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Responses to environmental xenobiotics: from endocrine disruption to lipid homeostasis imbalance

Giorgio Dimastrogiovanni

RESPONSES TO ENVIRONMENTAL XENOBIOTICS: FROM ENDOCRINE DISRUPTION TO LIPID HOMEOSTASIS IMBALANCE

Giorgio Dimastrogiovanni

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RESPUESTAS A XENOBIÓTICOS AMBIENTALES: DISRUPCIÓN ENDOCRINA Y ALTERACIÓN LIPÍDICA

Tesis doctoral

Giorgio Dimastrogiovanni

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RESPUESTAS A XENOBIOTICOS AMBIENTALES: DISRUPCIÓN ENDOCRINA Y ALTERACIÓN LIPÍDICA

Memoria presentada por

Giorgio Dimastrogiovanni

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Tutor:

Dr. Josep Planas Vilarnau

Profesor titular

Departamento de Fisiología

Facultad de Biología

Universidad de Barcelona

Directora:

Dra. Cinta Porte Visa

Profesora de Investigación

Codirectora:

Dra. Denise Fernandes

Investigadora Postdoctoral

Departamento de Química Ambiental

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ACRONYM LIST

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11βAD: 11β-Hydroxyandrostenedione

11βT: 11β-Hydroxytestosterone

17P4: 17α-Hydroxyprogesterone

3β,20-one: 3β-hydroxy-5α-pregnan-20-one

5α-DHP: 5α-pregnane-3,20-dione

ABCA1: ATP Binding-Cassette A-1

AD: Androstenedione

AhR: Aryl Hydrocarbon Receptor Protein

AHTN: Tonalide

APEs: Alkylphenol Ethoxylates

ARNT: Aryl Hydrocarbon Nuclear Translocator Protein

ATAT: Acyl-Coenzyme A:Steroid Acyltransferases

BPA: Bisphenol A

CYP: Cytochrome P450

CYP11β: 11β-Hydroxylases

CYP17: 17α-Hydroxylases/C17,20-Lyase

DAG: Diacylglycerol

DEHP: Di(2-Ethylhexyl) Phthalate

DES: Diethylstilbestrol

DHA: 5α-Dihydroandrostenedione

DHT: 5α-Dihydrotestosterone

E1: Estrone

E2: 17β-Estradiol

EDs: Endocrine Disruptors

EE2: Ethinyl Estradiol

EROD: 7-Ethoxyresorufin *O*-Deethylase

ESI: Electrospray Ionization

FA: Fatty Acid

FAS: Fatty Acid Synthase

FAT: Fatty Acid Translocase

FATP: Fatty Acid Transport Protein

GC: Gas Chromatography

GL: Glycerolipids

GP: Glycerophospholipids

GSH: Glutathione

HHCB: Galaxolide

HRMS: High-Resolution Mass Spectrometry

HSD: Hydroxysteroid Dehydrogenase

KCZ: Ketoconazole

LC: Liquid Chromatography

LPL: Lipoprotein Lipase

LXR: Liver X Receptor

NADPH: β-Nicotinamide Adenine Dinucleotide Phosphate

NP: Nonylphenol

NRs: Nuclear Receptors

P4: Progesterone

PC: Phosphatidylcholine

PPARs: Peroxisome Proliferator-Activated Receptors

PUFAs: Polyunsaturated Fatty Acids

RIA: Radioimmunoassay

RXRs: Retinoic X Receptors

SREBP: Sterol Response Element Binding Protein

STPs: Sewage Treatment Plants

T: Testosterone

TAG: Triacylglycerol

TBT: Tributyltin

TPT: Triphenyltin

UDPGA: UDP-Glucuronic Acid

UGT: UDP-Glucuronyltransferase

XREs: Xenobiotic-Responsive Element

1. GENERAL INTRODUCTION

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Modern society is nowadays highly dependent on the use and demand of chemicals for a wide range of services and products, and the chemical industry has become one of the largest manufacturing industries in the world. Chemical substances may enter the environment in different ways, and the aquatic environment is particularly susceptible to pollution and has been termed “the ultimate sink” for natural and man-made chemicals (Sumpter, 1998). Many compounds, such as pesticides normally released at their point of application, industrial chemicals unintentionally released by volatilization or by leaching, personal care products and pharmaceutical compounds excreted by humans and animals, find their way into rivers, lakes and the sea, mainly through urban and industrial effluents from sewage treatment plants (STPs) and partly due to accidental releases of chemicals through spills, land run-off, atmospheric deposition, etc. (Fig. 1). Considering that more than 100,000 chemicals are in everyday use, most of them disposed through sewer systems, it seems very likely that many chemicals, and their degradation products, are continuously entering into the aquatic environment (Sumpter, 2005). Subsequently, after the uneasy task of identifying and measuring these chemicals, the problem is to determine how they can affect the aquatic wildlife and its consequences.

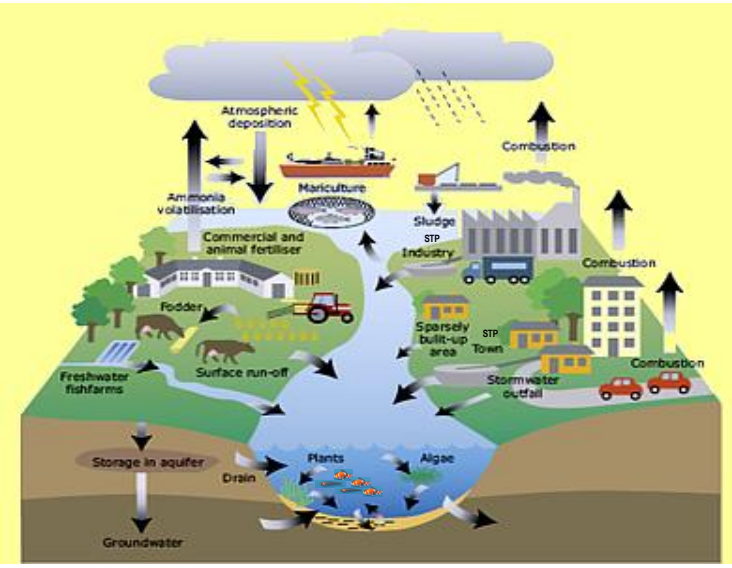


Figure 1. Scheme illustrating the potential sources and distribution of chemical into the aquatic environment (www.eea.europa.eu/themes/water/water-pollution)

1.1 Hepatic xenobiotic metabolism: enzymes involved in biotransformation

Although the concentrations of xenobiotics in the aquatic environment are normally low (ng to µg range), their continuous input and specific modes of action may lead to significant effects on aquatic organisms through acute or chronic exposure. After intake by organism, many environmental xenobiotics are metabolized or biotransformed. Thus, to improve the risk assessment of widespread contaminants, it is important to understand their metabolism and elimination in aquatic species.

Xenobiotics are usually excreted by organisms in two different ways: they can be eliminated in the original form (i.e. parent compound) or they can undergo several biotransformation reactions. Most of the compounds able to penetrate in the organisms are lipophilic molecules, as this characteristic allows them to easily permeate through cellular membranes and diffuse throughout the body. Biotransformation processes generally convert lipophilic compounds into more hydrophilic metabolites which are more easily excreted from the organism. In some cases, biotransformation can reduce the toxicity of the compound and increase its elimination; while in other cases, it can bioactivate the compound leading to a reactive metabolite which may be more toxic than the parent compound (Stegeman and Lech, 1991; Van der Oost et al., 2003). Since biotransformation reactions are also involved in the biosynthesis and elimination of endogenous molecules, such as steroids, bile acids, fatty acids, vitamins, etc. (Nebert and Russell, 2002), it is likely that xenobiotics sharing these common metabolic pathways may have the potential to interfere with the regular endogenous metabolism of exposed organisms.

Biotransformation usually involves two types of enzymatic reactions: phase I which is a non-synthetic alteration (i.e. through oxidation, reduction or hydrolysis) of the original foreign molecule, that is further conjugated in phase II. The majority of the phase II enzymes (e.g. UGTs, GSTs, SULTs, among others) catalyze synthetic conjugation reactions which facilitate the excretion of the phase I reaction products by the addition of an endogenous polar group to the molecule.

1.1.1 Phase I enzymes

The first step in the catabolism of lipophilic compounds is the functionalization process by the ubiquitous cytochrome P450 (CYP) system localized in the endoplasmatic reticulum of the liver and other tissues. The cytochrome P450 belongs to a superfamily of structurally and functionally related heme-thiolate isoenzymes, all consisting of a single polypeptide chain loosely bound to an iron-protoporphyrin IX (Fe^{3+}) as the prosthetic group (Stegeman and Hahn, 1994). The monomer of the enzyme has a molecular weight of 45 to 55 kDa. Another component of the CYP system is the flavoprotein NADPH-P450 reductase, which transfers electrons to the iron atom of the hemo group (Stegeman and Hahn, 1994). Apart from some primitive bacterial species, CYPs are present in all life forms (Lewis et al., 2006), and these enzymes are classified according to a systematic alphanumerical designation carried out strictly on the basis of primary sequence homology. Thus, cytochrome P450 superfamily is divided into families (structural homology at least 40%) specified by a number, which in turn are subdivided into subfamilies (structural homology at least 55%) denoted with a letter. The last number indicates the individual isoenzyme (Nebert et al., 1991). In general, cytochromes are present at high levels in the liver, accounting for 1 to 2% mass of hepatocytes (Lester et al., 1993). However, they are also expressed in a variety of extrahepatic tissues, such as intestine, kidney, gonads, lungs, brain, skin, etc. (Stegeman and Hahn, 1994; Ortiz-Delgado et al., 2002).

The pivotal role of the cytochrome P450 system in xenobiotic metabolism is confirmed by its broad substrate specificity, as each enzyme has a different substrate specificity (Ioannides et al., 2004), making it able to metabolize efficiently xenobiotics varying greatly in shape and size. In this thesis, we will focus on the CYP1 and CYP3 families which are primarily responsible for the metabolism of xenobiotics, but are also involved in endogenous metabolic processes.

CYP1A

Among the different CYP isoforms, the CYP1A subfamily has been extensively investigated because of its role in the biotransformation of a wide range of xenobiotic compounds, such are

dioxins, furans, pesticides, polychlorinated biphenyls (PCBs), polycyclic hydrocarbons (PAHs), petroleum products and pharmaceuticals, among others (Goksøyr and Förlin, 1992; Van der Oost et al., 2003). Induction of CYP1A is mediated through the activation of a cytosolic aryl hydrocarbon receptor protein (AhR) (Fig. 2, Route II). The xenobiotic binds to the AhR releasing a heat shock 90 protein (HSP90) which allows the AhR complex to bind to an aryl hydrocarbon nuclear translocator protein (ARNT). Then, the activated transcription factor is translocated into the nucleus, where it binds to specific regions of DNA near the promoter region of the CYP1A gene, designed as xenobiotic-responsive elements (XREs). This is followed by the transcription of CYP1A gene, the subsequent synthesis of new protein, and, finally, increased CYP1A catalytic activity (Sarasquete and Segner, 2000; Whyte et al., 2000). Thus, xenobiotics with a high binding affinity to AhR also have a high capacity to induce CYP1A (Chang and Puga, 1998; Billiard et al., 2002). The prototype ligand for the AhR is 2,3,7,8-tetra-chloro-dibenzo-*p*-dioxin (TCDD), which is one of the most potent CYP1A inducers. All steps of the CYP1A induction cascade (e.g. mRNA, protein and catalytic activity) can be used to measure CYP1A in fish (Stegeman and Hahn, 1994; Van der Oost et al., 2003).

7-Ethoxyresorufin *O*-deethylase (EROD) activity appears to be the most sensitive catalytic probe for determining the inductive response of CYP1A in fish (Goksøyr and Förlin, 1992). This activity is usually quantified in liver microsomes by estimating the increase in fluorescence of the reaction product 7-hydroxyresorufin (Burke and Mayer, 1974). Results of numerous laboratory and field studies have validated the use of CYP1A induction in fish as a sensitive biomarker of contamination in the aquatic environment (van der Oost et al., 1996; Fernandes et al., 2002). Enhanced EROD activity has been positively correlated with exposure to organic pollutants such as dioxins, PAHs, PCBs, petroleum hydrocarbons, in numerous field studies (Bucheli and Fent, 1995; Goksøyr, 1995; Ortiz-Delgado et al., 2002; Porte et al., 2002). Nonetheless, a relation between the concentration of an inducing chemical and CYP1A response is not always straightforward. Certain types of chemicals (e.g. organotins and metallic compounds) or mixtures of xenobiotics can also repress EROD induction or inhibit the catalytic activity in fish (Viarengo et al., 1997; Fent et al., 1998; Rice, 1998; Bozcaarmutlu and Arinç, 2004).

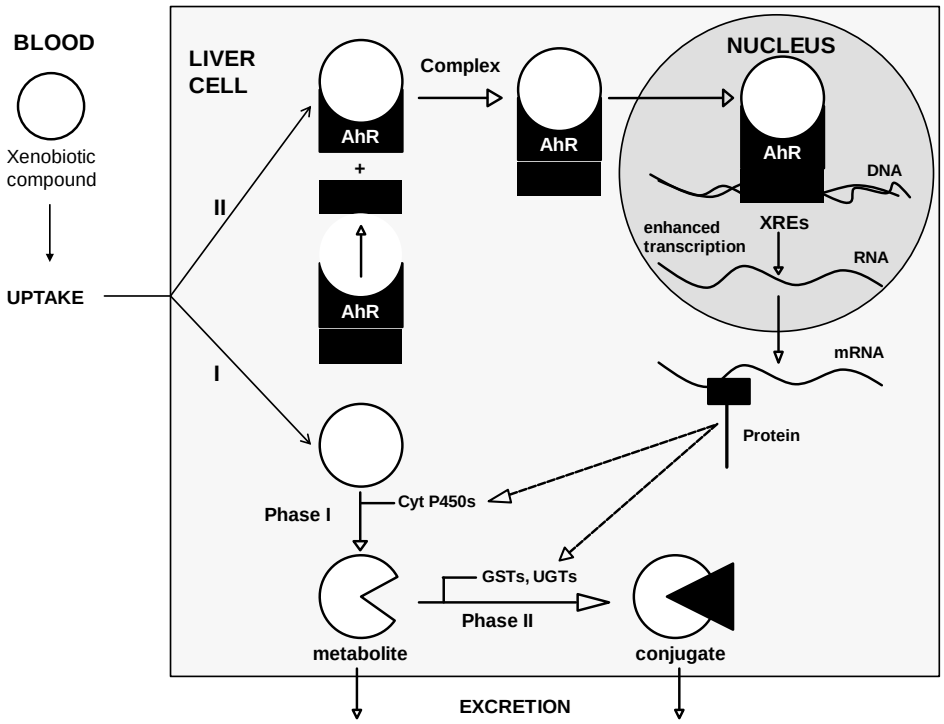


Figure 2. Schematic diagram presenting the fate of certain xenobiotic compounds in the liver cell. Route I: detoxification or toxication pathways; Route II: enzyme induction through binding to the AhR: aryl hydrocarbon receptor; HSP90: 90 KDa heat shock protein; ARNT: Ah receptor nuclear translocator; XREs: xenobiotic response elements; Cyt P450s: cytochrome P450 isoenzymes; GSTs: glutathione *S*-transferases; UGTs: UDP-glucuronosyltransferases (adapted from Van der Oost *et al.*, 2003).

CYP3A

As a consequence of its extraordinary functional diversity, the CYP3A subfamily is involved in the metabolism of endogenous hormones, bile acids, therapeutic drugs and environmental pollutants (Guengerich, 1999). The strategic localization of CYP3A isoenzymes in the gastrointestinal and respiratory tracts of mammals, makes this subfamily an important and diverse mechanism of defense against toxicity due to bioaccumulation of endogenous and exogenous lipophilic compounds (De Waziers et al., 1990; Celander et al., 2000; Hegelund and Celander, 2003). CYP3A subfamily has been extensively investigated in mammals, and catalytical and immunochemical studies pointed out the existence of CYP3A-like proteins also in non-mammalian species (Schuetz et al., 1992; Celander et al., 1996; Yawetz et al., 1998). Thus, interest has grown over the role and regulation of CYP3A enzymes in fish (McArthur et al., 2003). CYP3A30 from killifish liver (Celander and Stegeman, 1997) and CYP3A27 from

rainbow trout liver (Lee et al., 1998), were the first CYP3A family members reported in fish. Recently, novel isoenzymes of the CYP3A family have been cloned and sequenced from several fish species *viz*. CYP3A45 from rainbow trout (Lee and Buhler, 2003), CYP3A38 from medaka (Kullman et al., 2000) and the CYP3A65 from zebrafish larva (Tseng et al., 2005). Testosterone 6β-hydroxylation is a common catalytic probe to measure CYP3A activity (Waxman, 1988; Omiecinski et al., 1999). In fish, the CYP3A family is related to the activity of 6β-hydroxylation of testosterone and progesterone (Buhler and Wang-Buhler, 1998).

1.1.2 Phase II enzymes

Phase II enzymes catalyze conjugation reactions facilitating the excretion of chemicals by the covalent addition of polar groups (e.g. glucuronic acid (GA), glutathione (GSH), sulphate, etc) to the molecule, and thereby transforming a xenobiotic or endogenous compound into a more hydro-soluble product that can be excreted from the organism through the bile, urine or gills (Van der Oost et al., 2003). Since the Ah gene battery also comprises phase II genes, like UDP-glucuronosyltransferases (UGTs) and glutathione-S-transferases (GSTs), it seems that induction for most forms of phase II enzymes is probably regulated through the Ah-receptor (Nebert et al., 1990). Phase II enzymes play a key role in homeostasis maintenance of endogenous compounds, as well as in detoxification and clearance of xenobiotics and products of oxidative stress (Mulder, 2003). Some xenobiotic compounds undergo directly conjugative phase II reactions, while others need the prior metabolizing action of phase I enzymes (George, 1994) (Fig. 2, Route I). Conjugation with GSH represents the major route for electrophilic compounds, while conjugation with UDPGA is the preferred metabolic pathway for nucleophilic compounds. Other pathways, i.e. conjugation with sulphate, play a minor role in fish and are the preferred route for only a few compounds (George, 1994).

UDP-glucuronosyltransferases (UGTs)

Glucuronidation represents one of the most important phase II pathways for the inactivation and subsequent excretion of endogenous and xenobiotic compounds in fish (George, 1994; Mulder, 2003). UGTs catalyze the transfer of the glucuronide moiety from uridine 5'-diphosphoglucuronic acid (UDPGA) to acceptor substrates (aglycones) to form glucuronides. Most of these glucuronides are inactive; however, some may be bioactive or even toxic. UGTs are found in the endoplasmatic reticulum, unlike other phase II enzymes that are located in the cytosol (George, 1994; Mulder, 2003). UGTs conjugate a wide variety of endobiotics and xenobiotics containing different functional groups (e.g. –OH, -COOH, -NH_2 and –SH) (Ioannides, 2002). As expected for enzymes with a broad specificity for structurally diverse compounds, multiple UGT isoforms are present in fish (George, 1994), and they are generally named after their acceptor substrates, e.g. phenol, steroid, bilirubin (Van der Oost et al., 2003). In fish the UGT isoform that preferentially conjugates planar phenols (e.g. 1-naphthol, 4-nitrophenol) is induced by PAHs, inferring the involvement of the Ah receptor (Nebert et al., 1990; Clarke et al., 1992). Glucuronidation of xenobiotics in fish occurs mainly in the liver; however, UGT activity has also been detected in extrahepatic tissues, such as kidney, intestine and gills (George, 1994). An increase in UGT activity normally means an enhanced detoxification of the inducing compound; however, it may also have undesirable effects, such as alteration of steroid metabolism inasmuch that steroid hormones are among the UGT endogenous substrates (Stegeman et al., 1992). Increased hepatic UGT activities were found in fish exposed to PAHs, PCBs, PCDDs (Celander et al., 1993; Otto and Moon, 1995; Gadagbui and Goksrøyr, 1996; Fenet et al., 1998) and these findings are in agreement with field studies in which fish from polluted sites also showed an increased UGT activity (Gadagbui and Goksrøyr, 1996; Oost et al., 1998; Porte et al., 2002; Gaworecki et al., 2004). Although not as sensitive as phase I activities, UGT seems to be a valid biomarker, thanks to its good responsiveness to pollutant exposure (Van der Oost et al., 2003).

1.2 Environmental xenobiotics as endocrine and/or lipid disrupters

It is now generally accepted that many xenobiotics present in the environment may influence the reproductive capabilities of both vertebrates and invertebrates by adversely affecting endocrine signaling pathways (Colborn et al., 1993; Vos et al., 2000). These chemicals termed endocrine disruptors (EDs) may exert their effects by interfering with normal synthesis, storage, release, transport, metabolism, binding, action or elimination of endogenous hormones (Kavlock et al., 1996). Thus, they are a structurally diverse group of compounds with multiple modes of action, from hormone receptor-mediated action (agonists or antagonists) to disruption of hormone synthesis and/or metabolism (Bryan et al., 1987; Pickering and Sumpter, 2003). The effects of EDs are generally classified as estrogenic (compounds that mimic natural estrogen), androgenic (compounds that mimic natural testosterone), and thyroid (compounds with direct or indirect impacts to the thyroid receptor) (Snyder et al., 2003).

In the aquatic environment, several well-documented studies have described the impact of endocrine disrupters in wildlife. Perhaps, the clearest example of endocrine disruption unequivocally linked to a specific environmental xenobiotic is the case of imposex in marine gastropod mollusks which was related to tributyltin (TBT), a component widely used in antifouling paints and applied to ships and marine structures (Bryan et al., 1988; Matthiessen and Gibbs, 1998). Imposex describes a condition in which male sex organs (penis and vas deferens) are superimposed in a female gastropod, leading to complete reproduction failure, and hence local extinctions of species due to sterility of the females. Since then, a wide variety of other potential EDs have been discovered, amongst them, synthetic steroids such as diethylstilbestrol (DES) and ethinyl estradiol (EE2) (Desbrow et al., 1998; Lintelmann et al., 2003), pesticides such as DDT and diuron (Stahlschmidt-Allner et al., 1997; Lintelmann et al., 2003), dioxins (Depledge and Billinghurst, 1999), polycyclic aromatic hydrocarbons (PAHs) (Navas and Segner, 2000), phthalates (Latini et al., 2004), the industrial surfactant 4-nonylphenol (McCormick et al., 2005) and the plasticizer bisphenol A (Oehlmann et al., 2000; Rubin, 2011).

The focus of endocrine-disruptor research has been mainly on reproductive endpoints and sexual development. However, the fact that different species have different responsiveness to EDs may be taken as a sign of multiple mechanisms of action, and new potential areas of action for these compounds are being explored. EDs possess several modes of action, and one main route seems to be through interference with nuclear receptors (NRs), a class of transcription factors that regulate gene expression programs by interaction with small lipophilic hormones. The first studies linking nuclear receptors to ED-associated deleterious actions on sexual development and reproduction focused on their interference with estrogen and/or androgen signaling. However, in addition to the sex steroid receptors, the NR superfamily includes transcription factors playing pivotal roles in metabolic and energy regulation, such as glucocorticoid receptors (GRs), liver X receptor (LXR), and retinoic X receptors (RXRs), all potential targets for the action of EDs. In this respect, considering their key role in lipid homeostasis maintenance and their ability to bind a diverse range of compounds, the peroxisome proliferator-activated receptors (PPARs) represent a very interesting target for EDs. In fact, there are increasing evidences showing that environmental pollutants may disrupt these and other critical pathways involved in adipogenesis, lipid metabolism and energy balance (Tabb and Blumberg, 2006; Grün and Blumberg, 2007; Casals-Casas and Desvergne, 2011). Such compounds have been referred to as environmental obesogens, and the number of compounds reported to affect lipid-related pathways is continuously increasing.

Hence the need to further investigate the effects of potential endocrine and/or lipid disruptors in aquatic organisms is evident, and in this context the present thesis will focus on the selection of different compounds belonging to different chemical groups (e.g. hormones, personal care products, surfactants, biocides, plasticizers), that are known as environmental contaminants.

Natural and Synthetic hormones

Natural and synthetic hormones are considered very potent endocrine-disrupting compounds. They are excreted by humans and animals and are incompletely degraded in STPs. Although they are present at very low concentrations in the environment, such concentrations are

biologically active (Routledge et al., 1998; Johnson and Sumpter, 2001). The continuous consumption and release of these compounds into aquatic systems can certainly contribute to their prevalence in the environment and because synthetic hormones are designed specifically for their biological activity, their occurrence may result in latent risks to non-target aquatic organisms (Lange et al., 2002). For instance, natural hormones, such as 17β-estradiol (E2) and estrone (E1), and the synthetic estrogen ethinyl estradiol (EE2), which is an active ingredient of contraceptive pills, have been detected in STP effluents and related to endocrine alterations such as the 'feminization' of fish males (Desbrow et al., 1998; Belfroid et al., 1999; Körner et al., 2001). Apart from estrogens, of particular concern are those less studied chemicals capable of mimicking or antagonizing the action of progesterone. Progesterone (P4) is the steroid precursor of hormones such as 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) which act as pheromones and trigger the process of final maturation in eggs and sperm of fish (Nagahama, 1994). However, few studies have focused on the disruption of progesterone-mediated processes despite the importance of endogenous progestogens in the regulation of gametogenesis, secondary sex characteristics and reproductive behavior in aquatic organisms. Recently, Liang et al. (2015) showed that exogenous P4 and the synthetic progestin, norgestrel, may affect sex differentiation in zebrafish, likely by altering sex hormone levels and transcriptional expression profiles of genes related to steroidogenesis.

Polycyclic musks

Recently, focus has also been placed on new emerging contaminants, such as personal care products (PPCPs), which exert weak estrogenic and/or other hormonal activities. Amongst them, synthetic polycyclic musks are used in almost all scented consumer products, such as detergents, cleaning agents, soaps, shampoos and deodorants, because of their typical musky scent and their substantive property to bind fragrances to fabrics. The polycyclic musks are indane and tetraline derivatives highly substituted mainly by methyl groups. After their application in private households, they are dumped via STPs into the aquatic environment.

Galaxolide (**HHCB; 1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyrane**) (Figure 3) is by far the most widely used synthetic musk, with a production volume in Europe between 1,000 and 5,000 tons per year (OSPAR, 2004). STPs are the major sources of HHCB into the aquatic environment. Considerable amounts of HHCB have been detected in sewage effluents (0.2-6.0 μg/L), sewage sludge (5.4-21.2 μg/g d.w.), freshwater (10-314 ng/L) and freshwater fish sampled near STPs (0.04-3.6 μg/g w.w.) (Paxéus, 1996; Rimkus, 1999; Garcia-Jares et al., 2002; Gatermann et al., 2002; Moldovan, 2006; Zeng et al., 2005). Furthermore, the persistent nature of polycyclic musks and their tendency to bioaccumulate in fish and other aquatic organisms have been reported. Because of its highly lipophilic nature (log Kow = 5.90), HHCB bioaccumulates in aquatic organisms at concentrations ranging from <0.03 up to 160 μg/g lipid weight (Rimkus, 1999; Gatermann et al., 2002). Considering the high volume emissions, the intensive usage, the bioaccumulation tendency and the widespread distribution, the potential toxic effects of HHCB in biota are of great concern (Tanabe, 2005). In-vitro reporter gene assays have indicated potential estrogenic and antiestrogenic effects of HHCB towards human estrogen receptor, and in-vivo studies showed a dose-dependent antagonistic effect on zebrafish (*Danio rerio*) estrogen receptor (Seinen et al., 1999; Schreurs et al., 2004; Schreurs et al., 2005). Other studies revealed that polycyclic musks, and among them HHCB, had a high potential of interfering with the catalytic activity of CYP isoforms involved in the synthesis and metabolism of sex steroids in carp (*Cyprinus carpio*) (Schnell et al., 2009) and male medaka (*Oryzias latipes*) (Yamauchi et al., 2008). Nonetheless, information regarding the metabolism of polycyclic musks by aquatic organisms, the interference with endogenous and xenobiotic metabolizing enzymes and/or endocrine disrupting properties is still scarce.

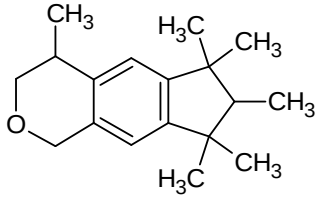


Figure 3. Chemical structure of the polycyclic musk Galaxolide.

Alkylphenol ethoxylates

Alkylphenol ethoxylates (APEs) are highly cost effective surfactants with exceptional performance, and widely used in domestic detergents, pesticide formulations and industrial products (Lorenc et al., 1992; Fiege et al., 2000). APEs are discharged to wastewater treatment facilities or directly released into the environment, where they are incompletely degraded to more persistent and toxic metabolites, such as **nonylphenol** (**NP**) (Figure 4) and octylphenol (OP) (Giger et al., 1984; Ahel et al., 1994a; Ahel et al., 1994b; Soto et al., 1991; Jobling and Sumpter, 1993). NP is also used in the manufacture of antioxidants and lubricating oil additives. Since its first synthesis in 1940, 500,000 tons of nonylphenol ethoxylates were produced worldwide, with 60% ending up in the aquatic environment (Renner, 1997; Solé et al., 2000). Thus, the presence of NP has been reported in surface waters (streams, rivers, lakes and estuaries), oceans and sediments, and in matrices such as sewage sludge and STP effluents, and although it has been demonstrated that its concentration in the aquatic environment is decreasing, it is still found at concentrations up to 4.0 µg/L in river waters and 1.0 mg/kg in sediments (Soares et al., 2008).

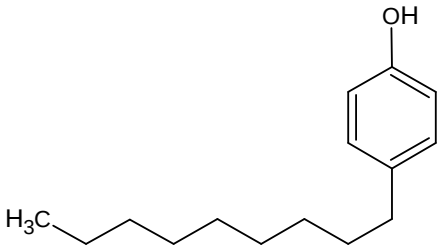


Figure 4. Chemical structure of 4-nonylphenol.

NP is characterized by low solubility in water and high hydrophobicity (log Kow = 4.48), therefore it accumulates in environmental compartments characterized by high organic content, typically sewage sludge and river sediments (John et al., 2000). In spite of its degradation by photolysis in natural waters (Ahel et al., 1994c), and a limited biodegradation in soil and sewage sludge through the action of microorganisms (Hesselsøe et al., 2001), NP has an estimated half-life of more than 60 years in sediments (Shang et al., 1999). Nowadays, alkylphenols are

considered environmental endocrine disrupters, consistent with their capacity for estrogenic and antiandrogenic activity (Lee et al., 2003; Bonefeld-Jorgensen et al., 2007). Adverse responses of the endocrine system to NP have been reported, such as: a) stimulation of vitellogenin production in trout hepatocytes, a female-specific protein and biomarker for exposure to estrogens (Sumpter and Jobling, 1995); b) disruption of sexual differentiation in carp (Gimeno et al., 1997); and c) increased frequencies of intersex in wild fish populations collected near sewage outfalls (Jobling et al., 1998; Petrovic et al., 2002; Lavado et al., 2004). Apart from these documented effects on the reproductive system, studies on possible disruption of lipid homeostasis and promotion of adiposity by NP are quite scarce and so far elusive. Thus, NP has been shown to stimulate the proliferation of fully differentiated adipocytes and the accumulation of triacylglycerols in 3T3-L1 murine cells, in a time- and concentration-dependent manner (Wada et al., 2007).

Bisphenol A

Bisphenol A (**BPA; 2,2-bis(4-hydroxyphenyl)propane**) is one of the highest volume chemicals produced worldwide, with more than 8 billion pounds produced every year and more than 100 tons released yearly into the atmosphere (Vandenberg et al., 2010). BPA is an organic compound composed of two phenol rings connected by a methyl bridge, with two methyl functional groups attached to the bridge (Figure 5).

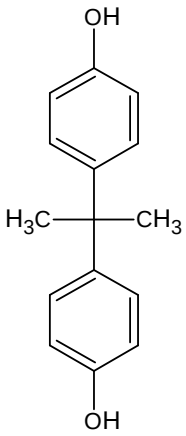


Figure 5. Chemical structure of Bisphenol A.

This compound is a building block of polycarbonate plastics and is found in numerous consumer products, such as reusable water bottles and food containers, paper, metallic cans, and also electrical and electronic equipment, automobiles, sports safety equipment, and optical lenses. The release into the environment is possible during the manufacturing process and by leaching from final products (Yamamoto and Yasuhara, 1999; Goodson et al., 2002). In this context, BPA has been detected in several environmental compartments, including landfill leachates (0.74-3.61 mg/L) (Coors et al., 2003; Kawagoshi et al., 2003), freshwater (0.5-20 ng/L) (Kuch and Ballschmiter, 2001; Brossa et al., 2005), seawater (20 ng/L) (Brossa et al., 2005), STP influents and effluents (0.047-1.51 µg/L) (Kuch and Ballschmiter, 2001; Céspedes et al., 2006), outdoor air samples (0.02-1.92 ng/m^3) (Matsumoto et al., 2005) and even in indoor air and dust samples (0.2-17.6 µg/g) (Rudel et al., 2003). Although BPA can be biodegraded or metabolized by organisms, generally leading to its detoxification (Kang et al., 2006), it is pseudo-persistent in the aquatic environment because of continual inputs, and organisms may be exposed to BPA chronically or during sensitive life stages. Although initially considered to be a weak environmental estrogen, subsequent studies demonstrated that BPA is as effective as estradiol in triggering some receptor responses (Stahlhut et al., 2009) and it may act as an androgen receptor antagonist (Krishnan et al., 1993; Lindholst et al., 2000; Goodman et al., 2006). In addition, new potential areas of BPA action are being explored. In-vivo studies have revealed that low doses of BPA (2.4-100 µg BPA/kg b.w./day) increased body weight in rats and mice exposed during gestation and/or lactation (Howdeshell et al., 1999; Rubin et al., 2001); and led to the accumulation of triglycerides and cholesteryl esters in mice liver (Marmugi et al., 2012).

Organotin compounds

Organotin compounds represent a group of organometallic moieties containing a Sn atom covalently bound to one or more organic substituents (e.g. methyl, ethyl, propyl, butyl and phenyl). Most prominent compounds of this group, **tributyltin (TBT)** and **triphenyltin (TPT)** (Figure 6) were primarily used as stabilizers in PVC plastics, as biocides in agrochemicals and antifouling paints, and as fungicides in timber and wood protection (Fent, 1996). These various

commercial and industrial applications increased drastically the worldwide production of organotin chemicals from less than 5,000 tons in 1955 up to about 50,000 tons in 1992 (Mercier et al., 1994; Omae, 2003), leading to the entrance of considerable concentrations of organotins into different compartments of the aquatic ecosystem, such as water (TBT: 2.1-1000 ng/L) (Dirkx et al., 1993; Shim et al., 2005), sediment (TBT: 73-15130 ng/g dry weight; TPT: 15-12272 ng/g dry weight) (Tolosa et al., 1992; Biselli et al., 2000) and biota (TBT: 6-11500 ng/g dry weight; TPT: 3-5930 ng/g wet weight) (Higashiyama et al., 1991; Stäb et al., 1994; Shim et al., 2005). Nowadays, although TBT is a strongly regulated chemical in most European countries, it is still detected in the aquatic environment due to its strong persistent nature and presence in municipal and industrial wastewater, sewage sludge and landfill leaches. Due to their lipophilic nature, these compounds can easily permeate through biological membranes and bioaccumulate in biota. Mollusks tend to exhibit a higher bioaccumulation factor for TBT due their filtering nature; whereas fish and crustaceans generally accumulate lower burdens of TBT, since they possess a more developed enzymatic metabolic system (Laughlin Jr, 1996). Nowadays, TBT and TPT are considered as model endocrine disruptors (Fent, 2006), and further studies have indicated them as potential obesogens. TBT was shown to promote lipid accumulation in adipose tissues and hepatic steatosis in laboratory mouse, and to induce ectopic adipocyte formation in the amphibian model *Xenopus* (Inadera and Shimomura, 2005; Grün et al., 2006).

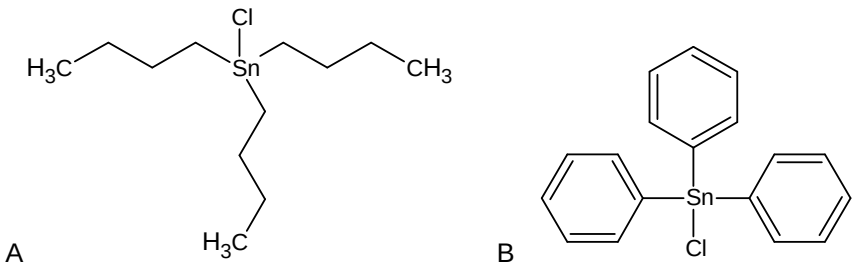


Figure 6. Chemical structure of A) tributyltin chloride, B) triphenyltin chloride.

Phthalates

Another class of commonly encountered chemicals that can negatively impact lipid and adipose tissue homeostasis are phthalate plasticizers. Phthalates are widely used industrial chemicals, serving as additives in the manufacture of PVC, in plasticizers for building materials and home furnishings, in food packaging and insect repellents, and they are also found in cosmetics, perfumes, and certain drugs (Staples et al., 1997). Phthalates can enter the environment through production, use, and disposal; furthermore, as they are non-covalently bonded to the polymeric matrix, they are also potentially released through losses during manufacturing processes and by leaching from final products (Bizzari et al., 2000; Fromme et al., 2002). Phthalates are characterized by low water solubility (0.04–0.4 mg/L) and, when released into the aquatic environment, they tend to adsorb strongly to suspended particles and sediments (Fromme et al., 2002). **Di(2-ethylhexyl) phthalate** (**DEHP**) (Figure 7) is among the most widely used phthalate esters with an annual worldwide production estimated around 2 million tons according to Swiss authorities (Federal Office of Public Health, www.bag.admin.ch/themen/chemikalien/00228/01378), and is by far the most abundant phthalate present in the aquatic environment (Shelby, 2006). Relatively high concentrations of DEHP have been reported in different aquatic compartments, such as surface water (0.33 to 97.8 mg/L), sewage effluents (1.74 to 182 mg/L), sewage sludge (27.9 to 154 mg/kg d.w.) and sediments (0.21 to 8.44 mg/kg) (Fromme et al., 2002). Hence, the biological effects of DEHP and its metabolites are of major concern. Indeed, experimental and epidemiological studies have shown the ability of some phthalates to alter lipid homeostasis, promoting obesity. Urine concentrations of four DEHP metabolites were positively correlated with abdominal obesity among adult U.S. men (Stahlhut et al., 2007; Hatch et al., 2008). Among these metabolites, mono-2-ethylhexyl phthalate (MEHP) promoted differentiation of 3T3-L1 murine cells into adipocytes and induced the expression of genes involved in lipogenesis and triglyceride synthesis (Feige et al., 2007).

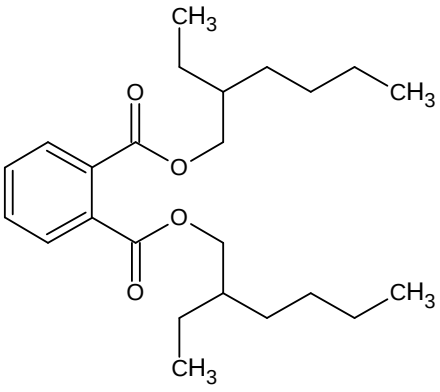


Figure 7. Chemical structure of di(2-ethylhexyl) phthalate (DEHP).

1.3 Endocrine disruption and potential non-genomic pathways

Steroid hormones bind to intracellular receptors and consequently regulate transcription and protein synthesis, triggering genomic events responsible for biological effects. Additionally, very rapid actions of steroids initiating intracellular signaling cascades have been recognized, which cannot be ascribed to transcriptional activation (Olsson et al., 1998; Falkenstein et al., 2000). These rapid effects are believed to occur through binding to membrane receptors, and have been named as non-genomic steroid actions.

It has been shown that environmental xenobiotics may act as endocrine disrupters not only through interaction with steroid nuclear receptors, but also by interfering with non-genomic pathways, such as steroid biosynthesis and metabolism. The biosynthesis of steroid hormones is the result of sequential hydroxylation and reduction reactions which convert cholesterol to physiological active androgens, estrogens and progestogens (Payne and Hales, 2004). Specific steroidogenic genes are differentially expressed in the somatic cells of ovary and testes, resulting in the production of a wide variety of sex steroids (Omura and Morohashi, 1995). Environmental chemicals may impair steroid synthesis and metabolism, consequently altering the bioavailability of active steroids within the organism and disrupting the physiological processes controlled by steroid hormones. Figure 8 illustrates a schematic diagram of both genomic and non-genomic key pathways in steroid action, which may be sensitive to disruption by environmental xenobiotics.

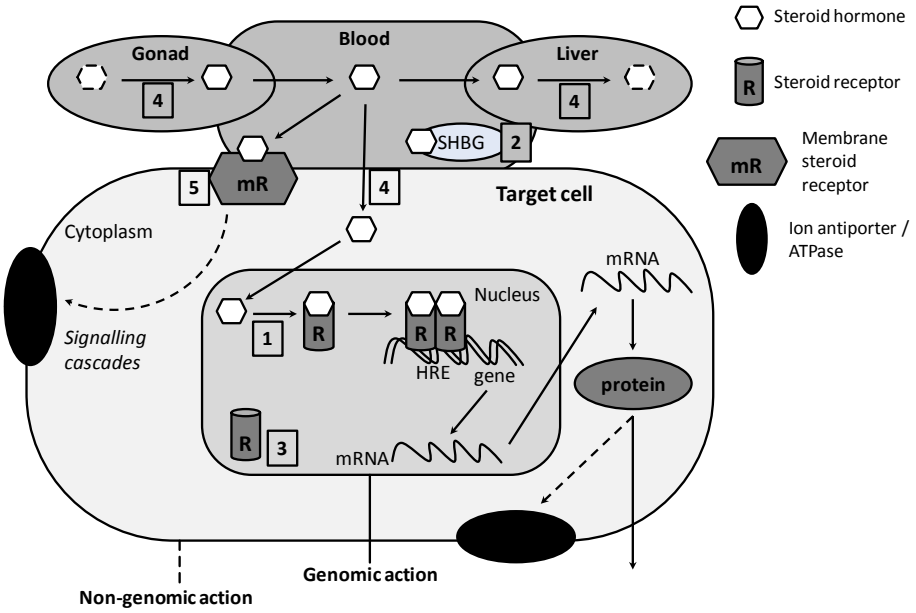


Figure 8. Schematic diagram showing several key pathways of steroid hormone action that may be targets of disruption by environmental xenobiotics. SHBG: steroid hormone binding globulin; HRE: hormone response elements. 1) Binding to steroid receptor; 2) Binding to SHBG or altering levels of SHBG; 3) Alteration of steroid receptor levels; 4) Alterations of biosynthesis and metabolism pathways, in target cell, liver and gonad; 5) Interaction with the non-genomic action of steroid hormones (adapted from Falkenstein et al., 2000).

1.3.1 Steroid biosynthesis in fish

In fish, sex steroids (i.e. androgens, estrogens and progestogens) play an important role in gametogenesis, ovulation and spermiation, resulting crucial in the gonadal differentiation and sexual maturation of the individuals (Devlin and Nagahama, 2002). The major categories of enzymes involved in the biosynthesis of gonadal steroid hormones are the cytochrome P450 superfamily (CYP) and the steroid dehydrogenase family (HSD) (Payne and Hales, 2004). The P450 isoenzymes involved in steroidogenesis (CYP11, CYP17, CYP19, and CYP21) are membrane-bound proteins associated with the mitochondrial membranes or the endoplasmatic reticulum (Payne and Hales, 2004). Unlike other CYP isoenzymes, these Cyt P450 families tend to have only one or two highly specific substrates as result of their endogenous role (Lewis, 2004). On the other hand, HSDs belong to the short-chain alcohol dehydrogenase reductase family. They are responsible for the reduction and oxidation of steroid hormones, using NAD+/NADP+ as acceptors and their reduced forms as donors of reducing equivalents (Payne

and Hales, 2004). HSD isoforms vary considerably in tissue distribution, catalytic activity (dehydrogenase vs. reductase), and substrate and cofactor specificity.

The first step in steroid biosynthesis is the conversion of cholesterol to pregnenolone, the starting precursor of all steroid hormones, accomplished by a mitochondrial cytochrome P450 enzyme named side chain cleavage (P450scc) (Fig. 9). Transport of free cholesterol from the cytoplasm into the mitochondria is facilitated by the steroidogenic acute regulatory protein (StAR). The StAR/P450scc system is considered the quantitative regulator of steroidogenesis, acting as a rate-limiting and hormonally regulated system (Miller, 2002). The steroid precursor pregnenolone (P5) is converted to progesterone (P4), which is further metabolized to 17α-hydroxyprogesterone (17P4) by 17α-hydroxylase (CYP17). 17P4 is then converted to androstenedione (AD) by C17,20-lyase (CYP17) or to 17α,20β-dihydroxyprogesterone (17α,20β-DP) by 20β-hydroxysteroid dehydrogenase (20β-HSD); 17α,20β-DP has been identified as the maturation-inducing hormone (MIH) in several teleost fish (Kime, 1993; Senthilkumaran et al., 2004; Scott et al., 2010). AD on the other hand is the precursor of T, and both T and AD can be transformed in testes to their respective 11β-hydroxylated metabolites, 11β-hydroxytestosterone (11βT) and 11β-hydroxyandrostenedione (11βAD), a reaction catalyzed by 11β-hydroxylases (CYP11β) (Kime, 1993; Liu et al., 2000). 11βT and 11βAD can be further metabolized to 11-ketoandrogens (11-ketotestosterone (11-KT) and 11-ketoandrostenedione (11-KAD)), by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) (Kusakabe et al., 2003). 11-KT is the most potent androgen in male teleost fish, being more effective than T in stimulating reproductive behavior, influencing spermatogenesis and stimulating secondary sexual characteristics (Borg, 1994; Devlin and Nagahama, 2002). Additionally, androgens (AD and T) are converted into estrogens (estrone and estradiol) through aromatization in female gonads; this reaction is catalyzed by cytochrome P450 aromatase (CYP19), a key enzyme regulating local and systemic levels of estrogens in the body (Cheshenko et al., 2008) (Fig. 9). Both, 17β-estradiol (E2) and estrone (E1) are the predominant estrogens in fish, being E2 responsible for ovarian development and maintenance (Yamamoto, 1969; Guiguen et al., 2010).

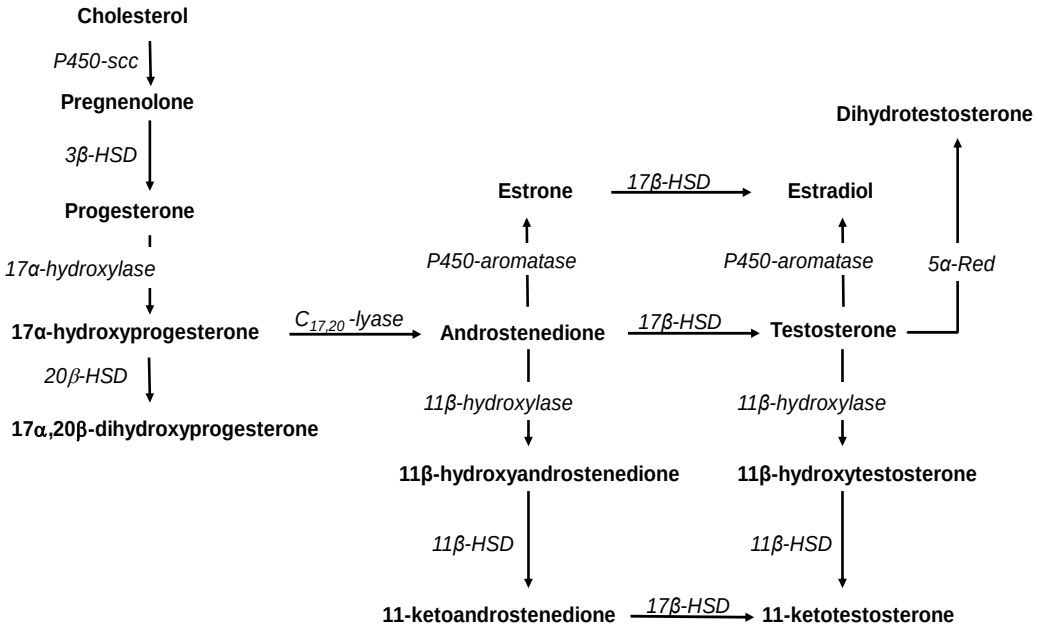


Figure 9. Scheme illustrating the main steroidogenic pathways involved in the biosynthesis of progestogens, androgens and estrogens in teleost fish. P450scc: P450 side chain cleavage; HSD: hydroxysteroid dehydrogenases; CYP17 with two enzymatic activities (17α-hydroxylase and C17,20-lyase); 11β-hydroxylase: CYP11β; P450 aromatase: CYP19; 5α-Red: 5α-reductase

Several studies have assessed the ability of EDs to alter steroid biosynthesis in fish. Monteiro et al. (2000) showed an inhibition of CYP17 (C17,20-lyase) and CYP19 activity in ovarian tissue of flounder (*Platichthys flesus)* exposed to high concentrations of PAHs (15 µM), that led to reduced secretion of AD and E2. Others found that exposure to pesticides and NP can inhibit 17β-HSD activity in carp, the key enzyme involved in the formation of T (Thibaut and Porte, 2004). Additionally, NP and the antifungal ketoconazole (KCZ) were shown to inhibit the activity of CYP17 (C17,20 lyase) and CYP11B reducing the formation of 11βAD in male sea bass (*Dicentrarchus labrax*) (Fernandes et al., 2007). Thus, an interference with steroid biosynthesis may alter bioavailable amounts of active hormones within the organism, and be a potential mechanism of endocrine disruption by affecting sexual differentiation, gamete growth and/or maturation of fish.

1.3.2 Steroid biosynthesis and metabolism in invertebrates

Most steps of the steroidogenic pathways described previously for vertebrates have been demonstrated to occur in invertebrates either by directly exposing the organisms to steroid precursors or by incubating homogenates with those steroid precursors (Figure 10). Thus, the key steps of steroidogenesis leading to progestogens, androgens and estrogens have been found in mollusks (De Longcamp et al., 1974; Lupo di Prisco and Dessi' Fulgheri, 1975; D'Aniello et al., 1996), echinoderms (Schoenmarkers and Voogt, 1980; Voogt et al., 1990; Le Curieux-Belfond et al., 2001; Lavado et al., 2006a) and to some extent in crustaceans (Swevers et al., 1991; Verslycke et al., 2002). In addition, several vertebrate-type sex steroids (e.g. P4, AD, T, and E2) have been reported for different invertebrate species (see Table 1), along with variations in their content during the reproductive cycle (Lupo di Prisco and Dessi' Fulgheri, 1975; Reis-Henriques and Coimbra, 1990; Reis-Henriques et al., 1990; Hines et al., 1996; Gauthier-Clerc et al., 2006). Although vertebrate-like steroids have been detected in many invertebrate species, together with several steps of steroid biosynthesis and metabolism, the biological role of these steroidal hormones in invertebrates is still unclear. The sole presence of vertebrate-like sex steroids cannot be taken as reliable proof of a reproductive role in invertebrates, since their presence may simply be the result of uptake from food or the environment, and there is still no firm evidence for steroid de-novo synthesis (Scott, 2012; Scott, 2013). Thus, it is important to understand the complexity of the invertebrate endocrine system, in order to progress on understanding the mechanisms of action of EDCs in invertebrates.

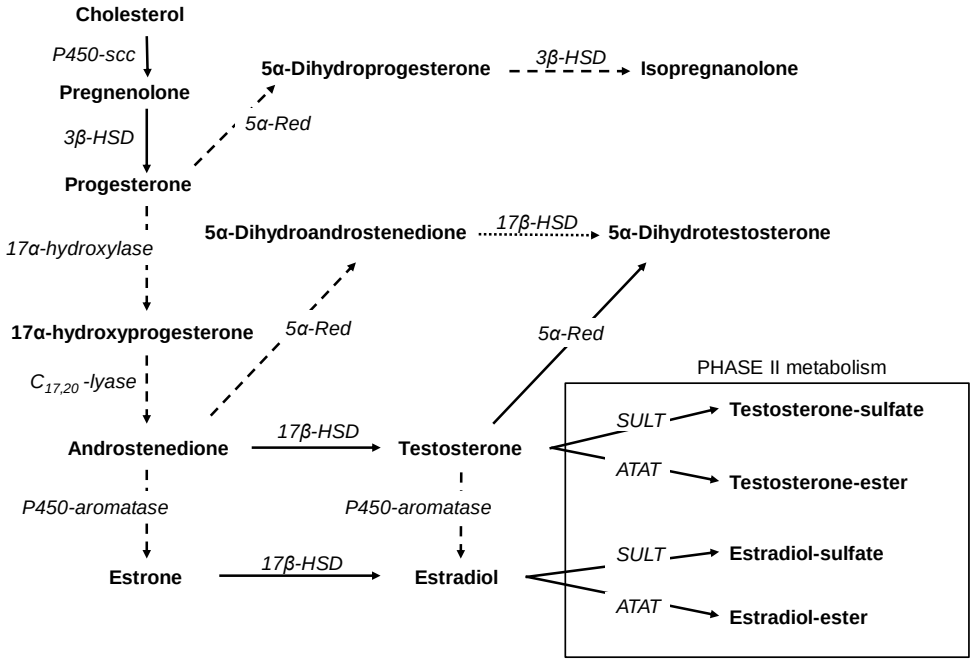


Figure 10. Scheme illustrating steroidogenic and metabolic pathways described in invertebrates through the measurement of the metabolism of labeled steroids. P450scc: P450 side chain cleavage; HSD: hydroxysteroid dehydrogenases; CYP17 with two enzymatic activities (17α-hydroxylase and C17,20-lyase); P450 aromatase: CYP19; 5α-Red: 5α-reductase; SULT: sulfotransferase; ATAT: fatty acid acyl-CoA acyltransferase. (- - - - - ▶) pathways not detected in crustaceans; (·····▶) pathways detected only in mollusks. (Adapted from: Porte et al., 2006; Janer and Porte, 2007; Fernandes et al., 2011).

Table 1. Vertebrate-like sex steroids detected in several invertebrate species using different methods (a) [^{14}C]-cholesterol label; (b) gas chromatography-mass spectrometry (GC-MS); (c) radioimmunoassay (RIA); (d) enzyme-linked immunosorbant assay (ELISA).

Phylum	Species	Matrix	Steroid(s)	Reference(s)
Mollusks	*Aplysia depilans*	Gonad, Hepatopancreas	[(a)] P5, P4, E1, E2, 17P4, T	Lupo di Prisco et al. (1973)
	Helix aspersa	Ovotestis	[(b)] AD, E1, E2	Le Guellec et al. (1987)
	Marisa cornuarietis	Digestive gland, gonad	[(c)] T, E2	Janer et al. (2006)
	Mya arenaria	Gonad	[(d)] P4, T, E2	Siah et al. (2002); Gauthier-Clerc et al. (2006)
	Mytilus edulis	Whole animal, Testes, ovaries	[(b)(c)] P4, AD, DHT, E2, E1, T	De Longcamp et al. (1974); Reis-Henriques et al. (1990)
	Mytilus galloprovincialis	Whole animal	[(c)] T, E2	Janer et al. (2005a)
	Octopus vulgaris	Gonad	[(c)] P4, T, E2	D'Aniello et al. (1996); Di Cosmo et al. (2001)
	Ruditapes decussatus	Gonad	[(c)] P4, T, E2	Ketata et al. (2007)
	Sepia officinalis	Hemolymph	[(c)] T	Carreau and Drosdowsky (1977)
	Thais clavigera	Gonad	[(b)] AD, T, E2, E1	Goto et al. (2012)
Arthropoda	*Artemia sp.*	Whole animal	[(b)(c)] P5, P4, DHT, T, E1, E2	Van Beek and De Loof (1988); Novak et al. (1990)
	Homarus americanus	Hemolymph, testes, ovary, hepatopancreas	[(c)] T, P4, E2	Burns et al. (1984); Couch et al. (1987)
	Nephrops norvegicus	Ovaries, eggs, hemolymph	[(b)] DHT, T, P5, E2	Fairs et al. (1989)
Echinodermata	*Asterias amurensis*	Ovaries	[(b)] P4	Ikegami et al. (1971)
	Asterias rubens	Ovary, pyloric caeca	[(c)] P4, E1	Dieleman and Schoenmakers (1979)

P5: pregnenolone; P4: progesterone; E1: estrone; E2: estradiol; 17P4: 17α-hydroxyprogesterone; T: testosterone; AD: androstenedione; DHT: 5α-dihydrotestosterone

The first step in analyzing sex steroids is to extract them from their matrix (e.g tissues). Several extraction and purification steps are usually necessary prior to the identification and/or quantification. Once extracted and partially purified, several methods are available for measuring steroid hormone levels. The technique most commonly used is radioimmunoassay (RIA). The advantages of this method include the availability of commercial reagents and kits, high sensitivity, and the capacity to analyze large numbers of samples relatively quickly and inexpensively (McMaster et al., 2001). Nonradioactive methods, including fluorescent immunoassay or enzyme-linked immunosorbant assay (ELISA), are also increasing in popularity. However, the lack of specificity is the major problem associated to immunoassays. Thus, the cross-reactivity of the antibodies with other steroids, and the interference of other substances with the antigen-antibody binding should be carefully revised. This is of special concern when working with invertebrates, due to the limited information available on the profile of endogenous steroids in the organisms under study, which might cross react with the antibodies used in the assay. Indeed, Zhu et al. (2003) reported that in addition to E2, extracts of *M. edulis* gonads contained a second molecule that had a high binding affinity for E2 antibodies. Chromatographic techniques on the other hand, although not as sensitive as biological techniques, enable simultaneous screening of different steroids. In addition, in contrast to immunoassays, some detection systems coupled to chromatographic techniques (e.g. mass spectrometry) provide a highly reliable identification of the steroids. Thus, the use of HPLC, gas chromatography-mass spectrometry (GC-MS), or liquid chromatography-mass spectrometry (LC-MS) has increased in recent years. Nonetheless, the limitations of using LC- or GC-MS for the determination of steroids in tissues are the interferences of the matrix and the high detection limits of these methods. Thus, mass spectrometry is still seldom used to quantify vertebrate-type sex steroids in invertebrates.

Together with steroidogenesis, steroid metabolism plays an important role in the regulation of endogenous steroid levels. Fatty acid conjugation (or esterification) transforms steroids into an apolar form which is retained within the lipoidal matrices of the body, reducing their bioactivity, bioavailability and their susceptibility to elimination (Borg et al., 1995). Thus, esterification might have a regulatory function by inactivating steroids. The esterification of steroids with fatty acids

is facilitated by the acyl-coenzyme A:steroid acyltransferases (ATAT), and it has been demonstrated to occur in several vertebrate (mammals) and invertebrate species. Steroid esters are considered long-acting steroids: they are not able to bind steroid receptors in the conjugated form, but the active steroid can be released by esterase when needed (Hochberg, 1998). While steroid esterification has not been reported in fish species, it is known to occur in invertebrates such as mollusks, echinoderms and crustaceans (Gooding and LeBlanc, 2001; Janer et al., 2004; Janer et al., 2005b; Janer et al., 2005c; Fernandes et al., 2010). In mollusks, exogenously provided T or E2 were converted to fatty acid esters by the mud snail *Ilyanassa obsoleta* (Gooding and LeBlanc, 2001) and the mussel *Mytilus galloprovincialis* (Janer et al., 2005a; Fernandes et al., 2010), while the amount of free steroid circulating in the tissues remained relatively constant. Nonetheless, future research is still required to study the mechanism of esterification of steroids as well as the esterases responsible for releasing steroids from the fatty acid moiety, and the process that affect/regulate the equilibrium between synthesis and hydrolysis of this family of steroids.

Nonetheless, similarly to vertebrates, some studies have shown the ability of xenobiotics to modulate steroid-related pathways in invertebrates. Thus, a decrease in P450-aromatase activity was detected in several mollusks exposed to TBT or inhabiting organotin polluted sites (Morcillo et al., 1999; Santos et al., 2002). Moreover, others have inferred that steroid conjugation may be a potential target for EDs. Esterification of T and T-sulfation have been considered as possible sites of action for TBT (Ronis and Mason, 1996; Gooding and LeBlanc, 2001; Verslycke et al., 2003; Janer et al., 2004; Janer et al., 2005c).

A part of this thesis will study the effects of EDs and/or natural hormones on steroid synthesis and/or levels, in fish and mollusks. The specific steroidogenic enzymes of concern considering both purposes are briefly described below.

CYP17

CYP17 is considered the qualitative regulator of steroidogenesis, determining which class of steroid will be synthesized thanks to both the 17α-hydroxylase and C17,20-lyase activity (Miller, 2002). Indeed, CYP17 (P450c17) catalyzes two reactions: the 17α-hydroxylation of the C21 steroids (pregnenolone or progesterone), yielding to 17α-hydroxypregnenolone or 17α-hydroxyprogesterone, and the following cleavage of the C17-20 bond to produce C19 steroids, dehydroepiandrosterone (DHEA) or androstenedione (AD). When CYP17 is absent, pregnenolone will be converted into mineralcorticoids, such as aldosterone. When CYP17 displays only 17α-hydroxylase activity, glucocorticoids such as cortisol will be produced, whereas when both the 17α-hydroxylase and 17,20 lyase activities are present, C19 precursors of sex steroids are synthesized.

CYP11β

CYP11β (P45011b or 11β-hydroxylase) catalyzes the hydroxylation at the C_{11} position of steroids in presence of molecular oxygen. CYP11β acts in the final step of biosynthesis of mineralcorticoids and glucocorticoids in vertebrates (Miller, 1988), and it is also involved in the synthesis of the 11-oxygenated androgens in fish testes (Kime, 1993). These androgens are considered the most potent ones in fish, and they are crucial for sex differentiation and spermatogenesis (Borg, 1994; Baroiller et al., 1998). Thus, 11β-hydroxylase catalyzes the hydroxylation of T or AD at C_{11}, yielding to 11β-hydroxytestosterone (11βT) or 11β-hydroxyandrostenedione (11βAD), respectively.

17β-Hydroxysteroid dehydrogenases (17β-HSD)

17β-HSDs are membrane-bound or soluble enzymes usually localized in steroidogenic cells, where they catalyze the last steps of steroid synthesis and their primary metabolism. Indeed, these enzymes catalyze the reduction of 17-ketosteroids to their respective 17β-hydroxysteroids and vice versa, as the interconversion of AD and T, or E1 and E2. Thanks to this dual activity (reduction vs. oxidation at position 17), 17β-HSDs modulate the biological potency of estrogens

and androgens in vertebrates: 17β-hydroxy forms are active and access the receptors, while keto forms have substantially less activity (Mindnich et al., 2004b).

Among the different forms of 17β-HSD identified, type 1 and type 3 participate in the activation of steroid hormones in human gonads (Labrie et al., 1997; Payne and Hales, 2004). Type 1, also known as estrogenic 17β-HSD, catalyzes the conversion of E1 to E2, and it is mainly expressed in female steroidogenic tissues but also in other tissues such as brain and adipose tissue (Luu-The, 2001). 17β-HSD type 3, also known as androgenic 17β-HSD, is exclusively expressed in microsomes of the testis, where it reduces AD, a weak androgen, to T, the potent androgen involved in sexual differentiation (Andersson et al., 1995). In fish, only the 17β-HSD type 1 homolog has been cloned from Japanese eel (Kazeto et al., 2000), zebrafish (Mindnich et al., 2004a), and Nile tilapia (Zhou et al., 2005).

3β-Hydroxysteroid dehydrogenases (3β-HSD)

3β-HSDs are membrane-bound enzymes distributed in both mitochondria and microsomes, and they are required for the synthesis of all classes of steroid hormones (Payne and Hales, 2004). 3β-Hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase (3β-HSD) catalyzes the sequential 3β-hydroxysteroid dehydrogenation and Δ5-Δ4 isomerization of the Δ5 C_{21}-C_{19} steroid precursors (Simard et al., 2005). Thus, 5-ene-3β-hydroxysteroids (Δ5 steroids), such as P5, 17α-hydroxypregnenolone (17P5) and dehydroepiandrosterone (DHEA), are converted into their respective 4-ene-3-oxosteroids (Δ4 steroids), namely P4, 17P4 and AD.

5α-Reductases

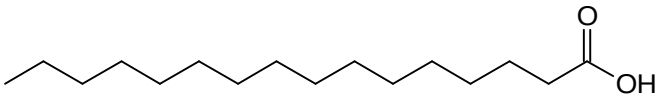
5α-Reductases are membrane-bound enzymes which act upon Δ4-3-ketosteroids by reducing the double bound in the A ring. Thus, AD and T are converted to 5α-dihydroandrostenedione (DHA) and 5α-dihydrotestosterone (DHT), respectively. DHT is the dominant androgen in many vertebrates, showing higher affinity than T for the androgen receptor (Chang, 2002) and being essential for the development and regulation of male secondary characteristics (Russell and Wilson, 1994). 5α-reduction precludes the aromatization of androgens to estrogens and promotes intracellular accumulation of androgens (Wilson, 2001).

1.4 Lipid functions and potential mechanisms of disruption

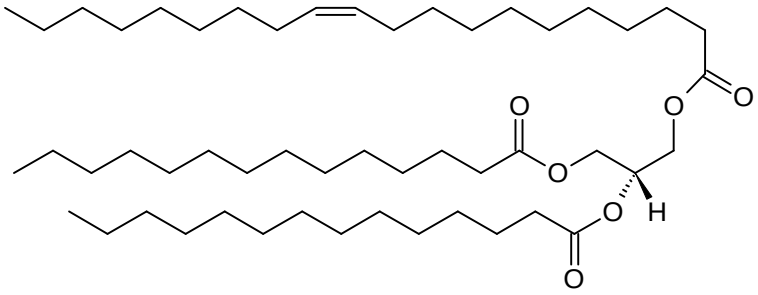
Lipids are a diverse and ubiquitous group of molecules that play a key role in different biological functions. They are part of the essential structure of cell membranes, they serve as body energy storage sources and they participate in different signaling pathways. The diversity in lipid function is reflected by the wide variation in the structure of lipid molecules. Lipids have been loosely defined as biological substances that are generally hydrophobic in nature and soluble in organic solvents (Smith et al., 2000). This definition covers a broad range of molecules, such as fatty acids, phospholipids, sterols, sphingolipids, and also others that do not belong to the lipid category.

Fatty acids (FA) represent a group of molecules synthesized by chain elongation of an acetyl-CoA primer with malonyl-CoA groups (Fig. 11). FA are precursors of bioactive molecules such as eicosanoids, prostaglandins and leukotrienes, and they also represent the building blocks for acylglycerols, sterol-esters and sphingolipids. Thus, different combinations of diverse fatty acids and functional head-groups, typically linked by a backbone, are responsible for the enormous structural diversity of lipids. Structures with a glycerol group are divided into two distinct classes, the glycerolipids (GL) and the glycerophospholipids (GP), while sphingolipids contain a long-chain nitrogenous base as core structure (Fig. 11).

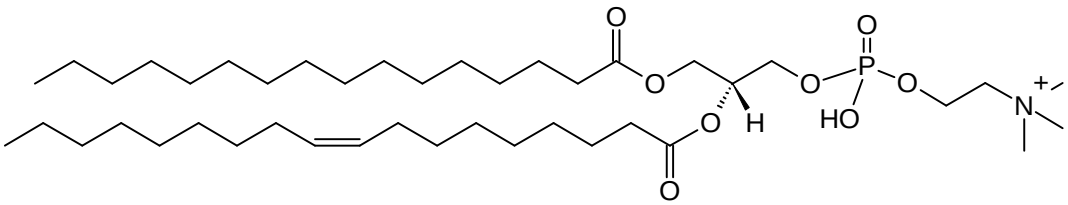
GP are polar lipids characterized by a common backbone of phosphatidic acid containing two esterified fatty acids. Phosphatic acid is esterified to the bases choline, ethanolamine, serine, and inositol to form the major subclasses of glycerophospholipids, viz. phosphatidylcholine (PC), phosphatidyletanolamine (PE), phopshatidylserine (PS), and phosphatidylinositol (PI). Among GL, triacylglycerols (TAG) represent a major class of neutral lipids and consist of three molecules of fatty acids esterified to the three alcohol groups of glycerol (Fig. 11). On the other hand, the most important simple lipid in all animals, including aquatic organisms, is cholesterol (Fig. 11). This is the most common of the compounds collectively called sterols, and can exist unesterified as an essential component of cell membranes or in a neutral lipid storage form esterified to a fatty acid (Tocher, 2003).



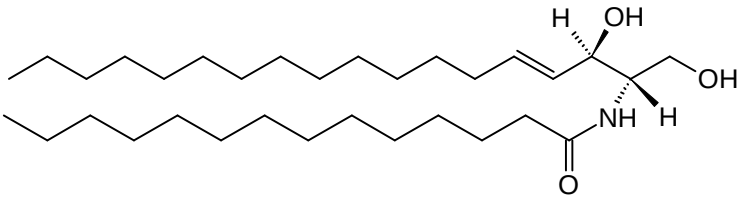
Fatty acids: hexadecanoic acid (palmitic acid)



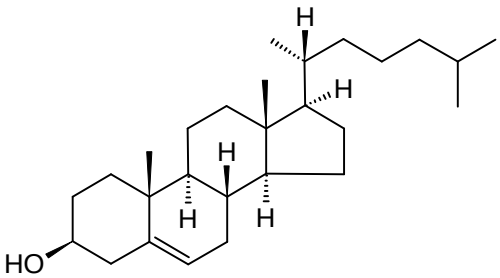
Glycerolipids: 1,2-ditetradecanoyl-3-(11Z-eicosenoyl)-sn-glycerol (TAG 48:1)



Glycerophospholipids: 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC 34:1)



Sphingolipids: N-(tetradecanoyl)-sphing-4-enine (Cer 32:1)



Sterols: cholest-5-en-3β-ol (Cholesterol)

Figure 11. Examples of lipid categories: representative structures from the five major lipid classes (in brackets the common name).

1.4.1 Lipids in aquatic organisms

Lipids and their constituent fatty acids are very important in the physiology and reproductive processes of aquatic organisms, and reflect the special biochemical and ecological conditions of the aquatic environment (Abad et al., 1995; Pazos et al., 1997). Neutral lipids, such as TAG and wax esters, play an important role in aquatic organisms as reserve of the FA destined either to provide energy or to be incorporated into phospholipids, the latest representing the building blocks for the membrane lipid bilayer (Bergé and Barnathan, 2005). Fatty acids form an impermeable barrier to water and polar molecules, providing the hydrophobic interior of cell membranes, and thus separating intracellular contents from the extracellular medium. In addition to the major polyunsaturated fatty acids (PUFAs) such as eicosapentanoic (EPA; 20:5n-3) and docosahexanoic (DHA; 22:6n-3) acids, a great variety of fatty acids occur in marine organisms, such as saturated, mono- and di-unsaturated, branched, halogenated, hydroxylated, methoxylated, non-methylene-interrupted dienoic (NMID) fatty acids (Bergé and Barnathan, 2005). Moreover, certain aquatic species (e.g. marine invertebrates) are especially rich in plasmalogens (Sargent, 1989), which differ from the GP group because the carbon chain is linked to the glycerol in the sn-1 position by a vinyl ether linkage instead of by the regular ester linkage. Plasmalogens are not only structural membrane components and a reservoir for lipid second messengers, they also facilitate membrane fusion, cholesterol efflux and store long-chain PUFA (Lesharpig and Fuchs, 2009), and it has been suggested that they may also act as membrane antioxidants (Hulbert et al., 2014).

One of the major roles of lipids in aquatic organisms is the storage and provision of metabolic energy in the form of ATP through the β-oxidation of fatty acids (Sargent, 1989; Tocher, 2003). Lipids and fatty acids provide energy for priority functions, such as growth and basal metabolism, in periods of food scarcity (Abad et al., 1995), and represent the major source of metabolic energy for reproduction (Rose, 2001; Sargent et al., 2002). Correlations between the reproductive stage of individuals and the lipid content of the gonads have been reported for aquatic organisms (Pazos et al., 1997; Morais et al., 2003; Palacios et al., 2007). However, lipids not only provide metabolic energy, but they also facilitate the absorption of fat-soluble vitamins (vitamins A, D, E, and K), and play an important role in the production and regulation of

eicosanoids (Bergé and Barnathan, 2005) which control several important physiological processes in aquatic organisms.

Overall, the maintenance of an adequate lipid composition in the cellular membranes is necessary to ensure membrane fluidity, activity of membrane-bound enzymes, exposure of surface proteins, and activation of specific signaling pathways. Thus, specific mechanisms exist to ensure the preservation of the lipid homeostasis in membrane composition.

1.4.2 Transcriptional control of lipid homeostasis

Lipid homeostasis can be defined as the balance between lipid uptake, transport, storage, biosynthesis, metabolism, and catabolism. A very large number of genes are involved in these processes, and there are mechanisms for regulating these genes at the transcriptional level, in order to maintain optimal lipid homeostasis. For example, fatty acids, and particularly PUFA, are able to control gene transcription by several direct and indirect mechanisms including changes in membrane composition, eicosanoid production, nuclear receptor activation, or alterations of specific transcription factors. Much work has been done into elucidating these mechanisms, and showing the pivotal importance of some nuclear receptors, such as the retinoid X receptors (RXR) and the peroxisome proliferator-activated receptors (PPAR), as key transcription factors regulating a growing list of genes involved in lipid homeostatic processes.

Retinoid X Receptors (RXR)

Retinoid X receptors (RXRs) have been among the most studied orphan nuclear receptor subfamilies, until the identification of their first endogenous ligand, the vitamin A derivative, 9-cis retinoic acid (Mangelsdorf and Evans, 1995). This subfamily of nuclear receptors includes three basic subtypes (RXRα, RXRβ and RXRγ) with different amino- and carboxy-terminal regions (Chambon, 1996). RXRs share structural features with the other members of the nuclear receptor superfamily. The N-terminal region of the receptor harbors a DNA-binding domain (DBD), which is composed of two zinc fingers which target the receptor to specific DNA

sequences, known as response elements. The large C-terminal portion of the receptor encompasses the ligand-binding domain (LBD), which serves as docking site for ligands and ensures both specificity and selectivity of the physiologic response (Fig. 12) (Robinson-Rechavi et al., 2003). Ligand binding provokes conformational modifications that trigger a cascade of events, such as the release of corepressor (CoR) complexes and the recruitment of coactivators (CoA), thus promoting transcriptional activation (Fig. 12) (Auwerx, 1999; Guan et al., 2005).

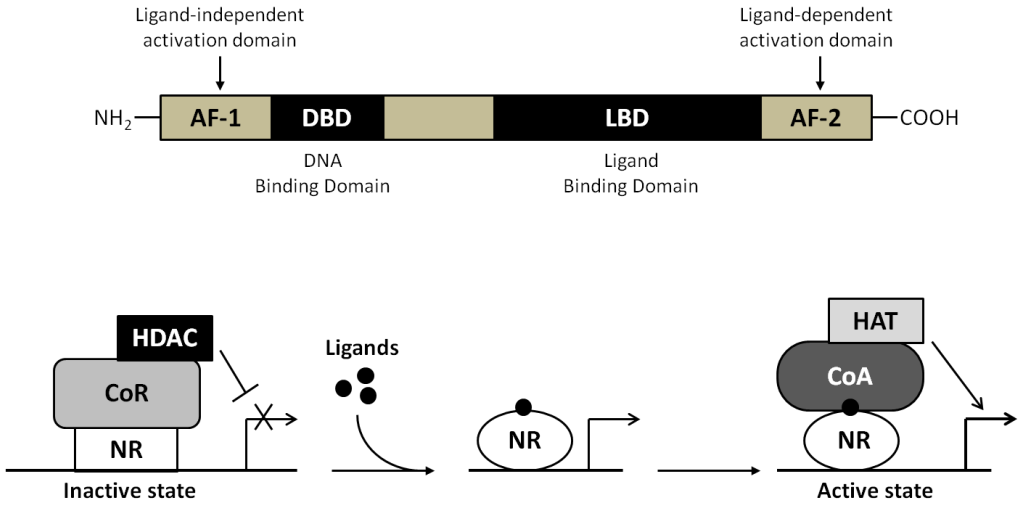


Figure 12. General structure and mechanism of action of NRs. CoR: corepressor complex; HDAC: histone deacetylase activity; CoA: coactivator complex; HAT: histone acetylase transferases (adapted from Auwerx, 1999).

Regarding the nuclear receptor signaling, RXRs do not function alone, but they rather form dimers with the second type of retinoid acid receptors (RARs) and other nuclear receptors (like thyroid, vitamin D, PPAR and liver X receptors) (Mangelsdorf and Evans, 1995; Blumberg and Evans, 1998). Thus, it seems that RXRs serve as master regulators of several key signaling pathways, and it is worthy of attention that receptors involved in lipid signaling are so far all RXR heterodimers (Chawla et al., 2001). Consequently, the finding that some RXR heterodimers are permissive for activation by RXR ligands has led to the possibility that RXR agonists and antagonists may affect lipid homeostasis (Repa et al., 2000a).

Peroxisome Proliferator-Activated Receptors (PPARs)

The peroxisome proliferator-activated receptors (PPARs) are transcription factors that play a key role in regulating the storage and catabolism of dietary fats. There are three different PPAR subtypes (PPAR-α, β and γ), and they perform a specific function in fatty acid homeostasis consistently with their distinct expression patterns. PPARα is mainly expressed in tissues characterized by a high rate of fatty acid catabolism (liver, kidney, heart, muscle) (Feige et al., 2006), while PPARγ is expressed in white and brown adipose tissue (tissues characterized by a high storage of fatty acids) and to a lesser extent in immune cells (Ferré, 2004). PPARγ is described as a "thrifty" gene (Auwerx, 1999), due to its key function in the promotion of adipocyte differentiation and lipid storage (Escher and Wahli, 2000). Finally, PPARβ is ubiquitously expressed, taking part in cell differentiation and survival, and also in systemic and tissue-specific fatty acid catabolism (Barish et al., 2006; Michalik and Wahli, 2007).

The PPARs form heterodimers with the 9-cis-retinoic acid receptor (RXR), then RXR/PPARs bind to DNA sequences containing direct repeats of the hexanucleotide sequence separated by one nucleotide (AGGTCAnAGGTCA), known as DR-1 response elements (Fig. 13) (Desvergne and Wahli, 1999). Interestingly, the PPAR/RXR heterodimer can be activated by ligands of both receptors, and it is synergistically activated in the presence of both ligands (Fig. 13). In contrast with other NRs, the LBD of PPARs can accommodate ligands with quite diverse structures, reflecting the large array of functions that have been described for this subfamily (Ferré, 2004). Indeed, PPARs are activated by naturally occurring or metabolized lipids derived from diet or from intracellular signaling pathways, ranging from saturated and unsaturated fatty acids to eicosanoids, such as prostaglandins and leukotrienes (Krey et al., 1997; Berger and Moller, 2002).

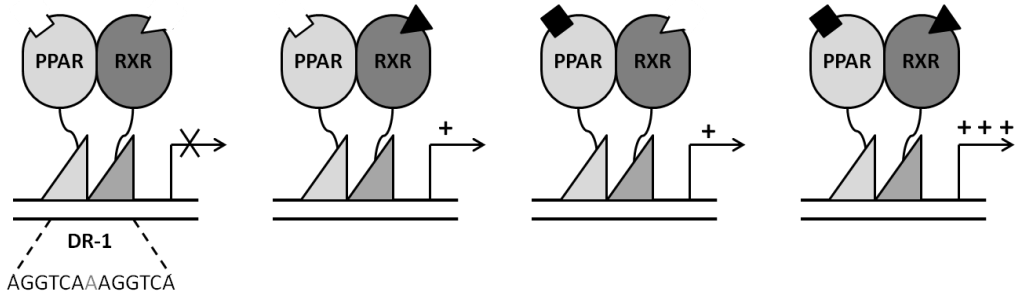


Figure 13. Gene transcription mechanisms of permissive heterodimer PPAR/RXR (adapted from Aranda and Pascual, 2001).

PPAR target genes and functions in lipid metabolism

PPARα and PPARγ promote fatty acid and cholesterol uptake by controlling the expression of fatty acid transporters (FAT, CD36), and by inducing the expression of fatty acid transport proteins (FATP) (Martin et al., 1997). The expression of lipoprotein lipase (LPL) is also regulated by both PPARα and PPARγ, thus enabling fatty acid release from lipoproteins and decreasing TAG levels (Schoonjans et al., 1996).

Furthermore, activation of PPARα and γ induces the expression of the cholesterol "efflux" pump, the ATP binding-cassette A-1 (ABCA1), through an indirect mechanism involving the liver X receptor (LXR) pathway (Chinetti et al., 2001; Knight et al., 2003). LXRs are members of the nuclear receptor superfamily and form heterodimers with the obligate partner 9-cis retinoic acid receptor (RXR) (Fievet and Staels, 2009); they act as cholesterol sensors that respond to elevated sterol concentrations, transactivating a cluster of genes that govern transport, catabolism, and elimination of cholesterol (Chawla et al., 2001). Besides their role in cholesterol homeostasis, LXRs also regulate a number of genes involved in fatty acid metabolism, and LXR activation raises plasma triglyceride levels (Schultz et al., 2000; Grefhorst et al., 2002). Indeed, LXRs indirectly regulate lipogenesis by controlling the expression of the sterol response element binding protein (SREBP), which stimulates the transcription of lipogenic genes such as fatty acid synthase (FAS) (Kim and Spiegelman, 1996; Repa et al., 2000b). FAS is considered the main enzyme involved in FA synthesis, as it catalyzes the NADPH-dependent synthesis of palmitate from acetyl-CoA and malonyl-CoA, generating long-chain saturated fatty acids. In

addition, it seems that LXRs are able to directly regulate the expression of lipogenic genes (Joseph et al., 2002), and to further inhibit the expression of PPARα target genes implicated in fatty acid oxidation, presumably through competition for binding to RXR (Ide et al., 2003).

Several studies showed that both peroxisomal and mitochondrial oxidation pathways are regulated by PPARα (Leone et al., 1999; Mandard et al., 2004), suggesting PPARα as a pivotal regulator of fatty acid catabolism (Fig. 14) (Feige et al., 2006). It is important to point out that regulating peroxisomal β-oxidation, PPARα contributes to the detoxification of endogenous and exogenous active molecules, such as eicosanoids and xenobiotics (Ferré, 2004).

In contrast to PPARα, PPARγ fosters fat storage in adipose tissue by inducing adipocyte differentiation and the transcription of several important lipogenic proteins (Fig. 14) (Chawla et al., 2001). Moreover, they also favor the esterification of fatty acids into triglycerides (Guan et al., 2002), and participate in the structural organization of lipid droplets (Dalen et al., 2004).

Despite the understanding of the functions of PPARα and γ, the role of PPARβ is still not well understood. It seems that PPARβ may act at the initial steps of adipocyte differentiation, partly by inducing the expression of PPARγ (Matsusue et al., 2004). However, its action is clearly weaker than that of PPARγ (Tontonoz et al., 1994) and there is still controversy about the adipogenic effects of PPARβ (Madsen et al., 2005).

		LIVER	MUSCLE	ADIPOSE TISSUE
Lipid utilization	**PPARα**	- Fatty acid oxidation - Response to fasting	- Fatty acid oxidation - Energy uncoupling	
	PPARβ		- Fatty acid oxidation - Energy uncoupling	- Fatty acid oxidation - Energy uncoupling
Lipid storage & Insulin sensitivity	**PPARγ**	- Lipogenesis - Insulin sensitivity	- Insulin sensitivity	- Adipocyte differentiation - Adipocyte survival - Lipogenesis - Adipokine secretion - Insulin sensitivity

Figure 14. Main metabolic functions regulated by PPARs (adapted from Feige et al., 2006).

1.4.3 Lipid profiling: available methodologies

Given the complexity of lipid classes and their distinct chemical properties, a full and comprehensive lipid analysis has always been challenging. One of the major obstacles in lipid research has been the lack of adequate and sensitive analytical techniques for the detection of intact lipid molecular species in biological systems. Thin-layer chromatography (TLC) and gas chromatography (GC) coupled to mass spectrometry were some of the most frequently used analytical methods to determine lipids. However, these traditional methodologies relied on analyses that were specific to a lipid class, or to a particular fatty acid composition, thus addressing only a small fraction of the lipid complexity important in the physiologic context. The development of soft ionization techniques (matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI)) has overcome these limitations, allowing the detection of non-volatile and high mass analytes, such as intact lipids (Wenk, 2005). In general, the use of liquid chromatography (LC) and the development of high-resolution mass spectrometry (HRMS) have increased the capabilities for the identification and quantification of hundreds of molecular lipid species (Ivanova et al., 2009). Thus, polar lipids, such as phospholipids and sphingolipids, and

even non-polar lipids, such as diacylglycerols (DAGs) and triacylglycerols (TAGs), have been successfully measured by ESI, and the combination of reversed phase HPLC with ESI-MS (HPLC-ESI-MS) is becoming a reliable method for profiling complex lipid mixtures (Han and Gross, 2001; Wenk, 2005).

Nevertheless, although these approaches are reasonably well established for bulk lipids, such as the abundant phospholipids that make up the majority of lipid mass in biomembranes, they have to be fine-tuned for the analysis of low-abundance signaling molecules. Furthermore, ESI/MS alone only provides information on the total carbon number and unsaturation index but does not identify the specific molecular composition of lipids due to the presence of multiple isobaric species, and absolute quantification with mass spectrometry is quite difficult to achieve. However, although lipid analysis is still full of challenges, these methodologies have increased the chance for a detailed analysis of individual lipid species, thus allowing studying the effects of environmental xenobiotics and endogenous compounds on these biomolecules and the pathways involved in their metabolism and homeostasis.

1.4.4 Environmental xenobiotics as obesogens

Recent findings have highlighted the existence of environmental obesogens; chemicals that are able to disrupt key pathways involved in adipogenesis and lipid metabolism, therefore leading to weight gain and metabolic disorders (Baillie-Hamilton, 2002; Heindel, 2003; Newbold et al., 2008; Grün and Blumberg, 2009a).

It was found that TBT and TPT were effective agonist ligands at environmentally relevant levels (nanomolar) for the retinoid X receptor (RXR) and peroxisome proliferator-activated receptor γ (PPARγ) (Nishikawa et al., 2004; Grün and Blumberg, 2006), thus disrupting homeostatic controls over adipogenesis and lipid homeostasis. Indeed, TBT- and TPT-treatment induced the differentiation of 3T3-L1 preadipocytes (mouse fibroblasts that can differentiate into adipocytes) as well as the expression of adipocyte marker genes in-vitro (Kanayama et al., 2005) In-vivo

exposure to TBT in mice induced the expression of lipogenic genes in adipose tissue and liver, resulting in a predisposition of mice to increase the fat mass (Grün et al., 2006).

In addition to organotins, several environmental xenobiotics showed the ability to disrupt the signaling pathways of nuclear receptors. Indeed, lipophilic compounds displaying substantial similarities to natural ligands (usually small lipophilic molecules like steroid hormones, thyroid hormones, vitamin A derivatives) may specifically bind to the related NRs, interfering with the corresponding endogenous ligand and the pathways they control. For instance, studies showed that DEHP was able to increase PPARα-dependent lipid mobilization and fatty acid oxidation, leading consequently to adipose tissue atrophy in rats and mice (Xie et al., 2002; Itsuki-Yoneda et al., 2007). Treatment with the xenoestrogen BPA increased the differentiation of 3T3-L1 preadipocytes by up-regulating genes required for adipocyte differentiation (Masuno et al., 2002; Masuno et al., 2005), although it is not clear if these effects were mediated exclusively through activation of the nuclear estrogen receptor (Phrakonkham et al., 2008). Other reports also showed PPAR activation by environmental pollutants such as pesticides (Takeuchi et al., 2006), perfluorooctane-based chemicals (Heuvel et al., 2006; Takacs and Abbott, 2007), and alkylphenols (Lopez-Espinosa et al., 2009). These findings suggest that exposure in-vivo to these environmental xenobiotics, and likely to other endocrine disrupters able to affect key signaling pathways involved in lipid homeostasis, could alter the normal adipocyte development and systemic lipid metabolism. For instance, among perfluoroalkyl compounds, perfluorooctanoic acid (PFOA) increases PPAR-dependent lipid mobilization, fatty acid oxidation and adipose tissue atrophy, resulting in alteration of systemic lipid metabolism in rodents (Gilliland and Mandel, 1996).

Recently, a certain number of studies have also indicated the ability of TBT to induce lipid accumulation in aquatic organisms. Thus, juvenile Chinook salmon (*Oncorhynchus tshawytscha*) exposed to TBT showed an increase in body weight and in the whole-body lipid content, along with a raise of lipid related plasma parameters (i.e. plasma triacylglycerols, cholesterol and lipase) (Meador et al., 2011). TBT also led to a remarkable increase in adiposity in zebra fish larvae (Tingaud-Sequeira et al, 2011). Furthermore, perturbations in fatty acid homeostasis and enhanced lipid accumulation have been observed in ramshorn snails *Marisa*

cornuarietis exposed to TBT and TPT (Janer et al., 2007; Lyssimachou et al., 2009). However, despite these evidences there is still a lack of studies investigating the ability of environmental pollutants to act as obesogens in aquatic organisms.

1.5 Organisms selected for this thesis

1.5.1 Fish

Fish species have attracted considerable interest in studies assessing biological and biochemical responses to environmental contaminants. They respond with great sensitivity to changes in the aquatic environment and play a major ecological role in aquatic food webs because of their function as a carrier of energy from lower to higher trophic levels (Beyer, 1996).

Sea bass (*Dicentrarchus labrax*) is a species of commercial interest that belongs to the family Moronidae, Order Perciformes (Fig. 15A). It is a demersal species distributed along the Easter Atlantic, the Mediterranean and the Black Sea. It feeds mainly on shrimps, mollusks and on fish, and its reproductive period is between January and March (Muzavor et al., 1993). It inhabits the littoral zone, on various kinds of bottoms in estuaries, lagoons and occasionally rivers, and thus it is often exposed to urban discharges.

1.5.2 Mussels

Mussels are often used in environmental toxicological studies because they possess several physiological attributes which render them particularly attractive as bioindicators, viz they are ubiquitous, they have a largely sedentary lifecycle, highly conserved pathways that are often homologous to vertebrate systems, they are filter feeders (a mussel of 5 cm in length can filter 5 liter/h) and sensitive to anthropogenic inputs. These characteristics make mussels perfect candidates for studying pollutant bioaccumulation (Porte and Albaigés, 1993; Weston and Maruya, 2002), reproductive alterations (Oberdorster et al., 1998) and morphological changes (Bauer et al., 1995). Additionally, mussels can be easily cultured, maintained and handled, which makes them valuable organisms for laboratory studies (Galloway et al., 2002).

Mytilus galloprovincialis is an ecological and commercial important marine species that belongs to the family Mytilidae, classe Bivalvia, phylum Mollusca (Fig. 15B). In Europe, it is found mainly in the Mediterranean, the Black Sea, and the Atlantic coasts. They feed on phytoplankton and organic matter.

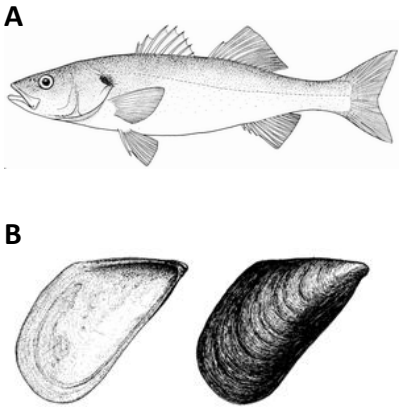


Figure 15. Selected organisms A) Sea bass (*Dicentrarchus labrax*); B) Mediterranean mussel (*Mytilus galloprovincialis*) (pictures obtained from FAO, Fisheries and Aquaculture Department).

1.6 In-vitro test models: alternative to in-vivo studies

The use of non-animal models, such as computer-based approaches and in-vitro studies, is supported by ethical, technical, scientific and economical reasons. In-vitro model systems, e.g. primary cell cultures, permanent cell lines, tissue slices, etc., are used principally for screening purposes and to obtain more comprehensive toxicological responses. However, they are also useful to evaluate local or tissue specific effects and to obtain mechanism-derived information (Eisenbrand et al., 2002).

One important limitation of in-vitro test systems is that they are not available for all tissues and organs. In addition, in-vitro systems lack the multiple and interactive actions of the immune, blood, endocrine and nervous systems, and also some of the systemic processes of absorption, penetration, distribution, and excretion are absent. Thus, no alternative in-vitro test can replace an in-vivo study looking at the complex nature of systemic toxicity. Moreover, in-vitro studies do not consider the processes in the ecosystem and overlook environmental factors which may influence toxicity (Fent, 2001).

On the other hand, in-vitro tests can help to determine doses and the best animal species to optimize conventional toxicity tests for a particular substance (e.g. the development of specific biomarkers). Moreover, the fact that in-vitro tests lack the influence of other organs and systems allows studying direct effects on a cell population. Therefore, to evaluate the impact of toxicants on individuals and ecosystems, it is necessary to integrate toxicological information through various levels of biological organization. This integration process begins with information from the simplest biological organization, from the molecular and cellular level. Thus, in-vitro assays represent a simplified test system that can provide important toxicological data on the activity of different compounds at the cellular level (Bols et al., 2005).

1.6.1 The use of cell cultures

Interactions between environmental xenobiotics and biota initially take place at the cellular level, making cellular responses not only the first manifestation of toxicity, but also valuable tools for an early and sensitive detection of chemical exposure (Fent, 2001). Cell cultures provide the best experimental system for studying these responses at the molecular and cellular levels, by allowing cells to be studied in a controlled and defined environment, independent of the multiple systemic and/or physiological controls. Whether chemical-induced alterations in cell structure and physiology will develop into adverse effects at higher levels of biological organization depends on many parameters, such as compensatory mechanisms and adaptative responses.

Cell cultures offer several advantages over studies with whole animals. The major advantage is likely the consistency and reproducibility of the acquired results, and such results are obtained more rapidly and with less cost (Bols et al., 2005). Another important advantage is that, through in-vitro assays, a wide range of chemicals and concentrations can be tested, thus reducing the use of animals in toxicology testing (Eisenbrand et al., 2002). Cell cultures allow also comparing different species for their relative sensitivity to environmental xenobiotics at the cellular and molecular level (Bols et al., 2005). Finally, although in-vivo tests may provide more accurate and detailed biological results, it has been shown that in-vitro models provide a quick and cost-effective option characterized by highly positive correlations with in-vivo results (Castano et al., 1996; Fent, 2001).

Cell lines vs. primary cultures

Primary cultures and cell lines are the two general types of cultures used for in-vitro studies. The two are interrelated because cell lines derive from primary cultures. Primary cultures are directly initiated from the cells, tissues and organs of animal and typically last for only few days. By convention, the primary culture ends and the cell line begins upon subcultivation or splitting of the primary culture into new culture vessels (Schaffer and Association, 1990). Then, the cell culture is propagated by repeating the cycle of letting the cell number to increase through cell

proliferation followed by splitting the cell population into new culture flasks. This cycle of growth and splitting, generally referred to as passaging, is limited in the case of finite cell lines, or can be done indefinitely in the case of continuous cell lines.

Primary cultures have the evident advantage that they are differentiated and express specialized functions, while cell lines, after a period of continuous growth, may lose some characteristics of the starting population. However, primary cultures have to be freshly isolated each time they are needed, and they can be very heterogeneous, as dissociation procedures are hard to repeat precisely and the physiological status of the donor species may change throughout the time. On the other hand, cell lines represent a convenient source of cells because, once established, they are quite homogenous and can be cryopreserved indefinitely. Cell lines have also the advantage to provide an unlimited amount of cells, whereas some organs may be too small to supply sufficient material for primary cultures. Finally, cell lines might be the only regularly available source of biological material for experiments on some species (Bols et al., 2005), viz the species of interest may be too small, only seasonally available, difficult to collect and/or costly to maintain in the laboratory. RTG-2 was the first permanent cell line of fish origin, it was established from gonads of juvenile rainbow trout (Wolf and Quimby, 1962). Since then, almost 300 cell lines have been established from fish (Lakra et al., 2011). Most of them are either fibroblast-like or epithelial-like, and originate mainly from the tissues of salmonids and cyprinids. Fish cell lines are grown similarly to mammalian cell lines using the same basic culture media supplemented with mammalian sera. However, most fish cell lines are cultivated at lower temperatures than mammalian cells, usually below 30ºC, and temperature ranges reflect the habitat temperature of the donor species.

1.6.2 Test models used in this study

In this study we have used subcellular fractions isolated from sea bass liver and gonads, and from digestive glands of mussel, as well as two different fish cell lines as test models. Subcellular fractions, such as microsomes, cytosol and mitochondria, are classical model systems allowing the evaluation of effects of xenobiotics on certain metabolic pathways without

any interference of other systems. They are commonly used to understand mechanisms involved in the action of toxicants in order to evaluate also the possible consequences to the organism health. On the other hand, two fish cell lines, RTL-W1 and ZFL, established from the liver of adult rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*), respectively (see Table 2 and Fig. 16), were used as in-vitro models to assess the ability of environmental xenobiotics to disrupt cellular lipids and lipid metabolism.

Table 2. Cell lines used in this thesis.

Cell line	Organism/tissue of origin	Morphology	Reference
RTL-W1	Rainbow trout - Liver	Epithelial	Lee et al. (1993)
ZFL	Zebrafish - Liver	Epithelial	Ghosh et al. (1994)

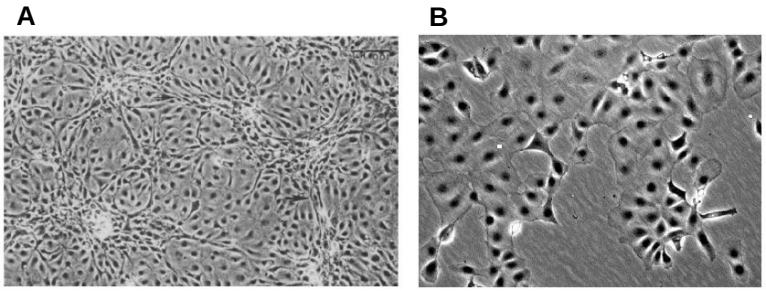


Figure 16. Morphological appearance (magnification 100x) of RTL-W1 (A) and ZFL (B) cell lines.

2. OBJECTIVES OF THE THESIS

2. OBJECTIVES OF THE THESIS

The aim of this thesis was to assess the potential endocrine/obesogenic effects of selected environmental contaminants on non-target aquatic organisms, by investigating: a) key enzymatic pathways involved in the metabolism of xenobiotic and endogenous compounds, and steroid biosynthesis; and b) effects of those chemicals on cellular lipids.

In order to accomplish these objectives the following studies were carried out and results are presented as a collection of papers addressing specific objectives:

Objective 1. To investigate the metabolism of the polycyclic musk galaxolide (HHCB) and its potential effects on biotransformation enzymes (e.g CYP1A, CYP3A, UGT) and the synthesis of active androgens (e.g CYP17, CYP11β) in the European sea bass (*Dicentrarchus labrax*).

> Paper 1: Fernandes, D., Dimastrogiovanni, G., Blázquez, M., Porte, C., 2013. Metabolism of the polycyclic musk galaxolide and its interference with endogenous and xenobiotic metabolizing enzymes in the European sea bass (*Dicentrarchus labrax*). Environmental Pollution 174, 214-221.

Objective 2. To describe the key enzymatic pathways involved in the metabolism of progesterone (P4) in the mantle/gonad tissues of the mussel *Mytilus galloprovincialis*; and to identify potential alterations on the endocrine system of mussels.

> Paper 2: Dimastrogiovanni, G., Fernandes D., Bonastre M., Porte, C., 2015. Progesterone is actively metabolized to 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one by the marine mussel *Mytilus galloprovincialis*. Aquatic Toxicology 165, 93-100.

Objective 3. To evaluate the ability of model endocrine disrupters such as TBT, TPT, NP, BPA and DEHP, to act as potential obesogens in aquatic organisms, by altering intracellular lipid homeostasis and markers of lipid metabolism, using two cellular models (RTL-W1 and ZFL).

Paper 3: Dimastrogiovanni, G., Córdoba, M., Navarro, I., Jáuregui, O., Porte, C., 2015. Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters. Aquatic Toxicology (in press)

Paper 4: Dimastrogiovanni, G., Jáuregui, O., Blumberg, B., Porte, C., 2015. TBT induces triglyceride accumulation and alters lipid profile in ZFL cells. (in preparation)

3. SUMMARY OF RESULTS AND DISCUSSION

3. SUMMARY OF RESULTS AND DISCUSSION

Many chemicals are continuously entering into the aquatic environment, as a result of their widespread use, disposal and accidental release. Several of these xenobiotics may disrupt reproductive functions of both vertebrates and invertebrates by adversely affecting endocrine signaling pathways, and may also lead to weight gain and metabolic disorders by altering critical pathways involved in lipid homeostasis and metabolism. However, knowledge about the effects of potential endocrine and lipid disrupters, together with information about their metabolism and elimination, in aquatic organisms is still limited. In this context, the present thesis investigated: a) the metabolism of the synthetic polycyclic musk HHCB and the alteration of the synthesis of active androgens in the European sea bass (*Dicentrarchus labrax*) (paper 1); b) the enzymatic pathways involved in the metabolism of the steroid precursor P4 in *Mytilus galloprovincialis* (paper 2); and c) the use of different liver cell lines (RTL-W1 and ZFL) as in-vitro models to assess the ability of known or suspected obesogenic compounds (i.e. TBT, TPT, 4-NP, BPA, and DEHP) to disrupt lipid homeostasis in fish cells, under different experimental conditions (papers 3 and 4).

The main results and conclusions obtained in this dissertation are summarized below.

3.1 Metabolism and mode of action of the synthetic musk galaxolide (HHCB) in the European sea bass (*Dicentrarchus labrax*)

In this study (paper 1) we investigated the metabolism and mode of action of galaxolide (HHCB) in sea bass (*Dicentrarchus labrax*), following a single intraperitoneal injection of 50 mg HHCB/kg body weight, during the period of 24, 48 and 96 hours post-injection. In addition, a group of fish was injected with 50 mg/kg body weight of ketoconazole (KCZ), a widely used fungicide that is known to interfere with different CYP isoenzymes, which was used in the experiment as a potential positive control.

3.1.1 Metabolism of HHCB in sea bass

Although several studies have reported the presence of HHCB in tissues of different fish species (Rimkus, 1999; Duedahl-Olesen et al., 2005; Zhang et al., 2013), data about its distribution within body compartments and its elimination kinetics in fish is still limited. In the present study, considering the levels of HHCB circulating in blood of the injected fish during the four day period (Table 3), we were able to estimate a half-life of HHCB in fish blood of approximately 10 days ($y = -0.0857x + 1.71$; $R^2 = 0.979$), which points out a certain clearance of the compound. In fact the analysis of bile samples confirmed the ability of sea bass to metabolize HHCB into a hydroxylated metabolite (OH-HHCB); reporting a significant increase in the concentration of OH-HHCB in bile over time that reached a maximum at 96 hour post-injection, while the concentration of HHCB significantly decreased (Table 3). Similarly, a half-life of HHCB of 2 to 3 days was reported in the bluegill sunfish (*Lepomis macrochirus*) exposed for 28 days to rather high nominal concentrations (1 and 10 mg/L) using a flow-through system (Balk and Ford, 1999).

Table 3. Levels of galaxolide (HHCB) and its hydroxylated metabolite (OH-HHCB) determined in plasma (n = 7-8) and bile (n = 6-7) of injected sea bass, after 24, 48 and 96 hours of exposure. Distinct letters indicate significant differences between exposure times ($p < 0.05$). n.d.: not detected

Sample	24h	48h	96h
Plasma (µg/mL)			
HHCB	1.61 ± 0.32 [a]	1.56 ± 0.47 [a]	1.36 ± 0.53 [a]
OH-HHCB	n.d.	n.d.	n.d.
Bile (µg/g)			
HHCB	72.05 ± 17.73 [a]	43.09 ± 16.89 [a,b]	18.15 ± 4.99 [b]
OH-HHCB	21.76 ± 2.54 [a]	40.91 ± 8.46 [b]	63.99 ± 19.51 [b]

To the best of our knowledge, this is the first study showing the presence of a hydroxylated-HHCB metabolite in fish bile. Commonly, metabolites are accumulated in the bile before

excretion which occurs mainly through alimentary tract. Therefore, bile metabolites are considered excellent markers for the assessment of exposure to those environmental contaminants that are readily metabolized by fish, such as polycyclic aromatic hydrocarbons, alkylphenols and others (Lavado et al., 2006b; Beyer et al., 2010). Biliary levels of galaxolide or its hydroxylated metabolite can be used as a marker of recent exposure to HHCB, and this method can probably be applied to monitor exposure to HHCB and other polycyclic musks in fish from field studies. In fact, recently, Bizarro et al. (2014) reported the presence of HHCB in bile of wild thicklip grey mullets (*Chelon labrosus*) sampled from several estuaries of the Basque Country.

3.1.2 Effects of HHCB on phase I and II enzymatic activities

An important function of metabolic degradation is the introduction of hydroxyl groups into unfunctionalized parts of molecules present in the organism (hydroxylation). A set of proteins, known as cytochrome P450 (CYP), belonging to the phase I metabolism, are vital biomolecules that are present in almost all living cells, which assist with the process of hydroxylation. In liver, the importance of hydroxylation (increase in the water solubility of compounds) can be signified by the excretion of xenobiotics to prevent toxic accumulation. Information on the enzymatic systems involved in the metabolism of HHCB and the persistence of the metabolite(s) formed is needed to fully assess the potential risks associated to environment exposure of this compound. In our study, hepatic EROD activity (CYP1A) remained unaltered following HHCB injection, while BFCOD activity (CYP3A) was induced 48 h post-injection, reaching a 133% induction (see Table 4); this suggests a possible involvement of the latter in HHCB metabolism. Interestingly, tonalide (AHTN), another synthetic polycyclic musk, also failed to induce EROD activity in exposed rats (Steinberg et al., 1999). Recently, Ribalta and Solé (2014) reported HHCB (at 100 μM) to be a poor to moderated in-vitro inhibitor of hepatic CYP1A and BFCOD activity in sea bass, as well as in other Mediterranean fish species, with the exception for *T. scabrus* that reported a 40% inhibition of CYP1A activity. Furthermore, HHCB was a poor CYP1A inhibitor (IC50 > 100 μM) in liver of *Cyprinus carpio* but a strong inhibitor of the CYP3A4 pathway (IC50=

68 ± 12 µM) (Schnell et al., 2009). Yamauchi et al. (2008) showed an induction of cyp3a40 mRNA expression in the liver of male medaka exposed to 500 µg/L of galaxolide for three days, although the mRNA expression levels of the pregnane X receptor (PXR) and of cyp3a38, another cyp3a paralog, remained unchanged. Thus, it seems that these CYP-associated activities are affected by HHCB in a species-dependent manner. In the present study, BFC was used as a catalytic probe to detect CYP3A induction in sea bass. Indeed, BFC is a mammalian CYP3A substrate, selectively metabolized by CYP3A4 in humans (Miller et al., 2000), but recent studies have suggested that it is a substrate for different CYP isoenzymes in fish (Scornaienchi et al., 2010; Smith and Wilson, 2010). In this regard, our results are not conclusive and the question remains on whether CYP3A and/or other CYP isoenzymes are involved in the metabolism of HHCB in the European sea bass. However, the significant induction of the phase II UGT-catalyzed activity 24 h post-injection (134.5%, see Table 4) suggests an early induction of UGT by HHCB and a comparatively slow phase I metabolism of the compound possibly by CYP3A, which seems to imply the involvement of these enzymes in the metabolic clearance of HHCB.

Table 4. Overview of the effects of galaxolide on the different enzymatic activities measured in sea bass liver and testes after 24, 48 and 96 hours of exposure. Values are expressed as % of the activity compared to that in vehicle-treated (corn oil) organisms set to 100%. ↑: significant higher activity than control; ↓: significant lower activity than control.

Enzymatic activity	24h	48h	96h
Liver			
Phase I			
CYP1A	103.89 ± 21.98	127.48 ± 17.43	118.77 ± 13.00
CYP3A	100.06 ± 10.57	132.63 ± 19.79	102.29 ± 14.45
Phase II			
UGT	134.52 ± 13.34 ↑	115.97 ± 17.09	90.67 ± 11.02
Testes			
CYP17	69.28 ± 2.92 ↓	79.82 ± 6.86	119.58 ± 17.90
CYP11β	90.76 ± 18.83	113.36 ± 15.28	69.08 ± 9.63 ↓

In contrast, ketoconazole, a PXR antagonist, induced CYP1A activity (2-fold increase) and acted as an inhibitor of both BFCOD (70-80%inhibition) and UGT (35-45% inhibition) activities, at 48 and 96 hours post-injection (see table 5). Although KCZ is commonly considered as a broad-spectrum CYP enzymatic inhibitor, our findings agree with previous studies which reported a 2- to 3-fold increase of EROD activity and an 80% decrease of BFCOD activity in rainbow trout (*Oncorhynchus mykiss*), 3 and 6 days after injection of 100 mg/kg KCZ (Hasselberg et al., 2008). Moreover, injection of 12 mg/kg of KCZ resulted in a 60% increase of CYP1A activity and 54% decrease of CYP3A activity in juvenile Atlantic cod (*Gadus morhua*) (Hasselberg et al., 2005). In addition to the cyp3a gene expression, PXR regulates a series of other genes including CYP enzymes such as CYP2C9, UGTs, and members of the drug transporters family such as P-glycoprotein (Iyer et al., 2006). Thus, the obtained results suggest the ability of KCZ to act as PXR antagonist in the European sea bass.

Table 5. Overview of the effects of ketoconazole on the enzymatic activities measured in sea bass liver and testes after 24, 48 and 96 hours of exposure. Values are expressed as % of the activity compared to that in vehicle-treated (corn oil) organisms set to 100%. ↑: significant higher activity than control; ↓: significant lower activity than control.

Enzymatic activity	48h	96h
Liver		
Phase I		
CYP1A	204.02 ± 20.78 ↑	181.50 ± 27.88 ↑
CYP3A	27.58 ± 3.99 ↓	19.21 ± 2.29 ↓
Phase II		
UGT	50.03 ± 3.61 ↓	58.81 ± 11.55 ↓
Testes		
CYP17	136.26 ± 16.71	190.79 ± 13.46 ↑
CYP11β	32.17 ± 11.72 ↓	45.02 ± 9.32 ↓

3.1.3 Effects of HHCB on male fish steroidogenic enzymes

The ability of HHCB to act as an endocrine disrupter in sea bass was investigated by assessing the effects on the synthesis of active androgens in male fish. CYP17 ($C_{17,20}$-lyase activity) catalyzes the conversion of 17α-hydroxyprogesterone (17P4) to androstenedione (AD), a precursor of testosterone (T) in male gonads. In teleost fish, CYP11β (11β-hydroxylase) converts both T and AD into their respective 11β-hydroxylated metabolites (βT and βAD), which are further metabolized to 11-ketoandrogens; being 11-ketotestosterone the main androgen in male teleost fish (Kime, 1993; Borg, 1994; Liu et al., 2000; Devlin and Nagahama, 2002). It has been reported that the synthesis of 11β-hydroxyandrostenedione (βAD), and thus the activity of CYP11β, in the European sea bass is elevated at the initial stages of sexual maturation, specifically in testes classified as early-spermatogenic, and that it significantly decreases with increasing maturation of the gonads (Fernandes et al., 2007). Thus, in the present study only male gonads classified as early maturing testes, containing all germ cell types from spermatogonia to sperm, were used to assess the activity of CYP17 and CYP11β, in order to avoid variability in activity responses, due to differences in gonad maturation stages.

The mitochondrial fractions isolated from gonads of male sea bass metabolized 17P4 to AD which was further converted to βAD. Moreover, other three minor metabolites were formed in testes of control and treated fish, but they could not be successfully identified. Interestingly, the metabolite profile observed for HHCB and KCZ varied significantly in treated fish, regardless of the time post-injection (Fig. 17). Thus, in HHCB-injected fish, all five metabolites were found when incubating testes with 17P4 as substrate, although the formation of the three unknown compounds and βAD was low. In contrast, no formation of βAD neither of two unknown metabolites was observed in testes of KCZ-injected fish. Nonetheless, in both cases AD was the most abundant metabolite (Fig. 17).

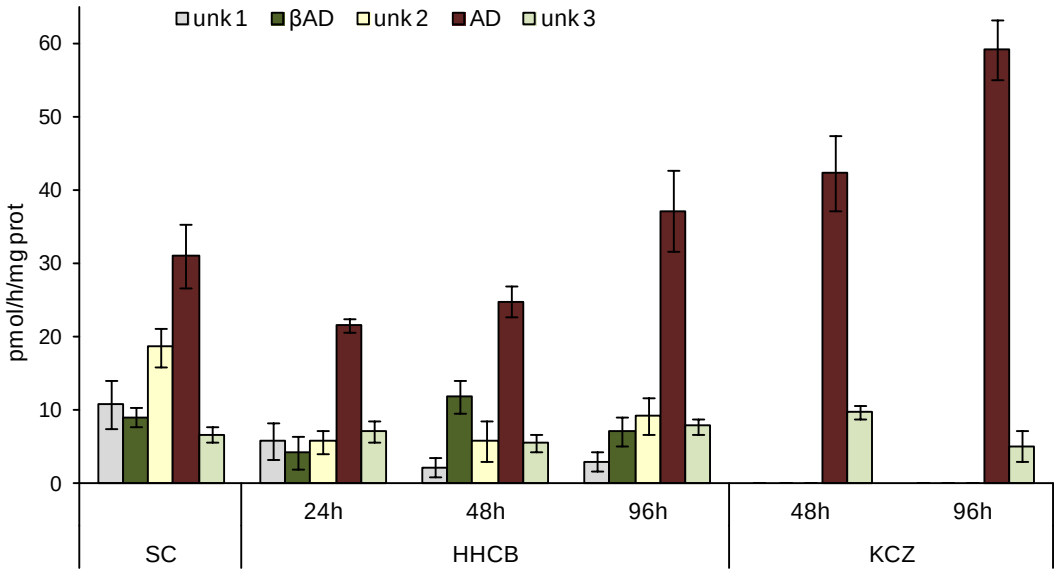


Figure 17. Metabolism of 17α-hydroxyprogesterone (17P4) in sea bass testes, after 24, 48 and 96 hours of exposure to galaxolide (HHCB) and ketoconazole (KCZ). SC: solvent control group (injected with corn oil); βAD: 11β-hydroxyandrostenedione; AD: androstenedione; Unk: unidentified metabolites.

HHCB led to a significant inhibition of CYP17 and of CYP11β activity in testes of sea bass 24 h and 96 h post-injection, respectively (Table 4). The observed effects agree with previous in-vitro findings in male carp gonads, where HHCB had an inhibitory effect on both CYP17 (IC50: 225 μM) and CYP11β (48 ± 7% at 1 mM) activities (Schnell et al., 2009). As far as we know, this is the first study reporting a weak inhibitory effect of HHCB on the synthesis of active androgens in fish in-vivo. Given the role of 11-oxygenated androgens in testicular recrudescence and spermatogenesis in many teleost fish (Borg, 1994; Miura et al., 1996), the inhibition of CYP17 and especially CYP11β enzymatic activities may alter the rate of androgen production, thus leading to impairment of androgen-related biological processes, such as spermatogenesis, reproductive behaviour and development of secondary sexual characteristics. Several studies have investigated the estrogenic potential of HHCB in fish, with often contradictory results. Thus, HHCB inhibited estradiol-induced vitellogenin (Vtg) production in rainbow trout (Simmons et al., 2010), whereas it induced the expression of hepatic estrogen receptor (type ERα) and Vtg

mRNA/protein in male medaka (Yamauchi et al., 2008). Van de Burg et al. (2008) proposed that the agonistic and/or anti-estrogenic activity of HHCB appears to be tissue and receptor subtype specific. In a recent study, Bizarro et al. (2014) found a significant correlation between the concentration of HHCB in the bile of wild thicklip grey mullets and the transcriptional levels of Vtg and aromatase-activity, which they suggested could be associated to the intersex condition reported in these fish.

As for KCZ, it strongly interfered with the synthesis of active androgens in sea bass testes when compared to HHCB, the effects being more evident 96 h post-injection *viz.* KCZ significantly induced the formation of AD (with an increase of 90% in CYP17 activity) and inhibited the synthesis of βAD (55-68% decrease in CYP11β activity; measured when incubating testes with AD as substrate) (see Table 5). Several studies have shown that exposure to KCZ is capable of interfering with steroidogenic CYP enzymes (e.g. CYP17, CYP19, CYP11β) in both mammals and fish, leading to reduced production of 17β-estradiol, testosterone, androstenedione, 11β-hydroxyandrostenedione and in some cases to a decrease in fecundity (Rocha et al., 2000; Fernandes et al., 2007; Villeneuve et al., 2007; Zhang et al., 2008).

Overall, the present study reveals that HHCB can be actively metabolized by sea bass, and acts as a weak inhibitor of the synthesis of oxyandrogens in gonads of male fish, when compared to the effects of the fungicide KCZ. Thus, HHCB can alter to some extent the synthesis of active androgens in sea bass through inhibition of CYP17 and CYP11β catalyzed activities in-vivo. This work contributes to the better understanding of the impact of synthetic musks on fish and proposes the determination of HHCB and/or its hydroxylated metabolite in bile as a tool to assess environmental exposure in wild fish.

3.2 Characterization of progesterone metabolism in mussel (*Mytilus galloprovincialis*) and potential pathways for endocrine alterations

It is already known that several natural hormones, and among them progesterone (P4), enter the aquatic environment through wastewater treatment plant effluents. Many of these hormones are often detected in water bodies at biologically active concentrations, which may represent latent risks to non-target aquatic organisms (Jobling et al., 1998; Johnson and Sumpter, 2001; Lange et al., 2002). Progesterone (P4) plays a key role as a precursor of several steroids in vertebrates, including estrogens and androgens; however, information on the metabolism of P4 in molluscs is still lacking. Thus, in this study (paper 2) we aimed to characterize the key enzymatic pathways involved in the metabolism of P4 in mussels (used as a model invertebrate species) and to further identify potential alterations of the endocrine system of mussels, as a consequence of exposure to different concentrations of exogenous progesterone (20, 200, 2000 ng/L and 10 μg/L for 7-days).

3.2.1 Characterization of P4 metabolism in mussels

The metabolism of progesterone (P4) was investigated in mitochondrial, microsomal and S12 fractions isolated from digestive glands. In the presence of NADPH (co-factor), all subcellular fractions readily converted P4 (substrate) into a major metabolite that was identified as 5α-pregnane-3,20-dione (5α-DHP) by comparison of retention time of authentic commercial standards (Fig. 18). The synthesis of 5α-DHP, which results from a reduction of the double bond of the A ring of P4, is catalyzed by the activity of the 5α-reductase (5αR) enzyme. The presence of this activity was confirmed by the 70% inhibition observed after exposure to 10 μM of dutasteride, a known inhibitor of 5αR (Margiotta-Casaluci et al., 2013). The highest metabolism of P4 was detected in digestive gland microsomal fractions (180.9 ± 9.4 pmol/h/mg protein) in comparison to S12 (86.5 ± 9.3 pmol/h/mg) or mitochondria (1.5 ± 0.1 pmol/h/mg protein), suggesting that 5αR is mainly a microsomal enzyme in mussels. Moreover, 5α-DHP was further metabolized to 3β-hydroxy-5α-pregnane-20-one (3β,20-one) by 3β-HSD (Fig. 18);

this reaction was significantly inhibited by trilostane (10 µM, 74% inhibition), a known 3β-HSD inhibitor (Ankley et al., 2011).

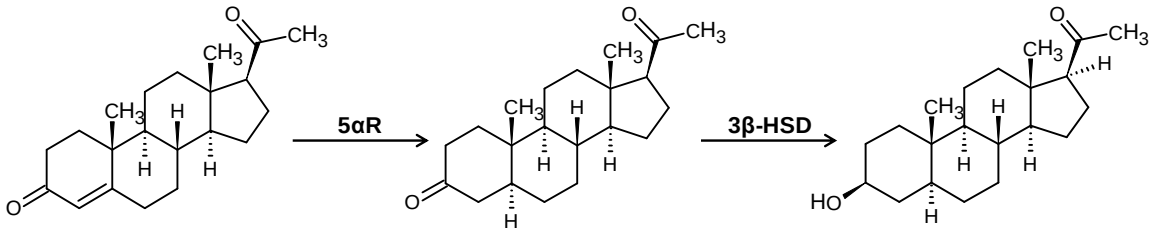


Figure 18. Metabolism of progesterone (P4) in the digestive gland of *Mytilus galloprovincialis*. 5αR: 5α-reductase; 3β-HSD: 3β-hydroxysteroid dehydrogenases.

In agreement with our findings, Hines et al. (1996) reported that P4 was predominantly metabolized in-vivo to 5α-DHP and 3β,20-one by the gastropod *Clione antarctica*. Also Wasson and Watts (2000) reported that P4 was primarily and rapidly metabolized into 5α-reduced metabolites, including 5α-DHP and 3β,20-one, in the ovaries and testes of the echinoid *Lytechinus variegatus.* This represents an important difference in what concerns to vertebrates, where P4 is converted into 17α-hydroxyprogesterone (17P4) by 17α-hydroxylases, which is further metabolized to androstenedione (AD). Thus, in the above mentioned studies no synthesis of 17P4 from P4 was reported (Hines et al., 1996; Wasson and Watts, 2000), neither in the present study was any synthesis of 17P4 or AD observed in the digestive gland or the gonad/mantle tissue of mussels when incubated with P4. However, when investigated the metabolism of AD in mussels, we observed that similarly to P4, digestive gland subcellular fractions metabolized AD (substrate) into 5α-androstane-3,17-dione (5α-DHA), a 5αR catalyzed pathway (Fig. 19), with the highest activity being detected in microsomes (45.8 ± 5.0 pmol/h/mg protein) in comparison to S12 (13.9 ± 3.4 pmol/h/mg). Interestingly, P4 showed to be a better substrate for 5αR than AD. Moreover, AD was further metabolized to T in S12 fraction, indicating the presence of a 17β-HSD catalyzed pathway (Fig. 19), in mussel digestive gland.

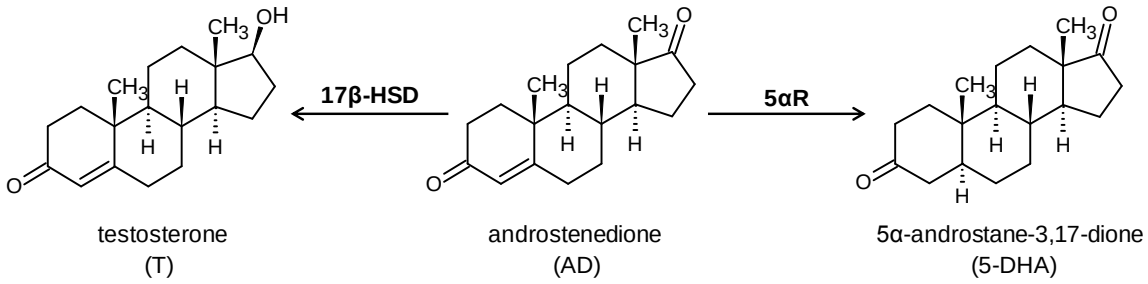


Figure 19. Metabolism of androstenedione (AD) in the digestive gland of *Mytilus galloprovincialis*. 5αR: 5α-reductase; 17β-HSD: 17β-hydroxysteroid dehydrogenases.

3.2.2 Effect of exogenous P4 on 5αR and 17β-HSD activities

Water analyses indicated that progesterone was taken up by mussels, since the concentration of P4 in the tanks sharply decreased (89 to 98% of nominal concentrations) 24 hours after dosing. However, we cannot discard the possibility that adherence of some P4 to the tank walls could partially have contributed to the significant decrease observed after 24 h. Concurrently, levels of P4 in mantle/gonad tissue of mussels indicated a dose response increase, particularly in those organisms exposed to 2000 ng/L and 10 μg/L of P4, showing tissue concentrations 5.8- and 27.4-fold higher, respectively, than unexposed mussels. Interestingly, the accumulation of P4 in mussels was lower than what we expected. In an analogous study, a 140-fold increase in testosterone (T) levels was observed in mussels exposed to 2000 ng/L of T for 5-days (Fernandes et al., 2010). A similar bioavailability in the exposure system is expected, as both steroids have comparable water solubility (16.8 (P4) and 23.4 (T) mg/L) and a similar behavior in the tanks, showing analogous water concentrations right after dosing and 24 hours later (Fernandes et al., 2010). Considering an equivalent uptake of both compounds, less retention of P4 than T in mussel tissues may explain the different accumulation pattern observed between studies, as P4 is not a substrate of acyl-coenzyme A acyltransferases. Indeed, these enzymes catalyze the conjugation of steroids having a hydroxyl group in position 17 (i.e. T and estradiol) with fatty acids, significantly reducing their elimination (Gooding and LeBlanc, 2001; Sternberg et al., 2010). Thus, exposure to exogenous T significantly increased the retention of T as fatty acid esters in the mud snail *Ilyanassa obsoleta* and the mussel *M. galloprovincialis* (Gooding

and LeBlanc, 2001; Fernandes et al., 2010). Similarly, exogenously administrated estradiol was extensively esterified by the mussel *M. galloprovincialis* and retained in the tissue (Janer et al., 2005a). Moreover, the rapid conversion of P4 to 5α-reduced metabolites, reported by our study, may also have contributed to an underestimation of P4 accumulation in exposed mussels.

The effect of P4 exposure on 5αR activity, measured as the synthesis of 5α-DHP and 5α-DHA, was investigated in digestive gland microsomal fractions of control and P4-exposed mussels. The conversion of P4 to 5α-DHP was not significantly altered by exposure despite a trend towards higher metabolism in P4-exposed organisms (195.9 – 248.8 pmol/h/mg protein) when compared to the control group (SC: 186.1 ± 8.4 pmol/h/mg protein); being the highest 5αR activity detected in mussels exposed to 200 ng/L P4. Similarly, the formation of 5α-DHA from AD was significantly increased in the same treated group (68.1 ± 8.5 *vs.* SC: 42.4 ± 1.8 pmol/h/mg protein). The metabolism of AD to T was not affected by P4 exposure (SC: 12.1 ± 1.84 pmol/h/mg protein vs. exposed organisms: 7.84 – 10.5 pmol/h/mg protein). This agrees with previous studies that demonstrated no change in T synthesis after exposure of mussels to either testosterone or estradiol (E2), but increased synthesis of 5α-reduced metabolites (5α-DHT and 5α-DHA) in mussels exposed to 2 µg/L E2 or a significant decrease in the formation of 5α-DHT in those exposed to 2 µg/L T (Janer et al., 2005a; Fernandes et al., 2010). Thus, exposure to exogenous steroids (P4, T and E2) modulates 5α-reductase activity with no significant effect on 17β-HSD, strengthening the hypothesis that 5α-reductase plays a more important role than 17β-HSDs in the metabolism of steroids in molluscs.

3.2.3 Effect of exogenous P4 on mussel gamete maturation

The analysis of steroids in mantle/gonad tissue of P4 exposed mussel suggests that the excess of P4 in mussels exposed to 10 µg P4/L is converted to immunoreactive T, resulting in a 5-fold increase in T levels, but not to E2, as no changes in E2 levels were found. However, we cannot rule out the cross-reactivity of P4 in the T assay, as the interference of compounds with structural similarity to the target steroid and/or being at very high concentration in the sample is a limitation of immunoassays (Krasowski et al., 2014). Indeed, when the cross-reactivity of P4 in

the T assay was examined, we detected very weak cross-reactivity (<0.1%) at concentrations of P4 < 100 pg/mL, while at concentrations of P4 > 5000 pg/mL, cross reactivity reached 1.8%. Thus, the increase of immunoreactive T was attributed to the presence of high concentrations of P4 in the tissue extract. Conversely, in fish, P4 administration significantly increased T production (3-fold) in both, testis and ovary explants, of the fathead minnow *Pimephales promelas*, while E2 production was not affected (Chishti et al., 2013; Chishti et al., 2014). Although RIA methods usually require less amount of sample, exhibit satisfactory precision and are quite useful for screening purposes, its accuracy and specificity can be limited in some cases by cross-reactivity with other steroids. On the other hand, although liquid chromatography coupled to a combination of different mass analyzers could be considered more specific and allow the simultaneous detection of several compounds in a single assay, its use for steroid quantification in invertebrate tissues is still limited, because of its high detection limits and matrix interferences (Gust et al., 2010). Therefore, analyzing vertebrate-type sex steroids in tissues of invertebrates still remains a complicated task due to the complex nature of the matrix that requires developing efficient extraction and detection methods, which we hope in the future will help to increase knowledge in invertebrate endocrinology.

Furthermore, gonads were examined to assess whether changes in gamete maturation had occurred as a consequence of progesterone exposure. The gonads in most of control mussels were classified as stage IIIA, thus 80% of the individuals had gonads in ripe stage, with follicles full of spermatozoa in males and full-grown oocytes in females. However, exposure to P4 led to a significant decrease in the number of mussels with gonads in stage IIIA, along with a higher number of individuals exhibiting gonads with follicles partially empty (stage IIIB) and in the restoration phase (stage IIIC) as a result of spawning (Fig. 20). This tendency towards a lesser number of mussels with ripe gonads was even more prevalent in individuals exposed to 10 µg/L P4, where most of the mussels had either partially empty gonads as a result of spawning (stage IIIB) or gonads in stages IIIC and IIID, corresponding to gonadal restoration after non-completed discharge of gametes and gonadal resorption, respectively. Thus, the results suggest that P4 exposure accelerated gonad maturation, resulting in a larger amount of mussels at spawning and post-spawning stages (stages IIIB, IIIC, IIID) (Fig. 20). Zucchi et al. (2013) reported

accelerated oocyte maturation and an impairment of ovarian physiology in female zebrafish exposed for 14 days to P4 (3.5 to 306 ng/L). Induced ovarian maturation and spawning was also observed in the greasyback shrimp (*Metapenaeus ensis*) after 1 month of exposure to 100 ng/g of P4 (Yano, 1985). Meanwhile, Hines et al. (1992) found the highest concentrations of P4 during spermatogenesis in testes of *Asterias vulgaris*, and Siah et al. (2002) reported increased P4 levels in gonads of *Mya arenaraia* during the ripe stage (male) and the spawning stage (female). Moreover, elevated levels of P4 were observed at the end of gametogenesis (spawning and spent stages) in both ovaries and testis of the clam, *Ruditapes decussatus* and in the sea urchin, *Lytechinus variegatus* (Wasson et al., 2000; Ketata et al., 2007). All of these evidences seem to suggest a role of P4 in gamete development in invertebrates.

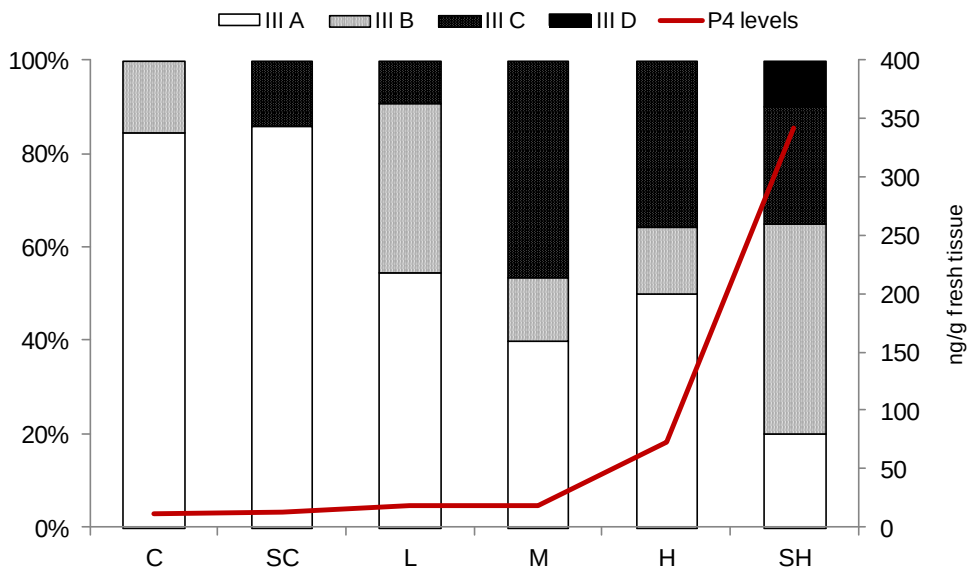


Figure 20. The relation between the distribution (%) of sexual maturation stages in control and experimental groups with progesterone (P4) levels detected in mussels. Stage IIIA: ripe gonads with follicles full of spermatozoa in males and full-grown oocytes in females. Stage IIIB: partially empty gonads as a result of spawning. Stage IIIC: gonadal restoration after non-completed discharge of gametes. Stage IIID: gonadal resorption, characterized by the presence of ruptured follicles and residual gametes, connective tissue reappears between follicles. n = 15-20 individuals. C: control; SC: solvent control; L: 20 ng/L P4; M: 200 ng/L P4; H: 2000 ng/L P4; SH: 10 µg/L P4.

In vertebrates the physiological function of P4 is mediated through nuclear receptors, as well as by membrane proteins unrelated to nuclear steroid receptors, such as progesterone membrane receptors (mPR) and progesterone membrane receptor component one (PGMRC1). Both proteins mediate rapid progestin actions initiated at the cell surface (a nongenomic mechanism) and have important physiological functions in a variety of reproductive tissues; for instance mPRα is an intermediary in progestin induction of oocyte maturation in fish (Thomas, 2008). However, so far mPRs have not been described in mussels and recently, Kaur et al. (2015) confirmed the lack of a progesterone receptor in gastropods and the absence of androgen, glucocorticoid and mineralocorticoid receptors, indicating that the endocrine system of gastropods (and probably molluscs) is fundamentally different of that of fish.

Overall, this work contributes to the better knowledge of the enzymatic pathways involved in the metabolism of progesterone in mussels and describes the ability of these invertebrates to actively metabolize P4 to 5α-DHP and 3β,20-one, with no evidence for the synthesis of 17α-hydroxyprogesterone or androstenedione. Moreover, exposure to high concentrations of P4 (10 μg/L) accelerates gamete maturation and release, but has no significant effect on steroid levels or steroid metabolizing enzymes. Thus, environmental concentrations of P4, detected in the ng/L range (up to 199 ng/L) in waste water treatment plant effluents and surface waters (Kolpin et al., 2002; Liu et al., 2012), are unlikely to have an endocrine action in mussels.

3.3 Lipid disruption as a response to environmental xenobiotics

Despite reported evidences of the existence of chemicals acting as obesogens in mammals (Elobeid and Allison, 2008; Grün and Blumberg, 2009b; Casals-Casas and Desvergne, 2011), little is known about the ability of these compounds to disrupt lipid metabolism in aquatic organisms. Xenobiotics displaying substantial similarities to natural ligands (usually small lipophilic molecules like steroid hormones, thyroid hormones, vitamin A derivatives) may specifically bind to related nuclear receptors, interfering with the corresponding endogenous ligand and the pathways they control. In this context, in order to evaluate the impact of potential obesogens on aquatic ecosystems, we investigated the ability of several chemical compounds to disrupt the synthesis and/or metabolism of lipids using two different fish liver cell lines. Therefore, in paper 3 was investigated the effect of environmentally relevant concentrations of known or suspected obesogenic compounds (TBT, TPT, NP, BPA and DEHP) on cellular lipids and different markers of lipid metabolism in the rainbow trout liver cell line (RTL-W1). In addition, in paper 4 the effect of different exposure conditions (range of concentrations and lipid provision) on hepatic cellular lipids was assessed using the model obesogen TBT in the zebrafish cell line (ZFL).

3.3.1 Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disruptors (EDs)

In this work (paper 3), we investigated the potentiality of several model EDs, namely TBT, TPT, 4-NP, BPA and DEHP, to modulate cellular lipids and markers of lipid metabolism in RTL-W1 cells for 24, 48 and 72 hours. Lipid composition of RTL-W1 cells was determined by high-performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS). Thus, a total of 29 lipid species was identified including 13 Phosphatidylcholines (PC), 6 PC-Plasmalogens and 10 triacylglycerols (TAG), and relative changes in their levels were determined comparing peak areas in exposed cells with their basal amounts in non-exposed cells. Additionally, expression of the following genes was investigated: (a) the ATP-binding

cassette transporter (ABCA1) related to cholesterol homeostasis; (b) two fatty acid transporters, namely the cluster of differentiation 36 (CD36) and the fatty acid transport protein 1 (FATP1), together with expression of the enzyme lipoprotein lipase (LPL) as markers of fatty acid uptake; (c) expression of the fatty acid synthase (FAS), a lipogenic enzyme; (d) liver X receptor (LXR) commonly involved in lipogenic pathways; and e) PPARα and PPARβ, promoters of fatty acids use in mammals, and PPARγ involved in lipid accumulation and adipogenesis in mammals and fish (Kota et al., 2005; Bouraoui et al., 2008). Thus, we observed that RTL-W1 cells growing on monolayer expressed detectable levels of genes involved in lipid metabolism. Among them, FA transporters -CD36 and FATP-1- and the lipogenic enzyme FAS were the most highly expressed, in agreement with a hepatic cell phenotype. Regarding PPARs, the β isotype was the most strongly expressed, while expression of isotypes α and γ were in comparison very low; this same pattern was described for the expression of PPARs in the liver of brown trout (*Salmo trutta*) (Batista-Pinto et al., 2005).

Under our experimental conditions, the expression of ABCA1 transporter was up-regulated by all the compounds tested, with the exception for DEHP (5 μM) (see table 6). ABCA1 protein belongs to a superfamily of membrane transporters that bind and hydrolyze ATP to drive diverse substrates across membranes (Wade and Owen, 2001). Specifically, ABCA1 is involved in cellular trafficking of cholesterol and phospholipids and in total body lipid homeostasis (Schmitz and Langmann, 2001), promoting clearance of excess cholesterol and phospholipids from hepatocytes and reducing hepatic lipid accumulation (Ma et al., 2014). Thus, it seems that an up-regulation of ABCA1 expression could be a first defense response of RTL-W1 cells to ED exposure. Studies about the regulation of ABCA1 gene expression have revealed that ABCA1 is induced in cholesterol-loaded cells as a result of activation of LXR/RXR heterodimers (Wang and Tall, 2003). Nuclear receptor LXR serves as cholesterol sensor and regulates genes for efflux, transport, and catabolism of cholesterol, as well as triglyceride production (Fievet and Staels, 2009). Cui et al. (2011) found that up to 100 nM of TBT activated LXR/RXR permissive heterodimer in a mouse macrophage-like cell line (RAW264 cells), increasing the expression of LXRs-target genes ABCA1 and FAS. Interestingly, there was no detectable alteration of LXR mRNA and protein levels by TBT treatment, and a PPARγ/RXR-mediated induction had to be

excluded, as PPARγ levels were negligible in the cell line used (Cui et al., 2011). Thus, it seems that TBT activated LXR/RXR by interacting with RXR, and our study strengthens this hypothesis. Indeed, organotin compounds, TBT and TPT (both at 100 nM), had no significant effect on LXR expression while they up-regulated the expression of FAS and ABCA1 in RTL-W1 cells, which are also characterized by a very low PPARγ expression.

On the other hand, TBT and TPT had different effects on the lipid composition of exposed RTL-W1 cells (see table 6). The up-regulation of FAS by both organotins indicates that cell lipid content can in part be increased by the synthesis of new fatty acids from lipogenic precursors present in the medium and inside the cells. However, cells exposed to TBT indicated a relative increase in some low unsaturated species of PC (32:1, 38:3) and PC-plasmalogens (34:1, 34:2), and up-regulation of LPL expression, whereas only TPT showed an up-regulation of FATP1 and a significant increase in TAG content, suggesting increased fatty acid uptake.

TBT has been shown to disturb lipid homeostasis in the filamentous fungus *Cunninghamella elegans*, leading to a decrease of phosphatidylethanolamine and phosphatidylserine, but increased the levels of phosphatidic acid, phosphatidylinositol and phosphatidylcholine (Bernat et al., 2014). These changes were observed together with a decrease in the overall unsaturation of phospholipids. TBT and TPT are known ligands of the RXR–PPARγ heterodimer and induce lipogenic PPARγ gene expression in liver and adipose tissue (Inadera and Shimomura, 2005). Pavlikova et al. (2010) reported up to 2-fold increase of PPARγ expression and PPARα/β isotypes in the liver of salmon exposed to rather high concentrations of TBT through the diet (10 mg/Kg). However, PPARγ expression in RTL-W1 cells is rather low, and exposure to organotin compounds did not alter the expression of PPARα/β, and led to minor alterations in cell lipids in comparison to the other endocrine disrupters tested (i.e. 4-NP, BPA and DEHP).

Table 6. Overview of the effects of potential obesogens (TBT, TPT, 4-NP, BPA, DEHP) on the mRNA expression of genes related with lipid metabolism and the lipid composition in RTL-W1 cells. ↑: significant induction/increment in comparison to control; ↓: significant inhibition/decrease in comparison to control; -: no effect

	TBT 100 nM	**TPT 100 nM**	**4-NP 20 µM**	**BPA 10 µM**	**DEHP 5 µM**
Gene expression					
ABCA1	↑	↑	↑	↑	-
CD36	-	-	↓	-	↓
FATP1	-	↑	-	↑	-
FAS	↑	↑	↓	↑	↓
LPL	↑	-	↓	-	↓
LXR	-	-	-	-	-
PPARα	-	-	-	-	-
PPARβ	-	-	-	-	-
PPARγ	-	-	-	-	-
Lipid classes					
PC	↑	-	↑	-	↑
PC-Plasmalogen	↑	-	↓	↑	↑
TAG	-	↑	-	↑	↑

Among the tested compounds, 10 µM BPA up-regulated the expression of FATP1 and FAS, which is consistent with an activation of fatty acid uptake and *de novo* lipogenesis in RTL-W1 cells. This being further supported by the significant increment observed in the levels of TAG (Table 6). Although the xenoestrogen BPA is usually considered a weak obesogenic compound, animal studies showed an increased body weight in offspring of mothers exposed to BPA during gestation and/or lactation period (Rubin et al., 2001; Rubin and Soto, 2009). Interestingly, the observed effects were stronger at low concentrations compared with high doses of exposure, and this nonmonotonic dose-response relationship has been reported for many other actions of BPA (Vandenberg et al., 2012). It has been suggested that down-regulation of receptors at higher hormone or xenoestrogen levels may lead to these nonmonotonic responses. Furthermore, it seems that these effects are not mediated exclusively by activation of the nuclear estrogen receptor (ER). Indeed, BPA has been shown to activate also membrane ER at

low doses, suggesting the possibility to act through nongenomic pathways (Alonso-Magdalena et al., 2005; Thomas and Dong, 2006). In-vitro studies reported an increased lipid accumulation and adipocyte differentiation in 3T3-L1 preadipocytes treated with BPA in the presence of insulin (Masuno et al., 2002; Masuno et al., 2005), although BPA failed to activate PPARγ (Riu et al., 2011; Taxvig et al., 2012). Additionally, in-vivo studies have shown an accumulation of liver triglycerides and cholesteryl esters with changes in hepatic FA composition in adult mice exposed to low BPA doses; an activation of lipogenesis and cholesterol biosynthesis were pointed out as the major mechanisms involved, potentially associated with an inhibition of FA oxidation (Marmugi et al., 2012).

On the other hand, LPL and FAS expression was significantly down-regulated in RTL-W1 cells exposed to 5 μM DEHP (Table 6). In-vivo exposure to DEHP partially protected wild-type mice from diet-induced obesity by reducing fat mass, likely a consequence of lower levels of TAG in liver and blood, and smaller adipocytes (Feige et al., 2010). This effect has been attributed to a PPARα-dependent activation of fatty acid catabolism in the liver, as DEHP induced the expression of PPARα target genes involved in the oxidation of FA and the protective effect was completely abolished in PPARα-null mice. Other in-vivo studies confirmed the ability of DEHP to stimulate mobilization of lipids and lipid utilization in liver (Xie et al., 2002; Itsuki-Yoneda et al., 2007), reporting a down-regulation of LPL activity that might explain the loss of fat due to decreased FA uptake from the circulation. Nonetheless, exposure to 5 μM DEHP caused a significant increase of TAGs and PCs in RTL-W1 cells (table 6), particularly of highly unsaturated PCs (> 5) of 38 and 40 C atoms, which is in agreement with other studies that reported DEHP (in this case at 50 μM) to enhance the accumulation of PCs in HRP-1 trophoblastic cells after 24 h exposure (Xu et al., 2006). However, 100 μM DEHP increased the expression of LPL and other adipogenic marker genes, and TAG content, in murine mesenchymal stem cells C3H/10T1/2, while it did not affect adipogenesis in pluripotent embryonic stem cell line (CGR8) (Biemann et al., 2012). Interestingly, the amount of mono[2-ethyl-hexyl] phthalate (MEHP) clearly increased in mesenchymal stem cells (MSCs) exposed to DEHP, and increased further during the process of adipogenic differentiation. MEHP is a known metabolite of DEHP that activate PPARγ adipogenic pathway, promoting 3T3-L1 adipocyte

differentiation and lipid accumulation (Hurst and Waxman, 2003; Feige et al., 2007). Biemann et al. (2012) suggested that adipocytes are able to store MEHP, and while PPARα-dependent stimulation of FA oxidation may require continuous exposure, episodic exposure to PPARγ activators, such as MEHP, may drive permanent changes in adipocyte differentiation (Grün and Blumberg, 2009b).

From all the compounds tested in this study, TBT (100 nM), DEHP (5 μM) and NP (20 μM) were the most effective compounds disturbing PCs (see table 6), the principal structural constituents of cellular membranes (Van Meer et al., 2008). In addition to PCs, Plasmalogens also participate in the structure of membranes, although they are further involved in other cellular functions and serve as reservoir for lipid second messengers and long-chain FAs (Lesharpig and Fuchs, 2009). A relative increase (up to 40%) of some PC-plasmalogens vas observed after exposure to BPA (34:2, 34:3, 40:7), DEHP (34:2, 34:3) and TBT (34:2, 34:1), while NP was the only compound to decrease (up to 50%) PC-plasmalogens (34:1, 34:2, 34:3; 36:2). Information about possible effects of NP and other alkylphenols on membrane lipids is scarce. Meier et al. (2007) found that alkylphenols can alter the fatty acid profile of polar lipids in the liver of Atlantic cod to more saturated fatty acids and less n-3 polyunsaturated fatty acids (n-3 PUFAs). They also demonstrated that alkylphenols increased the mean molecular areas of phospholipids in monolayers of native phospholipids extracted from cod liver. Thus, it is very likely that NP accumulates in the phospholipid bilayer of RTL-W1 cells and affects membrane fluidity. The modifications of PCs and PC-plasmalogens profiles in exposed cells may also be indicative of oxidative damage, as the higher the unsaturation indexes of a fatty acid molecule the higher its susceptibility to peroxidation (Hulbert et al., 2014).

Overall, this work shows that the selected endocrine disrupters have the potential to alter the expression of genes related to lipid metabolism in RTL-W1 cells as well as to produce significant changes in membrane lipids and TAGs. Among the tested compounds, BPA and DEHP significantly induced the intracellular accumulation of TAGs, the effect being more evident after shorter exposure times. The mode of action of these chemicals is multiple and complex and no clear association between expression of lipid related genes and TAG accumulation was detected in RTL-W1 cells. On the other hand, all the compounds tested apart

from TPT induced significant changes in membrane lipids –PCs and PC-plasmalogens–, indicating a strong interaction of the toxicants with cell membranes, and possibly with cell signaling. Interestingly, Malhão et al. (2013) described the spontaneous formation of three dimensional arrangements in RTL-W1 cells left in culture without being passaged. These RTL-W1 aggregates showed signs of hepatocytic differentiation, such as the development of RER and organization of bile canalicular-like structures. It is now well known that hepatocyte aggregates, also called spheroids, differ from monolayer cultures in many aspects, such as phenotype and gene expression (Lin and Chang, 2008), suggesting that these 3D aggregates may be more valuable in future metabolic studies (Malhão et al., 2013). Therefore, further studies regarding gene expression profile of RTL-W1 aggregates and its possible differentiation as hepatocytes or liver stromal cells would be of interest.

3.3.2 TBT induces TAG accumulation and alters lipid profile in ZFL cells

Here we investigated the effect of the model obesogen TBT on hepatic cellular lipids using the ZFL cell line, together with the influence of a lipid-enriched medium on its obesogenic properties (paper 4). Thus, TBT effects were studied at the concentrations of 10 nM, 25 nM, 50 nM, and 100 nM for 96 h in basal medium or in medium supplemented with a mixture of lipids (cholesterol: 4.5 g/L; cod liver oil FAME: 100 g/L; polyoxyethylenesorbitan monooleate: 250 g/L; D-α-tocopherol acetate: 20 g/L). The highest concentration of TBT chosen for this study has been reported to promote adipogenesis in vertebrates (Kanayama et al., 2005; Grün et al., 2006). Intracellular lipid accumulation was firstly estimated with Nile Red staining, and then the lipid profile of ZFL cells was characterized by ultrahigh performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS). Thus, a total of 72 lipid species were identified including 6 Lyso PCs, 17 PCs, 12 PC-Plasmalogens, 13 DAGs, 18 TAGs and 6 ChEs.

The first step was to investigate lipid accumulation in ZFL cells performing Nile Red staining, a lipid specific fluorescent dye. Nile red is commonly used to detect intracellular lipid droplets (LDs) in all kinds of cells, due to its specificity for hydrophobic environments (i.e., neutral lipid

droplets) and minimal interaction with components of cellular membranes (Greenspan et al., 1985; Genicot et al., 2005). LDs consist of a core of neutral lipids, predominantly triacylglycerols and sterol esters, separated from the aqueous cytosol by a monolayer of phospholipids and associated proteins (Martin and Parton, 2006; Walther and Farese Jr, 2012). This hydrophobic core of neutral lipids stores metabolic energy and membrane components, making LDs hubs for lipid metabolism. Additionally, LDs are implicated in a number of other cellular functions, such as protein storage and degradation. No enhancement of intracellular lipids was found in cells exposed to TBT in normal growth medium, and this is in agreement with our previous findings using rainbow trout hepatocytes (RTL-W1; see paper 3), where TBT perturbed PC levels but did not interfere with TAG levels. However, when medium was supplemented with cholesterol and fatty acids, it was observed a concentration-dependent induction of lipid accumulation, reaching a 250% increase in cells exposed to 100 nM TBT. This is one of the first in-vitro studies showing lipid accumulation in hepatocytes due to TBT presence, as previous findings demonstrated mostly adipogenic effects of TBT in the 3T3-L1 adipogenesis model and stem cells (Inadera and Shimomura, 2005; Kanayama et al., 2005; Grün and Blumberg, 2006; Kirchner et al., 2010). However, it is important to consider that only the amount of neutral lipids present in the droplets has been estimated, and that Nile Red staining does not allow the identification of lipid molecules (the qualitative composition of lipids). Thus, we further investigated the effects of TBT on the cellular lipid homeostasis by UHPLC-HRMS.

Results evidenced distinct changes in lipid levels within the several lipid classes identified, when comparing peak areas at the experimental condition (TBT-exposed cells) to the control condition (non-exposed cells). Different effects were observed when cells were exposed to TBT in basal medium or in medium supplemented with lipid mixture. In the first condition, we found that TBT-exposure affected mostly glycerophospholipids (GP), with an increase in the amount of Lyso PCs, PCs and PC-Plasmalogens in cells exposed to 25-100 nM TBT, whereas in supplemented medium no significant alteration of most of the GP were observed. GPs are the principal structural constituents of cellular membranes, and phosphatidylcholines accounts for > 50% of the phospholipids in most eukaryotic membranes (Van Meer et al., 2008). Several studies have demonstrated that TBT is a membrane-active molecule, and although its

mechanism of toxicity is not completely unveiled, the hydrophobicity of organotin compounds suggests that interaction with membranes may play an important role (Gray et al., 1987; Ortiz et al., 2005). Indeed, model membrane studies showed the incorporation of TBT in phosphatidylcholines, where it perturbs thermotropic and structural properties (i.e. the hydration degree of the carbonyl moiety), leading to membrane alteration and lysis (Morrow and Anderson, 1986; Ambrosini et al., 1991; Chicano et al., 2001). However, whether the observed changes in phospholipids might have occurred as the result of feedback mechanisms to counteract TBT toxic action, and the significance of its physiological consequences, can only be speculated. Interestingly, Ortiz et al. (2005) suggested that the ability of organotin compounds to transport organic anions across the phospholipid bilayer might be related to their toxicity mechanism. This transport was reduced by the presence of cholesterol, which could make the diffusion of triorganotin compounds more difficult given its known permeability-decreasing effect on membranes (Raffy and Teissié, 1999; Gabrielska et al., 2004). Furthermore, in the yeast *Saccharomyces cerevisiae*, the enrichment of the culture medium with the PUFA linoleic acid (C18:2n-6) was associated with an enhanced resistance to the toxic action of TBT, despite an increased TBT uptake by these cells (Masia et al., 1998). It is worth noticing that these two conditions (addition of cholesterol and/or PUFA) resulting in a reduction of TBT adverse effects, resemble our results.

On the other hand, when cells were exposed to 100 nM TBT in the presence of supplemented medium, levels of DAG, TAG and ChE suffered a dramatic increase. We infer that in the presence of cholesterol and several FAs, the TBT-exposed zebrafish hepatocytes were induced to accumulate more neutral lipids. This is in accordance with the results obtained through the Nile Red staining, where cells exposed to 100 nM TBT in presence of lipid-enriched medium showed an increase in the formation of lipid droplets, organelles usually considered as storage depots for neutral lipids. Several studies have demonstrated the ability of TBT to promote adipogenesis and lipid accumulation in-vitro and in-vivo in mammals (Kanayama et al., 2005; Grün et al., 2006; Iguchi et al., 2007). It has been hypothesized that TBT induces these alterations through interaction with the nuclear receptors RXR and PPARγ, which play pivotal roles in lipid homeostasis, because TBT binds to both receptors with high affinity binding (Grün

and Blumberg, 2006). Indeed, studies with human nuclear receptors have shown TBT and TPT to bind to RXR with similar affinity as the presumed natural ligand, 9-cis retinoic acid, and TBT binds to PPARγ with even higher affinity than the synthetic ligand troglitazone. Furthermore, TBT perturbed fatty acid homeostasis and enhanced lipid accumulation in ramshorn snails, which implicates RXR as a key player in this process, because snails lack a PPARγ ortholog (Janer et al., 2007), while RXR has been reported in several invertebrate species (Bouton et al., 2005). In addition to PPARs, RXR can form heterodimers with other nuclear receptors that are known to regulate lipid homeostasis, such as the liver X receptor (LXR) (Edwards et al., 2002). Archer et al. (2008) reported that zebrafish LXR is likely to be involved in regulation of cholesterol and lipid homeostasis, since treatment with a synthetic LXR ligand (GW3965) led to increased expression of FAS mRNA in ZFL cells, even though they were expressing low levels of LXR.

Interestingly, PC-Plasmalogens decreased consistently in cells exposed to 100 nM TBT in lipid-enriched medium, suggesting a connection with the simultaneous large accumulation of DAG-, TAG- and ChE molecules characterized by the presence of polyunsaturated fatty acids (DAG: 34:3, 34:4 and 36:3; TAG: 50:6, 52:4, 52:5, 54:4, 54:5 and 54:6; ChE: 18:3, 20:4 and 20:5). Indeed, plasmalogens are not only structural membrane components and a reservoir for lipid second messengers, they are also known to facilitate membrane fusion, to be involved in ion transport, cholesterol efflux and storage of long-chain PUFA (Lesharpig and Fuchs, 2009).

As shown in Figure 21, it is intriguing that within the series of lipid molecules with the same acyl chain length, the accumulation effect of 100 nM TBT is increasing in parallel with a raise in the unsaturation of the lipids. Interestingly, Puccia et al. (2005) found an increase in the content of PUFAs in ovaries of *Ciona intestinalis* exposed to TBT-Cl, suggesting a defense mechanism since PUFAs are essential in the synthesis of compounds such as prostaglandins, which are present in the ovary in a stress situation.

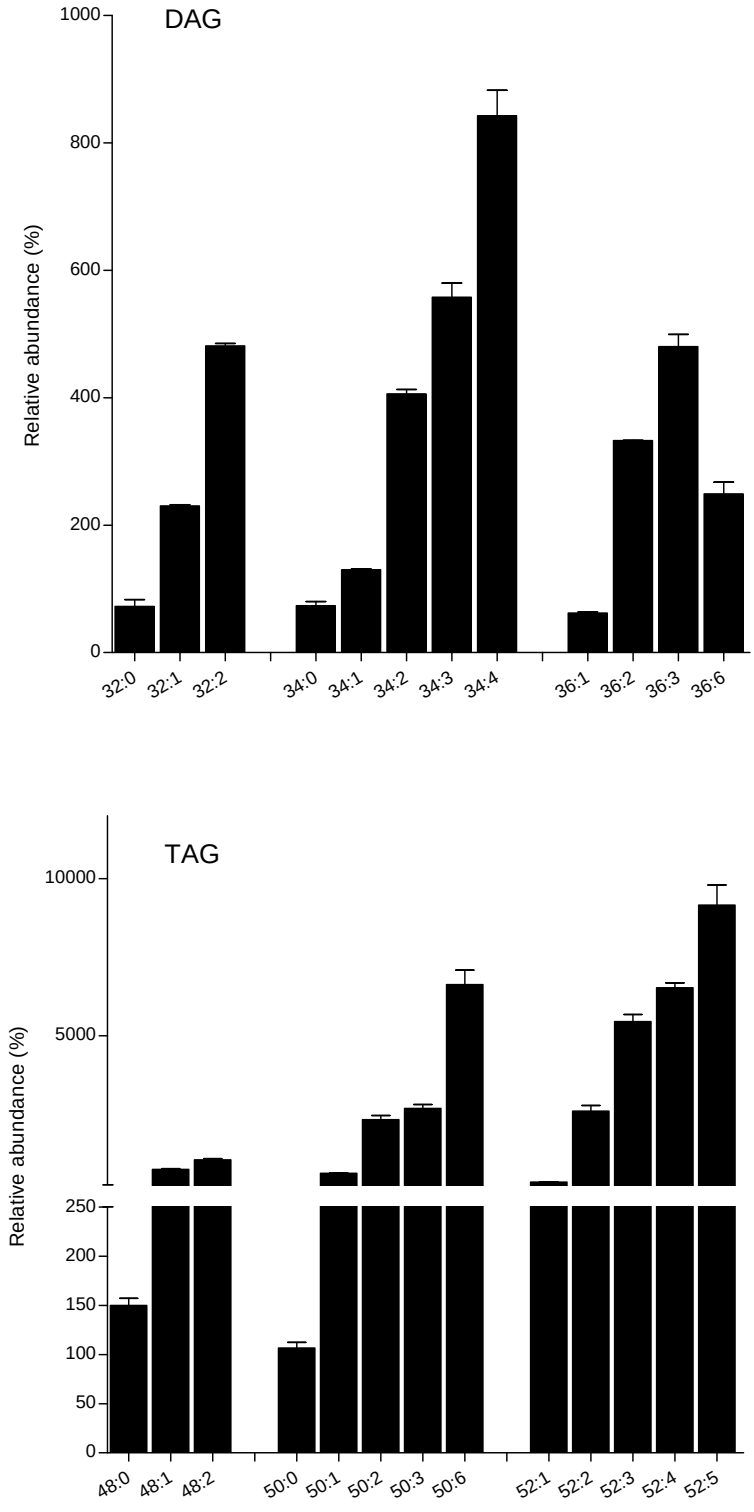


Figure 21. Relative abundance for different lipid molecules in ZFL cells exposed for 96 h to 100 nM TBT dissolved in lipid-enriched medium. DAG = diacylglycerols, TAG = triacylglycerol

Overall, this work highlights the ability of TBT to modify the intracellular lipid profile of ZFL cells but to induce steatosis only when the culture medium was supplemented with lipids. In addition, the results support the hypothesis that TBT may act more widely to disrupt multiple nuclear receptor mediated signaling pathways, eliciting adipogenic effects. Furthermore, we provide a basis for the use of ZFL cell line as an in-vitro model for the screening of environmental contaminants suspected to interfere with cellular lipids, allowing a pre-assessment of obesogenic effects on lipid homeostasis and accumulation.

In other respects, being the liver the main site for 'de novo' synthesis of lipids, the use of these fish cell lines (i.e. RTL-W1 and ZFL) can be a valuable in-vitro tool to estimate the potential of different compounds and their mixtures to interfere with lipid metabolism in hepatocytes under different exposure conditions (viz. supplementation of cell medium with specific lipids to emulate a lipid reach diet, different exposure lengths and concentrations). Finally, this approach could be used as a screening tool for the detection of metabolic disruptors that could lead to steatosis in fish and for the priorization of chemicals, as diseases associated with lipid metabolism, such as fatty liver syndrome, are found to increase in some species (e.g. cultured grass carp). Moreover, this study has benefited from the advancements in instrumentation and technology, such as the development of soft ionization techniques (matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI)), which has increased the capabilities for detection and analysis of a wide range of lipids (Wenk, 2005; Ivanova et al., 2009). Polar lipids, such as phospholipids, and non-polar lipids, such as DAGs and TAGs, were successfully measured by ESI, and the combination of reversed phase HPLC with ESI-MS (HPLC-ESI-MS) showed to be a reliable method for the characterization of cellular lipid profiles, as it allowed detailed analysis of individual lipid species.

4. CONCLUSIONS

4. CONCLUSIONS

I. HHCB had an estimated half-life in blood of European sea bass (*Dicentrarchus labrax*) of approximately 10 days, pointing out a certain clearance of the compound that bile analysis confirmed. The induction of CYP3A and UGT activities in the liver suggests an involvement of these enzymes in the metabolic clearance of HHCB.

II. HHCB inhibited the synthesis of androgens (CYP17 and CYP11β activities) in gonad of sea bass, which indicates the potential of HHCB to impair testicular steroid biosynthesis.

III. Mussels (*Mytilus galloprovincialis*) actively metabolized P4 to 5α-DHP, which was further converted to 3β,20-one, in digestive glands, with no evidence for the synthesis of 17P4 or AD. These findings are in accordance to that described for other invertebrate species (e.g gastropods, echinoids).

IV. Exposure to high concentrations of P4 (10 µg/L) accelerated gamete maturation and release, but had no significant effect on steroid levels or steroid metabolizing enzymes. Environmental concentrations of P4 (in the ng/L range) are unlikely to have an endocrine action in mussels.

V. Selected endocrine disruptors (TBT, NP, BPA and DEHP) induced significant alterations in PCs and PC-Plasmalogens of RTL-W1 cells, indicating a strong interaction of the chemicals with cell membranes and possibly with cell signaling. BPA and DEHP significantly increased the intracellular accumulation of TAGs; however, no clear association between expression of genes related to lipid metabolism (e.g. FAS, LPL, FATP1, CD36, ABCA1, LXR, PPAR-α, β and γ) and TAG accumulation was detected.

VI. TBT led to a significant increase in Lyso PCs, PCs and PC-Plasmalogens in ZFL cells exposed for 96 h in basal medium, whereas levels of DAG, TAG and ChE showed a dramatic increase when medium was enriched with lipids.

VII. Although further characterization is needed, the use of RTL-W1 and ZFL hepatic fish cell lines can be a valuable in-vitro tool for the screening of environmental contaminants suspected to interfere with lipid homeostasis and lipid metabolism in aquatic species, allowing a pre-assessment of obesogenic effects on hepatocytes under different exposure scenarios (viz. supplementation of cell medium with lipids, acute vs. chronic exposure).

5. RESUMEN DE LA TESIS

5. RESUMEN DE LA TESIS

1. Introducción

La sociedad moderna es altamente dependiente de los productos y servicios que las sustancias químicas proporcionan, pero, sin la debida precaución, éstas pueden llegar a representar una amenaza para la salud humana y ambiental. El ambiente acuático es especialmente susceptible a la contaminación, ya que los compuestos químicos pueden llegar a los ríos, lagos y mares, principalmente por escorrentía agrícola, vertido accidental, y plantas de tratamiento de agua residual. Por este motivo existe una gran necesidad de investigar como estos xenobióticos pueden afectar la fauna acuática y causar efectos adversos.

Si bien la concentración de compuestos sintéticos en los ambientes acuáticos es generalmente baja (en el rango de ng-µg), su entrada continúa junto con mecanismos de acción específicos, podría tener consecuencias negativas en organismos acuáticos tras una exposición reiterada. Una vez los xenobióticos entran en el organismo, pueden ser metabolizados o biotransformados. La biotransformación incluye dos tipos de reacciones enzimáticas: actividades de fase I, que catalizan una oxidación del compuesto; y actividades de fase II, que catalizan una conjugación de los productos de fase I para facilitar su solubilidad y eliminación. Así, para realizar una evaluación del riesgo de xenobióticos ambientales, es necesario entender su metabolismo y eliminación en organismos no diana y evaluar sus mecanismos de toxicidad, particularmente a través del sistema de citocromos P450 (CYP).

1.1 Contaminantes ambientales como disruptores endocrinos y lipídicos.

Varios xenobióticos ambientales tienen la capacidad de afectar las funciones reproductoras de vertebrados e invertebrados, alterando las vías de señalización endocrina (Colborn et al., 1993; Vos et al., 2000). Estos compuestos, definidos como disruptores endocrinos (EDs), pueden ejercer su acción interfiriendo con la síntesis, almacenamiento, liberación, transporte, metabolismo, enlace, acción y eliminación de las hormonas endógenas (Kavlock et al., 1996).

De este modo, los EDs representan un grupo de compuestos muy heterogéneo estructuralmente y con múltiples mecanismos de acción, desde la interferencia a nivel de receptores hormonales hasta la disrupción de la síntesis y/o metabolismo de las hormonas (Pickering and Sumpter, 2003).

Varios estudios ya han descrito el impacto y las consecuencias de los EDs sobre el medio ambiente y la fauna acuática. Probablemente, el ejemplo más conocido y claro de disrupción endocrina asociada de manera inequívoca a un contaminante ambiental específico, es el caso del imposex en gasterópodos marinos debido a la presencia del compuesto organoestánnico TBT (Matthiessen and Gibbs, 1998). Desde entonces, una amplia variedad de compuestos han sido identificados como EDs, entre ellos: esteroides sintéticos como el dietilestilbestrol (DES) y etinilestradiol (EE2) (Lintelmann et al., 2003), pesticidas como el DDT y el diuron (Stahlschmidt-Allner et al., 1997), dioxinas (Depledge and Billinghurst, 1999), ftalatos (Latini et al., 2004), tensioactivos industriales como el 4-nonylphenol (McCormick et al., 2005) y plastificantes como el bisphenol A (Oehlmann et al., 2000; Rubin, 2011).

Los EDs poseen diversos mecanismos de acción. Uno de los principales es la interferencia con los receptores nucleares (NRs), un grupo de factores de transcripción que regula la expresión de varios genes. Al principio, se investigaba principalmente los efectos perjudiciales de los EDs sobre la reproducción y la diferenciación sexual por su interferencia con la señalización estrogénica y androgénica. Sin embargo, la familia de receptores nucleares incluye también factores de transcripción relacionados con la regulación metabólica y energética, como los receptores glucocorticoides (GR), el receptor X hepático (LXR), los receptores X retinoides (RXRs), todos potenciales dianas de la acción de los EDs. En este aspecto, los receptores activados por proliferadores de peroxisomas (PPARs) representan una diana muy interesante para los EDs, considerando su papel esencial para el mantenimiento de la homeostasis lipídica y su capacidad de interactuar con una gama muy heterogénea de compuestos. Diferentes estudios han demostrado que varios contaminantes ambientales tienen la capacidad de alterar estas y otras vías clave involucradas en la adipogénesis, el metabolismo lipídico y el balance energético (Tabb and Blumberg, 2006; Grün and Blumberg, 2007; Casals-Casas and Desvergne, 2011). Estos compuestos han sido definidos como obesógenos ambientales, y su

número sigue creciendo continuamente. La necesidad de investigar los efectos de posibles EDs y/o disruptores lipídicos es evidente y, por esta razón, esta tesis se centra en el estudio de una selección de diferentes compuestos pertenecientes a diversos grupos químicos (hormonas, productos de cuidado personal, tensioactivos, biocidas, plastificantes), todos reconocidos como contaminantes ambientales.

1.2 Rutas alternativas de disrupción endocrina.

Los contaminantes ambientales pueden actuar como EDs tanto a través de la interacción con los receptores nucleares, como interfiriendo con rutas no genómicas, como la biosíntesis y el metabolismo de esteroides, alterando la disponibilidad de esteroides activos en el organismo y procesos fisiológicos controlados por éstos.

Los esteroides sexuales (andrógenos, estrógenos, progestágenos) juegan un papel importante en la gametogénesis, ovulación y espermiogénesis, siendo esenciales para la diferenciación sexual de los individuos. Las principales clases de enzimas involucrados en la biosíntesis de hormonas esteroides son la familia de citocromos P450 (CYP) y el grupo de esteroide deshidrogenasas (HSD). Varios estudios han revelado la capacidad de los EDs de modificar la síntesis de esteroides en peces. Monteiro et al. (2000) detectaron una inhibición de las actividades CYP17 y CYP19 en el tejido ovárico de platija (*Platichthys flesus*) expuesta a concentraciones elevadas de PAHs (15 µM), causando una reducción en la secreción de AD y E2. Pesticidas y NP inhibieron en carpa la actividad 17β-HSD, enzima clave para la síntesis de T (Thibaut and Porte, 2004). Además, NP y el fungicida ketoconazole disminuyeron las actividades CYP17 y CYP11β en machos de lubina (*Dicentrarchus labrax*), reduciendo así la formación de 11βAD (Fernandes et al., 2007). Por lo tanto, la interferencia de estos contaminantes ambientales con la biosíntesis hormonal representa un potencial mecanismo de disrupción endocrina, pudiendo afectar a la diferenciación sexual, al crecimiento y maduración de gametos. Varias etapas de la esteroidogénesis y algunos esteroides sexuales (ej. P4, AD, T, y E2) descritos en vertebrados han sido detectados en moluscos (De Longcamp et al., 1974; D'Aniello et al., 1996), equinodermos (Voogt et al., 1990; Le Curieux-Belfond et al., 2001;

Lavado et al., 2006a), y en parte también en crustáceos (Swevers et al., 1991; Verslycke et al., 2003). Sin embargo, el papel biológico de estas hormonas en invertebrados no está del todo claro; de hecho, no hay una firme evidencia de la síntesis *de novo* de estos esteroides en invertebrados, y la detección de estas hormonas podría ser simplemente el resultado de una absorción a través de la comida y/o el medio ambiente (Scott, 2012; Scott, 2013). Por ello, y para poder determinar los mecanismos de acción de los EDs en los invertebrados, es crucial entender el funcionamiento de su sistema endocrino. Varios trabajos han evidenciado la capacidad de xenobióticos de alterar la síntesis/metabolismo de esteroides en invertebrados. Moluscos expuestos al TBT, o viviendo en sitios contaminados por organoestánnicos, mostraron una disminución de la actividad P450-aromatasa (Morcillo et al., 1999; Santos et al., 2002). Para profundizar en este tema, una parte de esta tesis investiga los efectos de los EDs y las hormonas naturales sobre la síntesis y los niveles de esteroides en peces y moluscos.

1.3 Lípidos y posibles mecanismos de disrupción.

Los lípidos representan un grupo muy heterogéneo de moléculas con diferentes funciones: sirven de componentes estructurales de membrana, almacenan la energía necesaria para el organismo y participan en diferentes vías de señalización. Esta diversidad de funciones se refleja en la amplia variedad estructural de las moléculas lipídicas, que van desde las más sencillas (ácidos grasos), hasta los esteroles y esfingolípidos.

Los lípidos son muy importantes en los procesos fisiológicos y reproductivos de los organismos acuáticos. Los lípidos neutros, como triglicéridos, sirven de depósito de ácidos grasos destinados a proveer energía o para ser incorporados en nuevas moléculas lipídicas; y los fosfolípidos sirven de bloques de construcción de la bicapa de fosfolípidos de las membranas (Bergé and Barnathan, 2005).

Una de las principales funciones que cumplen los lípidos en organismos acuáticos es el almacenamiento y el abastecimiento de energía metabólica en forma de ATP por oxidación de los ácidos grasos (Tocher, 2003). De este modo, lípidos y ácidos grasos aportan la energía

necesaria para funciones fisiológicas primarias, como el crecimiento y el metabolismo basal, en épocas de escasez de comida (Abad et al., 1995), y representan la fuente principal de energía metabólica durante la reproducción (Rose, 2001; Sargent et al., 2002). Por ello, el mantenimiento de la homeostasis lipídica es crucial para la vida celular y del organismo, y existen varios mecanismos para asegurar la preservación de este equilibrio lipídico.

La homeostasis lipídica se define como el balance entre la captación, transporte, almacenamiento, síntesis, metabolismo y catabolismo lipídico. Numerosos genes están implicados en estos procesos y varios mecanismos regulan estos genes a nivel transcripcional, para mantener dicha homeostasis. Algunos de los receptores nucleares son cruciales en estos mecanismos de regulación lipídica. Entre ellos, los receptores X retinoides (RXRs) y los receptores activados por proliferadores de peroxisomas (PPARs) se han indicado como factores de transcripción clave en la regulación de genes implicados en los procesos de homeostasis lipídica. RXRs forman dímeros con receptores retinoides (RAR) y otros receptores nucleares (Blumberg and Evans, 1998), actuando como reguladores primarios de varias vías de señalización, incluyendo aquellas implicadas en el metabolismo y la síntesis lipídica (Chawla et al., 2001). Los PPARs desempeñan un papel esencial en la regulación del almacenamiento y catabolismo de lípidos. Existen tres diferentes subtipos de receptores PPAR: PPARα, presente especialmente en tejidos caracterizados por una alta tasa de catabolismo de ácidos grasos, PPARγ, implicado en la diferenciación de los adipocitos y almacenamiento de lípidos, y el PPARβ, que interviene en el catabolismo sistémico y de tejido de los ácidos grasos (Ferré, 2004; Feige et al., 2006; Michalik and Wahli, 2007).

En los últimos años, varios estudios han señalado la existencia de obesógenos ambientales, compuestos químicos capaces de afectar vías clave de adipogénesis y metabolismo lipídico, produciendo disfunciones metabólicas (Baillie-Hamilton, 2002; Newbold et al., 2008; Grün and Blumberg, 2009). Los organoestánnicos TBT y TPT actúan como agonistas de los receptores nucleares RXR y PPARγ a concentraciones detectadas en el medio ambiente (nanomolar) (Nishikawa et al., 2004; Grün and Blumberg, 2006), afectando la diferenciación de los preadipocitos 3T3-L1 y la expresión de genes relacionados con el metabolismo lipídico en estudios realizados in-vitro (Kanayama et al., 2005). Ratones expuestos al TBT manifestaron

una predisposición a incrementar la masa adiposa como consecuencia de la inducción de genes lipogénicos en el tejido adiposo e hígado (Grün et al., 2006). Además de los organoestánnicos, varios xenobióticos ambientales han mostrado capacidad de afectar las vías de señalización de los receptores nucleares: DEHP incrementó la oxidación PPARα-dependiente de ácidos grasos, causando atrofia en el tejido adiposo de rata y ratones (Xie et al., 2002; Itsuki-Yoneda et al., 2007); BPA indujo la diferenciación de los preadipocitos 3T3-L1 sobre-regulando genes relacionados con la diferenciación de adipocitos (Masuno et al., 2002; Masuno et al., 2005). Se han observado también casos de disrupción lipídica en organismos acuáticos: el salmón Chinook (*Oncorhynchus tshawytscha*) expuesto a TBT mostró un aumento en el peso corporal y en el contenido lipídico (Meador et al., 2011). Además, el TBT causó un aumento de la adiposidad en larvas de pez cebra (Tingaud-Sequeira et al., 2011), y aumentó la acumulación de lípidos en caracoles (*Marisa cornuarietis*) junto con el TPT (Janer et al., 2007; Lyssimachou et al., 2009). Sin embargo, las implicaciones toxicológicas de la presencia de obesógenos ambientales en los ambientes acuáticos permanecen en gran parte desconocidas, así como los posibles mecanismos de acción obesógena.

2. Objetivos

El objetivo de esta tesis ha sido evaluar los efectos obesogénicos y de disrupción endocrina de diferentes contaminantes ambientales sobre organismos acuáticos, estudiando: a) vías enzimáticas claves en el metabolismo de xenobióticos y de compuestos endógenos, y en la biosíntesis de esteroides; y b) los efectos sobre lípidos celulares.

Los resultados de esta tesis se presentan como una colección de publicaciones abordando los siguientes objetivos específicos:

1. Investigar el metabolismo de la fragancia policíclica galaxolide (HHCB) y sus efectos sobre enzimas de biotransformación (CYP1A, CYP3A, UGT) y síntesis de andrógenos activos (CYP17, CYP11β) en lubina (*Dicentrarchus labrax*).

Artículo 1. Fernandes, D., Dimastrogiovanni, G., Blázquez, M., Porte, C., 2013. Metabolism of the polycyclic musk galaxolide and its interference with endogenous and xenobiotic metabolizing enzymes in the European sea bass (*Dicentrarchus labrax*). Environmental Pollution 174, 214-221.

2. Describir las vías enzimáticas implicadas en el metabolismo de progesterona (P4) en gónadas y manto del mejillón (*Mytilus galloprovincialis*) e identificar alteraciones del sistema endocrino en mejillones expuestos.

Artículo 2. Dimastrogiovanni, G., Fernandes D., Bonastre M., Porte, C., 2015. Progesterone is actively metabolized to 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one by the marine mussel *Mytilus galloprovincialis*. Aquatic Toxicology 165, 93-100.

3. Evaluar la capacidad de disruptores endocrinos conocidos, como TBT, TPT, NP, BPA, DEHP de actuar como obesógenos en organismos acuáticos, alterando la homeostasis lipídica intracelular y los marcadores de metabolismo lipídico, utilizando dos modelos celulares (RTL-W1 y ZFL).

Artículo 3: Dimastrogiovanni, G., Córdoba, M., Navarro, I., Jáuregui, O., Porte, C., 2015. Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters. Aquatic toxicology (en prensa).

Artículo 4: Dimastrogiovanni, G., Jáuregui, O., Blumberg, B., Porte, C., 2015. TBT induces triglyceride accumulation and alters lipid profile in ZFL cells (en preparación).

3. Resumen de los resultados y discusión general

Existe poca información acerca del efecto, metabolismo y eliminación de los disruptores endocrinos y lipídicos en organismos acuáticos. Para su mayor conocimiento, esta tesis investigó: a) el metabolismo y mecanismo de acción de galaxolide (HHCB) en lubinas (*Dicentrarchus labrax*) (artículo 1); b) posibles alteraciones del sistema endocrino de mejillones (*Mytilus galloprovincialis)* expuestos a diferentes concentraciones de progesterona (P4) y de las vías enzimáticas involucradas en el metabolismo de este precursor (artículo 2); c) la utilización de dos líneas celulares (RTL-W1 y ZFL) para evaluar la capacidad de obesógenos ambientales (TBT, TPT, 4-NP, BPA, y DEHP) de modificar el perfil lipídico en células hepáticas de peces en diferentes condiciones experimentales (artículos 3 y 4).

3.1 Metabolismo y mecanismo de acción del galaxolide (HHCB) en lubinas (*Dicentrarchus labrax*).

Este trabajo investiga el metabolismo y mecanismo de acción de galaxolide (HHCB) en lubinas (*Dicentrarchus labrax*) tratadas con 50 mg HHCB/kg de peso corporal (inyección intraperitoneal) y analizadas 24, 48 y 96 horas pos-inyección. Además, se incluyó como control positivo un grupo de peces inyectados con ketoconazole (KCZ) (50 mg KCZ/kg de peso), un fungicida conocido por interferir con diferentes isoenzimas de CYP.

El análisis del compuesto en sangre reveló un tiempo de vida media del HHCB de aproximadamente 10 días. Además, el análisis de la bilis, mostró la capacidad de la lubina de metabolizar HHCB en un metabolito hidroxilado (OH-HHCB), observándose un aumento significativo de la concentración de OH-HHCB en bilis que llegó a su máximo 96 horas pos-inyección, mientras que la concentración de HHCB disminuyó significativamente. Los metabolitos biliares pueden utilizarse como indicadores de la exposición a contaminantes ambientales que son rápidamente metabolizados, tales como hidrocarburos aromáticos policíclicos, alquilfenoles y otros (Lavado et al., 2006b; Beyer et al., 2010). Por esto, la determinación de los niveles biliares de HHCB y/o OH-HHCB puede ser utilizada como

herramienta para evaluar la exposición de peces a este compuesto u a otras fragancias policíclicas. Este es el primer estudio que revela la presencia de un metabolito hidroxilado de HHCB en bilis de pez.

Para una mejor evaluación del riesgo asociado a la exposición a HHCB, se investigaron las posibles vías enzimáticas implicadas en su metabolismo. La actividad hepática EROD (CYP1A) no fue alterada por la inyección de HHCB, mientras que la actividad BFCOD (CYP3A) aumentó un 133% a las 48 horas post-inyección. En un estudio reciente se describe que HHCB (100 μM) es un inhibidor débil de las actividades CYP1A y CYP3A en lubina y en otras especies del Mediterráneo in-vitro, mientras que en *Trachyrincus scabrus* la inhibición de la actividad CYP1A fue del 40% (Ribalta and Solé, 2014). Además, HHCB inhibió levemente la actividad CYP1A (IC50 > 100 μM) en hígado de carpa (*Cyprinus carpio*), mientras que en la actividad CYP3A se observó una fuerte inhibición (IC50 = 68 ± 12 μM) (Schnell et al., 2009), sugiriendo que las actividades CYP puedan ser afectadas por HHCB dependiendo de la especie. En este respecto, nuestros resultados no aclaran completamente si la CYP3A u otras isoenzimas del CYP están involucrados en el metabolismo de HHCB en lubina. Sin embargo, la inducción significativa de la actividad de fase II UGT a las 24 horas pos-inyección sugiere que se produce una inducción temprana de este enzima por HHCB y, en comparación, un lento metabolismo de fase I, probablemente por CYP3A.

También se investigó la capacidad de HHCB de actuar como disruptor endocrino en lubina evaluando sus efectos sobre las actividades CYP17 y CYP11β, enzimas involucradas en la síntesis de andrógenos en machos. La fracción mitocondrial de las gónadas metabolizó 17α-hidroxiprogesterona (17P4) a androstenediona (AD), que fue posteriormente transformada en 11β-hidroxi-4-androstene-3,17-dione (βAD). HHCB inhibió significativamente las actividades CYP17 y CYP11β en testículos de lubina a las 24 y 96 horas después de la inyección, respectivamente. Los efectos observados en nuestro estudio concuerdan con resultados anteriores obtenidos in-vitro donde HHCB tenía un efecto inhibidor sobre ambas actividades (Schnell et al., 2009). Este estudio es el primero realizado in-vivo que indica un efecto inhibidor del HHCB sobre la síntesis de andrógenos en peces.

Por otro lado, KCZ indujo la actividad CYP1A (x2) y actuó como inhibidor de las actividades CYP3A (70-80% inhibición) y UGT (35-45% inhibición). Además, indujo significativamente la formación de AD (incremento del 90% en la actividad CYP17) e inhibió la síntesis de βAD (disminución del 55-68% en la actividad CYP11β). Varios estudios habían demostrado previamente la capacidad del KCZ de interferir con las enzimas esteroidogénicas (CYP) en mamíferos y peces (Rocha et al., 2000; Villeneuve et al., 2007; Zhang et al., 2008).

En resumen, el presente estudio revela que HHCB puede ser metabolizado activamente por lubina y que actúa como un inhibidor débil de las actividades CYP17 y CYP11β, evidenciando así su potencial de perjudicar la biosíntesis de esteroides relacionados con la función testicular. Este trabajo contribuye a comprender el impacto de fragancias sintéticas sobre peces, y propone la determinación en la bilis de HHCB y de su metabolito hidroxilado como herramienta para evaluar la exposición de peces a este compuesto.

3.2 Caracterización del metabolismo de progesterona en mejillones (*Mytilus galloprovincialis*) y potenciales vías de disrupción endocrina.

La progesterona (P4) y los progestágenos sintéticos entran en el medio acuático a través de las plantas de tratamiento de agua residual y por escorrentía agrícola, lo que conlleva un riesgo potencial para los organismos acuáticos, dada la actividad biológica de estos compuestos. Además, P4 es el precursor de diferentes esteroides en vertebrados, incluyendo estrógenos y andrógenos. Sin embargo, la información acerca del metabolismo de P4 en moluscos es escasa. Por esta razón, en este estudio (artículo 2) se caracterizaron las vías enzimáticas involucradas en el metabolismo de P4 en mejillones (elegidos como especie modelo de invertebrado) y se identificaron posibles alteraciones del sistema endocrino como consecuencia de la exposición durante 7 días a diferentes concentraciones de P4 (0.02-10 µg/L).

P4 se metabolizó activamente a 5α-pregnano-3,20-dione (5α-DHP) a través del enzima 5α-reductasa (5αR) y a 3β-hidroxi-5α-pregnano-20-one (3β,20-one) mediante 3β-HSD en fracciones subcelulares de glándula digestiva. El metabolismo de P4 más activo se detectó en

fracciones microsomales, sugiriendo que 5αR es principalmente una enzima microsomal. De acuerdo con nuestros resultados, otros estudios han mostrado que P4 es mayoritariamente metabolizada in-vivo a 5α-DHP y 3β,20-one en el gasterópodo *Clione antarctica* (Hines et al., 1996) y en el equinodermo *Lytechinus variegatus* (Wasson and Watts, 2000). Ésta representa una diferencia importante entre invertebrados y vertebrados, ya que estos últimos metabolizan P4 a 17α-hidroxiprogesterona (17P4) mediante el enzima 17α-hidroxilasa, y ésta a androstenediona (AD). Cuando se incubaron las fracciones subcelulares con AD para estudiar el metabolismo de esta hormona en mejillones, se observó la formación de 5α-DHA (catalizada por 5αR) y también la formación de T en la fracción citosólica, indicando la presencia de la vía enzimática 17β-HSD en la glándula digestiva de mejillones.

El análisis del agua mostró que P4 era absorbida por los mejillones, ya que la concentración de P4 en los tanques de exposición disminuyó drásticamente tras 24 horas de exposición. La posible adherencia de P4 a las paredes de los tanques puede también haber contribuido en parte a la reducción detectada. Asimismo, se observó una acumulación de P4 en el manto y gónadas de mejillones expuestos. Esta acumulación fue inferior a lo que se había descrito en mejillones para otra hormona (T) (Fernandes et al., 2010). La rápida conversión de P4 a metabolitos 5α-reducidos puede haber contribuido a una menor acumulación de P4 en los mejillones expuestos. No se observó un incremento significativo en el metabolismo de P4 a 5α-DHP en mejillones expuestos, aunque sí, una tendencia hacia un metabolismo mayor en individuos expuestos. La conversión de AD a 5α-DHA aumentó significativamente en mejillones expuestos, mientras que la conversión de AD a T no fue alterada por la exposición a P4. Estos resultados concuerdan con estudios anteriores, donde se observó que la exposición a esteroides exógenos (T y E2) alteraba la actividad 5αR sin ningún efecto sobre la 17β-HSD (Janer et al., 2005; Fernandes et al., 2010), sugiriendo que 5α-reductasa juega un papel más importante que 17β-HSD en el metabolismo de esteroides en moluscos.

Al analizar los niveles de esteroides endógenos en los mejillones expuestos, no se detectaron alteraciones significativas en los niveles de testosterona (T) y estradiol (E2) en el manto y gónadas, a pesar de un aumento de T atribuido a reactividad cruzada de P4 en los organismos expuestos a 10 µg P4/L. Por otro lado, el examen histológico de las gónadas mostró que la

exposición a 10 µg/L de P4 inducía la maduración y liberación de gametos. Una inducción de la maduración ovárica y del desove fue observada también en langostinos banana (*Metapenaeus ensis*) expuestos durante 1 mes a 100 ng/g P4 (Yano, 1985). Además, la concentración de P4 aumentó en las gónadas de *Mya arenaria* durante la maduración (machos) y el desove (hembras) (Siah et al., 2002) y elevados niveles de P4 fueron también detectados en los estadios finales de la gametogénesis (desove y estadio de recuperación) en *Ruditapes decussatus* y *Lytechinus variegatus* (Wasson and Watts, 2000; Ketata et al., 2007). Todos estos resultados, junto con los que se determinaron en este estudio, sugieren que P4 juega un papel en el desarrollo de gametos en invertebrados.

En general, este trabajo contribuye a la evaluación de las vías enzimáticas implicadas en el metabolismo de progesterona en mejillones y describe la capacidad de estos invertebrados de metabolizar activamente P4 a 5α-pregnano-3,20-dione (5α-DHP) y 3β-hidroxi-5α-pregnano-20-one (3β,20-one) en la glándula digestiva, sin observarse la síntesis de 17P4 o AD. Además, el examen histológico de las gónadas mostró que la exposición a 10 µg/L de P4 inducía la maduración y liberación de gametos en mejillones, pero no se detectó ningún efecto significativo sobre los niveles de esteroides y las enzimas relacionadas con el metabolismo de esteroides. Finalmente, las concentraciones de P4 detectadas en el medio ambiente (ng/L) es poco probable que produzcan alteraciones a nivel endocrino en mejillones.

3.3 Disrupción lipídica como respuesta a los xenobióticos ambientales.

Para evaluar el impacto de potenciales obesógenos en ecosistemas acuáticos, se investigó la capacidad de diferentes compuestos químicos de afectar la síntesis y/o metabolismo de lípidos usando dos líneas celulares de hígado de pez. Por eso, en el artículo 3 se investigó el efecto de concentraciones ambientales de reconocidos y/o presuntos obesógenos (TBT, TPT, NP, BPA y DEHP) sobre lípidos celulares y marcadores del metabolismo lipídico en la línea celular de hígado de trucha arco iris (RTL-W1). Además, en el artículo 4 se evaluó el efecto sobre los lípidos celulares de diferentes condiciones de exposición (medio suplementado con lípidos) en la línea celular de hígado de pez cebra (ZFL) expuesta al obesógeno modelo TBT.

3.3.1 Alteración de los lípidos celulares y marcadores del metabolismo lipídico en células RTL-W1 expuestas a EDs.

El perfil lipídico de las células RTL-W1 fue determinado por cromatografía líquida acoplada a espectrometría de masa de alta resolución (HPLC-HRMS). De este modo, se identificaron un total de 29 especies lipídicas, entre ellas: 13 fosfatidilcolinas (PC), 6 plasmalógenos de PC, y 10 triglicéridos (TAG). Se determinaron los cambios en los niveles de estos lípidos comparando las áreas de los picos en las células expuestas con los niveles basales correspondientes en células no-expuestas. Además, se analizó la expresión de los siguientes genes: a) el transportador dependiente de la unión de ATP (ABCA1), relacionado con la homeostasis del colesterol; b) dos transportadores de ácidos grasos, el cúmulo de diferenciación 36 (CD36) y la proteína transportadora de ácidos grasos (FATP1), junto con la expresión de la lipoproteinlipasa (LPL), como marcadores de la captación lipídica de ácidos grasos; c) la expresión de la ácido graso sintasa (FAS), una enzima lipogénica; d) el receptor X hepático (LXR) relacionado con las vías de lipogénesis; y e) los receptores activados por proliferadores de peroxisomas, PPARα y PPARβ, promotores de la utilización de ácidos grasos en mamíferos, y el PPARγ, implicado en la acumulación lipídica y adipogénesis en mamíferos y peces (Kota et al., 2005; Bouraoui et al., 2008).

Todos los compuestos, excepto DEHP, indujeron la expresión del gen ABCA1. Este transportador regula la circulación de colesterol y fosfolípidos, promoviendo la eliminación del exceso de estas moléculas y reduciendo la acumulación lipídica en hepatocitos (Ma et al., 2014). Así, un aumento de la expresión de ABCA1 puede ser una primera respuesta de defensa de las células RTL-W1 a la exposición a EDs.

Los compuestos organoestánnicos TBT y TPT (100 nM) mostraron efectos diferentes sobre la composición lipídica de las células RTL-W1. Ambos indujeron la expresión de FAS, pero sólo TPT indujo la expresión de FATP1 y un aumento significativo de TAG, sugiriendo un incremento de la captación de ácidos grasos y su acumulación intracelular en forma de TAGs, mientras que TBT indujo la expresión de LPL y aumentó los niveles de algunas especies de PC y plasmalógenos de PC. TBT y TPT son ligandos reconocidos del heterodímero RXR-PPARγ, y

ambos inducen la expresión del gen PPARγ en hígado y tejido adiposo (Inadera and Shimomura, 2005). Sin embargo, la expresión de PPARγ en RTL-W1 es relativamente baja, y la exposición a los compuestos organoestánnicos no alteró la expresión de los PPARα y β, causando modificaciones menores en los lípidos celulares en comparación con los otros compuestos analizados.

El plastificante BPA (10 µM) aumentó la expresión de FATP1 y FAS e incrementó los niveles de TAG tras 24 h de exposición, de acuerdo con una activación de la captación de ácidos grasos y de la lipogénesis en RTL-W1. Aunque BPA es comúnmente considerado un obesógeno débil, se ha descrito que crías de madres expuestas a este compuesto durante la gestación y lactancia mostraron un aumento del peso corporal (Rubin et al., 2001; Rubin and Soto, 2009). Es interesante el hecho que estos efectos obesogénicos eran mayores a las concentraciones más bajas de exposición, y que esta relación no monotónica dosis-respuesta ha sido referida también para otros efectos del BPA (Vandenberg et al., 2012). Además, estudios in-vivo indicaron una acumulación de TAGs y ésteres de colesterol en hígado de ratones adultos expuestos a bajas dosis de BPA. En estos estudios, la activación de lipogénesis y de la biosíntesis de colesterol se señalaron como mecanismos implicados junto con una inhibición de la oxidación de ácidos grasos (Marmugi et al., 2012).

Por otro lado, DEHP (5 µM) disminuyó significativamente la expresión de LPL y FAS en RTLW-1. La exposición in-vivo a DEHP protegió ratones de la obesidad inducida por la dieta, reduciendo la masa grasa (Feige et al., 2010), y este efecto fue atribuido a una activación a través del PPARα del catabolismo de ácidos grasos en el hígado. Sin embargo, exposición a 5 µM de DEHP causó un incremento significativo de los TAG (24 h) y las PC (48 y 72 h de exposición) en RTL-W1, en acuerdo con otro estudio que indicaba un acumulo de PC en células trofoblásticas (HRP-1) expuestas a 50 µM DEHP durante 24 h (Xu et al., 2006).

De todos los compuestos analizados en este estudio, TBT (100 nM), DEHP (5 µM) y NP (20 µM) fueron los más efectivos alterando los niveles de PC, los principales componentes estructurales de las membranas celulares (Van Meer et al., 2008). Además de PC, los plasmalógenos también participan en la estructura de las membranas. Un aumento relativo (hasta el 40%) de algunos plasmalógenos de PC se detectó en células expuestas a BPA,

DEHP y TBT, mientras que el NP fue el único compuesto que los redujo (hasta el 50%). Es muy probable que NP se acumule en la bicapa fosfolipídica de las células RTL-W1, alterando la fluidez de membrana. Además, las modificaciones de los niveles de PC y plasmalógenos de PC en células expuestas pueden indicar un daño oxidativo, cuanto mayor es el grado de insaturación de los ácidos grasos mayor es su susceptibilidad a la peroxidación (Hulbert et al., 2014).

En resumen, este estudio indica que los EDs estudiados son capaces de alterar la expresión de genes relacionados con el metabolismo lipídico en las células RTL-W1 y de causar cambios importantes en los lípidos de membrana y los TAG. Entre los compuestos estudiados, BPA y DEHP aumentaron significativamente la acumulación intracelular de triglicéridos (TAG), mientras que todos los compuestos, excepto TPT, modificaron los niveles de lípidos de membrana (PC y plasmalógenos de PC), evidenciando una fuerte interacción de estos xenobióticos con la membrana celular y la señalización celular. Además, este estudio ha evidenciado diferentes y complejos mecanismos de acción para estos compuestos, tanto a nivel de expresión de genes relacionados con el metabolismo lipídico como la modificación de los lípidos celulares.

3.3.2 TBT induce acumulación de TAG y modifica el perfil lipídico en células ZFL.

En este artículo se investigó el efecto del reconocido obesógeno TBT sobre los lípidos celulares de hepatocitos usando la línea celular de pez cebra ZFL. De este modo, células ZFL fueron expuestas a diferentes concentraciones de TBT (10, 25, 50 y 100 nM) durante 96 horas en medio de cultivo basal y en medio suplementado con una mezcla de lípidos. Se determinó la acumulación intracelular de lípidos con el colorante Rojo Nilo, y además se caracterizaron los lípidos celulares por cromatografía liquida de ultra-alta resolución acoplada a espectrometría de masas en tándem de alta resolución (UHPLC-HRMS-MS). De este modo, se identificaron 72 especies lipídicas, incluyendo 17 fosfatidilcolinas (PC), 6 lisofosfatidilcolinas (lyso-PC), 12 plasmalógenos de PC, 18 triglicéridos (TAG), 13 diglicéridos (DAG) y 6 ésteres de colesterol.

El primer paso fue evaluar la acumulación lipídica en células ZFL a través de la tinción con el Rojo Nilo, el cual se usa comúnmente para detectar los 'lipid droplets' (LD) intracelulares en todo tipo de células (Genicot et al., 2005). Los LDs están constituidos por un núcleo de lípidos neutros, mayoritariamente triglicéridos y ésteres de colesterol, que sirven de almacén de energía metabólica y de componentes estructurales de membrana. De acuerdo con nuestros resultados en las células RTL-W1, donde el TBT alteró los niveles de PC pero no interfirió con los de TAG, no se detectó ningún incremento significativo de lípidos intracelulares en células expuestas al TBT en medio de cultivo basal. Sin embargo, cuando el medio fue suplementado con colesterol y ácidos grasos, se observó una inducción de la acumulación lipídica, que alcanzó el 250% en células expuestas a 100 nM TBT. Estudios anteriores mostraron en su mayoría efectos adipogénicos del TBT en adipocitos y células madre (Inadera and Shimomura, 2005; Kanayama et al., 2005; Grün and Blumberg, 2006; Kirchner et al., 2010), pero éste es uno de los primeros trabajos in-vitro indicando acumulación lipídica por la presencia de TBT en hepatocitos.

Se observaron resultados diferentes cuando las células ZFL fueron expuestas a TBT en medio de cultivo basal o en medio suplementado con lípidos. En la primera condición experimental, la exposición al TBT afectó principalmente los glicerofosfolípidos (GP), con un incremento en los niveles de lyso-PC, PC y plasmalógenos de PC, mientras que en el medio suplementado no se detectó ninguna alteración de los GP. Diferentes estudios han demostrado que TBT es una molécula activa a nivel de membrana, mostrando la incorporación del TBT en las PC, donde perturba las propiedades estructurales y termotrópicas, llevando a la alteración y la lisis de la membrana (Ambrosini et al., 1991; Chicano et al., 2001; Ortiz et al., 2005). La presencia de colesterol disminuyó la acción toxica del TBT sobre la membrana (Ortiz et al., 2005), como también el suministro del ácido polinsaturado linoleíco al medio de cultivo de la levadura (*Saccharomyces cerevisiae*) (Masia et al., 1998). Estas dos condiciones (suministro de colesterol y PUFAs) reflejan las condiciones experimentales de nuestro estudio donde desaparece el efecto perturbador del TBT sobre los lípidos de membrana.

Por otro lado, los niveles de TAG, DAG y ésteres de colesterol sufrieron un incremento masivo en las células expuestas a 100 nM TBT en medio suplementado con lípidos. Esto está de

acuerdo con los datos obtenidos a través de la tinción con Rojo Nilo. Numerosos estudios han demostrado la habilidad del TBT de promover adipogénesis y acumulación lipídica in-vitro e in-vivo en los mamíferos (Kanayama et al., 2005; Grün et al., 2006; Iguchi et al., 2007). Se sugirió la hipótesis que el TBT induce estas alteraciones a través de la interacción con los receptores nucleares RXR y PPARγ, ya que se une a ambos receptores con una alta afinidad (Grün and Blumberg, 2006). Además, el TBT perjudicó la homeostasis de ácidos grasos e incrementó la acumulación lipídica en el caracol *Marisa cornuarietis*, lo que apunta al RXR como factor clave en este proceso, ya que los caracoles carecen del gen ortólogo PPARγ (Janer et al., 2007), mientras que RXR sí que ha sido detectado en invertebrados (Bouton et al., 2005).

Se observó que los plasmalógenos de PC disminuyeron significativamente en las células expuestas a 100 nM TBT en medio de cultivo enriquecido con lípidos, sugiriendo una relación con la gran acumulación de DAG, TAG y ésteres de colesterol caracterizados por la presencia de ácidos grasos polinsaturados. De hecho, los plasmalógenos no sirven solo de componentes estructurales, sino que están también implicados en el transporte de iones, movilización del colesterol y almacenamiento de ácidos grasos de cadena larga polinsaturados (Lesharpig and Fuchs, 2009).

En resumen, este estudio evidencia la capacidad del TBT de alterar el perfil lipídico de las células ZFL y de inducir acumulación de triglicéridos en hepatocitos sólo cuando el medio de cultivo es suplementado con una mezcla lipídica.

En general, siendo el hígado el principal órgano donde tiene lugar la síntesis *de novo* de lípidos, la utilización de estas líneas celulares de hígado de pez, RTL-W1 y ZFL, puede ser un modelo in-vitro apto para evaluar la capacidad de contaminantes ambientales de interferir con el metabolismo lipídico en especies acuáticas, permitiendo así una evaluación previa de los efectos obesogénicos en hepatocitos bajo diferentes condiciones de exposición (adición de lípidos al medio de cultivo para emular una dieta rica de grasas, diferentes tiempos y concentraciones de exposición).

4. Conclusiones

I. HHCB mostró un tiempo de vida media en la sangre de lubina (*Dicentrarchus labrax*) de aproximadamente 10 días. Asimismo, se demostró la excreción del compuesto y un metabolito hidroxilado (OH-HHCB) a través de la bilis. La inducción de las actividades CYP3A y UGT en hígado sugiere la participación de estos enzimas en el metabolismo de HHCB en lubina.

II. HHCB inhibió la síntesis de andrógenos (actividades CYP17 y CYP11β) en gónadas de lubina, evidenciando el potencial de HHCB de alterar la biosíntesis de andrógenos y posiblemente de la función testicular.

III. El mejillón (*Mytilus galloprovincialis*) metaboliza activamente P4 a 5α-DHP, y esta a 3β, 20-one en glándula digestiva, mientras que no se detectaron evidencias de la síntesis de 17P4 o AD. Estos resultados corroboran lo descrito en otras especies de invertebrados (gasterópodos, equinodermos, etc.)

IV. La exposición a concentraciones altas de P4 (10 µg/L) aceleró la maduración y liberación de gametos, pero no se detectó ningún efecto significativo sobre los niveles de esteroides ni las enzimas implicadas en el metabolismo de esteroides. Es poco probable que concentraciones ambientales de P4 (en el rango de ng/L) produzcan alteraciones significativas en el sistema endocrino de mejillones.

V. Reconocidos disruptores endocrinos (TBT, NP, BPA y DEHP) indujeron alteraciones significativas en PCs y Plasmalogénos de PC en las células RTL-W1, evidenciando una fuerte interacción de estos compuestos con las membranas celulares y posiblemente con la señalización celular. BPA y DEHP aumentaron significativamente la acumulación intracelular de TAG; sin embargo, no se evidenció una relación clara entre la expresión

de genes relacionados con el metabolismo lipídico (FAS, LPL, FATP1, CD36, ABCA1, LXR, PPARα, β y γ) y la acumulación de TAG.

VI. TBT incrementó significativamente los niveles de Lyso-PC, PC y Plasmalógenos de PC en células ZFL expuestas durante 96 horas en medio de cultivo basal, mientras los niveles de DAG, TAG y ésteres de colesterol aumentaron drásticamente cuando el medio de cultivo fue suplementado con lípidos.

VII. Las líneas celulares RTL-W1 y ZFL se han revelado como modelos in-vitro aptos para la investigación de contaminantes ambientales sospechosos de alterar la homeostasis lipídica y el metabolismo lipídico en especies acuáticas, permitiendo una evaluación previa de los efectos obesogénicos en hepatocitos bajo diferentes condiciones de exposición (adición de lípidos al medio de cultivo, diferentes concentraciones y tiempos de exposición).

6. REFERENCES

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Abad, M., Ruiz, C., Martinez, D., Mosquera, G., Sánchez, J., 1995. Seasonal variations of lipid classes and fatty acids in flat oyster, *Ostrea edulis,* from San Cibran (Galicia, Spain). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 110, 109-118.

Ahel, M., Giger, W., Koch, M., 1994a. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment—I. Occurrence and transformation in sewage treatment. *Water research*, 28, 1131-1142.

Ahel, M., Giger, W., Schaffner, C., 1994b. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment—II. Occurrence and transformation in rivers. *Water research*, 28, 1143-1152.

Ahel, M., Scully Jr, F.E., Hoigné, J., Giger, W., 1994c. Photochemical degradation of nonylphenol and nonylphenol polyethoxylates in natural waters. *Chemosphere*, 28, 1361-1368.

Alonso-Magdalena, P., Laribi, O., Ropero, A.B., Fuentes, E., Ripoll, C., Soria, B., Nadal, A., 2005. Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic α-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. *Environmental health perspectives*, 969-977.

Ambrosini, A., Bertoli, E., Zolese, G., Tanfani, F., 1991. Interaction of tributylin acetate and tributyltin chloride with dipalmitoyl phosphatidylcholine model membrane. *Chemistry and Physics of Lipids*, 58, 73-80.

Andersson, S., Geissler, W.M., Patel, S., Wu, L., 1995. The molecular biology of androgenic 17β-hydroxysteroid dehydrogenases. *The Journal of steroid biochemistry and molecular biology*, 53, 37-39.

Ankley, G.T., Cavallin, J.E., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Martinovic-Weigelt, D., Wehmas, L.C., Villeneuve, D.L., 2011. Temporal evaluation of effects of a model 3β-hydroxysteroid dehydrogenase inhibitor on endocrine function in the fathead minnow. *Environmental Toxicology and Chemistry*, 30, 2094-2102.

Aranda, A., Pascual, A., 2001. Nuclear hormone receptors and gene expression. *Physiological reviews*, 81, 1269-1304.

Archer, A., Lauter, G., Hauptmann, G., Mode, A., Gustafsson, J.Å., 2008. Transcriptional activity and developmental expression of liver X receptor (lxr) in zebrafish. *Developmental Dynamics*, 237, 1090-1098.

Auwerx, J., 1999. PPARγ, the ultimate thrifty gene. *Diabetologia*, 42, 1033-1049.

Baillie-Hamilton, P.F., 2002. Chemical toxins: a hypothesis to explain the global obesity epidemic. *The Journal of Alternative & Complementary Medicine*, 8, 185-192.

Balk, F., Ford, R.A., 1999. Environmental risk assessment for the polycyclic musks AHTN and HHCB in the EU: I. Fate and exposure assessment. *Toxicology Letters*, 111, 57-79.

Barish, G.D., Narkar, V.A., Evans, R.M., 2006. PPARδ: a dagger in the heart of the metabolic syndrome. *Journal of Clinical Investigation*, 116, 590-597.

Baroiller, J., Guiguen, Y., Iseki, K., Fostier, A., 1998. Physiological role of androgens on gonadal sex differentiation in two teleost fish, *Oncorhynchus mykiss* and *Oreochromis niloticus*. *J Exp Zool*, 281, 506-507.

Batista-Pinto, C., Rodrigues, P., Rocha, E., Lobo-da-Cunha, A., 2005. Identification and organ expression of peroxisome proliferator activated receptors in brown trout (Salmo trutta f. fario). *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, 1731, 88-94.

Bauer, B., Fioroni, P., Ide, I., Liebe, S., Oehlmann, J., Stroben, E., Watermann, B., 1995. TBT effects on the female genital system of *Littorina littorea*: a possible indicator of tributyltin pollution. *Hydrobiologia*, 309, 15-27.

Belfroid, A., Van der Horst, A., Vethaak, A., Schäfer, A., Rijs, G., Wegener, J., Cofino, W., 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Science of The Total Environment*, 225, 101-108.

Bergé, J.-P., Barnathan, G., 2005. Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. In: Marine biotechnology I. Springer, 49-125.

Berger, J., Moller, D.E., 2002. The mechanisms of action of PPARs. *Annual review of medicine*, 53, 409-435.

Bernat, P., Gajewska, E., Szewczyk, R., Słaba, M., Długoński, J., 2014. Tributyltin (TBT) induces oxidative stress and modifies lipid profile in the filamentous fungus Cunninghamella elegans. *Environmental Science and Pollution Research*, 21, 4228-4235.

Beyer, J., 1996. Fish biomarkers in marine pollution monitoring. Evaluation and validation in laboratory and field studies.

Beyer, J., Jonsson, G., Porte, C., Krahn, M.M., Ariese, F., 2010. Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: a review. *Environmental toxicology and pharmacology*, 30, 224-244.

Biemann, R., Navarrete Santos, A., Navarrete Santos, A., Riemann, D., Knelangen, J., Blüher, M., Koch, H., Fischer, B., 2012. Endocrine disrupting chemicals affect the adipogenic differentiation of mesenchymal stem cells in distinct ontogenetic windows. *Biochemical and biophysical research communications*, 417, 747-752.

Billiard, S.M., Hahn, M.E., Franks, D.G., Peterson, R.E., Bols, N.C., Hodson, P.V., 2002. Binding of polycyclic aromatic hydrocarbons (PAHs) to teleost aryl hydrocarbon receptors (AHRs). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 133, 55-68.

Biselli, S., Bester, K., Hühnerfuss, H., Fent, K., 2000. Concentrations of the antifouling compound Irgarol 1051 and of organotins in water and sediments of German North and Baltic Sea marinas. *Marine Pollution Bulletin*, 40, 233-243.

Bizarro, C., Ros, O., Vallejo, A., Prieto, A., Etxebarria, N., Cajaraville, M., Ortiz-Zarragoitia, M., 2014. Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (Chelon labrosus) from Basque estuaries (South-East Bay of Biscay). *Marine Environmental Research*, 96, 19-28.

Bizzari, S., Oppenberg, B., Iskikawa, Y., 2000. Plasticizers. Chemical Economics Handbook. . SRI International.

Blumberg, B., Evans, R.M., 1998. Orphan nuclear receptors—new ligands and new possibilities. *Genes & Development*, 12, 3149-3155.

Bols, N., Dayeh, V., Lee, L., Schirmer, K., 2005. Use of fish cell lines in the toxicology and ecotoxicology of fish. Piscine cell lines in environmental toxicology. *Biochemistry and molecular biology of fishes*, 6, 43-84.

Bonefeld-Jorgensen, E.C., Long, M., Hofmeister, M.V., Vinggaard, A.M., 2007. Endocrine-disrupting potential of bisphenol A, bisphenol A dimethacrylate, 4-n-nonylphenol, and 4-n-octylphenol in vitro: new data and a brief review. *Environmental health perspectives*, 115, 69.

Borg, B., 1994. Androgens in teleost fishes. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 109, 219-245.

Borg, W., Shackleton, C.H., Pahuja, S.L., Hochberg, R.B., 1995. Long-lived testosterone esters in the rat. *Proceedings of the National Academy of Sciences*, 92, 1545-1549.

Bouraoui, L., Gutiérrez, J., Navarro, I., 2008. Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). *Journal of Endocrinology*, 198, 459-469.

Bouton, D., Escriva, H., De Mendonça, R., Glineur, C., Bertin, B., Noël, C., Robinson-Rechavi, M., de Groot, A., Cornette, J., Laudet, V., 2005. A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. *Journal of molecular endocrinology*, 34, 567-582.

Bozcaarmutlu, A., Arinç, E., 2004. Inhibitory effects of divalent metal ions on liver microsomal 7-ethoxyresorufin O-deethylase (EROD) activity of leaping mullet. *Marine Environmental Research*, 58, 521-524.

Brossa, L., Marcé, R.M., Borrull, F., Pocurull, E., 2005. Occurrence of twenty-six endocrine-disrupting compounds in environmental water samples from Catalonia, Spain. *Environmental Toxicology and Chemistry*, 24, 261-267.

Bryan, G., Gibbs, P., Burt, G., Hummerstone, L., 1987. The effects of tributyltin (TBT) accumulation on adult dog-whelks, *Nucella lapillus*: long-term field and laboratory experiments. *Journal of the Marine Biological Association of the United Kingdom*, 67, 525-544.

Bryan, G., Gibbs, P., Burt, G., 1988. A comparison of the effectiveness of tri-n-butyltin chloride and five other organotin compounds in promoting the development of imposex in the dog-whelk, *Nucella lapillus*. *Journal of the Marine Biological Association of the United Kingdom*, 68, 733-744.

Bucheli, T.D., Fent, K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Reviews in Environmental Science and Technology*, 25, 201-268.

Buhler, D.R., Wang-Buhler, J.-L., 1998. Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 121, 107-137.

Burke, D.M., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabolism and Disposition*, 2, 583-588.

Burns, B., Sangalang, G., Freeman, H., McMenemy, M., 1984. Isolation and identification of testosterone from the serum and testes of the American lobster (*Homarus americanus*). *General and comparative endocrinology*, 54, 429-432.

Carreau, S., Drosdowsky, M., 1977. The in vitro biosynthesis of steroids by the gonad of the cuttlefish (*Sepia officinalis* L.). *General and comparative endocrinology*, 33, 554.

Casals-Casas, C., Desvergne, B., 2011. Endocrine disruptors: from endocrine to metabolic disruption. *Annual review of physiology*, 73, 135-162.

Castano, A., Cantarino, M., Castillo, P., Tarazona, J., 1996. Correlations between the RTG-2 cytotoxicity test EC50 and in vivo LC50 rainbow trout bioassay. *Chemosphere*, 32, 2141-2157.

Celander, M., Leaver, M.J., George, S.G., Förlin, L., 1993. Induction of cytochrome P450 1A1 and conjugating enzymes in rainbow trout (*Oncorhynchus mykiss*) liver: a time course study. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 106, 343-349.

Celander, M., Buhler, D.R., Förlin, L., Goksøyr, A., Miranda, C.L., Woodin, B.R., Stegeman, J.J., 1996. Immunochemical relationships of cytochrome P4503A-like proteins in teleost fish. *Fish physiology and biochemistry*, 15, 323-332.

Celander, M., Stegeman, J.J., 1997. Isolation of a Cytochrome P450 3A cDNA Sequence (CYP3A30) from the Marine Teleost *Fundulus heteroclitus* and Phylogenetic Analyses of *CYP3A* Genes. *Biochemical and biophysical research communications*, 236, 306-312.

Celander, M., Moore, M., Stegeman, J., 2000. Cellular localization of CYP3A proteins in various tissues from pilot whale (*Globicephala melas*). *Environmental toxicology and pharmacology*, 8, 245-253.

Céspedes, R., Lacorte, S., Ginebreda, A., Barceló, D., 2006. Chemical monitoring and occurrence of alkylphenols, alkylphenol ethoxylates, alcohol ethoxylates, phthalates and benzothiazoles in sewage treatment plants and receiving waters along the Ter River basin (Catalonia, N. E. Spain). *Analytical and Bioanalytical Chemistry*, 385, 992-1000.

Chambon, P., 1996. A decade of molecular biology of retinoic acid receptors. *The FASEB Journal*, 10, 940-954.

Chang, C.-Y., Puga, A., 1998. Constitutive activation of the aromatic hydrocarbon receptor. *Molecular and cellular biology*, 18, 525-535.

Chang, C., 2002. Androgens and androgen receptor: mechanisms, functions, and clinical applications. Springer.

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. *Science*, 294, 1866-1870.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and comparative endocrinology*, 155, 31-62.

Chicano, J.J., Ortiz, A., Teruel, J.A., Aranda, F.J., 2001. Organotin compounds alter the physical organization of phosphatidylcholine membranes. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1510, 330-341.

Chinetti, G., Lestavel, S., Bocher, V., Remaley, A.T., Neve, B., Torra, I.P., Teissier, E., Minnich, A., Jaye, M., Duverger, N., 2001. PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nature medicine*, 7, 53-58.

Chishti, Y.Z., Feswick, A., Munkittrick, K.R., Martyniuk, C.J., 2013. Transcriptomic profiling of progesterone in the male fathead minnow (Pimephales promelas) testis. *General and Comparative Endocrinology*, 192, 115-125.

Chishti, Y.Z., Feswick, A., Martyniuk, C.J., 2014. Progesterone increases ex vivo testosterone production and decreases the expression of progestin receptors and steroidogenic enzymes in the fathead minnow (Pimephales promelas) ovary. *General and Comparative Endocrinology*, 199, 16-25.

Clarke, D.J., George, S.G., Burchell, B., 1992. Multiplicity of UDP-glucuronosyltransferases in fish. Purification and characterization of a phenol UDP-glucuronosyltransferase from the liver of a marine teleost, *Pleuronectes platessa*. *Biochem. J*, 284, 417-423.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental health perspectives*, 101, 378.

Coors, A., Jones, P.D., Giesy, J.P., Ratte, H.T., 2003. Removal of estrogenic activity from municipal waste landfill leachate assessed with a bioassay based on reporter gene expression. *Environmental Science & Technology*, 37, 3430-3434.

Couch, E., Hagino, N., Lee, J., 1987. Changes in estradiol and progesterone immunoreactivity in tissues of the lobster, *Homarus americanus*, with developing and immature ovaries. *Comparative Biochemistry and Physiology Part A: Physiology*, 87, 765-770.

Cui, H., Okuhira, K., Ohoka, N., Naito, M., Kagechika, H., Hirose, A., Nishimaki-Mogami, T., 2011. Tributyltin chloride induces ABCA1 expression and apolipoprotein A-I-mediated cellular cholesterol efflux by activating LXRalpha/RXR. *Biochemical pharmacology*, 81, 819-824.

D'Aniello, A., Di Cosmo, A., Di Cristo, C., Assisi, L., Botte, V., Di Fiore, M., 1996. Occurrence of sex steroid hormones and their binding proteins in *Octopus vulgaris* Lam. *Biochemical and biophysical research communications*, 227, 782.

Dalen, K.T., Schoonjans, K., Ulven, S.M., Weedon-Fekjaer, M.S., Bentzen, T.G., Koutnikova, H., Auwerx, J., Nebb, H.I., 2004. Adipose tissue expression of the lipid droplet–associating proteins S3-12 and perilipin is controlled by peroxisome proliferator–activated receptor-γ. *Diabetes*, 53, 1243-1252.

De Longcamp, D., Lubet, P., Drosdowsky, M., 1974. The in vitro biosynthesis of steroids by the gonad of the mussel (*Mytilus edulis*). *General and comparative endocrinology*, 22, 116-127.

De Waziers, I., Cugnenc, P., Yang, C., Leroux, J., Beaune, P., 1990. Cytochrome P 450 isoenzymes, epoxide hydrolase and glutathione transferases in rat and human hepatic and extrahepatic tissues. *Journal of Pharmacology and Experimental Therapeutics*, 253, 387-394.

Depledge, M.H., Billinghurst, Z., 1999. Ecological significance of endocrine disruption in marine invertebrates. *Marine Pollution Bulletin*, 39, 32-38.

Desbrow, C., Routledge, E., Brighty, G., Sumpter, J., Waldock, M., 1998. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environmental Science & Technology*, 32, 1549-1558.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocrine Reviews*, 20, 649-688.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191-364.

Di Cosmo, A., Di Cristo, C., Paolucci, M., 2001. Sex steroid hormone fluctuations and morphological changes of the reproductive system of the female of *Octopus vulgaris* throughout the annual cycle. *Journal of Experimental Zoology*, 289, 33-47.

Dieleman, S., Schoenmakers, H., 1979. Radioimmunoassays to determine the presence of progesterone and estrone in the starfish *Asterias rubens*. *General and comparative endocrinology*, 39, 534.

Dirkx, W., Ceulemans, M., Adams, F., 1993. Determination of methyl-and butylin compounds in waters of the Antwerp harbour. *Science of The Total Environment*, 136, 279-300.

Duedahl-Olesen, L., Cederberg, T., Pedersen, K.H., Højgård, A., 2005. Synthetic musk fragrances in trout from Danish fish farms and human milk. *Chemosphere*, 61, 422-431.

Edwards, P.A., Kennedy, M.A., Mak, P.A., 2002. LXRs;: Oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. *Vascular pharmacology*, 38, 249-256.

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J.-C., Pieters, R., 2002. Methods of in vitro toxicology. *Food and Chemical Toxicology*, 40, 193-236.

Elobeid, M.A., Allison, D.B., 2008. Putative environmental-endocrine disruptors and obesity: a review. *Current opinion in endocrinology, diabetes, and obesity*, 15, 403.

Escher, P., Wahli, W., 2000. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 448, 121-138.

Fairs, N., Evershed, R., Quinlan, P., Goad, L., 1989. Detection of unconjugated and conjugated steroids in the ovary, eggs, and haemolymph of the decapod crustacean *Nephrops norvegicus*. *General and comparative endocrinology*, 74, 199.

Falkenstein, E., Tillmann, H.-C., Christ, M., Feuring, M., Wehling, M., 2000. Multiple actions of steroid hormones—a focus on rapid, nongenomic effects. *Pharmacological reviews*, 52, 513-556.

Feige, J.N., Gelman, L., Michalik, L., Desvergne, B., Wahli, W., 2006. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Progress in Lipid Research*, 45, 120-159.

Feige, J.N., Gelman, L., Rossi, D., Zoete, V., Métivier, R., Tudor, C., Anghel, S.I., Grosdidier, A., Lathion, C., Engelborghs, Y., 2007. The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor γ modulator that promotes adipogenesis. *Journal of Biological Chemistry*, 282, 19152-19166.

Feige, J.N., Gerber, A., Casals-Casas, C., Yang, Q., Winkler, C., Bedu, E., Bueno, M., Gelman, L., Auwerx, J., Gonzalez, F.J., 2010. The pollutant diethylhexyl phthalate regulates hepatic energy metabolism via species-specific PPARα-dependent mechanisms. *Environmental health perspectives (Online)*, 118, 234.

Fenet, H., Casellas, C., Bontoux, J., 1998. Laboratory and field-caging studies on hepatic enzymatic activities in European eel and rainbow trout. *Ecotoxicology and Environmental Safety*, 40, 137-143.

Fent, K., 1996. Ecotoxicology of organotin compounds. *CRC Critical Reviews in Toxicology*, 26, 3-117.

Fent, K., Woodin, B.R., Stegeman, J.J., 1998. Effects of triphenyltin and other organotins on hepatic monooxygenase system in fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 121, 277-288.

Fent, K., 2001. Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. *Toxicology in vitro*, 15, 477-488.

Fent, K., 2006. Worldwide occurrence of organotins from antifouling paints and effects in the aquatic environment. In: Antifouling paint biocides. Springer, 71-100.

Fernandes, D., Potrykus, J., Morsiani, C., Raldua, D., Lavado, R., Porte, C., 2002. The combined use of chemical and biochemical markers to assess water quality in two low-stream rivers (NE Spain). *Environmental research*, 90, 169-178.

Fernandes, D., Bebianno, M., Porte, C., 2007. Mitochondrial metabolism of 17alpha-hydroxyprogesterone in male sea bass (*Dicentrarchus labrax*): a potential target for endocrine disruptors. *Aquatic toxicology (Amsterdam, Netherlands)*, 85, 258.

Fernandes, D., Navarro, J.C., Riva, C., Bordonali, S., Porte, C., 2010. Does exposure to testosterone significantly alter endogenous metabolism in the marine mussel *Mytilus galloprovincialis*? *Aquatic toxicology*, 100, 313-320.

Fernandes, D., Loi, B., Porte, C., 2011. Biosynthesis and metabolism of steroids in molluscs. *The Journal of steroid biochemistry and molecular biology*, 127, 189-195.

Ferré, P., 2004. The biology of peroxisome proliferator-activated receptors relationship with lipid metabolism and insulin sensitivity. *Diabetes*, 53, S43-S50.

Fiege, H., Voges, H.W., Hamamoto, T., Umemura, S., Iwata, T., Miki, H., Fujita, Y., Buysch, H.J., Garbe, D., Paulus, W., 2000. Phenol derivatives. *Ullmann's encyclopedia of industrial chemistry*.

Fievet, C., Staels, B., 2009. Liver X receptor modulators: effects on lipid metabolism and potential use in the treatment of atherosclerosis. *Biochemical pharmacology*, 77, 1316-1327.

Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., Wenzel, A., 2002. Occurrence of phthalates and bisphenol A and F in the environment. *Water research*, 36, 1429-1438.

Gabrielska, J., Przestalski, S., Miszta, A., Soczyńska-Kordala, M., Langner, M., 2004. The effect of cholesterol on the adsorption of phenyltin compounds onto phosphatidylcholine and sphingomyelin liposome membranes. *Applied organometallic chemistry*, 18, 9-14.

Gadagbui, B.K.-M., Goksrøyr, A., 1996. CYP1A andother biomarker responses to effluents from a textile mill in the Volta River (Ghana) using caged tilapia (*Oreochromis niloticus*) andsediment-exposed mudfish (*Clarias anguillaris*). *Biomarkers*, 1, 252-261.

Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. *Aquatic toxicology*, 61, 169-180.

García-Jares, C., Llompart, M., Polo, M., Salgado, C., Macías, S., Cela, R., 2002. Optimisation of a solid-phase microextraction method for synthetic musk compounds in water. *Journal of Chromatography A*, 963, 277-285.

Gatermann, R., Biselli, S., Hühnerfuss, H., Rimkus, G., Hecker, M., Karbe, L., 2002. Synthetic musks in the environment. Part 1: Species-dependent bioaccumulation of polycyclic and nitro musk fragrances in freshwater fish and mussels. *Archives of Environmental Contamination and Toxicology*, 42, 437-446.

Gauthier-Clerc, S., Pellerin, J., Amiard, J.C., 2006. Estradiol-17β and testosterone concentrations in male and female *Mya arenaria* (Mollusca bivalvia) during the reproductive cycle. *General and comparative endocrinology*, 145, 133-139.

Gaworecki, K.M., Rice, C.D., van den Hurk, P., 2004. Induction of phenol-type sulfotransferase and glucuronosyltransferase in channel catfish and mummichog. *Marine Environmental Research*, 58, 525-528.

Genicot, G., Leroy, J.L.M.R., Soom, A.V., Donnay, I., 2005. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. *Theriogenology*, 63, 1181-1194.

George, S.G., 1994. Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. *Aquatic toxicology*, 4, 37-85.

Ghosh, C., Zhou, Y., Collodi, P., 1994. Derivation and characterization of a zebrafish liver cell line. *Cell biology and toxicology*, 10, 167-176.

Giger, W., Brunner, P.H., Schaffner, C., 1984. 4-Nonylphenol in sewage sludge: Accumulation of toxic metabolites from nonionic surfactants. *Science*, 225, 623-625.

Gilliland, F.D., Mandel, J.S., 1996. Serum perfluorooctanoic acid and hepatic enzymes, lipoproteins, and cholesterol: a study of occupationally exposed men. *American journal of industrial medicine*, 29, 560-568.

Gimeno, S., Komen, H., Venderbosch, P.W., Bowmer, T., 1997. Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. *Environmental Science & Technology*, 31, 2884-2890.

Goksøyr, A., Förlin, L., 1992. The cytochrome P4501A system in fish, aquatic toxicology and environmental monitoring. *Aquatic toxicology*, 22, 287-311.

Goksøyr, A., 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. In: Toxicology in Transition. Springer, 80-95.

Gooding, M.P., LeBlanc, G.A., 2001. Biotransformation and disposition of testosterone in the eastern mud snail *Ilyanassa obsoleta*. *General and comparative endocrinology*, 122, 172-180.

Goodman, J.E., McConnell, E.E., Sipes, I.G., Witorsch, R.J., Slayton, T.M., Yu, C.J., Lewis, A.S., Rhomberg, L.R., 2006. An updated weight of the evidence evaluation of reproductive and developmental effects of low doses of bisphenol A. *CRC Critical Reviews in Toxicology*, 36, 387-457.

Goodson, A., Summerfield, W., Cooper, I., 2002. Survey of bisphenol A and bisphenol F in canned foods. *Food Additives & Contaminants*, 19, 796-802.

Goto, Y., Kajiwara, M., Yanagisawa, Y., Hirose, H., Yoshimi, T., Umemura, M., Nakano, H., Takahashi, S., Shida, Y., Iguchi, T., 2012. Detection of vertebrate-type steroid hormones and their converting activities in the neogastropod *Thais clavigera* (Küster, 1858). *Journal of Molluscan Studies*, 78, 197-204.

Gray, B.H., Porvaznik, P., Flemming, C., Lee, L.H., 1987. Tri-n-butyltin: A membrane toxicant. *Toxicology*, 47, 35-54.

Greenspan, P., Mayer, E.P., Fowler, S.D., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. *The Journal of cell biology*, 100, 965-973.

Grefhorst, A., Elzinga, B.M., Voshol, P.J., Plösch, T., Kok, T., Bloks, V.W., van der Sluijs, F.H., Havekes, L.M., Romijn, J.A., Verkade, H.J., 2002. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *Journal of Biological Chemistry*, 277, 34182-34190.

Grün, F., Blumberg, B., 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology*, 147, s50-s55.

Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T., Blumberg, B., 2006. Endocrine-Disrupting Organotin Compounds Are Potent Inducers of Adipogenesis in Vertebrates. *Molecular Endocrinology*, 20, 2141-2155.

Grün, F., Blumberg, B., 2007. Perturbed nuclear receptor signaling by environmental obesogens as emerging factors in the obesity crisis. *Reviews in Endocrine and Metabolic Disorders*, 8, 161-171.

Grün, F., Blumberg, B., 2009a. Endocrine disrupters as obesogens. *Molecular and Cellular Endocrinology*, 304, 19-29.

Grün, F., Blumberg, B., 2009b. Minireview: the case for obesogens. *Molecular Endocrinology*, 23, 1127-1134.

Guan, H.-P., Li, Y., Jensen, M.V., Newgard, C.B., Steppan, C.M., Lazar, M.A., 2002. A futile metabolic cycle activated in adipocytes by antidiabetic agents. *Nature medicine*, 8, 1122-1128.

Guan, H.-P., Ishizuka, T., Chui, P.C., Lehrke, M., Lazar, M.A., 2005. Corepressors selectively control the transcriptional activity of PPARγ in adipocytes. *Genes & Development*, 19, 453-461.

Guengerich, F.P., 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annual review of pharmacology and toxicology*, 39, 1-17.

Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.-F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. *General and comparative endocrinology*, 165, 352-366.

Gust, M., Vulliet, E., Giroud, B., Garnier, F., Couturier, S., Garric, J., Buronfosse, T., 2010. Development, validation and comparison of LC–MS/MS and RIA methods for quantification of vertebrates-like sex-steroids in prosobranch molluscs. *Journal of Chromatography B*, 878, 1487-1492.

Han, X., Gross, R.W., 2001. Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. *Analytical biochemistry*, 295, 88-100.

Hasselberg, L., Grøsvik, B.E., Goksøyr, A., Celander, M.C., 2005. Interactions between xenoestrogens and ketoconazole on hepatic CYP1A and CYP3A, in juvenile Atlantic cod (Gadus morhua). *Comparative hepatology*, 4, 2.

Hasselberg, L., Westerberg, S., Wassmur, B., Celander, M.C., 2008. Ketoconazole, an antifungal imidazole, increases the sensitivity of rainbow trout to 17α-ethynylestradiol exposure. *Aquatic toxicology*, 86, 256-264.

Hatch, E., Nelson, J., Qureshi, M.M., Weinberg, J., Moore, L., Singer, M., Webster, T., 2008. Association of urinary phthalate metabolite concentrations with body mass index and waist circumference: a cross-sectional study of NHANES data, 1999-2002. *Environmental Health*, 7, 27.

Hegelund, T., Celander, M.C., 2003. Hepatic versus extrahepatic expression of CYP3A30 and CYP3A56 in adult killifish (*Fundulus heteroclitus*). *Aquatic toxicology*, 64, 277-291.

Heindel, J.J., 2003. Endocrine disruptors and the obesity epidemic. *Toxicological Sciences*, 76, 247-249.

Hesselsøe, M., Jensen, D., Skals, K., Olesen, T., Moldrup, P., Roslev, P., Krog Mortensen, G., Henriksen, K., 2001. Degradation of 4-nonylphenol in homogeneous and nonhomogeneous mixtures of soil and sewage sludge. *Environmental Science and Technology*, 35, 3695-3700.

Heuvel, J.P.V., Thompson, J.T., Frame, S.R., Gillies, P.J., 2006. Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-α,-β, and-γ, liver X receptor-β, and retinoid X receptor-α. *Toxicological Sciences*, 92, 476-489.

Higashiyama, T., Shiraishi, H., Otsuki, A., Hashimoto, S., 1991. Concentrations of organotin compounds in blue mussels from the wharves of Tokyo Bay. *Marine Pollution Bulletin*, 22, 585-587.

Hines, G.A., Watts, S.A., Sower, S.A., Walker, C.W., 1992. Sex steroid levels in the testes, ovaries, and pyloric caeca during gametogenesis in the sea star Asterias vulgaris. *General and comparative endocrinology*, 87, 451-460.

Hines, G.A., Bryan, P.J., Wasson, K.M., McClintock, J.B., Watts, S.A., 1996. Sex steroid metabolism in the antarctic pteropod *Clione antarctica* (Mollusca: Gastropoda). *Invertebrate Biology*, 115, 113-119.

Hochberg, R.B., 1998. Biological esterification of steroids. *Endocrine Reviews*, 19, 331-348.

Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G., Vom Saal, F.S., 1999. Environmental toxins: exposure to bisphenol A advances puberty. *Nature*, 401, 763-764.

Hulbert, A.J., Kelly, M.A., Abbott, S.K., 2014. Polyunsaturated fats, membrane lipids and animal longevity. *Journal of Comparative Physiology B*, 184, 149-166.

Hurst, C.H., Waxman, D.J., 2003. Activation of PPARα and PPARγ by environmental phthalate monoesters. *Toxicological Sciences*, 74, 297-308.

Ide, T., Shimano, H., Yoshikawa, T., Yahagi, N., Amemiya-Kudo, M., Matsuzaka, T., Nakakuki, M., Yatoh, S., Iizuka, Y., Tomita, S., 2003. Cross-talk between peroxisome proliferator-activated receptor (PPAR) α and liver X receptor (LXR) in nutritional regulation of fatty acid metabolism. II. LXRs suppress lipid degradation gene promoters through inhibition of PPAR signaling. *Molecular Endocrinology*, 17, 1255-1267.

Iguchi, T., Katsu, Y., Horiguchi, T., Watanabe, H., Blumberg, B., Ohta, Y., 2007. Endocrine disrupting organotin compounds are potent inducers of imposex in gastropods and adipogenesis in vertebrates. *Mol Cell Toxicol*, 3, 1-10.

Ikegami, S., Shirai, H., Kanatani, H., 1971. On the occurrence of progesterone in ovary of the starfish *Asterias amurensis*. *Zool. Mag.*, 26-28.

Inadera, H., Shimomura, A., 2005. Environmental chemical tributyltin augments adipocyte differentiation. *Toxicology Letters*, 159, 226-234.

Ioannides, C., 2002. Enzyme systems that metabolise drugs and other xenobiotics. J. Wiley.

Ioannides, C., Lewis, V., David, F., 2004. Cytochromes P450 in the bioactivation of chemicals. *Current topics in medicinal chemistry*, 4, 1767-1788.

Itsuki-Yoneda, A., Kimoto, M., Tsuji, H., Hiemori, M., Yamashita, H., 2007. Effect of a hypolipidemic drug, di (2-ethylhexyl) phthalate, on mRNA-expression associated fatty acid and acetate metabolism in rat tissues. *Bioscience, biotechnology, and biochemistry*, 71, 414-420.

Ivanova, P.T., Milne, S.B., Myers, D.S., Brown, H.A., 2009. Lipidomics: a mass spectrometry based systems level analysis of cellular lipids. *Current Opinion in Chemical Biology*, 13, 526-531.

Iyer, M., Reschly, E.J., Krasowski, M.D., 2006. Functional evolution of the pregnane X receptor.

Janer, G., Mesia-Vela, S., Porte, C., Kauffman, F.C., 2004. Esterification of vertebrate-type steroids in the Eastern oyster (*Crassostrea virginica*). *Steroids*, 69, 129-136.

Janer, G., Lavado, R., Thibaut, R., Porte, C., 2005a. Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*: A possible regulating role for steroid acyltransferases. *Aquatic toxicology*, 75, 32-42.

Janer, G., LeBlanc, G., Porte, C., 2005b. Androgen metabolism in invertebrates and its modulation by xenoandrogens: a comparative study. *Annals of the New York Academy of Sciences*, 1040, 354-356.

Janer, G., Sternberg, R., LeBlanc, G., Porte, C., 2005c. Testosterone conjugating activities in invertebrates: are they targets for endocrine disruptors? *Aquatic toxicology*, 71, 273-282.

Janer, G., Lyssimachou, A., Bachmann, J., Oehlmann, J., Schulte-Oehlmann, U., Porte, C., 2006. Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds. *Steroids*, 71, 435.

Janer, G., Navarro, J.C., Porte, C., 2007. Exposure to TBT increases accumulation of lipids and alters fatty acid homeostasis in the ramshorn snail *Marisa cornuarietis*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146, 368-374.

Janer, G., Porte, C., 2007. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. *Ecotoxicology*, 16, 145-160.

Jobling, S., Sumpter, J.P., 1993. Detergent components in sewage effluent are weakly oestrogenic to fish: an in vitro study using rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic toxicology*, 27, 361-372.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. *Environmental Science & Technology*, 32, 2498-2506.

John, D.M., House, W.A., White, G.F., 2000. Environmental fate of nonylphenol ethoxylates: differential adsorption of homologs to components of river sediment. *Environmental Toxicology and Chemistry*, 19, 293-300.

Johnson, A.C., Sumpter, J.P., 2001. Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environmental Science & Technology*, 35, 4697-4703.

Joseph, S.B., Laffitte, B.A., Patel, P.H., Watson, M.A., Matsukuma, K.E., Walczak, R., Collins, J.L., Osborne, T.F., Tontonoz, P., 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *Journal of Biological Chemistry*, 277, 11019-11025.

Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T., Nishikawa, J.-i., 2005. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor γ/retinoid X receptor pathway. *Molecular pharmacology*, 67, 766-774.

Kang, J.-H., Katayama, Y., Kondo, F., 2006. Biodegradation or metabolism of bisphenol A: from microorganisms to mammals. *Toxicology*, 217, 81-90.

Kaur, S., Jobling, S., Jones, C.S., Noble, L.R., Routledge, E.J., Lockyer, A.E., 2015. The nuclear receptors of Biomphalaria glabrata and Lottia gigantea: Implications for developing new model organisms.

Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Kaattari, S., Lucier, G., Luster, M., Mac, M.J., Maczka, C., 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the US EPA-sponsored workshop. *Environmental health perspectives*, 104, 715.

Kawagoshi, Y., Fujita, Y., Kishi, I., Fukunaga, I., 2003. Estrogenic chemicals and estrogenic activity in leachate from municipal waste landfill determined by yeast two-hybrid assay. *Journal of Environmental Monitoring*, 5, 269-274.

Kazeto, Y., Ijiri, S., Matsubara, H., Adachi, S., Yamauchi, K., 2000. Cloning of 17β-hydroxysteroid dehydrogenase-I cDNAs from Japanese eel ovary. *Biochemical and biophysical research communications*, 279, 451-456.

Ketata, I., Guermazi, F., Rebai, T., Hamza-Chaffai, A., 2007. Variation of steroid concentrations during the reproductive cycle of the clam *Ruditapes decussatus*: A one year study in the gulf of Gabès area. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 147, 424-431.

Kim, J.B., Spiegelman, B.M., 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes & Development*, 10, 1096-1107.

Kime, D.E., 1993. 'Classical' and 'non-classical' reproductive steroids in fish. *Reviews in Fish Biology and Fisheries*, 3, 160-180.

Kirchner, S., Kieu, T., Chow, C., Casey, S., Blumberg, B., 2010. Prenatal exposure to the environmental obesogen tributyltin predisposes multipotent stem cells to become adipocytes. *Molecular Endocrinology*, 24, 526-539.

Knight, B.L., Patel, D.D., Humphreys, S.M., Wiggins, D., Gibbons, G.F., 2003. Inhibition of cholesterol absorption associated with a PPARα-dependent increase in ABC binding cassette transporter A1 in mice. *Journal of Lipid Research*, 44, 2049-2058.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology*, 36, 1202-1211.

Körner, W., Bolz, U., Triebskorn, R., Schwaiger, J., Negele, R.-D., Marx, A., Hagnemaier, H., 2001. Steroid analysis and xenosteroid potentials in two small streams in southwest Germany. *Journal of Aquatic Ecosystem Stress and Recovery*, 8, 215-229.

Kota, B.P., Huang, T.H.-W., Roufogalis, B.D., 2005. An overview on biological mechanisms of PPARs. *Pharmacological Research*, 51, 85-94.

Krasowski, M.D., Drees, D., Morris, C.S., Maakestad, J., Blau, J.L., Ekins, S., 2014. Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. *BMC clinical pathology*, 14, 33.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M.G., Wahli, W., 1997. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Molecular Endocrinology*, 11, 779-791.

Krishnan, A.V., Stathis, P., Permuth, S.F., Tokes, L., Feldman, D., 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology*, 132, 2279-2286.

Kuch, H.M., Ballschmiter, K., 2001. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environmental Science & Technology*, 35, 3201-3206.

Kullman, S., Hamm, J., Hinton, D., 2000. Identification and characterization of a cDNA encoding cytochrome P450 3A from the fresh water teleost medaka (*Oryzias latipes*). *Archives of biochemistry and biophysics*, 380, 29-38.

Kusakabe, M., Nakamura, I., Young, G., 2003. 11β-Hydroxysteroid dehydrogenase complementary deoxyribonucleic acid in rainbow trout: cloning, sites of expression, and seasonal changes in gonads. *Endocrinology*, 144, 2534-2545.

Labrie, F., Luu-The, V., Lin, S.-X., Claude, L., Simard, J., Breton, R., Bélanger, A., 1997. The key role of 17β-hydroxysteroid dehydrogenases in sex steroid biology. *Steroids*, 62, 148-158.

Lakra, W.S., Swaminathan, T.R., Joy, K.P., 2011. Development, characterization, conservation and storage of fish cell lines: a review. *Fish physiology and biochemistry*, 37, 1-20.

Lange, I.G., Daxenberger, A., Schiffer, B., Witters, H., Ibarreta, D., Meyer, H.H.D., 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. *Analytica Chimica Acta*, 473, 27-37.

Latini, G., Verrotti, A., De Felice, C., 2004. Di-2-ethylhexyl phthalate and endocrine disruption: a review. *Current Drug Targets-Immune, Endocrine & Metabolic Disorders*, 4, 37-40.

Laughlin Jr, R.B., 1996. Bioaccumulation of TBT by aquatic organisms. In: Organotin. Springer, 331-355.

Lavado, R., Thibaut, R., Raldúa, D., Martín, R., Porte, C., 2004. First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicology and Applied Pharmacology*, 196, 247-257.

Lavado, R., Sugni, M., Candia Carnevali, M.D., Porte, C., 2006a. Triphenyltin alters androgen metabolism in the sea urchin *Paracentrotus lividus*. *Aquatic toxicology*, 79, 247-256.

Lavado, R., Ureña, R., Martin-Skilton, R., Torreblanca, A., del Ramo, J., Raldúa, D., Porte, C., 2006b. The combined use of chemical and biochemical markers to assess water quality along the Ebro River. *Environmental Pollution*, 139, 330-339.

Le Curieux-Belfond, O., Moslemi, S., Mathieu, M., Séralini, G., 2001. Androgen metabolism in oyster *Crassostrea gigas*: evidence for 17beta-HSD activities and characterization of an aromatase-like activity inhibited by pharmacological compounds and a marine pollutant. *The Journal of steroid biochemistry and molecular biology*, 78, 359.

Le Guellec, D., Thiard, M., Remy-Martin, J., Deray, A., Gomot, L., Adessi, G., 1987. In vitro metabolism of androstenedione and identification of endogenous steroids in *Helix aspersa*. *General and comparative endocrinology*, 66, 425.

Lee, H.J., Chattopadhyay, S., Gong, E.-Y., Ahn, R.S., Lee, K., 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. *Toxicological Sciences*, 75, 40-46.

Lee, L.E., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K.-B., Pasitschniak-Arts, M., Mosser, D.D., Bols, N.C., 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. *Cell biology and toxicology*, 9, 279-294.

Lee, S.-J., Wang-Buhler, J.-L., Cok, I., Yu, T.-S., Yang, Y.-H., Miranda, C.L., Lech, J., Buhler, D.R., 1998. Cloning, sequencing, and tissue expression of CYP3A27, a new member of the CYP3A subfamily from embryonic and adult rainbow trout livers. *Archives of biochemistry and biophysics*, 360, 53-61.

Lee, S.-J., Buhler, D.R., 2003. Cloning, tissue distribution, and functional studies of a new cytochrome P450 3A subfamily member, CYP3A45, from rainbow trout (*Oncorhynchus mykiss*) intestinal ceca. *Archives of biochemistry and biophysics*, 412, 77-89.

Leone, T.C., Weinheimer, C.J., Kelly, D.P., 1999. A critical role for the peroxisome proliferator-activated receptor α (PPARα) in the cellular fasting response: the PPARα-null mouse as a model of fatty acid oxidation disorders. *Proceedings of the National Academy of Sciences*, 96, 7473-7478.

Lesharpig, J., Fuchs, B., 2009. Plasmalogens in biological systems: their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis. *Current medicinal chemistry*, 16, 2021-2041.

Lester, S.M., Braunbeck, T.A., Teh, S.J., Stegeman, J.J., Miller, M.R., Hinton, D.E., 1993. Hepatic cellular distribution of cytochrome P-450 IA1 in rainbow trout (Oncorhynchus mykiss): an immunohisto-and cytochemical study. *Cancer research*, 53, 3700-3706.

Lewis, D.F., 2004. 57 varieties: the human cytochromes P450. *Pharmacogenomics*, 5, 305-318.

Lewis, D.F., Ito, Y., Goldfarb, P.S., 2006. Cytochrome P450 structures and their substrate interactions. *Drug development research*, 66, 19-24.

Liang, Y.-Q., Huang, G.-Y., Liu, S.-S., Zhao, J.-L., Yang, Y.-Y., Chen, X.-W., Tian, F., Jiang, Y.-X., Ying, G.-G., 2015. Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*). *Aquatic toxicology*, 160, 172-179.

Lin, R.Z., Chang, H.Y., 2008. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology journal*, 3, 1172-1184.

Lindholst, C., Pedersen, K.L., Pedersen, S.N., 2000. Estrogenic response of bisphenol A in rainbow trout (*Oncorhynchus mykiss*). *Aquatic toxicology*, 48, 87-94.

Lintelmann, J., Katayama, A., Kurihara, N., Shore, L., Wenzel, A., 2003. Endocrine disruptors in the environment (IUPAC Technical Report). *Pure and applied chemistry*, 75, 631.

Liu, S., Govoroun, M., D'Cotta, H., Ricordel, M.-J., Lareyre, J.-J., McMeel, O.M., Smith, T., Nagahama, Y., Guiguen, Y., 2000. Expression of cytochrome $P450_{11\beta}$ (11β-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. *The Journal of steroid biochemistry and molecular biology*, 75, 291-298.

Liu, S., Ying, G.-G., Zhao, J.-L., Zhou, L.-J., Yang, B., Chen, Z.-F., Lai, H.-J., 2012. Occurrence and fate of androgens, estrogens, glucocorticoids and progestagens in two different types of municipal wastewater treatment plants. *Journal of Environmental Monitoring*, 14, 482-491.

Lopez-Espinosa, M., Freire, C., Arrebola, J., Navea, N., Taoufiki, J., Fernandez, M., Ballesteros, O., Prada, R., Olea, N., 2009. Nonylphenol and octylphenol in adipose tissue of women in Southern Spain. *Chemosphere*, 76, 847-852.

Lorenc, J.F., Lambeth, G., Scheffer, W., 1992. Alkylphenols. *Kirk-Othmer Encyclopedia of Chemical Technology*.

Lupo di Prisco, C., Fulgheri, F.D., Tomasucci, M., 1973. Identification and biosynthesis of steroids in the marine mollusc *Aplysia depilans*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 45, 303-310.

Lupo di Prisco, C., Dessi' Fulgheri, F., 1975. Alternative pathways of steroid biosynthesis in gonads and hepatopancreas of *Aplysia depilans*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 50, 191-195.

Luu-The, V., 2001. Analysis and characteristics of multiple types of human 17β-hydroxysteroid dehydrogenase. *The Journal of steroid biochemistry and molecular biology*, 76, 143-151.

Lyssimachou, A., Navarro, J.C., Bachmann, J., Porte, C., 2009. Triphenyltin alters lipid homeostasis in females of the ramshorn snail *Marisa cornuarietis*. *Environmental Pollution*, 157, 1714-1720.

Ma, D., Liu, W., Wang, Y., 2014. ApoA-I or ABCA1 expression suppresses fatty acid synthesis by reducing 27-hydroxycholesterol levels. *Biochimie*, 103, 101-108.

Madsen, L., Petersen, R.K., Kristiansen, K., 2005. Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1740, 266-286.

Malhão, F., Urbatzka, R., Navas, J., Cruzeiro, C., Monteiro, R., Rocha, E., 2013. Cytological, immunocytochemical, ultrastructural and growth characterization of the rainbow trout liver cell line RTL-W1. *Tissue and Cell*, 45, 159-174.

Mandard, S., Müller, M., Kersten, S., 2004. Peroxisome proliferator-activated receptor α target genes. *Cellular and Molecular Life Sciences CMLS*, 61, 393-416.

Mangelsdorf, D.J., Evans, R.M., 1995. The RXR heterodimers and orphan receptors. *Cell*, 83, 841-850.

Margiotta-Casaluci, L., Hannah, R.E., Sumpter, J.P., 2013. Mode of action of human pharmaceuticals in fish: The effects of the 5-alpha-reductase inhibitor, dutasteride, on reproduction as a case study. *Aquatic toxicology*, 128, 113-123.

Marmugi, A., Ducheix, S., Lasserre, F., Polizzi, A., Paris, A., Priymenko, N., Bertrand-Michel, J., Pineau, T., Guillou, H., Martin, P.G., 2012. Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver. *Hepatology*, 55, 395-407.

Martin, G., Schoonjans, K., Lefebvre, A.-M., Staels, B., Auwerx, J., 1997. Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARα and PPARγ activators. *Journal of Biological Chemistry*, 272, 28210-28217.

Martin, S., Parton, R.G., 2006. Lipid droplets: a unified view of a dynamic organelle. *Nature Reviews Molecular Cell Biology*, 7, 373-378.

Masia, A., Avery, S.V., Zoroddu, M.A., Gadd, G.M., 1998. Enrichment with a polyunsaturated fatty acid enhances the survival of *Saccharomyces cerevisiae* in the presence of tributyltin. *FEMS microbiology letters*, 167, 321-326.

Masuno, H., Kidani, T., Sekiya, K., Sakayama, K., Shiosaka, T., Yamamoto, H., Honda, K., 2002. Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. *Journal of Lipid Research*, 43, 676-684.

Masuno, H., Iwanami, J., Kidani, T., Sakayama, K., Honda, K., 2005. Bisphenol A accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. *Toxicological Sciences*, 84, 319-327.

Matsumoto, H., Adachi, S., Suzuki, Y., 2005. Bisphenol A in ambient air particulates responsible for the proliferation of MCF-7 human breast cancer cells and Its concentration changes over 6 months. *Archives of Environmental Contamination and Toxicology*, 48, 459.

Matsusue, K., Peters, J.M., Gonzalez, F.J., 2004. PPARβ/δ potentiates PPARγ-stimulated adipocyte differentiation. *The FASEB Journal*, 18, 1477-1479.

Matthiessen, P., Gibbs, P.E., 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environmental Toxicology and Chemistry*, 17, 37-43.

McArthur, A.G., Hegelund, T., Cox, R.L., Stegeman, J.J., Liljenberg, M., Olsson, U., Sundberg, P., Celander, M.C., 2003. Phylogenetic analysis of the cytochrome P450 3 (CYP3) gene family. *Journal of molecular evolution*, 57, 200-211.

McCormick, S.D., O'Dea, M.F., Moeckel, A.M., Lerner, D.T., Björnsson, B.T., 2005. Endocrine disruption of parr-smolt transformation and seawater tolerance of Atlantic salmon by 4-nonylphenol and 17β-estradiol. *General and comparative endocrinology*, 142, 280-288.

McMaster, M.E., Jardine, J.J., Ankley, G.T., Benson, W.H., Greeley, M.S., Gross, T.S., Guillette, L.J., MacLatchy, D.L., Orlando, E.F., Van Der Kraak, G.J., 2001. An interlaboratory study on the use of steroid hormones in examining endocrine disruption. *Environmental Toxicology and Chemistry*, 20, 2081-2087.

Meador, J.P., Sommers, F.C., Cooper, K.A., Yanagida, G., 2011. Tributyltin and the obesogen metabolic syndrome in a salmonid. *Environmental research*, 111, 50-56.

Meier, S., Andersen, T.C., Lind-Larsen, K., Svardal, A., Holmsen, H., 2007. Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from female Atlantic cod (Gadus morhua). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 145, 420-430.

Mercier, A., Pelletier, É., Hamel, J.-F., 1994. Metabolism and subtle toxic effects of butyltin compounds in starfish. *Aquatic toxicology*, 28, 259-273.

Michalik, L., Wahli, W., 2007. Peroxisome proliferator-activated receptors (PPARs) in skin health, repair and disease. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1771, 991-998.

Miller, V.P., Stresser, D.M., Blanchard, A.P., Turner, S., Crespi, C.L., 2000. Fluorometric High-Throughput Screening for Inhibitors of Cytochrome P450. *Annals of the New York Academy of Sciences*, 919, 26-32.

Miller, W.L., 1988. Molecular Biology of Steroid Hormone Synthesis. *Endocrine Reviews*, 9, 295-318.

Miller, W.L., 2002. Androgen biosynthesis from cholesterol to DHEA. *Molecular and Cellular Endocrinology*, 198, 7-14.

Mindnich, R., Deluca, D., Adamski, J., 2004a. Identification and characterization of 17β-hydroxysteroid dehydrogenases in the zebrafish, *Danio rerio*. *Molecular and Cellular Endocrinology*, 215, 19-30.

Mindnich, R., Möller, G., Adamski, J., 2004b. The role of 17 beta-hydroxysteroid dehydrogenases. *Molecular and Cellular Endocrinology*, 218, 7-20.

Miura, C., Miura, T., Yamashita, M., Yamauchi, K., Nagahama, Y., 1996. Hormonal induction of all stages of spermatogenesis in germ-somatic cell coculture from immature Japanese eel testis. *Development, growth & differentiation*, 38, 257-262.

Moldovan, Z., 2006. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. *Chemosphere*, 64, 1808-1817.

Monteiro, P.R.R., Reis-Henriques, M.A., Coimbra, J., 2000. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (Platichthys flesus L.). *Aquatic toxicology*, 48, 549-559.

Morais, S., Boaventura, D., Ré, P., Hawkins, S.J., 2003. Gonad development and fatty acid composition of *Patella depressa* Pennant (Gastropoda: Prosobranchia) populations with different patterns of spatial distribution, in exposed and sheltered sites. *Journal of experimental marine biology and ecology*, 294, 61-80.

Morcillo, Y., Albalat, A., Porte, C., 1999. Mussels as sentinels of organotin pollution: Bioaccumulation and effects on P450-mediated aromatase activity. *Environmental Toxicology and Chemistry*, 18, 1203-1208.

Morrow, J.S., Anderson, R.A., 1986. Shaping the too fluid bilayer. *Laboratory investigation; a journal of technical methods and pathology*, 54, 237-240.

Mulder, G.J., 2003. Conjugation reactions in drug metabolism: an integrated approach. CRC Press.

Muzavor, S., Arruda, L., Andrade, J., 1993. Roteiro Ecológico da Ria Formosa II Peixes. *Algarve em Foco, Faro, Portugal*.

Nagahama, Y., 1994. Endocrine regulation of gametogenesis in fish. *International Journal of Developmental Biology*, 38, 217-217.

Navas, J.M.a., Segner, H., 2000. Antiestrogenicity of β-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. *Aquatic toxicology*, 51, 79-92.

Nebert, D., Nelson, D., Coon, M., Estabrook, R., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F., Guengerich, F., Gunsalus, I., Johnson, E., 1991. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA and cell biology*, 10, 1.

Nebert, D.W., Petersen, D.D., Fornace Jr, A.J., 1990. Cellular responses to oxidative stress: the [Ah] gene battery as a paradigm. *Environmental health perspectives*, 88, 13.

Nebert, D.W., Russell, D.W., 2002. Clinical importance of the cytochromes P450. *The Lancet*, 360, 1155-1162.

Newbold, R.R., Padilla-Banks, E., Jefferson, W.N., Heindel, J.J., 2008. Effects of endocrine disruptors on obesity. *International Journal of Andrology*, 31, 201-208.

Nishikawa, J.-i., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., Horiguchi, T., 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environmental Science & Technology*, 38, 6271-6276.

Novak, F., Vanbeek, E., Lambert, J., De Loof, A., 1990. Pregnenolone and estradiol identification in the brine shrimp, *Artemia sp*., by means of gas chromatographical mass spectrometrical analysis. *Comparative biochemistry and physiology b-biochemistry & molecular biology*, 95, 565-569.

Oberdorster, E., Rittschof, D., McClellan-Green, P., 1998. Testosterone metabolism in imposex and normal *Ilyanassa obsoleta*: comparison of field and TBTA Cl-induced imposex. *Marine Pollution Bulletin*, 36, 144-151.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M., Markert, B., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. *Ecotoxicology*, 9, 383-397.

Olsson, P.-E., Borg, B., Brunstrom, B., Hakansson, H., Klasson-Wehler, E., 1998. Endocrine disrupting substances. *Stockholm: Swedish Environmental Protection Agency*.

Omae, I., 2003. Organotin antifouling paints and their alternatives. *Applied organometallic chemistry*, 17, 81-105.

Omiecinski, C.J., Remmel, R.P., Hosagrahara, V.P., 1999. Concise review of the cytochrome P450s and their roles in toxicology. *Toxicological Sciences*, 48, 151-156.

Omura, T., Morohashi, K.-i., 1995. Gene regulation of steroidogenesis. *The Journal of steroid biochemistry and molecular biology*, 53, 19-25.

Oost, R., Lopes, S., Komen, H., Satumalay, K., Bos, R., Heida, H., Vermeulen, N., 1998. Assessment of environmental quality and inland water pollution using biomarker responses in caged carp (*Cyprinus carpio*): Use of a bioactivation: detoxication ratio as a biotransformation index (BTI).

Ortiz-Delgado, J.B., Sarasquete, C., Behrens, A., González de Canales, M.L., Segner, H., 2002. Expression, cellular distribution and induction of cytochrome P4501A (CYP1A) in gilthead seabream, *Sparus aurata,* brain. *Aquatic toxicology*, 60, 269-283.

Ortiz, A., Teruel, J.A., Aranda, F.J., 2005. Effect of triorganotin compounds on membrane permeability. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1720, 137-142.

OSPAR background document on musk xylene and other musks. 2004. OSPAR Commission, London.

Otto, D.M., Moon, T.W., 1995. 3, 3′, 4, 4′-Tetrachlorobiphenyl Effects on Antioxidant Enzymes and Glutathione Status in Different Tissues of Rainbow Trout. *Pharmacology & toxicology*, 77, 281-287.

Palacios, E., Racotta, I., Arjona, O., Marty, Y., Le Coz, J.-R., Moal, J., Samain, J.-F., 2007. Lipid composition of the pacific lion-paw scallop, *Nodipecten subnodosus*, in relation to gametogenesis: 2. Lipid classes and sterols. *Aquaculture*, 266, 266-273.

Pavlikova, N., Kortner, T.M., Arukwe, A., 2010. Peroxisome proliferator-activated receptors, estrogenic responses and biotransformation system in the liver of salmon exposed to tributyltin and second messenger activator. *Aquatic toxicology*, 99, 176-185.

Paxéus, N., 1996. Organic pollutants in the effluents of large wastewater treatment plants in Sweden. *Water research*, 30, 1115-1122.

Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews*, 25, 947-970.

Pazos, A.J., Román, G., Acosta, C.P., Sánchez, J.L., Abad, M., 1997. Lipid classes and fatty acid composition in the female gonad of *Pecten maximus* in relation to reproductive cycle and environmental variables. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 117, 393-402.

Petrovic, M., Solé, M., López De Alda, M.J., Barceló, D., 2002. Endocrine disruptors in sewage treatment plants, receiving river waters, and sediments: Integration of chemical analysis and biological effects on feral carp. *Environmental Toxicology and Chemistry*, 21, 2146-2156.

Phrakonkham, P., Viengchareun, S., Belloir, C., Lombes, M., Artur, Y., Canivenc-Lavier, M.-C., 2008. Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis. *The Journal of steroid biochemistry and molecular biology*, 110, 95-103.

Pickering, A.D., Sumpter, J.P., 2003. Peer Reviewed: Comprehending endocrine disruptors in aquatic environments. *Environmental Science & Technology*, 37, 331A-336A.

Porte, C., Albaigés, J., 1993. Bioaccumulation patterns of PCB congeners in bivalves, crustaceans and fishes from the Mediterranean coast. Implications in biomonitoring studies. *Arch. Environ. Contam. Toxicol*, 26, 273-281.

Porte, C., Escartín, E., García de la Parra, L.M., Biosca, X., Albaigés, J., 2002. Assessment of coastal pollution by combined determination of chemical and biochemical markers in *Mullus barbatus*. *Marine ecology progress series*, 235, 205-216.

Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 143, 303-315.

Puccia, E., Messina, C., Cangialosi, M., D'Agati, P., Mansueto, C., Pellerito, C., Nagy, L., Mansueto, V., Scopelliti, M., Fiore, T., 2005. Lipid and fatty acid variations in Ciona intestinalis ovary after tri-n-butyltin (IV) chloride exposure. *Applied organometallic chemistry*, 19, 23-29.

Raffy, S., Teissié, J., 1999. Control of Lipid Membrane Stability by Cholesterol Content. *Biophysical Journal*, 76, 2072-2080.

Reis-Henriques, M.A., Coimbra, J., 1990. Variations in the levels of progesterone in *Mytilus edulis* during the annual reproductive cycle. *Comparative Biochemistry and Physiology Part A: Physiology*, 95, 343-348.

Reis-Henriques, M.A., Le Guellec, D., Remy-Martin, J.P., Adessi, G.L., 1990. Studies of endogenous steroids from the marine mollusc *Mytilus edulis* L. by gas chromatography and mass spectrometry. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 95, 303-309.

Renner, R., 1997. European bans on surfactant trigger transatlantic debate. *Environmental Science & Technology*, 31, 316A-320A.

Repa, J., Turley, S., Lobaccaro, J.-M., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R., Dietschy, J., Mangelsdorf, D., 2000a. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science*, 289, 1524-1529.

Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.-M.A., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L., Mangelsdorf, D.J., 2000b. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. *Genes & Development*, 14, 2819-2830.

Ribalta, C., Solé, M., 2014. In Vitro Interaction of Emerging Contaminants with the Cytochrome P450 System of Mediterranean Deep-Sea Fish. *Environmental Science & Technology*, 48, 12327-12335.

Rice, L.E.R., Charles D 1998. Tributyltin modulates 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126)-induced hepatic CYP1A activity in channel catfish, *Ictalurus punctatus*. *Journal of Toxicology and Environmental Health Part A*, 55, 197-212.

Rimkus, G.G., 1999. Polycyclic musk fragrances in the aquatic environment. *Toxicology Letters*, 111, 37-56.

Riu, A., Grimaldi, M., le Maire, A., Bey, G., Phillips, K., Boulahtouf, A., Perdu, E., Zalko, D., Bourguet, W., Balaguer, P., 2011. Peroxisome proliferator-activated receptor gamma is a target for halogenated analogs of bisphenol A. *Environmental health perspectives*, 119, 1227-1232.

Robinson-Rechavi, M., Garcia, H.E., Laudet, V., 2003. The nuclear receptor superfamily. *Journal of cell science*, 116, 585-586.

Rocha, M.P., Reis-Henriques, M., Coimbra, J., 2000. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). *Aquatic toxicology (Amsterdam, Netherlands)*, 48, 549.

Ronis, M.J.J., Mason, A.Z., 1996. The metabolism of testosterone by the periwinkle (*Littorina littorea*) *in vitro* and *in vivo*: effects of tributyl tin. *Marine Environmental Research*, 42, 161-166.

Rose, G., 2001. Can Newfoundland cod stocks recover without capelin. *ICES Newsletter*, 38.

Routledge, E., Sheahan, D., Desbrow, C., Brighty, G., Waldock, M., Sumpter, J., 1998. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environmental Science & Technology*, 32, 1559-1565.

Rubin, B.S., Murray, M.K., Damassa, D.A., King, J.C., Soto, A.M., 2001. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. *Environmental health perspectives*, 109, 675.

Rubin, B.S., Soto, A.M., 2009. Bisphenol A: Perinatal exposure and body weight. *Molecular and Cellular Endocrinology*, 304, 55-62.

Rubin, B.S., 2011. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. *The Journal of steroid biochemistry and molecular biology*, 127, 27-34.

Rudel, R.A., Camann, D.E., Spengler, J.D., Korn, L.R., Brody, J.G., 2003. Phthalates, alkylphenols, pesticides, polybrominated diphenyl ethers, and other endocrine-disrupting compounds in indoor air and dust. *Environmental Science & Technology*, 37, 4543-4553.

Russell, D.W., Wilson, J.D., 1994. Steroid 5alpha-reductase: two genes/two enzymes. *Annual review of biochemistry*, 63, 25-61.

Santos, M., ten Hallers-Tjabbes, C., Vieira, N., Boon, J., Porte, C., 2002. Cytochrome P450 differences in normal and imposex-affected female whelk *Buccinum undatum* from the open North Sea. *Marine Environmental Research*, 54, 661.

Sarasquete, C., Segner, H., 2000. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. *Science of The Total Environment*, 247, 313-332.

Sargent, J.R., 1989. Ether-linked glycerides in marine animals. *Marine biogenic lipids, fats and oils*, 1, 175-197.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids. *Fish nutrition*, 3, 181-257.

Schaffer, W.I., 1990. Terminology associated with cell, tissue and organ culture, molecular biology and molecular genetics. *In vitro cellular & developmental biology*, 26, 97-101.

Schmitz, G., Langmann, T., 2001. Structure, function and regulation of the ABC1 gene product. *Current opinion in lipidology*, 12, 129-140.

Schnell, S., Martin-Skilton, R., Fernandes, D., Porte, C., 2009. The interference of nitro-and polycyclic musks with endogenous and xenobiotic metabolizing enzymes in carp: an in vitro study. *Environmental Science & Technology*, 43, 9458-9464.

Schoenmarkers, H., Voogt, P., 1980. In vitro biosynthesis of steroids from progesterone by the ovaries and pyloric ceca of the starfish *Asterias rubens*. *General and comparative endocrinology*, 41, 408.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.-M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B., Auwerx, J., 1996. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *The EMBO journal*, 15, 5336.

Schreurs, R.H., Legler, J., Artola-Garicano, E., Sinnige, T.L., Lanser, P.H., Seinen, W., Van der Burg, B., 2004. In vitro and in vivo antiestrogenic effects of polycyclic musks in zebrafish. *Environmental Science & Technology*, 38, 997-1002.

Schreurs, R.H., Sonneveld, E., Jansen, J.H., Seinen, W., van der Burg, B., 2005. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. *Toxicological Sciences*, 83, 264-272.

Schuetz, E.G., Schuetz, J.D., Grogan, W.M., Naray-Fejes-Toth, A., Fejes-Toth, G., Raucy, J., Guzelian, P., Gionela, K., Watlington, C.O., 1992. Expression of cytochrome P450 3A in amphibian, rat, and human kidney. *Archives of biochemistry and biophysics*, 294, 206-214.

Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D.J., 2000. Role of LXRs in control of lipogenesis. *Genes & Development*, 14, 2831-2838.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010. Cytochrome P450-mediated 17β-estradiol metabolism in zebrafish (Danio rerio). *Journal of Endocrinology*, 206, 317-325.

Scott, A., Sumpter, J., Stacey, N., 2010. The role of the maturation-inducing steroid, 17, 20β-dihydroxypregn-4-en-3-one, in male fishes: a review. *Journal of Fish Biology*, 76, 183-224.

Scott, A.P., 2012. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. *Steroids*, 77, 1450-1468.

Scott, A.P., 2013. Do mollusks use vertebrate sex steroids as reproductive hormones? II. Critical review of the evidence that steroids have biological effects. *Steroids*, 78, 268-281.

Seinen, W., Lemmen, J.G., Pieters, R.H., Verbruggen, E.M., van der Burg, B., 1999. AHTN and HHCB show weak estrogenic—but no uterotrophic activity. *Toxicology Letters*, 111, 161-168.

Senthilkumaran, B., Yoshikuni, M., Nagahama, Y., 2004. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. *Molecular and Cellular Endocrinology*, 215, 11-18.

Shang, D.Y., Macdonald, R.W., Ikonomou, M.G., 1999. Persistence of nonylphenol ethoxylate surfactants and their primary degradation products in sediments from near a municipal outfall in the strait of Georgia, British Columbia, Canada. *Environmental Science & Technology*, 33, 1366-1372.

Shelby, M.D., 2006. NTP-CERHR monograph on the potential human reproductive and developmental effects of di (2-ethylhexyl) phthalate (DEHP). *NIH Publication*, 06.

Shim, W.J., Yim, U.H., Kim, N.S., Hong, S.H., Oh, J.R., Jeon, J.K., Okamura, H., 2005. Accumulation of butyl-and phenyltin compounds in starfish and bivalves from the coastal environment of Korea. *Environmental Pollution*, 133, 489-499.

Siah, A., Pellerin, J., Benosman, A., Gagné, J.P., Amiard, J.C., 2002. Seasonal gonad progesterone pattern in the soft-shell clam *Mya arenaria*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 132, 499-511.

Simard, J., Ricketts, M.-L., Gingras, S., Soucy, P., Feltus, F.A., Melner, M.H., 2005. Molecular biology of the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase gene family. *Endocrine Reviews*, 26, 525-582.

Simmons, D.B.D., Marlatt, V.L., Trudeau, V.L., Sherry, J.P., Metcalfe, C.D., 2010. Interaction of Galaxolide® with the human and trout estrogen receptor-α. *Science of The Total Environment*, 408, 6158-6164.

Smith, A., Datta, S.P., Smith, G.H., Campbell, P.N., Bentley, R., McKenzie, H., 2000. Oxford dictionary of biochemistry and molecular biology. Oxford University Press (OUP).

Smith, E.M., Wilson, J.Y., 2010. Assessment of cytochrome P450 fluorometric substrates with rainbow trout and killifish exposed to dexamethasone, pregnenolone-16α-carbonitrile, rifampicin, and β-naphthoflavone. *Aquatic toxicology*, 97, 324-333.

Snyder, S.A., Westerhoff, P., Yoon, Y., Sedlak, D.L., 2003. Pharmaceuticals, personal care products, and endocrine disruptors in water: implications for the water industry. *Environmental Engineering Science*, 20, 449-469.

Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., Lester, J., 2008. Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environment International*, 34, 1033-1049.

Solé, M., De Alda, M.J.L., Castillo, M., Porte, C., Ladegaard-Pedersen, K., Barceló, D., 2000. Estrogenicity determination in sewage treatment plants and surface waters from the catalonian area (NE Spain). *Environmental Science and Technology*, 34, 5076-5083.

Soto, A.M., Justicia, H., Wray, J.W., Sonnenschein, C., 1991. p-Nonyl-phenol: an estrogenic xenobiotic released from" modified" polystyrene. *Environmental health perspectives*, 92, 167.

Stäb, J., Cofino, W., Van Hattum, B., Brinkman, U., 1994. Assessment of transport routes of triphenyltin used in potato culture in the Netherlands. *Analytica Chimica Acta*, 286, 335-341.

Stahlhut, R.W., van Wijngaarden, E., Dye, T.D., Cook, S., Swan, S.H., 2007. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult US males. *Environmental health perspectives*, 115, 876.

Stahlhut, R.W., Welshons, W.V., Swan, S.H., 2009. Bisphenol A data in NHANES suggest longer than expected half-life, substantial nonfood exposure, or both. *Biomedical Sciences publications (MU)*.

Stahlschmidt-Allner, P., Allner, B., Römbke, J., Knacker, T., 1997. Endocrine disrupters in the aquatic environment. *Environmental Science and Pollution Research*, 4, 155-162.

Staples, C.A., Peterson, D.R., Parkerton, T.F., Adams, W.J., 1997. The environmental fate of phthalate esters: a literature review. *Chemosphere*, 35, 667-749.

Stegeman, J., Brouwer, M., Richard, T., Förlin, L., Fowler, B., Sanders, B., Van Veld, P., 1992. Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. *Biomarkers: Biochem., Physiol. and Histolog. markers of Anthropogen. Stress. Lewis Publishers, Chelsea, MI, USA*, 235-335.

Stegeman, J.J., Lech, J.J., 1991. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environmental health perspectives*, 90, 101.

Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenases: current perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species. *Aquatic toxicology: molecular, biochemical, and cellular perspectives*, 87, 206.

Steinberg, P., Fischer, T., Arand, M., Park, E., Elmadfa, I., Rimkus, G., Brunn, H., Dienes, H.-P., 1999. Acute hepatotoxicity of the polycyclic musk 7-acetyl-1, 1, 3, 4, 4, 6-hexamethyl-1, 2, 3, 4-tetrahydronaphthaline (AHTN). *Toxicology Letters*, 111, 151-160.

Sternberg, R.M., Gooding, M.P., Hotchkiss, A.K., LeBlanc, G.A., 2010. Environmental-endocrine control of reproductive maturation in gastropods: implications for the mechanism of tributyltin-induced imposex in prosobranchs. *Ecotoxicology*, 19, 4-23.

Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental health perspectives*, 103, 173.

Sumpter, J.P., 1998. Xenoendocrine disrupters—environmental impacts. *Toxicology Letters*, 102, 337-342.

Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: an overview. *Acta hydrochimica et hydrobiologica*, 33, 9-16.

Swevers, L., Lambert, J., De Loof, A., 1991. Metabolism of vertebrate-type steroids by tissues of three crustacean species. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 99, 35-41.

Tabb, M.M., Blumberg, B., 2006. New Modes of Action for Endocrine-Disrupting Chemicals. *Molecular Endocrinology*, 20, 475-482.

Takacs, M.L., Abbott, B.D., 2007. Activation of mouse and human peroxisome proliferator–activated receptors (α, β/δ, γ) by perfluorooctanoic acid and perfluorooctane sulfonate. *Toxicological Sciences*, 95, 108-117.

Takeuchi, S., Matsuda, T., Kobayashi, S., Takahashi, T., Kojima, H., 2006. In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR) α and PPARγ and quantitative analysis of in vivo induction pathway. *Toxicology and Applied Pharmacology*, 217, 235-244.

Tanabe, S., 2005. Synthetic musks--arising new environmental menace? *Marine Pollution Bulletin*, 50, 1025-1026.

Taxvig, C., Dreisig, K., Boberg, J., Nellemann, C., Schelde, A.B., Pedersen, D., Boergesen, M., Mandrup, S., Vinggaard, A.M., 2012. Differential effects of environmental chemicals and food contaminants on adipogenesis, biomarker release and PPARγ activation. *Molecular and Cellular Endocrinology*, 361, 106-115.

Thibaut, R., Porte, C., 2004. Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *The Journal of steroid biochemistry and molecular biology*, 92, 485-494.

Thomas, P., Dong, J., 2006. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *The Journal of steroid biochemistry and molecular biology*, 102, 175-179.

Thomas, P., 2008. Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Frontiers in neuroendocrinology*, 29, 292-312.

Tingaud-Sequeira, A., Ouadah, N., Babin, P.J., 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. *Journal of Lipid Research*, 52, 1765-1772.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science*, 11, 107-184.

Tolosa, I., Bayona, J., Albaiges, J., Merlini, L., De Bertrand, N., 1992. Occurrence and fate of tributyl-and triphenyltin compounds in western mediterranean coastal enclosures. *Environmental Toxicology and Chemistry*, 11, 145-155.

Tontonoz, P., Hu, E., Spiegelman, B.M., 1994. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell*, 79, 1147-1156.

Tseng, H.-P., Hseu, T.-H., Buhler, D.R., Wang, W.-D., Hu, C.-H., 2005. Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. *Toxicology and Applied Pharmacology*, 205, 247-258.

Van Beek, E., De Loof, A., 1988. Radioimmunological determinations of concentrations of six c-21, c-19 and c-18 steroids during the reproductive-cycle of female *Artemia sp.* (Crustacea, Anostraca). *Comparative biochemistry and physiology a-physiology*, 89, 595-599.

Van Der Burg, B., Schreurs, R., Van Der Linden, S., Seinen, W., Brouwer, A., Sonneveld, E., 2008. Endocrine effects of polycyclic musks: do we smell a rat? *International Journal of Andrology*, 31, 188-193.

Van der Oost, R., Goksøyr, A., Celander, M., Heida, H., Vermeulen, N.P., 1996. Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*) II. Biomarkers: pollution-induced biochemical responses. *Aquatic toxicology*, 36, 189-222.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental toxicology and pharmacology*, 13, 57-149.

Van Meer, G., Voelker, D.R., Feigenson, G.W., 2008. Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology*, 9, 112-124.

Vandenberg, L.N., Chahoud, I., Heindel, J.J., Padmanabhan, V., Paumgartten, F.J., Schoenfelder, G., 2010. Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environmental health perspectives*, 1055-1070.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.-H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses. *Endocrine Reviews*, 33, 378-455.

Verslycke, T., Poelmans, S., De Wasch, K., Vercauteren, J., Devos, C., Moens, L., Sandra, P., De Brabander, H.F., Janssen, C.R., 2003. Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea) following tributyltin exposure. *Environmental Toxicology and Chemistry*, 22, 2030-2036.

Viarengo, A., Bettella, E., Fabbri, R., Burlando, B., Lafaurie, M., 1997. Heavy metal inhibition of EROD activity in liver microsomes from the bass *Dicentrarchus labrax* exposed to organic xenobiotics: role of GSH in the reduction of heavy metal effects. *Marine Environmental Research*, 44, 1-11.

Villeneuve, D.L., Ankley, G.T., Makynen, E.A., Blake, L.S., Greene, K.J., Higley, E.B., Newsted, J.L., Giesy, J.P., Hecker, M., 2007. Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine-active chemicals. *Ecotoxicology and Environmental Safety*, 68, 20-32.

Voogt, P., Den Besten, P., Jansen, M., 1990. The Δ5-pathway in steroid metabolism in the sea star *Asterias rubens* L. *Comparative biochemistry and physiology. B. Comparative biochemistry*, 97, 555-562.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambré, C., Tarazona, J.V., Brandt, I., Vethaak, A.D., 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. *CRC Critical Reviews in Toxicology*, 30, 71-133.

Wada, K., Sakamoto, H., Nishikawa, K., Sakuma, S., Nakajima, A., Fujimoto, Y., Kamisaki, Y., 2007. Life style-related diseases of the digestive system: endocrine disruptors stimulate lipid accumulation in target cells related to metabolic syndrome. *Journal of Pharmacological Sciences*, 105, 133-137.

Wade, D.P., Owen, J.S., 2001. Regulation of the cholesterol efflux gene, ABCA1. *The Lancet*, 357, 161-163.

Walther, T.C., Farese Jr, R.V., 2012. Lipid droplets and cellular lipid metabolism. *Annual review of biochemistry*, 81, 687-714.

Wang, N., Tall, A.R., 2003. Regulation and Mechanisms of ATP-Binding Cassette Transporter A1-Mediated Cellular Cholesterol Efflux. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 23, 1178-1184.

Wasson, K.M., Watts, S.A., 2000. Progesterone metabolism in the ovaries and testes of the echinoid Lytechinus variegatus Lamarck (Echinodermata). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 127, 263-272.

Waxman, D.J., 1988. Interactions of hepatic cytochromes P-450 with steroid hormones: regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochemical pharmacology*, 37, 71-84.

Wenk, M.R., 2005. The emerging field of lipidomics. *Nature Reviews Drug Discovery*, 4, 594-610.

Weston, D.P., Maruya, K.A., 2002. Predicting bioavailability and bioaccumulation with in vitro digestive fluid extraction. *Environmental Toxicology and Chemistry*, 21, 962-971.

Whyte, J.J., Jung, R., Schmitt, C., Tillitt, D., 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *CRC Critical Reviews in Toxicology*, 30, 347-570.

Wilson, J.D., 2001. The role of 5a-reduction in steroid hormone physiology. *Reproduction, Fertility and Development*, 13, 673-678.

Wolf, K., Quimby, M., 1962. Established eurythermic line of fish cells in vitro. *Science*, 135, 1065-1066.

Xie, Y., Yang, Q., Nelson, B.D., DePierre, J.W., 2002. Characterization of the adipose tissue atrophy induced by peroxisome proliferators in mice. *Lipids*, 37, 139-146.

Xu, Y., Knipp, G.T., Cook, T.J., 2006. Effects of di-(2-ethylhexyl)-phthalate and its metabolites on the lipid profiling in rat HRP-1 trophoblast cells. *Archives of toxicology*, 80, 293-298.

Yamamoto, T.-O., 1969. Sex Differentiation. *Fish physiology*, 3, 117-175.

Yamamoto, T., Yasuhara, A., 1999. Quantities of bisphenol A leached from plastic waste samples. *Chemosphere*, 38, 2569-2576.

Yamauchi, R., Ishibashi, H., Hirano, M., Mori, T., Kim, J.-W., Arizono, K., 2008. Effects of synthetic polycyclic musks on estrogen receptor, vitellogenin, pregnane X receptor, and cytochrome P450 3A gene expression in the livers of male medaka (*Oryzias latipes*). *Aquatic toxicology*, 90, 261-268.

Yano, I., 1985. Induced ovarian maturation and spawning in greasyback shrimp, Metapenaeus ensis, by progesterone. *Aquaculture*, 47, 223-229.

Yawetz, A., Woodin, B., Stegeman, J., 1998. Cytochromes P450 in liver of the turtle *Chrysemys picta picta* and the induction and partial purification of CYP1A-like proteins. *Biochimica et biophysica acta*, 1381, 12.

Zeng, X., Sheng, G., Xiong, Y., Fu, J., 2005. Determination of polycyclic musks in sewage sludge from Guangdong, China using GC–EI-MS. *Chemosphere*, 60, 817-823.

Zhang, X., Hecker, M., Jones, P.D., Newsted, J., Au, D., Kong, R., Wu, R.S., Giesy, J.P., 2008. Responses of the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. *Environmental Science & Technology*, 42, 6762-6769.

Zhang, X., Xu, Q., Man, S., Zeng, X., Yu, Y., Pang, Y., Sheng, G., Fu, J., 2013. Tissue concentrations, bioaccumulation, and biomagnification of synthetic musks in freshwater fish from Taihu Lake, China. *Environmental Science and Pollution Research*, 20, 311-322.

Zhou, L., Wang, D., Senthilkumaran, B., Yoshikuni, M., Shibata, Y., Kobayashi, T., Sudhakumari, C., Nagahama, Y., 2005. Cloning, expression and characterization of three types of 17β-hydroxysteroid dehydrogenases from the Nile tilapia, *Oreochromis niloticus*. *Journal of molecular endocrinology*, 35, 103-116.

Zhu, W., Mantione, K., Jones, D., Salamon, E., Cho, J.J., Cadet, P., Stefano, G.B., 2003. The presence of 17-beta estradiol in *Mytilus edulis* gonadal tissues: evidence for estradiol isoforms. *Neuroendocrinology Letters*, 24, 137-140.

Zucchi, S., Castiglioni, S., Fent, K., 2013. Progesterone alters global transcription profiles at environmental concentrations in brain and ovary of female zebrafish (Danio rerio). *Environmental Science & Technology*, 47, 12548-12556.

7. IMPACT FACTOR OF PUBLISHED ARTICLES

MINISTERIO
DE ECONOMIA
Y COMPETITIVIDAD

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
INSTITUTO DE DIAGNOSTICO
AMBIENTAL Y ESTUDIOS DEL AGUA
(IDAEA)

Cinta Porte Visa, directora de la Tesis doctoral presentada por **Giorgio Dimastrogiovanni**, EXPONE:

Que el doctorando ha participado activamente en la realización de todos los artículos presentados en la memoria, tal y como se refleja en la distribución de autores, siendo primer autor en 3 de los 4 artículos presentados y segundo autor en 1 de ellos. En general, ha participado en la discusión del diseño experimental, y ha contribuido de manera significativa al análisis de muestras, elaboración y redacción de los trabajos.

A continuación se detallan los artículos que forman parte de esta tesis, el grado de participación del doctorando y el Factor de Impacto (F.I.) de las revistas en las cuales han sido publicados o enviados para su publicación:

1. Fernandes, D., Dimastrogiovanni, G., Blázquez, M., Porte, C., 2013. Metabolism of the polycyclic musk galaxolide and its interference with endogenous and xenobiotic metabolizing enzymes in the European sea bass (*Dicentrarchus labrax*). Environmental Pollution 174, 214-221. **F.I.: 3,902**

   Grado de participación: Realización del trabajo experimental junto con D. Fernandes. Realización de todos los ensayos enzimáticos excepto el análisis histológico.

2. Dimastrogiovanni, G., Fernandes D., Bonastre M., Porte, C., 2015. Progesterone is actively metabolized to 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one by the marine mussel *Mytilus galloprovincialis*. Aquatic Toxicology 165, 93-100. **F.I.: 3.513**
   Grado de participación: Realización del trabajo experimental junto con M. Bonastre. Realización de los ensayos enzimáticos y análisis de esteroides. Discusión de resultados y elaboración del manuscrito.

C/ Jordi Girona, 18-26
08034 BARCELONA
Telf.: 93 400 61 00
Fax.: 93 204 59 04

3. Dimastrogiovanni, G., Córdoba, M., Navarro, I., Jáuregui, O., Porte, C., 2015. Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters. Aquatic Toxicology (en prensa) **F.I.: 3.513**

   Grado de participación: Realización del trabajo experimental. Discusión de resultados y elaboración del manuscrito.

4. Dimastrogiovanni, G., Jáuregui, O., Blumberg, B., Porte, C., 2015. TBT induces triglyceride accumulation and alters lipid profile in ZFL cells (en preparación).

   Grado de participación: Realización del trabajo experimental. Discusión de resultados y elaboración del manuscrito.

Barcelona, 19 de junio de 2015

Cinta Porte Visa
Directora de la tesis

C/ Jordi Girona, 18-26
08034 BARCELONA
Telf.: 93 400 61 00
Fax.: 93 204 59 04

8. PUBLICATIONS

Paper 1

METABOLISM OF THE POLYCYCLIC MUSK GALAXOLIDE AND ITS INTERFERENCE WITH ENDOGENOUS AND XENOBIOTIC METABOLIZING ENZYMES IN THE EUROPEAN SEA BASS

D. Fernandes[a], G. Dimastrogiovanni[a], M. Blázquez[b,c] and C. Porte[a]

[a] Environmental Chemistry Department, IDAEA-CSIC, C/ Jordi Girona 18, 08034 Barcelona, Spain

[b] Instituto de Ciencias del Mar (ICM-CSIC), Passeig Maritim 37-49, 08003 Barcelona, Spain

[c] Instituto de Acuicultura Torre la Sal (IATS-CSIC), Ribera de Cabanes, 12595 Castellón, Spain

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Resumen

Este estudio investiga el metabolismo y mecanismo de acción del galaxolide (HHCB) en lubinas (*Dicentrarchus labrax*) tratadas con 50 mg HHCB/kg del peso corporal (inyección intraperitoneal). Además, se incluyó un grupo de peces inyectados con ketoconazole (KCZ) (50 mg KCZ/kg de peso), un fungicida conocido por interferir con diferentes isoenzimas del CYP. HHCB fue convertido por la lubina en su metabolito hidroxilado (OH-HHCB) y actuó como un inhibidor débil de la síntesis de oxi-andrógenos en gónadas de machos. Ambos compuestos (HHCB y OH-HHCB) fueron detectados en la bilis. El fungicida ketoconazole actuó como un inhibidor fuerte de las actividades CYP11β y CYP3A. Este trabajo contribuye a comprender el impacto de fragancias sintéticas sobre peces, y propone la determinación en la bilis de HHCB y de su metabolito hidroxilado como herramienta para evaluar la exposición de peces a este compuesto.

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Metabolism of the polycyclic musk galaxolide and its interference with endogenous and xenobiotic metabolizing enzymes in the European sea bass (*Dicentrarchus labrax*)

Denise Fernandes [a,1], Giorgio Dimastrogiovanni [a], Mercedes Blázquez [b,c], Cinta Porte [a,*]

[a] Enviromental Chemistry Department, IDAEA-CSIC, C/ Jordi Girona 18, 08034 Barcelona, Spain
[b] Instituto de Ciencias del Mar (ICM-CSIC), Passeig Maritim 37-49, 08003 Barcelona, Spain
[c] Instituto de Acuicultura Torre la Sal (IATS-CSIC), Ribera de Cabanes, 12595 Castellón, Spain

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ABSTRACT

This study investigates the metabolism and mode of action of galaxolide (HHCB) in the European sea bass -*Dicentrarchus labrax*- following a single intraperitoneal injection of 50 mg HHCB/kg body weight. In addition, a group of fish was injected with 50 mg/kg of ketoconazole (KCZ), a fungicide that is known to interfere with different Cyp isoenzymes. HHCB was actively metabolised by sea bass and acted as a weak inhibitor of the synthesis of oxyandrogens in gonads of male fish. Both, HHCB and a hydroxylated metabolite were detected in bile. The fungicide ketoconazole was a strong inhibitor of Cyp11β and Cyp3a-catalyzed activities. The work contributes to the better understanding of the impact of synthetic musks on fish and proposes the determination of HHCB and/or its hydroxylated metabolite in bile as a tool to assess environmental exposure in wild fish.

1. Introduction

Galaxolide (HHCB; 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran), is a polycyclic musk commonly used as a fragrance ingredient in a wide variety of personal care and consumer products, including cosmetics, cleaning agents, detergents, air fresheners and perfumes (Rimkus, 1999). Galaxolide is by far the most widely used polycyclic musk, with a production volume in Europe between 1000 and 5000 tons per year (OSPAR, 2004). It has been reported that 77% of the used musks drain into the sewer system and reach sewage treatment plants (STPs), which are major sources of these compounds into the aquatic environment. Considerable amounts of HHCB have been detected in sewage effluents (0.2–6.0 μg/L), sewage sludge (13.9–27.9 μg/g d.w.), freshwater (36–314 ng/L) and freshwater fish sampled near STPs (0.04–3.6 μg/g w.w.) (Winkler et al., 1998; Rimkus, 1999; Gatermann et al., 2002; Moldovan, 2006). Due to its highly lipophilic nature (log *K*ow = 5.90), HHCB bioaccumulates in aquatic organisms at concentrations ranging from <0.03 up to 160 μg/g lipid weight (Rimkus, 1999; Gatermann et al., 2002). In this regard, high HHCB concentrations have been detected in sharks and mammals (Nakata, 2005; Kannan et al., 2005), as well as in coastal bird species and otters (Rimkus, 2004), indicating food chain transfer in aquatic ecosystems.

Nonetheless, despite its wide occurrence and bioaccumulation by aquatic fauna, information regarding the metabolism of this compound by aquatic organisms, the interference with endogenous and xenobiotic metabolizing enzymes and/or endocrine disrupting properties is still scarce. In vitro reporter gene assays have indicated potential estrogenic and antiestrogenic effects of HHCB towards human estrogen receptor (Seinen et al., 1999; Schreurs et al., 2005), and a dose-dependent antagonistic effect on zebrafish (*Danio rerio*) estrogen receptor (Schreurs et al., 2004). Schreurs et al. (2005) also reported HHCB (≥1 μM) to be an antagonist of the androgen and progesterone receptors in U2-OS human cells. Other studies revealed that polycyclic musks, and among them HHCB, had a high potential of interfering with the catalytic activity of Cyp isoforms involved in the synthesis and metabolism of sex steroids, including Cyp3a (cytochrome P450, family 3, subfamily A), Cyp17 (cytochrome P450, family 17, subfamily A, 17α–hydroxylase/17,20-lyase activity) and Cyp11β (cytochrome P450, family 11, subfamily B, 11β-hydroxylase activity) while having a minor inhibitory effect

* Corresponding author.
E-mail addresses: cinta.porte@idaea.csic.es, cpvqam@cid.csic.es (C. Porte).
[1] Present address: C.I.M.A., FCT, University of Algarve, Campus de Gambelas 8005-139, Faro, Portugal.

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on Cyp1a (Schnell et al., 2009). Particularly interesting was the finding that 100–200 μM HHCB inhibited Cyp17 and Cyp11β-catalyzed activities in mitochondrial fractions isolated from gonads of male carp (*Cyprinus carpio*). Cyp17 catalyzes the conversion of 17α-hydroxyprogesterone (17P4) to androstenedione (AD), a precursor of testosterone (T) in male gonads; both T and AD can be transformed into their respective 11β-hydroxylated metabolites by Cyp11β, and further metabolized to 11-ketotestosterone (11-KT), the main androgen in male teleosts (Kime, 1993; Borg, 1994; Liu et al., 2000; Devlin and Nagahama, 2002).

Within this context, the present study aimed at investigating the metabolism of the polycyclic musk HHCB and its potential effects on biotransformation enzymes and the synthesis of active androgens in the European sea bass (*Dicentrarchus labrax*), a marine fish inhabiting coastal and estuarine zones and thus, often exposed to urban discharges. To this end, adult fish was injected with 50 mg/kg body weight of HHCB, and circulating levels of HHCB in plasma were determined 24, 48 and 96 h post-injection. Additionally, HHCB was monitored in the bile of injected individuals to assess the potential metabolism and excretion of the compound. Cyp1a- and Cyp3a-catalyzed activities were determined by measuring 7-ethoxyresorufin *O*-deethylase (EROD) and 7-benzyloxy-4-[trifluoromethyl]-coumarin *O*-dibenzyloxylase (BFCOD) activity, respectively. UDP-glucuronyltransferase (UGT) was determined by using *p*-nitrophenol as a substrate. The potential of HHCB to act as an endocrine disrupter was investigated by looking at the metabolism of 17P4 (Cyp17) and AD (Cyp11β) in mitochondrial fractions isolated from male gonads of control and injected fish. In addition, a group of fish was injected with the fungicide ketoconazole (KCZ), a well known inhibitor of several Cyp isoenzymes, including Cyp1a, Cyp3a and steroidogenic enzymes (Miranda et al., 1998; Guengerich, 1999; Monteiro et al., 2000; Fernandes et al., 2007).

2. Material and methods

2.1. Chemicals

17α-Hydroxyprogesterone (17P4), androst-4-ene-3,17-dione (AD), *p*-nitrophenol, 7-ethoxyresorufin, 7-hydroxyresorufin, 7-hydroxy-4-(trifluoromethyl)-coumarin, galaxolide (HHCB), ketoconazole (KCZ), nicotinamide adenine dinucleotide phosphate (NADPH), uridine5′-diphosphoglucuronic acid (UDPGA) and β-glucuronidase from *Helix pomatia* Type H-5, were obtained from Sigma–Aldrich (Steinhem, Germany). 7-Benzyloxy-4-trifluoromethyl-coumarin was purchased from Cypex (Dundee, Scotland, UK). [1,2,6,7-^{3}H]17α-Hydroxyprogesterone (60–100 Ci/mmol) and [1β-^{3}H]-androst-4-ene-3,17-dione (15–30 Ci/mmol) were from Perkin–Elmer Life Sciences (Boston, MA). All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany).

2.2. Experimental design

Adult 3 year-old European sea bass reared in the facilities of the Institute of Marine Sciences (Barcelona, 41°23′N; 2°11′E) were kept in 2500 L round aerated aquaria (90% dissolved O_2) under natural simulated conditions of photoperiod and temperature (15 °C). Filtered sea water pH ~8.0, salinity (37.8 ppt) was set at a flow rate of 3 L/min (~25% renewal total water volume/h). In December, coinciding with the onset of the reproductive season in the European sea bass (December–March), 88 fish from a mixed-sex population (weight 261 ± 36 g and length 24.4 ± 1.2 cm) were transferred to 700 L round fibreglass tanks ($n = 15$ fish/tank) and acclimated to the new tanks for two days before injections in order to avoid possible stress-related effects that could interfere with the results. One tank was used for rearing the sham control group, another one for the KCZ-injected group, two for the vehicle-control group and the remaining two tanks for the HHCB-injected group. All fish were treated in agreement with the regulations of animal welfare (European convention for the protection of vertebrate animals used for experimental and other scientific purposes; ETS N° 123, 01/01/91).

After the acclimatization period, fish were anaesthetised with 0.2% phenoxyethanol and treated with a single intra-peritoneal (i.p.) injection of HHBC or KCZ dissolved in corn oil at a final concentration of 50 mg/kg body weight (b.w.). The vehicle-treated group received a single i.p. injection of corn oil and a non-injected control group was also included in the study. Fish were left in recovery tanks until they became fully reactive to visual stimulus, and carefully transferred back to the experimental tanks. Fish samples were taken at 24, 48 and 96 h after injection. Sea bass were anaesthetised with 0.2% phenoxyethanol, total length and weight recorded, and blood (~3 mL) taken from the caudal vein of each individual fish. The blood was transferred to heparinised tubes and after centrifugation (1000 × *g*; 15 min) the plasma was separated, snap-frozen in liquid nitrogen and stored at −80 °C. Target tissues (liver, gonads) and bile (directly taken from the gallbladder with a micropipette) were immediately frozen in liquid nitrogen and stored at −80 °C until further analyses. Subsamples from the central part of male gonads were fixed in 4% paraformaldehyde for histological confirmation of sexual maturation stage.

2.3. Blood and bile analyses

Bile samples were hydrolyzed by a modification of the method described in Escartín and Porte (1999). Briefly 100 μL of bile were incubated for 1 h at 40 °C in the presence of 1 mL 0.4 M acetic acid/sodium acetate buffer pH 5.0 containing 2000 units of β-glucoronidase and 50 units of sulphatase. Hydrolyzed samples were extracted with 1 mL of ethyl acetate (×3), the extracts recombined and reduced to 100 μL under a nitrogen stream. Plasma samples (1 mL) were extracted with 2 mL of ethyl acetate (×3). The recombined extracts were reduced to ~100 μL under a gentle stream of nitrogen. Both, bile and plasma extracts were derivatized by the addition of 100 μL of bis-(trimethylsilyl)trifluoroacetamide (BSTFA), heated for 1 h at 70 °C, dried under a nitrogen stream, and analyzed by gas chromatography–mass spectrometry electron impact mode (GC–MS-EI). The equipment was an Agilent 6890 series GC system with an Agilent 5973 Network mass selective detector. The column, a HP-5MS 30 m × 0.25 mm i.d., film thickness 0.25 μm (Agilent J&W), was programmed from 90 °C to 140 °C at 10 °C/min and from 140 to 300 °C at 4 °C/min. The carrier gas was Helium at 80 Kpa. The injector temperature was 280 °C and the ion source and the analyzer were maintained at 230 °C and 150 °C, respectively. Analysis was performed in full scan mode (m/z: 50–500) with a scan time of 1.68 s. HHCB standard was used to identify the presence of the compound in bile and plasma samples. Both HHCB and its hydroxylated metabolite were quantified in selected ion register (SIR) mode, by using the following ions: m/z 243, 258 for HHCB, and m/z 301, 346 for the metabolite. Concentrations were expressed in μg/g of bile or μg/mL of plasma; the concentration of the metabolite expressed as HHCB equivalents.

2.4. Subcellular fractionation

Hepatic microsomal fractions were prepared as follows. Livers from both males and females (1 g) were flushed with ice-cold 1.15% KCl and homogenized in 1:5 w/v of cold homogenization buffer containing 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 × *g* for 15 min, and the obtained supernatant centrifuged at 12,000 × *g* for 20 min. The resulting supernatant was further centrifuged at 100,000 × *g* for 60 min to obtain the microsomal fraction. Microsomal pellets were resuspended in a ratio of 0.5 mL buffer/g of liver in 100 mM potassium–phosphate buffer pH 7.4, containing 150 mM KCl, 20% (w/v) glycerol, 1 mM DTT, 0.1 mM PMSF and 1 mM EDTA.

Gonad mitochondrial fractions were prepared as described in Fernandes et al. (2007). Individual male gonads (0.5 g) were flushed with ice-cold 1.15% KCl and homogenized in 2 mL ice-cold homogenization buffer. Homogenates were centrifuged twice at 500 × *g* for 15 min, the fatty layer removed and the supernatant centrifuged at 12,000 × *g* for 20 min. The resulting pellets were resuspended in homogenization buffer and further centrifuged at 12,000 × *g* for 20 min. Mitochondrial pellets were resuspended in the same buffer as liver microsomes, and both, mitochondrial and microsomal proteins were measured by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

2.5. Cyp1a- and Cyp3a catalyzed activities

EROD activity was determined in the microsomal fraction of the liver and was assayed by incubating 0.1–0.4 mg of microsomal protein with 3.7 μM of 7-ethoxyresorufin and 225 μM of NADPH in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 (final volume of 250 μL) at 30 °C for 10 min. The reaction was stopped by adding 400 μL of ice-cold acetonitrile (ACN) and after centrifugation (2000 × *g*/10 min) an aliquot (200 μL) of the supernatant was transferred to a 96-multiwell plate. Fluorescence was read at the excitation/emission wavelength pairs of 537/583 using a Varioskan microplate reader (Thermo Electron Corporation). Quantification was performed using a 7-hydroxyresorufin calibration curve and the activity calculated as the amount of 7-hydroxyresorufin (pmol) generated per milligram of protein per minute of reaction time.

BFCOD activity was analyzed according to the procedure described by BD Gentest™ and optimized for sea bass liver microsomes (Thibaut et al., 2006). The assay consisted in incubating 0.1–0.4 mg of liver microsomal protein with 200 μM of 7-benzyloxy-4-trifluoromethyl-coumarin (BFC) and 22.5 μM of NADPH in 100 mM potassium phosphate buffer pH 7.4 (final volume of 250 μL), at 30 °C for 10 min. The reaction was stopped by addition of 75 μL of 0.5 M Tris-base/ACN (20:80, v/v), and the fluorescence was directly read in a 200 μL aliquot transferred to a 96-multiwell plate at the excitation/emission wavelength pairs of 409 and 530 nm, using

a Varioskan microplate reader (Thermo Electron Corporation). Quantification was made using the calibration curve of the 7-hydroxy-4-(trifluoromethyl)-coumarin authentic standard. The activity was calculated as the amount of 7-hydroxy-4-(trifluoromethyl)-coumarin (pmol) generated per milligram of protein per minute of reaction time.

2.6. UDP-glucuronosyltransferase (UGT)

Hepatic UGT was assayed as described in Fernandes et al. (2002). Briefly, 0.25 mg of microsomal liver proteins (pretreated for 15 min with 0.2% TritonX-100 on ice) were incubated with 3.15 mM of UDPGA in 33 mM Tris/$MgCl_2$ buffer pH 7.4. The reaction was initiated by the addition of 81 μM *p*-nitrophenol (*p*NP) and run for 30 min at 30 °C in a shaking water bath. The reaction was stopped by the addition of 0.2 M ice-cold trichloracetic acid (TCA), centrifuged (1500 × *g*/15 min), alkalinized with 0.1 mL of 10 N KOH and the remaining *p*NP was measured spectrophotometrically at 405 nm. Activity was calculated as the amount of *p*NP (nmol) consumed per milligram of protein per minute of reaction time.

2.7. Histological analysis

Gonads were fixed in 4% paraformaldehyde in phosphate buffered saline for 24 h, dehydrated through a graded ethanol series and embedded in paraffin. Tissue sections (6 μm thick) were stained with methylene blue/azure II/basic fuchsin (Bennett et al., 1976). Testes were classified according to their stage of development (Begtashi et al., 2004). Since all testes were in stage V, a further classification, based on the amount of sperm present in the gonads, resulted in two subgroups: early stage V and late stage V. Early stage V contained all germ cell types from spermatogonia A to sperm. The testicular lobules still preserve their outer structure of connective tissue, however only a part of them had sperm in their lumen. Late stage V was characterized by the first signs of disorganization in the surrounding connective tissue. Moreover, the majority of these lobules were filled with sperm that was also present in the efferent duct. Only those testes classified as early stage V were used to study Cyp17 and Cyp11β enzymes.

2.8. Metabolism of 17α-hydroxyprogesterone and androstenedione in male gonads

The metabolism of 17α-hydroxyprogesterone (17P4) was assessed by incubating the mitochondrial fraction (0.8–1.0 mg protein) isolated from male gonads (early stage V) in 50 mM Tris–HCl buffer pH 7.4, with 0.20 μM [^{3}H]17α-hydroxyprogesterone, 10 mM $MgCl_2$, and 1 mM NADPH in a total volume of 250 μL. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 30 min at 30 °C. Similarly, the metabolism of androstenedione (AD) was assessed by incubating the mitochondrial fraction (0.8–1.0 mg protein) in 50 mM Tris–HCl buffer pH 7.4, with 0.21 μM [^{3}H]androstenedione, 10 mM $MgCl_2$, and 1 mM NADPH in a total volume of 250 μL. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 30 min at 30 °C. Both incubations were stopped by adding 250 μL of ice-cold ACN and after centrifugation (1500 × *g*; 10 min), 100 μL of supernatant was injected into a reverse-phase HPLC system coupled with a radiometric detector, as described in Fernandes et al. (2007).

2.9. Statistical analysis

Enzymatic activities were determined in duplicate in 5–8 fish per assay. Values are presented as mean ± SEM. Statistical significance was assessed by using one-way ANOVA analysis of variance with Dunnett's test for differences from vehicle-treated control. Data were tested for normality and homogeneity of variance. Since no statistically significant differences were detected between sham control and vehicle-treated fish, only data for vehicle-treated fish are shown. Data for males and females are pooled as no significant sex-related differences were found for the different enzymatic activities determined in the liver (Student's *t*-test; $p < 0.05$). All statistical analyses were performed with the software package SPSS/PC™ version 19.0 (SPSS Inc., Chicago, IL); *p* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Blood and bile analyses

The analysis of plasma and hydrolyzed bile samples by GC–MS allowed the identification of HHCB 24, 48 and 96 h after injection, together with a hydroxylated-metabolite of HHCB that was only detected in bile samples (Fig. 1). The mass spectra *m/z* 346 (M^+) indicated a trimethylsilyl derivative (TMS) of a hydroxylated HHCB metabolite with a molecular weight of 274 (346 − 73 + 1); *m/z* 211 and 241 originated from the loss of TMS-OH groups (−90); the *m/z* 227, 301 and 331 ions indicated the loss of a methyl group (−15, CH_3) from different benzylic positions, whereas *m/z* 316 emerges from the loss of a neutral formaldehyde (−30, CH_2O). The base peak at *m/z* 103 originated from the fragmentation at the cyclic acetal pyrane ring with a rearrangement of the TMS group and the second most abundant ion *m/z* 73 corresponds to the loss of the TMS group [$(CH_3)_3Si$].

HHCB concentration in plasma remained constant 24 and 48 h after injection (1.56–1.61 μg/mL), and slightly decreased 96 h post-injection (1.36 ± 0.53 μg/mL) (Fig. 2A). HHCB was excreted through the bile: a concentration as high as 72 μg/g was detected 24 h post-injection, but excretion of the parent compound sharply decreased over time (18 μg/g, 96 h post-injection) (Fig. 2B). In contrast, increased amounts of the hydroxylated metabolite (OH-HHCB) were determined over time, its concentration increased from 21 μg/g 24 h post-injection to 64 μg/g 96 h after, which indicates an active metabolism and excretion of HHCB in sea bass (Fig. 2B). The hydroxylated metabolite was not detected in plasma of injected fish.

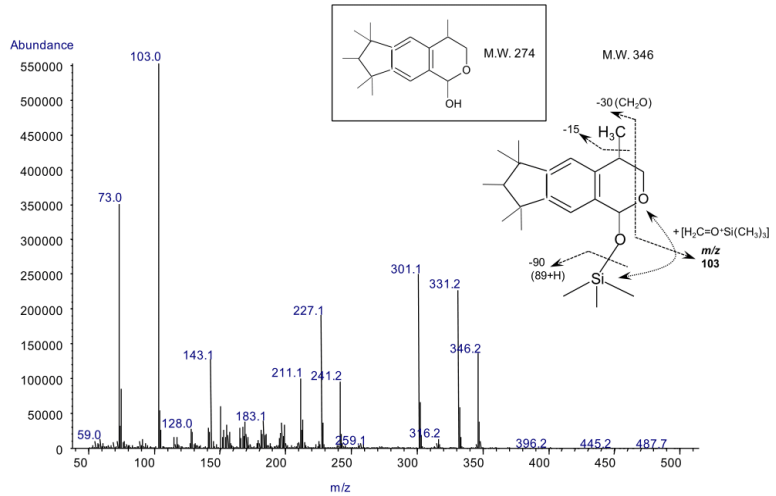

Fig. 1. Mass spectra and chemical structure of the derivatized hydroxylated-metabolite of galaxolide (OH-HHCB) detected in bile of injected fish and proposed fragmentation.

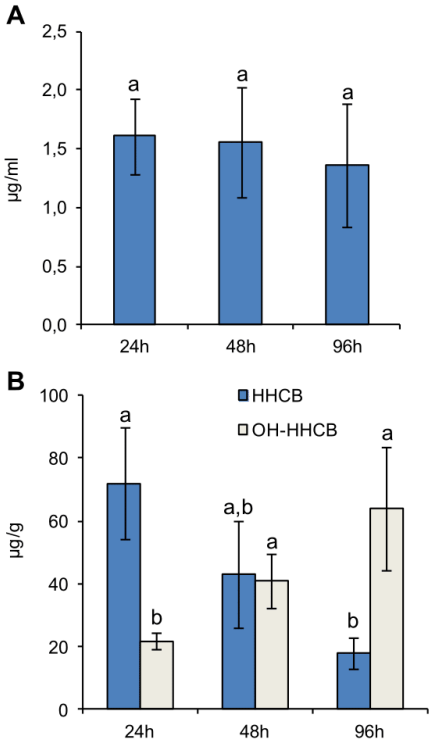

Fig. 2. Metabolism of galaxolide determined by GC–MS-SIM mode. (A) HHCB levels in plasma (n = 7–8); (B) HHCB and OH-HHCB levels in bile (n = 6–7). Distinct letters indicate significant differences between exposure times ($p < 0.05$).

3.2. EROD, BFCOD and UGT activities

EROD and BFCOD activities were determined in microsomal fractions isolated from liver of control and injected fish. These activities were not significantly altered in sea bass following injection of 50 mg/kg b.w. HHCB, despite of a certain increase of BFCOD activity 48 h after i.p. injection (Fig. 3A and B). In contrast, UGT activity was significantly induced by HHCB 24 h post-injection (0.582 nmol/min/mg protein) when compared to control fish (0.392 nmol/min/mg), but the activity decreased over time to reach control values after 96 h (Fig. 3C).

As for fish treated with KCZ, EROD activity significantly increased 48 and 96 h after administration of the compound, reaching a significant 2-fold induction 48 h after injection when compared to vehicle-treated fish (15.22 vs 7.46 pmol/min/mg protein). In contrast, hepatic BFCOD activity was strongly inhibited by KCZ; a 72% and 81% inhibition was observed 48 and 96 h post injection (Fig. 3B). Similarly, KCZ had a significant inhibitory effect on UGT activity, yielding to a 45% inhibition 48 h after injection (Fig. 3C). This significant inhibition was still observed 96 h after injection.

3.3. Metabolism of 17α-hydroxyprogesterone and androstenedione in male gonads

Mitochondrial fractions isolated from gonads of male sea bass at early spermatogenic stage V metabolized 17P4 to a polar metabolite,

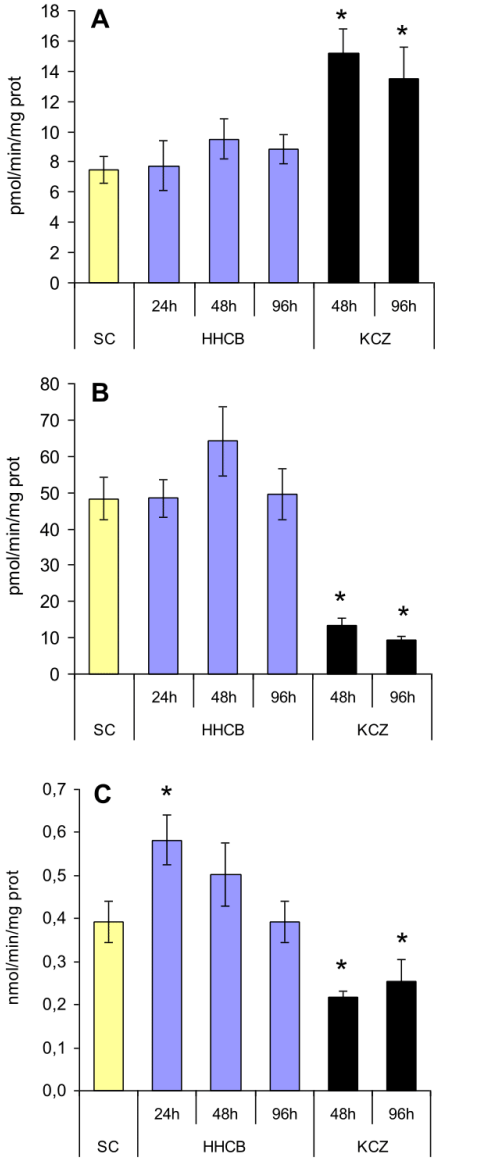

Fig. 3. (A) EROD activity, (B) BFCOD activity and (C) UGT activity determined in liver microsomal fraction of sea bass (*Dicentrarchus labrax*) 24, 48 and 96 h after a single injection of 50 mg/kg b.w. of galaxolide (HHCB) or ketoconazole (KCZ). Values are mean ± SEM (n = 5–8). *Statistically significant differences relative to control ($p < 0.05$).

eluting at approximately 7.75 min and identified as 11β-hydroxy-4-androstene-3,17-dione (βAD), and a second major metabolite (retention time = 11.65 min) identified as androstenedione (AD). Both metabolites were identified by comparison of the retention time of commercial standards, which were shown to co-elute in the chromatographic system. Other three minor metabolites (retention time = 5.6, 8.3 and 16.4 min) were formed, but they could not be

successfully identified. The profile of metabolites formed varied significantly among HHCB and KCZ injected fish and those injected with corn oil (SC), regardless of the time post-injection (Fig. 4). AD was the most abundant metabolite in testes of KCZ-injected fish, while two of the unknown metabolites (retention time = 5.6 and 8.3 min) were not formed; those peaks were also rather low in HHCB-injected fish. In contrast, the formation of the third unknown metabolite (retention time = 16.4) did not differ among treatments.

Differences in the metabolism of 17P4 to AD (Cyp17) and the metabolism of AD to βAD (Cyp11β) were further quantified and determined over time (Fig. 5). HHCB led to a significant inhibition in the metabolism of 17P4 to AD (Cyp17 activity) in testes 24 h post-injection (21.5 pmol/h/mg protein) when compared to vehicle-treated organisms (31.03 pmol/h/mg protein) (Fig. 5A). Moreover, the conversion of AD to 11β-hydroxyandrostenedione (βAD), a Cyp11β catalyzed reaction, was inhibited by 30% 96 h after HHCB injection (Fig. 5B). Regarding KCZ, the synthesis of AD from 17P4 increased by 90% (59.21 ± 4.18 pmol/h/mg protein) 96 h post-injection (Fig. 5A), while the formation of βAD was significantly inhibited 48 h (8.21 ± 2.99 pmol/h/mg protein) and 96 h (11.48 ± 2.38 pmol/h/mg protein) after KCZ i.p. injection, when compared to control fish (25.51 ± 2.85 pmol/h/mg protein) (Fig. 5B).

4. Discussion

Although several studies have reported the predominance of HHCB over other synthetic musks in tissues of both wild and cultured fish (Rimkus, 1999; Duedahl-Olesen et al., 2005), data on the distribution within body compartments and elimination

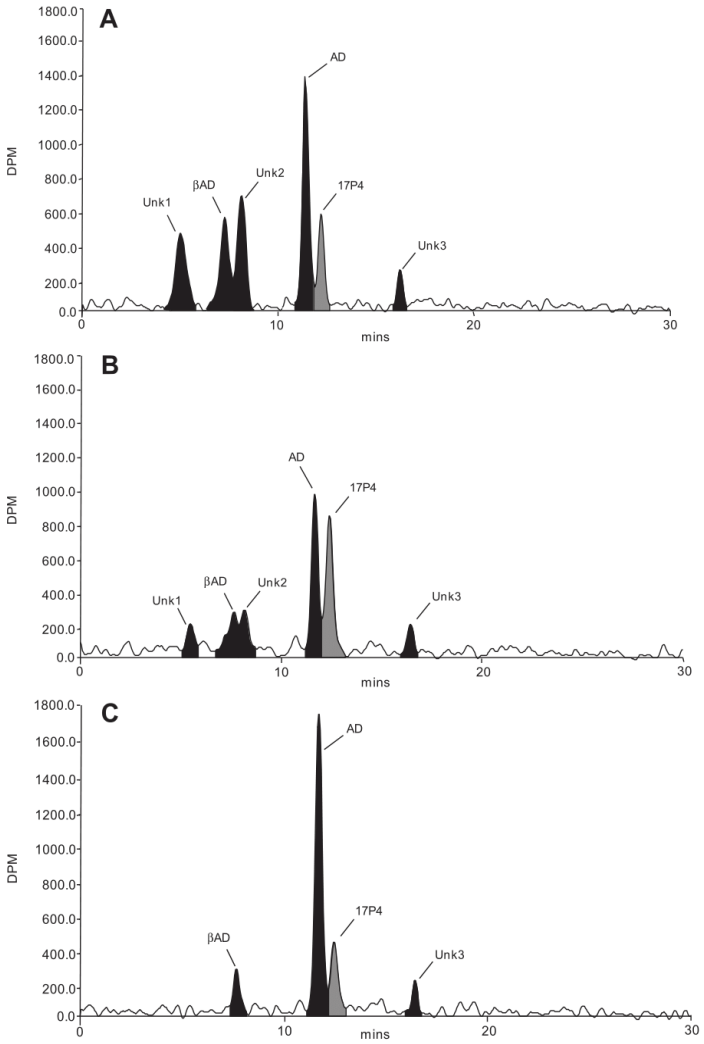

Fig. 4. Representative HPLC radiochromatograms of 17α-hydroxyprogesterone (17P4) metabolism by mitochondrial fractions isolated from testis of sea bass. (A) i.p injection of corn oil (B) i.p. injection of 50 mg/kg HHCB; (C) i.p. injection of 50 mg/kg KCZ. βAD: 11β-hydroxyandrostenedione; AD: androstenedione. Unk: unidentified metabolites.

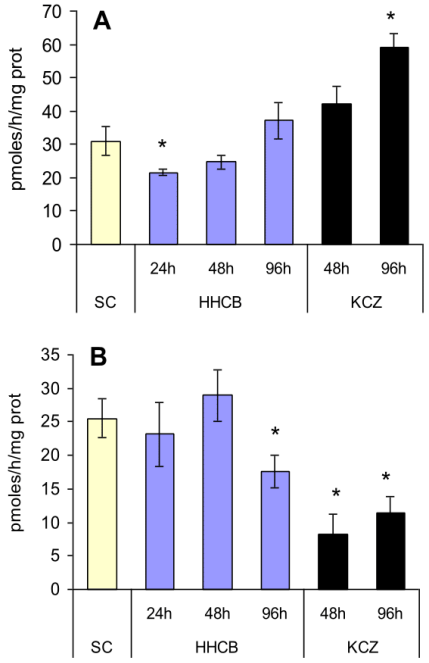

Fig. 5. Effect of HHCB and KCZ injection on (A) Cyp17 activity –metabolism of 17P4 to AD–, and (B) Cyp11β activity –metabolism of AD to βAD– determined in mitochondrial fractions isolated from testis of sea bass. Values are mean ± SEM (n = 5–8). *Statistically significant differences relative to control ($p < 0.05$).

kinetics of musk fragrances in fish are still scarce. In the present study, levels of HHCB circulating in the blood of injected European sea bass remained almost unaltered over the studied period (1.61–1.36 μg/mL). A rough estimation indicates a half-life of HHCB in blood of approximately 10 days ($y = -0.0857x + 1.71$; $R^2 = 0.979$), which points out a certain clearance of the compound. The analysis of bile samples confirmed the ability of sea bass to metabolize HHCB into a hydroxylated metabolite (Fig. 1), which was excreted through the bile together with the parental compound; the concentration of this hydroxylated metabolite in bile increased over time to reach a maximum 96 h post-injection, while the concentration of HHCB decreased (Fig. 2). Similarly, a half-life of HHCB of 2–3 days was reported in the bluegill sunfish (*Lepomis macrochirus*) exposed for 28 days to rather high nominal concentrations (1 and 10 mg/L) using a flow-through system (Balk and Ford, 1999). The authors reported the presence of an identical polar metabolite fraction in both the water and the organic extracts of bluegill sunfish exposed to HHCB, and suggested a relatively high metabolic rate of HHCB that was apparent after 3 days of exposure. Metabolic clearance of HHCB was further confirmed by the significant induction of UGT activity 24 h post-injection (Fig. 3C), which suggest an early induction of UGT by HHCB, and a comparatively slow Phase I metabolism of the compound possibly by Cyp3a (Fig. 3B).

To the best of our knowledge, this is the first identification of a hydroxylated-HHCB metabolite in fish bile. Fish bile metabolites are seen as excellent markers for the assessment of exposure to those pollutants that are readily metabolized by fish, among them polycyclic aromatic hydrocarbons, alkylphenols and others (Lavado et al., 2006; Beyer et al., 2010). These metabolites are accumulated in the bile before further elimination occurs, mainly via the alimentary tract. Thus, biliary levels of HHCB or its hydroxylated metabolite can be used as a marker of recent exposure to HHCB, and this technique can probably be applied to monitor exposure to other polycyclic musks in fish.

Despite the studies indicating metabolism of HHCB by fish, further information on the enzymatic systems involved and the toxicity and persistence of the metabolite(s) formed is needed to fully assess the potential risks associated to environmental exposure to this compound. In the present work, hepatic EROD activity (Cyp1a) remained unaltered following HHCB injection, while the activity BFCOD (Cyp3a-mediated) slightly increased 48 h post-injection, which suggests a possible involvement of the latter in HHCB metabolism. Interestingly, tonalide (AHTN), another synthetic polycyclic musk, also failed to induce EROD activity in exposed rats (Steinberg et al., 1999). Yamauchi et al. (2008) reported an induction of *cyp3a40* mRNA expression in the liver of male medaka exposed to 500 μg/L of HHCB for 3 days, although the mRNA expression levels of the pregnane X receptor (PXR) and of *cyp3a38*, another *cyp3a* paralog, remained unchanged. In the present study, BFC was used as a catalytic probe to detect Cyp3a induction in sea bass. Indeed, BFC is a mammalian Cyp3a substrate, selectively metabolized by Cyp3a4 in humans (Miller et al., 2000), but recent studies have suggested that it is a substrate for different Cyp isoenzymes in fish (Scornaienchi et al., 2010; Smith and Wilson, 2010). In this regard, our results are not conclusive and the question remains on whether Cyp3a and/or other Cyp isoenzymes are involved in HHCB metabolism in the European sea bass.

In contrast, KCZ, a PXR antagonist, induced Cyp1a activity (2-fold increase) and acted as an inhibitor of Cyp3a (BFCOD activity; 70–80% inhibition) and UGT activity (35–45% inhibition), 48 and 96 h post-injection. This agrees with previous results by Hasselberg et al. (2008) that reported 2- to 3-fold increase of EROD activity and 80% decrease of BFCOD activity in rainbow trout (*Oncorhynchus mykiss*), 3 and 6 days after injection of 100 mg/kg KCZ. Moreover, injection of 12 mg/kg of KCZ resulted in 60% increase of Cyp1a activity in juvenile Atlantic cod (*Gadus morhua*) and a 54% decrease of Cyp3a-mediated BFCOD activity (Hasselberg et al., 2005). In addition to *cyp3a* gene expression, PXR regulates a series of other genes including Cyp enzymes such as *cyp2c9*, UGTs, and members of the drug transporters family such as P-glycoprotein (Iyer et al., 2006). Thus, the obtained results suggest the ability of KCZ to act as PXR antagonist in the European sea bass.

Additionally, KCZ and to a lesser extent HHCB interfered with Cyp17 and Cyp11β, both enzymes involved in the synthesis of active androgens in male fish. It has been reported that the synthesis of 11β-hydroxyandrostenedione (Cyp11β) in the European sea bass is elevated at the initial stages of sexual maturation, specifically in testes classified as early spermatogenic, and significantly decreases with increasing maturation of the gonads (Fernandes et al., 2007). Therefore, only male gonads classified as early stage V were selected for the study to avoid variability in the enzymatic activity due to differences in gonad maturation. Under these conditions, HHCB led to a 30% decrease in the synthesis of both AD (Cyp17) 24 h post-injection and βAD (Cyp11β) 96 h post-injection. The observed effects agree with previous in vitro findings in male carp gonads, where HHCB had an inhibitory effect on Cyp17 (IC50 = 225 ± 20 μM) and Cyp11β (48 ± 7% at a concentration of 1 mM) (Schnell et al., 2009). As far as we know, this is the first study reporting a weak inhibitory effect of HHCB on the synthesis of active androgens in fish in-vivo. Several studies have investigated the estrogenic potential of HHCB in fish, with often contradictory results. Thus, HHCB inhibited estradiol-induced vitellogenin (Vtg) production in rainbow trout, whereas it induced the expression of hepatic estrogen receptor (type ERα) and Vtg mRNA/protein in male medaka (Yamauchi et al., 2008; Simmons et al., 2010).

Given the role of 11-oxygenated androgens in testicular recrudescence and spermatogenesis in many teleost fish (Borg, 1994; Miura et al., 1996), the inhibition of Cyp17 but particularly Cyp11β enzymatic activities may alter the rate of androgen production, disturbing the local and systemic level of androgens, and thus leading to disruption of androgen-related biological processes, such as spermatogenesis, reproductive behaviour and development of secondary sexual characteristics, among others. In this sense, KCZ strongly interfered with the synthesis of active androgens in sea bass testis, the effects being more evident 96 h post-injection; KCZ significantly induced the formation of AD (Cyp17) and inhibited the synthesis of βAD (Cyp11β), the inhibition on Cyp11β was stronger than the one observed for HHCB. Several studies, have shown that exposure to KCZ is capable of interfering with steroidogenic Cyp enzymes (e.g. Cyp17, Cyp19, Cyp11β) in both mammals and fish, leading to reduced production of 17β-estradiol, testosterone, androstenedione, 11β-hydroxy-androstenedione and in some cases a decrease in fecundity (Kan et al., 1985; Monod et al., 1993; Monteiro et al., 2000; Fernandes et al., 2007; Villeneuve et al., 2007; Zhang et al., 2008).

Overall, the present study reveals that KCZ and to some extent HHCB can alter the synthesis of active androgens in sea bass through activation/inhibition of Cyp17 and Cyp11β catalyzed activities in vivo and contributes to the better understanding of the impact of these compounds on fish species. The determination of HHCB and/or its hydroxylated metabolite in bile can be a sensitive tool to assess environmental exposure to synthetic musks on fish populations.

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References

Balk, F., Ford, R.A., 1999. Environmental risk assessment for the polycyclic musks AHTN and HHCB in the EU: I. Fate and exposure assessment. Toxicology Letters 111, 57–79.

Begtashi, I., Rodríguez, L., Moles, G., Zanuy, S., Carrillo, M., 2004. Long-term exposure to continuous light inhibits precocity in juvenile male European sea bass (*Dicentrarchus labrax*, L.). I. Morphological aspects. Aquaculture 241, 539–559.

Bennett, H.S., Wyrick, A.D., Lee, S.W., McNeil, J.H., 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives, and simple stains. Stain Technology 51, 71–94.

Beyer, J., Jonsson, G., Porte, C., Krahn, M.M., Ariese, F., 2010. Analytical methods for determining metabolites of polycyclic aromatic hyrocarbon (PAH) pollutants in fish bile: a review. Environmental Toxicology and Pharmacology 30, 224–244.

Borg, B., 1994. Androgens in teleost fishes. Comparative Biochemistry and Physiology 109C, 219–245.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological and environmental influences. Aquaculture 208, 191–364.

Duedahl-Olesen, L., Cederberg, T., Pedersen, K.H., Højgård, A., 2005. Synthetic musk fragrances in trout from Danish fish farms and human milk. Chemosphere 61, 422–431.

Escartín, E., Porte, C., 1999. Assessment of PAH pollution in coastal areas from the NW Mediterranean through the analysis of fish bile. Marine Pollution Bulletin 38, 1200–1206.

Fernandes, D., Potrykus, J., Morsiani, C., Raldua, D., Lavado, R., Porte, C., 2002. The combined use of chemical and biochemical markers to assess water quality in two low-stream rivers (NE Spain). Environmental Research 90, 169–178.

Fernandes, D., Bebianno, M.J., Porte, C., 2007. Mitochondrial metabolism of 17α-hydroxyprogesterone in male sea bass (*Dicentrarchus labrax*): a potential target for endocrine disruptors. Aquatic Toxicology 85, 258–266.

Gatermann, R., Biselli, S., Hühnerfuss, H., Rimkus, G.G., Hecker, M., Karbe, L., 2002. Synthetic musks in the environment. Part 1: Species-dependent bioaccumulation of polycyclic and nitro musk fragrances in freshwater fish and mussels. Archives of Environmental Contamination and Toxicology 42, 437–446.

Guengerich, F.P., 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. Annual Reviews of Pharmacology and Toxicology 39, 1–17.

Hasselberg, L., Grøsvik, B.E., Goksøyr, A., Celander, M.C., 2005. Interactions between xenoestrogens and ketoconazole on hepatic CYP1A and CYP3A, in juvenile Atlantic cod (*Gadus morhua*). Comparative Hepatology 4 (2).

Hasselberg, L., Westerberg, S., Wassmur, B., Celander, M.C., 2008. Ketoconazole, an antifungal imidazole, increases the sensitivity of rainbow trout to 17α-ethynylestradiol exposure. Aquatic Toxicology 86, 256–264.

Iyer, M., Reschly, E.J., Krasowski, M.D., 2006. Functional evolution of the pregnane X receptor. Expert Opinion on Drug Metabolism and Toxicology 2, 381–397.

Kan, P.B., Hirst, M.A., Feldman, D., 1985. Inhibition of steroidogenic cytochrome P-450 enzymes in rat testis by ketoconazole and related imidazole anti-fungal drugs. Journal of Steroid Biochemistry 23, 1023–1029.

Kannan, K., Reiner, J.L., Yun, S.H., Perrotta, E.E., Tao, L., Johnson-Restrepo, B., Rodan, B.D., 2005. Polycyclic musk compounds in higher trophic level aquatic organisms and humans from the United States. Chemosphere 61, 693–700.

Kime, D.E., 1993. "Classical" and "non-classical" reproductive steroids in fish. Reviews in Fish Biology and Fisheries 3, 160–180.

Lavado, R., Ureña, R., Martin-Skilton, R., Torreblanca, A., del Ramo, J., Raldúa, D., Porte, C., 2006. The combined use of chemical and biochemical markers to assess water quality along the Ebro River. Environmental Pollution 139, 330–339.

Liu, S., Govoroun, M., D'Cotta, H., Ricordel, M.-J., Lareyre, J.-J., McMeel, O.M., Smith, T., Nagahama, Y., Guiguen, Y., 2000. Expression of cytochrome P45011β (11β-hydroxylase) gene during gonadal sex differentiation and spermatogenesis in rainbow trout, *Oncorhynchus mykiss*. Journal of Steroid Biochemistry and Molecular Biology 75, 291–298.

Miller, V.P., Stresser, D.M., Blanchard, A.P., Turner, S., Crespi, C.L., 2000. Fluorometric high-throughput screening for inhibitors of cytochrome P450. Annals New York Academy of Sciences 919, 26–32.

Miranda, C.L., Henderson, M.C., Buhler, D.R., 1998. Evaluation of chemicals as inhibitors of trout cytochrome P450s. Toxicology and Applied Pharmacology 148, 237–244.

Miura, C., Miura, T., Yamashita, M., Yamauchi, K., Nagahama, Y., 1996. Hormonal induction of all stages of spermatogenesis in germ-somatic cell coculture from immature Japanese eel testis. Development Growth and Differentiation 38, 257–262.

Moldovan, Z., 2006. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. Chemosphere 64, 1808–1817.

Monod, G., Mones, A., Fostier, A., 1993. Inhibition of ovarian microsomal aromatase and folicular oestradiol secretion by imidazole fungicides in rainbow trout. Marine Environmental Research 35, 153–157.

Monteiro, P.R.R., Reis-Henriques, M.A., Coimbra, J., 2000. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). Aquatic Toxicology 48, 549–559.

Nakata, H., 2005. Occurrence of synthetic musk fragrances in marine mammals and sharks from Japanese coastal waters. Environmental Science and Technology 39, 3430–3434.

OSPAR Commission, 2004. Ospar Background Document on Musk Xylene and Other Musks. In: Hazardous Substances Series. OSPAR Commission, London.

Rimkus, G.G., 1999. Polycyclic musk fragrances in the aquatic environment. Toxicology Letters 111, 37–56.

Rimkus, G.G., 2004. The handbook of environmental chemistry. In: Rimkus, G. (Ed.), Synthetic Musk Fragrances in the Environment. Springer, Berlin, p. 333.

Schnell, S., Martin-Skilton, R., Fernandes, D., Porte, C., 2009. The interference of nitro- and polycyclic musks with endogenous and xenobiotic metabolizing enzymes in carp: an in vitro study. Environmental Science Technology 43, 9458–9464.

Schreurs, R.H.M.M., Legler, J., Artola-Garicano, E., Sinnige, T.L., Lanser, P.H., Seinen, W., van der Burg, B., 2004. In vitro and in vivo antiestrogenic effects of polycyclic musks in Zebrafish. Environmental Science Technology 38, 997–1002.

Schreurs, R.H.M.M., Sonneveld, E., Jansen, J.H.J., Seinen, W., van der Burg, B., 2005. Interaction of polycyclic musks and UV filters with the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR) in reporter gene bioassays. Toxicological Sciences 83, 264–272.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010. Cytochrome P450-mediated 17β-estradiol metabolism in zebrafish (*Danio rerio*). Journal of Endocrinology 206, 317–325.

Seinen, W., Lemmen, J.G., Pieters, R.H.H., Verbruggen, E.M.J., van der Burg, B., 1999. AHTN and HHCB show weak estrogenic – but no uterotrophic activity. Toxicology Letters 111, 161–168.

Simmons, D.B.D., Marlatt, V.L., Trudeau, V.L., Sherry, J.P., Metcalfe, C.D., 2010. Interaction of Galaxolide® with the human and trout estrogen receptor-α. Science of the Total Environment 408, 6158–6164.

Smith, E.M., Wilson, J.Y., 2010. Assessment of cytochrome P450 fluorometric substrates with rainbow trout and killifish exposed to dexamethasone, pregnenolone-16[α]-carbonitrile, rifampicin, and [β]-naphthoflavone. Aquatic Toxicology 97, 324–333.

Steinberg, P., Fisher, T., Arand, M., Park, Eunju, Elmadfa, I., Rimkus, G., Brunn, H., Dienes, H.-P., 1999. Acute hepatotoxicity of the polycyclic musk 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthaline (AHTN). Toxicology Letters 111, 151–160.

Thibaut, R., Schnell, S., Porte, C., 2006. The interference of pharmaceuticals with endogenous and xenobiotic metabolizing enzymes in carp liver: an in-vitro study. Environmental Science and Technology 40, 5154–5160.

Villeneuve, D.L., Ankley, G.T., Makynen, E.A., Blake, L.S., Greene, K.J., Higley, E.B., Newsted, J.L., Giesy, J.P., Hecker, M., 2007. Comparison of fathead minnow ovary explant and H295R cell-based steroidogenesis assays for identifying endocrine related chemicals. Ecotoxicology and Environmental Safety 68, 20–32.

Winkler, M., Kopf, G., Hauptvogel, C., Neu, T., 1998. Fate of artificial musk fragrances associated with suspended particulate matter (SPM) from the river Elbe (Germany) in comparison to other organic contaminants. Chemosphere 37, 1139–1156.

Yamauchi, R., Ishibashi, H., Hirano, M., Mori, T., Kim, J.-W., Arizono, K., 2008. Effects of synthetic polycyclic musks on estrogen receptor, vitellogenin, pregnane X receptor, and cytochrome P450 3A gene expression in the livers of male medaka (*Oryzias latipes*). Aquatic Toxicology 90, 261–268.

Zhang, X., Hecker, M., Jones, P.D., Newsted, J., Au, D., Kong, R., Wu, R.S.S., Giesy, J.P., 2008. Responses of the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. Environmental Science and Technology 42, 6762–6769.

Paper 2

PROGESTERONE IS ACTIVELY METABOLIZED TO 5α-PREGNANE-3,20-DIONE AND 3β-HYDROXY-5α-PREGNAN-20-ONE BY THE MARINE MUSSEL *MYTILUS GALLOPROVINCIALIS*

G. Dimastrogiovanni[a], D. Fernandes[a,b], M. Bonastre[a] and C. Porte[a]

[a] Environmental Chemistry Department, IDAEA-CSIC, C/ Jordi Girona 18, 08034 Barcelona, Spain

[b] FCT, CIMA, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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Resumen

Progesterona (P4) y progestágenos sintéticos entran en el medio acuático a través de las plantas de tratamiento de agua residual y por escorrentía agrícola, lo que conlleva un riesgo potencial para los organismos acuáticos dada la actividad biológica de estos compuestos. P4 es el precursor de diferentes esteroides en vertebrados, incluyendo estrógenos y andrógenos. Mejillones (*Mytilus galloprovincialis*) fueron expuestos durante 7 días a diferentes concentraciones de progesterona (0.02-10 µg/L) con el objetivo de: a) evaluar alteraciones en los niveles de esteroides endógenos como consecuencia de la exposición, y b) describir las vías enzimáticas involucradas en el metabolismo de progesterona en mejillones. No se detectaron alteraciones significativas en los niveles de testosterona (T) y estradiol (E2) en el manto y gónadas de mejillones expuestos, a pesar de un aumento de T atribuido a reactividad cruzada de P4 en los organismos expuestos a 10 µg P4/L. P4 se metabolizó activamente a 5α-pregnano-3,20-dione (5α-DHP) y 3β-hidroxi-5α-pregnano-20-one (3β,20-one) en la glándula digestiva, mientras que no se observó síntesis de 17α-hidroxiprogesterona o androstenediona. El metabolismo de P4 a 5α-DHP no fue afectado por la exposición. El examen histológico de las gónadas mostró que la exposición a 10 µg/L P4 inducía la maduración y liberación de gametos en mejillones. Las concentraciones de progesterona detectadas en el medio ambiente (en el rango ng/L) es poco probable que produzcan alteraciones a nivel endocrino en mejillones.

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Progesterone is actively metabolized to 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one by the marine mussel *Mytilus galloprovincialis*

Giorgio Dimastrogiovanni[a], Denise Fernandes[a,b,*], Marta Bonastre[a], Cinta Porte[a,*]

[a] Environmental Chemistry Department, IDAEA-CSIC, C/Jordi Girona 18-26, 08034 Barcelona, Spain
[b] FCT, CIMA, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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ABSTRACT

Progesterone (P4) and synthetic progestins enter the aquatic environment through wastewater treatment plant effluents and agricultural run-off, posing potential risks to aquatic organisms due to their biological activity. P4 is a precursor of a number of steroids in vertebrates, including estrogens and androgens. Mussels *Mytilus galloprovincialis* were exposed to P4 at the ng to low μg/L range (0.02–10 μg/L) for 7 days with the aim of (a) assessing potential alterations on endogenous steroids as a consequence of exposure, and (b) describing the enzymatic pathways involved in P4 metabolism in mussels. No significant alteration of the levels of testosterone (T) and estradiol (E2) was observed in mantle/gonad tissue of exposed mussels, in spite of a 5.6-fold increase in immunoreactive T in those exposed to 10 μg P4/L, which was attributed to cross-reactivity. P4 was actively metabolized to 5α-pregnane-3,20-dione (5α-DHP) and 3β-hydroxy-5α-pregnan-20-one (3β,20-one) in digestive gland, with no evidence for the synthesis of 17α-hydroxyprogesterone or androstenedione. The metabolism of P4 to 5α-DHP was not altered by exposure. Histological examination of the gonads suggested that exposure to 10 μg/L P4 induced gamete maturation and release in mussels. Nonetheless, environmental concentrations of P4 are unlikely to have an endocrine action in mussels.

1. Introduction

A wide range of progestins, including progesterone (P4), are used nowadays in human and veterinary medicine. P4, as other natural and synthetic hormones, continuously enters into aquatic systems through sewage discharge and animal waste disposal, reaching concentrations in the range of 1.5–200 ng P4/L in waste water treatment plant effluents and surface waters (Kolpin et al., 2002; Liu et al., 2012; Kumar et al., 2015). These concentrations might be biologically active and pose a risk to fish: e.g., alter sex differentiation in zebra fish (Liang et al., 2015). However, no information regarding potential targets and mechanisms of action of P4 and progestins in aquatic invertebrates, such as bivalves, is so far available.

The presence of vertebrate-type sex steroids (e.g., progesterone (P4), androstenedione (AD), testosterone (T), and estradiol (E2)) together with some enzymes involved in sex steroid biosynthesis (e.g., 3β/17β-hydroxysteroid dehydrogenases – 3β/17β-HSD –, 5α-reductase) have been described in molluscs (Janer and Porte, 2007; Lafont and Mathieu, 2007; Fernandes et al., 2011). However, studies are still needed to better understand the role of steroids in mollusc's reproduction, to establish their origin (endogenous vs. exogenous) and their mechanisms of action (Scott, 2012, 2013).

Annual variations in steroid content, which are often considered as an argument for the role of steroids in the control of reproductive activity, have often been reported. Thus, Reis-Henriques and Coimbra (1990) reported high levels of P4 in mussels – *Mytilus edulis* – during the spawning season. An increase of P4 with the advancement of sexual maturity was reported in *Sepia officinalis* (Henry and Boucaud-Camou, 1994). Siah et al. (2002) observed increased P4 levels in gonads of *Mya arenaria* during gametogenic periods and suggested that P4 might be involved in the regulation of gametogenesis in marine bivalves. Wang and Croll (2004) reported that injections of P4, E2, T and dehydroepiandrosterone accelerated gonad differentiation and increased male/female ratio in the sea scallop *Placopecten magellanicus*, with all four steroids inducing masculinisation.

* Corresponding authors. Tel.: +34 934006175; fax: +34 93 204 5904.
E-mail addresses: deniseferna@gmail.com (D. Fernandes), cinta.porte@cid.csic.es (C. Porte).

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Apart from steroids, steroidogenic enzymes such are 5α-reductases and 3β/17β-hydroxysteroid dehydrogenases (3β/17β-HSD) have been described in molluscs (Fernandes et al., 2011). The androgen precursor androstenedione (AD) is mainly metabolized to 5α-reduced metabolites (5α-dihydroandrostenedione (5α-DHA) and 5α-dihydrotestosterone (5α-DHT)) and minor amounts of T in *Mytilus galloprovincialis* (Janer et al., 2005; Fernandes et al., 2010). Significant differences, both in terms of activity and synthesis of metabolites, have been reported for the metabolism of AD in other mollusc species. Thus, AD was mainly metabolized to 5α-DHA by microsomal fractions isolated from the visceral coil of the gastropods *Bolinus brandaris* and *Marisa cornuarietis*, whereas no evidence of 5α-reductase activity was detected in *Hexaplex trunculus* that actively metabolized AD to T, a 17β-HSD catalyzed pathway (Janer et al., 2006; Lyssimachou et al., 2009). Changes in 17β-HSD activity have been associated with the reproductive cycle of the scallop *Patinopecten yessoensis* (Matsumoto et al., 1997) and the oyster *Crassostrea gigas* (Le Curieux-Belfond et al., 2001); the activity increased with sexual maturation and declined after spawning, suggesting a role of 17β-HSD in steroid biosynthesis in bivalves. Nonetheless, HSDs are multifunctional enzymes that can metabolize different substrates, including steroids, bile and fatty acids, retinoids and xenobiotics (Prehn et al., 2009). Recently, Lima et al. (2013) cloned the Hsd17b12 orthologue in the gastropod *Nucella lapillus* and pointed out that this enzyme is likely to be involved in lipid metabolism. Thus, the observed association of 17β-HSD with the reproductive cycle of molluscs may be driven by lipid dynamics, as in bivalves, lipids and fatty acids are stored during gametogenesis, they reach a peak at the beginning of the spawning and gradually decline to reach a minimum at post-spawning (Lazzara et al., 2012).

As pointed out by Scott (2012), information on the metabolism of earlier steroid precursors, such as P4 and pregnenolone, is lacking in molluscs. Hines et al. (1996) described the ability of the gastropod *Clione antarctica* to synthesize several progestin and androgen metabolites, including 5α-pregnane-3,20-dione (5α-DHP), 3β-hydroxy-5α-pregnan-20-one (3β,20-one), 3β-hydroxy-4-pregnen-20-one, 5α-androstane-3,17-dione, 3α-hydroxy-5α-androstan-17-one, and 3β-hydroxy-5α-androstan-17-one, from P4 and AD. The synthesis or accumulation of T and E2 was not reported. Wasson and Watts (2000) reported that P4 is metabolized to 5α-reduced metabolites including 5α-DHP and 3β,20-one, among others, by both the ovaries and testes of the echinoid *Lytechinus variegatus*.

The aim of this work was (a) to identify potential alterations of the endocrine system of the mussel *M. galloprovincialis* as a consequence of exposure to different concentrations of exogenous P4 and (b) to describe key enzymatic pathways involved in the metabolism of this steroid precursor. To this end, mussels were exposed to exogenous P4 (20, 200, 2000 & 10,000 ng/L for 7-days) to assess potential alteration of the levels of P4, testosterone and estradiol in the mantle/gonad tissue as a consequence of exposure, histological changes in the gonads, and the metabolism of both, P4 and AD, by digestive gland subcellular fractions.

2. Materials and methods

2.1. Chemicals

Unlabelled steroids were obtained from Sigma (Steinheim, Germany) and Steraloids (Wilton, NH, USA). [1β-^{3}H]-Androstenedione (15–30 Ci/mmol), [1,2,6,7-^{3}H] progesterone (90–115 Ci/mmol) and [4-^{14}C]-testosterone (45–60 mCi/mmol) were purchased from PerkinElmer Life Science Inc. (Boston, MA, USA). Palmitoyl-CoA and trilostane were purchased from Sigma (Steinheim, Germany). Dutasteride (CAS number 164656-23-9; 99.9% pure) was a gift from GlaxoSmithkline (GSK). Radioimmunoassay (RIA) kits for progesterone, testosterone and estradiol were obtained from Beckmann Coulter (Marseille, France). All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany).

2.2. Experimental design

Mussels (*M. galloprovincialis*) (4–7 cm; $n = 177$) were collected in December 2011 from the bivalve farms located in the Ebro Delta (NE Spain), carried to the laboratory and randomly placed into 50 L glass aquaria (30 mussels/tank) filled with filtered sea-water and fitted with constant air bubbling. The mussels were acclimated in the laboratory for 2 days. Environmental conditions, i.e., temperature (18 °C), salinity (35‰) and photoperiod (12 L:12 D) stimulated the original conditions of mussels.

After acclimation, mussels were exposed to different concentrations of progesterone: 20 ng/L (L), 200 ng/L (M), 2000 ng/L (H) and 10 μg/L (SH) for 7 days. There were two sets of controls: non-exposed mussels (C; control tank) and mussels exposed to 0.002% (v/v) triethylene glycol that was used as a carrier (SC; solvent control). Water was changed every day and fresh progesterone added. Mussels were fed every other day with a commercially available plankton preparation (NutraPlus Reef Feed, Cranswick Pet & Aquatics Plc Portugal). After 7-days exposure, the digestive gland and the mantle/gonad tissue were dissected and stored at −80 °C for the determination of enzyme activities and steroid levels, respectively. Gonads of 20 individuals per tank were dissected and placed in buffered formalin 10% (potassium phosphate buffer 100 mM, pH 7.4) for histological examination.

Water samples were collected immediately after dosing and 24 h later (on days 1st and 5th). Water samples (50 to 80 mL) were filtered (0.45 μm), extracted with dichloromethane (3 × 20 mL), evaporated to dryness under nitrogen and stored at −20 °C until RIA analyses.

2.3. Steroid analysis

Progesterone, testosterone and estradiol were measured as described in Fernandes et al. (2010). Briefly, tissue samples (0.5 g wet weight) were homogenized in 2 ml ethanol and frozen overnight at −80 °C. Homogenates were then extracted with 2 ml of ethyl acetate (×3), the organic extract was evaporated under nitrogen, resuspended in 1 ml methanol containing 1% KOH and incubated at 45 °C for 3 h. After the saponification step, Milli-Q water (4 ml) was added, and the sample extracted with dichloromethane (3 × 3 ml). The efficiency of the extraction procedure was 65.4 ± 1.3% for testosterone, 62.4 ± 0.8% for estradiol and 52.0 ± 3.8% for progesterone.

Dry extracts (tissue and water samples) were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatin, and assayed for progesterone, estradiol and testosterone concentrations using commercial RIA kits. Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were 30 pg/g for T, 10 pg/g for E2 and 40 pg/g for P4. The limit of detection of P4 in water was 0.2 ng/L. Cross-reactivity of P4 was 0.03% in the testosterone-RIA kit (IM1119) and <0.0038% in the estradiol-RIA kit (A21854). Intra-assay coefficients of variations were 14.8% (T), 12.1% (E2) and 6.5% (P4). Inter-assay coefficients of variations were 15% (T), 11.2% (E2) and 7.2% (P4).

2.4. Subcellular fractionation

Digestive glands (each sample a pool of 2 individuals) were homogenized in 4 ml of ice cold homogenization buffer containing 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were centrifuged at 500 × *g* for 15 min, the fatty layer removed and the obtained supernatant centrifuged at 12,000 × *g* for 45 min. The resulting pellet, termed mitochondrial fraction, was resuspended in a small volume of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4, containing 0.15 M KCl, 20% (w/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.1 mM PMSF. An aliquot of the supernatant, termed S12, was collected and stored at −80 °C until its use, and the remaining supernatant centrifuged at 100,000 × *g* for 60 min to obtain the microsomal fraction. The resulting pellet (microsomal fraction) was resuspended in homogenization buffer and further centrifuged at 100,000 × *g* for 30 min (washing step). Microsomal pellets were resuspended in the same buffer as mitochondrial pellets and both fractions were stored at −80 °C until assays were performed. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

2.5. Progesterone and androstenedione metabolism

The metabolism of P4 was first investigated by incubating S12, mitochondrial and microsomal fraction isolated from mussel digestive gland (0.5 mg protein) in the presence of 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$ with 0.2 μM [^{3}H]-P4 (0.1 μCi) as substrate and 1 mM NADPH as co-factor in a total volume of 250 μL. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 15 min at 30 °C. Similarly, the metabolism of AD was determined by incubating digestive gland S12 and microsomal fractions (0.4–0.5 mg protein) in 50 mM Tris–HCl buffer pH 7.4, 10 mM $MgCl_2$, with 0.2 μM [^{3}H]-AD (0.075 μCi) and 0.32 mM NADPH in a total volume of 250 μL. The reaction was initiated by the addition of NADPH and incubated in constant shaking for 60 min at 30 °C. Both incubations were stopped by adding 250 μL of acetonitrile, and after centrifugation (1500 × *g*, 10 min), 100 μL of the supernatant were injected into a reverse-phase HPLC system coupled with a radiometric detector.

2.6. HPLC analysis

HPLC analyses were performed on a Agilent Technologies 1200 series system equipped with an analytical Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm reversed-phase column (Agilent, USA) protected by a guard column Eclipse XDB-C18 4.6 × 12.5 mm, 5 μm (Agilent, USA). Separation of [^{14}C]-T and its palmitoyl-ester was performed at 1.2 ml/min with a mobile phase composed of (A) 56% water containing 0.1% acetic acid (pH 3), 13% acetonitrile and 31% methanol, and (B) 100% methanol. The run consisted of 9 min isocratic 100% A, 6 min of linear gradient from 100% A to 0% A, and 25 min isocratic 0% A. Separation of [^{3}H]-P4 metabolites was performed at 1 ml/min with a mobile phase composed of (A) 80% water, 10% acetonitrile and 10% methanol, and (B) 50% acetonitrile and 50% methanol. The run consisted of a 28 min linear gradient from 60% A to 0% A, 28–33 min at 0% A (isocratic mode), 33–38 min linear gradient from 0% A to 60% A and 38–48 min 60% A. Separation of [^{3}H]-AD metabolites was performed at 1 ml/min with a mobile phase composed of (A) 75% water and 25% acetonitrile, and (B) 25% water and 75% acetonitrile. The run consisted of a 30 min linear gradient from 100% A to 0% A, 5 min at 0% A (isocratic mode), 5 min linear gradient from 0% A to 100% A, and 5 min at 100% A. Chromatographic peaks were monitored by on-line radioactivity detection with a Flow Scintillation Analyzer Radiomatic 610TR (PerkinElmer) using Ultima-Flo M (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks.

2.7. Histological analysis

Gonad tissue fixed in 10% formaldehyde in 100 mM potassium phosphate buffer pH 7.4 was dehydrated with ethanol, cleared in Histo-Clear (National Diagnostic, Atlanta, GA, USA), and embedded in paraplast (Sigma–Aldrich, Steinheim, Germany). Sections (7 μm) were stained with Haematoxylin-Eosin Y (Shandon Inc., Pittsburgh, PA, USA), mounted and examined by light microscopy. Mussels were then sorted into distinct stages of gonadal development according to Lubet (1959).

2.8. Statistical procedures

Results are mean values ± SEM of $n = 6$ for steroid analysis, and $n = 5$–8 for pooled samples of 2 digestive glands for steroid metabolism. Statistical significance was assessed by using one-way ANOVA, followed by Dunnett's test for differences from control. Level of significance was $p < 0.05$ or $p < 0.001$. No statistically significant differences were detected among the two control sets (C and SC).

3. Results

3.1. Progesterone concentration in water

The concentration of P4 determined in the tanks right after dosing was close to nominal, viz. 27 ± 0.2, 145 ± 42, 2057 ± 705 and 12,539 ± 2867 ng/L (mean ± range of two different sampling days). However, 24 h after dosing, the concentration of P4 in water sharply decreased to 3 ± 0.8, 12 ± 3.8, 84 ± 22 and 267 ± 67 ng/L. P4, in the range of 0.3–1.5 ng/L, was detected in control tanks.

3.2. Tissue steroid levels

Steroid levels were determined in the mantle/gonad tissue of control and exposed mussels. No differences in P4 levels were observed between controls and *L* & *M* exposure groups (*C*: 11.6 ± 3.4; SC: 12.5 ± 2.7; *L*: 18 ± 4.5; *M*: 17.9 ± 6 ng/g w.w.), whereas organisms from tanks *H* (73 ± 12.1 ng/g w.w.) and SH (342.4 ± 31.3 ng/g w.w) exhibited a statistically significant increase in P4 levels (Fig. 1A). Moreover, a 5-fold increase in immunoreactive T was detected in organisms from the SH-group (SC: 0.94 *vs.* SH: 5.26 ng/g w.w.) (Fig. 1B). However, no significant differences in estradiol levels were observed between control and exposed organisms (3.38–5.20 ng/g w.w.) (Fig. 1C). When the cross-reactivity of P4 in the T assay was examined, we detected a very weak cross-reactivity (<0.1%) at concentrations of P4 < 100 pg/mL, in agreement to what is reported by the manufacturer (0.03%). However, at concentrations of P4 > 5000 pg/mL, cross-reactivity reached 1.8%. Thus, the increase of immunoreactive T in organisms from tanks *H* & SH was attributed to the presence of high concentrations of P4 in the tissue extract.

3.3. Progesterone and androstenedione metabolism

Digestive gland subcellular fractions readily converted P4 into a major metabolite that was identified as 5α-pregnane-3,20-dione (5α-DHP) and minor amounts of a second metabolite, tentatively identified as 3β-hydroxy-5α-pregnane-20-one (3β,20-one) by comparison of the retention time of authentic commercial standards (Fig. 2AA)). No metabolites were formed in the absence of NADPH. Approximately 75% of DHP was metabolized into

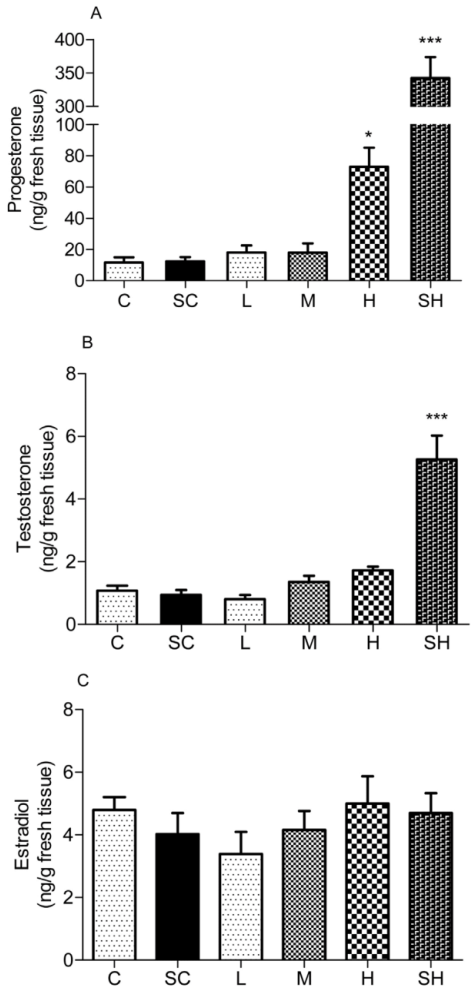

Fig. 1. Levels of (A) progesterone, (B) testosterone and (C) estradiol in mantle/gonad tissue of control and P4-exposed mussels. C: control; SC: solvent control; *L*: 20 ng/L; *M*: 200 ng/L; *H*: 2000 ng/L; SH: 10 μg/L. Data expressed as mean ± SEM ($n = 6$). A one-way ANOVA followed by Dunnett's test was used to estimate significant differences relative to control. p Values: $^{*}p < 0.05$, $^{***}p < 0.001$.

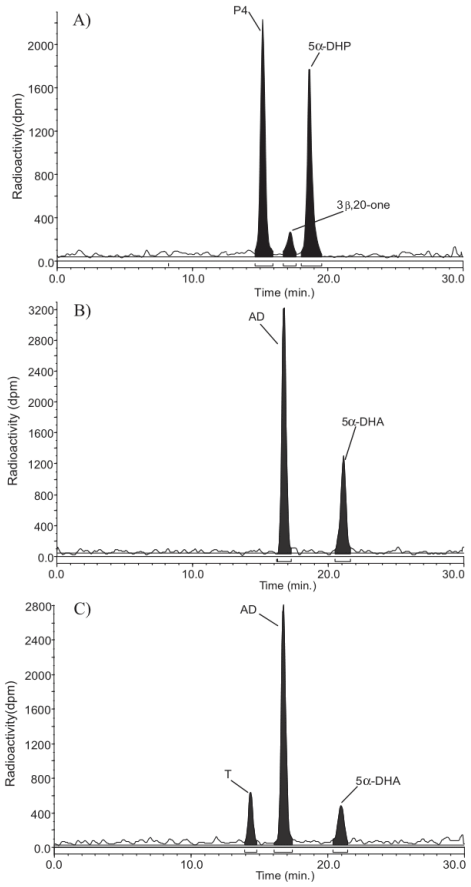

Fig. 2. Representative radiochromatograms for the metabolism of (A) [^{3}H]-P4 in digestive gland microsomal fraction after 15 min incubation; and [^{3}H]-AD in (B) microsomal and (C) S12 fraction after 60 min incubation. P4, progesterone; 5α-DHP, 5α-pregnane-3,20-dione; 3β,20-one, 3β-hydroxy-5α-pregnane-20-one; AD, androstenedione; 5α-DHA, 5α-androstane-3,17-dione; T, testosterone.

3β,20-one (25.6 ± 0.4 pmol/h/mg protein) after 3 h incubation. The formation of this metabolite was inhibited (74 ± 3%) by 10 μM of trilostane, a known inhibitor of 3β-HSD.

The synthesis of 5α-DHP, which results from a reduction of the double bond of the A ring of P4, is a 5α-reductase (5αR) catalyzed pathway. The highest 5αR activity was detected in microsomal fractions (180.9 ± 9.4 pmol/h/mg protein) followed by S12 (86.5 ± 9.3 pmol/h/mg) and mitochondria (1.5 ± 0.1 pmol/h/mg protein), suggesting that 5αR is mainly a microsomal enzyme in mussels. Likewise, digestive gland subcellular fractions metabolized AD into 5α-androstane-3,17-dione (5α-DHA) through 5αR, the highest activity being detected in microsomes (45.8 ± 5.0 pmol/h/mg protein) in comparison to S12 (13.9 ± 3.4 pmol/h/mg) (Fig. 2B and C). Thus, P4 was a better substrate for 5αR than AD. Dutasteride (10 μM), an inhibitor of 5αR, strongly inhibited (70 ± 5%) the formation of both 5α-DHP and 5α-DHA.

The conversion of P4 to 5α-DHP was not significantly altered by exposure despite a trend towards higher metabolism in P4-exposed organisms (195.9–248.8 pmol/h/mg protein) when compared to SC (186.1 ± 8.4 pmol/h/mg protein). The highest 5αR activity was detected in mussels from tank M (Fig. 3.

Similarly, the formation of 5α-DHA from AD was significantly increased in organisms from tank *M* (*M*: 68.1 ± 8.5 *vs.* SC: 42.4 ± 1.8 pmol/h/mg protein) (Fig. 3B). AD was metabolized to both, T and DHA, by S12 fractions (Fig. 3C), which indicates the presence of 17β-HSD in the cytosolic fraction. The metabolism of AD to T was not affected by P4-exposure, whereas the formation of 5α-DHA was significantly increased in mussels from tank *M* as observed in the microsomal fraction.

3.4. Histological analysis

Gonads were examined to assess whether changes in gamete maturation had occurred as a consequence of progesterone

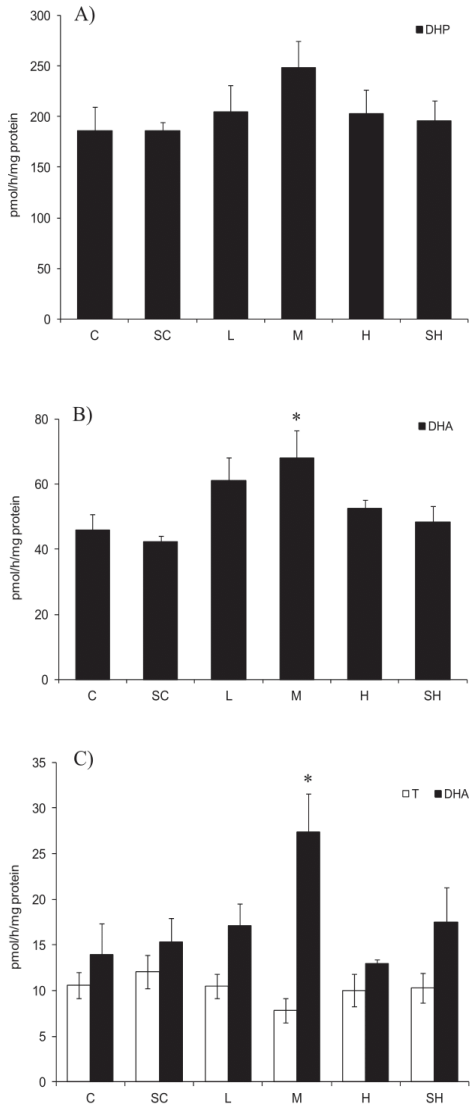

Fig. 3. (A) Metabolism of P4 to DHP, and (B) metabolism of AD to DHA by microsomal fractions isolated from digestive glands of control and P4-exposed mussels. (C) Metabolism of AD to T and DHA in S12 fractions of the same organisms. DHP: 5α-pregnane-3,20-dione; T: testosterone; DHA: 5α-androstane-3,17-dione. C: control; SC: solvent control; *L*: 20 ng/L; *M*: 200 ng/L; *H*: 2000 ng/L; SH: 10 μg/L. Data expressed as mean ± SEM ($n = 8$). A one-way ANOVA followed by Dunnett's test was used to estimate significant differences relative to control. p Values: $^*p < 0.05$.

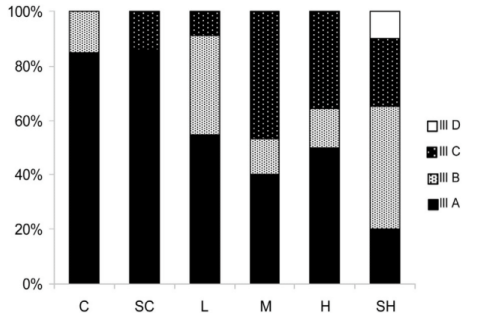

Fig. 4. Distribution of sexual maturation stages in the experimental groups. Stage IIIA: ripe gonads with follicles full of spermatozoa in males and full-grown oocytes in females. Stage IIIB: partially empty gonads as a result of spawning. Stage IIIC: gonadal restoration after non-completed discharge of gametes. Stage IIID: gonadal resorption, characterized by the presence of ruptured follicles and residual gametes, connective tissue reappears between follicles. $n = 15–20$ individuals examined per tank.

exposure (Fig. 4). The gonads in most of the individuals (12 out of 15) in tanks *C* & SC were classified as stage IIIA, thus 80% of the individuals had gonads in ripe stage, with follicles full of spermatozoa in males and full-grown oocytes in females. Exposure to P4 lead to a significant decrease in the number of mussels with gonads classified as SMS IIIA along with a higher number of individuals exhibiting gonads with follicles partially empty (stage IIIB) and in the restoration phase (stages IIIC) as a result of spawning. This tendency towards a lesser number of mussels with ripe gonads was more prevalent in tank SH, where only 4 organisms out of 20 were on the brink of gamete release (stage IIIA); most of the mussels had either partially empty gonads as a result of spawning (stage IIIB) or gonads in stages IIIC and IIID, corresponding to gonadal restoration after non-completed discharge of gametes and gonadal resorption, respectively. No clear evidence of increased frequency of atretic oocytes was observed in exposed mussels.

4. Discussion

The analysis of P4 in water revealed daily oscillations in concentrations, which right after dosing were close to nominal, but sharply decreased up to 89–98% of nominal concentrations 24 h later. Indeed, P4 was taken up by mussels, and its concentration in mantle increased with exposure, particularly in mussels exposed to 2000 and 10,000 ng/L (tanks *H* & SH), which showed P4 concentrations 5.8- and 27.4-fold higher than unexposed mussels (Fig. 1A). However, the adherence of P4 to the tank walls might have also contributed to the almost complete disappearance of P4 from the water over 24 h. For future experiments, the inclusion of a control tank with only the test compound (no mussels) is advisable to ascertain whether the disappearance of the compound is due to uptake by mussels or to other abiotic factors.

The accumulation of P4 in exposed mussels was lower than expected. In an analogous study, a 140-fold increase in T levels was observed in mussels exposed to 2000 ng/L of T for 5-days against the 5.8-fold detected in the present study (Fernandes et al., 2010). Both steroids have similar water solubility (16.8 (P4) and 23.4 (T) mg/L) and a similar behavior in the tanks, with analogous water concentrations right after dosing and 24 h later (Fernandes et al., 2010); thus, a similar bioavailability in the exposure system is expected. However, P4 is not a substrate of acyl-coenzyme A acyltransferases; these enzymes catalyze the conjugation of steroids having a hydroxyl group in position 17 (i.e., T and E2) with fatty acids, significantly reducing their elimination from the body (Gooding and LeBlanc, 2001; Sternberg et al., 2010), and consequently, less accumulation of P4 than T or other 17-hydroxysteroids in mussel's lipids is expected. Thus, exposure to exogenous T significantly increased the retention of T as fatty acid esters in the mud snail *Ilyanassa obsoleta* and the mussel *M. galloprovincialis* (Gooding and LeBlanc, 2001; Fernandes et al., 2010). Similarly, exogenously administrated estradiol was extensively esterified by the mussel *M. galloprovincialis* and retained in the tissue (Janer et al., 2005). Additionally, the rapid metabolism of P4 into 5α-DHP

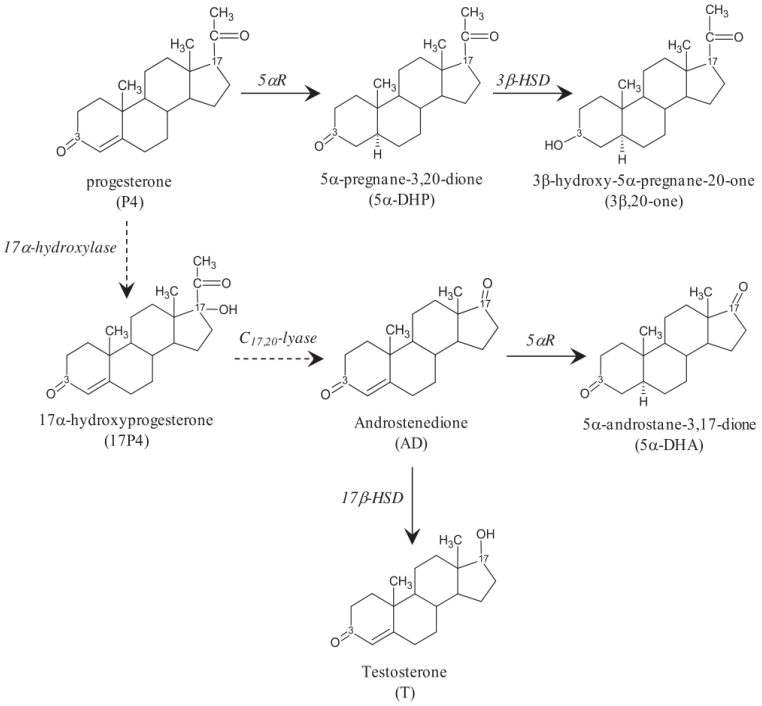

Fig. 5. Schematic summary of progesterone and androstenedione metabolism identified in digestive gland of *Mytilus galloprovincialis*. Solid arrows represent steroid metabolic pathways observed in mussels. Broken arrows represent non-observed pathways. 5αR: 5α-reductase; 3β- or 17β-HSD: 3β- or 17β-hydroxysteroid dehydrogenases.

(186–249 pmol/h/mg protein) might also have contributed to the low P4 accumulation detected in exposed mussels.

P4 metabolism was catalyzed by a 5α-reductase enzyme detected in the digestive gland of mussels that was (a) significantly inhibited (70%) in the presence of 10 μM dutasteride, a known inhibitor of 5α-reductase (Margiotta-Casaluci et al., 2013), (b) tightly associated to microsomal membranes, and (c) P4 was a better substrate than AD, in agreement to what has been described for vertebrates (Levy et al., 1995). The metabolite, 5α-DHP was further metabolized to 3β-hydroxy-5α-pregnane-20-one (3β,20-one) by 3β-HSD; this reaction was significantly inhibited by trilostane (10 μM, 74% inhibition), a known 3β-HSD inhibitor (Ankley et al., 2011). In agreement with our findings, Hines et al. (1996) reported that P4 was predominantly metabolized in vivo to 5α-DHP and 3β,20-one by the gastropod *C. antarctica*. Also Wasson and Watts (2000) reported that P4 was primarily and rapidly metabolized into 5α-reduced metabolites, including 5α-DHP and 3β,20-one, in the ovaries and testis of the echinoid *L. variegatus*.

In vertebrates, P4 is a metabolic precursor of a number of steroids, including androgens and estrogens. It is converted into 17α-hydroxyprogesterone (17P4) by 17α-hydroxylases, which is further metabolized to androstenedione and T (Fig. 5). However, the synthesis of 17P4 from P4 was not reported in the above mentioned studies (Hines et al., 1996; Wasson and Watts, 2000). Neither in the present study, was no synthesis of 17P4 or AD observed in the digestive gland or the gonad/mantle tissue of mussels.

Chishti et al. (2013, 2014),) showed that P4 administration significantly increased T production (3-fold) in both testis and ovary explants, of the fathead minnow *Pimephales promelas*, while E2 production was not affected. In contrast to what has been described for fish, the analysis of steroids in P4 exposed mussels suggests no significant alteration of T or E2 levels, despite the observed increase in immunoreactive T (tanks *H* & SH) that was attributed to cross-reactivity. In fact, the interference of compounds with structural similarity to the target steroid and/or being at very high concentration in the sample is a limitation of immunoassays (Krasowski et al., 2014).

The synthesis of T from AD (a 17β-HSD catalyzed pathway) was detected in digestive gland S12 fraction, but it was not altered by exposure. This agrees with previous studies that demonstrated no change in T synthesis after exposure of mussels to either T or E2, but increased synthesis of 5α-reduced metabolites (5α-DHT and 5α-DHA) in mussels exposed to 2 μg/L E2 or a significant decrease in the formation of 5α-DHT in those exposed to 2 μg/L T (Janer et al., 2005; Fernandes et al., 2010). Thus, exposure to exogenous steroids (P4, T and E2) modulates 5α-reductase activity with no significant effect on 17β-HSD, strengthening the hypothesis that 5α-reductase plays a more important role than 17β-HSDs in the metabolism of steroids in molluscs.

The histological analysis of the gonads allowed the detection of a higher percentage of gonads with partially empty follicles (stage IIIB) and in the restoration phase (stages IIIC), with new gonial mitosis. Thus, P4 exposure accelerated gonad maturation, resulting in a larger amount of mussels at spawning and post-spawning stages (stages IIIB, IIIC, IIID) in comparison to control mussels. Zucchi et al. (2013) reported accelerated oocyte maturation and an impairment of ovarian physiology in female zebrafish exposed for 14 days to P4 (3.5–306 ng/L). Also, induced ovarian maturation and spawning was observed in the greasyback shrimp (*Metapenaeus ensis*) after 1 month of exposure to 100 ng/g of P4 (Yano, 1985). The physiological function of progesterone is mediated through nuclear receptors, but also, two types of membrane proteins unrelated to

nuclear steroid receptors, namely, progesterone membrane receptors (mPR) and progesterone membrane receptor component one (PGMRC1). These two proteins mediate rapid progestin actions initiated at the cell surface that are often nongenomic and have important physiological functions in a variety of reproductive tissues: viz. mPRα is an intermediary in progestin induction of oocyte maturation in fish (Thomas, 2008). Nonetheless, mPRs have not been described in mussels so far, and recently, Kaur et al. (2015) report no evidence of the presence of a progesterone receptor in two gastropod species – *Biomphalaria glabrata* and *Lottia gigantean* – (Kaur et al., 2015). No convincing homologues of the androgen receptor, glucocorticoid receptor and mineralocorticoid receptor were either found, which indicates that the endocrine system of gastropods (and probably molluscs) is fundamentally different of that of fish.

Overall, this work contributes to the better knowledge of the enzymatic pathways involved in the metabolism of P4 in mussels and describes the ability of these invertebrates to actively metabolize P4 to 5α-DHP and 3β,20-one, with no evidence for the synthesis of 17α-hydroxyprogesterone or androstenedione. Exposure to high concentrations of P4 (10 μg/L) accelerates gamete maturation and release, but has no significant effect on steroid levels or steroid metabolizing enzymes. Environmental concentrations of P4, detected in the ng/L range (up to 199 ng/L) in waste water treatment plant effluents and surface waters are unlikely to have an endocrine action in mussels.

Acknowledgments

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References

Ankley, G.T., Cavallin, J.E., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Martinovic-Weigelt, D., Wehmas, L.C., Villeneuve, D.L., 2011. Temporal evaluation of effects of a model 3β-hydroxysteroid dehydrogenase inhibitor on endocrine function in the fathead minnow. Environ. Toxciol. Chem. 30, 2094–2102.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chishti, Y.Z., Feswick, A., Munkittrick, K.R., Martyniuk, C.J., 2013. Transcriptomic profiling of progesterone in the male fathead minnow (*Pimephales promelas*) testis. Gen. Comp. Endocrinol. 192, 115–125.

Chishti, Y.Z., Feswick, A., Martyniuk, C.J., 2014. Progesterone increases ex vivo testosterone production and decreases the expression of progestin receptors and steroidogenic enzymes in the fathead minnow (*Pimephales promelas*) ovary. Gen. Comp. Endocrinol. 199, 16–25.

Fernandes, D., Loi, B., Porte, C., 2011. Biosynthesis and metabolism of steroids in molluscs. J. Steroid Biochem. Mol. Biol. 127, 189–195.

Fernandes, D., Navarro, J.C., Riva, C., Bordonali, S., Porte, C., 2010. Does exposure to testosterone significantly alter endogenous metabolism in the marine mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 100, 313–320.

Gooding, M.P., LeBlanc, G.A., 2001. Biotransformation and disposition of testosterone in the Eastern mud snail *Ilyanassa obsoleta*. Gen. Comp. Endocrinol. 122, 172–180.

Henry, J., Boucaud-Camou, E., 1994. In vitro stimulation by progesterone of the main nidamental glands biosyntheses in the mollusc cephalopod *Sepia officinalis* L. Comp. Biochem. Physiol. 108A, 25–30.

Hines, G.A., Bryan, P.J., Wasson, K.M., McClintock, J.B., Watts, S.A., 1996. Sex steroid metabolism in the antarctic pteropod *Clione antarctica* (Mollusca: Gastropoda). Invertebr. Biol. 115, 113–119.

Janer, G., Lavado, R., Thibaut, R., Porte, C., 2005. Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*: a possible regulating role for steroid acyltransferases. Aquat. Toxicol. 75, 32–42.

Janer, G., Bachmann, J., Oehlmann, J., Schulte-Oehlmann, U., Porte, C., 2006. The effect of organotin compounds on gender specific androstenedione metabolism in the freshwater ramshorn snail *Marisa cornuarietis*. J. Steroid Biochem. Mol. Biol. 99, 147–156.

Janer, G., Porte, C., 2007. Sex steroids and potential mechanisms of non-genomic endocrine disruption in invertebrates. Ecotoxicology 16, 145–160.

Kaur, S., Jobling, S., Jones, C.S., Noble, L.R., Routledge, E.J., Lockyer, A.E., 2015. The nuclear receptors of *Biomphalaria glabrata* and *Lottia gigantea*: implications for developing new model organisms. PLoS One 10 (4), e0121259, http://dx.doi.org/10.1371/journal.pone.0121259

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams: 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36, 1202–1211.

Krasowski, M.D., Drees, D., Morris, C.S., Maakestad, J., Blau, J.L., Ekins, S., 2014. Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. BMC Clin. Pathol. 14, 33.

Kumar, V., Johnson, A.C., Trubiroha, A., Tumovaí, J., Ihara, M., Grabic, R., Kloas, W., Tanaka, H., Kroupovaí, H.K., 2015. The challenge presented by progestins in ecotoxicological research: a critical review. Environ. Sci. Technol. 49, 2625–2638.

Lafont, R., Mathieu, M., 2007. Steroids in aquatic invertebrates. Ecotoxicology 16, 109–130.

Lazzara, R., Fernandes, D., Faria, M., López, J.F., Tauler, R., Porte, C., 2012. Changes in lipid content and fatty acid composition along the reproductive cycle of the fresh water mussel *Dreissena polymorpha*: its modulation by clofibrate exposure. Sci. Total Environ. 432, 195–201.

Le Curieux-Belfond, O., Moslemi, S., Mathieu, M., Söralini, G.E., 2001. Androgen metabolism in oyster *Crassostrea gigas*: evidence for 17β-HSD activities and characterization of an aromatase-like activity inhibited by pharmacological compounds and a marine pollutant. J. Steroid Biochem. Mol. Biol. 78, 359–366.

Levy, M.A., Brandt, M., Sheedy, K.M., Holt, D.A., Heaslip, J.I., Trill, J.J., Ryan, P.J., Morris, R.A., Garrison, L.M., Bergsma, D.J., 1995. Cloning, expression and functional characterization of type 1 and type 2 steroid 5-reductases from cynomolgus monkey: comparisons with human and rat isoenzymes. J. Steroid Biochem. Mol. Biol. 52, 307–319.

Liang, Y.-Q., Huang, G.-Y., Liu, S.-S., Zhao, J-l., Yang, Y.-Y., Chen, X.-W., Tian, F., Jiang, Y.-X., Ying, G.-G., 2015. Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*). Aquat. Toxicol. 160, 172–179.

Lima, D., Machado, A., Reis-Henriques, M.A., Rocha, E., Santos, M.M., Castro, L.F.C., 2013. Cloning and expression analysis of the 17β hydroxysteroid dehydrogenase type 12 (HSD17B12) in the neogastropod *Nucella lapillus*. J. Steroid Biochem. Mol. Biol. 134, 8–14.

Liu, S., Ying, G.-G., Zhao, J.-L., Zhou, L.-J., Yang, B., Chen, Z.-F., Lai, H.-J., 2012. Occurrence and fate of androgens, estrogens, glucocorticoids and progestagens in two different types of municipal wastewater treatment plants. J. Environ. Monit. 14, 482–491.

Lubet, P., 1959. Recherches sur le cycle sexuel et l'emission des gamètes chez les Mytiloidae et les Pectinidae (mollusques bivalves). Revue Travaux l'Institut Peches Maritimes 23, 387–548.

Lyssimachou, A., Ramón, M., Porte, C., 2009. Comparative study on the metabolism of the androgen precursor androstenedione in two gastropod species: in vitro alterations by TBT and TPT. Comp. Biochem. Physiol. 149C, 409–413.

Margiotta-Casaluci, L., Hannah, R.E., Sumpter, J.P., 2013. Mode of action of human pharmaceuticals in fish: the effects of the 5-alpha-reductase inhibitor, dutasteride, on reproduction as a case study. Aquat. Toxicol. 128, 113–123.

Matsumoto, T., Osada, M., Osawa, Y., Mori, K., 1997. Gonadal estrogen profile and immunohistochemical localization of steroidogenic enzymes in the oyster and scallop during sexual maturation. Comp. Biochem. Physiol. 118B, 811–817.

Prehn, C., Möller, G., Adamski, J., 2009. Recent advances in 17beta-hydroxysteroid dehydrogenases. J. Steroid Biochem. Mol. Biol. 114, 72–77.

Reis-Henriques, M.A., Coimbra, J., 1990. Variations in the levels of progesterone in *Mytilus edulis* during the annual reproductive cycle. Comp. Biochem. Physiol. 95A, 343–348.

Scott, A.P., 2012. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. Steroids 77, 1450–1468.

Scott, A.P., 2013. Do mollusks use vertebrate sex steroids as reproductive hormones? II. Critical review of the evidence that steroids have biological effects. Steroids 78, 268–281.

Siah, A., Pellerin, J., Benosman, A., Gagné, J.P., Amiard, J.C., 2002. Seasonal gonad progesterone pattern in the soft-shell clam *Mya arenaria*. Comp. Biochem. Physiol. 132A, 499–511.

Sternberg, R.M., Gooding, M.P., Hotchkiss, A.K., LeBlanc, G.A., 2010. Environmental-endocrine control of reproductive maturation in gastropds: implications for the mechanism of tributyltin-induced imposex in prosobranchs. Ecotoxicology 19, 4–23.

Thomas, P., 2008. Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component one (PGMRC1) and their roles in mediating rapid progestin actions. Front. Neuroendocrinol. 29, 292–312.

Wang, C., Croll, R.P., 2004. Effects of sex steroids on gonadal development and gender determination in the sea scallop, *Placopecten magellanicus*. Aquaculture 238, 483–498.

Wasson, K.M., Watts, S.A., 2000. Progesterone metabolism in the ovaries and testes of the echinoid *Lytechinus variegatus* Lamarck (Echinodermata). Comp. Biochem. Physiol. 127C, 263–272.
Yano, I., 1985. Induced ovarian maturation and spawning in greasyback shrimp, *Metapenaeus ensis*, by progesterone. Aquaculture 47, 223–229.
Zucchi, S., Castiglioni, S., Fent, K., 2013. Progesterone alters global transcription profiles at environmental concentrations in brain and ovary of female zebrafish (*Danio rerio*). Environ. Sci. Technol. 47, 12548–12556.

Paper 3

ALTERATION OF CELLULAR LIPIDS AND LIPID METABOLISM MARKERS IN RTL-W1 CELLS EXPOSED TO MODEL ENDOCRINE DISRUPTERS

G. Dimastrogiovanni[a], M. Córdoba[b], I. Navarro[b], O. Jáuregui[c], and C. Porte[a]

[a] Environmental Chemistry Department, IDAEA-CSIC, C/ Jordi Girona 18, 08034 Barcelona, Spain

[b] Department of Physiology and Immunology, Faculty of Biology, University of Barcelona, Avda. Diagonal 643, Barcelona 08028, Spain

[c] Scientific and Technological Centers, University of Barcelona, CCiTUB, Baldiri i Reixac 10-12, 08028 Barcelona, Spain

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Resumen

Este estudio investiga la utilización de la línea celular de hígado de trucha arco iris (RTL-W1) para evaluar la capacidad de disruptores endocrinos (TBT, TPT, 4-NP, BPA y DEHP) de modificar el metabolismo de lípidos y el perfil lipídico de células expuestas. Entre los compuestos estudiados, BPA y DEHP aumentaron significativamente la acumulación intracelular de triglicéridos (TAG), mientras todos los compuestos, excepto TPT, modificaron los niveles de lípidos de membrana (fosfatidilcolinas (PC) y plasmalógenos de PC), evidenciando una fuerte interacción de estos xenobióticos con la membrana celular y la señalización celular. Se detectaron diferentes genes involucrados en el metabolismo lipídico en RTL-W1, y la expresión de estos genes se alteró por exposición a BPA, TBT y TPT (incremento de FATP1 y FAS) y a 4-NP y DEHP (disminución de FAS y LPL). En resumen, este estudio ha evidenciado diferentes y complejos mecanismos de acción para estos compuestos, tanto a nivel de expresión de genes relacionados con el metabolismo lipídico como la modificación de los lípidos celulares. La línea celular RTL-W1 podría ser un modelo apto para la detección en peces de compuestos químicos que causan esteatosis, u otras patologías relacionadas con el metabolismo lipídico, aunque se recomienda una caracterización más extensa.

Abstract

This work investigates the suitability of the rainbow trout liver cell line (RTL-W1) as an in-vitro model to study the ability of model endocrine disrupters, namely TBT, TPT, 4-NP, BPA and DEHP, to act as metabolic disrupters by altering cellular lipids and markers of lipid metabolism. Among the tested compounds, BPA and DEHP significantly increased the intracellular accumulation of triacylglycerols (TAGs), while all the compounds -apart from TPT-, altered membrane lipids –phosphatidylcholines (PCs) and plasmalogen PCs–, indicating a strong interaction of the toxicants with cell membranes and cell signaling. RTL-W1 expressed a number of genes involved in lipid metabolism that were modulated by exposure to BPA, TBT and TPT (up-regulation of FATP1 and FAS) and 4-NP and DEHP (down-regulation of FAS and LPL). Multiple and complex modes of action of these chemicals were observed in RTL-W1 cells, both in terms of expression of genes related to lipid metabolism and alteration of cellular lipids. Although further characterization is needed, this might be a useful model for the detection of chemicals leading to steatosis or other diseases associated with lipid metabolism in fish.

Keywords: RTL-W1; endocrine disrupters; lipids; phospholipids; triacylglycerols

Introduction

The hypothesis that chemicals in our environment may act as obesogens, perturbing mechanisms involved in body weight control and lipid homeostasis is currently being investigated. Several endocrine disrupters have been identified in recent years to act as obesogenic compounds: organotins such as tributyltin (TBT) and triphenylthin (TPT), estrogenic compounds such as diethylstilbestrol and bisphenol A (BPA), phthalates, perfluorooctanoates and nonylphenols (Elobeid and Allison 2008, Grün and Blumberg 2009; Casals-Casas and Desvergne, 2011).

TBT and TPT promote adipogenesis by binding to the retinoid X receptor (RXRα) and peroxisome proliferator-activated receptor (PPARγ), and they consequently induce the differentiation of murine preadipocyte cells (3T3-L1) to adipocytes (Inadera and Shimomura, 2005; Kanayama et al., 2005). Furthermore, in-utero exposure to TBT led to liver steatosis and increased lipid accumulation and maturation of adipocytes in mouse models and induced ectopic adipocyte formation in *Xenopus* (Grün et al., 2006; Iguchi et al., 2007).

Other studies revealed increased body weight in rats and mice exposed during gestation and/or lactation to low doses of BPA, an endocrine disruptor highly prevalent in our environment (Rubin, 2011). Soon after, Marmugi et al. (2012) reported the accumulation of triglycerides and cholesteryl esters in the liver of mice exposed to 50 and 500 µg BPA/kg/day. Interestingly, micromolar concentrations of BPA and 4-nonylphenol (4-NP) triggered the differentiation of 3T3-L1 cells in adipocytes (Masuno et al., 2002; 2003), and BPA induced the expression of adypocite-specific genes (such are FAS and leptin) (Phrakonkham et al., 2008).

Phthalates, widely used as plasticizers and stabilizers in the manufacture of consumer products, are also shown to alter lipid homeostasis. Thus, urine concentrations of four phthalate metabolites deriving from di-2-ethylhexyl phthalate (DEHP) were positively correlated with abdominal obesity among adult U.S. males (Stahlhut et al., 2007). Among these metabolites, mono-2-ethylhexyl phthalate (MEHP), a known activator of PPARγ promoted differentiation of 3T3-L1 cells into adipocytes and induced the expression of genes involved in lipogenesis, triglyceride synthesis and adipokines (Feige et al., 2007).

Despite the reported evidences in mammals, little is known on the ability of these chemicals to disrupt lipid metabolism in aquatic organisms, which are often exposed to significant concentrations of

these compounds. Interestingly, an increase in whole-body lipid content along with a raise of lipid related plasma parameters (triacylglycerols, cholesterol and lipase) was observed in juvenile chinook salmon (*Oncorhynchus tshawytscha*) exposed to TBT (Meador et al., 2011). Also, long term exposure to TBT induced lipid and fatty acid accumulation in the ramshorn snail *Marisa cornuarietis* (Janer et al., 2007). TBT, tetrabromobisphenol-A (TTBPA), tetrachlorobisphenol-A and TTBPA-sulfate induced lipid accumulation in zebrafish larvae, which are proposed as a screening model (Tingaud-Sequeira et al., 2011; Riu et al., 2014).

As a result of the above observations and because the liver is the predominant site of de novo synthesis of lipids, this study aimed at investigating the use of the rainbow trout (*Oncorhynchus mykiss*) liver cell line (RTL-W1) as a in-vitro model to assess the ability of known or suspected obesogenic compounds, namely TBT, TPT, 4-NP, BPA and DEHP, to disrupt cellular lipids and lipid metabolism. RTL-W1 is predominantly epithelial-like in shape and appears to be a liver stem cell (Lee et al., 1993; Malhao et al., 2013). The lipid composition of RTL-W1 cells was determined by high-performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS). Phosphatidylcholines (PC), plasmalogen PCs and triacylglycerols (TAG) were identified and analyzed under positive electrospray ionization mode (ESI), and relative changes in their levels were determined comparing peak areas in exposed cells with their basal amounts in non-exposed cells. Additionally, expression of the following genes was investigated: (a) the ATP-binding cassette transporter (ABCA1) related to cholesterol homeostasis; (b) two fatty acid transporters -cluster of differentiation 36 (CD36) and fatty acid transport protein 1 (FATP1)- and the enzyme lipoprotein lipase (LPL) as markers of fatty acid uptake; (c) the expression of the lipogenic enzyme, fatty acid synthase (FAS); (d) liver X receptor (LXR) commonly involved in lipogenic pathways; PPARs α and β, promoters of fatty acids use in mammals, and PPARγ involved in lipid accumulation and adipogenesis in mammals and fish (Kota et al., 2005; Bouraoui et al., 2008).

Materials and Methods

Fish cell culture and exposure

The rainbow trout liver cell line (RTL-W1) was kindly supplied by Professor N.C. Bols, University of Waterloo, Canada. RTL-W1 cells were routinely cultured in 75-cm^2 culture flasks at 20°C in Leibovitz's L-15 culture medium (Sigma–Aldrich, Steinheim, Germany) supplemented with 5% fetal bovine serum (FBS, Sigma–Aldrich, Steinheim, Germany) and 1% penicillin–streptomycin solution (10000 units/ml penicillin, 10 mg/ml streptomycin, Sigma–Aldrich, Steinheim, Germany). When 90% of confluence was reached, cells were dissociated with 0.05% (w/v) trypsin and 0.5 mM EDTA for subculturing and exposure experiments.

Tributyltin chloride 96% (TBT), triphenyltin chloride 95% (TPT), 4-nonylphenol (4-NP), bisphenol A 99% (BPA), and bis-(2-ethylhexyl)-phthalate (DEHP) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions were prepared and diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

For exposure experiments, confluent flasks were used to seed pre-coated (1% gelatine) 6-well Costar culture plates (Corning Inc.) at a cell density of 10^6 cells per well. Cells were allowed to attach for 48 h prior to exposure, and cells were exposed to test compounds diluted in culture medium. Contaminated medium was replaced by new one after 48 h exposure. The final concentration applied in wells was 100 nM TBT and TPT, 20 µM 4-NP, 10 µM BPA and 5 µM DEHP and cells were exposed for 24, 48 and 72 h. The selected concentrations did not affect cell viability in RTL-W1 cells (84.1 – 98.9 % cell viability) (Thibaut et al., unpublished results), and were shown to induce lipid droplet formation and to promote adipocyte differentiation in 3T3-L1 cells (Masuno et al., 2003; Inadera and Shimomura, 2005; Hao et al., 2012).

The final concentration of DMSO in culture wells was 0.5% (v/v). For each assay a control was performed by adding only the solvent (DMSO) to the cells. The number of independent experiments, using cells of different passage, was of three for gene analysis and four for lipid profiling. Three technical replicates were performed in each experiment.

RNA extraction and cDNA synthesis

Total RNA was extracted by the Tri-reagent method (Ambion, Applied Biosystems, Spain). The quantity and quality of isolated RNA was determined spectrophotometrically with a NanoDrop® ND-1000 UV-Vis (Labtech Int., U.K.). For cDNA synthesis, 1 µg of RNA, 3 µl of a blend 2:1 random hexamers (600 µM)/oligodT (50 µM), 2 µl dNTP (10 mM), 0.5 µl of reverse transcriptase (20 U/µl), and 0.5 µl of RNAse inhibitor were mixed with the kit buffer in a final volume of 20 µl (Transcriptor fist strand cDNA synthesis kit, Roche, Germany), and incubated at 50°C for 6 min, followed by the inactivation of the enzymes at 85°C for 5 min. The cDNA obtained was stored at -20°C for qRT-PCR.

Real-time PCR analysis

PCR measurements were performed by applying the primers at 0.35 µM with one-fortieth of the cDNA synthesis reaction and SYBR-Green PCR mix (Bio-Rad, Spain) in a total volume of 20 µl. The RT-PCR primer sequences for target genes (ABCA1, LXR, CD36, PPARβ, PPARγ, PPARα, FAS, FATP1 and LPL) and the reference gene (EF1α) are shown in Table 1. Reactions were performed in an iQCycler IQ Real-time Detection System (Bio-Rad, Spain). Each PCR product was sequenced to confirm identity, and each one was found to be 100% identical to its respective sequence. Primers sequences were obtained from our previous studies (references in Table 1) and were designed against sequences from the following databases: http://compbio.dfci.harvard.edu/tgi/; http://www.ncbi.nlm.nih.gov/dbEST/; http://www.sigenae.org/index.php?id=21. Prior to the analyses a dilution curve with a pool of cDNA samples was run to calculate primer efficiency and the values obtained ranged from 91.3% to 112.5%. Reactions were performed in duplicate, and the fluorescence data acquired during the extension phase were determined using the delta-delta-CT method (Livak and Schmittgen, 2001) using EF1α as endogenous reference gene after confirming that its expression did not differ between treatments.

Extraction and analysis of lipids

Lipids were extracted with a modification of the method of Christie (1985). A solution of methanol:chloroform (1:2) containing 0.01% of butylated hydroxytoluene (BHT) as an antioxidant was

added to the cell pellets, vortexed (1 min) and after 30 min incubation at room temperature, extracted in an ultrasonic bath for 5 min (x 2). The extracts were evaporated to dryness, reconstituted with 400 µL of acetonitrile and stored in an argon atmosphere.

High-performance liquid chromatography coupled with electrospray ionization high resolution mass spectrometry (HPLC-ESI-HRMS) has been used for the identification of lipids. The chromatographic separation was performed on an Agilent 1200 RRLC (Agilent, Waldbronn, Germany) using a Zorbax Eclipse Plus RRHD C8 column (1.8 µm particle size, 100 mm x 2.1 mm, Agilent, USA). Flow rate was 0.3 ml/min, column temperature 60ºC, and injection volume 10 µL. Mobile phases consisted of acetonitrile:water (60:40) with 10 mM ammonium acetate (A) and isopropanol:acetonitrile (90:10) with 10 mM ammonium acetate (B). Gradient elution started at 40% of B, increased to 100% B in 15 min and maintained for 5 min. Then, initial conditions were attained in 1 min and the system was stabilized for 9 min. The HPLC system was coupled to a hybrid quadrupole time-of-flight QSTAR Elite (ABSciex). TOF-MS data acquisition between *m/z* 100 and 1000 Da was performed in positive ionization mode. The TOF was calibrated with reserpine (1 pmol/µL) using the ions at *m/z* 195.0651 and *m/z* 609.2812. Processing of acquired mass spectra, identification and relative quantification of detected molecular species were performed with Analyst QS 2.0 software (AB Sciex).

Phosphatidylcholines (PC), plasmalogen PCs, and triacylglycerols (TAG) were analyzed under positive ESI. An inventory of lipids, including 40 phosphatidylcholines (PCs), 14 plasmalogen PCs, and 12 triacylglycerols (TAGs) with their theoretical exact mass and molecular formula was generated and used as a reference database. Identification of lipids was carried out after comparison of registered lipids in the database and the ones encountered in cells, under the criteria of exact mass with an error of ±5 mDa. To verify the registered lipids, internal standards representative of each lipid class were included in each sample, namely, 1,2,3-17:0 TG (TAG 51:0; m/z=866.8171), 17:0 Lyso PC (m/z=510.356), 16:0 D31-18:1 PC (C42H51D31NO8P, m/z=791.267), and PC(P-18:0/18:1) (m/z=772.6215) for PC-plasmalogens. To quantify lipid profile alterations in exposed cells, peak areas were compared with those in non-exposed cells set to 100%.

Statistical analysis

For gene expression, statistical significance was assessed by using non-parametric Kruskal-Wallis and Mann-Whitney U test. For lipid composition, differences were assessed by using one way ANOVA with Dunnett's post hoc test. Statistical analysis was carried out with SPSS Statistics v.21 Chicago, IL. Results are presented as mean values ± SEM (n = 4 independent experiments).

Results

Gene transcription

Gene expression levels of selected key transcription factors and target genes involved in lipid metabolism were detected in RTL-W1 cells by qPCR. Relative measurement in control cells cultured in the absence and presence of 0.5% DMSO over time (24 to 72 h culture) suggested differences in expression among the selected genes, being CD36 and FAS mRNA expression the highest followed by FATP-1 and PPARβ, while the expression of PPARγ and PPARα was rather low (Table 2). The presence of 0.5% DMSO in culture medium had no significant effect on expression patterns, apart from PPARγ, which was not further considered due to rather low expression (Ct values >33) and high variability. Over time differences were observed for FAS and PPARβ, the expression increased in control cells from 24 to 48-72 h in culture.

Exposure of RTL-W1 cells to the selected chemicals had a transient effect in the expression of cell transcription factors, since changes were observed following 24 and/or 48 h of exposure, but no statistically significant effects were observed after 72 h exposure, regardless of the compound tested (Figs. 1 and 2). The expression of ABCA1 transporter, which encodes for a protein that mediates cholesterol efflux, was up-regulated by all the compounds tested, except DEHP, following 24 h exposure (Fig. 1). On the other hand, the expression of the fatty acid transporter CD36 was down-regulated by 4-NP and DEHP after 48 h exposure, while an up-regulation of FATP1, the other fatty acid transporter examined, was detected after 24 h exposure of RTL-W1 cells to BPA and TPT (Fig. 1). The lipogenic enzyme FAS was up-regulated by TBT, TPT and BPA after 24 h exposure, but down-regulated by 4-NP

and DEHP, both at 24 and 48 h. NP and DEHP also down-regulated the expression of LPL in RTL-W1 cells, while this gene was significantly up-regulated by TBT and BPA after 24 h exposure (Fig. 1).

Regarding the transcriptional factors studied, no significant differences in the levels of expression of LXR between exposed and control cells were observed, despite of an up-regulation of BPA and TPT after 24 h of exposure in comparison to longer exposure times (Figure 2). Similar results were obtained for the modulation of PPARα and PPARβ mRNA levels, with relative increases after BPA treatment (24 h), together with a down regulation of PPARβ by 4-NP (48 h).

Lipid class composition

The analysis of RTL-W1 lipids by HPLC-ESI-MS allowed the identification and relative quantification of several PCs, plasmalogen PCs and TAGs under positive ESI. Individual chromatographic peaks were isolated from the full scan MS spectra when lipids' exact mass was selected. A total of 29 lipid species were identified including 13 PCs, 6 Plasmalogen PCs and 10 TAGs. The compounds identified with their molecular formula, accurate mass, error and retention times are shown in Table 3. The lipid composition of RTL-W1 cells under normal culture conditions (control and solvent control) and their over time variation is indicated in Table 4. PCs (36:2, 34:1, 34:2), plamalogen PCs (36:2, 38:5) and TAG (52:2, 50:1, 50:2 and 48:1) were the most abundant. The presence of DMSO in the culture medium had no significant effect on lipid composition apart from a relative increase of TAG (48:1) and a decrease of PC (32:1). Lipid composition did not significantly changed over time (24 to 72 h), with the exception of TAG (50:2), which significantly increased in cells cultured for 48-72 h and TAG (54:3) that decreased after 72 h culture.

The chromatographic profiles of exposed and non-exposed cells were further analyzed by comparison of peak areas of the identified lipids in control (DMSO) and exposed cells. The first outcome of this comparison is presented in Figures 3-5, which show changes in lipid classes in exposed cells compared with their basal levels in non-exposed cells, set to 100%. Significant changes in cell lipids were observed following exposure to the selected chemicals. An increase in PCs was observed in cells exposed to DEHP –up to 1.5-fold- (Fig. 3), the highest increase detected for PCs 38:6, 38:5, 40:6, 40:7, 32:1 and 32:2 after 48 and 72 h exposure. Exposure to 4-NP also led to significant changes in cell PCs, with a significant increase of some species (38:6, 38:5, 40:7, 40:6), and a significant decrease of others (36:2,

36:3). 4-NP exposure also led to a significant decrease in the amount of Plasmalogen PC (up to 50%), which was particularly evident for some species (34:1, 34:2, 34:3, 36:2) after 72 h exposure, while the longer chain ones (38:5 and 40:7) were not significantly altered (Fig. 4). Conversely, exposure to TBT led to a significant increase of Plasmalogen PC 34:1 after 72 h exposure, with no significant alteration of the other species (Fig. 4). The most significant alterations were observed for TAGs that increased up to 2-fold following 24 h exposure to BPA and DEHP (Fig. 5). TPT and 4-NP led to a significant increase in most of the detected TAGs after 48 h exposure (up to 36% for TPT and 27% for 4-NP exposure), apart from TAG 54:3 (Fig. 5).

Discussion

RTL-W1 has been mostly used in toxicological studies, including induction of CYP1A (EROD activity) and evaluation of DNA damage (Segner et al., 2000; Schirmer et al., 2000; Schnell et al., 2009; Boettcher et al., 2010). The metabolism of lauric acid and the glucuronidation of 1-naphthol and testosterone by RTL-W1 cells have also been described (Thibaut et al., 2009). Malhão et al. (2013) recently characterized the phenotype of RTL-W1 cells growing in monolayer or aggregates, and concluded that the former were similar to bile preductular epithelial cells, which are considered as stem cells in teleost liver. Interestingly, organelle content in monolayer cells was constituted basically by mitochondria and abundant free ribosomes, with no (cytochemically) detectable peroxisomes and lysosomes. In the present study, we observed that RTL-W1cells growing on monolayer expressed detectable levels of genes involved in lipid metabolism. Among them, FA transporters -CD36 and FATP-1- and the lipogenic enzyme FAS appear to be the most highly expressed, in agreement with a hepatic cell phenotype. Regarding PPARs, PPARβ was the most strongly expressed isotype, while expression of PPARα and PPARγ was very low, in accordance to the expression pattern described in the liver of brown trout (*Salmo trutta*) (Batista-Pinto et al., 2005). PPARβ takes part in cell differentiation and survival, systemic and tissue specific fatty acid catabolism, and it is ubiquitously expressed in different fish tissues (Michalik and Wahli, 2007; He et al., 2012), while PPARγ is mainly expressed in tissues characterized by a high storage of fatty acids, such as white and brown adipose tissue (Feige, 2006).

Under our experimental conditions, the expression of ABCA1 transporter was up-regulated by all the compounds tested, apart from DEHP. ABCA1 promotes clearance of excess cholesterol and

phospholipids from hepatocytes, reduces hepatic lipid accumulation and prevents endoplasmic reticulum stress (Ma et al., 2014), suggesting that an up-regulation of ABCA1 expression could be a first defense response of RTL-W1 cells to ED exposure.

Among the tested compounds, an up-regulation of the expression of FATP1 and FAS was caused by BPA exposure, which is consistent with an activation of fatty acid uptake and *de novo* lipogenesis in RTL-W1 cells, and supported by the 2-fold increase in TAG detected after 24 h exposure, an increase that was still significant (1.3-fold) after 48 h exposure (Fig. 5). It has been reported that the main products of FAS in fish are the saturated fatty acids 16:0 (palmitic acid) and 18:0 (stearic acid), which can be desaturated to yield, respectively, 16:1n-7 (palmitoleic acid) and 18:1n-9 (oleic acid) (Tocher, 2003). Although, ESI/HRMS only provides information on the total carbon number and unsaturation index, and does not identify molecular composition due to the presence of multiple isobaric species, combinations of the above mentioned fatty acids are very likely to occur in the TAGs detected in RTL-W1 cells (Table 4). BPA has usually shown a weak obesogenic response in the 3T3-L1 lipid accumulation assay and was a weak activator of PPARγ receptor (Pereira-Fernandes et al., 2013). However, in-vivo studies have shown an accumulation of liver triglycerides and cholesteryl esters with changes in hepatic FA composition in adult mice exposed to low BPA doses; an activation of lipogenesis and cholesterol biosynthesis were pointed out as the major mechanisms involved (Marmugi et al., 2012).

Similarly, organotin compounds, TBT and TPT, up-regulated the expression of FAS, FATP1 (TPT), and LPL (TBT). The up-regulation of FAS indicates that cell lipid content can be at least in part increased by the synthesis of new fatty acids from lipogenic precursors present in the medium and inside the cells. However, only cells exposed to TPT showed an up-regulation of FATP1 and a significant increase in TAG content, suggesting increased fatty acid uptake (Fig. 5), whereas those exposed to TBT had significant changes in PC and plasmalogen-PCs. TBT has been shown to disturb lipid homeostasis in the filamentous fungus *Cunninghamella elegans*, it led to a decrease of phosphatidylethanolamine and phosphatidylserine, but increased the levels of phosphatidic acid, phosphatidylinositol and phosphatidylcholine (Bernat et al., 2014). These changes were observed together with a decrease in the overall unsaturation of phospholipids. In the present study, TBT caused a relative increase of some PCs (32:1, 38:3) and plasmalogen-PCs (34:1, 34:2) while highly unsaturated species remain almost unchanged (Figs. 3&4). TBT and TPT are known ligands of the RXR–PPARγ heterodimer and induce lipogenic PPARγ gene expression in liver and adipose tissue (Inadera and Shimomura, 2005). Pavlikova et al.

(2010) reported up to 2-fold increase of PPARγ expression and PPARα/β isotypes in the liver of salmon exposed to rather high concentrations of TBT through the diet (10 mg/Kg). However, PPARγ expression in RTL-W1 cells is rather low, and exposure to organotin compounds did not alter the expression of PPARα/β, and led to minor alterations in cell lipids in comparison to the other endocrine disrupters tested.

4-NP and DEHP down-regulated CD36, LPL and FAS expression in RTL-W1 cells. This contrasts with results in other cell models where LPL expression increased in DEHP (100 μM) treated murine mesenchymal stem cells C3H/10T1/2. Mono-(2-ethylhexyl), a metabolite of DEHP, also increased LPL expression in mice adipose tissue (Hao et al., 2012). Nonetheless, exposure to DEHP caused a significant increase of TAGs (24 h) and PCs (48 and 72 h exposure), particularly of highly unsaturated PCs (> 5) of 38 and 40 C atoms, which is in agreement with other studies that reported DEHP (50 μM) to enhance the accumulation of PCs in HRP-1 trophoblastic cells after 24 h exposure (Xu et al., 2006). Similarly, NP exposure increased the relative abundance of TAGs and PCs in RTL-W1 cells.

Overall, DEHP, NP and TBT were the most effective compounds disturbing PCs, the principal structural constituents of cellular membranes (Van Meer et al., 2008). Plasmalogens are structural membrane components, but also a reservoir for lipid second messengers; they facilitate membrane fusion, and are involved in ion transport, cholesterol efflux and store long-chain PUFA (Lesharpig and Fuchs, 2009). A relative increase (up to 40%) of some plasmalogen-PCs vas observed after exposure to BPA (34:2, 34:3, 40:7), DEHP (34:2, 34:3) and TBT (34:2, 34:1), while NP was the only compound to decrease (up to 50%) plasmalogen-PCs (34:1, 34:2, 34:3; 36:2). Information about possible effects of 4-NP and other alkylphenols on membrane lipids is scarce. Meier et al. (2007) found that alkylphenols can alter the fatty acid profile of polar lipids in the liver of Atlantic cod to more saturated fatty acids and less n-3 polyunsaturated fatty acids (n-3 PUFA). They also demonstrated that alkylphenols increased the mean molecular areas of phospholipids in monolayers of native phospholipids extracted from cod liver. Thus, it is very likely that 4-NP accumulates in the phospholipid bilayer of RTL-W1 cells and affects membrane fluidity. The modifications of PCs and plasmalogen-PCs profiles in exposed cells may be also be indicative of oxidative damage, as the higher the unsaturation index of a fatty acid molecule the higher its susceptibility to peroxidation (Hulbert et al., 2007).

Overall, this work shows that the selected endocrine disrupters have the potential to alter the expression of genes related to lipid metabolism in RTL-W1 cells as well as to produce significant changes in membrane lipids and triacylglicerols. All the compounds tested, apart from TPT, induced significant

changes in membrane lipids –PCs and plasmalogen PCs-, indicating a strong interaction of the toxicants with cell membranes, and possibly with cell signaling. Among the tested compounds, BPA and DEHP significantly induced the intracellular accumulation of TAGs, the effect being more evident after shorter exposure times (24 and 48 h). The mode of action of these chemicals is multiple and complex and no clear association between expression of lipid related genes and TAG accumulation has been detected. Although further characterization is needed, the use of this fish cell line can be a valuable in-vitro tool to estimate the potential of different compounds and their mixtures to interfere with lipid metabolism in hepatocytes under different exposure conditions (viz. supplementation of cell medium with specific lipids, presence/absence of FBS in culture medium, different concentrations and exposure lenght). It is particularly interesting the fact that although RTL-W1 is an epithelial-like cell line, it has been reported that, especially cell aggregates, had signs of hepatocytic differentiation (Malhão et al, 2013). Therefore, further studies regarding gene expression profile of cell aggregates and its possible differentiation as hepatocytes or liver stromal cells would be of interest.

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References

Batista-Pinto, C., Rodrigues, P., Rocha, E., Lobo-da-Cunha, A., 2005. Identification and organ expression of peroxisome proliferator activated receptors in brown trout (*Salmo trutta f. fario*). Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression, 1731, 88-94.

Bernat, P, Gajewska, E., Szewczyk, R., Słaba, M., Długoński, J. 2014. Tributyltin (TBT) induces oxidative stress and modifies lipid profile in the filamentous fungus *Cunninghamella elegans*. Environmental Science Pollution Research 21, 4228–4235.

Boettcher, M., Grund, S., Keiter, S., Kosmehl, T., Reifferscheid, G., Seitz, N., Rocha, P., Hollert, H., Braunbeck, T. 2010. Comparison of *in vitro* and *in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test. Mutation Research 700, 11-17.

Bouraoui, L., Gutiérrez, J., Navarro, I. 2008. Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). Journal of Endocrinology 198, 459-469.

Casals-Casas, C., Desvergne, B. 2011. Endocrine disruptors: from endocrine to metabolic disruption. Annual Reviews of Physiology 73, 135-162.

Christie, W.W. 1985. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. Journal of Lipid Research 26, 507-512.

Cruz-Garcia, L., Minghetti, M., Navarro, I., Tocher, D.R. 2009. Molecular cloning, tissue expression and regulation of Liver X Receptor (LXR) transcription factors of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology 153B,81-88.

Cruz-Garcia, L., Sánchez-Gurmaches, J., Gutiérrez, J., Navarro, I. 2011. Regulation of LXR by fatty acids, insulin, growth hormone and tumor necrosis factor α in rainbow trout myocytes. Comparative Biochemistry and Physiology 160A, 125-136.

Elobeid, M.A., Allison, D.B. 2008. Putative environmental-endocrine disruptors and obesity: a review. Current Opinion in Endocrinology, Diabetes and Obesity 15, 403-408.

Feige, J.N., Gelman, L., Michalik, L., Desvergne, B., Wahli, W. 2006. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Progress in Lipid Research 45, 120-159.

Feige, J.N., Gelman, L., Rossi, D., Zoete, V., Métivier, R., Tudor, C., Anghel, S.I., Grosdidier, A., Lathion, C., Engelborghs, Y. 2007. The endocrine disruptor monoethyl-hexyl-phthalate is a selective

peroxisome proliferator-activated receptor γ modulator that promotes adipogenesis. Journal of Biological Chemistry 282, 19152-19166.

Grün, F., Blumberg, B. 2009. Minireview: the case for obesogens. Molecular Endocrinology 23, 1127-1134.

Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T., Blumberg, B. 2006. Endocrine-Disrupting Organotin Compounds Are Potent Inducers of Adipogenesis in Vertebrates. Molecular Endocrinology 20, 2141-2155.

Hao, C.J., Cheng, X.J., Xia, H.F., Ma, X. 2012. The Endocrine Disruptor 4-Nonylphenol Promotes Adipocyte Differentiation and Induces Obesity in Mice. Cellular Physiology and Biochemistry 30, 382-394.

Hulbert, A.J., Pamplona, R., Buffenstein, R., Buttemer, W.A. 2007. Life and death: metabolic rate, membrane composition, and life span of animals. Physiological Reviews 87, 1175–1213.

Iguchi, T., Katsu, Y., Horiguchi, T., Watanabe, H., Blumberg, B., Ohta, Y. 2007. Endocrine disrupting organotin compounds are potent inducers of imposex in gastropods and adipogenesis in vertebrates. Molecular Cellular Toxicology 3, 1-10.

Inadera, H., Shimomura, A. 2005. Environmental chemical tributyltin augments adipocyte differentiation. Toxicology Letters 159, 226-234.

Janer, G., Navarro, J.C., Porte, C. 2007. Exposure to TBT increases accumulation of lipids and alters fatty acid homeostasis in the ramshorn snail *Marisa cornuarietis*. Comparative Biochemistry and Physiology 146C, 368-374.

Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T., Nishikawa, J.-I. 2005. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor γ/retinoid X receptor pathway. Molecular Pharmacology 67, 766-774.

Kota, B.P., Huang, T.H.-W., Roufogalis, B.D. 2005. An overview on biological mechanisms of PPARs. Pharmacological Research 51, 85-94.

Lee, L.E., Clemons, J.H., Bechtel, D.G., Caldwell, S.J., Han, K.-B., Pasitschniak-Arts, M., Mosser, D.D., Bols, N.C. 1993. Development and characterization of a rainbow trout liver cell line expressing cytochrome P450-dependent monooxygenase activity. Cell Biology and Toxicology 9, 279-294.

Lesharpig, J., Fuchs, B. 2009. Plasmalogens in biological systems: their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis. Current Medicinal Chemistry 16, 2021-2041.

Livak, K.J., Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.

Ma, D., Liu, W., Wang, Y. 2014. ApoA-I or ABCA1 expression suppresses fatty acid synthesis by reducing 27 hydroxycholesterol levels. Biochimie 103, 101-108

Malhão, F., Urbatzka, R., Navas, J., Cruzeiro, C., Monteiro, R., Rocha, E. 2013. Cytological, immunocytochemical, ultrastructural and growth characterization of the rainbow trout liver cell line RTL-W1. Tissue and Cell 45, 159-174.

Marmugi, A., Ducheix, S., Lasserre, F., Polizzi, A., Paris, A., Priymenko, N., Bertrand-Michel, J., Pineau, T., Guillou, H., Martin, P.G. 2012. Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver. Hepatology 55, 395-407.

Masuno, H., Kidani, T., Sekiya, K., Sakayama, K., Shiosaka, T., Yamamoto, H., Honda, K. 2002. Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. Journal of Lipid Research 43, 676-684.

Masuno, H., Okamoto, S., Iwanami, J., Honda, K., Shiosaka, T., Kidani, T., Sakayama, K., Yamamoto, H. 2003. Effect of 4-nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. Toxicological Sciences 75, 314-320.

Meador, J.P., Sommers, F.C., Cooper, K.A., Yanagida, G. 2011. Tributyltin and the obesogen metabolic syndrome in a salmonid. Environmental Research 111, 50-56.

Meier, S., Andersen, T.C., Lind-Larsen, K., Svardal, A., Holmsen, H. 2007. Effects of alkylphenols on glycerophospholipids and cholesterol in liver and brain from female Atlantic cod (*Gadus morhua*). Comparative Biochemistry and Physiology Part 145C, 420-430.

Michalik, L., Wahli, W. 2007. Peroxisome proliferator-activated receptors (PPARs) in skin health, repair and disease. Biochimica Biophysica Acta 1771, 991-998.

Pavlikova, N., Kortner, T.M., Arukwe, A. 2010. Peroxisome proliferator-activated receptors, estrogenic responses and biotransformation system in the liver of salmon exposed to tributyltin and second messenger activator. Aquatic Toxicology 99, 176-185.

Pereira-Fernandes, A., Demaegdt, H., Vandermeiren, K., Hectors, T. L., Jorens, P. G., Blust, R., and Vanparys, C. 2013. Evaluation of a screening system for obesogenic compounds: Screening of endocrine disrupting compounds and evaluation of the PPAR dependency of the effect. PloS One 8, e77481.

Phrakonkham, P., Viengchareun, S., Belloir, C., Lombes, M., Artur, Y., Canivenc-Lavier, M.-C. 2008. Dietary xenoestrogens differentially impair 3T3-L1 preadipocyte differentiation and persistently affect leptin synthesis. The Journal of Steroid Biochemistry and Molecular Biology 110, 95-103.

Riu, A., McCollum, C.W., Pinto, C.L., Grimaldi, M., Hillenweck, A., Perdu, E., Zalko, D., Bernard, L., Laudet, V., Balaguer, P., Bondesson, M., Gustafsson, J.A. 2014. Halogenated Bisphenol-A Analogs Act as Obesogens in Zebrafish Larvae (*Danio rerio*). Toxicological Sciences 139, 48–58.

Rubin, B.S. 2011. Bisphenol A: An endocrine disruptor with widespread exposure and multiple effects. The Journal of Steroid Biochemistry and Molecular Biology 127, 27-34.

Sánchez-Gurmaches, J., Cruz-Garcia, L., Gutiérrez, J., Navarro, I. 2012. mRNA expression of fatty acid transporters in rainbow trout: in vivo and in vitro regulation by insulin, fasting and inflammation and infection mediators. Comparative Biochemistry and Physiology 163A, 177–188.

Schnell, S., Bols, N.C., Barata, C., Porte, C. 2009. Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. Aquatic Toxicology 93, 244-252.

Schirmer, K., Chan, A.G.J., Bols, N.C. 2000. Transitory metabolic disruption and cytotoxicity elicited by benzo(a)pyrene in two cell lines from rainbow trout liver. Journal of Biochemistry and Molecular Toxicology 14, 262-276.

Segner, H., Behrens, A., Joyce, E.M., Schirmer, K., Bols, N.C. 2000. Transient induction of 7-ethoxyresorufin-*O*-deethylase (EROD) activity by medium change in the rainbow trout liver cell line, RTL-W1. Marine Environmental Research 50, 489-493.

Stahlhut, R.W., van Wijngaarden, E., Dye, T.D., Cook, S., Swan, S.H. 2007. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult US males. Environmental Health Perspectives 115, 876-882.

Thibaut, R., Schnell, S., Porte, C. 2009. Assessment of metabolic capabilities of PLHC-1 and RTL-W1 fish liver cell lines. Cell Biology and Toxicology 25, 611-622.

Tingaud-Sequeira, A., Ouadah, N., Babin, P.J. 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. Journal of Lipid Research 52, 1765-1772.

Tocher, D.R. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11, 107-184.

Van Meer, G., Voelker, D.R., Feigenson, G.W. 2008. Membrane lipids: where they are and how they behave. Nature Reviews Molecular Cell Biology 9, 112-124.

Xu, Y., Knipp, G.T., Cook, T.J. 2006. Effects of di-(2-ethylhexyl)-phthalate and its metabolites on the lipid profiling in rat HRP-1 trophoblast cells. Archives of Toxicology 80, 293-298.

Figure 1. mRNA expression of ABCA1, CD36, FATP1, LPL and FAS in RTL-W1 cells following exposure to TBT and TPT (100 nM), 4-NP (20 µM), BPA (10 µM) and DEHP (5 µM) for 24, 48 or 72 h. Values are presented as relative units (RU) using EloF1α as a reference gene. Data are shown as mean ± SEM (n= 3). Results were analyzed by non-parametric Kruskal-Wallis and Mann-Whitney U test. Values that do not share a common letter are significantly different ($p<0.05$). *Significant differences respect to control (DMSO)

Figure 2. mRNA expression of LXR, PPARα and PPARβ in RTL-W1 cells following exposure to TBT and TPT (100 nM), 4-NP (20 µM), BPA (10 µM) and DEHP (5 µM) for 24, 48 or 72 h. Values are presented as relative units (RU) using EloF1α as a housekeeping gene. Data are shown as mean ± SEM (n= 3). Results were analyzed by non-parametric Kruskal-Wallis and Mann-Whitney U test. Values that do not share a common letter are significantly different ($p<0.05$). *Significant differences respect to control (DMSO).

Figure 3. Changes in phosphatidylcholines detected in RTL-W1 cells exposed for 24, 48 and 72 h to TBT and TPT (100 nM), 4-NP (20 µM), BPA (10 µM) and DEHP (5 µM). Values expressed as relative abundance compared with control cells as mean ± SEM (n= 4). *Significant differences respect to control (DMSO).

Figure 4. Changes in plasmalogen-PCs detected in RTL-W1 cells exposed for 24, 48 and 72 h to TBT and TPT (100 nM), 4-NP (20 µM), BPA (10 µM) and DEHP (5 µM). Values expressed as relative abundance compared with control cells as mean ± SEM (n= 4). *Significant differences respect to control (DMSO).

Figure 5. Changes in triacylglycerosl detected in RTL-W1 cells exposed for 24, 48 and 72 h to TBT and TPT (100 nM), 4-NP (20 µM), BPA (10 µM) and DEHP (5 µM). Values expressed as relative abundance compared with control cells as mean ± SEM (n= 4). *Significant differences respect to control (DMSO).

Figure 1.

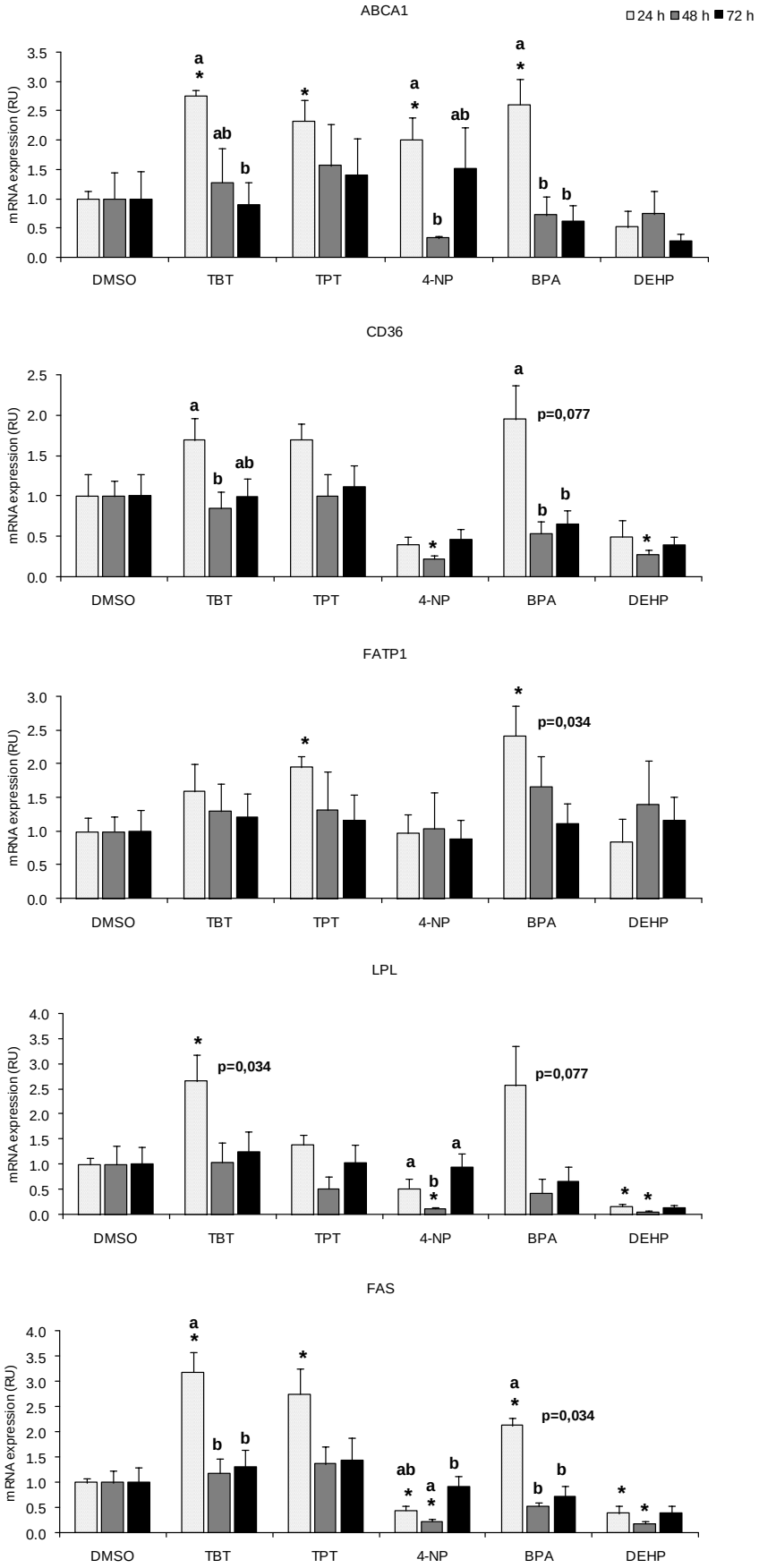
ABCA1
24 h 48 h 72 h
mRNA expression (RU)
DMSO TBT TPT 4-NP BPA DEHP
CD36
p=0,077
FATP1
p=0,034
LPL
p=0,034
p=0,077
FAS
p=0,034

Figure 2.

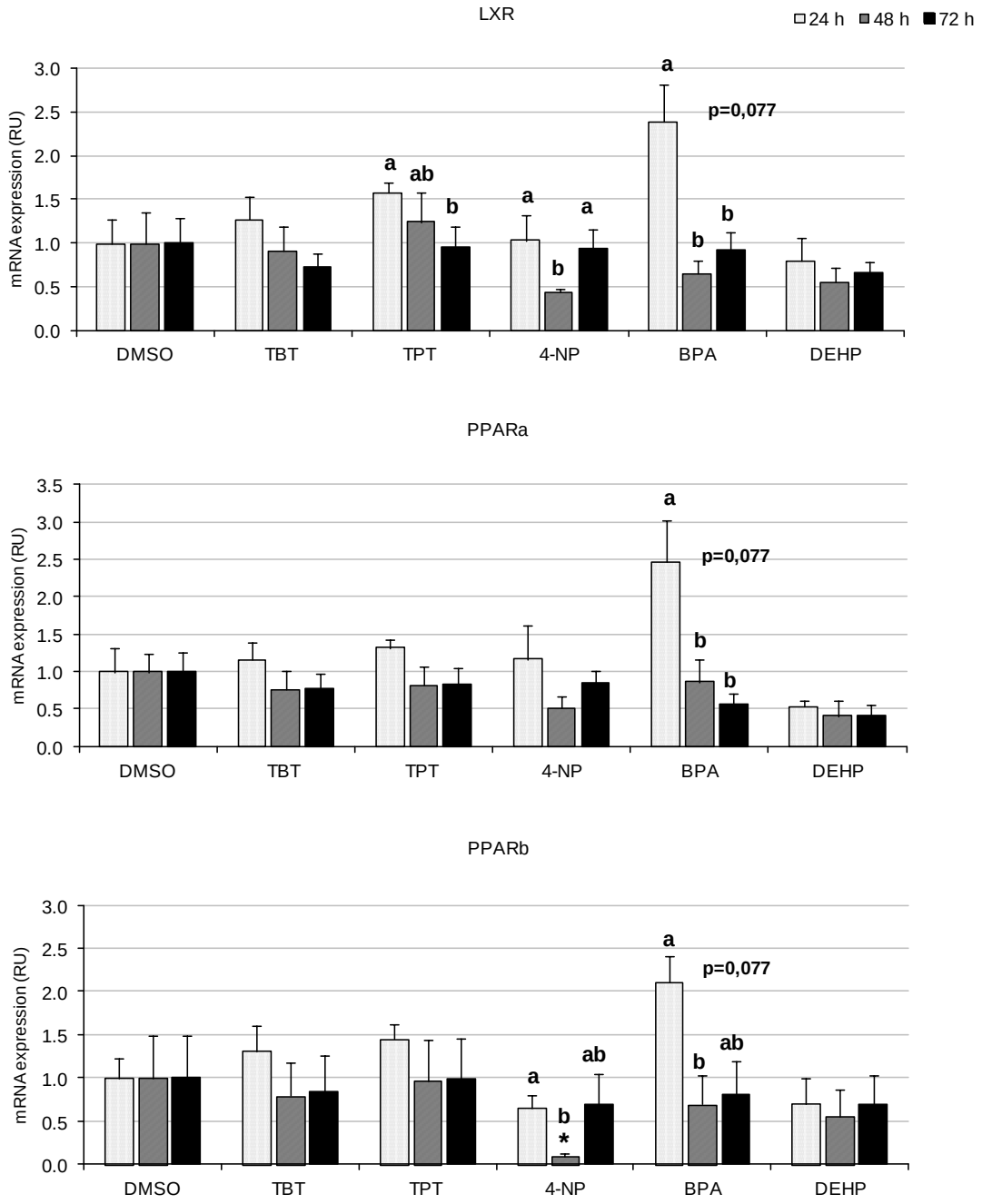
LXR
24 h
48 h
72 h
mRNA expression (RU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
a
b
a
p=0,077
b
b
DMSO
TBT
TPT
4-NP
BPA
DEHP
PPARa
mRNA expression (RU)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
p=0,077
b
b
DMSO
TBT
TPT
4-NP
BPA
DEHP
PPARb
mRNA expression (RU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
p=0,077
a
b
*
ab
b
ab
DMSO
TBT
TPT
4-NP
BPA
DEHP

Figure 3.

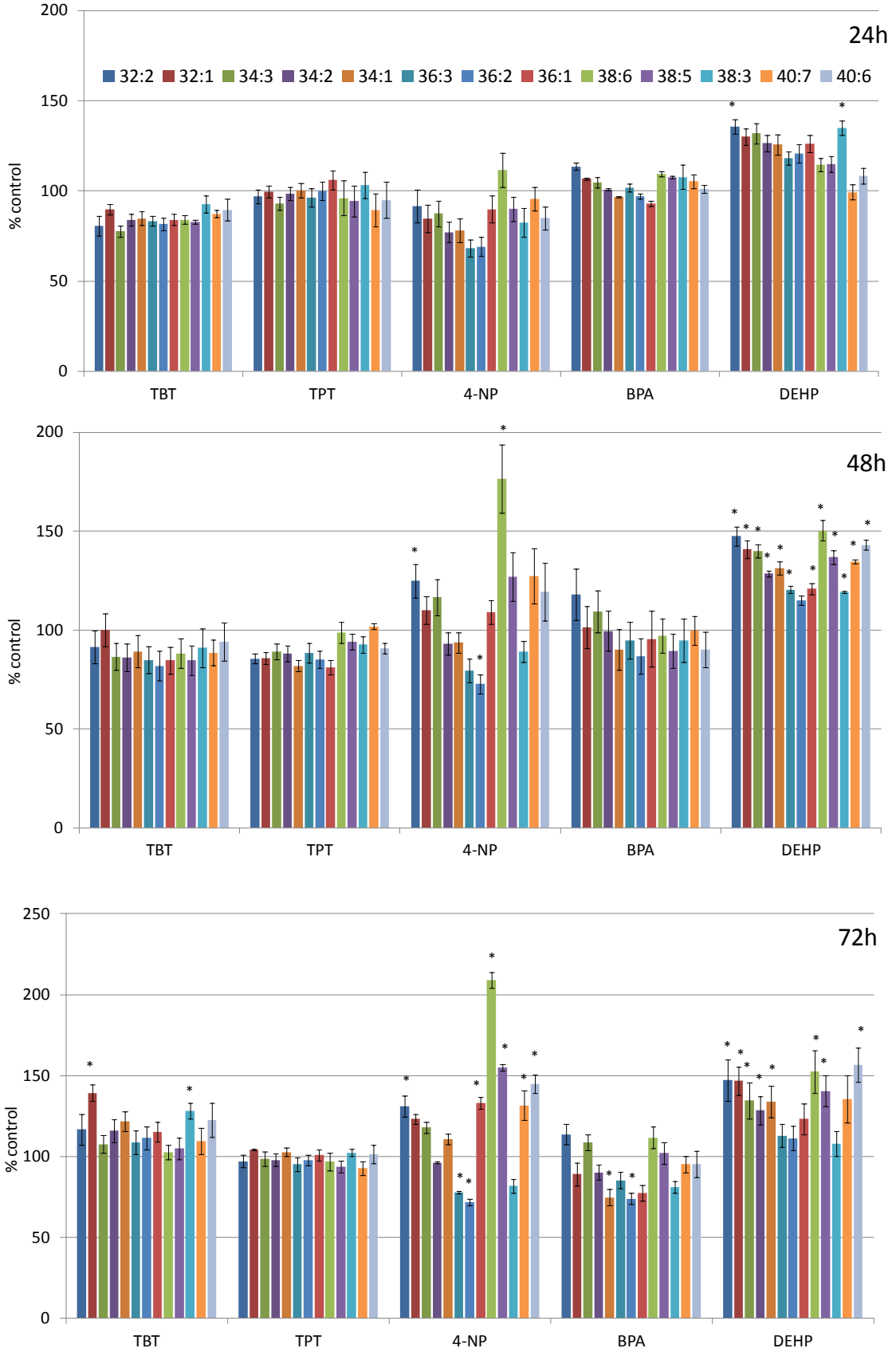
24h
32:2 32:1 34:3 34:2 34:1 36:3 36:2 36:1 38:6 38:5 38:3 40:7 40:6
% control
200
150
100
50
0
TBT
TPT
4-NP
BPA
DEHP
48h
% control
200
150
100
50
0
TBT
TPT
4-NP
BPA
DEHP
72h
% control
250
200
150
100
50
0
TBT
TPT
4-NP
BPA
DEHP

Figure 4.

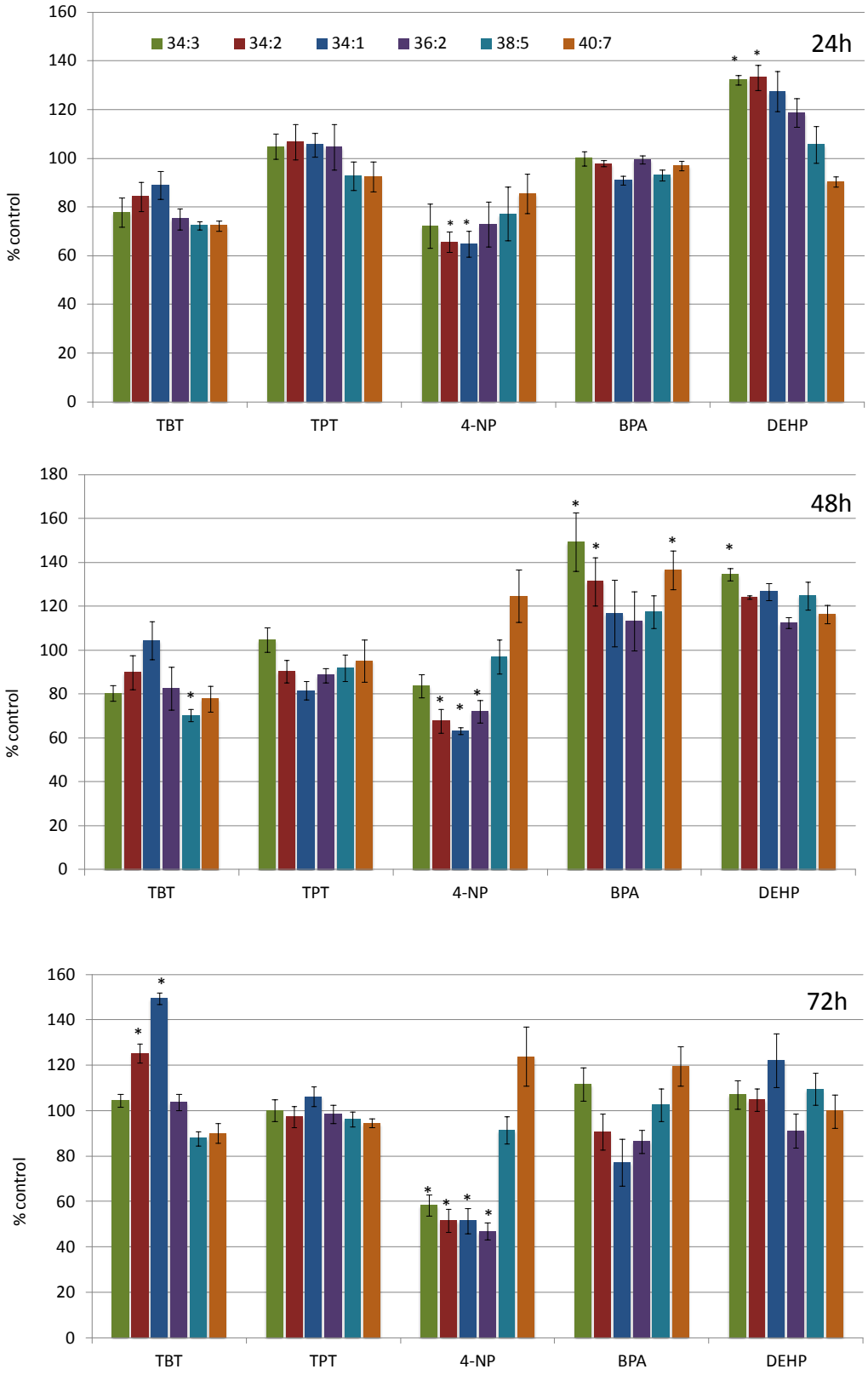
34:3
34:2
34:1
36:2
38:5
40:7
24h
48h
72h
% control
TBT
TPT
4-NP
BPA
DEHP

Figure 5.

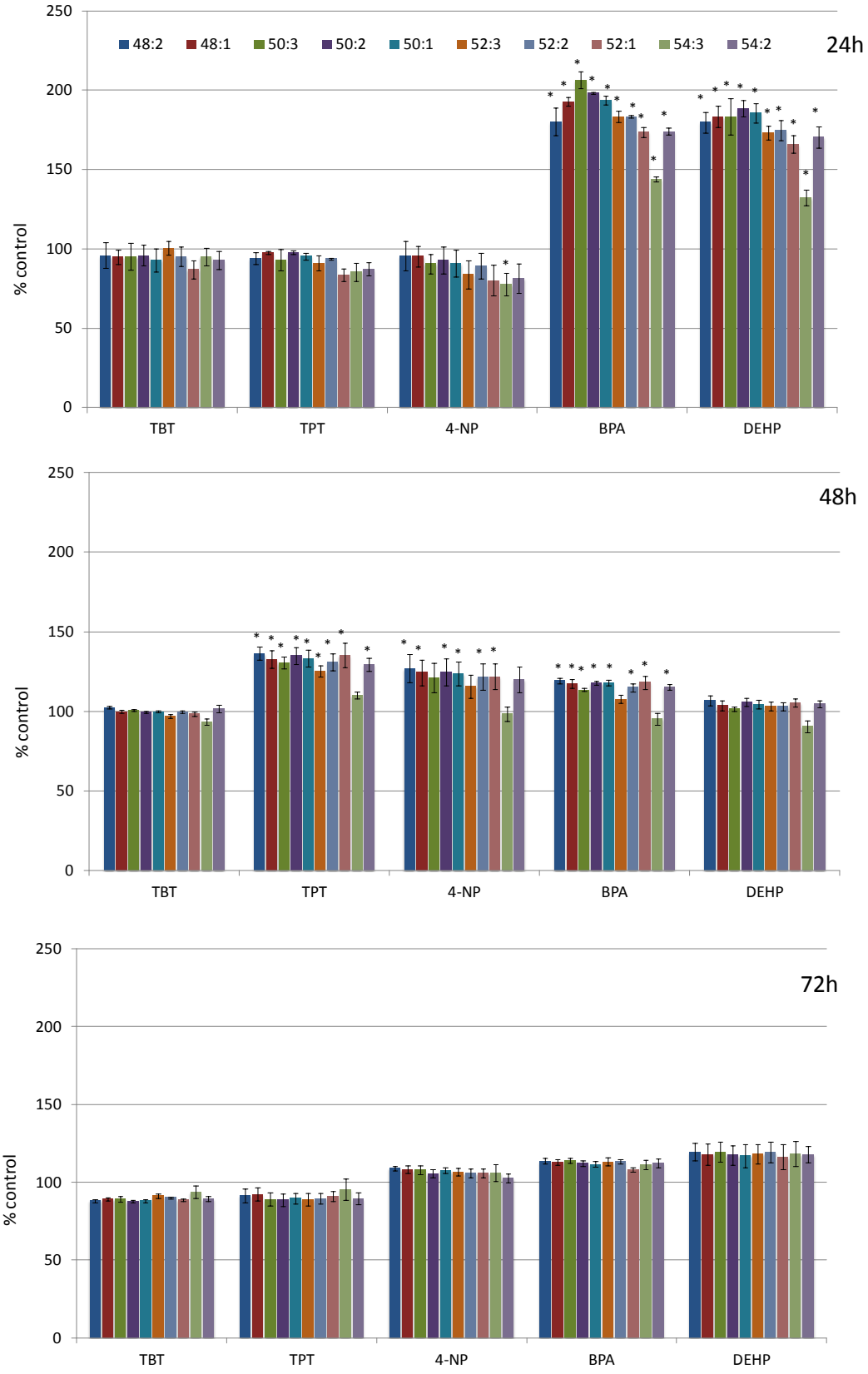
48:2
48:1
50:3
50:2
50:1
52:3
52:2
52:1
54:3
54:2
24h
48h
72h
% control
250
200
150
100
50
0
TBT
TPT
4-NP
BPA
DEHP

Table 1. Rainbow trout primer sequences and PCR conditions for semiquantitative RT-PCR analysis.

mRNA		Primer sequence (5´→ 3´)	Accession No.	Annealing Tª (°C)	Amplicon size (bp)	Database	Reference
ABCA1	F	CAGGAAAGACGAGCACCTTC	TC169876	58	229	DFCI	Cruz-Garcia et al.,
	R	TCTGCCACCTCACACACTTC					2011
LXR	F	TGCAGCAGCCGTATGTGGA	NM0011593	62	171	GenBank	Cruz-Garcia et al.,
	R	GCGGCGGGAGCTTCTTGTC	38				2011
CD36	F	CAAGTCAGCGACAAACCAGA	AY606034.1	62	106	DFCI	Sanchez-Gurmaches
	R	ACTTCTGAGCCTCCACAGGA					et al., 2012
PPARβ	F	CTGGAGCTGGATGACAGTA	AY356399.1	60	195	GenBank	Cruz-Garcia et al.,
	R	GTCAGCCATCTTGTTGAGCA					2011
PPARγ	F	GACGGCGGGTCAGTACTTTA	HM536192.1	60	171	DFCI	Cruz-Garcia et al.,
	R	ATGCTCTTGGCGAACTCTGT					2011
PPARα	F	CTGGAGCTGGATGACACAGTGA	AY494835	54	195	GenBank	Cruz-Garcia et al.,
	R	GGCAAGTTTTTGCAGCAGAT					2011
FAS	F	GAGACCTAGTGGAGGCTGTC	Tcaa0001c.m	54	186	Sigenae	Cruz-Garcia et al.,
	R	TCTTGTTGATGGTGAGCTGT	.06_5.1.om.4				2011
FATP1	F	AGGAGAGAACGTCTCCACCA	CA373015	60	157	DFCI	Sanchez-Gurmaches
	R	CGCATCACAGTCAAATGTCC					et al., 2012
LPL	F	TAATTGGCTGCAGAAAACAC	AJ224693	59	164	GenBank	Cruz-Garcia et al.,
	R	CGTCAGCAAACTCAAAGGT					2011
EF1α	F	TCCTCTTGGTCGTTTCGCTG	AF498320	58	159	GenBank	Cruz-Garcia et al.,
	R	ACCCGAGGGACATCCTGTG					2011

Table 2. Basal expression of lipid metabolism related genes in RTL-W1 cells cultured in the absence or in the presence of 0.5% DMSO over time. Values are mRNA copies relative to 10^3 copies EF1α (mean ± SEM, n=3). *significant effect of DMSO, same time in culture; different letters denote significant differences over time.

	- DMSO			+ DMSO		
	24h	48h	72h	24h	48h	72h
ABCA1	6.49 ± 2.54	15.31 ± 2.57	15.07 ± 2.82	11.20 ± 1.61	41.93 ± 18.73	50.39 ± 24.18
LXR	17.67 ± 2.03	25.04 ± 0.39	18.84 ± 3.13	20.64 ± 5.56	28.66 ± 10.18	29.64 ± 8.29
CD36	155.62 ± 24.24	160.98 ± 2.34	111.93 ± 19.83	109.52 ± 29.44	210.60 ± 40.29	175.75 ± 47.04
PPARβ	23.04 ± 11.34	35.35 ± 1.20	29.12 ± 5.55	38.21 ± 8.73 [a]	169.26 ± 82.11 [b]	154.49 ± 75.69 [a,b]
PPARγ	60.46 ± 23.14 [a]	4.59 ± 0.88 [a]	147.86 ± 41.24 [b]	0.82 ± 0.42 *	1.98 ± 1.11	0.73 ± 0.22 *
PPARα	1.27 ± 0.43	2.64 ± 0.34	4.54 ± 0.97	1.43 ± 0.45	2.85 ± 0.69	2.92 ± 0.73
FAS	37.43 ± 30.21 [a]	118.40 ± 9.09 [b]	90.55 ± 18.35 [a,b]	64.29 ± 5.17 [a]	207.60 ± 48.98 [b]	217.03 ± 64.53 [b]
FATP1	37.11 ± 16.55	54.38 ± 5.30	74.28 ± 17.06	44.58 ± 8.72	46.67 ± 9.95	44.97 ± 14.15
LPL	9.82 ± 0.41	23.92 ± 2.91	37.77 ± 7.18	9.01 ± 1.20	37.72 ± 13.65	34.32 ± 11.69

Table 3. Lipid molecular species identified in RTL-W1 cells with their molecular formula (MF), accurate mass, mass difference obtained as (theoretical mass−detected mass) and retention time (T_r). All identified compounds in accordance to criteria of maximum permissible mass error of 5 mDa. PC = Phosphatidylcholines, TAG = triacylglycerols.

Lipid subclass	Molecular ion	Lipid specie	MF	Accurate mass (*m/z*)	Mass difference (mDa)	T_r (min)
PC	$[M + H]^+$	32:1	$C_{40}H_{78}NO_8P$	732.5538	-2.6	7.5
		32:2	$C_{40}H_{76}NO_8P$	730.5381	-3.0	7
		34:1	$C_{42}H_{82}NO_8P$	760.5851	-3.0	8.5
		34:2	$C_{42}H_{80}NO_8P$	758.5694	-2.6	7.8
		34:3	$C_{42}H_{78}NO_8P$	756.5538	-3.3	7.2
		36:1	$C_{44}H_{86}NO_8P$	788.6164	-3.0	9.5
		36:2	$C_{44}H_{84}NO_8P$	786.6007	-3.5	8.7
		36:3	$C_{44}H_{82}NO_8P$	784.5851	-1.6	8.05
		38:3	$C_{46}H_{86}NO_8P$	812.6164	-3.4	8.9
		38:5	$C_{46}H_{82}NO_8P$	808.5851	-3.8	7.7
		38:6	$C_{46}H_{80}NO_8P$	806.5694	-3.1	7.1
		40:6	$C_{48}H_{84}NO_8P$	834.6007	-2.2	8.1
		40:7	$C_{48}H_{82}NO_8P$	832.5851	-3.0	7.4
Plasmalogen PC	$[M + H]^+$	34:1	$C_{42}H_{84}NO_7P$	746.6058	-0.8	9.2
		34:2	$C_{42}H_{82}NO_7P$	744.5902	0.0	8.45
		34:3	$C_{42}H_{80}NO_7P$	742.5745	-3.6	8
		36:2	$C_{44}H_{86}NO_7P$	772.6215	-0.9	9.4
		38:5	$C_{46}H_{84}NO_7P$	794.6058	-3.1	8.4
		40:7	$C_{48}H_{84}NO_7P$	818.6058	-3.6	8.1
TAG	$[M + NH_4]^+$	48:1	$C_{51}H_{96}O_6$	822.7545	-1.0	13.9
		48:2	$C_{51}H_{94}O_6$	820.7389	-1.9	13.6
		50:1	$C_{53}H_{100}O_6$	850.7858	-2.7	14.3
		50:2	$C_{53}H_{98}O_6$	848.7702	-0.9	14
		50:3	$C_{53}H_{96}O_6$	846.7545	-1.1	13.7
		52:1	$C_{55}H_{104}O_6$	878.8171	0.1	14.6
		52:2	$C_{55}H_{102}O_6$	876.8015	-0.6	14.4
		52:3	$C_{55}H_{100}O_6$	874.7858	-0.9	14.1
		54:2	$C_{57}H_{106}O_6$	904.8328	-0.5	14.7
		54:3	$C_{57}H_{104}O_6$	902.8171	1.1	14.4

Table 4. Relative abundance of the different lipid molecules in RTL-W1 control cells cultured with and without 0.5% DMSO over time (mean ± SEM, n=4). *indicates significant effect of DMSO, same time in culture; different letters denote significant differences over time.

	- DMSO			+ DMSO		
	24h	48h	72h	24h	48h	72h
TAG						
48:2	8.94 ± 0.04	8.74 ± 0.18	9.31 ± 0.26	8.79 ± 0.17	8.74 ± 0.36	8.81 ± 0.23
48:1	13.00 ± 0.38	13.23 ± 0.30	12.35 ± 0.24	13.12 ± 0.26	13.45 ± 0.32	13.32 ± 0.25 *
50:3	3.46 ± 0.12	4.19 ± 0.03	4.20 ± 0.07	3.37 ± 0.19	4.20 ± 0.13	4.22 ± 0.14
50:2	17.56 ± 0.35	18.12 ± 0.54	18.81 ± 0.45	17.60 ± 0.31 [a]	18.3 ± 0.44 [a,b]	19.03 ± 0.48 [b]
50:1	16.36 ± 0.63	17.75 ± 0.43	16.80 ± 0.25	16.58 ± 0.52	16.87 ± 0.48	17.39 ± 0.42
52:3	6.72 ± 0.11	5.93 ± 0.02	6.68 ± 0.56	6.54 ± 0.18	6.33 ± 0.20	6.24 ± 0.21
52:2	18.55 ± 0.65	18.53 ± 0.77	17.82 ± 0.58	18.65 ± 0.53	18.35 ± 0.62	18.11 ± 0.50
52:1	5.75 ± 0.61	5.25 ± 0.11	5.77 ± 0.12	5.86 ± 0.63	5.20 ± 0.12	5.34 ± 0.17
54:3	6.20 ± 0.15	5.14 ± 0.13	4.92 ± 0.25	6.10 ± 0.12 [a]	5.45 ± 0.32 [a,b]	4.27 ± 0.18 [b]
54:2	3.45 ± 0.08	3.13 ± 0.05	3.34 ± 0.17	3.41 ± 0.13	3.11 ± 0.07	3.27 ± 0.10
PC						
32:2	2.77 ± 0.26	2.96 ± 0.26	3.46 ± 0.17	3.03 ± 0.28	2.90 ± 0.06	3.06 ± 0.24
32:1	7.26 ± 1.12	8.28 ± 0.21	7.65 ± 0.74	6.95 ± 0.53	7.04 ± 0.28 *	6.81 ± 0.41
34:3	3.33 ± 0.39	3.17 ± 0.11	3.23 ± 0.24	3.34 ± 0.3	3.07 ± 0.09	3.33 ± 0.33
34:2	12.40 ± 0.80	13.95 ± 1.51	13.18 ± 0.79	13.42 ± 1.12	13.29 ± 0.41	13.56 ± 0.96
34:1	29.93 ± 1.18	32.26 ± 3.74	32.23 ± 3.47	30.43 ± 2.30	32.65 ± 1.52	32.04 ± 2.15
36:3	7.12 ± 0.78	6.38 ± 0.67	6.84 ± 0.17	7.12 ± 0.69	6.88 ± 0.28	7.23 ± 0.52
36:2	15.77 ± 1.34	15.55 ± 0.62	17.14 ± 0.78	15.22 ± 1.35	16.38 ± 0.66	17.88 ± 1.03
36:1	7.75 ± 0.72	6.92 ± 0.62	7.27 ± 0.73	6.70 ± 0.43	7.24 ± 0.31	7.09 ± 0.5
38:6	2.77 ± 0.09	1.63 ± 0.07	1.41 ± 0.14	2.74 ± 0.16	1.81 ± 0.05	1.47 ± 0.09
38:5	4.99 ± 0.48	4.09 ± 0.35	3.35 ± 0.12	5.32 ± 0.38	4.09 ± 0.06	3.41 ± 0.21
38:3	1.88 ± 0.13	1.72 ± 0.12	1.72 ± 0.07	1.70 ± 0.09	1.80 ± 0.05	1.56 ± 0.1
40:7	2.02 ± 0.06	1.56 ± 0.07	1.31 ± 0.02	2.10 ± 0.14	1.46 ± 0.1	1.39 ± 0.08
40:6	2.02 ± 0.21	1.53 ± 0.13	1.20 ± 0.11	1.93 ± 0.11	1.40 ± 0.03	1.16 ± 0.07
Plasmalogen-PC						
34:1	11.68 ± 1.16	15.46 ± 1.45	15.91 ± 1.98	11.52 ± 0.77	12.95 ± 0.73	12.98 ± 0.77
34:2	14.90 ± 1.15	17.61 ± 1.62	17.12 ± 0.95	15.71 ± 1.33	17.26 ± 1.03	18.27 ± 1.41
34:3	6.75 ± 0.70	6.98 ± 0.09	8.20 ± 0.79	7.49 ± 0.65	7.61 ± 0.32	8.24 ± 1.00
36:2	28.52 ± 2.07	30.16 ± 1.48	30.88 ± 2.77	28.61 ± 1.91	30.35 ± 1.24	32.07 ± 1.60
38:5	24.86 ± 2.83	21.11 ± 1.60	19.15 ± 1.04	24.44 ± 2.01	22.79 ± 0.99	20.37 ± 1.62
40:7	13.28 ± 0.77	8.68 ± 0.63	8.74 ± 1.35	12.23 ± 0.95	9.04 ± 0.76	8.06 ± 0.71

Paper 4

TBT INDUCES TRIGLYCERIDE ACCUMULATION AND ALTERS LIPID PROFILE IN ZFL CELLS

G. Dimastrogiovanni[a], O. Jáuregui[b], B. Blumberg[c], and C. Porte[a]

[a] Environmental Chemistry Department, IDAEA-CSIC, C/ Jordi Girona 18, 08034 Barcelona, Spain

[b] Scientific and Technological Centers, University of Barcelona, CCiTUB, Baldiri i Reixac 10-12, 08028 Barcelona, Spain

[c] Departments of Developmental and Cell Biology and Pharmaceutical Sciences, University of California, Irvine, California 92697-2300 USA

In preparation

Resumen

El compuesto organoestánnico tributil estaño (TBT) promueve la diferenciación de adipocitos, la acumulación lipídica y esteatosis hepática en ratones. Sin embargo, hay poca información acerca de posibles efectos obesogénicos de este compuesto, y potencial disrupción del metabolismo lipídico en peces. Este trabajo investiga la utilización de la línea celular de hígado de pez cebra (ZFL) para evaluar el efecto de concentraciones ambientales del TBT sobre perfiles lipídicos de hepatocitos. Las células ZFL se expusieron a diferentes concentraciones de TBT (10, 25, 50 y 100 nM) durante 96 horas en medio de cultivo basal y en medio suplementado con una mezcla de lípidos. La acumulación intracelular de lípidos, determinada con el colorante Rojo Nilo, aumentó de 2.5 veces en células expuestas a 100 nM TBT en presencia de lípidos. Además, los lípidos celulares fueron caracterizados por cromatografía liquida de ultra-alta resolución acoplada a espectrometría de masas en tándem de alta resolución (UHPLC-HRMS-MS). De este modo, se identificaron 72 especies lipídicas, incluyendo fosfatidilcolinas (PC), lisofosfatidilcolinas (lyso-PC), plasmalógenos de PC, triglicéridos (TAG), diglicéridos (DAG), y esteres de colesterol. Al exponer las células al TBT en medio basal se observó un aumento de 2 veces de lípidos de membrana (lyso-PC, PC y plasmalógenos). Sin embargo, cuando se suplementó el medio de cultivo con lípidos, se detectó un incremento de TAG de 20 veces, y ninguna alteración en lípidos de membrana. Este estudio evidencia la capacidad del TBT de alterar el perfil lipídico de las células ZFL y de inducir acumulación de triglicéridos en hepatocitos solo cuando el medio de cultivo es suplementado con una mezcla lipídica.

Abstract

The organotin compound tributyltin (TBT) is known to induce adipocyte differentiation and to increase lipid accumulation and hepatic steatosis in mice. However, information regarding its obesogenic effects and/or disruption of lipid metabolism in fish is scarce. This work investigates the suitability of the ZFL cell line as an in-vitro model to study the effect of environmentally relevant concentrations of TBT on hepatic cellular lipids. ZFL cells were exposed to 10, 25, 50 & 100 nM TBT for 96 h in basal medium or in medium supplemented with a mixture of lipids. Nile Red was used to estimate intracellular lipid accumulation, which increased up to 2.5-fold in cells exposed to 100 nM TBT when culture medium was supplemented with lipids. Cellular lipids were further characterized by ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-MS). A total of 72 lipid species including phosphatidylcholines (PC), PC-plasmalogen, lyso-PC, diacylglycerols, triacylglycerols and cholesterol esters were identified. A relative increase (2-fold) in membrane lipids (PC, plasmalogen PC and lyso PC) was detected in TBT-exposed cells cultured in basal medium. However, if basal medium was supplemented with lipids, TBT-exposed cells showed a 20-fold increase in triacylglycerols, with no alteration in membrane lipids. This work highlights the ability of TBT to modify the lipidome of ZFL cells and to induce triacylglycerols accumulation only when culture medium was supplemented with lipids. The model can be useful for the screening of environmental contaminants suspected to disrupt lipid metabolism.

Keywords: ZFL cells; tributyltin; lipidomics; mass spectrometry

Introduction

Some studies have suggested a role of environmental pollutants in the obesity epidemic and related diseases observed in the industrialized countries by disrupting mechanisms involved in body weight control and metabolic efficiency (Baillie-Hamilton, 2002; Newbold et al., 2008). Indeed, Grün et al. (2006) pointed out the existence of a number of chemicals, termed obesogens, which inappropriately regulate lipid metabolism and promote adipogenesis. Since then, several studies have supported this hypothesis, and the number of compounds categorized as obesogens has been continuously increasing. Thus, bisphenol A and nonylphenol enhance adipogenesis in murine preadipocyte cells (3T3-L1) (Masuno et al., 2002; Masuno et al., 2003). Phthalates and perfluoroalkyl compounds can activate primary regulators of lipid metabolism, such as the nuclear receptors PPARα and PPARγ (Heuvel et al., 2006; Maloney and Waxman, 1999). Monoethyl-hexyl-phthalate directly activates PPARγ and promotes adipogenesis in 3T3-L1 (Feige et al., 2007). Increased concentrations of several prevalent urinary phthalate metabolites have been associated with increased abdominal obesity and insulin resistance in adult males (Stahlhut et al., 2007).

Many studies have indicated organotins as potential obesogens. Thus, tributyltin (TBT) induced adipocyte differentiation in 3T3-L1 cells, increased lipid accumulation in mouse models and induced ectopic adipocyte formation in the amphibian model *Xenopus* (Grün et al., 2006; Inadera and Shimomura, 2005). Both, TBT and triphenyltin (TPT) were found to be selective activators at the low nanomolar range of the two nuclear receptors RXR and PPARγ, which play a key role in adipocyte differentiation and energy storage (Grün and Blumberg, 2006; Kanayama et al., 2005). Moreover, TBT and TPT altered fatty acid homeostasis, enhancing lipid accumulation in the ramshorn snail *Marisa cornuarietis* (Janer et al., 2007; Lyssimachou et al., 2009). TBT was shown to induce an increase in body weight and whole-body lipid content as well as to increase lipid related plasma parameters (plasma triacylglycerols, cholesterol and lipase) in juvenile Chinook salmon (Meador et al., 2011).

Relatively few studies have addressed the effects of obesogenic compounds in fish and the disruption of mechanisms involved in lipid homeostasis. Zebrafish exposed to mixtures of persistent organic pollutants (POPs), comprising polybrominated diphenyl ethers (PBDE), polychlorinated biphenyls (PCB), and dichlorodiphenyltrichloroethane metabolites (DDT), showed higher body mass coupled with alterations in gene transcription profiles of PPARα and PPARγ (Lyche et al., 2010;

Nourizadeh-Lillabadi et al., 2009). Zebrafish larvae treated with TBT exhibited a noticeable increase in adiposity, while anti-obesogenic compounds, the PPARγ antagonist T0070907 and the α1-adrenergic receptor agonist phenylephrine, promoted an adiposity reduction in the fasting state (Tingaud-Sequeira et al., 2011). Her et al. (2013) showed that hepatic lipid accumulation in the liver of transgenic zebrafish was related to the up-regulation of genes involved in lipid metabolism, such as C/EBP-α and PPAR-γ; the expression of these genes was also detected in the zebrafish liver cell line, ZFL. The ZFL cell line has been used in diverse toxicological studies (Chan et al., 2006; Chen et al., 2011; Kling and Förlin, 2009; Teng et al., 2013). Although, expression levels of several hepatic genes in ZFL cells are lower than those found in the zebrafish liver (Eide et al., 2014), ligand-binding studies showed an increased expression of transcription factors and nuclear receptors involved in the energy balance maintenance and lipid metabolism (Archer et al., 2008; Pomati et al., 2007).

The aim of this study was to investigate the suitability of the ZFL cell line as an in-vitro model to study the effect of environmentally relevant concentrations of obesogens on hepatic cellular lipids using the model obesogen TBT. The ZFL cell line was established from adult zebrafish liver and behaves similarly to healthy zebrafish liver cells, in culture (Ghosh et al., 1994). Therefore, it can be a valuable tool for the screening of those hazardous chemicals that have been classified as potential obesogens in other animal models. Intracellular lipid accumulation will be estimated with Nile Red staining and ZFL lipidome characterized by ultra-high performance liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS), by comparing lipid profiles of TBT-exposed and non-exposed cells. We conclude that TBT is able to affect intracellular lipid homeostasis in ZFL cells, and to promote triacylglycerols accumulation when cells were provided with lipids.

Materials and Methods

Chemicals

Tributyltin chloride (TBT) was purchased from Sigma Aldrich (Steinheim, Germany). Stock standard solutions of TBT were prepared in DMSO and stored at -20ºC. HPLC grade water, ethanol (> 99.8%) and acetonitrile (> 99.8%) were purchased from Merck (Darmstadt, Germany); 9-diethylamino-5H-benzo[α]phenoxa-]phenoxazine-5-one (Nile Red) and bisBenzimide H 33342 trihydrochloride (Hoechst

33342) were obtained from Aldrich Chemical (Milwaukee, USA). All solvents and reagents were of analytical grade.

Cell culture and exposure design

Zebrafish liver cells (ZFL) were purchased from American Type Culture Collection (ATCC CRL-2643). They were grown in a medium of 50% L-15, 35% Dulbecco's minimum essential medium high glucose (DMEM HG) and 15% Ham's F-12 supplemented with 0.15 g/L sodium bicarbonate, 15 mM HEPES, 10 mg/L insulin, 5% fetal bovine serum (FBS), 0.5% trout serum, 50 ng/ml mouse EGF, 100 U/ml of penicillin and 100 μg/ml of streptomycin, in a humidified incubator at 28 ºC. Cells were routinely cultured in 75 cm^2 polystyrene flasks. When subconfluent (80-90%), cells were dissociated with 0.25% (w/v) trypsin/0.53 mM EDTA solution for subculturing, and experiments were carried out on confluent cell monolayers.

Cells were seeded at a rate of 200,000 cells per well (24-well plate) and allowed to grow for 48 h. Then, the culture medium was changed and cells were treated for 96 h with TBT dissolved in basal medium or in medium supplemented with lipid mixture 1000x (2.5 μL/mL medium), which contained 45 mg/ml cholesterol, 100 mg/ml cod liver oil fatty acids, 250 mg/ml polyoxyethylene sorbitan monooleate, and 20 mg/ml D-α-tocopherol acetate. Medium was replaced every 2 days. The final concentration of DMSO in culture wells was 0.1% (v/v), and final concentrations of TBT were 10, 25, 50 and 100 nM. At the end of the experiment, cells were fixed for staining or collected for lipid extraction and analysis. For each assay a control was performed by adding the solvent (0.1% DMSO) to cells.

Intracellular lipid droplets staining

Intracellular lipid accumulation was determined as described by Greenspan et al. (1985), with slight modifications. Stock solutions of 1 mg/ml Nile Red (NR), a lipid specific fluorescent dye, and 1 mg/ml Hoechst 33342, a nucleic acid stain, were prepared in DMSO and Milli-Q water, respectively, and stored protected from light. Just before use, the working solutions were prepared by dilution in PBS (1:1000). Cells were fixed in 10% formalin for 30 min, rinsed with PBS, and stained with the working solution (1 μg/ml NR, 1 μg/ml Hoechst 33342) in the dark for 30 min at room temperature. The fluorescence

intensity of each well was measured using a plate reader at an excitation/emission wavelength of 355/460 nm for Hoechst 33342, and 485/590 nm for Nile Red. Fluorescence measurement was performed twice, once before the staining to measure the background values, and afterwards. The specific lipid content per well was calculated as the ratio of fluorescence obtained for NR to fluorescence of Hoechst 33342. Data are reported as fold induction over vehicle (0.1% DMSO) controls (mean ± SEM) of four biological replicates.

Extraction and analysis of lipids

Lipids were extracted with a modification of the method of Christie (1985). A solution of methanol:chloroform (1:2) containing 0.01% of butylated hydroxytoluene (BHT) as an antioxidant was added to the cell pellets, vortexed and after 30 min at room temperature, extracted in an ultrasonic bath for 5 min (x2). The extracts were evaporated to dryness and reconstituted with 400 µL of acetonitrile.

Lipid analysis was performed with an ultra-high performance liquid chromatography (UHPLC) system Accela (Thermo Fisher Scientific) coupled to a LTQ-Orbitrap Velos mass spectrometer from Thermo Fisher Scientific (Bremen, Germany) equipped with heated electrospray ionization (H-ESI) source. The chromatographic separation was performed on an Acquity UPLC BEH C_8 column (1.7 µm particle size, 100 mm x 2.1 mm, Waters, Ireland) at a flow rate of 0.3 ml/min and a column temperature of 30ºC. The mobile phase was methanol with 1 mM ammonium formiate and 0.2% formic acid (A)/ water with 2 mM ammonium formiate and 0.2% formic acid (B). Gradient elution started at 85% of A, increased to 90% A in 10 min, held for 2 min, increased to 99% A in 6 min and held for 2 min. Initial conditions were attained in 1 min and the system was stabilized for 3 min. Ten microlitres were injected.

Mass spectra were acquired in profile mode with a setting of 30000 resolution at *m/z* 400. Operation parameters were as follows: source voltage, 3.5 kV; sheath gas, 50 (arbitrary units); auxiliary gas, 20 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 300°C, S-Lens RF Level 60%. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target $1 \cdot 10^6$ for MS mode and $5 \cdot 10^4$ for MS^n mode). The mass range in FTMS mode was from *m/z* 395 to 1000. The data analysis was achieved using Xcalibur software 2.1.0 (Thermo Fisher Scientific). An external calibration for mass accuracy was carried out before the analysis using the commercial standard Pierce® LTQ Velos ESI Positive Ion Calibration Solution (#88323) for positive ESI, and

Pierce® ESI Negative Ion Calibration Solution (#88324) for negative ESI. Phosphatidylcholines (PC), lyso PCs, plasmalogen PCs, diacylglycerols (DAG), triacylglycerols (TAG) and cholesterol esters (ChE) were analyzed under positive ESI. Processing of acquired mass spectra, identification and relative quantification of detected molecular species was performed with Xcalibur 2.1.0 software (Thermo Scientific). Identification of lipids was possible after comparison of registered lipids from the database and the ones encountered in cells, under the criteria of exact mass. To quantify lipid profile alterations in TBT-exposed cells, peak areas were compared with their basal amounts in non-exposed cells (control samples) set to 100%, and changes were expressed as relative abundance.

Statistical analysis

Results are mean values ± SEM. Statistical significance between exposure groups and solvent control groups (0.1% DMSO) was assessed by using one way ANOVA with Dunnett's post hoc test. Statistical analysis used the software IBM SPSS Statistics v.21.

Results

Intracellular lipid droplets staining

Lipid accumulation in ZFL cells following 96 h exposure to increasing concentrations of TBT is shown in Figure 1. No significant accumulation of intracellular lipids was observed in the absence of lipid mixture (Fig. 1A). However, when the culture medium was supplemented with lipids, cells exposed to 50 and 100 nM TBT showed a significant enhancement in the accumulation of intracellular lipid droplets, reaching a 250% increase after 96 h exposure to 100 nM TBT (Fig. 1B).

Lipid profile changes following TBT exposure

The analysis of cellular lipids by UHPLC-HRMS allowed the identification and relative quantification of Lyso PCs, PCs, plasmalogen PCs, DAGs, TAGs and ChEs under positive ESI. Lyso PC eluted in the first 2 min of the chromatogram, followed by PCs, plasmalogen PCs and DAGs that eluted in the subsequent

10 minutes, while TAGs and ChEs appeared at the final minutes (Fig. 2). A total of 72 lipid species were identified including 6 Lyso PCs, 17 PCs, 12 Plasmalogen PCs, 13 DAGs, 18 TAGs and 6 ChEs. The compounds identified with their molecular formula, accurate mass, error and retention times are shown in Table 1. The use of 30,000 resolution with the Orbitrap would allow the resolution of isobaric species, although in our specific case, such species were chromatographically resolved.

The chromatographic profiles of exposed and non-exposed cells were further analyzed in terms of compounds identified, and their relative amount by comparison of their peak areas in control and exposed cells. The first outcome of this comparison is presented in Figure 3, which shows the changes in lipid classes in TBT-exposed cells compared with their basal levels of non-exposed cells, set to 100%. As shown in Fig. 3, TBT exposure led to significant alterations in lipid profiles. As observed with Nile Red, the response of ZFL cells to TBT exposure was remarkably different in absence or in presence of lipids in the culture medium. When cells were grown in basal medium, a significant increase of PCs (2.4 and 2.3-fold, respectively) and Plasmalogen PCs (2.6 and 2.5-fold, respectively) was observed in cells exposed to 25 nM and 50 nM TBT, whereas Lyso PC increased by 2.2 and 1.9-fold in the presence of 25 nM and 100 nM TBT, respectively. In contrast, a small increase in the amount of DAGs (1.3 to 1.5-fold) and no significant effect on TAG and ChE was observed (Fig. 3A). Interestingly, when growth medium was supplemented with a lipid mixture, no significant alteration of most of the glycerophospholipids was observed (Fig. 3B). Furthermore, in plasmalogen PC group, lipid levels of cells exposed to 100 nM TBT were 50% lower than in control samples. In contrast, the TBT exposure strongly affected the glycerolipids groups. DAGs increased significantly up to 2.1-fold in cells exposed to TBT 50 nM and 100 nM, and ChE levels were increased up to 3.2-fold at the highest TBT concentration. The greatest alteration was detected in the TAG group, with a 19.8-fold enhancement in 100 nM TBT exposed cells.

Table 2 shows the relative abundance of all the identified lipid molecules. A generalized increase of all the Lyso PC (up to 2-fold), PC (up to 3-fold) and plasmalogen PC species (up to 4-fold) was detected in the absence of lipid mixture. Interestingly, the greatest enhancement was detected in cells exposed to 25 nM and 50 nM TBT, while at 100 nM TBT, the increase was less evident. Regarding glycerolipids, most of the detected diacylglycerols increased (32:1, 32:2, 34:1, 34:2, 34:3, 36:2, 36:3, 38:5); the major increase was usually detected in cells exposed to 100 nM TBT, although an increase of DAG 32:1, 34:2 and 36:2 (1.3 to 1.7-fold) was already observed in cells exposed to 10 nM TBT. In contrast, some TAG (48:2, 50:3, 50:6) were observed to increase with TBT-exposure, whereas others

(52:1, 54:4, 54:5. 54:6) slightly decreased. Cholesterol esters remained unchanged with the exception of ChE 22:6. When the medium was supplemented with lipids, TBT exposure had little effect on glycerophospholipids. Relative abundance of Lyso PC species remained unaltered, while relative levels of PCs (34:1, 36:1, 38:2) and several Plasmalogen PCs diminished with increasing of TBT concentration (25 nM to 100 nM TBT). An in-depth look at the TAG profile evidenced that the sharp increment in TAG content was due to an impressive increase in all the lipid molecules identified (apart from 50:0) but only observed after exposure to the highest concentration of TBT (100 nM), while the raise in DAG (32:1, 32:2, 34:1, 34:2, 34:3, 34:4, 36:2, 36:3, 36:6) was already observed at 50 nM TBT.

Discussion

This is one of the first *in vitro* studies showing lipid accumulation in hepatic cells following exposure to environmental concentrations of TBT, as previous in-vitro works have mostly demonstrated lipogenic effects of TBT in the 3T3-L1 adipogenesis model (Grün et al., 2006; Inadera and Shimomura, 2005; Kanayama et al., 2005). Interestingly, lipid accumulation in ZFL cells was only induced by TBT exposure when cell medium was supplemented with a lipid mixture, consisting of cholesterol and fatty acids (Fig. 1B), reaching a 250% increase in cells exposed to 100 nM TBT. Nile red is commonly used to detect intracellular lipid droplets (LDs), as it proved to be very specific for hydrophobic environments (i.e., neutral lipid droplets) whereas fluorescence due to interaction with cellular membranes is minimal (Genicot et al., 2005; Greenspan et al., 1985). However, lipid droplets consist of a core of neutral lipids, predominantly triacylglycerols and sterol esters, separated from the aqueous cytosol by a monolayer of phospholipids and associated proteins (Martin and Parton, 2006; Walther and Farese Jr, 2012). Therefore, to further investigate the effects of TBT on ZFL lipidome ultra-high performance liquid chromatography – high resolution mass spectrometry (UHPLC-HRMS) was used. Liquid chromatography-mass spectrometry (LC-MS) has evolved with important advancements in instrumentation and technology, such as the development of soft ionization techniques (matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI)), increasing the capabilities for detection and analysis of a wide range of lipids (Ivanova et al., 2009; Wenk, 2005). Indeed, polar lipids, such as phospholipids and sphingolipids, and even non-polar lipids, such as DAGs and TAGs, have been successfully measured by ESI, and the combination of reversed phase HPLC with ESI-MS (HPLC-ESI-

MS) is becoming a reliable method for profiling complex lipid mixtures, as it allows detailed analysis of individual lipid species (Han and Gross, 2001; Wenk, 2005).

Using this approach, we found distinct changes in lipid levels within several lipid classes identified. TBT effects were studied at 0.1 µM and lower concentrations (50 nM, 25 nM and 10 nM) since these were the levels at which TBT promoted adipogenesis in vertebrates. Opposed effects were observed when cells were exposed to TBT in basal medium or in medium supplemented with lipid mixture (Fig. 3). In the first condition, we found that TBT-exposure affected mostly glycerophospholipids (GP), with an increase in the amount of Lyso PCs, PCs and PC-Plasmalogens (Fig. 3A). GPs are the principal structural constituents of cellular membranes, and phosphatidylcholines accounts for >50% of the phospholipids in most eukaryotic membranes (Van Meer et al., 2008). Plasmalogens are not only structural membrane components and a reservoir for lipid second messengers, they are also known to facilitate membrane fusion, and are involved in ion transport, cholesterol efflux and store long-chain PUFA (Lesharpig and Fuchs, 2009). Several studies have demonstrated that TBT is a membrane-active molecule, and although its mechanism of toxicity is not completely unveiled, the hydrophobicity of organotin compounds suggests that interaction with membranes may play an important role (Gray et al., 1987; Ortiz et al., 2005). Indeed, model membrane studies showed the incorporation of TBT in phosphatidylcholines, where it perturbs thermotropic and structural properties (i.e. the hydration degree of the carbonyl moiety), leading to membrane alteration and lysis (Ambrosini et al., 1991; Chicano et al., 2001). However, whether the observed changes in phospholipids might have occurred as the result of feedback mechanisms to counteract TBT toxic action and the significance of the physiological consequences remains unknown. Interestingly, Ortiz et al. (2005) suggested that the ability of organotin compounds to transport organic anions across the phospholipid bilayer might be related to their mechanism of toxicity. This transport was reduced in the presence of cholesterol, which could make the diffusion of triorganotin compounds more difficult given its known permeability-decreasing effect on membranes (Gabrielska et al., 2004; Raffy and Teissié, 1999). Furthermore, in the yeast *Saccharomyces cerevisiae*, the enrichment of the culture medium with a polyunsaturated fatty acid (linoleic acid, 18:2) was associated with an enhanced resistance to the toxic action of TBT, despite an increased TBT uptake by these cells (Masia et al., 1998). It is worth noting that these two conditions (addition of Cholesterol and/or PUFA) resulting in a reduction of the adverse effects of TBT resembles the experiment where basal medium was supplemented with lipid mixture (Fig. 3B), experimental condition in which TBT-increasing effect on phospholipids disappears.

When cells were exposed to TBT in the presence of lipid mixture supplemented medium (Fig. 3B), levels of DAG, TAG and ChE suffered a dramatic increase. We infer that in the presence of cholesterol and several FAs the TBT-exposed zebrafish hepatocytes were induced to accumulate more neutral lipids. This is in accordance with the results obtained through the Nile Red staining, where cells exposed to 100 nM TBT in presence of lipid-enriched medium showed an increase in the formation of lipid droplets, organelles usually considered as storage depots for neutral lipids. Several studies demonstrated the ability of TBT to promote adipogenesis and lipid accumulation *in vitro* and *in vivo* (Grün et al., 2006; Iguchi et al., 2007; Kanayama et al., 2005). It was concluded that TBT induces these alterations in vertebrates through interaction with the nuclear receptors, RXR and PPARγ (which play pivotal roles in lipid homeostasis) because TBT binds to both receptors with high affinity binding (Grün and Blumberg, 2006). Indeed, studies with human nuclear receptors have showed TBT as well as triphenyltin (TPT), bind to RXR with similar affinity as the presumed natural ligand, 9-cis retinoic acid. TBT binds to PPARγ with higher affinity than the synthetic ligand troglitazone. TBT also affected lipid homeostasis in salmonids, tropical fishes and invertebrate species (Meador et al., 2011; Oliveira Ribeiro et al., 2002; Puccia et al., 2005). Interestingly, TBT perturbed fatty acid homeostasis and enhanced lipid accumulation in ramshorn snails, which implicates RXR as a key player in this process, because snails lack a PPARγ ortholog (Janer et al., 2007). RXR has been reported in several invertebrate species (Bouton et al., 2005). Vertebrate RXRs form homodimers that can bind to peroxisome proliferator response elements and induce transactivation of adipocyte-specific genes (IJpenberg et al., 2004). In addition to PPARs, RXR can form heterodimers with other nuclear receptors that are known to regulate lipid homeostasis, such as the liver X receptor (LXR) (Edwards et al., 2002). It should be mentioned that Archer et al. (2008) concluded that as in mammals, zebrafish LXR is likely to be involved in regulation of cholesterol and lipid homeostasis, and treatment with the synthetic ligand GW3965 led to increased expression of *fasn* (zebrafish FA synthase*)* mRNA in ZFL cells, even though they were expressing low levels of LXR.

Interestingly, Plasmalogen PCs decreased consistently in cells exposed to TBT 100 nM in lipid-enriched medium, suggesting a connection with the simultaneous large accumulation of DAG-, TAG- and ChE molecules characterized by the presence of polyunsaturated fatty acids (DAG: 34:3, 34:4 and 36:3; TAG: 50:6, 52:4, 52:5, 54:4, 54:5 and 54:6; ChE: 18:3, 20:4 and 20:5). As shown in Table 2 and Figure 4, it is intriguing that within the series of lipid molecules with the same acyl chain length, the accumulation effect of TBT 100 nM is increasing in parallel with a raise in the unsaturation of the lipids.

Overall, this work highlights the ability of TBT to modify the intracellular lipid profile of ZFL cells but to induce steatosis only when the culture medium was supplemented with lipids. Furthermore, the results support the hypothesis that TBT may act more widely to disrupt multiple nuclear receptor mediated signaling pathways, eliciting adipogenic effects. In conclusion, in this study we provide a basis for the use of ZFL cell line as an in-vitro model for the screening of environmental contaminants suspected to interfere with cellular lipids, allowing a pre-assessment of obesogenic effects on lipid homeostasis and accumulation.

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References

Ambrosini, A., Bertoli, E., Zolese, G., Tanfani, F., 1991. Interaction of tributylin acetate and tributyltin chloride with dipalmitoyl phosphatidylcholine model membrane. Chemistry and Physics of Lipids 58, 73-80.

Archer, A., Lauter, G., Hauptmann, G., Mode, A., Gustafsson, J.Å., 2008. Transcriptional activity and developmental expression of liver X receptor (lxr) in zebrafish. Developmental Dynamics 237, 1090-1098.

Baillie-Hamilton, P.F., 2002. Chemical toxins: a hypothesis to explain the global obesity epidemic. The Journal of Alternative & Complementary Medicine 8, 185-192.

Bouton, D., Escriva, H., De Mendonça, R., Glineur, C., Bertin, B., Noël, C., Robinson-Rechavi, M., de Groot, A., Cornette, J., Laudet, V., 2005. A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. Journal of molecular endocrinology 34, 567-582.

Chan, K.M., Ku, L.L., Chan, P.C.Y., Cheuk, W.K., 2006. Metallothionein gene expression in zebrafish embryo-larvae and ZFL cell-line exposed to heavy metal ions. Marine Environmental Research 62, Supplement 1, S83-S87.

Chen, D., Zhang, D., Yu, J.C., Chan, K.M., 2011. Effects of Cu_2O nanoparticle and $CuCl_2$ on zebrafish larvae and a liver cell-line. Aquatic Toxicology 105, 344-354.

Chicano, J.J., Ortiz, A., Teruel, J.A., Aranda, F.J., 2001. Organotin compounds alter the physical organization of phosphatidylcholine membranes. Biochimica et Biophysica Acta (BBA) - Biomembranes 1510, 330-341.

Christie, W.W., 1985. Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. Journal of lipid research 26, 507-512.

Edwards, P.A., Kennedy, M.A., Mak, P.A., 2002. LXRs;: Oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis. Vascular pharmacology 38, 249-256.

Eide, M., Rusten, M., Male, R., Jensen, K.H.M., Goksøyr, A., 2014. A characterization of the ZFL cell line and primary hepatocytes as in vitro liver cell models for the zebrafish (*Danio rerio*). Aquatic Toxicology 147, 7-17.

Feige, J.N., Gelman, L., Rossi, D., Zoete, V., Métivier, R., Tudor, C., Anghel, S.I., Grosdidier, A., Lathion, C., Engelborghs, Y., 2007. The endocrine disruptor monoethyl-hexyl-phthalate is a selective peroxisome proliferator-activated receptor γ modulator that promotes adipogenesis. Journal of Biological Chemistry 282, 19152-19166.

Gabrielska, J., Przestalski, S., Miszta, A., Soczyńska-Kordala, M., Langner, M., 2004. The effect of cholesterol on the adsorption of phenyltin compounds onto phosphatidylcholine and sphingomyelin liposome membranes. Applied organometallic chemistry 18, 9-14.

Genicot, G., Leroy, J.L.M.R., Soom, A.V., Donnay, I., 2005. The use of a fluorescent dye, Nile red, to evaluate the lipid content of single mammalian oocytes. Theriogenology 63, 1181-1194.

Ghosh, C., Zhou, Y., Collodi, P., 1994. Derivation and characterization of a zebrafish liver cell line. Cell biology and toxicology 10, 167-176.

Gray, B.H., Porvaznik, P., Flemming, C., Lee, L.H., 1987. Tri-n-butyltin: A membrane toxicant. Toxicology 47, 35-54.

Greenspan, P., Mayer, E.P., Fowler, S.D., 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. The Journal of cell biology 100, 965-973.

Grün, F., Blumberg, B., 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology 147, s50-s55.

Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T., Blumberg, B., 2006. Endocrine-Disrupting Organotin Compounds Are Potent Inducers of Adipogenesis in Vertebrates. Molecular Endocrinology 20, 2141-2155.

Han, X., Gross, R.W., 2001. Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. Analytical biochemistry 295, 88-100.

Her, G.M., Pai, W.-Y., Lai, C.-Y., Hsieh, Y.-W., Pang, H.-W., 2013. Ubiquitous transcription factor YY1 promotes zebrafish liver steatosis and lipotoxicity by inhibiting CHOP-10 expression. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids.

Heuvel, J.P.V., Thompson, J.T., Frame, S.R., Gillies, P.J., 2006. Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-α,-β, and-γ, liver X receptor-β, and retinoid X receptor-α. Toxicological Sciences 92, 476-489.

Iguchi, T., Katsu, Y., Horiguchi, T., Watanabe, H., Blumberg, B., Ohta, Y., 2007. Endocrine disrupting organotin compounds are potent inducers of imposex in gastropods and adipogenesis in vertebrates. Mol Cell Toxicol 3, 1-10.

IJpenberg, A., Tan, N.S., Gelman, L., Kersten, S., Seydoux, J., Xu, J., Metzger, D., Canaple, L., Chambon, P., Wahli, W., 2004. In vivo activation of PPAR target genes by RXR homodimers. The EMBO journal 23, 2083-2091.

Inadera, H., Shimomura, A., 2005. Environmental chemical tributyltin augments adipocyte differentiation. Toxicology Letters 159, 226-234.

Ivanova, P.T., Milne, S.B., Myers, D.S., Brown, H.A., 2009. Lipidomics: a mass spectrometry based systems level analysis of cellular lipids. Current Opinion in Chemical Biology 13, 526-531.

Janer, G., Navarro, J.C., Porte, C., 2007. Exposure to TBT increases accumulation of lipids and alters fatty acid homeostasis in the ramshorn snail *Marisa cornuarietis*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 146, 368-374.

Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T., Nishikawa, J.-i., 2005. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor γ/retinoid X receptor pathway. Molecular pharmacology 67, 766-774.

Kling, P., Förlin, L., 2009. Proteomic studies in zebrafish liver cells exposed to the brominated flame retardants HBCD and TBBPA. Ecotoxicology and Environmental Safety 72, 1985-1993.

Lesharpig, J., Fuchs, B., 2009. Plasmalogens in biological systems: their role in oxidative processes in biological membranes, their contribution to pathological processes and aging and plasmalogen analysis. Current medicinal chemistry 16, 2021-2041.

Lyche, J.L., Nourizadeh-Lillabadi, R., Almaas, C., Stavik, B., Berg, V., Skåre, J.U., Alestrøm, P., Ropstad, E., 2010. Natural Mixtures of Persistent Organic Pollutants (POP) Increase Weight Gain, Advance Puberty, and Induce Changes in Gene Expression Associated with Steroid Hormones and Obesity in Female Zebrafish. Journal of Toxicology and Environmental Health, Part A 73, 1032-1057.

Lyssimachou, A., Navarro, J.C., Bachmann, J., Porte, C., 2009. Triphenyltin alters lipid homeostasis in females of the ramshorn snail *Marisa cornuarietis*. Environmental Pollution 157, 1714-1720.

Maloney, E.K., Waxman, D.J., 1999. *trans*-Activation of PPARα and PPARγ by Structurally Diverse Environmental Chemicals. Toxicology and applied pharmacology 161, 209-218.

Martin, S., Parton, R.G., 2006. Lipid droplets: a unified view of a dynamic organelle. Nature Reviews Molecular Cell Biology 7, 373-378.

Masia, A., Avery, S.V., Zoroddu, M.A., Gadd, G.M., 1998. Enrichment with a polyunsaturated fatty acid enhances the survival of *Saccharomyces cerevisiae* in the presence of tributyltin. FEMS microbiology letters 167, 321-326.

Masuno, H., Kidani, T., Sekiya, K., Sakayama, K., Shiosaka, T., Yamamoto, H., Honda, K., 2002. Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. Journal of Lipid Research 43, 676-684.

Masuno, H., Okamoto, S., Iwanami, J., Honda, K., Shiosaka, T., Kidani, T., Sakayama, K., Yamamoto, H., 2003. Effect of 4-nonylphenol on cell proliferation and adipocyte formation in cultures of fully differentiated 3T3-L1 cells. Toxicological sciences 75, 314-320.

Meador, J.P., Sommers, F.C., Cooper, K.A., Yanagida, G., 2011. Tributyltin and the obesogen metabolic syndrome in a salmonid. Environmental research 111, 50-56.

Newbold, R.R., Padilla-Banks, E., Jefferson, W.N., Heindel, J.J., 2008. Effects of endocrine disruptors on obesity. International Journal of Andrology 31, 201-208.

Nourizadeh-Lillabadi, R., Lyche, J.L., Almaas, C., Stavik, B., Moe, S.J., Aleksandersen, M., Berg, V., Jakobsen, K.S., Stenseth, N.C., Utne Skåre, J., Alestr⊘m, P., Ropstad, E., 2009. Transcriptional Regulation in Liver and Testis Associated with Developmental and Reproductive Effects in Male Zebrafish Exposed to Natural Mixtures of Persistent Organic Pollutants (POP). Journal of Toxicology and Environmental Health, Part A 72, 112-130.

Oliveira Ribeiro, C.A., Schatzmann, M., Silva de Assis, H.C., Silva, P.H., Pelletier, E., Akaishi, F.M., 2002. Evaluation of Tributyltin Subchronic Effects in Tropical Freshwater Fish (*Astyanax bimaculatus*, Linnaeus, 1758). Ecotoxicology and Environmental Safety 51, 161-167.

Ortiz, A., Teruel, J.A., Aranda, F.J., 2005. Effect of triorganotin compounds on membrane permeability. Biochimica et Biophysica Acta (BBA) - Biomembranes 1720, 137-142.

Pomati, F., Cotsapas, C.J., Castiglioni, S., Zuccato, E., Calamari, D., 2007. Gene expression profiles in zebrafish (*Danio rerio*) liver cells exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. Chemosphere 70, 65-73.

Puccia, E., Messina, C., Cangialosi, M., D'Agati, P., Mansueto, C., Pellerito, C., Nagy, L., Mansueto, V., Scopelliti, M., Fiore, T., 2005. Lipid and fatty acid variations in *Ciona intestinalis* ovary after tri-n-butyltin (IV) chloride exposure. Applied organometallic chemistry 19, 23-29.

Raffy, S., Teissié, J., 1999. Control of Lipid Membrane Stability by Cholesterol Content. Biophysical Journal 76, 2072-2080.

Stahlhut, R.W., van Wijngaarden, E., Dye, T.D., Cook, S., Swan, S.H., 2007. Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult US males. Environmental health perspectives 115, 876.

Teng, Q., Ekman, D.R., Huang, W., Collette, T.W., 2013. Impacts of 17α-ethynylestradiol exposure on metabolite profiles of zebrafish (*Danio rerio*) liver cells. Aquatic Toxicology 130–131, 184-191.

Tingaud-Sequeira, A., Ouadah, N., Babin, P.J., 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. Journal of lipid research 52, 1765-1772.

Van Meer, G., Voelker, D.R., Feigenson, G.W., 2008. Membrane lipids: where they are and how they behave. Nature reviews molecular cell biology 9, 112-124.

Walther, T.C., Farese Jr, R.V., 2012. Lipid droplets and cellular lipid metabolism. Annual review of biochemistry 81, 687-714.

Wenk, M.R., 2005. The emerging field of lipidomics. Nature Reviews Drug Discovery 4, 594-610.

Table 1. Lipid molecular species identified in ZFL cells with their molecular formula (MF), accurate mass, mass difference obtained as (theoretical mass−detected mass), and retention time (Tr). All identified compounds in accordance to criteria of maximum permissible mass error of 5 mDa. PC = Phosphatidylcholines, DAG = diacylglycerols, TAG = triacylglycerols, ChE = cholesterol esters.

Lipid subclass	Molecular ion	Lipid specie	MF	Accurate mass (*m/z*)	Mass difference (mDa)	T_r (min)
Lyso PC	$[M + H]^+$	16:0	$C_{24}H_{50}NO_7P$	496.3398	0.0	1.4
		18:0	$C_{26}H_{54}NO_7P$	524.3711	0.7	1.8
		18:1	$C_{26}H_{52}NO_7P$	522.3554	0.2	1.4
		18:2	$C_{26}H_{50}NO_7P$	520.3398	0.1	1.3
		20:3	$C_{28}H_{52}NO_7P$	546.3554	0.1	1.4
		20:4	$C_{28}H_{50}NO_7P$	544.3398	0.0	1.3
PC	$[M + H]^+$	31:0	$C_{39}H_{78}NO_8P$	720.5538	0.4	7.7
		32:0	$C_{40}H_{80}NO_8P$	734.5694	0.8	8.3
		32:1	$C_{40}H_{78}NO_8P$	732.5538	0.2	7.6
		34:0	$C_{42}H_{84}NO_8P$	762.6007	0.6	10.5
		34:1	$C_{42}H_{82}NO_8P$	760.5851	0.6	8.8
		34:2	$C_{42}H_{80}NO_8P$	758.5694	0.2	7.6
		34:3	$C_{42}H_{78}NO_8P$	756.5538	0.6	7.4
		36:1	$C_{44}H_{86}NO_8P$	788.6164	0.5	10.7
		36:2	$C_{44}H_{84}NO_8P$	786.6007	0.2	9.8
		36:3	$C_{44}H_{82}NO_8P$	784.5851	0.5	8.2
		36:5	$C_{44}H_{78}NO_8P$	780.5538	0.2	7.2
		36:6	$C_{44}H_{76}NO_8P$	778.5381	1.1	5.6
		38:2	$C_{46}H_{88}NO_8P$	814.632	1.4	11.1
		38:5	$C_{46}H_{82}NO_8P$	808.5851	0.5	8.7
		38:6	$C_{46}H_{80}NO_8P$	806.5694	0.9	7.5
		40:6	$C_{48}H_{84}NO_8P$	834.6007	0.7	9.8
		40:7	$C_{48}H_{82}NO_8P$	832.5851	0.5	7.9
Plasmalogen PC	$[M + H]^+$	30:0	$C_{38}H_{78}NO_7P$	692.5589	0.6	7.7
		32:1	$C_{40}H_{80}NO_7P$	718.5745	0.6	8.6
		34:1	$C_{42}H_{84}NO_7P$	746.6058	0.8	10.0
		34:4	$C_{42}H_{78}NO_7P$	740.5589	0.5	7.2
		36:2	$C_{44}H_{86}NO_7P$	772.6215	1.1	10.5
		36:4	$C_{44}H_{82}NO_7P$	768.5902	2.0	9.0
		36:5	$C_{44}H_{80}NO_7P$	766.5745	1.1	7.9
		38:4	$C_{46}H_{86}NO_7P$	796.6215	0.6	10.3
		38:6	$C_{46}H_{82}NO_7P$	792.5902	0.5	8.8
		38:7	$C_{46}H_{80}NO_7P$	790.5745	0.2	8.7
		40:7	$C_{48}H_{84}NO_7P$	818.6058	0.6	9.2

		40:8	$C_{48}H_{82}NO_7P$	816.5902	0.9	9.2
DAG	$[M + NH_4]^+$	32:1	$C_{35}H_{66}O_5$	584.5248	0.9	9.0
		32:0	$C_{35}H_{68}O_5$	586.5405	1.0	10.2
		32:2	$C_{35}H_{64}O_5$	582.5092	0.5	7.7
		34:0	$C_{37}H_{72}O_5$	614.5718	0.4	12.3
		34:1	$C_{37}H_{70}O_5$	612.5561	0.9	10.8
		34:2	$C_{37}H_{68}O_5$	610.5405	0.9	9.5
		34:3	$C_{37}H_{66}O_5$	608.5248	0.4	8.4
		34:4	$C_{37}H_{64}O_5$	606.5092	0.3	7.6
		36:1	$C_{39}H_{74}O_5$	640.5874	0.8	12.9
		36:2	$C_{39}H_{72}O_5$	638.5718	0.9	11.4
		36:3	$C_{39}H_{70}O_5$	636.5561	0.8	10.1
		36:6	$C_{39}H_{64}O_5$	630.5092	0.0	7.4
		38:5	$C_{41}H_{70}O_5$	660.5561	-0.1	9.8
TAG	$[M + NH_4]^+$	48:0	$C_{51}H_{98}O_6$	824.7702	1.1	20.1
		48:1	$C_{51}H_{96}O_6$	822.7545	1.7	19.3
		48:2	$C_{51}H_{94}O_6$	820.7389	2.1	18.8
		50:0	$C_{53}H_{102}O_6$	852.8015	0.9	21.2
		50:1	$C_{53}H_{100}O_6$	850.7858	1.7	20.3
		50:2	$C_{53}H_{98}O_6$	848.7702	1.8	19.5
		50:3	$C_{53}H_{96}O_6$	846.7545	1.9	18.9
		50:6	$C_{53}H_{90}O_6$	840.7076	0.9	17.6
		52:1	$C_{55}H_{104}O_6$	878.8171	2.3	21.4
		52:2	$C_{55}H_{102}O_6$	876.8015	2.7	20.5
		52:3	$C_{55}H_{100}O_6$	874.7858	1.6	19.8
		52:4	$C_{55}H_{98}O_6$	872.7702	2.0	19.2
		52:5	$C_{55}H_{96}O_6$	870.7545	0.9	18.9
		54:2	$C_{57}H_{106}O_6$	904.8328	2.8	21.6
		54:3	$C_{57}H_{104}O_6$	902.8171	2.7	20.8
		54:4	$C_{57}H_{102}O_6$	900.8015	0.6	20.1
		54:5	$C_{57}H_{100}O_6$	898.7858	0.4	19.4
		54:6	$C_{57}H_{98}O_6$	896.7702	0.7	19.3
ChE	$[M + NH_4]^+$	18:1	$C_{45}H_{78}O_2$	668.634	1.1	20.3
		18:2	$C_{45}H_{76}O_2$	666.6183	1.2	19.4
		18:3	$C_{45}H_{74}O_2$	664.6027	0.7	18.7
		20:4	$C_{47}H_{76}O_2$	690.6183	1.3	19.0
		20:5	$C_{47}H_{74}O_2$	688.6027	1.2	18.4
		22:6	$C_{49}H_{76}O_2$	714.6183	1.7	18.7

Table 2. Relative abundance for different lipid molecules in ZFL cells exposed for 96 h to increasing concentrations of TBT dissolved in basal medium or lipid mixture supplemented medium. PC = Phosphatidylcholines, DAG = diacylglycerols, TAG = triacylglycerols, ChE = cholesterol esters. Values expressed as relative abundance compared with control cells (0.1% DMSO) set to 100% ± SEM (n=3). *$p < 0.05$ indicate significant difference relative to vehicle controls.

Lipid subclass	Lipid specie	MF	− Lipid mixture				+ Lipid mixture			
			10 nM	25 nM	50 nM	100 nM	10 nM	25 nM	50 nM	100 nM
Lyso PC	16:0	$C_{24}H_{50}NO_7P$	119.4 ± 25.2	213.0 ± 14.0 *	178.3 ± 11.6	200.1 ± 29.9 *	98.8 ± 4.6	106.8 ± 12.8	124.3 ± 9.8	123.7 ± 14.0
	18:0	$C_{26}H_{54}NO_7P$	85.7 ± 9.3	218.7 ± 26.6 *	154.2 ± 21.4	182.4 ± 33.1	84.7 ± 1.8	96.2 ± 10.3	76.5 ± 4.5	97.5 ± 13.9
	18:1	$C_{26}H_{52}NO_7P$	128.5 ± 21.1	219.6 ± 22.5 *	206.7 ± 18.8 *	186.6 ± 25.1	99.9 ± 11.6	96.9 ± 10.8	105.9 ± 7.9	88.7 ± 9.8
	18:2	$C_{26}H_{50}NO_7P$	169.2 ± 49.0	249.7 ± 31.4	255.9 ± 20.7 *	226.2 ± 48.0	114.6 ± 12.7	117.2 ± 9.9	147.5 ± 22.2	175.1 ± 20.2
	20:3	$C_{28}H_{52}NO_7P$	148.8 ± 29.8	224.6 ± 23.6 *	240.2 ± 20.3 *	206.6 ± 40.2	107.0 ± 16.6	110.3 ± 10.6	114.3 ± 11.4	103.0 ± 14.9
	20:4	$C_{28}H_{50}NO_7P$	165.4 ± 33.0	225.3 ± 23.8	244.9 ± 24.3 *	226.6 ± 49.1	112.4 ± 19.3	117.4 ± 6.9	110.6 ± 13.8	93.2 ± 13.1
PC	31:0	$C_{39}H_{78}NO_8P$	84.2 ± 6.9	245.1 ± 15.1 *	198.5 ± 49.5	198.7 ± 38.5	88.6 ± 4.6	82.5 ± 5.4	75.8 ± 6.7	69.4 ± 15.2
	32:0	$C_{40}H_{80}NO_8P$	108.0 ± 6.6	285.2 ± 5.9 *	264.2 ± 63.8 *	271.5 ± 45.4 *	93.8 ± 5.5	93.2 ± 5.7	85.8 ± 8.6	82.6 ± 18.9
	32:1	$C_{40}H_{78}NO_8P$	127.3 ± 5.4	270.3 ± 5.3 *	291.8 ± 68.3 *	278.7 ± 44.3 *	99.4 ± 6.5	94.5 ± 6.8	91.9 ± 6.7	76.1 ± 10.5
	34:0	$C_{42}H_{84}NO_8P$	105.4 ± 7.0	325.9 ± 15.8 *	278.3 ± 76.4	263.0 ± 57.8	93.5 ± 9.3	88.9 ± 3.4	81.1 ± 6.1	90.5 ± 22.8
	34:1	$C_{42}H_{82}NO_8P$	110.4 ± 1.7	223.5 ± 7.7 *	219.1 ± 49.1 *	185.3 ± 29.0	98.7 ± 6.8	70.2 ± 14.8	70.0 ± 7.5	50.3 ± 7.7 *
	34:2	$C_{42}H_{80}NO_8P$	125.3 ± 3.4	259.6 ± 6.7 *	279.0 ± 65.1 *	258.7 ± 39.8 *	98.9 ± 7.0	97.2 ± 11.3	90.5 ± 0.9	90.6 ± 13.5
	34:3	$C_{42}H_{78}NO_8P$	107.3 ± 7.5	257.0 ± 6.3 *	260.9 ± 70.5	284.7 ± 54.3 *	103.4 ± 6.6	107.3 ± 14.3	108.6 ± 5.0	129.4 ± 20.6
	36:1	$C_{44}H_{86}NO_8P$	101.2 ± 2.0	224.8 ± 15.5 *	204.7 ± 49.0	152.6 ± 30.9	90.4 ± 5.6	77.7 ± 4.8 *	67.1 ± 4.9 *	51.6 ± 6.9 *
	36:2	$C_{44}H_{84}NO_8P$	117.2 ± 2.6	236.6 ± 8.0 *	238.8 ± 54.8 *	191.4 ± 32.7	100.9 ± 9.9	101.5 ± 12.2	93.4 ± 4.5	86.7 ± 12.9
	36:3	$C_{44}H_{82}NO_8P$	108.5 ± 1.7	240.2 ± 11.9 *	225.3 ± 52.2 *	194.1 ± 32.1	101.3 ± 9.3	104.6 ± 15.3	97.0 ± 2.8	110.7 ± 16.9
	36:5	$C_{44}H_{78}NO_8P$	100.3 ± 6.5	231.8 ± 10.3 *	209.3 ± 51.3	196.2 ± 36.6	96.8 ± 5.5	88.4 ± 12.6	71.6 ± 3.8	73.1 ± 12.6
	36:6	$C_{44}H_{76}NO_8P$	115.7 ± 10.5	277.6 ± 13.0	270.4 ± 73.2	297.4 ± 72.7 *	106.7 ± 7.0	114.6 ± 24.4	98.2 ± 3.3	93.6 ± 17.8
	38:2	$C_{46}H_{88}NO_8P$	108.4 ± 4.2	273.1 ± 22.4 *	279.0 ± 75.7 *	200.5 ± 45.7	94.3 ± 8.8	88.9 ± 10.1	74.7 ± 3.5	66.6 ± 10.4 *
	38:5	$C_{46}H_{82}NO_8P$	102.9 ± 4.4	227.4 ± 12.4 *	208.0 ± 48.8	182.0 ± 31.2	92.6 ± 10.0	94.7 ± 15.0	77.5 ± 2.5	67.7 ± 10.6
	38:6	$C_{46}H_{80}NO_8P$	107.5 ± 4.9	228.9 ± 12.6 *	208.3 ± 46.6 *	186.5 ± 29.7	103.4 ± 8.8	102.3 ± 14.1	81.4 ± 3.3	68.2 ± 11.1
	40:6	$C_{48}H_{84}NO_8P$	93.7 ± 3.0	201.6 ± 23.4 *	163.8 ± 33.4	137.0 ± 24.2	102.3 ± 11.2	96.9 ± 13.6	72.1 ± 5.1	61.8 ± 10.1
	40:7	$C_{48}H_{82}NO_8P$	106.3 ± 5.0	214.7 ± 11.1 *	188.3 ± 40.8	161.2 ± 25.7	104.3 ± 12.1	114.9 ± 26.1	90.9 ± 2.9	82.8 ± 12.2
Plasmalogen PC	30:0	$C_{38}H_{78}NO_7P$	63.7 ± 3.5	203.0 ± 26.1 *	139.5 ± 36.5	118.3 ± 25.3	86.4 ± 1.7	74.8 ± 5.2	64.4 ± 6.7 *	52.8 ± 11.9 *
	32:1	$C_{40}H_{80}NO_7P$	110.5 ± 4.6	257.4 ± 11.5 *	263.5 ± 67.7 *	229.3 ± 41.1	93.3 ± 9.3	89.6 ± 9.4	82.0 ± 12.0	52.7 ± 8.1 *

	34:1	$C_{42}H_{84}NO_7P$	114.5 ± 5.4	271.1 ± 14.2 *	284.6 ± 72.8 *	233.7 ± 45.2	101.0 ± 16.9	98.3 ± 14.2	92.7 ± 14.8	54.9 ± 9.9
	34:4	$C_{42}H_{78}NO_7P$	103.5 ± 6.8	231.9 ± 15.9	218.7 ± 53.2	215.0 ± 49.7	95.7 ± 8.5	91.7 ± 9.2	79.7 ± 4.4	63.6 ± 11.4 *
	36:2	$C_{44}H_{86}NO_7P$	138.6 ± 9.4	304.5 ± 10.6 *	351.3 ± 91.7 *	276.9 ± 52.3	105.0 ± 13.1	114.7 ± 19.1	106.0 ± 16.5	91.1 ± 15.7
	36:4	$C_{44}H_{82}NO_7P$	103.8 ± 2.0	216.9 ± 17.1 *	194.2 ± 41.2	169.9 ± 27.1	95.9 ± 10.9	87.7 ± 9.2	70.6 ± 7.0	49.3 ± 6.7 *
	36:5	$C_{44}H_{80}NO_7P$	85.4 ± 4.5	192.1 ± 20.8	149.1 ± 35.3	147.5 ± 34.8	90.4 ± 5.2	79.9 ± 6.1	65.7 ± 5.7 *	40.2 ± 6.0 *
	38:4	$C_{46}H_{86}NO_7P$	101.2 ± 3.7	246.7 ± 21.7 *	209.3 ± 53.8	158.7 ± 28.2	85.0 ± 10.8	64.1 ± 7.5 *	43.1 ± 3.5 *	21.8 ± 3.4 *
	38:6	$C_{46}H_{82}NO_7P$	108.1 ± 0.9	225.7 ± 24.7 *	194.6 ± 41.6	179.1 ± 29.9	96.9 ± 13.5	89.4 ± 13.1	70.1 ± 8.5	42.6 ± 6.2 *
	38:7	$C_{46}H_{80}NO_7P$	125.3 ± 6.7	273.2 ± 25.0 *	274.5 ± 57.7 *	286.7 ± 50.0 *	96.1 ± 12.3	97.8 ± 12.4	97.8 ± 12.2	81.0 ± 10.6
	40:7	$C_{48}H_{84}NO_7P$	147.7 ± 6.5	310.2 ± 25.3 *	300.0 ± 65.7 *	292.1 ± 48.3 *	110.0 ± 21.8	120.0 ± 24.6	96.8 ± 15.7	72.3 ± 12.4
	40:8	$C_{48}H_{82}NO_7P$	162.5 ± 15.9	345.2 ± 31.2 *	384.1 ± 78.5 *	446.7 ± 80.5 *	101.1 ± 14.8	103.8 ± 14.8	107.2 ± 14.8	84.9 ± 15.2
DAG	32:0	$C_{35}H_{68}O_5$	132.6 ± 9.6	118.4 ± 13.4	143.7 ± 18.2	153.3 ± 15.2	102.1 ± 15.3	110.5 ± 8.5	123.1 ± 7.3	72.3 ± 10.9
	32:1	$C_{35}H_{66}O_5$	131.6 ± 6.1 *	129.6 ± 8.9 *	155.3 ± 8.2 *	198.1 ± 5.1 *	126.1 ± 10.4	134.7 ± 34.2	248.5 ± 21.8 *	230.4 ± 1.8 *
	32:2	$C_{35}H_{64}O_5$	143.4 ± 3.5	107.0 ± 4.8	144.1 ± 8.6	255.4 ± 15.6 *	141.6 ± 13.3	158.9 ± 50.5	350.6 ± 71.4 *	481.4 ± 3.8 *
	34:0	$C_{37}H_{72}O_5$	115.8 ± 7.6	116.51 ± 21.2	122.4 ± 11.2	110.8 ± 7.2	120.3 ± 15.9	111.0 ± 3.2	99.8 ± 8.2	73.6 ± 6.5
	34:1	$C_{37}H_{70}O_5$	120.3 ± 6.7	120.0 ± 3.6	135.9 ± 9.7 *	137.9 ± 2.3 *	106.7 ± 5.5	102.3 ± 19.9	168.9 ± 1.6 *	130.1 ± 1.2
	34:2	$C_{37}H_{68}O_5$	172.5 ± 15.1 *	168.6 ± 12.4 *	175.4 ± 11.1 *	253.9 ± 13.2 *	122.2 ± 7.9	132.6 ± 48.1	299.2 ± 54.5 *	406.1 ± 7.1 *
	34:3	$C_{37}H_{66}O_5$	125.3 ± 7.6	95.5 ± 4.2	131.6 ± 9.6	255.5 ± 27.8 *	133.1 ± 13.0	147.6 ± 56.7	365.5 ± 97.1 *	557.7 ± 22.5 *
	34:4	$C_{37}H_{64}O_5$	–	–	–	–	146.0 ± 18.0	171.3 ± 68.0	439.1 ± 130.9 *	842.9 ± 39.7 *
	36:1	$C_{39}H_{74}O_5$	121.1 ± 12.9	114.7 ± 10.7	113.4 ± 11.3	88.0 ± 5.7	103.4 ± 3.5	91.0 ± 13.5	121.3 ± 7.9	62.0 ± 1.5
	36:2	$C_{39}H_{72}O_5$	140.0 ± 11.1 *	138.5 ± 5.8 *	156.8 ± 11.9 *	148.6 ± 5.1 *	137.9 ± 6.2	135.6 ± 42.1	281.0 ± 47.7 *	332.8 ± 0.2 *
	36:3	$C_{39}H_{70}O_5$	110.6 ± 13.8	120.1 ± 4.1	140.8 ± 12.5	158.5 ± 9.9 *	127.9 ± 6.6	146.9 ± 54.2	328.9 ± 82.3 *	480.2 ± 19.1 *
	36:6	$C_{39}H_{64}O_5$	–	–	–	–	155.0 ± 10.8	175.0 ± 57.1	285.6 ± 39.4 *	249.1 ± 18.2
	38:5	$C_{41}H_{70}O_5$	155.2 ± 9.9 *	154.8 ± 7.3 *	163.9 ± 16.5 *	147.2 ± 8.8 *	134.1 ± 9.5	114.4 ± 39.4	203.5 ± 31.1	190.1 ± 10.2
TAG	48:0	$C_{51}H_{98}O_6$	84.6 ± 3.1	85.7 ± 7.7	77.7 ± 5.0	73.8 ± 2.9	100.7 ± 3.7	97.0 ± 5.6	97.2 ± 5.0	149.9 ± 7.3 *
	48:1	$C_{51}H_{96}O_6$	79.5 ± 12.0	96.9 ± 8.5	94.4 ± 11.4	90.6 ± 6.1	102.6 ± 7.6	101.6 ± 26.4	132.7 ± 31.1	743.9 ± 25.0 *
	48:2	$C_{51}H_{94}O_6$	110.1 ± 6.5	120.3 ± 5.5	136.2 ± 7.4 *	160.1 ± 8.3 *	106.4 ± 5.6	106.2 ± 14.3	117.3 ± 12.7	1047.9 ± 39.2 *

	50:0	$C_{53}H_{102}O_6$	95.8 ± 9.2	90.2 ± 12.8	79.2 ± 8.7	67.7 ± 1.9	97.9 ± 5.6	94.4 ± 3.5	88.6 ± 4.1	106.6 ± 5.7
	50:1	$C_{53}H_{100}O_6$	92.0 ± 5.2	99.3 ± 12.3	88.4 ± 9.0	63.1 ± 5.5 *	104.9 ± 3.7	104.4 ± 34.2	112.3 ± 32.5	616.6 ± 9.7 *
	50:2	$C_{53}H_{98}O_6$	121.2 ± 0.7 *	129.8 ± 4.9 *	134.3 ± 8.0 *	125.9 ± 7.3 *	97.8 ± 5.9	106.2 ± 24.9	119.8 ± 15.6	2328.9 ± 127.2 *
	50:3	$C_{53}H_{96}O_6$	127.4 ± 6.2	130.4 ± 8.4 *	170.6 ± 5.8 *	182.0 ± 5.8 *	94.6 ± 5.4	98.2 ± 14.7	121.3 ± 18.8	2682.7 ± 130.4 *
	50:6	$C_{53}H_{90}O_6$	117.9 ± 17.2	150.4 ± 14.6	186.6 ± 16.4	324.1 ± 14.8 *	93.8 ± 6.5	114.8 ± 73.0	127.3 ± 67.5	6629.2 ± 458.6 *
	52:1	$C_{55}H_{104}O_6$	99.5 ± 10.6	105.5 ± 15.1	85.8 ± 11.3	57.7 ± 4.4 *	108.4 ± 2.4	100.7 ± 26.1	117.8 ± 28.1	342.1 ± 4.1 *
	52:2	$C_{55}H_{102}O_6$	109.8 ± 3.5	112.8 ± 4.9	111.1 ± 8.1	88.5 ± 6.4	104.4 ± 3.8	117.1 ± 29.3	122.6 ± 22.9	2601.8 ± 174.9 *
	52:3	$C_{55}H_{100}O_6$	92.9 ± 22.2	88.8 ± 18.0	146.6 ± 20.0	145.5 ± 3.7	103.9 ± 6.9	152.2 ± 38.8	137.1 ± 18.7	5447.2 ± 232.0 *
	52:4	$C_{55}H_{98}O_6$	107.1 ± 5.3	112.8 ± 4.7	118.1 ± 9.2	111.8 ± 5.9	100.4 ± 4.5	137.4 ± 42.8	130.0 ± 25.2	6527.9 ± 155.6 *
	52:5	$C_{55}H_{96}O_6$	111.4 ± 2.4	120.1 ± 5.0	126.3 ± 5.2 *	136.7 ± 5.1 *	100.4 ± 9.6	141.3 ± 94.7	142.3 ± 57.3	9163.6 ± 638.6 *
	54:2	$C_{57}H_{106}O_6$	103.0 ± 5.0	113.4 ± 12.5	97.8 ± 8.9	70.7 ± 2.8	107.2 ± 3.3	112.4 ± 28.0	138.2 ± 19.0	1252.8 ± 20.1 *
	54:3	$C_{57}H_{104}O_6$	87.0 ± 1.5	88.8 ± 3.8	89.0 ± 4.6	74.8 ± 1.4	113.4 ± 10.9	114.2 ± 10.8	126.9 ± 25.0	2346.6 ± 108.9 *
	54:4	$C_{57}H_{102}O_6$	92.6 ± 1.1	96.1 ± 3.1	96.5 ± 4.6	80.3 ± 2.9 *	101.0 ± 3.9	116.3 ± 22.8	112.6 ± 13.6	2775.3 ± 193.8 *
	54:5	$C_{57}H_{100}O_6$	97.2 ± 1.0	102.1 ± 3.2	96.6 ± 3.9	81.8 ± 3.1 *	102.8 ± 5.3	120.3 ± 43.1	114.3 ± 28.1	3097.1 ± 188.5 *
	54:6	$C_{57}H_{98}O_6$	104.2 ± 0.6	110.7 ± 4.2	102.9 ± 3.6	84.4 ± 3.3 *	108.0 ± 7.2	108.6 ± 68.4	116.9 ± 45.6	3066.3 ± 258.6 *
ChE	18:1	$C_{45}H_{78}O_2$	79.3 ± 10.5	95.1 ± 5.4	111.0 ± 4.4	91.0 ± 1.8	116.0 ± 10.7	128.8 ± 12.6	163.8 ± 28.7	207.0 ± 16.1 *
	18:2	$C_{45}H_{76}O_2$	83.8 ± 10.1	101.1 ± 8.1	119.1 ± 4.3	100.1 ± 1.9	130.7 ± 18.7	147.3 ± 26.1	198.6 ± 44.3	335.7 ± 47.0 *
	18:3	$C_{45}H_{74}O_2$	73.4 ± 19.9	105.5 ± 15.9	127.1 ± 4.8	103. ± 12.0	108.9 ± 10.1	149.3 ± 30.5	293.2 ± 127.6	611.5 ± 34.0 *
	20:4	$C_{47}H_{76}O_2$	83.1 ± 13.0	98.6 ± 10.6	129.5 ± 3.4	102.6 ± 2.2	113.1 ± 11.3	139.5 ± 31.1	209.3 ± 72.7	352.3 ± 58.3 *
	20:5	$C_{47}H_{74}O_2$	91.5 ± 13.7	107.6 ± 7.7	143.4 ± 8.0	116.3 ± 4.8	99.8 ± 7.7	138.4 ± 51.9	311.3 ± 173.2	767.9 ± 189.4 *
	22:6	$C_{49}H_{76}O_2$	91.6 ± 12.0	106.7 ± 9.1	142.0 ± 4.4 *	111.2 ± 2.8	100.4 ± 7.6	114.3 ± 11.8	119.1 ± 14.3	116.9 ± 10.5

Fig. 1. Total lipid accumulation determined in ZFL cells exposed for 96 h to different concentrations of TBT dissolved in (A) basal medium, (B) lipid mixture supplemented medium. All data are expressed as mean fold change ± SEM in four replicates. One-way ANOVA test (Dunnett's post hoc test) was used to estimate the P values: *$p < 0.05$ indicate significant difference relative to vehicle (0.1% DMSO) controls.

Fig. 2. UHPLC-HRMS total ion chromatogram of extracted lipids from ZFL cells.

Fig. 3. Changes in lipid class composition of ZFL cells exposed for 96 h to different concentrations of TBT dissolved in (A) basal medium, (B) lipid mixture supplemented medium. The content of individual lipid classes was determined by summing up relative abundances of all identified species. Values expressed as relative abundance compared with control cells (0.1% DMSO) set to 100%. One-way ANOVA test (Dunnett's post hoc test) was used to estimate the P values: *$p < 0.05$. Error bars correspond to SEM (n=3).

Fig. 4. Relative abundance for different lipid molecules in ZFL cells exposed for 96 h to TBT 100 nM dissolved in lipid-enriched medium. DAG = diacylglycerols, TAG = triacylglycerols. Values expressed as relative abundance compared with control cells (0.1% DMSO) set to 100% ± SEM (n=3).

Figure 1.

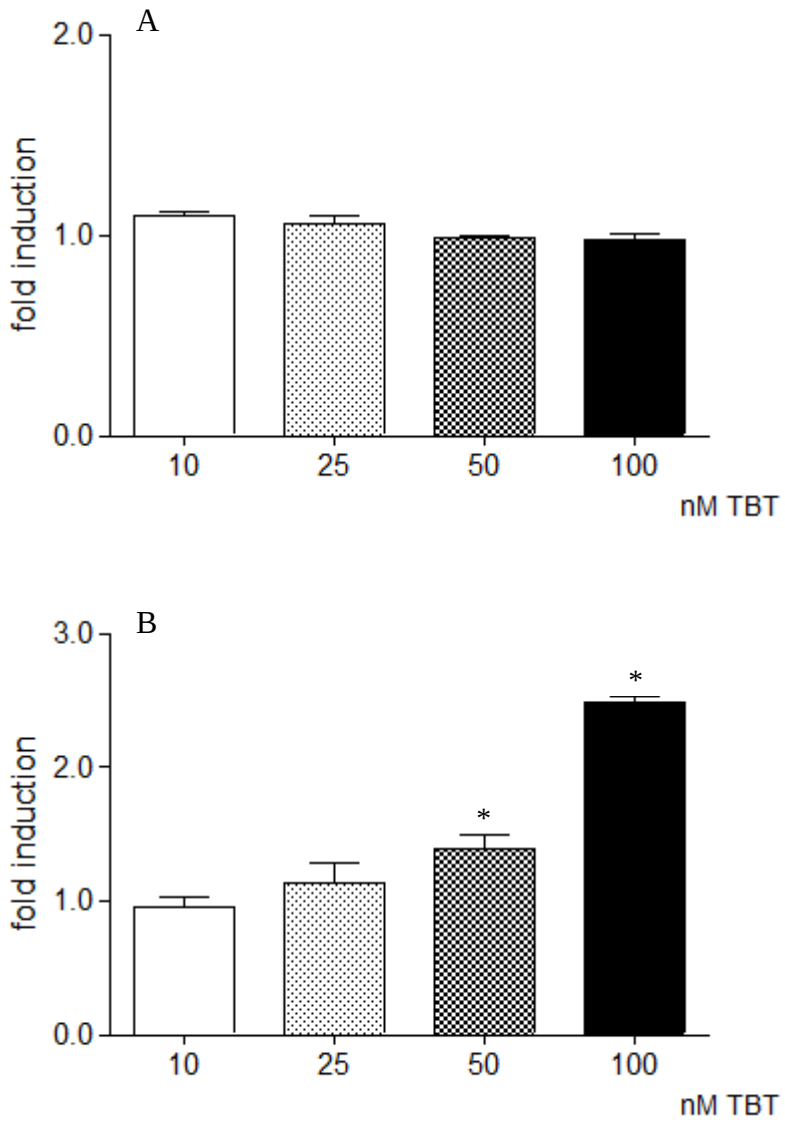

A
2.0
1.0
0.0
fold induction
10
25
50
100
nM TBT
B
3.0
2.0
1.0
0.0
fold induction
*
*
10
25
50
100
nM TBT

Figure 2.

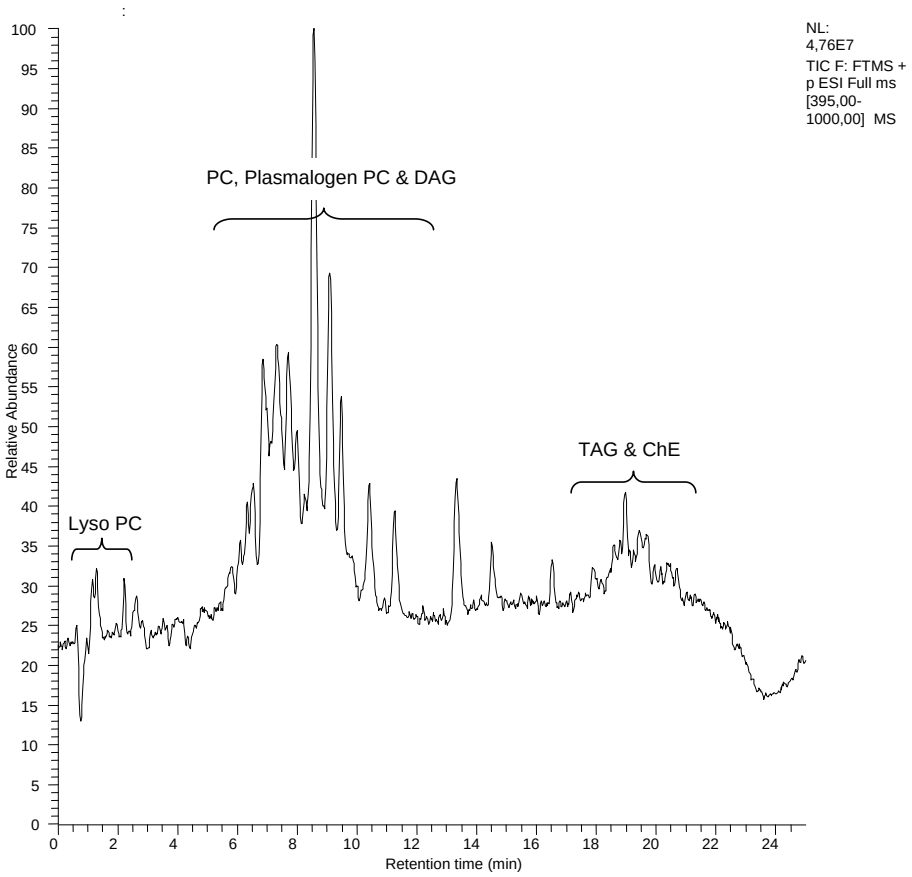

NL:
4,76E7
TIC F: FTMS +
p ESI Full ms
[395,00-
1000,00] MS
PC, Plasmalogen PC & DAG
TAG & ChE
Lyso PC
Relative Abundance
Retention time (min)

Figure 3.

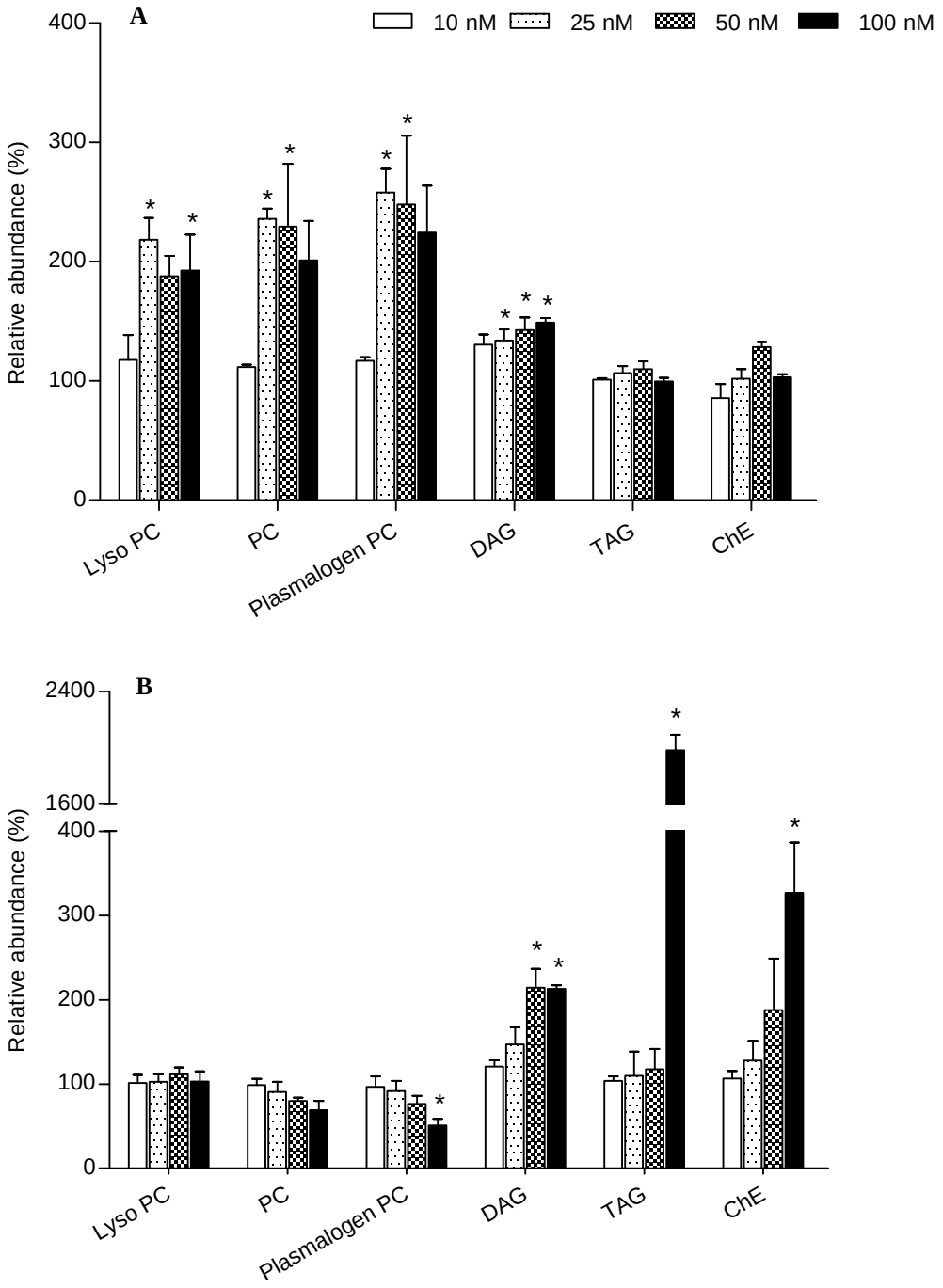
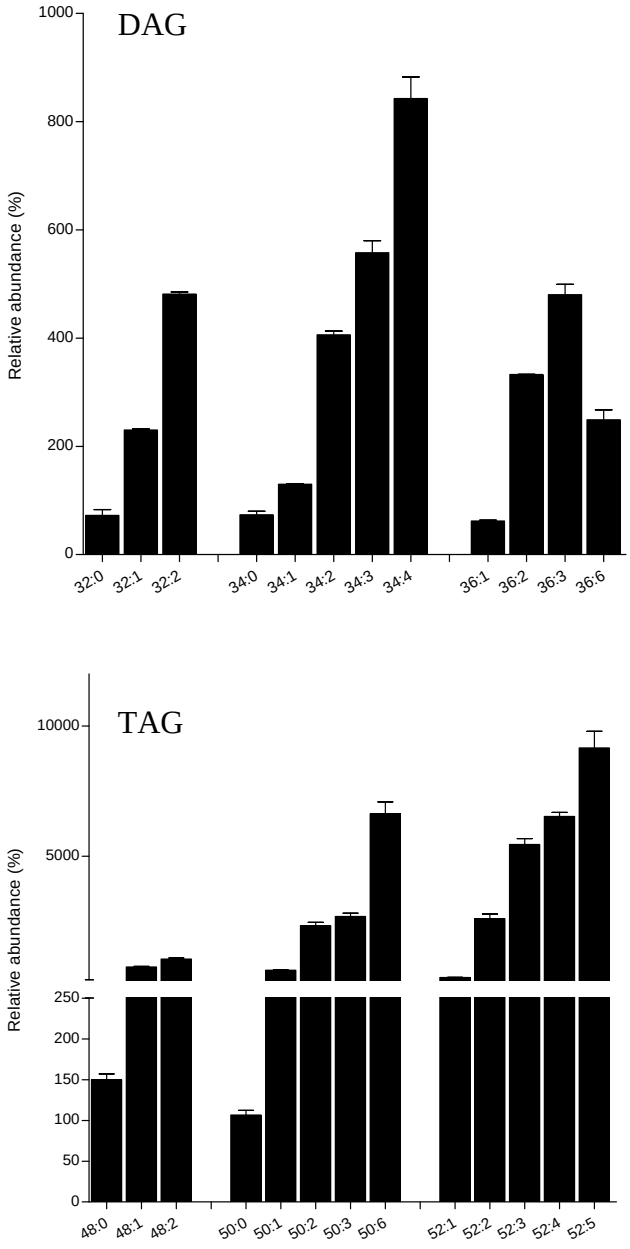

A
10 nM
25 nM
50 nM
100 nM
Relative abundance (%)
400
300
200
100
0
Lyso PC
PC
Plasmalogen PC
DAG
TAG
ChE
B
2400
1600
400
300
200
100
0
Relative abundance (%)
Lyso PC
PC
Plasmalogen PC
DAG
TAG
ChE

Figure 4.


DAG
Relative abundance (%)
1000
800
600
400
200
0
32:0
32:1
32:2
34:0
34:1
34:2
34:3
34:4
36:1
36:2
36:3
36:6
TAG
Relative abundance (%)
10000
5000
250
200
150
100
50
0
48:0
48:1
48:2
50:0
50:1
50:2
50:3
50:6
52:1
52:2
52:3
52:4
52:5