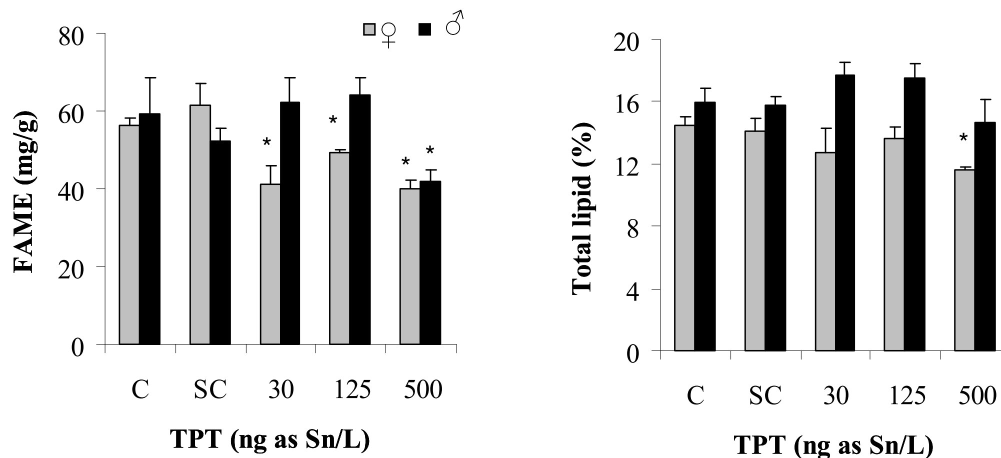

Figure 8-2. Fatty acid methyl esters (FAME, mg/g dry mass) and total lipids (% dry weight) in the digestive gland/gonad complex of *Marisa cornuarietis* exposed to TPT for 7-days. Values are mean ± SEM (n=4). *Significant differences respect to control (p<0.05).

at the highest TPT concentration (Figure 8-2). Furthermore, TPT exposure resulted in a significant decrease (20%) in the percentage of lipids in the digestive gland/gonad complex of females at the highest TPT concentration (500 ng/L) but had no significant effect in exposed males (Figure 8-2).

8.4 Discussion

One week exposure to TPT had a significant effect on the percentage of lipids, fatty acid content and fatty acid metabolism in the digestive gland/gonad complex of females of *M. cornuarietis*, whereas males demonstrated very few significant alterations. Percentage of lipids, total fatty acid content as well as carbon chain length and unsaturation degree, all decreased significantly in TPT-exposed females. In parallel, the activity of peroxisomal AOX, the enzyme responsible for the break down of C14-C18 and C>20 fatty acids was significantly induced, which supports the observed decrease in fatty acid content.

In vertebrates, the peroxisomal AOX gene is transcriptionally activated by PPARα (Reddy and Hashimoto, 2001). Activation of the enzymes involved in the peroxisomal β-oxidation pathway, including AOX, with a parallel increase in volume and density of peroxisomes is a phenomenon known as peroxisome proliferation that has been related to hepatocarcinogenesis in rats and mice (Yu et al., 2003). Although

PPARs have not been identified in invertebrates (Thornton, 2003), existing data demonstrate that peroxisome proliferation in response to organic contaminant exposure occurs. Thus, induction of AOX activity with a parallel increase in peroxisomal volume density have been observed in mussels *Mytilus edulis* exposed to specific peroxisome proliferators (fibrates and phthalates) and various organic pollutants (PAHs and PCBs) (Cajaraville and Ortiz-Zarragoitia, 2006; Ortiz-Zarragoitia and Cajaraville, 2006), in slugs *Arion ater* exposed to a Cd-kerosene mixture (Zaldibar et al., 2007) and in the land snail *Helix aspera* exposed to air-born urban pollutants (Regoli et al., 2006). Therefore, a mechanism of peroxisome-proliferation analogous to the one promoted by PPARα activation in vertebrates also exists in invertebrates.

The fact that total lipids and almost all fatty acid groups decreased in such a short exposure period in exposed females is of special concern, taking into account the multi-functional role of fatty acids in cell structure and function, energy metabolism and storage, bioactive signalling and synthesis of various compounds involved in physiological regulation (e.g. steroids, eicosanoids, etc.) (Benatti et al., 2004). Toxicity of organotin compounds has been related to their interference with cell's membrane permeability, fluidity and signalling (Ortiz et al., 2005). Thus, exposure of ovaries of *Ciona intestinalis* to TBT for 5 hours caused a reduction in total lipids and triglycerides but an increase in phospholipids and PUFA, including HUFA and arachidonic acid (Puccia et al., 2005); phospholipids and PUFA are involved in maintaining membrane fluidity and the authors suggest that this increase is an adaptive mechanism to TBT toxicity. Enrichment of yeast *Saccharomyces cerevisiae* with linoleic acid (18:2n-6) caused resistance of the membranes to the toxic action of TBT (Masia et al., 1998), suggesting that although membrane fluidity was enhanced, toxicity of TBT was blocked probably by an increase of the lipophilicity of the membrane that would prevent the passive diffusion of TBT. In the present work, a relative increase of arachidonic acid was observed in 7-days exposed females (Table 8-1). Arachidonic acid is required in cell signalling and specifically as a substrate for eicosanoids synthesis (Nakamura and Nara, 2004). Eicosanoids, which include prostaglandins, thromboxanes, leukotrienes, hydroxyl FA and lipoxines are also critical in a very wide range of physiological processes in invertebrates; these include regulating egg-production, egg-laying, spawning and hatching, mediating immunological responses to infections, and regulating neurophysiology among other processes (Stanley-Samuelson 1994). Thus, the relative increase of arachidonic acid (30%) may be a short-term response in

exposed females in order to maintain endogenous levels stable (2.0-2.4 mg/g) and minimize the effect of TPT on physiological functions.

Additionally, the proportion of arachidonic acid was markedly increased in females exposed to 125 and 500 ng/L TPT-Sn (Table 8-1), but not in males. These changes paralleled the alterations observed in total lipid and fatty acid levels, suggesting a link between the relative increase of this potential regulator of lipogenesis (Yoshikawa et al., 2002) and the observed decrease in total lipid and fatty acid levels in exposed individuals.

In vertebrates, fatty acids are endogenous ligands of various nuclear steroid receptors and control transcript signalling. All three isoforms of PPAR are activated by fatty acids, specifically PUFAs, regulating an extensive network of genes involved in glucose and lipid metabolism (Benatti et al., 2004). Palmitic (16:0), stearic (18:0), palmitoleic (16:1n-7), oleic (18:1n-9), linoleic (18:2n-6), arachidonic acid (20:4n-6) and EPA (20:5n-3) are endogenous ligands of PPARα which is involved in fatty acid oxidation and catabolism, whereas linoleic, arachidonic acid and eicosanoids are endogenous ligands of PPARγ which plays a central role in adipocyte differentiation and storage of fatty acids (Willson and Wahli, 1997; Reddy and Hashimoto, 2001; Kota et al., 2005; Mochizuki et al., 2006). Other transcriptional factors have been identified to be targets of fatty acid regulation such as the Liver X receptor and RXR, which are both involved in lipid regulation (Benatti et al., 2004). Since fatty acids appear to be PPAR ligands at a concentration range that is consistent with their physiological circulating levels (Braissant et al., 1996), alterations in the abundance of endogenous fatty acids may trigger different mechanisms of lipid regulation further down the cascade of events. Indeed, Janer et al. (2007) found a significant increase in the percentage of total lipids and total fatty acid content in females of *M. cornuarietis* exposed to 500 ng TBT-Sn/L for 100 days. Furthermore, the percentage of PUFAs, including HUFAs, decreased and MUFAs increased. The discrepancies with the present study are probably a reflection of long- and short-term effects of organotin compounds on lipid homeostasis rather than a different effect of TBT and TPT, and deserve further investigation. Interestingly, both studies indicated higher susceptibility of females than males of *M. cornuarietis* to lipid alterations.

In vertebrates and some invertebrate species, steroids are conjugated with fatty acids to form apolar esters that are retained in the lipoidal matrices of the body from where they can be hydrolysed by esterases and liberate the active steroids upon

demand (Borg et al., 1995). In *M. cornuarietis* most of the estradiol and testosterone have been found to exist in the esterified form (Janer et al., 2006). Esterification of steroids occurs upon acyl-CoA moieties, which activation is depended on the concentration of the corresponding fatty acids (Hochberg, 1998). In the oyster *Crassostrea virginica*, estradiol esters formation was achieved using the fatty acid moieties C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 (Janer et al., 2004). Exposure of mussels *Mytilus edulis* to estradiol resulted in the formation of estradiol esters with C16:0, C16:1 and C16:2 fatty acid moieties (Labadie et al., 2007). Additionally, acyl-CoAs are substrates of AOX enzyme. Interestingly, in the present experiment, one week exposure to TPT resulted in a significant increase of AOX activity in exposed females together with a significant increase in esterified testosterone levels (60-85%) and a concomitant decrease in esterified estradiol (50-84%) (Lyssimachou et al., 2008). The observed alterations in esterified steroids were not directly related to changes in P450 aromatase activity or to changes in 17β-HSD and 5α-reductase, involved in the metabolism of the androgen precursor androstenedione. Thus, the hypothesis that changes in fatty acid availability might trigger alterations in endogenous steroid levels is a challenging one. In molluscs, the esterification of steroids with fatty acids appears to be an important regulation mechanism of endogenous steroid levels (Gooding and LeBlanc, 2001; Janer et al., 2005).

Overall, short-term exposure of *M. cornuarietis* to environmentally relevant doses of TPT lead to a decrease of total lipids and fatty acid content and an increase in AOX activity, which is involved in fatty acid catabolism. Since fatty acids have a pivotal role in organisms (cell membrane composition, bioactive signalling, steroid and eicosanoid synthesis), the observed effects are of special concern. Further research should focus on the higher sensitivity of females in comparison to males, the potential link of these alterations with the development of imposex and the role of fatty acid composition on the control of adipogenesis in different species. Finally, being TPT a high affinity ligand of RXR, the obtained data further support the hypothesis that RXR may also be implicated in lipid homeostasis in gastropods (alone or in combination with putative PPARs) as it is in vertebrates.

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Chapter 9. General Discussion

9. GENERAL DISCUSSION

9.1 General remarks

The present thesis has investigated the different effects, at molecular and subcellular level, of specific EDCs present in the environment, namely EE2, TBT, GEM and TPT, on the endocrine system of fish or gastropod molluscs following laboratory exposures. It has been demonstrated that by using specific and sensitive molecular and biochemical assays it is possible to detect alterations in the endocrine system of either fish or gastropods indicating that the health of these aquatic organisms might be at risk. These molecular and biochemical alterations are understood as early signs which indicate that the organisms are experiencing endocrine disruption. In reality, such alterations may be recompensed by the endocrine's systems homeostasis and not lead to permanent alterations on the endocrine system. However, taking into account that in the field aquatic organisms are continuously exposed to low levels of EDCs, the observed effects in this work, following short term exposure to the selected EDCs, may persist leading to adverse health effects. Especially in the case of juvenile fish (Chapters 3-5), changes in the endocrine system may lead to abnormal production of hormones and result in abnormal sex determination and reproduction organ growth later in adulthood.

Furthermore, the present work has detected new targets of EDCs in either fish or gastropods providing further information on the mode of action of these chemicals. Hence, it was shown that the human xenoestrogen EE2, used in contraceptive pills modulates not only estrogenic responses in juvenile fish (Chapter 3) but the early steps of steroidogenesis as well, as it was indicated by the alterations caused on brain and interrenal StAR protein and P450*scc* mRNA levels and protein content (Chapter 4). Additionally, the human pharmaceutical and lipid regulator GEM interferes not only with typical peroxisome proliferation enzymes (AOX and CAT) in the liver of juvenile fish but with the Cytochrome P450 system as well (Chapter 5), indicating that this compound leads to different effects in fish than what it has been observed in mammals. In terms of gastropod molluscs, the present work has demonstrated that different species of this family metabolise the androgen precursor AD in a different manner (Chapter 6 and 7) and show different sensitivity to TBT and TPT after in-vitro exposure (Chapter 6). During the research to detect the first targets of the organotin TPT on the endocrine system of gastropod molluscs, it was shown that 7-days in-vivo exposure of the

ramshorn snail *M. cornuarietis*, a period which is insufficient for the development of the imposex phenomenon in this species, modulated esterified sex steroid levels in females (Chapter 7) as well as lipid content, fatty acid profile and metabolism (Chapter 8). In the following sections these results are discussed further.

9.2 Mechanisms of endocrine disruption by EE2, TBT and GEM on juvenile fish.

Chapter 3 investigated the effects of EE2 and TBT, two compounds with opposite mechanism of action, on neurosteroidogenesis. Alterations in neurosteroidogenesis of juvenile fish may lead to disturbed production of neurosteroids such as testosterone and 17β-estradiol and affect brain sexual differentiation, reproductive organ development and mating behaviour (Lephart, 1996; Fenske and Segner, 2004).

Firstly, it was shown that both P450aromA and P450aromB genes were expressed in the brain of juvenile salmon fish, with P450aromB showing higher abundance than P450aromA confirming previous studies demonstrating the expression of P450aromB in the brain of fish with high levels of mRNA and the expression of P450aromA in the ovary with relatively low mRNA levels (Tchoudakova and Callard, 1998; Callard et al., 2001). Despite the documented differential expression of the isoforms A and B in neural and gonadal tissues, the present work shows that there is a degree of overlap, as it has been observed before in other teleost fish (Callard et al., 2001).

EE2 was shown to up-regulate the expression of both P450arom genes and enzyme activity in the brain of juvenile salmon after 3-days exposure indicating that a higher amount of neuroestrogens is being produced compared to the control group. This is further supported by the observation that brain ERα mRNA and plasma VTG levels, two typical xenoestrogen responses were also induced by EE2. The up-regulation of brain and whole tissue P450aromB mRNA levels and aromatase activity by estrogens and xenoestrogens (Gelinas et al., 1998; Kishida et al., 2001; Mandiki et al., 2005), among them EE2 (Trant et al., 2001; Kazeto et al., 2004) has been demonstrated in different fish species. Thus, the observed induction of brain P450aromB and ERα mRNA expression by 3-day exposure to EE2 in the study of Chapter 3 confirms that the P450aromB isoform in the brain of fish is under the control of an auto-regulatory feedback loop, which is driven by the products of aromatisation, the estrogens (Figure 9-1). This is further supported by the identification of EREs in the

promoter region of the P450aromB gene of the zebra fish, which shows a direct responsiveness of P450aromB to estrogens (Callard et al., 2001). Therefore, the present work shows that the synthetic estrogen EE2 is also capable to up-regulate the P450aromB gene in a similar way as the natural estrogens and therefore P450aromB gene induction may be used as an indication of exposure of fish to estrogenic compounds.

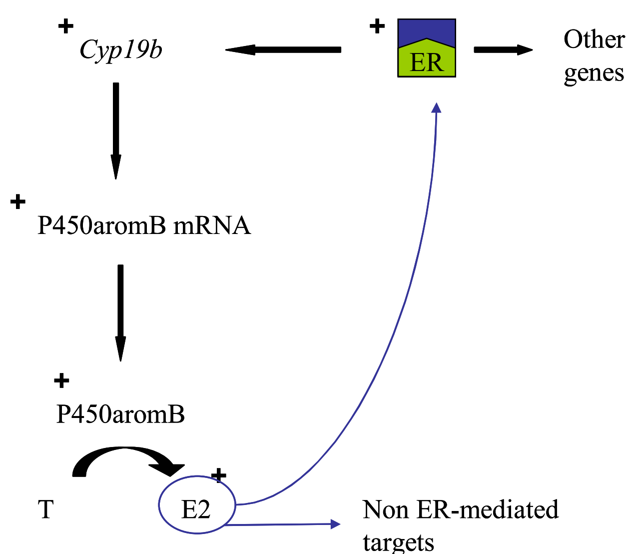

Figure 9-1. Autoregulation of brain P450aromB expression driven by the products of aromatase, the estrogens and by xenoestrogens such as EE2.

Short-term exposure of juvenile salmon to EE2 showed to interfere not only with neuroestrogen regulation and biosynthesis but with the entire hypothalamus–pituitary–interrenal (HPI) axis as well (Chapter 4), which has the key role to control the endocrine response to stress, through the production of corticoids. Thus, 3-days exposure of juvenile salmon to EE2 induced brain and head kidney StAR protein, P450*scc* and CYP11β expression. An increase in StAR and P450*scc* mRNA and protein levels in response to trophic hormone stimulation indicates acute steroid regulation in steroidogenic tissues (Stocco, 2001). Therefore, the observed induction in StAR and P450*scc* mRNA and protein levels in juvenile salmon caused by EE2 exposure reveals that this compound promotes acute synthesis and regulation of neural and interrenal steroids in juvenile fish. Furthermore, this stimulation in acute neurosteroid biosynthesis in the brain of fish promoted by EE2 could explain partly the

higher amounts of estrogens produced as indicated by the up-regulation of P450aromB and ERα gene expression, aromatase activity and plasma Vtg levels (Chapter 3).

There is limited amount of scientific data on the effects of xenobiotics on the expression of StAR protein, P450*scc* and CYP11β mRNA, especially in fish. It has been documented that exposure of juvenile salmon fish to the xenoestrogen nonylphenol resulted in induced brain StAR protein and CYP11β mRNA levels but not of P450*scc* (Arukwe, 2005). Furthermore, exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to EE2 or to the androgenic compound 11β-hydroxyandrostenedione resulted in inhibition of testicular P450*scc* and CYP11β mRNA levels (Baron et al., 2005), whereas 10-days exposure to E2 inhibited CYP11β mRNA levels but not P450*scc* (Govoroun et al., 2001). These results in combination with the results of the present study suggest that brain and interrenal StAR protein, P450*scc* and CYP11β mRNA expression in fish are modulated by xenobiotics but this modulation is independent to the estrogenic or androgenic character of the compounds.

Although enzymatic activities and steroid hormone levels were not measured due to the limited amount of sample, the induction of CYP11β mRNA levels by EE2 may result in induction of CYP11β synthesis and consequently activity. CYP11β catalyses the conversion of 11-deoxycortisol to the glucocorticoid cortisol but in fish CYP11β also catalyses the synthesis of the potent androgen 11-KT (Alam et al., 2005; Socorro et al., 2007). 11-KT has a central role in sexual maturation, development of secondary sexual characteristics and reproductive behaviour in male teleost fish (Borg, 1994) and scientific data indicate it has a central role in the reproduction of female fish as well (Lokman et al., 2002; Matsubara et al., 2003). Therefore, the up-regulation of CYP11β mRNA expression by EE2 particularly in the brain of juvenile salmon fish of the present study may result in higher production of 11-KT leading to disturbed steroidogenesis which may affect the physiology of the juvenile fish.

The trialkyltin TBT, which has been suggested to inhibit aromatase activity in gastropod snails (Bettin et al., 1996; Santos et al., 2002) and human cells (Saitoh et al., 2001), down-regulated the expression of both P450arom isoforms in the brain of juvenile salmon fish following 7-days exposure but aromatase activity although reduced, it was not statistically significant (Chapter 3). The potential of TBT to interfere with reproduction success and fertilisation in fish has been demonstrated before (Haubruge et al., 2000; McAllister and Kime, 2003; Nakayama et al., 2004) but the

interference of TBT with the regulation of neurosteroidogenesis in juvenile fish has not been previously reported.

Although the present data supports previous documentations on the action of TBT as an aromatase inhibitor, the fact that brain ERα mRNA and plasma Vtg showed a tendency to increase indicate that the mechanism of action of TBT is not limited to aromatase inhibition. A direct inhibition of aromatase should result to a lower production of estrogens and a parallel decrease in ERα mRNA levels and Vtg levels. For example, when adult fathead minnow (*Pimephales promelas*) were exposed to the aromatase inhibitor fadrozole for 21 days at concentrations up to 50 ng/L, aromatase activity was significantly inhibited in both males and females (Ankley et al., 2002), and this inhibition was accompanied by a significant decrease in E2 and Vtg levels in the plasma, which was not caused by TBT in the present work. However, the fact that the solvent control appeared to interfere with neurosteroidogenesis following 7-day exposure (Chapter 3 and 4) may have obscured the effects of TBT.

During the work of Chapter 4 the effects of TBT on StAR protein, P450*scc* and CYP11β mRNA levels in the brain and head kidney of juvenile salmon following 7-days exposure were examined (ANEX I). However, due to the strong effect of the carrier solvent DMSO on the expression of these enzymes (Chapter 4) the results are inconclusive. Therefore, DMSO should be avoided as a carrier solvent during in-vivo studies, especially when the study is related to molecular alterations in the brain.

In the case of the human lipid regulator and rodent peroxisome proliferator GEM, the study of Chapter 5 demonstrates that injection exposure of juvenile eels to the compound leads to alterations in the activity of hepatic endogenous and xenobiotic metabolising enzymes and induces typical peroxisome proliferation enzymatic pathways.

Hence, GEM inhibited the activity of three hepatic cytochrome P450 enzymes, namely CYP1A, CYP3A-like and CYP2K-like, in the liver of immature ells. This inhibition was detected 24-h post-injection and to a concentration range of 0.1-10 mg/kg, whereas 96-h post-injection to a higher concentration range (2-200 mg/kg), resulted in no significant alterations of CYP-catalysed activities suggesting that fish were able to recover their CYP-activities during this time. On the contrary, GEM had a minor effect on the phase II enzymatic activities examined despite the fact that glucuronidation catalysed by UGT is a major biotransformation pathway of GEM in mammals (Knauf et al., 1990; Sallustio and Fairchild, 1995).

Taking into account that fish in the field may be continuously exposed to low concentrations of GEM, the observed inhibition of CYP-catalysed activities 24h post-injection is of concern. The role of CYP metabolism in fish has been reviewed extensively indicating the importance of this enzymatic system in the de-activation or in some cases activation of xenobiotics (Goksøyr and Förlin, 1992; Buhler and Wang-Buhler, 1998; Arinc et al., 2000). Induction of CYP1A activity and protein content in the liver of eels (*A. anguilla*) has been observed in sites contaminated with various pollutants among them PAHs, dioxins, and PCBs (van der Oost et al., 1996; Livingstone et al., 2000). In terms of CYP3A, induction of hepatic CYP3A-like protein content has also been observed in eels from contaminated sides (van der Oost et al., 1996). Furthermore, in humans CYP3A- isoenzymes participate in the oxidation of approximately 60% of all clinically used drugs in humans, making it the most important enzyme in drug metabolism (Anzenbacher and Anzenbacherova, 2001; Tredger and Stoll, 2002). Finally, the CYP2K-like enzyme measured in the present study catalyses the ω- and ω-1 hydroxylation of fatty acids and plays a key role in lipid homeostasis of fish (Buhler and Wang-Buhler, 1998).

Following scientific evidence that GEM causes peroxisome proliferation and hepatocarcinogenesis in rodents (Hashimoto et al., 1995), the present work aimed to measure AOX and CAT activity, two inducible enzymes during peroxisome proliferation (Lazarow and Duve, 1976). 96-h post-injection to GEM, AOX activity was significantly induced in liver peroxisomal fractions, whereas CAT activity although induced, was statistically significant only at low dose (2 μg/g) exposure. Similar increases in AOX and CAT activity have been observed in rainbow trout following exposure to ciprofibrate (Yang et al., 1990) and to GEM (Scarano et al., 1994). Although the present study provides evidence that GEM is capable of inducing typical peroxisome proliferation responses in juvenile fish, the induction observed in AOX activity is of a much lower magnitude (15- to 17-fold) than what it has been observed in rodents (Sausen et al., 1995). Thus, peroxisome proliferation in eels caused by GEM is considerably lower than the one observed in rodents and quantitative morphometric analysis of peroxisomes is suggested as a complementary measurement for peroxisome proliferation. Nevertheless, the induction of AOX activity indicates that GEM, being a hypolipogenic drug for humans, induces to a certain extent peroxisomal β-oxidation of fatty acids in the liver of fish as well and consequently may stimulate lipolysis; thus the implications of aquatic contamination by this drug should be considered.

Peroxisome proliferation leading to hepatocarcinogenesis has been observed in rodents but not in humans or other mammalian species such as hamsters, guinea pigs and rhesus monkeys (Bentley et al., 1993). The enzymes reported to get induced by PPs (AOX, CAT, acyl-CoA: steroid acyltransferase, CYP4A) are regulated to some extent by PPARα in rodents, as in PPARα null mice such effects do not occur (Reddy and Hashimoto, 2001; Xu et al., 2001b). In the work presented in Chapter 5 these enzymes in the liver of eels were either mildly induced (AOX, CAT) or remained unaltered (ATAT, CYP2K- and CYP2M-like) following GEM exposure. Rodents have high concentration of hepatic PPARα compared to humans and other mammals, which could explain their high susceptibility to these compounds (Palmer et al., 1998). Furthermore, it has been shown that typical mammalian peroxisome proliferators may bind weakly or not at all to PPARα of some fish (Kondo et al., 2007), which could also explain the low observed response of PPARα regulated enzymes to GEM in eels of the work of Chapter 5.

9.3 Mechanisms of endocrine disruption of organotins on gastropod molluscs

In Chapter 6 it was shown that imposex occurred in both *B. brandaris* and *H. trunculus* collected from the Ebro Delta (North West Mediterranean), despite the regulations for the use of TBT containing antifouling paints in Spain. The fact that no further imposex stages were observed confirms previous studies that the populations of these species may not be in danger of extinction (Ramón and Amor, 2001). Although *B. brandaris* showed a higher imposex frequency (100%) than *H. trunculus* (80%), the number of samples was insufficient to conclude that the species *B. brandaris* is more susceptible to imposex than *H. trunculus*. In fact, during surveys carried out on the western Iberian peninsula and Sicilian coast, speciments of *B. brandaris* and *H. trunculus* revealed very similar imposex degree and frequency (Chiavarini et al., 2003; Gómez-Ariza et al., 2006) suggesting that both species are equally susceptible to imposex.

The metabolism of AD to testosterone through the action of 17β-HSD is a common feature across vertebrate species and some invertebrates (Payne and Hales, 2004; Thibaut and Porte, 2004; Janer and Porte, 2007). However, as Chapters 6 and 7 demonstrate microsomal fractions isolated from the digestive gland/gonad complex of three different gastropod molluscs metabolised AD in a different manner, indicating significant differences in androgen synthesis between these species. Figures 9-2, 9-3

and 9-4 illustrate the AD metabolic pathway of *M. cornuarietis*, *B. brandaris* and *H. trunculus*, respectively. Thus, *M. cornuarietis* metabolised AD in a gender specific manner, leading to a high rate of formation of 5α-dihydroandrostenedione (DHA)- a 5α-reductase catalytic pathway- in females, and of 5α -dihydrotestosterone (DHT) and testosterone (T)- two 17β-HSD catalytic pathways- in males (Figure 9-2). In *B. brandaris* AD metabolism was also gender specific leading to a higher formation of DHA and to a minor extent T in females than males but with no differences in the formation of DHT between sexes (Figure 9-3). The metabolism of AD to DHA has been detected in other molluscs, such as the mussel *Mytilus galloprovinciallis* and the slug *Ariolimax californicus* (Gottfried and Dorfman, 1970; Janer et al., 2005a) indicating that 5α -reductases play a more important role than 17β-HSDs in the metabolism of AD in these species. In *H. trunculus* both males and females metabolised AD principally to T showing that 17β-HSD is the main AD metabolising enzyme in this species (Figure 9-4), as it has been observed in vertebrates and some invertebrate species as well (Janer and Porte, 2007). Thus, substrate specificity of 17β -HSDs is higher for DHA than for AD in the gastropods *M. cornuarietis* and *B. brandaris*, and this contrasts with the high affinity of *H. trunculus* and vertebrates 17β -HSDs for AD.

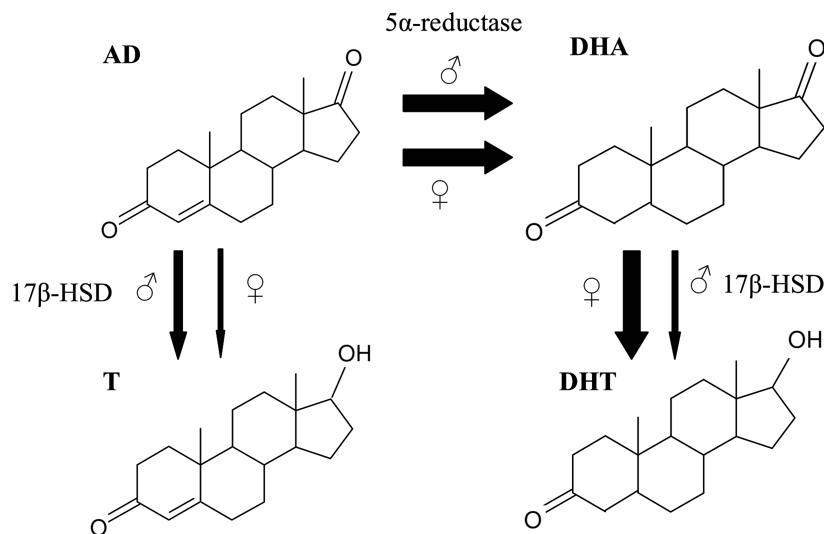

Figure 9-2. Androstenedione metabolism in digestive gland/gonad complex of *Marisa cornuarietis*.

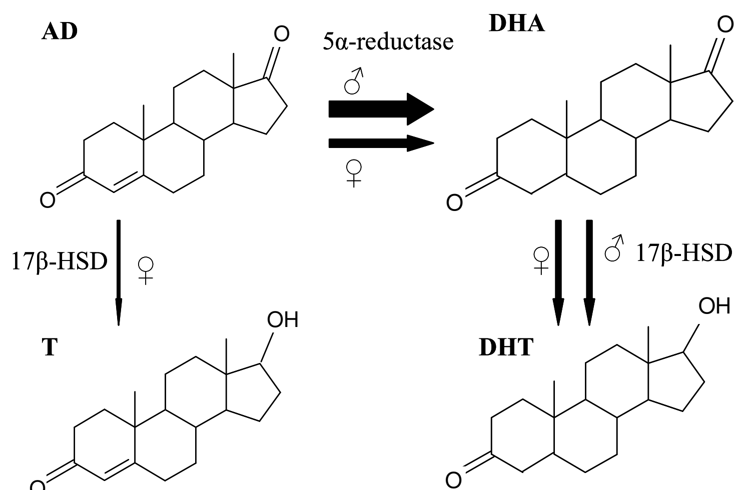

Figure 9-3. Androstenedione metabolism in digestive gland/gonad complex of *Bolinus brandaris*.

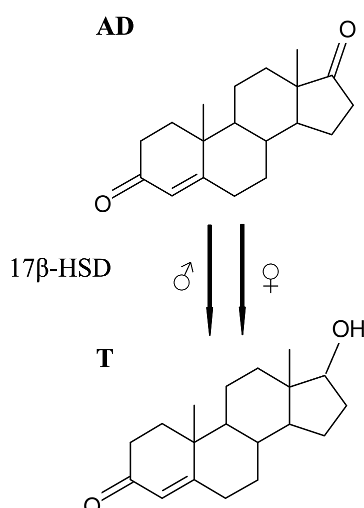

Figure 9-4. Androstenedione metabolism in the digestive gland/gonad complex of *Hexaplex trunculus*.

Additionally, AD metabolic rate varied between the studied species. Therefore, activity of AD metabolism across species followed the trend *B. brandaris* >> *H. trunculus* > *M. cornuarietis* (Chapters 6 and 7). Thus, the metabolism of the androgen precursor AD strongly differs among gastropod species both in terms of activity and metabolic profile, confirming the wide diversity of gastropod molluscs.

In-vitro exposure to TBT or TPT interfered with the metabolism of AD in *B. brandaris* and *H. trunculus*, particularly in females (Chapter 6). Table 9-1 shows the

statistically significant effects of TBT and TPT on three gastropod species following in-vitro exposures from the present thesis (Chapter 6) and from the literature. The susceptibility of 5α-reductase to inhibition by organotin compounds has already been reported in several gastropod (Ronis and Mason, 1996; Janer et al., 2006a) and non-gastropod species (Thibaut and Porte, 2004), whereas in the case of 17β-HSD contradictory data has been reported. Thus in-vitro exposure of *M. cornuarietis* and the clam *R. decussata* to TBT or TPT inhibited 17β-HSD activity (Table 9-1; Morcillo et al., 1998), whereas in the echinoderm *Paracentrotus lividus* TPT had no effect (Lavado et al., 2006).

Table 9-1. In-vitro effects of TBT and TPT on androstenedione metabolism in digestive gland/gonad complex of *Bolinus brandaris*, *Hexaplex trunculus* and *Marisa cornuarietis*.

		TBT (μM)			TPT (μM)		
		0.1	1	10	0.1	1	10
Bolinus brandaris							
5α-reductase	♂	-	-	-	-	-	-
	♀	-	-	↓	↓	↓	↓
Hexaplex trunculus							
17β-HSD	♂	-	-	-	-	-	-
	♀	-	-	↑	-	-	-
Marisa cornuarietis[1]							
5α-reductase	♂	-	-	↓	-	-	↓
	♀	-	-	↓	-	-	↓
17β-HSD	♂	-	-	↓	-	↓	↓
	♀	-	-	↓	-	↓	↓

[1]Janer et al. (2006a)

The last two chapters of the present thesis aimed to investigate the potential mechanisms of action of TPT on the freshwater ramshorn snail *M. cornuarietis* following in-vivo exposure (Chapters 7 and 8). The exposure period was 7-days and the selected compound was TPT. TPT is known to be a potent imposex inducer in this

species, with EC10 values for increased vas deferens and mean penis length of 18 and 23.4 ng TPT/L (as Sn), respectively, following 4-month exposure (Schulte-Oehlmann et al., 2000).

One of the first theories on the mechanism of toxicity of organotins on female gastropods is that they increase testosterone levels by inhibiting aromatase activity. A literature summary of the observed in-vivo effects of TBT on testosterone and/or 17β-estradiol levels in different gastropod species, including the results from Chapter 7, is given in Table 9-2. Although most of the studies indicate that TBT induces free testosterone levels in imposex females, this increase is detected after the induction of imposex and in some cases only at the high exposure group. Additionally, an increase in endogenous free testosterone levels is not always evident in female gastropods experiencing imposex due to TBT exposure, as it was observed in the case of *M. cornuarietis* (Table 9-2) or in imposex females from the field (Morcillo and Porte, 1999). Following the data from the present work showing that short-term exposure of *M. cornuarietis* to TPT did not alter free testosterone content, it becomes evident that the documented alterations in free testosterone levels in imposex gastropod females may be a secondary effect which could be species specific.

The reported increase in free testosterone levels in imposex females (Table 9-2) is unlikely to be due to a direct inhibition of aromatase activity since such an increase would be accompanied by a reduction in 17β-estradiol levels, which as it can be seen from Table 9-2, is not always the case. This is further supported by the results of Chapter 7 where neither aromatase activity nor the total metabolism of AD were altered in *M. cornuarietis* following 7-days exposure to TPT. Similarly, the metabolism of AD in male and female of *M. cornuarietis* was not modulated by 50-days in-vivo exposure to TPT or TBT (Janer et al., 2006a). Thus, unlike what it has been observed during in-vitro exposures (Chapter 6; Table 9-1), AD metabolism is not a primary target of TBT or TPT in this species following in-vivo exposure.

Both testosterone and 17β-estradiol measured in the digestive gland/gonad complex of *M. cornuarietis* during the work of Chapter 7 were found predominately in the esterified form confirming previous scientific data showing that the conjugation of steroids with fatty acids plays a critical role in the regulation of free steroids levels in gastropod molluscs (Gooding and LeBlanc, 2001, 2004; Janer et al., 2006b). The second theory on the mechanism of toxicity of organotins on female gastropod molluscs is that TBT inhibits the esterification of testosterone (Table 9-2) leading to a

Table 9-2. Observed effects on testosterone (T) and 17β-estradiol (E2) content in various gastropod species after in-vivo exposure to TBT or TPT.

Gastropod species	Ref.	Compound	Duration	Imposex	Effect on steroid levels			
					T		E2	
					Free	Ester	Free	Ester
Nassarius reticulates	(1)	TBT	4-months	✓	↑		-	
Nucella lapillus	(1)	TBT	4-months	✓	↑		↑	
	(2)	TBT	3-months	✓	↑	-	↑	-
	(3)	TBT	1-month	✓	↑			
Ilyanassa obsoleta	(4)	TBT	3-months	✓	↑	-		-
Marisa cornuarietis	(5)	TBT	3-months	✓	↑	↓	↓	
	(6)	TBT	100-days	✓	-	↓	-	↓
	(7)	TPT	7-days	×	-	↑	-	↓

(1) Bettin et al. (1996); (2) Santos et al. (2005); (3) Spooner et al. (1991); (4) Gooding et al. (2003); (5) Schulte-Oehlmann et al. (1995); (6) Janer et al. (2006b); (7) This thesis.

higher percentage of free testosterone in the organism (Gooding et al., 2003; Janer et al., 2006b). This contrasts the results of Chapter 7 where TPT caused induction of esterified testosterone levels in females of *M. cornuarietis*. Although, in-vitro studies show that TBT inhibits ATAT activity at environmentally relevant concentrations, TPT has not been shown to have an effect (Janer et al., 2005b; Sternberg and LeBlanc, 2006). Furthermore, in-vivo exposure of the female gastropods to TBT for 3 months had no effect on ATAT activity, even though esterified testosterone levels decreased (Gooding et al., 2003; Janer et al., 2006b).

Following the observation that esterified 17β-estradiol and testosterone in mammals have a prolonged action compared to their parent steroids (Hochberg, 1991), the fact that TPT increased esterified testosterone and decreased esterified 17β-estradiol in *M. cornuarietis* in such a short time can be understood as a “masculinising”

effect. However, this implies that the vertebrate steroids testosterone and 17β-estradiol function as sex steroids in invertebrates as well. Although evidence from laboratory studies suggests that these hormones may have a role in the development of secondary sexual characteristics in gastropod molluscs (Chapter 6 and 7) the mode of action of vertebrate hormones in invertebrates remains unclear. For a classical hormone signalling to occur, androgens and estrogens must bind to their equivalent receptor. A study on the role of steroidal androgens and estrogens in the recrudescence of the mud snail *I. obsoleta* males showed that although testosterone levels (free and total) in males varied with reproductive status, it was not possible to identify any androgen receptor analogue (Sternberg et al., 2008a). Testosterone in females and 17β-estradiol in both sexes did not follow a trend through the reproductive status. Interestingly, an ER-like receptor has been identified in some gastropod species, with high similarity to ERs of other molluscs, which does not bind 17β-estradiol and does not require a ligand to activate gene transcription (Thornton et al., 2003; Kajiwara et al., 2006). This apparent ligand-independence of the molluscan ER does not support the hypothesis that estrogens regulate reproduction in these organisms. Therefore it becomes apparent that a classical steroid signalling pathway does not exist in gastropods and therefore the hypothesis that organotin-induced testosterone levels will induce androgenic responses remains inconclusive. Nevertheless, a molluscan-specific steroid signalling pathway with the possible regulation by exogenous testosterone and estradiol cannot be excluded.

It has been suggested that fatty acid esterification of testosterone and estradiol is catalysed by the same enzymes since in-vitro studies indicate that estradiol is an inhibitor of the esterification of testosterone in microsomal fractions from mammalian steroidogenic tissues (Martyn et al., 1988) and in microsomal fractions from the digestive gland/gonad complex of gastropods as well (Sternberg and LeBlanc, 2006). Furthermore, ATAT and AEAT activities in microsomes from snails collected from a field site at different times of the year were significantly correlated supporting further that a single enzyme is responsible for the biotransformation of both steroids (Sternberg and LeBlanc, 2006). Following these observations, a direct interaction of organotins with this enzyme should result in a similar effect on the esterification of estradiol and testosterone. However, as shown in Chapter 7, TPT induced esterified testosterone levels but increased esterified 17β-estradiol, suggesting that the action of TPT may not be directly on the enzyme.

Even if acyl-CoA : steroid transferases are not steroid specific, evidence suggests that the rate of formation of the fatty acid esters varies both in terms of the steroid substrate and the fatty acid acyl donor. Therefore, testosterone palmitoate rate of formation was about 35% higher than estradiol palmitoate in microsomal fractions of the mudsnail *I. obsolete* (Sternberg and LeBlanc, 2006), whereas in rat liver microsomal fractions testosterone oleoate rate of formation was approximately 35% lower than estradiol oleoate (Xu et al., 2002). In terms of the fatty acid moiety of acyl-CoAs, in-vitro studies on mammals and molluscs show that the rate of formation of estradiol esters varies according to the fatty acid acyl donor (Figure 9-5). Thus, in liver rat microsomal fractions, incubation of E2 in the presence of different fatty acid acyl-CoAs showed that formation of E2-arachidonoate was the highest, whereas formation of E2-palmitoate was the lowest with a 2-fold difference (Xu et al., 2001a). In the case of bovine placental microsomes, E2-oleoate showed the highest rate of formation, whereas E2-arachidonoate showed the lowest rate of formation being 5-fold less (Martyn et al., 1988). Interestingly, microsomes from the digestive gland of the oyster *Crassostera virginica* also revealed variations in the formation of estradiol esters according to the fatty acyl-CoA used. Thus, E2-palmitoate showed the highest rate of formation and E2-linoleote the lowest with a 2-fold difference (Janer et al., 2004). It becomes clear that the rate of formation of fatty acid steroids varies in terms of the steroid being esterified, the fatty acid moiety of acyl-CoAs, the tissue and the organism.

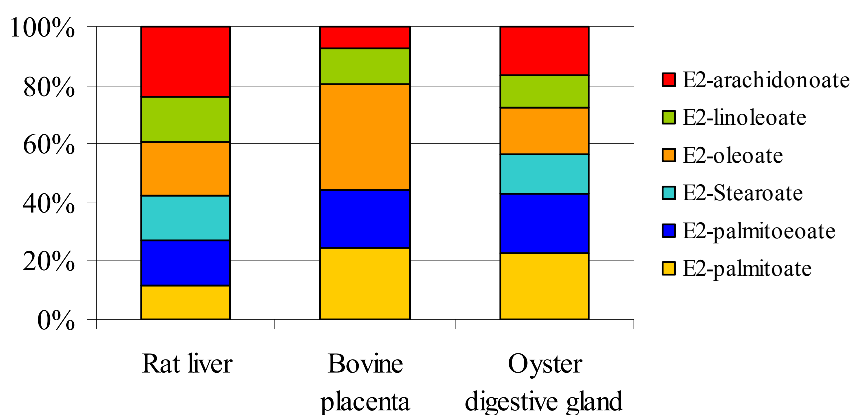

Figure 9-5. In-vitro formation of 17β-estradiol (E2) fatty acid esters in microsomal fraction from different animal tissues following incubation with various fatty acid acyl-CoAs.

Additionally, in-vivo synthesis of fatty acid steroid esters does not necessarily reflect the results obtained from in-vitro synthesis. Thus, E2-arachidonate and E2-linoleate are abundant in bovine placenta but arachidonyl-CoA and linolenoyl-CoA are poor substrates for E2 esterification in-vitro (Martyn et al., 1988). Therefore, it must be the availability of fatty acids and the balance between synthesis and breakdown of steroid fatty acid esters by esterases that determine the nature of the fatty acid acyl moiety with which steroids are esterified.

Following the above observations the work in Chapter 8 investigated whether TPT interferes with the metabolism of FA in the gastropod *M. cornuarietis*, which would affect all parts of lipid homeostasis including esterification of steroids. Indeed as Table 9-3 illustrates, 7-days exposure to TPT altered MUFA and HUFA levels, decreased the total lipids and FA content in the digestive gland/gonad complex of females of *M. cornuarietis* and induced peroxisomal β-oxidation of FA. Therefore TPT interferes directly with the lipid homeostasis of female gastropods of this species. Alterations in the fatty acid profile and lipid content were also observed in *M. cornuarietis* following 100-days exposure to TBT (Janer et al., 2007). However, the authors found exactly the opposite effects from what it was observed in the present study (Table 9-3).

The fact that 7-days exposure to TPT resulted in decreased lipid and FA content in females is in agreement with the observed induction of AOX activity, which catalyses the peroxisomal break-down of long chain and very long chain FA. These alterations are of special concern taking into account the critical multi-functional role of FA in cell structure and function, energy metabolism and storage, bioactive signalling and synthesis of various compounds involved in physiological regulation (e.g. steroids, eicosanoids etc) (Benatti et al., 2004). Eicosanoids that are synthesised by the enzymatic oxygenation of three HUFA namely linolenic, arachidonic and eicosapentanoic acid, play a critical role in the physiological function of invertebrates regulating egg-production, egg-laying, spawning and hatching, mediating immunological immunological responses to infections, and regulating epithelial ion and water flux, temperature set points, and neurophysiology (Stanley-Samuelson, 1994a; 1994b; Stanley and Howard, 1998). Therefore, the observed increase in HUFAs abundance caused by 7-days exposure to TPT may indicate an effect on the reproduction and development of the snails through alterations in eicosanoid synthesis.

Table 9-3. Effects of TPT on fatty acid metabolism, profile and lipid content in the digestive gland/gonad complex of *Marisa cornuarietis* following 7-days exposure.

	TPT ng as Sn/L (7-days)						TBT ng as Sn/L (100-days)[a]					
	Males			Females			Males			Females		
	30	125	500	30	125	500	30	125	500	30	125	500
AOX activity	↑	-	-	↑	-	↑						
SFA[b]	-	-	-	-	-	-	-	-	-	↓	-	-
MUFA[b]	-	-	-	-	↓	↓	-	-	↑	-	-	↑
PUFA[b]	-	-	-	-	-	-	-	-	-	-	-	↓
HUFA[b]	-	-	-	-	↑	↑	-	-	-	-	-	↓
%Lipids	-	-	-	-	-	↓	-	-	-	-	-	↑
Total FA[c]	-	-	↓	↓	↓	↓	-	-	-	-	-	↑

[a]data from Janer et al. (2007); [b]percentage of total FA; [c]calculated as FAME (fatty acid methyl esters)

AOX synthesis in mammals is controlled by the PPARα gene (Lee et al., 1995). Induction of AOX activity in *M. cornuarietis* by TPT suggests that a mechanism of peroxisome-proliferation analogous to the one promoted by PPARα activation in vertebrates may also exist in invertebrates. Additionally, fatty acids and eicosanoids activate transcriptional factors which play a role in lipid regulation, such as PPARβ, PPARγ, LXR and RXR (Willson and Wahli, 1997; Reddy and Hashimoto, 2001; Benatti et al., 2004; Kota et al., 2005; Mochizuki et al., 2006). Since fatty acids appear to be ligands for the PPARs at a concentration range that is consistent with their physiological circulating levels (Braissant et al., 1996), alterations in the abundance of endogenous fatty acids may trigger different mechanisms of lipid regulation further down the cascade of events. Although all PPARs appear to have emerged later in the evolution of the nuclear receptor family and up to today have only been identified in vertebrates (Thornton, 2003), a lipid regulatory mechanism in invertebrates triggered by the most abundant fatty acids cannot be excluded. Therefore, the alterations in lipid homeostasis caused by short-term exposure to TPT may trigger differential lipid regulatory mechanisms that would explain the opposite results obtained by Janer et al. (2007) following 100-days exposure to TBT (Table 9-3).

Cloning of the RXR orthologue in the gastropods *T. clavigera*, *N. lapillus, I. obsoleta* and *B. glabrata* revealed that 9-*cis* RA is a high affinity ligand for this receptor as it has been observed in vertebrates (Nishikawa et al., 2004; Bouton et al., 2005; Castro et al., 2007; Sternberg et al., 2008b). Interestingly, ligand binding assays show that TBT and TPT bind to the *T. clavigera* RXR with high affinity as well comparable to the one of 9-*cis* RA, the proposed endogenous ligand (Nishikawa et al., 2004). Furthermore, injections of 9-*cis* RA, TBT and TPT resulted in equal induction of imposex in females of *T. clavigera* and *N. lapillus* (Nishikawa et al., 2004; Castro et al., 2007a). It was found that RXR gene expression in *T. clavigera* was significantly higher in the penises of males and imposex-exhibiting females than in the penis-forming areas of normal females, whereas all the other reproductive organs showed very low RXR gene expression (Horiguchi et al., 2007). The above data strongly suggest that induction of imposex by TBT and TPT in female gastropods may be mediated by activation of RXR. Additionally, in a recent study it was shown that RXR mRNA levels increased appreciably in both males and females of *I. obsoleta* during recrudescence but the time of recrudescence and RXR mRNA expression was different between sexes (Sternberg et al., 2008b). Thus RXR appears to have a role in the reproduction of gastropod species.

Recently, independent in-vitro cell based nuclear receptor ligand binding assays identified TBT and TPT as high affinity ligands for RXR, PPARγ and for a number of additional permissive RXR-heterodimeric partners such as RXR:PPARγ, RXR:PPARβ and RXR:LXR (Kanayama et al., 2005; Nakanishi et al., 2005; Grün et al., 2006). PPARγ plays a central role in adipocyte differentiation and triglyceride storage (Ferré, 2004) and treatment of 3T3-L1 cells with TPT or TBT promoted adipocyte differentiation and PPARγ mRNA expression to the same extent as synthetic RXR and PPARγ ligands (Kanayama et al., 2005; Grün et al., 2006). Furthermore, in-utero exposure of pregnant mice to TBT increased adiposity in newborn mouse liver, testis and adipose depots and in-vivo exposure of *Xenopus laevis* tadpoles to TBT, RXRα or PPARγ synthetic ligands during metamorphosis resulted in ectopic adipocyte formation in and around the gonadal tissues (Grün et al., 2006). These results suggest that developmental exposure to TBT stimulates adipogenesis in vertebrates through RXR and PPARγ activation.

The activation of RXR in vertebrates and invertebrates indicates that there must be a conserved transcriptional mechanism for TBT and TPT action across phyla. Since

PPARs have not been identified in invertebrates, the observed changes in fatty acid profile and lipids following in *M. cornuarietis* following exposure to TPT or TBT in the present work cannot be linked to the activation of RXR:PPARs heterodimers as it has been suggested in vertebrates. However, RXR alone may have a role in lipid regulation. Different research groups aiming to discover the endogenous natural RXR ligand in vertebrates, if any, have shown that apart from 9-*cis*-RA, other fatty acids such as oleic acid (Bourguet et al., 2000), docohexaenoic acid (de Urquiza et al., 2000) and n-3 PUFAs (Fan et al., 2003) bind to the LBD of RXR as well. All the cloned gastropod RXRs resemble a high homology with vertebrate RXRs in the LBD, where 82-84% of the amino acid residues are identical (Nishikawa et al., 2004; Bouton et al., 2005). Hence, gastropod RXR may be activated by endogenous FA as well and may have a role in gastropod lipid homeostasis. Future studies should investigate whether the observed changes in lipid homeostasis in female gastropods following exposure to organotins are related to the activation of the RXR receptor.

Modulations in RXR maybe linked to changes in aromatase activity as well. Treatment of human ovarian granulosa cells (Mu et al., 2000; Mu et al., 2001), breast adipose stromal cells (Rubin et al., 2002) and MCF-7 human breast cancer cells (Yanase et al., 2001) with a PPARγ and/or a RXR synthetic ligand, resulted in a significant inhibition of aromatase activity and P450arom mRNA expression. In addition, exposure of human ovarian glanulosa-like cells (KGN) to TBT and TPT, inhibited significantly the aromatase activity and P450arom mRNA expression as well (Saitoh et al., 2001). In-vivo exposures of *X. laevis* to either TBT, or RXR or PPARγ ligands resulted in repressed aromatase expression during their development stage with a parallel increase in adipocyte formation suggesting that aromatase is regulated by a RXR:PPARγ heterodimer (Grün et al., 2006). These results bring questions to whether gastropod RXR also has a role in the regulation of the aromatase enzyme which would explain partly the observed elevated testosterone levels in imposex-induced female gastropods.

An important observation of the present thesis is that all three gastropod species showed that females were more sensitive to organotin exposure than males (Chapters 6-7; tables 9-2 and 9-3). There is very limited information on the differences of the endocrine system between females and males and therefore it becomes difficult to conclude why females are more susceptible to TBT and TPT exposure than males. However, some studies have reported similar results using different gastropod and

invertebrate species. Indeed, injections of *N. lapillus* with TBT (1 µg/g body weight) for 2 months induced the development of imposex in females and increased the penis length of males; however, the effect in females was more severe as penis growth reached about 1.2 mm whereas in males, penis increased only by approximately 0.4 mm (Castro et al., 2007). Furthermore, exposure of the abalone *Haliotis gigantean* to 100 ng/L of TBT or TPT caused significant spermatogenesis in ovaries of exposed females, whereas no significant histological changes were observed in the testis of exposed males (Horiguchi et al., 2002). Moreover, 4-week exposure of *P. lividus* to TPT significantly induced 5α-reductase and 3β-HSD activity in females, but in males, the effect was only observed at the high exposure dose (225 ng TPT/L) (Lavado et al., 2006). Therefore, when examining the effects of organotins on aquatic organisms it is important to take into account that these effects may be gender specific.

The present thesis clearly demonstrates that although androgen metabolism is a target of TBT and TPT in female gastropods following in-vitro exposure (Chapter 6), this is not the case after in-vivo exposure (Chapter 7). The fact that one-week exposure of female gastropods of *M. cornuarietis* to TPT resulted in altered testosterone and estradiol fatty acid esters (Chapter 7), lower lipid and fatty acid content and altered fatty acid profile (Chapter 8) indicates that the lipid metabolism may be a primary target of TPT as it has been observed in vertebrates. Nevertheless, the present work confirms previous studies on the diverse toxicity of organotin compounds both in fish (Chapter 3) and in invertebrates (Chapters 6, 7 and 8) and therefore provides further evidence to support the total ban of this compound from the market and use.

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Chapter 10. Conclusions and Future Perspectives

5. CONCLUSIONS AND FUTURE PERSPECTIVES

10.1 Conclusions

1. EE2 interferes not only with typical ER mediated mechanism (plasma Vtg content and brain ERα gene expression) but with neurohormone biosynthesis in the brain of juvenile salmon as indicated by the up-regulation of brain P450arom isoform genes expression and aromatase activity. The observed alterations in the brain are an indication that fish are experiencing impaired neurosteroidogenesis which could have serious consequences on their development and sex differentiation.

2. EE2 interferes with the entire hypothalamus-pituitary-interrenal axis of juvenile salmon by up-regulating brain and head kidney StAR, P450*scc* and Cyp11β mRNA levels. Therefore, EE2 promoted acute synthesis and regulation of neural and interrenal steroids.

3. TBT was shown to down-regulate P450arom gene expression in the brain of juvenile salmon indicating its interference with neurosteroidogenesis but not with brain aromatase activity or other ER-mediated mechanisms suggesting that inhibition of aromatase activity may not be a primary target of TBT in fish.

4. GEM, the human lipid regulator and rodent peroxisome proliferator, interferes with phase I metabolism of juvenile eels and it induces typical peroxisome proliferation enzymes but to a lesser extent than what it has been observed in rodents. Therefore, fish are not as susceptible to this PP as rodents.

5. Androgen metabolism in gastropod molluscs appears to vary between different species. Hence, androstenedione was metabolised in a different manner among the three studied gastropods (*B. brandaris*, *H. trunculus* and *M. cornuarietis*) revealing the high diversity of this phylum. Furthermore, TPT and TBT had a different effect on this metabolism following in-vitro exposures.

6. Short term exposure to the organotin compound TPT interferes with the esterification of 17β-estradiol and testosterone in females of *M. cornuarietis* but not with endogenous levels of free steroids, androgen biosynthesis and aromatase activity indicating that the later are not primary targets of TPT in *M. cornuarietis*.

7. Short term exposure to TPT interferes with fatty acid and lipid homeostasis mainly in females of *M. cornuarietis* suggesting that lipid metabolism is a primary target of this compound.

10.2 Future perspectives

The fact that the xenoestrogen EE2 was shown to modulate brain and head kidney StAR protein and P450*scc* mRNA expression in juvenile salmon (Chapter 4), which are involved in the early steps of steroidogenesis, brings questions on whether other ECDs produce such responses. This could partly explain the altered observed hormone levels in some fish following exposure to EDCs that cannot be directly related to an androgenic or estrogenic action of the compounds. The significance of such alterations on the physiology and health of juvenile fish should be further investigated.

The present thesis gives evidence that the primary action of organotins on gastropod molluscs may not be the metabolism of androgens as it has been suggested before but the lipid metabolism. Further research is necessary on the effects of organotins on lipid homeostasis and the relation of such effects with the reproduction of the snails.

It should be further investigated whether the effect of organotins on the esterification of testosterone and estradiol in gastropod molluscs is restricted to these sex steroids or whether these compounds have an effect on the esterification of other steroid hormones and molecules as well, such as the esterification of retinol by the catalytic activity of acyl-CoA: retinol acyltransferase.

Since gastropods have an RXR receptor, the role of this receptor in the reproduction of the gastropod snails should be examined in detail. Additionally, it should be elucidated whether gastropods have the capacity for retinoic acid biosynthesis and whether 9-*cis*-RA is the natural ligand of gastropod RXR. The

possibility of gastropod RXR to function as a ligand independent receptor should also be examined.

ANEX I

ANEX I

Complementary work on Chapter 4.

Apart from the fish groups that were exposed to EE2, brain and head kidney StAR protein, P450*scc* and CYP11β mRNA levels were also measured in juvenile salmon exposed to TBT for 7 days. However, since the solvent carried, DMSO, interfered with brain StAR protein, P450*scc* and CYP11β mRNA levels, as well as head kidney P450*scc* mRNA levels, following 7-days exposure compared to 3-days exposure (Chapter 4), the results from 7-days TBT exposure were disregarded.

Experimental

Two groups of (twelve individual per group) salmon fish *Salmon salar* (mean weight and length 10 ± 2.5 g and 9± 2 cm, respectively) were exposed once for 7 days to waterborne TBT at 50 and 250 ng/L. In addition, a solvent control group was included in which fish were exposed to the carrier vehicle DMSO at 7.5 ppb. Experimental conditions were identical to the ones explained in the studies of Chapter 3 and 4.

The methodology used for total RNA isolation and quantitative (real-time) RT-PCR for StAR protein, P450*scc* and CYP11β was identical to the one described in Chapter 4.

Results and Discussion

As it can be seen from Figures 1 and 2, 7-days exposure to the carrier solvent DMSO induced StAR protein, P450*scc* and CYP11β gene expression in the brain of juvenile salmon and P450*scc* gene expression in the head kidney compared to the 3-days exposed group (please refer to Chapter 4 for further discussion). 7-days exposure to 250ng TBT/L, caused a 0.4-fold decrease of brain StAR mRNA levels compared to the solvent control (Figure 1). No other statistically significant alterations were observed either in the brain or in the head kidney of juvenile salmon following TBT exposure when compared to the solvent control. However, it is not possible to conclude that TBT does not have any other effect on the expression of the proteins in this study due to the interference of the carrier solvent DMSO.

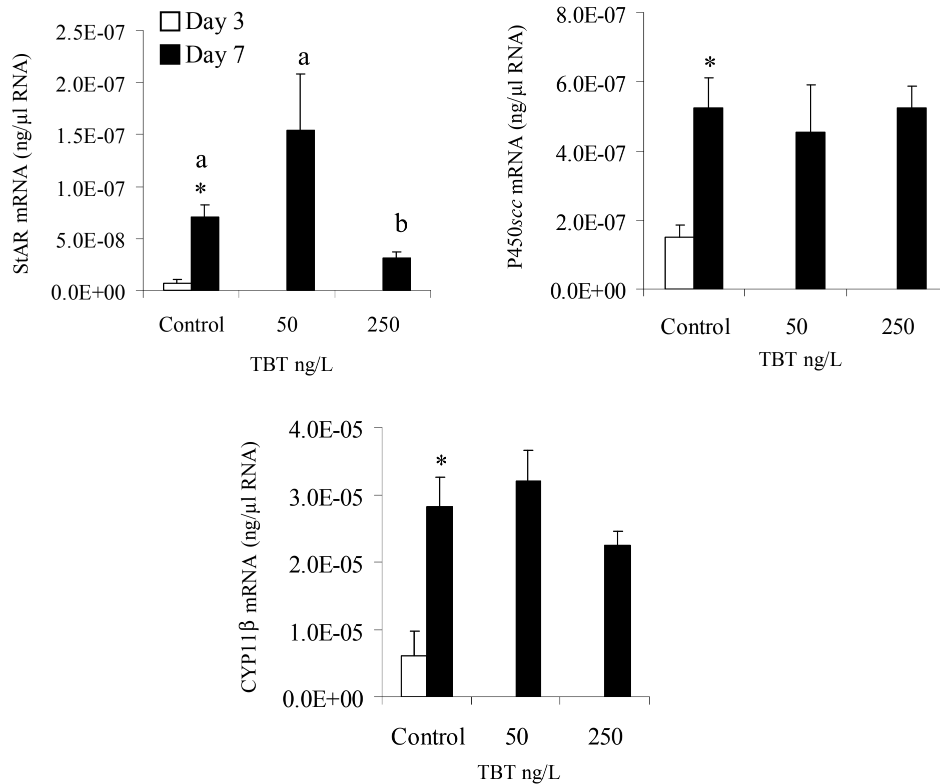

Figure 1. Changes in brain StAR, P450*scc* and CYP11β mRNA levels of juvenile Atlantic salmon after exposure to tributyltin (TBT) for 7 days. Data for solvent control is shown for 3 and 7 days. All values represent the mean (n=6) ± standard error of the mean (SEM). Different letters denote TBT exposure groups that are significantly different ($p<0.05$) compared to the carrier vehicle (DMSO) treated group, analysed using one-way ANOVA Dunnett's test; asterisk denotes differences of controls between 3 and 7 days (Student's *t*-test, $p<0.05$).

Nevertheless, the fact that 250 ng TBT/L inhibited the StAR protein gene expression (Figure 1) following 7-days exposure, which is the opposite effect than the one caused by DMSO alone, suggests that TBT interferes with the first step in the acute synthesis of neurosteroids. An inhibition in StAR protein mRNA levels, may lead to reduced production of steroid following acute stimulation (Stocco, 2001). For example, studies with Leydig mouse cells have shown that the organochloride insecticide Lindane, the organophosphate insecticide Dimethoate and the pesticide Roundup reduce StAR protein expression and inhibit progesterone production (Walsh et al., 2000; Walsh and Stocco, 2000). In a different study Mimeault et al. (2005) found that after 96h waterborne exposure of male goldfish to the pharmaceutical GEM, StAR mRNA levels were reduced by 50% in the testes of the fish, which was concomitant with a 50% reduction in plasma testosterone. Interestingly, in the study of Chapter 3, 7-days exposure to TBT resulted in inhibition of the gene expression of the two

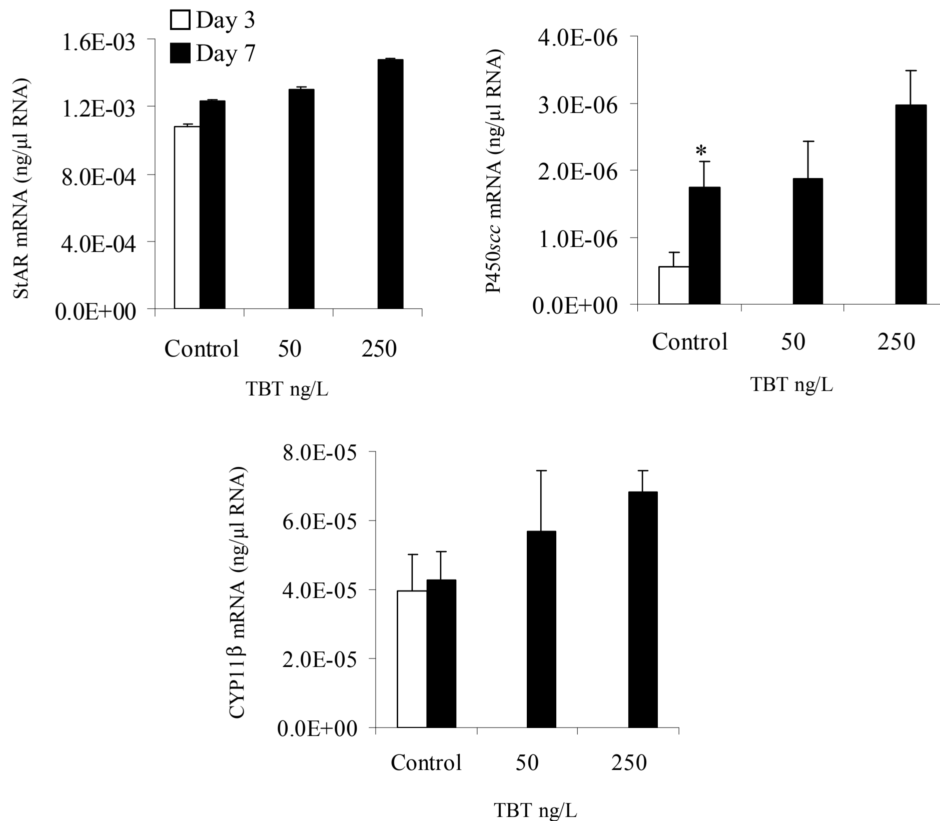

Figure 2. Changes in head kidney StAR, P450*scc* and CYP11β mRNA levels of juvenile Atlantic salmon after exposure to tributyltin (TBT) for 7 days. Data for solvent control is shown for 3 and 7 days. All values represent the mean (n=6) ± standard error of the mean (SEM). Asterisk denotes differences of controls between 3 and 7 days (Student's *t*-test; $p < 0.05$).

P450arom isoforms in the brain of juvenile salmon and aromatase activity was reduced as well, however not being statistically significant. Although steroid levels were not measured due to limited amount of blood samples, a reduction in P450arom expression and activity may lead in reduced production of estrogens. Whether a reduction in StAR gene expression causes a reduction in the production of steroid hormones is an issue that should be further investigated. Nevertheless, the present work indicates that TBT interferes with the acute synthesis of neurosteroids and more studies are necessary to elucidate the significance of such alterations in the physiology and health of fish.

References

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