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Studies of androgenic and estrogenic effects on PPARs, estrogen receptors and some related gene and phenotypic targets, using brown trout primary hepatocytes as the model system

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SEDE ADMINISTRATIVA
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
FACULDADE DE MEDICINA

CÉLIA CRISTINA CARREIRAS LOPES

STUDIES OF ANDROGENIC AND ESTROGENIC EFFECTS ON PPARs, ESTROGEN RECEPTORS AND SOME RELATED GENE AND PHENOTYPIC TARGETS, USING BROWN TROUT PRIMARY HEPATOCYTES AS THE MODEL SYSTEM

Tese de Candidatura ao grau de Doutor em Patologia e Genética Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto e Faculdade de Medicina da Universidade do Porto

Orientador – Eduardo Jorge Sousa Rocha
Categoria – Professor Catedrático
Afiliação – Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto

Coorientador – Tânia Vieira Madureira
Categoria – Investigadora Auxiliar
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto
Categoria – Professora Auxiliar Convidada
Afiliação – Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto

Para a minha mãe

Pelo que me ensinaste

Porque me fizeste o que sou

Por TUDO

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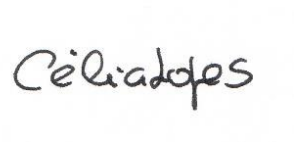
DECLARAÇÃO DE HONRA

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.



List of Publications

The author declares that in the Thesis are included 4 published articles and 1 submitted manuscript.

The doctoral candidate participated in the conception, execution of the experimental work, data analysis and wrote the original draft of all manuscripts included in the Thesis, listed below. The publications from Chapters 2, 3 and 4 are fully reproduced in the Thesis and all are Elsevier articles. Thus, as author, the candidate has the right to include them in the doctoral Thesis, as stated by the publisher.

Publications included in this Thesis:

1- **Lopes, C.,** Madureira, T.V., Ferreira, N., Pinheiro, I., Castro, L.F.C., Rocha, E., 2016. Peroxisome proliferator-activated receptor gamma (PPARγ) in brown trout: interference of estrogenic and androgenic inputs in primary hepatocytes. Environmental Toxicology and Pharmacology 46, 328-336. doi: 10.1016/j.etap.2016.08.009.

2- **Lopes, C**., Malhão, F., Guimarães, C., Pinheiro, I., Gonçalves, J.F., Castro, L.F.C., Rocha, E., Madureira, T.V., 2017. Testosterone-induced modulation of peroxisomal morphology and peroxisome-related gene expression in brown trout (*Salmo trutta f. fario*) primary hepatocytes. Aquatic Toxicology 193, 30-39. doi: 10.1016/j.aquatox.2017.09.026.

3- **Lopes, C**., Madureira, T.V., Gonçalves, J.F., Rocha, E., 2020. Disruption of classical estrogenic targets in brown trout primary hepatocytes by the model androgens testosterone and dihydrotestosterone. Aquatic Toxicology, 227,105586. doi: 10.1016/j.aquatox.2020.105586.

4- **Lopes, C**., Rocha, E, Pereira, I.L., Madureira, T.V. Deciphering interferences of testosterone and dihydrotestosterone on lipid metabolism genes using brown trout primary hepatocytes. Submitted for publication in an international journal.

Abstract

The synthesis, mode of action and functions of sex-steroid hormones are overall similar among vertebrates. Therefore, animal models are often used to study the effects of hormone mimic compounds, the so-called endocrine-disrupting chemicals (EDCs). The regulatory organizations have been defining assays with distinct fish species, using *in vivo* and *in vitro* experimental approaches. To investigate the mechanisms of action of chemicals on specific endocrine processes *in vitro* models are recognized as advantageous since they allow the exclusion of confounding factors, inherent to *in vivo* studies. Fish primary hepatocytes, cultured in monolayer, retain for days their morphofunctional differentiation and metabolization properties and respond promptly to hormonal stimuli.

Research using brown trout (*Salmo trutta* f. *fario*) as a model organism suggested that peroxisome proliferator-activated receptors (PPARs) expression and peroxisomal morphology are influenced by sex-steroids. This Thesis aimed to explore further the regulation (and potential disruption) of PPARs and related targets, including peroxisomes, by estrogenic and androgenic hormones, using juvenile brown trout primary hepatocytes. The Thesis also aimed to get new insights on the interferences of androgens in estrogen receptor (ER) signaling and lipid metabolism pathways.

First, it was investigated the effects of 17α-ethinylestradiol (EE2) and testosterone (T) on the expression of PPARγ. For that purpose, the complementary deoxyribonucleic acid (cDNA) sequence of PPARγ was isolated. The cloned sequence presented ~ 98% identity with a previously described in Atlantic salmon (*Salmo salar*). It was found that estrogens and androgens changed PPARγ expression. The messenger ribonucleic acid (mRNA) levels of PPARγ, measured by quantitative real-time polymerase chain reaction (qRT-PCR), followed a non-monotonic curve after EE2 exposure, with increased expression after the lower concentration (1 μM) and a decrease after the higher dose (50 μM). PPARγ expression declined after exposure to 10 and 50 μM of T.

Next, it was assessed the effects of T on the mRNA levels of PPARα and two peroxisomal genes, catalase (Cat) and urate oxidase (Uox). As T is an aromatizable androgen, the known biomarker of estrogenic signaling, vitellogenin A (VtgA), was also included. The effects of T on peroxisomes morphology were also studied, by transmission electron microscopy (TEM) and stereological techniques, after Cat staining by immunofluorescence (IF). PPARα and Cat mRNA levels were not altered by the androgenic exposure, but Uox was down-regulated, after 10 and 50 μM of T. On the contrary, VtgA was up-regulated by all androgen concentrations (1, 10 and 50 μM).

Smaller peroxisome profiles were noted in TEM, and a reduction in peroxisome relative volumes was found in the stereological analysis. To explore mechanistically the effects of T, primary hepatocytes were exposed to 10 μM of T, in combination with two concentrations (10 and 50 μM) of ER and androgen receptor (AR) antagonists, ICI 182,780 (ICI) and flutamide (F), respectively. The smaller peroxisome profiles observed after T exposure were less abundant in the mixture conditions with both receptor antagonists in TEM. ICI reversed Vtg mRNA and the peroxisomal relative volumes to control levels but Uox expression was still down-regulated in the T plus ICI mixture conditions. F interfered with the expression of Cat and Uox. A concentration-dependent reduction of Vtg mRNA was observed between the two mixtures of T and F. Thus, data from this study suggest that T acted through both ARs and ERs to modulate functional and structural peroxisomal targets.

Androgenic interferences in classical estrogenic targets were explored thereafter. Hepatocytes were exposed to T and dihydrotestosterone (DHT), which is a non-aromatizable androgen, from 1 nM to 100 μM concentrations. The mRNA levels of VtgA, ERs alpha (ERα) and beta 1 (ERβ1) and two zona pellucida proteins (ZPs), zona pellucida glycoprotein 2.5 (ZP2.5) and zona pellucida glycoprotein 3a.2 (ZP3a.2), were assessed. Moreover, AR and the ovarian-type aromatase CYP19a1 were also included in the gene portfolio. DHT and T increased VtgA, ZPs and ERα expression and decreased ERβ1 mRNA levels. CYP19a1 expression was not altered by any androgen. The AR mRNA levels tended to up-regulate after the micromolar concentrations of DHT, while T only up-regulated AR after the 10 μM concentration, compared with control levels. For both androgens, significant and positive correlations were found between the mRNA levels of Vtg an ERα, and between ZPs and ERα, which evidences the relevance of this receptor in the process of Vtg and ZPs induction by T and DHT. Phenotypic alterations were complementarily assessed by immunohistochemistry (IHC), which was quantified using an HScore system. Vtg and ZPs HScores increased after exposure to both androgens, following a similar pattern to that observed for the mRNA levels. The HScores of AR, ERα, ERβ and CYP19 showed increasing trends after the micromolar concentrations of DHT. Thereby, the IHC data were highly consistent with the molecular findings, concerning Vtg and ZPs.

Finally, the effects of T and DHT (at the same concentrations described previously) were studied in the mRNA levels of genes from key pathways in lipid metabolism, many known to be regulated by PPARs. The targets included: PPARγ, involved in the regulation of adipogenesis and peroxisomal β-oxidation; apolipoprotein A1 (ApoA1) and fatty acid binding protein 1 (Fabp1), with functions in lipid transport; long-chain acyl-coenzyme A

(CoA) synthetase 1 (Acsl1), essential for fatty acid activation; lipoprotein lipase (LPL), mainly related to lipolysis and fatty acid up-take; steroidogenic acute regulatory protein (StAR), important in steroidogenesis, bile acids synthesis and cholesterol metabolism; acyl-CoA oxidase 1 3I (Acox1-3I), rate-limiting in peroxisomal β-oxidation and, at last, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), main enzymes in *de novo* lipogenesis. Acsl1 was up-regulated by T and DHT, while the lipogenic enzymes FAS and ACC were up-regulated by 100 μM of T and DHT, respectively. Acox1-3I was down-regulated by T and PPARγ by both androgens. ApoA1 was decreased by T and DHT, while Fabp1, StAR and LPL mRNA levels were not altered by any of the androgens. From the data, we suggest that androgens may be shifting lipid metabolism towards lipogenesis in this model system. Additionally, androgens seem to have the potential to impact on lipoprotein metabolism.

Overall, the Thesis findings disclosed estrogenic and androgenic impacts in PPARγ expression. Further, they uncovered new androgenic interferences in peroxisomes morphology and in the expression of peroxisomal, estrogenic and lipid-related targets, which are still poorly explored topics in fish. The Thesis data added new knowledge about androgenic and estrogenic effects on nuclear receptors that are master transcriptional regulators and in some linked gene and phenotypic targets, which may help to understand the regulation and disruption of key pathways in fish, by hormones and EDCs.

Resumo

A síntese, modo de ação e funções das hormonas esteroides sexuais são, em geral, semelhantes entre os vertebrados. Assim, os modelos animais são frequentemente usados para estudar os efeitos causados por compostos que mimetizam tais hormonas, os designados compostos desreguladores endócrinos (EDCs). As entidades reguladoras têm vindo a implementar ensaios com diferentes espécies de peixes, usando metodologias experimentais *in vivo* e *in vitro*. Para investigar os mecanismos de ação de um composto químico em processos endócrinos específicos, os modelos *in vitro* são reconhecidamente mais adequados, uma vez que permitem a exclusão de fatores de confundimento inerentes aos estudos *in vivo*. Os hepatócitos primários de peixes, cultivados em monocamada, retêm durante dias a sua diferenciação morfofuncional e capacidade de metabolização, respondendo prontamente a estímulos hormonais.

Estudos prévios usando a truta fário (*Salmo trutta* f. *fario*) como organismo modelo sugerem que a expressão dos recetores ativados por proliferadores de peroxissomas (PPARs) e a morfologia dos peroxissomas são regulados por esteroides sexuais. Esta Tese teve como objetivo explorar a regulação (e potencial disrupção) dos PPARs e alguns alvos relacionados, incluindo os peroxissomas, por hormonas estrogénicas e androgénicas, usando hepatócitos primários de truta fário juvenil. A Tese teve igualmente como objetivo obter novos conhecimentos acerca das interferências de androgénios na sinalização dos recetores de estrogénios (ERs) e nas vias do metabolismo lipídico.

Inicialmente, foram investigados os efeitos do 17α-etinilestradiol (EE2) e da testosterona (T) na expressão do PPARγ. Para esse fim, foi isolada a sequência de ácido desoxirribonucleico complementar (cDNA) do PPARγ. A sequência clonada apresentava ~ 98% de identidade com uma descrita anteriormente em salmão do Atlântico (*Salmo salar*). A expressão do PPARγ foi alterada pela exposição a estrogénios e androgénios. Os níveis do ácido ribonucleico mensageiro (mRNA) do PPARγ, medidos pela quantificação em tempo real da reação em cadeia da polimerase (qRT-PCR), seguiram uma curva não monotónica após exposição a EE2. Houve um aumento da expressão com a menor concentração (1 μM) e uma diminuição após a maior concentração (50 μM). A expressão do PPARγ diminuiu com a exposição a 10 e 50 μM de T.

Em seguida, determinamos os efeitos da T nos níveis de mRNA do PPARα e de dois genes peroxissomais, a catalase (Cat) e a urato oxidase (Uox). Como a T é um

androgénio aromatizável, foi também incluído o gene da vitelogenina A (VtgA), um reconhecido biomarcador de sinalização estrogénica. Foram também estudados os efeitos da T na morfologia quantitativa dos peroxissomas, por microscopia eletrónica de transmissão (TEM) e técnicas estereológicas, após marcação da Cat por imunofluorescência (IF). Os níveis de mRNA do PPARα e da Cat não foram alterados pela exposição ao androgénio, mas a expressão da Uox foi reduzida pela T, a 10 e 50 μM. Pelo contrário, a VtgA foi regulada positivamente por todas as concentrações de androgénio (1, 10 e 50 μM). Após a exposição, observaram-se perfis menores de peroxissomas em TEM, a par de uma redução do volume relativo dos organelos na célula. Para explorar mecanisticamente os efeitos da T, os hepatócitos primários foram expostos a 10 μM de T, em combinação com duas concentrações (10 e 50 μM) de antagonistas do ER e do recetor de androgénios (AR), ICI 182,780 (ICI) e flutamida (F), respetivamente. Na TEM, os perfis menores de peroxissomas observados após a exposição à T foram menos abundantes nas misturas de T com ambos os antagonistas. O ICI reverteu para níveis controlo as quantidades de mRNA da Vtg e as alterações morfológicas nos volumes relativos dos peroxissomas, mas a expressão da Uox manteve-se diminuída nas condições correspondentes às misturas de T e ICI. A F interferiu na expressão da Cat da Uox. Observou-se uma redução significativa na expressão da VtgA entre duas misturas de T e F, dependente da concentração. Assim sendo, os resultados deste estudo sugerem que a T atuou através do AR e do ER para modular os alvos funcionais e estruturais dos peroxissomas.

Foram em seguida exploradas interferências androgénicas em alvos classicamente considerados como estrogénicos. Os hepatócitos foram expostos a T e dihidrotestosterona (DHT), que é um androgénio não aromatizável, em concentrações compreendidas entre 1 nM e 100 μM. Foram avaliados os níveis de mRNA da VtgA, dos ERs alfa (ERα) e beta 1 (ERβ1) e de duas proteínas da zona pelúcida (ZPs), a glicoproteína da zona pelúcida 2.5 (ZP2.5) e a glicoproteína da zona pelúcida 3a.2 (ZP3a.2). Além disso, o AR e a variante ovárica da aromatase, CYP19a1, também foram incluídos no portfólio de genes. A DHT e a T aumentaram a expressão da VtgA, ZPs e ERα e diminuíram os níveis de mRNA do ERβ1. A expressão do CYP19a1 não foi alterada por nenhum dos androgénios. Os níveis de mRNA do AR foram tendencialmente aumentados pelas concentrações micromolares de DHT, enquanto que a T só aumentou a expressão do AR na concentração de 10 μM, comparando com os níveis controlo. Para ambos os androgénios, verificaram-se correlações significativas e positivas entre os níveis de mRNA da VtgA e do ERα e entre as ZPs e ERα, o que evidencia a relevância deste recetor no processo de indução de Vtg e ZPs pela T e DHT.

Complementarmente, foram avaliadas as alterações fenotípicas por imunohistoquímica (IHC), usando um sistema de HScore semi-quantitativo. Os HScores da Vtg e ZPs aumentaram após a exposição a ambos os androgénios, seguindo um padrão semelhante ao observado para os níveis de mRNA. Os HScores do AR, ERα, ERβ e CYP19 mostraram uma tendência crescente após as concentrações micromolares de DHT. Portanto, os dados da IHC foram bastante concordantes com os achados moleculares, no que diz respeito à Vtg e ZPs.

Por fim, foram estudados os efeitos da T e DHT (nas mesmas concentrações descritas anteriormente) nos níveis de mRNA de genes de vias chave do metabolismo lipídico, alguns dos quais se sabe serem regulados por PPARs. Os genes alvo incluíam: o PPARγ, envolvido na regulação da adipogénese e da β-oxidação peroxissomal; a apolipoproteína A1 (ApoA1) e a proteína de ligação a ácidos gordos 1 (Fabp1), com funções no transporte lipídico; a acil-coenzima A (CoA) sintetase de cadeia longa 1 (Acsl1), essencial para a ativação de ácidos gordos; a lipoproteína lipase (LPL), principalmente relacionada com a lipólise e captação de ácidos gordos; a proteína reguladora aguda da esteroidogénese (StAR), importante na esteroidogénese, síntese de ácidos biliares e no metabolismo do colesterol; a acil-CoA oxidase 1 isoforma 3l (Acox1-3l), limitante na β-oxidação peroxissomal; e por fim a acetil-CoA carboxilase e a sintase de ácidos gordos (ACC e FAS), enzimas da lipogénese *de novo*. A expressão da Acsl1 foi aumentada pela T e pela DHT, enquanto que a expressão das enzimas lipogénicas FAS e ACC aumentou após exposição às concentrações de 100 μM de T e DHT, respetivamente. Os níveis de mRNA da Acox1-3l diminuíram com a T e os do PPARγ com ambos os androgénios. A expressão da ApoA1 foi diminuída por ambos os androgénios, enquanto que os níveis de mRNA dos genes Fabp1, StAR e LPL não foram alterados por nenhum dos androgénios. Perante estes dados, sugerimos que neste modelo *in vitro* os androgénios podem estar a direcionar o metabolismo lipídico para a lipogénese. Adicionalmente, os androgénios parecem ter potencial para afetar o metabolismo das lipoproteínas.

De uma maneira geral, os resultados da Tese evidenciam impactos estrogénicos e androgénicos na expressão do PPARγ. Para além disso, descobrimos interferências androgénicas na morfologia dos peroxissomas e na expressão de alvos peroxissomais, estrogénicos e lipídicos, tópicos ainda pouco explorados em peixes. Os achados desta Tese adicionam novos conhecimentos acerca dos efeitos dos estrogénios e androgénios em recetores nucleares que são importantes reguladores da transcrição, assim como em alvos moleculares e fenotípicos relacionados, que podem ajudar a entender a regulação e disrupção de vias chave em peixes, por hormonas e EDCs.

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Chapter 1

General introduction

1. Endocrine disruption and fish as models in ecotoxicology

1.1. Endocrine disruption chemicals (EDCs) and their molecular targets

Endocrine-disrupting chemicals (EDCs) are defined as “exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes” (Kavlock et al., 1996), or, simplifying, “endocrine disruptors are chemicals, or chemical mixtures, that interfere with normal hormone action” (WHO, 2013).

Humans and all living organisms are exposed to a great diversity of EDCs, naturally occurring or human-made, and thus a lot of effort has been focused in the investigation of the consequences of such exposures in the past decades (Ankley and Johnson, 2004; Navas and Segner, 2006; OECD, 2014; Sumpter, 2008) and in the development of adequate methods for the detection and characterization of those substances (Castaño et al., 2003; Navas and Segner, 2006). Because estrogens and androgens control key reproduction processes, it has been given great emphasis to compounds that interfere with normal hormonal signaling and may consequently affect populations viability and maintenance (Ankley and Johnson, 2004; Scholz and Mayer, 2008). Endogenous hormones, namely 17β-estradiol (E2) and testosterone (T), in females, and T, dihydrotestosterone (DHT) and 11-ketotestosterone (11-KT), in males (depending on species), determine secondary sex characteristics and influence gonadal development and maturation (Martyniuk et al., 2013; Scholz and Mayer, 2008; Scholz et al., 2013). Mechanistically, EDCs can modify, for instance, the synthesis and metabolism of hormones, hormone plasma concentrations or mimic or antagonize the action of endogenous hormones (Scholz et al., 2013). EDCs can also disrupt hormonal signaling by interfering with hormone receptors, for example, estrogen receptor (ER) and androgen receptor (AR), and with other interconnected nuclear receptors/signaling pathways. Peroxisome proliferator-activated receptors (PPARs), for example, are nuclear receptors with major roles in regulating body metabolism, but also reproduction and cell differentiation (Rosen and Spiegelman, 2001; Vitti et al., 2016). Resembling hormonal receptors, PPARs seem to be targeted by diverse classes of pollutants (e.g., phthalate monoesters, tributyltin), some known to also interfere with ERs and ARs (Grimaldi et al., 2015; Grün et al., 2006; Toporova and Balaguer, 2020).

1.2. Fish models and their biomarkers for assessing endocrine disruption

The synthesis, action of endogenous hormones and the overall responses to toxics are highly conserved among vertebrates (Ankley et al., 2004; Scholz et al., 2013), making fish attractive models for research purposes. Specifically, small fish species with short life cycles, such as the fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) or Japanese medaka (*Oryzias latipes*), allow large scale screening studies, and the assessment of full life cycle or even multigenerational effects (Schartl, 2014; Scholz and Mayer, 2008). Although a lot of work in ecotoxicology has been done with small fish models, they are mainly asynchronous spawners, which does not reflect the distinct reproductive strategies of most wild fish populations (Hutchinson et al., 2006). That can be a limitation for the determination of effects of chemicals on the reproductive processes. Salmonids, for example, are annual spawners and experience drastic changes in hormonal levels along a reproductive cycle (Soivio et al., 1982; Wallaert and Babin, 1994). Rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) have been shown to be very sensible species to hormonal disturbances (Körner et al., 2008; Tyler et al., 2005; Van den Belt et al., 2003), confirming that salmonids are suitable models for endocrine disruption studies.

EDCs are ubiquitously present and, eventually, reach distinct aquatic environments (Sumpter, 2005; Vulliet and Cren-Olivé, 2011). The presence of hormonal compounds, pharmaceuticals and various other chemicals in the ground, surface, and drinking waters has been shown in several studies (aus der Beek et al., 2016; Backe et al., 2011; Chang et al., 2011; Vulliet and Cren-Olivé, 2011; Yang et al., 2017). These compounds may cause deleterious effects in humans and wildlife, even at nanomolar concentrations (Diamanti-Kandarakis et al., 2009; Guillette and Gunderson, 2001; Larsson et al., 1999; Vandenberg et al., 2012). Much of the knowledge on the harmful effects caused by environmental contaminants came in fact from wildlife, including fish, observations (Guillette and Gunderson, 2001; Larsson et al., 2000). Due to the presence of such chemicals in the water, fish are considered as primary targets of potential disruptions (Scholz and Mayer, 2008). Fish are also employed for environmental surveillance and monitoring, acting as sentinels for the presence of EDCs in their ecosystems (Behrens and Segner, 2005; Triebskorn et al., 2002).

Biological markers that respond readily to hormonal inputs are usually used as indicators of endocrine disruption and are of utmost importance in the risk assessment of chemicals. Fish have reliable biomarkers that can be used to assess interferences with hormonal (ER an AR) signaling. Spiggin and vitellogenin (Vtg) are widely used biomarkers of androgenic and estrogenic exposure, respectively. The spiggin is produced by the three-spined

stickleback (*Gasterosteus aculeatus*) males, in kidney cells, in response to increased androgen levels, by interaction with AR (Jolly et al., 2006; Katsiadaki et al., 2002). As for Vtg, it is an endogenous glycolipoprotein (the egg-yolk precursor protein) produced either physiologically by female fish after increased E2 levels, which interact with ER (Gunnarsson et al., 2007; Sumpter and Jobling, 1995), or by estrogenic induction in the male and female fish liver (Celius et al., 2000; Scholz et al., 2004). The complete or partial Vtg protein sequence is known from a wide number of fish species (237, according to the National Center for Biotechnology Information (NCBI) records). It can be detected (and quantified) in its protein form by standardized methods, including enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and western blot (WB). Alternatively, Vtg can be detected at messenger ribonucleic acid (mRNA) level by polymerase chain reaction (PCR) methods. Independently of the detection method, Vtg has been a widely accepted and used biomarker of estrogenic exposure (Arukwe and Goksøyr, 2003; Hultman et al., 2015a; Martyniuk et al., 2020; Sumpter and Jobling, 1995).

The ERs govern Vtg induction. At least three different ERs – ERα or Esr1, ERβ1 or Esr2b and ERβ2 or Esr2a have been described in fish (Menuet et al., 2004; Mouriec et al., 2009). Since it is consistently induced by estrogens, increased ERα expression is also considered an estrogen-exposure biomarker (Feswick et al., 2017).

Other recognized biological markers of estrogen exposure are the zona pellucida proteins (ZPs), also commonly known as zona radiata proteins (ZRPs). The zona pellucida is also called vitelline envelope, chorion or egg envelope and constitutes the extracellular matrix that surrounds fish eggs (Litscher and Wassarman, 2018). Similarly to Vtg, ZPs are produced after E2 stimulation, and can be induced by exogenous estrogens (Arukwe et al., 2000; Arukwe et al., 2002; Hultman et al., 2015b; Uren Webster et al., 2015).

2. Regulation of estrogenic biomarkers by estrogens and androgens in the liver - Evidence from fish *in vivo* studies

2.1. Regulation of estrogenic targets by estrogens

Since the early *in vivo* studies that showed Vtg induction in rainbow trout exposed to wastewater treatment plant effluents (Purdom et al., 1994), much attention has been given to identifying the adverse outcomes of estrogenic substances (Sumpter, 2005). ZPs and Vtg are target genes that are consistently induced by estrogens *in vivo* (Cleveland and Manor, 2015; Doyle et al., 2013; Gunnarsson et al., 2007; Uren Webster et al., 2015). Several *in vivo* studies have even shown that ZPs are earlier and more responsive

biomarkers (to low concentrations of estrogens), than Vtg (Gunnarsson et al., 2007; Thomas-Jones et al., 2003; Uren Webster et al., 2015). Despite that, and even knowing the inter-individual variability of Vtg inductions (Feswick et al., 2017), Vtg is universally used as a biomarker of estrogen exposure (Martyniuk et al., 2020).

From a mechanistic point of view, in fish, the induction of Vtg by estrogenic compounds is generally accompanied by an up-regulation of ERα mRNA levels, as shown in brown trout (Madureira et al., 2018; Uren Webster et al., 2015), rainbow trout (Benninghoff and Williams, 2008) or goldfish (*Carassius auratus*) (Marlatt et al., 2008) exposed to natural (E2) and to synthetic estrogens, such as 17α-ethinylestradiol (EE2). Further, positive correlations between Vtg induction and ERα mRNA levels were found in juvenile Atlantic salmon (*Salmo salar*) after waterborne exposure to 4-nonylphenol (NP) (Meucci and Arukwe, 2006), in brown trout juveniles after water exposure to EE2 (Körner et al., 2008), and in Japanese medaka after exposure to E2, EE2, estrone (E1) and estriol (E3) (Yost et al., 2014). Therefore, from the ERs, ERα is assumed to be the most related to Vtg induction.

Increases in ZPs expression were also associated with increased ERα levels in male brown trout exposed to E2 (Arukwe and Røe, 2008; Uren Webster et al., 2015). Moreover, high correlations were found between the mRNA levels of ERα and ZPs in male Japanese medaka (Yost et al., 2014) and juvenile Atlantic salmon (Meucci and Arukwe, 2006).

Contrasting with ERα, the ERβ subtypes are commonly down-regulated after exposure to estrogens, as in goldfish and zebrafish exposed to E2 (Marlatt et al., 2008; Menuet et al., 2004) or Japanese medaka after different estrogens (Yost et al., 2014). A decrease of ERβ1 mRNA was also found in juvenile brown trout after EE2 (50 µg/L) exposure (Madureira et al., 2018). Likewise, ERβ2 mRNA levels lowered in rainbow trout after intraperitoneal (IP) injection of 5 µg E2/g body weight and 50 µg/g body weight of genistein (Cleveland and Manor, 2015). This is not, however, a unique pattern since others have found no effect in ERβ isoforms after exposure to estrogenic compounds, in both rainbow and brown trout (Boyce-Derricott et al., 2009; Uren Webster et al., 2015), or even up-regulation (Benninghoff and Williams, 2008). The regulation of the ERβ subtypes in fish is complex and influenced by different factors, including sex, reproductive status, tissue and developmental stage (Marlatt et al., 2014).

In Japanese medaka, exposure to E2 down-regulated ERα mRNA, while ERβ2 levels increased and positively correlated with Vtg1 (Chakraborty et al., 2011). This study, and others, evidence the role of the ERβ subtypes in the dynamic of Vtg production. For instance, in Japanese medaka, the extent of Vtg induction by several estrogens was in line with the binding strength of the same estrogens to the ERβ subtypes (Yost et al., 2014).

Using gene knockdown strategies, in zebrafish (Griffin et al., 2013) and rainbow trout (Leaños-Castañeda and Van Der Kraak, 2007) it was established that the ERβ subtypes were engaged in the estrogenic response, including in Vtg secretion, probably with different timings in the dynamics of the reaction. Accordingly, when the expression of the ERs was studied along a reproductive cycle in rainbow trout (Nagler et al., 2012), ERβ2 was highly expressed in early vitellogenesis and ERα increased in the latter stages of oocyte maturation, while ERβ1 levels correlated positively with the changes in ERα (suggesting a role of this isotype in maintaining basal levels of ERα).

2.2. Regulation of estrogenic targets by androgens

Comparing with the number of studies that explored the effects of estrogens and their potential as endocrine disruptors in fish, a limited number of studies have addressed *in vivo* androgenic effects. Despite this discrepancy, evidence exists on the potential of androgens to disrupt estrogenic signaling. The results of a few studies that studied androgenic influences in distinct estrogenic targets, such as Vtg, ZPs and ERs are summarized in Table 1. Most studies were performed with small fish species, such as the fathead minnow (Ankley et al., 2004; Ankley et al., 2003; Hornung et al., 2004; Panter et al., 2004), Eastern and Western mosquitofish (*Gambusia holbrooki* and *Gambusia affinis*, respectively) (Brockmeier et al., 2013; Huang et al., 2016; Huang et al., 2013), Japanese medaka (Chikae et al., 2004; Örn et al., 2006; Seki et al., 2004) or zebrafish (Andersen et al., 2006; Örn et al., 2003; Örn et al., 2006).

Table 1. Effects of androgens on known estrogenic targets in fish *in vivo* studies.

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Nile tilapia (*Oreochromis niloticus*) Female	MT IP injection 10 days	20 mg/kg	↓ Vtg	Gel electrophoresis Northern and Slot blot Plasma Liver	(Lazier et al., 1996)
Fathead minnow (*Pimephales promelas)* Male/female	MT Water exposure 21 days	0.2 mg/L	↑ Vtg (males, females)	ELISA Plasma	(Ankley et al., 2001)
Fathead minnow (*Pimephales promelas*) N/D (undifferentiated)	MT Water exposure 21 days	10, 50, 100 µg/L	↑ Vtg ER no effect MT+ fadrozole no Vtg induction (14 days exposure)	ELISA qRT-PCR Whole-body homogenate	(Zerulla et al., 2002)
Zebrafish (*Danio rerio*) Male/female	MT Water exposure 20 dph until 60 dph	26, 50, 100, 260, 500, 1000 ng/L	↓ Vtg (males, females)	ELISA Whole-body homogenate	(Örn et al., 2003)
Fathead minnow (*Pimephales promelas*) Male/female	MT Water exposure 7 days	20, 200 µg/L	↑Vtg (males, females)	ELISA Plasma	(Hornung et al., 2004)
Fathead minnow (*Pimephales promelas*) Male/female	MT Water exposure 3 weeks	0.1, 1, 5, 50 µg/L	↑ Vtg 1, 5, 50 µg/L (males) ↑ Vtg 50 µg/L (females)	ELISA Plasma	(Pawlowski et al., 2004)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Japanese medaka (*Oryzias latipes*) Male/female	MT Diet exposure 7 days	0.02, 0.2, 2, 20 mg/g diet	↑ Vtg 0.02, 0.2 mg/g (males) ↓ Vtg 20 mg/g (females)	ELISA Plasma	(Chikae et al., 2004)
Japanese medaka (*Oryzias latipes*) Male/female	MT Water exposure 12 hpf until 101 days	0.35, 1.09, 3.29, 9.98, 27.75 ng/L	F0 ↑ Vtg (males) (non significant) F1 ↑ Vtg 9.98 ng/L (males) ↓ Vtg 0.35, 1.09, 3.29, 9.98 ng/L (females)	ELISA Liver homogenate	(Seki et al., 2004)
Mummichog (*Fundulus heteroclitus*) Male/female	MT Water exposure 7 days	250, 1000 ng/L	↓ Vtg 1000 ng/L (females) Males no effect	ELISA Plasma	(Sharpe et al., 2004)
Mummichog (*Fundulus heteroclitus*) Male/female	MT Water exposure 14 days	1, 10, 100 ng/L	↓ Vtg 100 ng/L (females) Males no effect	ELISA Plasma	(Sharpe et al., 2004)
Zebrafish (*Danio rerio*) Male	MT Water exposure 7 days	4.5, 6.6, 8.5, 19.8, 35.9, 62.3 ng/L	↑ Vtg 4.5 ng/L	ELISA Whole-body homogenate	(Andersen et al., 2006)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Japanese medaka (*Oryzias latipes*) Male/female	MT Water exposure 3 weeks	22.5, 46.8, 88.1, 188, 380 ng/L	↓ Vtg 188, 380 ng/L (females)	ELISA Liver homogenate Males undetectable levels	(Kang et al., 2008)
Mangrove killifish (*Kryptolebias marmoratus*) Hermaphrodite	MT IP injection 30 days	0.1, 1, 5, 10, 50, 100 µg/g body weight	↓ Vtg and ERα at day 7 ↓ Vtg at day 30 ERβ no effect	qRT-PCR Liver Sampling day 7 and 30 post injection	(Park et al., 2013)
Pengze crucian carp (*Carassius auratus* var. pengze) Female (immature)	MT Water exposure 4 weeks	50 µg/L	↑ VtgB weeks 1,3,4 ↓ ERα1 weeks 3,4 ↓ ERα2 weeks 1,4 ↓ ERβ1 weeks 1,2	qRT-PCR Body trunk	(Zheng et al., 2013)
Pengze crucian carp (*Carassius auratus* var. pengze) Female (immature)	MT Water exposure 8 weeks	50, 100 µg/L	↑ VtgB	qRT-PCR Liver Sampling week 2,4, and 8 ↓ ovarian aromatase weeks 4,8	(Zheng et al., 2016)
Brook stickleback (*Culaea inconstans*) Male	MT Water exposure 7 days	100 ng/L	No effect on Vtg	qRT-PCR Liver	(Muldoon et al., 2016)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Western mosquitofish (*Gambusia affinis*) Female	T Water exposure 8 days	1 µg/L	↓ VtgA, VtgB, VtgC ↓ ERα, ERβ	qRT-PCR Liver	(Huang et al., 2012)
Western mosquitofish (*Gambusia affinis*) Male	T Water exposure 8 days	1 µg/L	↑ VtgA, VtgB, VtgC (non-significant) ERα no effect ERβ no effect	qRT-PCR Liver	(Huang et al., 2013)
Brown trout *(Salmo trutta* f. *fario*) N/D (immature)	T Water exposure 28 days	50 µg/L	↑ VtgA ERα no effect ERβ1 no effect	qRT-PCR Liver	(Madureira et al., 2018)
Goldfish (*Carassius auratus* L.) N/D (immature)	MT, T, MDHT Dietary exposure 2 weeks	1 mg/g diet	↑ Vtg	Polyacrilamide gel Serum	(Hori et al., 1979)
Mummichog (*Fundulus heteroclitus*) Male/female	MT, DHT Water exposure 14 days	DHT – 10, 100 µg/L MT – 0.1, 1 µg/L	↑ Vtg1 100 µg/L DHT (males) ↓ Vtg1 100 µg/L DHT (females) MT no effect	qRT-PCR Liver MT induced ovarian aromatase	(Rutherford et al., 2015)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Rainbow trout (*Oncorhynchus mykiss*) Female	T, DHT IP injection 72h	5.0 µg/g body weight	T ↑Vtg 24 and 72h DHT no effect on Vtg ERα1 ↓DHT 72h ↑T 24h ERα2 ↓DHT 24 and 72h ERβ no effect (T and DHT)	qRT-PCR Liver Sampling 24 and 72h	(Cleveland and Weber, 2015)
Rainbow trout (*Oncorhynchus mykiss*) Female	T, DHT Dietary exposure 1 month	30 mg/kg diet	No effect on Vtg No effect on ERs	qRT-PCR Liver	(Cleveland and Weber, 2016)
Black goby (*Gobius niger* L.) Male/female	DHT IP injection Every two days for 2 weeks	2.4 mg/fish	↑ Vtg	Immunoprecipitation Plasma Tamoxifen inhibited Vtg induction	(Le Menn et al., 1980)
Fathead minnow (*Pimephales promelas*) Males/Female	DHT Water exposure 21 days	10 (6.0), 32 (6.1) 100 (8.6) µg/L	↑ Vtg 6.1 and 8.6 µg/L (males) ↓ 6.0 (females)	ELISA Plasma	(Panter et al., 2004)
Rainbow trout (*Oncorhynchus mykiss*) N/D (immature)	DHT Dietary exposure 14 days	5 ppm/wet weight	↑ Vtg ↑ ZRP No effect on ERs	Microarray qRT-PCR Liver	(Benninghoff and Williams, 2008)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Murray rainbowfish (*Melanotaenia fluviatilis*) Male/female	DHT Water exposure 35 days	25 µg/L	↑ Vtg (males, females) F ↑ Vtg (males, females) DHT + flutamide ↑ Vtg (males, females)	ELISA Tail	(Bhatia and Kumar, 2016)
Fathead minnow (*Pimephales promelas*) Male/female	MDHT Water exposure Until 63 and 114 dph	0.1, 0.32, 1 µg/L	↓ Vtg 0.1 µg/L 63 and 114 dph (pooled fish) ↑ Vtg 1.0 µg/L 114 dph (males) Females no effect	ELISA Plasma Exposure when embryos (blastula stage)	(Bogers et al., 2006)
Zebrafish (*Danio rerio*) Female	MDHT Water exposure 168 h	0.1, 0.7, 4.9 µg/L	↓ Vtg protein 0.1µg/L (24h) ↑ Vtg protein 0.7 and 4.9 µg/L (24h) No effect at 168h ↓ Vtg mRNA dose dependent at 24h	ELISA qRT-PCR Plasma Sampling 24 and 168 h	(Hoffmann et al., 2008)
Largemouth bass (*Micropterus salmoides*) Male	DHT, 11-KT IP injection N/A	DHT 62.5 µg/g 11-KT 2 µg/g	↑ Vtg DHT/11-KT	cDNA array Liver	(Blum et al., 2004)
Fathead minnow (*Pimephales promelas*) Male/female	17β-TB Water exposure 21 days	0.0015 – 41 µg/L	↓ Vtg from 0.026 µg/L (females) ↑ Vtg 41 µg/L (males)	ELISA Plasma	(Ankley et al., 2003)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Fathead minnow (*Pimephales promelas*) Female	17β-TB Water exposure 14 days	0.5 µg/L	↓ Vtg (females)	qRT-PCR Liver	(Ankley et al., 2004)
Fathead minnow (*Pimephales promelas*) Female	17β-TB Water exposure 24 h	50, 500 ng/L	↓ Vtg1, Vtg3	qRT-PCR Liver	(Miracle et al., 2006)
Zebrafish (*Danio rerio*) Japanese medaka (*Oryzias latipes)* Male/female	17β-TB Water exposure 20 dph until 60 dph	10, 50 ng/L	↓ Vtg 50 ng/L	ELISA Whole-body homogenate Vtg measured 38 dph	(Örn et al., 2006)
Fathead minnow (*Pimephales promelas*) Male/female	17α-TB Water exposure 21 days	0.003, 0.01, 0.03, 0.1 µg/L	↓ Vtg 0.03 and 0.1 µg/L females No effect on males	ELISA Plasma	(Jensen et al., 2006)
Sheepshead minnow (*Cyprinodon variegatus*) Male/female	17β-TB Water exposure 21 days	0.005, 0.05, 5.0 µg/L	↓ Vtg in females (non-significant)	ELISA Plasma Males undetectable levels	(Hemmer et al., 2008)

Table 1. (Cont.)

Species **Sex (maturation status)**	**Compound** **Administration** **Exposure time**	**Concentration**	**Main effects**	**Detection method** **Sample type** **Observations**	**Reference**
Fathead minnow (*Pimephales promelas*) Female	17β-TB Water exposure 4 days	0.1, 1.0 µg/L	↓ Vtg1	qRT-PCR Liver ↓aromatase expression (brain)	(Dorts et al., 2009)
Sheepshead minnow (*Cyprinodon variegatus*) Male/female	17β-TB Water exposure 42 weeks	0.007, 0.027, 0.13, 0.87, 4.1 µg/L	↓ Vtg 0.87 and 4.1 µg/L (females)	ELISA Plasma Males undetectable levels	(Cripe et al., 2010)
Eastern mosquitofish (*Gambusia holbrooki*) Female	17β-TB Water exposure 21 days	0.1, 1, 10 µg/L	↓ Vtg 10 µg/L all time points	qRT-PCR Liver Time points: 3, 7, 14, 21 days	(Brockmeier et al., 2013)
Eastern mosquitofish (*Gambusia holbrooki*) Female	17β-TB Water exposure 14 days	1 µg/L	↓ Vtg ↓ ZP2 ↓ ZP3a.1	Gene Microarray	(Brockmeier et al., 2013)

11-ketotestosterone, 11-KT; dihydrotestosterone, DHT; flutamide, F; methyldihydrotestosterone, MDHT; methyltestosterone, MT; testosterone, T; trenbolone, TB.

Enzyme-linked immunosorbent assay, ELISA; real-time quantitative polymerase chain reaction, qRT-PCR.

Vitellogenin, Vtg; estrogen receptor alpha, ERα; estrogen receptor beta, ERβ; zona pellucida protein, ZP; zona radiata protein, ZRP.

Complementary deoxyribonucleic acid, cDNA; decrease, ↓; days post-hatch, dph; hours post-fertilization, hpf; increase, ↑; information not available, N/A; intraperitoneal, IP; not determined, N/D; parts per million, ppm.

Vtg was the most often monitored biomarker, either measured as protein, by ELISA, in plasma, whole-body (Andersen et al., 2006; Örn et al., 2006; Zerulla et al., 2002) and liver homogenates (Seki et al., 2004) or as mRNA levels by using real-time quantitative polymerase chain reaction (qRT-PCR) or other molecular techniques (Benninghoff and Williams, 2008; Blum et al., 2004).

Trenbolone (TB) is considered a pure, non-aromatizable androgen and also a potent AR agonist (Ankley et al., 2018). TB effects on Vtg levels have been evaluated in several species, such as the fathead minnow (Ankley et al., 2003; Brockmeier et al., 2013; Miracle et al., 2006), sheepshead minnow (*Cyprinodon variegatus*) (Cripe et al., 2010) or zebrafish (Örn et al., 2006). Overall, 17β-TB always decreased Vtg in females, in concentrations ranging from 0.026 µg/L to 41 µg/L (Ankley et al., 2003). 17α-TB also lowered the plasma Vtg levels in female fathead minnow, after 0.03 and 0.1 µg/L. (Jensen et al., 2006).

Exposure to the non-aromatizable androgens DHT and methyldihydrotestosterone (MDHT) produced less homogeneous results on Vtg levels than TB. For instance, diet administration (5 ppm/wet weight) of DHT to juvenile rainbow trout (Benninghoff and Williams, 2008) or IP injection (62.5 µg/g body weight) in largemouth bass (*Micropterus salmoides*) males (Blum et al., 2004) up-regulated Vtg mRNA levels. DHT also increased Vtg protein levels in black goby (*Gobius niger* L.) males and females (IP 2.4 mg/fish) (Le Menn et al., 1980) and juvenile Murray rainbowfish (*Melanotaenia fluviatilis*) (water exposure, 25 µg/L) (Bhatia and Kumar, 2016). Others, however, found dissimilar effects between sexes, with an increase in Vtg levels in males and a decrease in females (Panter et al., 2004; Rutherford et al., 2015). Vtg induction also varied according to concentration and time of exposure in zebrafish, with decreased Vtg plasma levels after 0.1 µg/L of MDHT and increased levels at higher concentrations at 24 h exposure, but no effects after 168 h (Hoffmann et al., 2008).

Effects of the aromatizable androgens methyltestosterone (MT) and T on Vtg expression also showed a non-uniform pattern. These compounds increased Vtg protein (Ankley et al., 2001; Hornung et al., 2004; Pawlowski et al., 2004; Zerulla et al., 2002) or mRNA levels (Madureira et al., 2018), but were also shown to decrease both (Lazier et al., 1996; Örn et al., 2003). An increase of Vtg in males and a decrease in females were described in Japanese medaka and Western mosquitofish (Chikae et al., 2004; Huang et al., 2013; Huang et al., 2012; Seki et al., 2004), revealing sex-specific effects. Some studies found a decrease in Vtg protein levels only in females (Kang et al., 2008; Sharpe et al., 2004), but Vtg mRNA induction in females was noted in other studies (Zheng et al., 2016; Zheng et al., 2013).

Other estrogenic biomarkers, namely ZPs and ERs have been much less studied under androgenic inputs. However, up-regulation of mRNA levels of ZPs was observed in rainbow trout exposed to DHT (Benninghoff and Williams, 2008), while a down-regulation was found after exposure to 17β-TB in Western mosquitofish (Brockmeier et al., 2013). As it happened with Vtg and ZPs, the expression of liver ERs presented different patterns under androgenic stimulation, but no-change or down-regulation profiles were the most common. For instance, no changes in the mRNA levels of ERs were found in brown trout (Madureira et al., 2018), rainbow trout (Benninghoff and Williams, 2008) or fathead minnow (Zerulla et al., 2002) juveniles exposed to T, DHT and MT, respectively. In Western mosquitofish, T did not affect ERα and ERβ mRNA levels in males (Huang et al., 2013), but a significant down-regulation of both was obtained in females (Huang et al., 2012). Down-regulations of ERα (Park et al., 2013; Zheng et al., 2013) and ERβ1 (Zheng et al., 2013) by MT were reported in the mangrove killifish (*Kryptolebias marmoratus*) and in juvenile Pengze crucian carp (*Carassius auratus* var. pengze).

3. *In vitro* models for endocrine disruption studies in fish

3.1. Monolayer cultures of primary hepatocytes

Within the ecotoxicological context, the evaluation of single or mixture effects of EDCs requires *in vitro* and *in vivo* assay designs (Dang, 2014; OECD, 2014; Scholz et al., 2013). *In vitro* approaches appear as an alternative to *in vivo* testing, implementing the principles of the 3Rs (replacement, refinement, reduction), but are also rapid and cost-effective methods for screening purposes, compared with *in vivo* studies (Rehberger et al., 2018; Tollefsen et al., 2003). *In vitro*, the role of selected parameters can be studied individually, under a controlled environment (Iguchi et al., 2006; Segner, 1998b), and confounding factors from other organs and systems are absent, which makes these models ideal for the investigation of specific mechanisms of action (Dang, 2014; Mommsen and Lazier, 1986). Because the vertebrate liver is the prime metabolization and target site for xenobiotics and hormones, *in vitro* methods based on liver cells or sub-fractions have been long used and emerged as valuable tools for ecotoxicological studies (Iguchi et al., 2006; Scholz et al., 2013; Segner and Cravedi, 2001). Amongst the *in vitro* techniques, primary hepatocytes present advantages as to other cell-based methods e.g., receptor binding, transactivation, or reporter gene assays. An obvious benefit is the possibility to determine the effects of intermediate metabolites of xenobiotics and the effects of compounds that require metabolization to be active (e.g., pro-estrogens or pro-carcinogens) (Navas and Segner, 2006). Despite the advantages of using primary cell cultures, there are also known

limitations, which mainly relate to the fact that the hepatocytes are studied isolated, without their normal cellular interactions (Mommsen et al., 1994).

The first double perfusion method to isolate parenchymal liver cells was described in Sprague Dawley rat (*Rattus norvegicus*) (Berry and Friend, 1969). The method consisted of continuous perfusion of the liver *in situ*, via the portal vein, first with a calcium-free buffered salt solution, to clear all blood from the liver, and then with a buffered solution containing the enzymes collagenase and hyaluronidase. The method was afterwards improved and modified to other species, including either freshwater and marine fish (Klaunig et al., 1985; Mommsen et al., 1994; Tollefsen et al., 2003). In fish, the liver can be perfused either *in situ* or after removal from the abdominal cavity, most frequently with a Hank's type salt buffer, and the extracellular matrix digested with collagenase from *Clostridium histolyticum* (Braunbeck and Segner, 2000; Mommsen et al., 1994) The first buffer is calcium-free to help the subsequent process of cell disaggregation; but calcium is a cofactor for collagenase and must be added to the collagenase-containing buffer (Baksi and Frazier, 1990; Mommsen et al., 1994). The perfusion buffer should be adapted according to the plasma composition of the donor fish, adjusting the osmolality with sodium chloride for marine fish or urea for elasmobranchs (Pesonen and Andersson, 1997). Also, the pH of the perfusion buffer can vary according to the fish species to be used, with a reported range between 7.3 and 8.0, even though for most fish the optimal pH is around 7.4 (Baksi and Frazier, 1990). A cell suspension is obtained after mechanical dissociation, which is then filtered through decreasing size mesh nylon filters (from 250 to 50 μM). Non-parenchymal and damaged cells are removed by low speed (50-60 × g) centrifugation (Flouriot et al., 1995; Mommsen et al., 1994), and then the hepatocyte pellet is washed (in buffer or culture medium) and finally suspended in culture media. The most employed media for hepatocyte culture are similar to the ones used for mammalian cultures, including Leibowitz L15, Eagle's minimal essential medium (MEM), Dulbecco's modified Eagle's Medium (DMEM) and medium 199 (Braunbeck and Segner, 2000; Flouriot et al., 1993; Pesonen and Andersson, 1997).

Although hepatocytes can be used as a cell suspension, most studies with primary hepatocytes employ the so-called monolayer cultures. In the latter technique, hepatocytes adhere to a physical support, usually glass or plastic, forming two-dimensional (2D) structures. In some fish species, including rainbow trout (Klaunig et al., 1985), hepatocytes have been shown to attach poorly to culture vessels, and thus several matrices were used to facilitate cell adhesion, including laminin, fibronectin or poly-L-lysine (Madureira et al., 2015b; Mommsen et al., 1994). Cells must be maintained at the temperatures of the donor

fish habitat or at the acclimation temperature prior to the isolation procedures (Pesonen and Andersson, 1997).

Baksi and Frasier (1990) and Segner (1998) reviewed the first studies with fish hepatocytes. The earlier publications are from the late seventies and focused on distinct aspects of normal physiological and biochemical processes of the hepatocyte function, namely energy, lipid and glucose metabolism, protein synthesis, and endocrinology. Fewer studies also addressed the toxicological and genotoxic effects of chemicals and xenobiotic metabolism (Baksi and Frazier, 1990; Segner, 1998b).

In optimized monolayer culture conditions, hepatocytes were shown to re-establish cell-cell connections and recover from membrane damaging derived from the isolation procedures (Maitre et al., 1986; Segner, 1998b). Cultured primary hepatocytes have been shown to maintain their viability, up to eight days (Braunbeck and Storch, 1992; Mommsen and Lazier, 1986), functional differentiation and metabolization properties (Segner, 1998b; Segner and Cravedi, 2001). They also retain the capacity to respond to hormonal stimuli and therefore can be routinely used for toxicity assessment and mechanistic studies with hormonally-active compounds (Madureira et al., 2015b; Maitre et al., 1986; Petersen et al., 2017; Trombley et al., 2015).

Primary hepatocytes are a very useful tool to study interferences with estrogen-regulated pathways (Navas and Segner, 2006). Much work has been devoted to the optimization/validation of *in vitro* assays to measure Vtg (Hultman et al., 2015a; Kim and Takemura, 2002; Kim and Takemura, 2003; Maitre et al., 1986; Mommsen and Lazier, 1986), the master biomarker of estrogenic exposure. In cultured hepatocytes, Vtg protein can be measured in culture media or hepatocyte homogenates but can also be monitored by quantification of mRNA levels by qRT-PCR methods. Therefore, measuring Vtg levels in primary hepatocytes has been used in the last decades for: 1) screening the potential effect of EDCs, namely for their estrogenic or anti-estrogenic properties (Pelissero et al., 1993; Segner et al., 2003; Smeets et al., 1999); 2) assessment of estrogenic potency of chemicals (Rani et al., 2010; Smeets et al., 1999); 3) evaluation of the estrogenic disruption potential of complex environmental mixtures, such as wastewater effluents (Li et al., 2005; Pawlowski et al., 2003). The *in vitro* model has also shown to be a valuable tool for mechanistic studies on the pathways underneath disruptions caused by EDCs (Madureira et al., 2015b; Madureira et al., 2017b; Maradonna et al., 2013; Pesonen and Andersson, 1997; Petersen and Tollefsen, 2011).

Primary hepatocytes were shown to replicate responses obtained *in vivo*, after hormonal (estrogenic) exposure (Hultman et al., 2015b). The number of substances that need nowadays to be screened for their toxic and endocrine disruption properties, namely for

regulatory purposes, calls for high throughput techniques. Using salmonid fish as a source for primary hepatocytes may reduce the number of animals necessary for the experiments, following the 3Rs principle, since a single fish can yield enough hepatocytes to several experimental conditions. This is not so feasible with small-sized fish like Japanese medaka or zebrafish, where pooling hepatocytes from several fish is often necessary (Forsgren et al., 2014; Kordes et al., 2002; Maradonna et al., 2013). The use of cryopreserved primary hepatocytes from rainbow trout is now included in the Organization for Economic Co-operation and Development (OECD) official guidelines for *in vitro* biotransformation assays (Rehberger et al., 2018).

Recent guidelines on ecotoxicology and risk assessment are based on integrated approaches. Therefore, data on the adverse effects of a certain chemical or mixture are obtained with different models, increasing the evidence on its toxicological effects. In addition to monolayer cultures of primary hepatocytes, other *in vitro* models have been implemented on ecotoxicological research. These include permanent cell lines, liver fragments and slices and three-dimensional (3D) cultures.

3.2. Fish cell lines

When primary cells are maintained by passaging, or sub-division to new culture vessels, they become cell lines which can in some situations be maintained for prolonged times in culture, or even infinitely (Bols et al., 2005; Segner, 1998a). Thus, a great advantage of cell lines, in comparison with primary cultures, is the almost unlimited supply of cells that allow high throughput testing. Fish cell lines represent a versatile tool in ecotoxicology for being easy to maintain, standardizable, with reproducible results and less variable than primary cultures (Castaño et al., 2003). The cell line RTG-2, derived from rainbow trout gonad was the earliest permanent fish cell line to be established (Wolf and Quimby, 1962). A large variety of fish cell lines were thereafter developed, mostly derived from normal tissues, which differs from mammalian cell lines that are mostly derived from tumoral tissues. Most fish cell lines are grown at temperatures below 37 °C, reflecting the normal range of temperatures from the habitats of the donor fishes (Fent, 2001; Nicholson, 1989). The amino acid-rich medium Leibovitz L15, which does not require CO_2 buffering is the most employed for fish cell culture and fetal bovine serum (FBS) the most used medium supplement (Nicholson, 1989).

Most fish cell lines were developed for the study of fish viruses, but their use was widespread to applications such as cytotoxicity, carcinogenesis, genotoxicity, and

metabolism/toxic mechanisms of xenobiotics (Nicholson, 1989; Rehberger et al., 2018; Segner, 1998a). The first study with a fish cell line goes back to 1968, where the effects of zinc were studied in a fathead minnow epithelial cell line (Rachlin and Perlmutter, 1968). A wealthy of fish cell lines have been developed, but few are commercially available and effectively used in ecotoxicology research (Lakra et al., 2011; Rehberger et al., 2018). Examples of the most used, are RTG-2 (gonad-derived), RTL-W1 (from liver) and RTgill-W1 (from gills), all from rainbow trout, and the PLHC1 hepatoma cell line from the clearfin livebearer (*Poeciliopsis lucida*) (Fent, 2001; Rehberger et al., 2018). Fish cell lines have been mostly used in ecotoxicological studies for three fundamental applications: 1) investigation on the mechanisms of action of toxicants; 2) rating the relative toxicity of chemicals; and 3) assessing the toxicological potential of environmental samples (Dayeh et al., 2013; Fent, 2001). Cell lines have thus proven to be a reliable tool for the relative ranking of the toxic potential of individual compounds and effluents (Rehberger et al., 2018; Tanneberger et al., 2013).

A limitation of cell lines, compared to primary cells, is that they may be less differentiated and have lower metabolic capacity, making them less suitable for physiological and mechanistic studies (Castaño et al., 2003; Fent, 2001). For example, in the ZFL zebrafish liver cell line, cells expressed lower mRNA levels of almost all genes and showed lower transcriptional induction of estrogenic targets (e.g., ERs), comparing with liver samples (Eide et al., 2014b). Brown trout (*Salmo trutta lacustris*) primary hepatocytes were also more sensitive to Vtg synthesis upon E2 stimulation than the RTH-149 and ZFL liver cell lines (Christianson-Heiska and Isomaa, 2008).

3.3. Three-dimensional (3D) culture – multicellular aggregates or spheroids

When adherent cells are cultured in low adhesion plates, under a gyratory shaker or in specific scaffolds, cells tend to aggregate and reorganize into 3D, multicellular, tissue-like structures. These 3D arrangements have been called multicellular aggregates or spheroids and can be generated using the same cell types cultured in 2D, including primary cells or continuous cell lines (Achili et al., 2012; Lauschke et al., 2016). Comparing with 2D culture, the prime advantage of the 3D culture is the greatest proximity to the *in vivo* microenvironment, including cell-cell and cell-matrix interactions and to the *in vivo* cellular responses (Achilli et al., 2012; Edmondson et al., 2014). In mammals, 3D cultures have been used in different applications, such as fundamental biology (of intercellular and cell-matrix interactions), cancer research, drug screening, tissue engineering and toxicological studies (Achilli et al., 2012; Bell et al., 2018; Lauschke et al., 2016; Ryu et al., 2019).

In fish, very few studies used 3D culture systems as models. The first were published in the 90's (Cravedi et al., 1996; Flouriot et al., 1993), both consisting of spheroids from rainbow trout primary hepatocytes. A several years gap existed until further works were published on the optimization and use of fish hepatocyte spheroids (Baron et al., 2012; Uchea et al., 2015; Uchea et al., 2013). Spheroids from fish hepatocyte normal (RTL-W1) (Lammel et al., 2019) and tumoral (PLHC-1) cell lines were also optimized (Rodd et al., 2017).

Rainbow trout hepatocyte spheroids have been generated and cultured with agitation (50-70 rpm) in Leibovitz L15 or DMEM nutrient mixture F12 (DMEM-F12) culture medium, at temperatures between 15 °C and 18 °C (Baron et al., 2017; Cravedi et al., 1996; Hultman et al., 2019; Uchea et al., 2013). Under these conditions, they have been shown to hold in culture for longer periods, compared to monolayer cultures (for up to one month) (Baron et al., 2012; Pesonen and Andersson, 1997; Uchea et al., 2015), to preserve their metabolic properties (Cravedi et al., 1996; Flouriot et al., 1995; Uchea et al., 2013) and to not des-differentiate (Flouriot et al., 1993; Uchea et al., 2015), making them a promising model for prolonged exposures (Hultman et al., 2019).

3D fish hepatocyte models have been used to study toxicological effects and the metabolism of xenobiotics (Baron et al., 2017; Baron et al., 2012; Hultman et al., 2019; Uchea et al., 2015). Hepatocyte rainbow trout aggregates were shown to retain functional ERs and Vtg secretion after estrogenic stimulation, equivalent to *in vivo*, for up to 1 month and to metabolize T (Cravedi et al., 1996; Flouriot et al., 1993), suggesting that this model can be a useful tool for endocrine disruption studies. A 3D spheroid model of brown trout hepatocytes was also recently implemented in our laboratory and is responsive to androgenic stimuli (Pereira, 2019).

3.4. Liver fragments and slices

The culture of either liver fragments or slices is considered the interface between *in vitro* methods and *in vivo* studies (Shilling and Williams, 2000). It represents the compromise of being an *in vitro* technique where the architecture, the different cellular components, as well as intercellular communications, are maintained similar to the original tissue (Navas and Segner, 2006; Shilling and Williams, 2000). Manually cut liver fragments were first employed, with obvious limitations due to their excessive thickness and lack of uniformity (Ekins, 1996). These problems were overpassed after the development of an apparatus to cut thin and homogeneous slices (Krumdieck et al., 1980). These precision-cut liver slices (PCLS), ranging from 100 to 300 μm in thickness, are now used in most such type studies.

Tissue slices were shown to maintain viability in culture for up to 9 days (Eide et al., 2014a; Harvey et al., 2019; Shilling and Williams, 2000). PCLS have been used to study xenobiotics metabolism (Serrano et al., 2020; Tapper et al., 2018), lipid metabolism (Harvey et al., 2019) and for mechanistic studies (Serrano et al., 2020; Yadetie et al., 2018). Liver fragments and slices have also been used for the screening of estrogenicity and anti-estrogenic potential of chemicals, by measuring Vtg, in fish such as rainbow trout (Schmieder et al., 2000; Shilling and Williams, 2000), Atlantic cod (*Gadus Morhua*) (Eide et al., 2014a), Mozambique tilapia (*Oreochromis mossambicus*) (Swart and Pool, 2009), three-spined stickleback (Björkblom et al., 2007) and roach (*Rutilus rutilus*) (Gerbron et al., 2010).

4. Regulation of biomarkers of estrogenic exposure by estrogens and androgens in fish primary hepatocytes

4.1. Effects of estrogenic exposure on Vtg and other estrogenic targets

Induction of Vtg (mRNA or protein) by endogenous and exogenous estrogens in fish primary hepatocytes has been extensively reviewed (Hultman et al., 2015a; Iguchi et al., 2006; Navas and Segner, 2006; Scholz et al., 2013). Methods to measure Vtg and thereafter screen for the estrogenic and anti-estrogenic potential of chemicals have been optimized in a variety of model species including the European eel (*Anguilla anguilla* L.) (Peyon et al., 1998), Japanese medaka (Kordes et al., 2002; Scholz et al., 2004), common carp (*Cyprinus carpio*) (Bickley et al., 2009; Smeets et al., 1999), common bream (*Abramis brama*) (Rankouhi et al., 2004), zebrafish (Wang et al., 2017), channel catfish (*Ictalurus punctatus*) (Monteverdi and Di Giulio, 1999), Mozambique tilapia and Nile tilapia (*Oreochromis niloticus*) (Kim and Takemura, 2003; Liu et al., 2007; Riley et al., 2004) and goldfish (Li et al., 2005; Nelson and Habibi, 2010).

As to salmonids, the dynamics of Vtg induction in response to estrogens has been mostly studied in rainbow trout (Hultman et al., 2015a; Islinger et al., 1999; Pawlowski et al., 2000; Petersen and Tollefsen, 2011). However, Atlantic salmon (Celius et al., 1999; Tollefsen et al., 2003; Trombley et al., 2015) and brown trout (forma *lacustris* and forma *fario*) (Christianson-Heiska and Isomaa, 2008; Madureira et al., 2015b) have also been used. In primary hepatocytes from salmonids, the lowest reported observed effect concentrations (LOEC) of E2 and EE2 to cause Vtg induction were in the order of 10^{-12} M to 10^{-10} M (Hultman et al., 2015a; Scholz et al., 2013).

In what concerns ZPs, fewer *in vitro* studies have addressed the expression of ZPs after exposure to estrogens. At the protein level, ZPs were induced in primary hepatocytes from

Atlantic salmon juveniles after treatment with 0.1, 1 and 10 nM of E2 (Celius and Walther, 1998) and with the xenoestrogens NP and bisphenol A (BPA) (1, 5 and 10 μM) (Celius et al., 1999). ZPs were also shown to be quite susceptible to estrogenic exposure in these studies and to be detected in the culture media before Vtg, suggesting that changes in ZPs could be more precocious biomarkers of estrogenic exposure than Vtg. Protein levels of ZPs were also increased in adult male spotted snakehead (*Channa punctate*) after exposure to E1, E2 and E3 (0.05 to 10 μM), but with different potencies (Rani et al., 2010). At last, ZPs mRNA may also be induced by estrogens. For example, the two ZPs (ZRP3 and ZRP4) were increased after EE2 (0.03, 0.3, 3 and 30 nM) exposure in juvenile rainbow trout hepatocytes (Hultman et al., 2015b).

As to ERs expression, ERα mRNA was induced by E2 (100 nM) in goldfish (Nelson and Habibi, 2010). Similar results were obtained after exposure to nanomolar (Hultman et al., 2015b) and micromolar (Madureira et al., 2015b) concentrations of EE2 in juvenile rainbow trout and brown trout, respectively. In hepatocytes isolated from male juvenile Atlantic salmon exposed to the phytoestrogen genistein and to BPA (0.1 to 100 μM), ERα gene expression was also increased by genistein (1, 10, 100 μM) and BPA (10, 100 μM) (Olsvik et al., 2017). A substantial increase in ER protein in nuclear extracts of primary hepatocytes was observed in Atlantic salmon, after E2 exposure (Mommsen and Lazier, 1986). The behaviour of the ERβ genes after estrogenic stimulus in primary hepatocytes has been less studied. For example, ERβ1 was down-regulated by 1, 10 and 50 μM EE2 in juvenile brown trout (Madureira et al., 2015b), and ERβ2 was also down-regulated by 100 μM of genistein in male juvenile Atlantic salmon (Olsvik et al., 2017). Different modulation profiles were reported in primary zebrafish hepatocytes obtained from female and male individuals (Eide et al., 2014b; Maradonna et al., 2013). Specifically, the exposure to 10 nM of EE2 did not change ERβ1 but increased and decreased ERβ2 gene expression in females and males, respectively (Maradonna et al., 2013). In another study, 100 nM of EE2 increased and decreased ERβ1 in male and female hepatocytes, respectively, and down-regulated ERβ2 only in females (Eide et al., 2014b). The latter results reinforce evidence of sex-specific regulation of ERs by estrogens in fish models.

It is known that Vtg induction is governed by the ERs, and that there is a role for the α and β subtypes in the process, but little is still known about the dynamics and functional involvement of each specific isoform. This was studied *in vitro* in mixed-sex goldfish hepatocytes, by specific knockdown of the ER subtypes (Nelson and Habibi, 2010). Silencing of ERα reduced Vtg induction by E2 in both sexes, while inhibition of the ERβ isoforms completely blocked Vtg induction in males, but only partially in females. Thus, it appears that the ERβ isoforms have a more preponderant role in the process of Vtg

induction than ERα, especially in males. Moreover, the knockdown of ERβ1 and ERβ2 decreased the expression of ERα (although this reduction was not significant in the case of ERβ2), sustaining a role for the ERβ isoforms, especially ERβ1, in maintaining ERα levels. The roles of the different isotypes in the process of Vtg induction were hypothesized to be related to the different basal expression levels of ERα between males and females, and to the changes along the reproductive cycle in females (Nelson and Habibi, 2010, 2013). Nelson and Habibi (2010) proposed a model were the ERβ subtypes contribute directly for E2 induction of Vtg and ERα (Fig. 1).

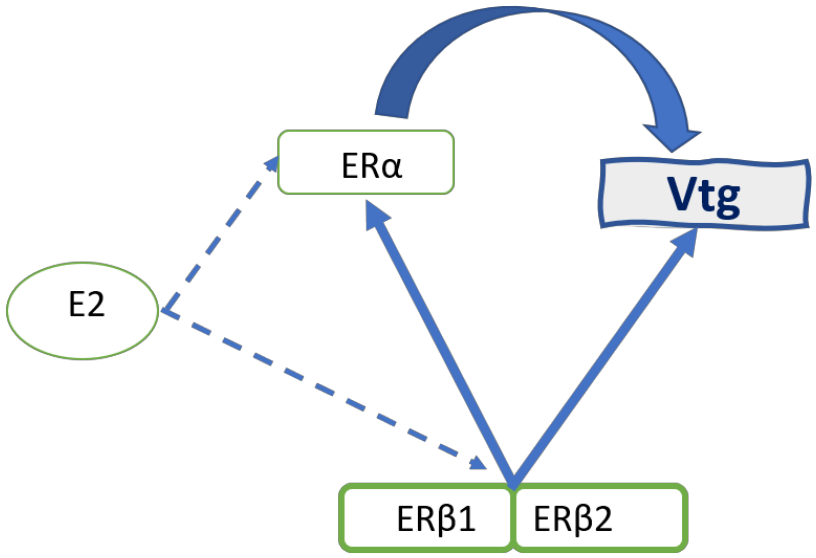

Fig. 1. Proposed model for estradiol (E2) mediated vitellogenesis. E2, acting through both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), induces vitellogenin (Vtg). E2, through the ERβ subtypes also increases ERα expression. With increased ERα expression, the hepatocyte is sensitized to further stimulation by E2 and the role of ERα becomes more preponderant in the E2-induction of vitellogenesis. Adapted, with permission of Oxford University Press, from Nelson and Habibi, 2010.

4.2. Effects of androgenic exposure on Vtg and other estrogenic targets

The effects of androgens on estrogenic targets in fish hepatocytes are limited to Vtg, as detailed in Table 2.

Table 2. Effects of androgens on Vtg in fish primary hepatocytes.

Species **Sex (maturation status)** **Weight**	**Model**	**Androgen** **Exposure time**	**Concentration (M)**	**Vitellogenin detection**	**Main effects on Vtg**	**Reference**
Rainbow trout (*Onchorhynchus mykiss*) Male (N/A) 150-250 g	Hepatocytes 2D	T Androstenedione 48 h	$1x10^{-6}$	RIA Dot Blot	No effect	(Vaillant et al., 1988)
Rainbow trout (*Onchorhynchus mykiss*) Female (immature) ~ 500 g	Hepatocytes 3D 4-6 day-old aggregates	T 11-KT 6 days	$1x10^{-6}$ – $1x10^{-4}$	ELISA	↑ $1x10^{-5}$ M Tamoxifen (10^{-6} M) inhibited Vtg stimulation by T and 11-KT(10^{-5} M)	(Pelissero et al., 1993)
European eel (*Anguilla anguilla* L.) Female (N/A) N/A	Hepatocytes 2D	T 10 days	$1x10^{-9}$ – $1x10^{-5}$	ELISA	↑ after $1x10^{-6}$ and $1x10^{-5}$ M Tamoxifen ($1x10^{-5}$ M) inhibited Vtg induction by T ($1x10^{-5}$ M) E2 + T induced Vtg more than E2 alone High doses of T inhibited Vtg induction by high doses of E2	(Peyon et al., 1997)

Table 2. (Cont.)

Species **Sex (maturation status)** **Weight**	**Model**	**Androgen** **Exposure time**	**Concentration (M)**	**Vitellogenin detection**	**Main effects on Vtg**	**Reference**
Rainbow trout (*Onchorhynchus mykiss*) Male (immature) 150-250 g	Hepatocytes 2D	T MT Androsterone 24 h	$2x10^{-9}$ – $2x10^{-5}$	Dot Blot Northern Blot	↑ Tamoxifen ($1x10^{-5}$ M) ↓ 50 % of E2 ($2x10^{-5}$ M) induced Vtg Tamoxifen ($1x10^{-5}$ M) ↓ 100 % of androgen ($2x10^{-5}$ M) induced Vtg	(Mori et al., 1998)
Rainbow trout (*Onchorhynchus mykiss*) N/A	Hepatocytes 3D 5-day-old aggregates	T 48 h	$1x10^{-6}$ $1x10^{-5}$	Dot Blot	No effect	(Le Guevel and Pakdel, 2001)
Siberian sturgeon (*Acipenser baeri*) Male/female (immature) ~ 600 g	Hepatocytes 3D 4-day-old aggregates	T 14 days	$1x10^{-6}$ – $5x10^{-5}$	ELISA	↑ after $5x10^{-6}$ M	(Latonnelle et al., 2002)
Atlantic salmon (*Salmo salar*) Male ~ 500 g	Hepatocytes 2D	T 4 days	$1x10^{-12}$ – $1x10^{-7}$	ELISA	No effect	(Tollefsen et al., 2003)

Table 2. (Cont.)

Species **Sex (maturation status)** **Weight**	**Model**	**Androgen** **Exposure time**	**Concentration (M)**	**Vitellogenin detection**	**Main effects on Vtg**	**Reference**
Mozambique tilapia (*Oreochromis mossambicus*) Male/female (mature) 100-500g	Hepatocytes 2D	DHT T MT 2-4 days	$1x10^{-7}$ – $1x10^{-5}$	ELISA	↑ after $5x10^{-6}$ M (females) DHT more induction than T or MT ↑ after E2 priming (males) Potentiation of E2 effect Tamoxifen ↓ Vtg induction by DHT	(Kim et al., 2003)
Mozambique tilapia (*Oreochromis mossambicus*) Male/female (mature) 150–200g	Hepatocytes 2D	DHT 48 h	$1x10^{-7}$ – $1x10^{-4}$	ELISA	↑ Vtg after $1x10^{-4}$ M (females) No effect (males)	(Riley et al., 2004)
Japanese eel (*Anguilla japonica*) (N/D) (immature) 200-250g	Hepatocytes 2D	MT 6 days	$1x10^{-9}$ – $1x10^{-5}$	SDS - PAGE	↑ after $1x10^{-6}$ M MT (in presence of E2) Synthesis ↓ by tamoxifen and F (tamoxifen more effective)	(Kwon et al., 2005)

Table 2. (Cont.)

Species **Sex (maturation status)** **Weight**	**Model**	**Androgen** **Exposure time**	**Concentration (M)**	**Vitellogenin detection**	**Main effects on Vtg**	**Reference**
Rainbow trout (*Onchorhynchus mykiss*) N/A	Hepatocytes 2D	Trendione 17α-TB 17β-TB 24 h	3.70×10^{-9} (trendione) 3.70×10^{-9} (17α-TB) 3.72×10^{-9} (17β-TB)	qRT-PCR	E2 equivalencies/Vtg induction Trendione and 17β-TB with estrogenic activity	(Forsgren et al., 2014)
Atlantic salmon (*Salmo salar*) Male/female (immature) N/A	Hepatocytes 2D	T 4 days	1×10^{-6}	qRT-PCR	↑	(Trombley et al., 2015)

11-ketotestosterone, 11-KT; dihydrotestosterone, DHT; estradiol, E2; flutamide, F; methyltestosterone, MT; testosterone, T; trenbolone, TB.

Enzyme-linked immunosorbent assay, ELISA; radioimmunoassay, RIA; real-time quantitative polymerase chain reaction, qRT-PCR; sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE.

Decrease ↓; not determined, N/D; increase, ↑; information not available, N/A; two-dimensional, 2D; three-dimensional, 3D.

In eel hepatocytes, the production of Vtg seemed to be dependent on estrogenic action (Kwon et al., 2005; Peyon et al., 1997), that is, it only happened in the presence of E2 or after E2 priming. However, when combined with E2, androgens (T and MT) were shown to potentiate estrogenic effects (Kwon et al., 2005; Peyon et al., 1997). An additive effect of E2 and androgens (T, DHT, MT), meaning that co-treatment with E2 and androgen increases Vtg more than E2 alone, was also shown in the Mozambique tilapia (Kim et al., 2003).

Two studies with Mozambique tilapia (Kim et al., 2003; Riley et al., 2004) used hepatocytes from sexually mature animals and showed Vtg induction, but only in females, after micromolar concentrations of T, MT and DHT (5 and 100 μM). However, when the hepatocytes from male Mozambique tilapia had been previously exposed to E2, subsequent exposure to T ($1x10^{-6}$ and $1x10^{-5}$, but not $1x10^{-7}$ M) maintained Vtg protein above the control levels, denoting a positive action of T on Vtg synthesis (Kim et al., 2003).

Most studies showed that micromolar androgen concentrations were required to induce Vtg, except for two rainbow trout experiments. Mori (1998) reported induction of Vtg mRNA using $2x10^{-9}$ M (2nM) of T, androsterone and 17α-MT in rainbow trout (Mori et al., 1998). Another rainbow trout study also showed that some of the metabolites of the potent model androgen TB acetate (17β-TB and trendione) had estrogenic properties at nanomolar levels, by inducing Vtg (mRNA) *in vitro* over control levels (Forsgren et al., 2014). *In vivo,* rainbow trout was more sensitive than zebrafish to the induction of Vtg by xenoestrogens (Van den Belt et al., 2003), denoting the greater sensitivity of salmonids to estrogenic insult. However, other studies using hepatocytes from rainbow trout have found no induction of Vtg, at mRNA and protein level, after exposure to 1 and 10 μM of androgens (Le Guevel and Pakdel, 2001; Vaillant et al., 1988).

4.3. Mechanistic insights into the regulation of estrogenic targets by androgens

Interferences of androgens with targets considered as estrogenic biomarkers have been shown *in vitro* and *in vivo*, and various hypotheses were already pointed as possible underlying mechanisms. Mechanistic studies have showed that when hepatocytes were co-exposed with tamoxifen (ER antagonist) and androgens, the ER antagonist reduced or completely inhibited the Vtg induction caused by androgens (Kim et al., 2003; Kwon et al., 2005; Mori et al., 1998; Pelissero et al., 1993; Peyon et al., 1997), indicating that an ER-mediated mechanism was involved. However, several hypotheses could be raised to explain such a result.

One of the most suggested mechanisms is the conversion of androgens to estrogenic compounds, which can occur by aromatization of MT or T, for example (Hornung et al., 2004; Zerulla et al., 2002). The product of MT aromatization (17α-methylestradiol) was detected in the plasma of fathead minnow exposed to the androgen (Hornung et al., 2004). Moreover, an aromatase inhibitor (fadrozole) blocked Vtg induction by MT (Zerulla et al., 2002). Changes in brain and ovary aromatase activities have been used as arguments in favour of the aromatase involvement in the process of Vtg induction by androgens. Actually, decreases in brain and ovary aromatase activities were shown after *in vivo* exposure to MT (Hornung et al., 2004). *In vitro*, fadrozole did not affect Vtg induction in co-exposure with T (Kim et al., 2003). These data suggest that aromatase may have a more relevant role in Vtg production/induction *in vivo* than *in vitro*. However, it should be noted that changes in aromatase expression were also observed after exposure to non-aromatizable androgens, for example, DHT (Dorts et al., 2009; Mouriec et al., 2009).

Direct binding of androgens to the ERs is another major mechanism proposed to explain androgenic interferences in estrogenic targets. Binding of androgens to fish ERs was shown in the black goby (Le Menn et al., 1980). T, DHT and MDHT transactivate human (Gaido et al., 1997), and fish (rainbow trout) ERs (Le Dréan et al., 1995; Matthews et al., 2000). In addition, ICI 164,384 (an ER antagonist) was effective at blocking the transactivation produced by T (Le Dréan et al., 1995). The described data strengthened the notion that Vtg production through the ERs directly is a common mechanism to both aromatizable and non-aromatizable androgens, at least *in vitro*.

Vtg induction through the ARs was also hypothesized in one study where flutamide (F), in co-treatment with androgens, had an inhibitory effect on Vtg induction (Kwon et al., 2005). Contrarily, cyproterone acetate (another AR inhibitor) enhanced DHT-induced Vtg levels (Kim et al., 2003). *In vivo*, however, F did not revert Vtg induction, when in combination with DHT in male and female Murray rainbowfish (Bhatia and Kumar, 2016). In female fathead minnow, F also did not counteract β-TB effects, which reduced Vtg gene expression (Ankley et al., 2004). Quite the opposite, in single exposures, F was already shown to modulate (increase or reduce) Vtg levels in fish (Bhatia et al., 2014a; Bhatia et al., 2014b; Jensen et al., 2004). The exact mechanism by which F interferes with Vtg is unknown, but F was reported to weakly bind ERs in fish (Ankley et al., 2003; Le Guevel and Pakdel, 2001). Moreover, F increased ERα and ERβ1/ERβ2 mRNA levels in male Murray rainbowfish (Bhatia et al., 2014b) and male fathead minnow (Filby et al., 2007). F also interfered with the AR, decreasing AR mRNA levels in the liver of female fathead minnow (Filby et al., 2007) and male Murray rainbowfish (Bhatia et al., 2014b).

It has been shown that Vtg can be induced by androgens in the absence of changes in the mRNA levels of the ERs (Benninghoff and Williams, 2008; Madureira et al., 2018) and even ERs (ERα and ERβ) inhibition (Zheng et al., 2013). This is intriguing, considering the current knowledge about the dynamics and the role of ERs in Vtg induction.

5. Peroxisomes and peroxisome proliferator-activated receptors (PPARs)

5.1. Peroxisomes and peroxisomal functions

Peroxisomes are multitasked organelles ubiquitously present in eukaryotic cells. Since they were discovered in the mouse (*Mus musculus*) kidney (Rhodin, 1954) and then in rat liver (Bernhard and Rouiller, 1956), their biological roles in crucial metabolic functions have been uncovered. Much of our current knowledge on these fascinating organelles owes to the development of cytochemical techniques that allowed their identification in light and electron microscopy. The most used peroxisome enzyme marker is catalase (Cat), which enables univocal peroxisome recognition through alkaline staining with 3,3'-diaminobenzidine (DAB) (Fahimi and Baumgart, 1999). Peroxisomes are enclosed by a single membrane and, despite their apparent simple architecture, they govern several metabolic (anabolic and catabolic) pathways, along with non-metabolic functions. Among their metabolic roles are included: α- and β-oxidation of fatty acids; biosynthesis of ether-phospholipids (plasmalogens), polyunsaturated fatty acids (PUFA) and bile acids; and the metabolism of reactive oxygen species and purines (Depreter et al., 2002; Smith and Aitchison, 2013; Wanders et al., 2016). Other distinct functions are related to cellular stress responses and the defence against pathogens (Islinger et al., 2018). Some of the peroxisomal functions are shared with other cellular compartments while others, mainly related to lipid metabolism, are exclusive to peroxisomes. The latter include the initial steps of the β-oxidation of very-long-chain fatty acids (VLCFA), α-oxidation of branched-chain fatty acids and synthesis of ether lipids (Lodhi and Semenkovich, 2014; Wanders et al., 2016). Peroxisomes carry a diversity of enzymes to face such diverse metabolic and non-metabolic functions. Around 85 genes encoding peroxisomal proteins have been identified in humans (Schrader and Fahimi, 2008). Peroxisomal proteins comprehend either the structural ones, the peroxins (Pex), and those for the enzymatic reactions held by peroxisomes. The most well-known are Cat, the acyl-coenzyme A (CoA) oxidases (Acox), which are involved in peroxisomal β-oxidation and the enzymes involved purine catabolism, xanthine oxidase, urate oxidase (Uox) (and allantoinase and allantoicase in some teleosts). Some of the main peroxisomal pathways, with the respective enzymes, are represented in Fig. 2.

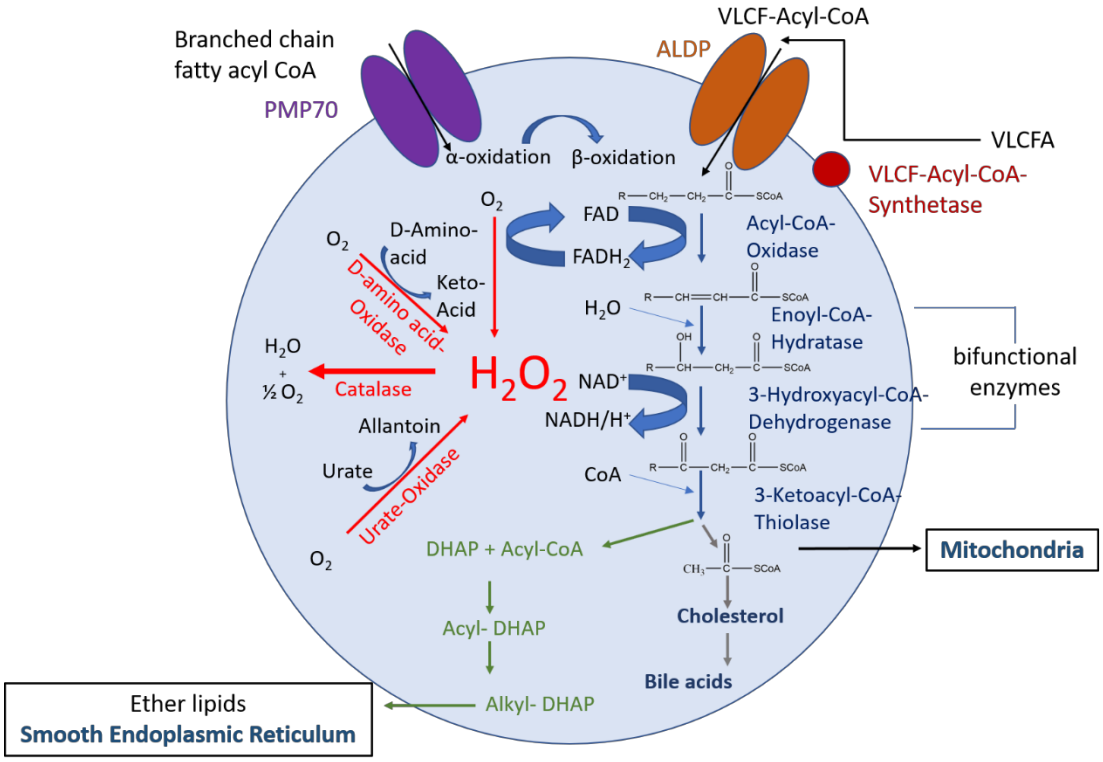

Fig. 2. Major pathways that occur in peroxisomes of the mammalian liver. The very-long-chain fatty acids (VLCFA) are transported by membrane proteins into the matrix, where they are oxidized by β-oxidation enzymes, consisting of multiple acyl-coenzyme A (CoA) oxidases and thiolases, as well as two distinct multifunctional (hydratase/3-hydroxyacyl-CoA dehydrogenase) enzymes. The products of the β-oxidation serve as substrates for the biosynthesis of ether glycerolipids, from its precursor dihydroxyacetone phosphate (DHAP), cholesterol and bile acids, or may exit the peroxisome for further oxidation in mitochondria. Peroxisomal β-oxidation and the activity of other peroxisomal oxidases produces hydrogen peroxide (H_2O_2), which is broken down by catalase. Branched-chain fatty acids enter in the peroxisomal matrix and go through α-oxidation. The products from α-oxidation can thereafter be channelled to β-oxidation in peroxisomes or mitochondria. 70 kDa peroxisomal membrane protein, PMP70; adrenoleukodystrophy protein, ADLP. Adapted from Schrader and Fahimi, 2008.

The critical importance of peroxisomes is evident by the existence of very severe human diseases caused by defects in genes coding for distinct peroxisomal structural proteins and enzymes (Wanders et al., 2016). One paradigmatic example is the Zellweger syndrome, also called cerebro-hepato-renal syndrome, caused by a mutation in one or more of the Pex enzymes, leading to the complete absence of functional peroxisomes in the most severe forms of the disease. These patients accumulate VLCFA in plasma and have a deficit of plasmalogens, resulting in multiple severe conditions, such as developmental delay, hypotonia and liver dysfunction (Islinger et al., 2018; Wanders et al., 2016).

The peroxisomes are more abundant in organs where occur detoxifying reactions, such as the liver and kidney. Another characteristic of peroxisomes is their plasticity, as they adapt their shape and can alter size and number when subjected to certain stimuli or external conditions (Schrader and Fahimi, 2008; Smith and Aitchison, 2013). These morphological variations accommodate the induction of specific metabolic pathways or changes in the rates of reactions occurring within the organelle (Smith and Aitchison, 2013). Peroxisomes can proliferate by the stimulus of diet PUFAs, but also by the influence of synthetic substances named peroxisome proliferators, for instance, the hypolipidemic drugs WY-14,643 (WY) and fibrates, the adrenal hormone dehydroepiandrosterone (DHEA) and some industrial chemicals, such as plasticizers (Corton et al., 2018; Islinger et al., 2010). The severity of effects resulting from exposure to peroxisome proliferators varies amongst species. Rodents from the Muridae family are the most susceptible (Islinger et al., 2010). In these species, peroxisome proliferators induce enzymes from the β-oxidation pathway, peroxisome proliferation and even liver tumours (Reddy, 2004), while other species, including human (Corton et al., 2018; Gonzalez, 2002) and fish (Orner et al., 1995; Scarano et al., 1994) seem to be refractory to peroxisomal proliferation.

Changes in the expression of peroxisomal proteins that occur in parallel with peroxisomal proliferation are controlled at the transcription level, mainly by a family of nuclear receptors, the PPARs, with particular emphasis to PPARα (Corton et al., 2018; Reddy, 2004).

5.2. The PPAR family in mammals and fish

PPARs belong to the nuclear receptor superfamily, that also includes the steroid, thyroid, retinoic acid, glucocorticoid and vitamin D receptors (Tyagi et al., 2011). In mammals, the PPAR (NR1C) family comprises 3 members: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). PPAR proteins exhibit a structure analogous to other nuclear receptors, as shown in Fig. 3, with: a) N-terminal A/B – ligand-independent transcriptional activation domain (with the activation function-1); b) C – deoxyribonucleic acid (DNA) binding domain, important for binding to the DNA of target genes; c) D – hinge domain; and d) E/F – ligand-binding domain (with the ligand-dependent activation function-2) located at the C-terminus, responsible for the binding of specific ligands (Corton et al., 2000; Zieleniak et al., 2008).

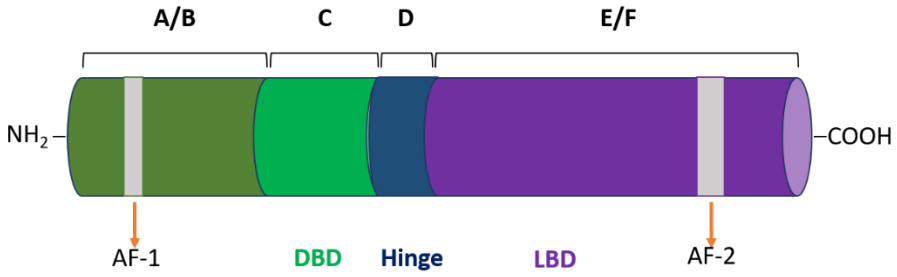

Fig. 3. Structure of the peroxisome proliferator-activated receptor protein. The functional regions correspond to an N-terminal A/B domain, with the activation function-1 (AF-1), a C-domain or deoxyribonucleic acid (DNA)-binding domain (DBD), a D-domain or hinge domain, and a C-terminal E/F domain or ligand-binding domain (LBD), with the activation function-2 (AF-2).

It was the knockdown of PPARα that demonstrated its implication on the effects of peroxisome proliferators in mouse (Lee et al., 1995). PPARs heterodimerize with the retinoid X receptor (RXR) and, in the absence of ligands, are associated with corepressor proteins (Lodhi and Semenkovich, 2014). The binding of a receptor agonist leads to the recruitment of coactivation factors and translocation to the nucleus, to initiate the transcription of target genes (Corton et al., 2018; Den Broeder et al., 2015). PPAR/RXR heterodimers bind to specific DNA sequences on the target genes, the peroxisome proliferator response elements (PPREs), usually present in the gene's promoter or enhancer regions (Corton et al., 2018; Lodhi and Semenkovich, 2014).

The β-oxidation enzymes, such as Acox1, bifunctional enzymes and thiolases, all possess peroxisome proliferator response elements (PPREs), and thus the regulation of β-oxidation genes by PPARα is thought to happen by this direct mechanism (Cancio and Cajaraville, 2000; Schrader et al., 2016). PPRE independent mechanisms have also been described for PPAR transcriptional modulation, including the interaction with other transcription factors, which in turn bind directly to the target genes (McMullen et al., 2014; Schrader et al., 2016).

In mammals, PPARs have different (but sometimes overlapping) tissue expressions, intimately related to their respective functions (Corton et al., 2018). The most ubiquitously expressed is PPARβ, while PPRAα is expressed predominantly in tissues with high rates of β-oxidation (e.g., liver, kidney, skeletal muscle, and brown adipose tissue) and PPARγ is mainly expressed in the adipose tissue, large intestine, spleen and immune system (Grygiel-Górniak, 2014; Schrader et al., 2016).

PPARα and PPARγ are the most studied PPARs, for being the main target receptors of respectively, the hypolipidemic drugs and the antidiabetic drugs thiazolidinediones (Corton

et al., 2000). The natural activators of PPARs are lipophilic molecules, unsaturated fatty acids and oxidized derivatives (e.g., eicosanoids) (Leaver et al., 2008; Tyagi et al., 2011). PPARα regulates lipid and glucose homeostasis (Rakhshandehroo et al., 2010), whereas PPARγ is mainly associated with adipogenesis and energy (fatty acid) storage (Tyagi et al., 2011). In human and mouse, the PPARγ gene originates two protein variants by alternative splicing sites – PPARγ1 and PPARγ2. The two proteins differ in the N-terminal extreme (Tontonoz and Spiegelman, 2008; Zieleniak et al., 2008). PPARγ2 expression is more restricted to adipose tissue and has more adipogenic action (Tontonoz et al., 1994; Tontonoz and Spiegelman, 2008).

The three different PPAR isotypes (α, β and γ) have also been described in several fish species, such as gilthead sea bream (*Sparus aurata*) (Leaver et al., 2005), plaice (*Pleuronectes platessa*) (Leaver et al., 2005), European sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004), Japanese sea bass (*Lateolabrax japonicas*) (Dong et al., 2015), grass carp (*Ctenopharyngodon idella*) (He et al., 2012), cobia (*Rachycentron canadum*) (Tsai et al., 2008), Japanese medaka (Kondo et al., 2009) and rabittfish (*Siganus canaliculatus*) (You et al., 2017).

For specific fish species, it was reported more than one of each PPAR isoform, for example, two PPARα and PPARβ genes were found in zebrafish (Den Broeder et al., 2015), and two PPARα in Japanese sea bass (Dong et al., 2015), and Japanese medaka (Kondo et al., 2009). Phylogenetic analysis showed that, in some species, the two PPARα genes appear in separate branches, for example, in zebrafish and Japanese sea bass (Den Broeder et al., 2015; Dong et al., 2017; Dong et al., 2015). The duplication of PPARα genes probably occurred in the teleost-specific genome duplication (Round 3), originating two PPARα orthologs (Dong et al., 2015).

In Salmonids, PPARα genes seem to have a different pattern, since the two genes found in brown trout (Madureira et al., 2017a) and rainbow trout (Dong et al., 2017) appear in the same branch in the phylogenetic analysis, exhibiting more similarity than the orthologs described in other fish species. As salmonids went through a 4th round of whole-genome duplication it would be expectable to find in some situations 4 identical genes and, accordingly, four PPARβ were described in Atlantic salmon (Leaver et al., 2007). After the salmonid-specific genome duplication event, the process of reverting to a stable diploid status (rediploidization) is still ongoing (Allendorf and Thorgaard, 1984).

A single PPARγ gene has been described in fish, similarly to what was reported for mammals (Tontonoz and Spiegelman, 2008; Zieleniak et al., 2008). However, different mRNA subtypes have been described in some fish species, including Nile tilapia or Atlantic

salmon (Andersen et al., 2000; He et al., 2015; Sundvold et al., 2010; Todorčević et al., 2008). In addition, PPARγ has been shown to have a broader tissue expression than the mammalian counterpart (Leaver et al., 2005) and different ligand-binding properties – for example, it was not activated by PUFA or the thiazolidinedione rosiglitazone – probably arising from differences in the ligand-binding domain (Kondo et al., 2007; Leaver et al., 2005). Fish PPARγ was also shown to be activated by PPARα agonists, such as clofibrate and bezafibrate (Ibabe et al., 2005; Ruyter et al., 1997; You et al., 2017), similar to what happens in mammals (Corton et al., 2000), which can imply overlapping functions of both PPARs.

5.3. Overview of the major pathways from lipid metabolism

The liver is pivotal in lipid metabolism, in which PPARs have an essential role. All PPARs regulate endocrine pathways and promote energy homeostasis, adapting body metabolism according to nutritional status and energy requirements (Wang et al., 2020). Despite the existing differences between fish and terrestrial vertebrate metabolism, for example, the less efficient utilization of carbohydrates by fish (Leaver et al., 2008), in general, the main pathways in lipid metabolism are thought to be analogous between mammals and fish (Tocher and Glencross, 2015). Lipids (and proteins) are major components of the fish body and are fundamental for fish physiology, including for growth and reproduction (Tocher, 2003).

Lipid transport, that includes the extracellular and intracellular transport of lipids, is an essential aspect of lipid metabolism. The extracellular trafficking is done in the form of lipoproteins, which carry triglycerides (TG) and cholesterol esters and deliver them to target organs. Lipoproteins are classified according to increasing density and decreased fat to protein ratio (which correlates negatively with size) as ultra-low-density lipoproteins (ULDL or chylomicrons), very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Babin and Vernier, 1989). The apolipoproteins, along with phospholipids and cholesterol, constitute the outer layer that surrounds the lipoprotein core made of TG and cholesteryl esters and regulate lipoprotein metabolism (Babin et al., 1997). Apolipoproteins are differently distributed among lipoproteins, for example, the apolipoprotein of B type (ApoB) is the major species in VLDL and LDL, while apolipoprotein A (ApoA) are major constituents of HDL (Babin et al., 1997; Tocher, 2003).

The intracellular lipid transport is facilitated by fatty acid-binding proteins (Fabp), which enable lipid transport within cells by binding long-chain fatty acids (LCFA), eicosanoids and

other hydrophobic ligands (Furuhashi and Hotamisligil, 2008; Tocher, 2003). Fabp1 is the liver-type Fabp, even though it can be expressed in other tissues, e.g., intestine (Furuhashi and Hotamisligil, 2008).

The TG hydrolase gene family comprises lipoprotein lipase (LPL), hepatic lipase (LIPC) and endothelial lipase (LIPG) (Holmes et al., 2011), which are extracellular enzymes with important roles in lipid metabolism and transport (Mead et al., 2002; Tocher, 2003). LPL is generally found in the endothelium of blood capillaries, and has a major role on lipid mobilization, hydrolyzing lipids from remnants of TG/phospholipid-rich lipoproteins, such as VLDL or chylomicrons (Holmes et al., 2011). Released fatty acids are up-taken by tissues and then used for generating energy through β-oxidation, re-esterified and stored, or secreted (Tocher, 2003).

Fatty acid synthesis occurs in the cytoplasm, whereas lipid oxidation is a mitochondrial and peroxisomal function. Hepatic lipid synthesis consists of two main processes, the synthesis of fatty acids (also called *de novo* lipogenesis – DNL) and the esterification of fatty acids into glyceride species including TG (the richest form of energy store) and phospholipids (Tocher, 2003; Tocher and Glencross, 2015). Acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) are the major enzymes in DNL. ACC catalyzes the first, rate-limiting and committed step of DNL catalyzing the carboxylation of acetyl-CoA into malonyl-CoA (Leaver et al., 2008; Tocher and Glencross, 2015). FAS is a multi-enzyme protein that catalyzes fatty acid synthesis from acetyl-CoA and malonyl-CoA, in the presence of NADPH (Leaver et al., 2008; Tocher and Glencross, 2015). Fatty acid synthesis results primarily in palmitic and stearic acids that can be desaturated and elongated (Leaver et al., 2008; Tocher, 2003). Fatty acid β-oxidation represents the reverse process of fatty acid synthesis, where fatty acids are broken down to originate energy, in the form of adenosine triphosphate (ATP) (Boukouvala et al., 2004). LCFA need to be activated by long-chain acyl-CoA synthetases (Acsl), becoming acyl-CoA, to be channelled either to incorporation into more complex lipids or to go through β-oxidation (Digel et al., 2009; Li et al., 2010).

6. Hormonal regulation of PPAR signaling and lipid metabolic pathways

6.1. PPAR and lipid metabolism as hormonally regulated pathways in mammals and fish

Liver physiology is regulated by sex hormones in health and disease (Kur et al., 2020; Shen and Shi, 2015). In humans, sex hormones decrease with age, which has been linked to

increased risk of obesity and related diseases (Palmisano et al., 2018; Shen and Shi, 2015). In agreement, lower levels of androgens and estrogens were associated with higher levels of lipid deposition in liver (Shen and Shi, 2015) and reduced protein levels of ERα and AR with severe lipid accumulation (steatosis) in the human liver (Zhang et al., 2013). On the opposite, one study reported that higher E2 levels were associated with fatty liver in both men and women (Lazo et al., 2015). In the same cross-sectional study, high T titers were associated with an increased risk of fatty liver in women and decreased risk in man (Lazo et al., 2015).

Hepatic lipid accumulation can result from various scenarios, including increased lipid transport to the liver, decreased hepatic export of lipoproteins, increased lipogenesis and reduced fatty acid oxidation (Lin et al., 2008; Nasiri et al., 2015). Several paths of hepatic fatty acid, TG and cholesterol metabolism, including lipogenesis and β-oxidation, are modulated by estrogens and androgens, in humans and rodents (Kelly et al., 2016; Nasiri et al., 2015; Shen and Shi, 2015; Zhang et al., 2013). The regulation of energy metabolism in the liver is complex and involves several key transcription factors, including PPARs, liver X receptor (LXR) and the sterol regulatory element-binding proteins (SREBPs) (Kelly et al., 2016; Kelly and Jones, 2013). The regulation of liver metabolism may thus happen by direct regulation of the enzymes involved in the different lipidic pathways, or indirectly by the regulation the referred transcription factors, which then orchestrate the expression of either lipogenic or catabolic enzymes (Kelly and Jones, 2013). Some data exist on the regulation of lipogenesis by estrogenic and androgenic signaling in human and rodent models. For example, obese male mouse with hepatic AR knockout fed with a high-fat diet, showed increased liver TG levels, an effect that was accompanied by a decrease in the mRNA levels of PPARα and an increase of PPARγ and ACC (Lin et al., 2008). In the same study, in primary hepatocytes, DHT increased the expression of PPARα only in mice with functional AR, suggesting that the observed effects were AR-mediated (Lin et al., 2008). An AR independent action of T was reported in another study, where liver lipid deposition and protein levels of ACC and FAS were increased by a cholesterol-enriched diet in mice with non-functional AR (testicular feminized mouse – Tfm), but also in orchiectomized mice (Kelly et al., 2016; Kelly et al., 2014). T treatment decreased lipid deposition and FAS and ACC protein expression to wild-type levels (Kelly et al., 2016; Kelly et al., 2014). Tfm mice under normal diet treatment also had a slight increase in lipid accumulation and expression of PPARγ (Kelly et al., 2016). Furthermore, co-treatment with T and an ERα antagonist inhibited (but not completely) the beneficial effects of T on lipid deposition, linking some of the effects of T to estrogenic signaling (Kelly et al., 2014). Also in a rat model of non-alcoholic fatty liver disease (NAFLD), the gene expression of FAS was decreased by E2

and DHT (Zhang et al., 2013). Moreover, both steroids reversed NAFLD histopathological alterations, with additive effects (Zhang et al., 2013). Contrarily to the presented evidence, in the human hepatoma C3A cell line, T and DHT increased the expression of ACC, FAS and of the mitochondrial β-oxidation enzyme carnitine palmitoyl transferase 1 (CPT1) (Nasiri et al., 2015).

An interesting link between PPAR, peroxisomes and hormonal signaling is the existing data on DHEA and its sulfated form, DHEA-S. DHEA is a weak androgen produced mainly in the adrenal cortex, that can be metabolized to originate other androgens (androstenedione, T, DHT) or estrogens (e.g., E1, E2) (Prough et al., 2016). DHEA activates PPARα (Peters et al., 1996; Prough et al., 2016) and it has been shown to alter lipid metabolism, especially in mouse and rat models (de Heredia et al., 2009). Specifically, DHEA feeding changed the serum fatty acid profile of female rats (de Heredia et al., 2009), had a protector effect on the development of liver steatosis (Hakkak et al., 2017) and reduced liver TG and cholesterol levels (along with inhibiting of SREBP1, FAS, Acox and PPARα gene expression) (Chen et al., 2016). DHEA is also a potent inducer of peroxisome proliferation in rodents and increases PPARα and the expression of peroxisomal enzymes (Depreter et al., 2002; Frenkel et al., 1990).

PPARs and lipid metabolism are therefore strongly intermingled (Reddy, 2004), and so are sex steroids in the regulation of lipid metabolic processes. Several aspects of peroxisome proliferation have been shown to differ among sexes, for example, the higher induction of PPAR, β-oxidation enzymes and P450 IVA in male rodents (Beier et al., 1997; Kawashima et al., 1989; Nakajima et al., 2000). The expression of PPARs also varies between males and females, with the former expressing higher levels, in mammals and fish (Eide et al., 2014b; Jalouli et al., 2003; Manor et al., 2015). The fluctuation of PPARs along the reproductive cycle of fish was reported in brown trout (Batista-Pinto et al., 2009), and the same has been shown in respect to peroxisomal enzymes, including 17β-hydroxysteroid dehydrogenase type 4 (17β-HSD4), Pex11, and purine catabolism enzymes (Castro et al., 2013; Castro et al., 2009; Resende et al., 2005), whose expressions negatively correlate with the levels of the female sex steroid E2.

Peroxisomal morphology has also been shown to change along the reproductive cycle in brown trout, with dissimilar patterns between males and males, stressing a hypothetical role for sex hormones in peroxisomes physiology (Rocha et al., 1999). PPARs regulate the maintenance of a systemic balance between energy storage and expenditure, including in the demanding process of reproduction (Maradonna et al., 2013). This combined evidence points to a possible regulation by sex-steroid hormones (and their chemical mimics) of PPAR signaling and PPAR mediated pathways.

6.2. Estrogenic regulation of PPARs and peroxisomal signaling in fish liver

The effect of estrogens in PPARs liver expression was addressed in some fish studies. PPARα mRNA was up-regulated after waterborne exposure to E2 (10^{-8} M) in juvenile sole (*Sole sole*) (Cocci et al., 2013), and 50 µg EE2/L in juvenile brown trout (Madureira et al., 2018). Contrarily, PPARβ and PPARγ decreased in juvenile rainbow trout treated with E2 (IP) injection (Cleveland and Manor, 2015), and PPARγ also lowered after EE2 exposure in juvenile brown trout (Madureira et al., 2018). EDCs with known estrogenic properties also modulated PPARs expression, *in vivo*, since diet exposure to BPA, NP and 4-tert-octylphenol (t-OP) increased all PPAR isotypes in juvenile sea bream (Maradonna et al., 2015).

In vitro, EE2 (10 nM) increased the mRNA expression of all PPARs in female zebrafish primary hepatocytes (Maradonna et al., 2013) and PPARγ in hepatocytes from male fish (Maradonna et al., 2013). An opposite pattern was obtained in primary hepatocytes from rainbow trout juveniles, where EE2 decreased the expression of PPARα (at 3 and 30 nM concentrations) and PPARγ (30 nM) (Hultman et al., 2015b).

In mammals, negative and reciprocal crosstalk has been described between ERs and PPARs (Nuñez et al., 1997). This crosstalk was validated in several models for PPARα (Jeong and Yoon, 2007; Yoon, 2010) and PPARγ (Lin et al., 2013; Wang and Kilgore, 2002). Mechanistically, this crosstalk has been justified to occur by either competitive binding to each other response elements (Keller et al., 1995; Nuñez et al., 1997) or inhibition of coactivator recruitment (Yoon, 2010). Whatever mechanism, the evidence unveiled a clear interplay between estrogenic and PPAR signaling, in mammals and fish.

The expression and activity of peroxisomal enzymes were also altered by exposure to estrogens. Cat mRNA/protein expressions were decreased after waterborne exposure to 30 ng EE2/L in male zebrafish (De Wit et al., 2010) and juvenile brown trout (50 µg/L) (Madureira et al., 2018). Cat enzymatic activity was either increased (Maradonna et al., 2014; Sun et al., 2019) or decreased (Batista-Pinto et al., 2015; Qiu et al., 2016) by exposure to estrogens. The mRNA of the peroxisomal β-oxidation enzymes – Acox1 isoform 3l (Acox1-3l) and Acox3 – was down-regulated *in vivo* after exposure to EE2 (50 µg/L) in juvenile brown trout (Madureira et al., 2018), while in rainbow trout triploid females, E2 also down-regulated the peroxisomal β-oxidation L-bifunctional enzyme (ehhadh) (Cleveland and Weber, 2016). At last, purine metabolism, represented by the Uox gene, was down-regulated *in vivo* by waterborne exposure to EE2 and E2 in juvenile brown trout (Batista-Pinto et al., 2015; Madureira et al., 2018).

In vitro, EE2-exposed primary hepatocytes from juvenile brown trout showed a down-regulation profile of the peroxisomal enzymes Acox1-3I, Acox1-3II, Cat and Uox (Madureira et al., 2016; Madureira et al., 2015b). In contrast, 17β-HSD4, also involved in peroxisomal β-oxidation, and Acox3 had increased mRNA levels (Madureira et al., 2016; Madureira et al., 2015b). Similarly, Acox3 was also up-regulated by EE2 (30 nM) in juvenile rainbow trout hepatocytes (Hultman et al., 2015b).

Peroxisomes are adaptive organelles in what concerns their number and size. Therefore, it would be expectable that a stimulus that alters the expression of transcription factors and their target peroxisomal enzymes would as well produce morphological changes. Although this is an unexplored subject in fish literature, both natural estrogens (E2) and xenoestrogens (EE2, dibutylphthalate (DBP), t-OP and methoxychlor (MXC) showed peroxisome proliferation effects (increased surface and numerical density) in zebrafish (Ortiz-Zarragoitia and Cajaraville, 2005; Ortiz-Zarragoitia et al., 2006). Peroxisome morphology was also modified by EE2 in juvenile brown trout *in vitro* and *in vivo* but, in this case, the peroxisomal volume density was decreased, as likewise determined by stereological analysis (Madureira et al., 2015a; Madureira et al., 2015b; Madureira et al., 2018).

6.3. Estrogenic regulation of lipid metabolism in fish liver

Lipid metabolic pathways have been shown to be modulated by estrogens in fish studies. For example, microarray studies identified that pathways from lipid and fatty acid metabolism are commonly altered by estrogenic exposure, either *in vitro* (Hultman et al., 2015b) and *in vivo* (De Wit et al., 2010; Doyle et al., 2013; Hoffmann et al., 2006; Ruggeri et al., 2008).

Genes from distinct pathways such as lipogenesis, β-oxidation (mitochondrial and peroxisomal) and lipolysis are altered by estrogens. For example, from DNL, ACC was mainly down-regulated after estrogenic exposure (Cleveland and Manor, 2015; Cleveland and Weber, 2016). ACC mRNA levels were decreased in triploid female rainbow trout after E2-contaminated diet (Cleveland and Weber, 2016), and after IP injection of the phytoestrogens genistein and daidzein in rainbow trout juveniles (Cleveland and Manor, 2015). On the contrary, FAS was up-regulated by E2 (water exposure) in zebrafish (Ruggeri et al., 2008; Sun et al., 2019). Industrial xenoestrogens such as BPA and NP also increased FAS expression in juvenile sea bream (dietary exposure) (Maradonna et al., 2015) and zebrafish (water exposure) (Santangeli et al., 2018; Sun et al., 2019). In rare minnow (*Gobiocypris rarus*), different profiles were obtained for gene expression and enzymatic

activities, for example, ACC mRNA levels decreased whereas its enzymatic activity increased in females after BPA exposure (Guan et al., 2016).

LPL (involved in fatty acid hydrolysis and up-take) mRNA was decreased by waterborne exposure to EE2 in male zebrafish (De Wit et al., 2010) and by dietary exposure to E2 in triploid female rainbow trout (Cleveland and Weber, 2016), but was increased by E2 and genistein (IP) in juvenile rainbow trout (Cleveland and Manor, 2015) and by BPA (diet exposure) in sea bream juveniles (Maradonna et al., 2015).

Lipid transport and cholesterol metabolism are probably the main pathways affected by estrogens since ApoA1 mRNA is commonly down-regulated by estrogenic exposures (De Wit et al., 2010; Hoffmann et al., 2006; Martyniuk et al., 2007; Moens et al., 2006), as it was shown in brown trout juveniles exposed to 50 µg/L of EE2 (Madureira et al., 2018). The same occurs for fatty acid intracellular transport. For instance, the liver-type Fabp1 was down-regulated by EE2 in juvenile brown trout and male zebrafish (De Wit et al., 2010; Madureira et al., 2018) and Fabp1a was up-regulated in male zebrafish after water exposure to E2 and BPA (Sun et al., 2019).

Fatty acid activation, represented by Acsl1 gene, was up-regulated by EE2 in brown trout (Madureira et al., 2018). A similar result was shown for Acsl6, after water exposure to BPA (76 µg/L), in male marine (Java) medaka (*Oryzias javanicus*) (Kim et al., 2018).

In brown trout, EE2 changed the lipid profiles in the liver, increased the relative volume of lipid droplets in hepatocytes and enhanced the plasma levels of TG and VLDL. A similar result was reported in other fish studies, for instance in male zebrafish and rainbow trout juveniles, where E2 increased hepatic lipid, includingTG levels (Cakmak et al., 2006; Sun et al., 2019).

BPA and other xenoestrogens (e.g., phthalates, alkylphenols) have been studied by their obesogenic effects, as reviewed elsewhere (Capitão et al., 2017; Olivares-Rubio and Vega-López, 2016). For example, BPA and NP (50 mg/kg/body weight) had a steatogenic effect in the liver (Maradonna et al., 2015). Recently, in zebrafish, BPA was shown to have biphasic effects on lipid handling, promoting fatty acid synthesis and TG storage at the lowest dose (5 µg/L), and DNL and cholesterologenesis at the higher (20 µg/L) concentration (Santangeli et al., 2018). Also, in zebrafish, total liver fat and TG levels were increased after BPA exposure, with more pronounced effects after the lower concentration (100 µg/L) (Sun et al., 2019).

6.4. Androgenic regulation of PPARs and peroxisomal signaling in fish liver

The androgenic interferences on liver PPAR/peroxisomal signaling is still an unexplored subject in fish studies and, consequently, data on the subject are almost non-existing. Still, diet exposure to DHEA induced hepatic carcinomas in trout and decreased Cat enzymatic activity, with no alterations in liver β-oxidation (Orner et al., 1996; Orner et al., 1995). Diet exposure to T and DHT, however, did not alter the mRNA expression PPARs or peroxisomal β-oxidation enzymes (Acox1 and ehhadh) in triploid female rainbow trout (Cleveland and Weber, 2016).

6.5. Androgenic regulation of lipid metabolism in fish liver

The regulation of lipid metabolism by androgens has also been a subject of very few research studies. Nevertheless, a transcriptomic analysis identified that biological processes such as lipid, lipoprotein, and cholesterol metabolism were altered by 17β-TB (1 µg/L) in female mosquitofish (Brockmeier et al., 2013). In the latter work, ApoA1 transcripts were up-regulated, along with other apolipoproteins, fatty acid desaturases, and lecithin-cholesterol acyltransferase (LCAT), involved in cholesterol esterification (Brockmeier et al., 2013). Fatty acid biosynthesis and lipid binding were also biological functions influenced by exposure to 1 µg/L TB, in female rainbow trout and again ApoA1 was up-regulated, as well as a fatty acid desaturase (Hook et al., 2006). Thus, similarly to what had happened with the exposure to estrogens, the metabolism of apolipoproteins seems to be one of the most affected by TB exposure, but in this case with an up-regulation pattern. The effects of T and DHT were explored in a set of genes related to lipid metabolism in adult triploid female rainbow trout fed with androgen-containing diets (30 mg steroid/kg diet) (Cleveland and Weber, 2016). The ACC gene was down-regulated by both androgens whereas other lipogenic genes, FAS, SREBP1 and stearoyl-CoA desaturase (SCD1) were not changed.

7. The brown trout

Brown trout belongs to the Salmonidae family, which includes salmon, trout, charr, freshwater whitefishes, ciscos and graylings. It was first named by Linnaeus in 1758 and it was originally a strictly European species, but it has been nowadays introduced in all continents except Antarctica (Elliott, 1994; Lobón-Cerviá, 2017). Linnaeus first described three species, *Salmo eriox*, *Salmo fario* and *Salmo trutta* consisting of trout living in the sea, stream or river, respectively (Elliott, 1994). Brown trout have been categorized into several

species and subspecies, most now grouped into a single, polymorphic, species complex (*Salmo trutta* L.) (Klemetsen et al., 2003; Lobón-Cerviá, 2017). Brown trout habitats are broad, varying from brooks, rivers, lakes, estuaries and coastal sea although they can migrate among habitats or live permanently in freshwaters (Winfield, 2011). Since brown trout morphology differs according to the habitats, a sub-characterization into different morphotypes, "morpha" or "forma" (f.) has been historically used, including the *Salmo trutta* f. *trutta*, *Salmo trutta* f. *fario* (Fig. 4*)* or *Salmo trutta* f. *lacustris*, for sea-trout, stream trout and lake trout, respectively (Elliott, 1994; Winfield, 2011).

Fig. 4. The brown trout (*Salmo trutta* f. *fario*). A, three-year-old male; B, three-year-old female; C, one-year-old juvenile. Bar = 10 cm.

From the freshwater populations, the f. *fario* is typically strictly riverine, whereas the f. *lacustris* inhabits lakes and usually migrates to the original river for reproduction. The sea-trout is the anadromous form, which resides in the sea for variable periods but returns to the parent river for reproduction (Klemetsen et al., 2003; Lobón-Cerviá, 2017). Different migration patterns may co-exist in the same population, and the different populations interbreed (Elliott, 1994). Brown trout reproduce in autumn or winter, depending on latitude and altitude; happening earlier at higher latitudes and altitudes because of the lower water

temperature (Klemetsen et al., 2003). In the Northern hemisphere, most populations breed in November and December (Elliott, 1994).

Brown trout is not only an ecologically but also an economically relevant species (Marlatt et al., 2014; Winfield, 2011). Even though brown trout is not a representative species in aquaculture, it is widely used for recreational fishing and is a food source for specific communities (Davidson et al., 2010). As a highly adaptative species, brown trout colonize many habitats and are even considered as invasive species (Lobón-Cerviá, 2017; Winfield, 2011). They tolerate a wide range of temperatures for growth, from 4 ºC to 19.5 ºC (Elliott, 1994), but require well-oxygenated waters, and cold water for egg development (between 0 ºC and 13 ºC) (Elliott, 1994; Klemetsen et al., 2003). Albeit they are not globally considered as an endangered species (according to the International Union for Conservation of Nature (IUCN) Red Book) specific populations, such as the *Salmo trutta aralensis*, from the Aral Sea and Amu Darya river, are considered critically endangered (Birstein, 2000). In the Red Book of Vertebrates in Portugal, the migratory form is also considered critically endangered (Cabral et al., 2006). Other local populations have been also reported has critically reduced according to literature reports, for example, the one from the Llobregat River, Spain (Vera et al., 2019) or the Caspian brown trout (*Salmo trutta caspius*) from the Caspian sea, Iran (Vera et al., 2011). Moreover, their dependence on cold waters represents a susceptibility due to the global warming scenario, including in the Iberian Peninsula (Almodóvar et al., 2012; Winfield, 2011).

The fact that brown trout is a species with wide distribution, which colonizes even remote sites, plus its steady response to hormonal stimuli, makes it an attractive model species for ecotoxicological studies and, as a sentinel species, for monitoring the presence of hormonally active compounds in their habitats (Behrens and Segner, 2005; Jarque et al., 2015; Triebskorn et al., 2002; Zezza et al., 2020). Brown trout has been used as a model for investigating the effects of EDCs, particularly for their estrogenic modes of action and effects on reproduction (Bjerregaard et al., 2008; Körner et al., 2008; Uren Webster et al., 2015), but also in the immune system (Casanova-Nakayama et al., 2011). Brown trout primary hepatocytes were used in mechanistic studies targeting EDCs effects in estrogenic and PPAR signaling (Christianson-Heiska and Isomaa, 2008; Madureira et al., 2016; Madureira et al., 2015b; Madureira et al., 2017b), and for basic research on lipid metabolism (Li and Olsen, 2017).

8. Background and objectives of the Thesis

The early studies in our research team using brown trout focused on liver morphology and seasonal morphological variations, including in peroxisomes. Estrogenic interferences in PPAR signaling have also been explored *in vivo* and *in vitro*. Opposite patterns were observed in peroxisome dimensions in male and female adult brown trout. Female peroxisomes were smaller when the gonad was mature, while male peroxisomes were smaller when the gonad was regressed (Rocha et al., 1999). The expression of PPARα was found to be higher in early vitellogenesis than in late vitellogenesis and pre-spawning periods in females, while in males a decreasing trend was noted in early vitellogenesis and the following periods (when compared with the post-spawning stage) (Batista-Pinto et al., 2009). Interestingly, PPARβ levels were only altered in males, being significantly higher in the spawning period, comparing with the other seasons. Considering PPARγ, a significant decrease of mRNA levels was observed during late vitellogenesis and pre-spawning periods, in both male and female individuals. In females, similar trends to the ones observed for PPARα expression were found for the peroxisomal enzymes 17β-HSD4 and Pex11α, suggesting an inverse correlation between, on the one hand, estrogen levels and gonadal maturation, and, on the other hand, peroxisomal size and the expressions of PPARα, 17β-HSD4 and Pex11α (Castro et al., 2013). The activity of the peroxisomal enzymes xanthine oxidase, Uox and allantoinase presented a similar pattern in females, with decreased activity during the late vitellogenic and spawning stages, compared with the early vitellogenesis period (Resende et al., 2005). A decreasing pattern was also evident in males, in the same stages, for Uox (Resende et al., 2005). These main findings from previous seasonal *in vivo* studies with brown trout are summed-up in Fig. 5.

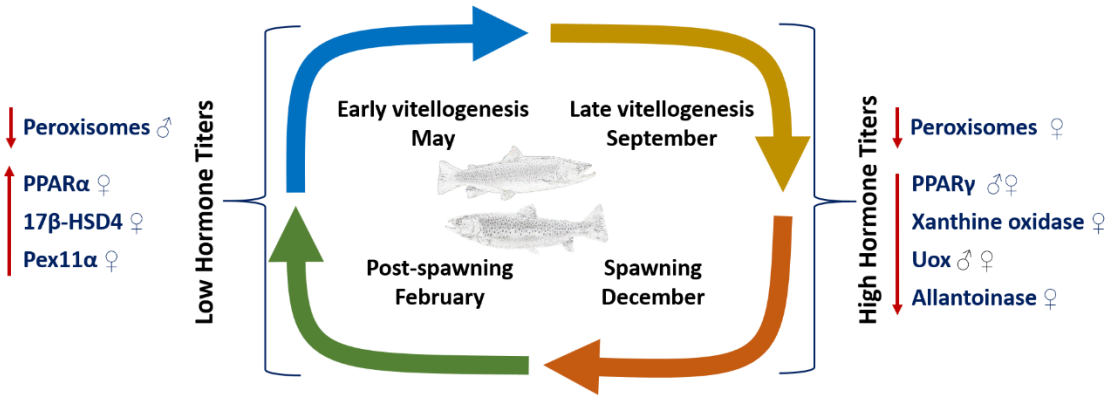

Fig. 5. Peroxisome proliferator-activated receptors (PPARs) and peroxisomal endpoints along the reproductive cycle in brown trout. 17β-hydroxysteroid dehydrogenase type 4, 17β-HSD4; decrease, ↓; increase, ↑; peroxin 11 alpha, Pex11α; peroxisome proliferator-activated receptor alpha, PPARα; peroxisome proliferator-activated receptor gamma, PPARγ and urate oxidase, Uox.

Hence, *in vivo* data revealed differences in peroxisomal-related parameters, including PPARs expression along the reproductive cycle, that were very consistent (presenting an inverse pattern), with the variation of estrogen levels experienced by females during gonadal development. As for males, some parameters including PPARγ expression and the activities of enzymes involved in purine metabolism, followed similar trends to females, while for instance PPARα expression and peroxisomes dimensions, presented distinct patterns in males and females. Trout experience extreme hormonal (especially in T and E2) fluctuations during the reproductive cycle (Rocha et al., 2004; Soivio et al., 1982; Wallaert and Babin, 1994). Because correlation does not prove causation, whether the differences observed in PPAR levels are owed to the variations of E2, T, both sex-hormones (or eventually others such as 11-KT or DHT) is currently unknown.

To explore the mechanistic interplays between hormonal and PPAR signaling, an *in vitro* model, consisting of isolated primary hepatocytes from juvenile brown trout, was previously optimized by our research team. Primary hepatocytes were found to respond readily to estrogens by increasing the expression of Vtg and ERα, evidencing the potential to be used as a suitable model to investigate the existing interconnections between estrogenic and PPAR signaling (Madureira et al., 2016; Madureira et al., 2015b; Madureira et al., 2017b). In brown trout hepatocytes, EE2 caused a reduction in peroxisome relative volumes and changed the expression of some peroxisomal target genes, including Cat and Uox, but not PPARα (Madureira et al., 2015a; Madureira et al., 2015b). In the same *in vitro* model, clofibrate (PPARα agonist) was shown to alter peroxisomal (decrease of Uox mRNA) and lipid (decrease of ApoA1 mRNA) signaling (Madureira et al., 2018). WY, another PPARα agonist, increased Vtg and ERα mRNA levels (Madureira et al., 2017b).

Similarly to other teleosts, it was expectable that more than one isoform of PPARα could exist in brown trout. Thus, the two PPARα isoforms were sequenced and were differently regulated by EE2 exposure, that is, the mRNA of the Ba isoform was increased and the Bb isoform decreased (Madureira et al., 2017a). The expression of Acox enzymes, known as PPARα targets involved in peroxisomal cdfatty acid β-oxidation, was likewise shown to change after EE2 exposure. Acox1 expression decreased and Acox3 mRNA increased after estrogenic stimulus (Madureira et al., 2016). The main results from the *in vitro* studies using juvenile brown trout primary hepatocytes exposed to EE2 are represented in Fig. 6.

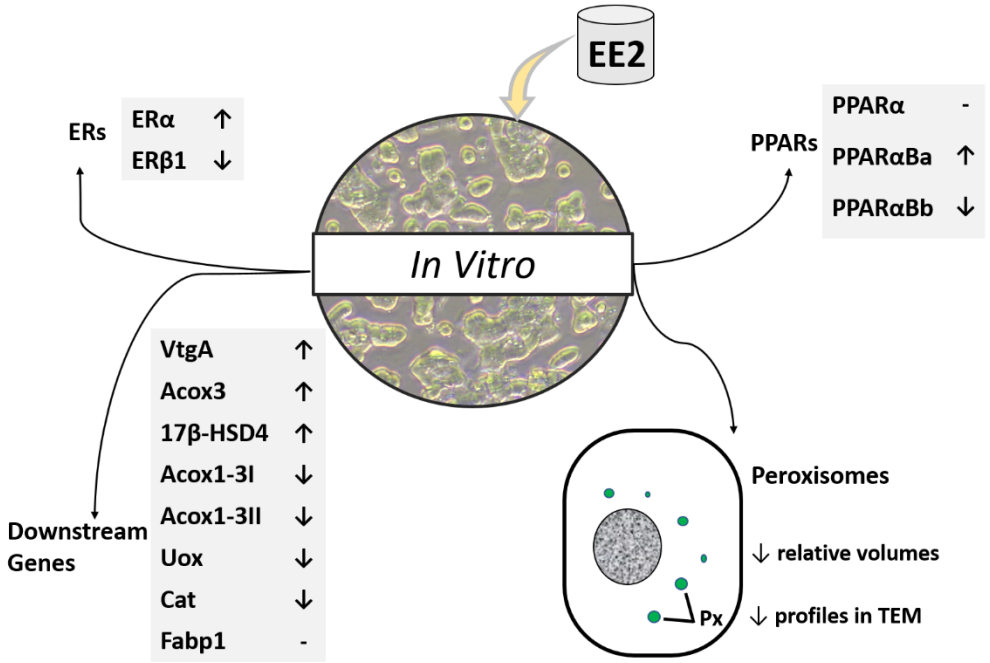

Fig. 6. Gene expression and peroxisome morphology after 17α-ethinylestradiol (EE2) exposure in juvenile brown trout primary hepatocytes. Decrease, ↓; increase, ↑; peroxisome, Px; transmission electron microscopy, TEM. 17β-hydroxysteroid dehydrogenase type 4, 17β-HSD4; acyl-CoA oxidase 1 3I, Acox1–3I; acyl-CoA oxidase 1 3II, Acox1–3II; acyl-CoA oxidase 3, Acox3; catalase, Cat; estrogen receptor alpha, ERα; estrogen receptor beta 1, ERβ-1; fatty acid-binding protein 1, Fabp1; peroxisome proliferator-activated receptor alpha, PPARα; peroxisome proliferator-activated receptor alpha Ba, PPARαBa, peroxisome proliferator-activated receptor alpha Bb, PPARαBb; urate oxidase, Uox and vitellogenin A, VtgA.

In this doctoral Thesis, we proposed to fill some of the missing gaps on the existing knowledge concerning the hormonal regulation of peroxisomes and PPAR signaling in brown trout (and fish models in general), using the optimized primary hepatocyte model. The purpose was to explore this model in different experimental scenarios, with the objective of elucidating mechanisms underlying the potential interferences caused by estrogenic and androgenic compounds along PPAR regulated pathways. We started by enlarging the brown trout PPAR portfolio, cloning the complete complementary deoxyribonucleic acid (cDNA) sequence of PPARγ, and studied estrogenic and androgenic influences in this gene. Then, the androgenic effects on PPAR regulated pathways, which have been seldom explored in brown trout and endocrine disruption studies in general, were assessed. The new results are expectedly important in understanding the physiological and pathological effects of sex-hormones and their mimics, including in the context of endocrine disruption.

In the course of this Thesis, hormonal (estrogenic and androgenic) effects in estrogenic, peroxisomal and lipid signaling were further explored by our research team in an *in vivo* study, also using juvenile brown trout (Madureira et al., 2018). In this work, the previously established interferences *in vitro* between estrogenic and peroxisomal signaling were mostly corroborated by the *in vivo* results. Moreover, EE2 was found to change the plasma lipid profile and to increase the liver lipid content. T also interfered with estrogenic signaling, increasing Vtg levels, and changing peroxisomes morphology. Because the results of that work are relevant in the context of this Thesis, they are summarized in Fig. 7.

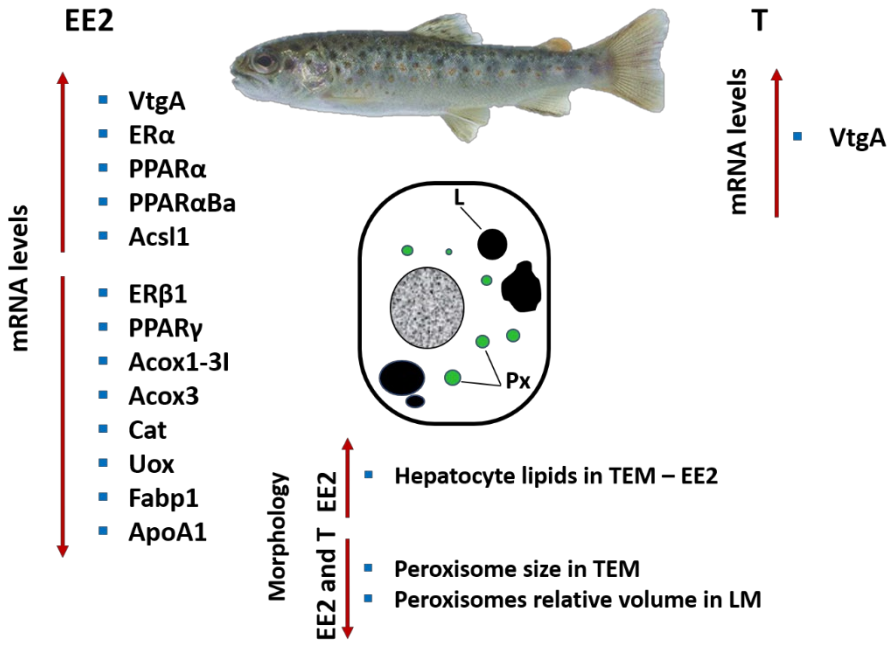

Fig. 7. Gene expression and peroxisome morphology after *in vivo* exposure of juvenile brown trout to 17α-ethinylestradiol (EE2) and testosterone (T). Decrease, ↓; increase, ↑; lipid, L; light microscopy, LM; peroxisome, Px; transmission electron microscopy, TEM. Acyl-coenzyme A long chain synthetase 1, Acsl1; acyl-coenzyme A oxidase 1 3l, Acox1–3l; acyl-coenzyme A oxidase 3, Acox3; apolipoprotein A1, ApoA1; catalase, Cat; estrogen receptor alpha, ERα; estrogen receptor beta 1, ERβ1; fatty acid-binding protein 1, Fabp1; peroxisome proliferator-activated receptor alpha, PPARα; peroxisome proliferator-activated receptor alpha Ba, PPARαBa; urate oxidase, Uox and vitellogenin A, VtgA.

Within the described context and purpose, the main specific objectives of this Thesis were to:

I. Isolate and characterize the PPARγ gene from brown trout;

II. Assess the effects of EE2 and T in the expression of PPARγ in juvenile brown trout isolated primary hepatocytes;

III. Evaluate the influence of T in peroxisomes morphology and in the expression of a selection of peroxisome related genes (PPARα, Uox and Cat), by using the same *in vitro* model;

IV. Investigate the potential interferences of aromatizable and non-aromatizable androgens in classical estrogenic targets, namely Vtg and ZPs;

V. Explore the transcriptional effects of androgens in selected lipid metabolic pathways, including in the expression of PPAR regulated genes.

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Chapter 2

Peroxisome proliferator-activated receptor gamma (PPARγ) in brown trout: interference of estrogenic and androgenic inputs in primary hepatocytes

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Peroxisome proliferator-activated receptor gamma (PPARγ) in brown trout: Interference of estrogenic and androgenic inputs in primary hepatocytes

Célia Lopes[a,b,1], Tânia Vieira Madureira[a,b,*,1], Nádia Ferreira[a,b], Ivone Pinheiro[a,b], L. Filipe C. Castro[a,c,2], Eduardo Rocha[a,b,2]

[a] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), U.Porto – University of Porto, Rua dos Bragas 289, P 4050-123 Porto, Portugal
[b] Institute of Biomedical Sciences Abel Salazar (ICBAS), U.Porto – University of Porto, Laboratory of Histology and Embryology, Department of Microscopy, Rua Jorge Viterbo Ferreira 228, P 4050-313 Porto, Portugal
[c] Faculty of Sciences (FCUP), U.Porto – University of Porto, Department of Biology, Rua do Campo Alegre, P 4169-007 Porto, Portugal

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ABSTRACT

Peroxisome proliferator-activated receptor gamma (PPARγ) is a pivotal regulator of lipid and glucose metabolism in vertebrates. Here, we isolated and characterized for the first time the PPARγ gene from brown trout (*Salmo trutta* f. *fario*). Hormones have been reported to interfere with the regulatory function of PPARγ in various organisms, albeit with little focus on fish. Thus, primary hepatocytes isolated from juveniles of brown trout were exposed to 1, 10 and 50 μM of ethinylestradiol (EE2) or testosterone (T). A significant (3 fold) decrease was obtained in response to 50 μM of EE2 and to 10 and 50 μM of T (13 and 14 folds), while a 3 fold increase was observed at 1 μM of EE2. Therefore, trout PPARγ seems a target for natural/synthetic compounds with estrogenic or androgenic properties and so, we advocate considering PPARγ as another alert sensor gene when assessing the effects of sex-steroid endocrine disruptors.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs), a group of the well-known nuclear receptor superfamily, are ligand-activated transcription factors, involved in such diverse pathways as lipid and glucose homeostasis, control of cellular proliferation and differentiation (Rosen and Spiegelman, 2001). These receptors are structurally similar to steroid or thyroid hormone receptors, and their natural activating ligands are lipid derived substrates (Tyagi et al., 2011). PPAR activating ligands comprise: (a) endogenous compounds, such as fatty acids and eicosanoids; (b) drugs, like fibrates and thiazolidinediones; and (c) environmental chemicals, such as phthalates or organotins (Berger and Moller, 2002; Forman et al., 1997; Grün et al., 2006; Hurst and Waxman, 2003; Krey et al., 1997). Three isotypes (PPARα, PPARβ/δ and PPARγ), encoded by different genes, have been identified among a variety of species (Desvergne and Wahli, 1999; Kliewer et al., 1994), including distinct fish, such as European plaice (*Pleuronectes platessa*) (Leaver et al., 2005) and European sea bass (*Dicentrarchus labrax*) (Boukouvala et al., 2004). From the three PPARs, PPARγ [NR1C3] is the most studied one, in the contexts of energy, lipid and glucose control and insulin sensitivity (Ahmadian et al., 2013; Zieleniak et al., 2008). With functions in both physiologic and pathological conditions, it has been associated with normal cell differentiation and the pathophysiology of carcinogenesis (Grabacka et al., 2008; Rosen et al., 1999; Strand et al., 2012; Tachibana et al., 2008), type

Abbreviations: aa, amino acids; AR, androgen receptor; CODEHOP, consensus-degenerate hybrid oligonucleotide primer; DHT, dihydrotestosterone; dNTP, deoxyribonucleotide triphosphate; E2, 17β-estradiol; EDTA, ethylenediaminetetraacetic acid; EE2, 17α-ethinylestradiol; ER, estrogen receptor; FBS, fetal bovine serum; IPTG, isopropyl-beta-D-thiogalactopyranoside; LB medium, Luria-Bertani medium; ORF, open reading frame; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; qRT-PCR, quantitative real time polymerase chain reaction; RACE, rapid amplification of cDNA ends; SIAS, sequence identity and similarity; T, testosterone; Xgal, 5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside.

* Corresponding author at: CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, U.Porto – University of Porto, Rua dos Bragas 289, P 4050-123 Porto, Portugal.
E-mail addresses: tvmadureira@icbas.up.pt, taniavmad@hotmail.com (T.V. Madureira).
[1] Joint first authors. These authors contributed equally to this manuscript.
[2] Joint senior researchers.

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2 diabetes and atherosclerosis (Berger et al., 2005; Dong et al., 2015), inflammation and immunity (Kim et al., 2015). PPARγ was first cloned in the African clawed frog *(Xenopus laevis)* (Dreyer et al., 1992), subsequently in mouse (Zhu et al., 1993) and human (Greene et al., 1997), and later on in fish species, including grass carp (*Ctenopharyngodon idella*) (He et al., 2012), olive flounder (*Paralichthys olivaceus*) (Cho et al., 2009), yellow catfish (*Pelteobagrus fulvidraco*) (Zheng et al., 2015) and Atlantic salmon (*Salmo salar*) (Andersen et al., 2000; Sundvold et al., 2010).

Hormonal imbalances, either estrogenic or androgenic, have been reported to affect PPARγ expression. A negative crosstalk between estrogens and PPARγ expression levels is now well established in the literature, in both *in vivo* and *in vitro* conditions, mostly in human and mouse species, and particularly in cancer tissues and cell lines (*e.g.*, Bonofiglio et al., 2005; Chu et al., 2014; Foryst-Ludwig et al., 2008; Lin et al., 2013; Wang and Kilgore, 2002). In fish, a similar pattern has also been shown in cultured hepatocytes from zebrafish (*Danio rerio*) (Ibabe et al., 2005) and rainbow trout (*Oncorhynchus mykiss*) (Hultman et al., 2015a). Equally in fish, an *in vivo* descriptive study (Batista-Pinto et al., 2009) using female mature adult brown trout (*Salmo trutta* f. *fario*) showed a significant decrease of PPARγ mRNA levels during late vitellogenesis and prespawning periods. In contrast, the molecular dialog between androgens and PPARγ expression has been much less explored. Inhibition of PPARγ by T or DHT was reported in a human kidney (Du et al., 2009) and prostate cancer cell lines (Olokpa et al., 2016), in mouse 3T3-L1 preadipocytes (Sato et al., 2013) and in C3H 10T1/2 mouse pluripotent cells (Singh et al., 2003). In the above-mentioned *in vivo* seasonal study in brown trout, the decrease in the PPARγ mRNA levels was significant in male individuals in early vitellogenesis, late vitellogenesis and prespawning stages (Batista-Pinto et al., 2009). The similarity of results between sexes raised the hypothesis that both estrogens and androgens may have a role in the modulation of PPARγ function in this species. Moreover, as a nuclear receptor, PPARγ may be a potential target of xenobiotics, with either estrogenic or androgenic properties (Grimaldi et al., 2015; Sonnenschein and Soto, 1998), and therefore with potential endocrine disrupting effects.

Being the liver the primary site of steroids metabolism, it is herein proposed to investigate, *in vitro*, the possible influence of estrogenic (EE2) and androgenic (T) stimuli in the expression of PPARγ, in brown trout isolated primary hepatocytes, testing the hypothesis brought up by the *in vivo* results (Batista-Pinto et al., 2009). For this purpose, the complete gene sequence of the brown trout PPARγ was isolated prior to experimental assays. Overall, our data bring new insights about the regulation and eventual disruption of PPARγ by estrogenic and androgenic compounds.

2. Materials and methods

2.1. Animals

The fish used in the experiments were purchased from a public Aquaculture Station (Torno, Portugal) and allowed to acclimate during at least four weeks, with a natural photoperiod, at ±16 °C. Animals were fed every day (except on the day before the experiments) with dry granules for salmonids (T-4 Optiline, Skretting). Water quality parameters were measured periodically to grant suitable standards. All the animal procedures were performed in compliance with the Portuguese Decree-Law No. 113/2013 implementing EU Directive No. 2010/63 on animal protection for scientific purposes.

Juveniles of brown trout (*Salmo trutta* f. *fario*), approximately one year old, were used for all procedures, with mean mass (±standard deviation) of 87.1 (±9.5) g and a mean total length of 20.0 (±1.2) cm.

2.2. RNA extraction and synthesis of cDNA and cDNA RACE

Total RNA from liver and cell pellets was purified with the illustra™ RNAspin Mini Isolation Kit (GE Healthcare), according to manufacturer's instructions. The obtained RNA was quantified using the Qubit™ fluorometer (LifeTechnologies), with the Quant-iT RNA BR assay kit (Invitrogen) and loaded on a 1% agarose gel stained with GelRed (Biotium) for quality assessing. cDNA was synthetized with the iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer instructions, using 1 μg of total RNA from the liver and 0.25 μg from the cell pellets per 20 μL of total reaction volume. The SMARTer™ RACE cDNA Amplification Kit (Clontech) was used for the synthesis of cDNA RACE.

2.3. Isolation, characterization and phylogenetics of PPARγ sequence from brown trout

Degenerate primers were designed in the CODEHOP software using protein sequences from zebrafish, Atlantic salmon and rainbow trout (GenBank ABI30002.1, ACZ62641.1 and ADN92686.1, respectively). Two sets of primers were selected from the generated blocks (Table 1). Each PCR reaction mixture (total volume of 20 μL) included 6 μL of PCR grade water, 10 μL of 2 x Phusion™ Flash High Fidelity PCR Master Mix (Finnzymes), 1 μL of primer forward (100 μM), 1 μL of primer reverse (100 μM) and 2 μL of liver cDNA. The PCR reaction included an initial denaturation step (98 °C/10 s), 35 cycles of 98 °C/1 s; 55 °C/5 s; 72 °C/15 s and a final extension step (72 °C/1 min). The resulting bands were purified with the GRS PCR & Gel Band Purification Kit (Grisp), following the protocol for gel band purification.

RACE and Nested RACE primers (Table 1) were designed on the obtained specific sequences using Primer3 (v. 0.4.0) software (Koressaar and Remm, 2007; Untergasser et al., 2012). The SMARTer™ RACE cDNA Amplification Kit and the Advantage® 2 PCR Kit (Clontech) were used following the general instructions with minor modifications. The RACE PCR reaction mixture (total volume of 25 μL) included: 1.25 μL of 3′ or 5′ cDNA RACE, 2.5 μL of universal primer, 0.5 μL of 3′ or 5′ RACE primer, 17.25 μL of PCR grade water, 2.5 μL of 10× cDNA PCR buffer, 0.5 μL dNTP's and 0.5 μL of 50× Advantage cDNA Polymerase. The Nested PCR reaction had 2.5 μL of RACE PCR product diluted 1/50 with tricine EDTA, 0.5 μL of Nested universal primer, 0.5 μL of specific 3′ or 5′ Nested primer, 18 μL of PCR grade water, 2.5 μL of cDNA PCR buffer, 0.5 μL of dNTP's and 0.5 μL of Advantage cDNA Polymerase. The resulting bands from the Nested PCR were purified and sent for sequencing (STABVIDA, Portugal).

The complete PPARγ ORF was amplified with specific primers designed with Primer3 software (Table 1), using the Phusion™ Flash High-Fidelity PCR Master Mix with minor modifications to the protocol above-mentioned (40 cycles of amplification, annealing temperature of 59 °C and 30 s of extension per cycle). The retrieved band was isolated and ligated overnight at 4 °C, using the pGEM® T Easy Vector System (Promega). NZY5α competent cells (Nzytech) were transformed according to the protocol included in the instructions for the vector system employed. The colony selection was carried on LB/ampicillin/IPTG/X-Gal plates and the illustra™ plasmidPrep Mini Spin Kit (GE Healthcare) was used to extract and purify the plasmid DNA from the selected colonies. The plasmids were then digested with EcoRI for 1 h at 37 °C and the purified product was sequenced with M13 primers (STABVIDA, Portugal). Sequence identities and similarity were checked using the SIAS software (http://imed.med.ucm.es/Tools/sias.html). To determine the orthology of the brown trout sequence a maximum likelihood

Table 1
List of primer sequences used throughout the experiments.

Primer	Sequence 5′-3′	Purpose
StPPARgF1	CGGAAGCCCTTCTGCgaratgatgga	First species specific fragments
StPPARgR1	CCAGCTGCCGCAGGtcngtcatytt	
StPPARgF2	GACAAGGCCTCCGGCttycaytaygg	
StPPARgR2	CGCGGTCAGGGACttcatrtcrtg	
StPPARg_5RACE	GACAGTACTGGCACTTATTCCGGCTCTT	RACE and Nested RACE
StPPARg_3RACE	CCATCGAGGACCTCCAGGAGACAGT	
StPPARg_5RACE_Nested	GGTCATACACCAGTTTTAACCGCACTGG	
StPPARg_3RACE_Nested	AGTTGAAGACCATCCACCCAGACTGC	
StPPARg_cloneF	CTGCAACAACGGGATCAAG	Cloning of the total cDNA
StPPARg_cloneR	AACAGGTCTAGGACCAGGCTAA	
StPPARgRT_F	CGGAATAAGTGCCAGTAC	qRT-PCR
StPPARgRT_R	GGGTCCACATCCATAAAC	

phylogenetic tree was performed, with 1000 bootstrap replicates, based on the JTT matrix-based model. The analysis involved 29 amino acid sequences, listed on Fig. 1. All positions containing gaps and missing data were eliminated. There were a total of 387 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

A final alignment was done with BioEdit software using the following sequences: human (NP619725.2), zebrafish (ABI30002.1), rainbow trout (ADN92686.1) and Atlantic salmon (ACZ62641.1) (Fig. 2.).

2.4. Hepatocyte isolation

For the hepatocyte isolation, fish were anesthetized with ethylene glycol monophenyl ether at 0.6 mL/L (Merck). Two animals were used for each experiment. After withdrawal of the circulating blood trough the caudal vein, the liver was excised from the abdominal cavity and a two-step collagenase perfusion technique was performed in order to obtain the hepatocytes for culture, as previously described (Madureira et al., 2015). The dissociated hepatocytes were pelleted and then resuspended in Leibovitz's L15 medium, without phenol red, supplemented with 5% charcoal stripped FBS (Sigma-Aldrich), 100 μg/mL of streptomycin and 100 U/mL of penicillin (PAA Laboratories GmbH), before plating. Cell viability was assessed with the Countess™ Automated Cell Counter (Invitrogen), based on the trypan blue exclusion technique. Hepatocytes were cultured in 24 well plates (SPL Life Sciences), coated with poly-L-lysine (Sigma-Aldrich) at a density of 1×10^6/mL, in 500 μL of L15 medium and kept at 19 °C (without additional O_2/CO_2).

2.5. Hepatocyte exposure

After 24 h on plate, primary hepatocytes were exposed to EE2 (CAS 57-63-6, Sigma-Aldrich) and T (CAS 58-22-0, Sigma-Aldrich), in independent experiments, for a period of 96 h, with renewal of solutions every 24 h. The exposure time and renewal schedule were validated in previous assays (Madureira et al., 2015), and are in line with other *in vitro* published data (Hultman et al., 2015b).

Stock solutions of the compounds were prepared in ethanol p.a. (Merck) and the working solutions in L15 medium, in order to obtain a maximum ethanol concentration of 0.1%. For the EE2 exposure, the experimental design included the following conditions: control (L15 medium), solvent control (0.1% ethanol in L15 medium), EE2 (1, 10 and 50 μM). As to the experiment with T, five treatments were tested: control, (L15 medium), solvent control (0.1% ethanol in L15 medium) and T (1, 10 and 50 μM). In both experiments, each condition was accessed in six different wells distributed along three plates.

At the end of each exposure, cells were trypsinized using trypsin-EDTA (1×) 0.05%/0.02% in PBS (PAA Laboratories GmbH) and viability re-measured. Hepatocytes were pelleted by centrifugation (160*g* for 5 min at 4 °C), snap frozen in liquid nitrogen and kept at −80 °C until RNA extraction.

2.6. qRT-PCR

The relative mRNA expression levels of PPARγ were determined by qRT-PCR, using a Bio-Rad IQ5 equipment. Primers were designed using the Beacon Designer™ software, in two exons flanking one of the biggest conserved introns. The forward primer was designed at the predicted exon 4 and the reverse at the predicted exon 5, using the zebrafish sequence as a reference (Table 1).

A calibration curve was performed using a series of known dilutions of the purified plasmid containing PPARγ, starting at 1/10 000. The program included 30 s at 95 °C, followed by 40 cycles of 95 °C/5 s and 56 °C/10 s. To verify the specificity of the PCR product a melting curve was done at the end of the run (from 55 °C to 95 °C with 0.5 °C increments, 30 s per step). Each plate design comprised duplicates of each sample, and no template controls. As a reference gene *rpl8* was chosen, showing a steadily expression profile under the chosen experimental conditions, as already reported by others (Körner et al., 2008; Madureira et al., 2015).

The reaction mixtures (total volume of 20 μL) consisted of 5 μL of diluted (1/5) cDNA, 10 μL of SsoFast™ EvaGreen® Supermix (Biorad) and 0.6 μL (300 nM) of specific PPARγ primers (Table 1) or *rpl8* primers. The *rpl8* primers were the following: forward TCAGCTGAGCTTTCTTGCCAC and reverse AGGACTGAGCTGTTCATTGCG (Körner et al., 2008). The PCR program was similar to the one used for PPARγ except for the annealing temperature (59 °C). Gene expression was normalized by the Pfaffl method (Pfaffl, 2001), considering the respective PCR efficiencies (98.1 and 99% for PPARγ and r*pl8*, respectively).

2.7. Statistical analysis

The STATISTICA software (version 12.0, StatSoft Inc.) was used for the statistical analysis. Graphic presentation was designed with GraphPad Prism 6. The normality and homogeneity of variances of all data were inferred by using Shapiro-Wilk's W-test and Levene's test, respectively, with assumptions met with transformed data (logarithm). The Tukey test was used for comparisons between treatments groups, for both experiments. The statistical significance threshold was set at $p \leq 0.05$.

3. Results

3.1. Isolation and characterization of PPARγ sequence in brown trout

We isolated the full ORF of PPARγ in brown trout. The coding region contains 562 aa, with high protein identity and

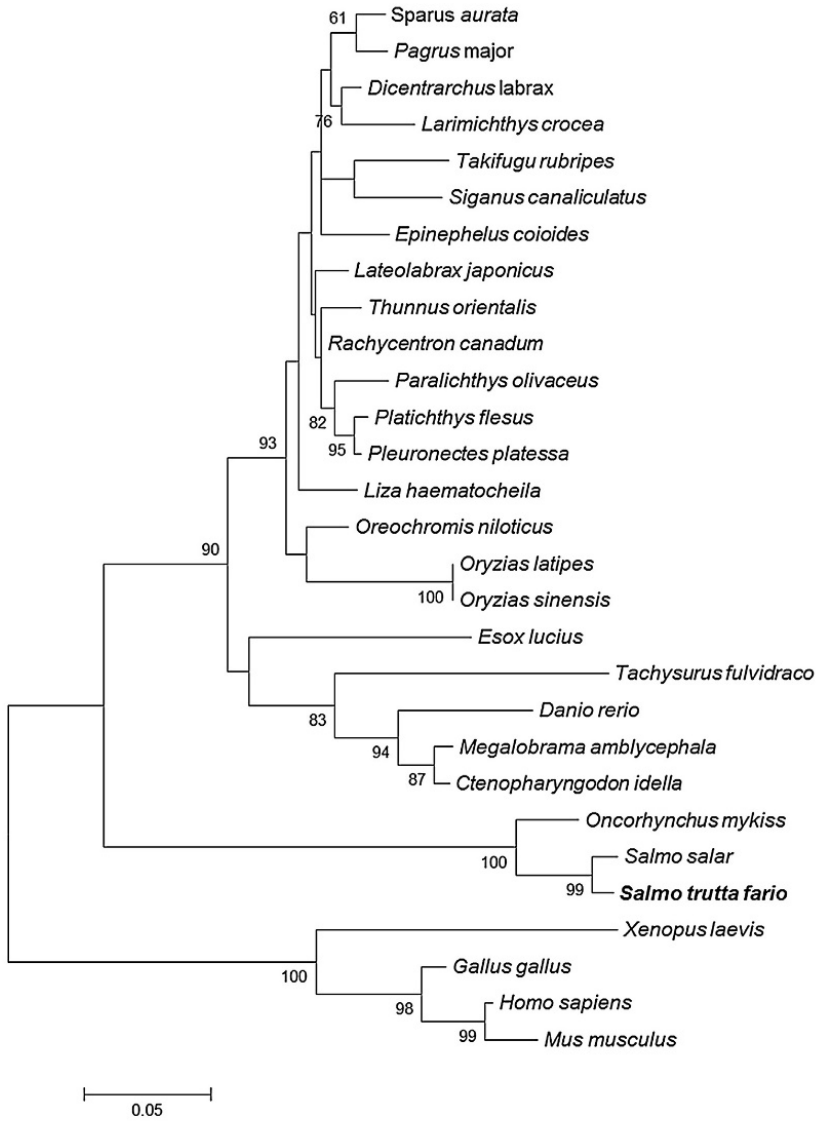

Fig. 1. Phylogenetic analysis of PPARγ sequences. Values at nodes represent percentage values of 1000 replicates. Values below 50% are not shown. *Homo sapiens* – NP619725.2; *Mus musculus* – AAA62110.1; *Gallus gallus* – NP001001460.1; *Xenopus laevis* – NP001081312.1; *Esox lucius* – XP010872599.1; *Danio rerio* – NP571542.1; *Platichthys flesus* – CAB51396.1; *Takifugu rubripes* – NP001091096.1; *Liza haematocheila* – AIK22389.1; *Epinephelus coioides* – AJF19172.1; *Megalobrama amblycephala* – ADH95692.2; *Sparus aurata* – AAT85618.1; *Dicentrarchus labrax* – AAT85617.1; *Rachycentron canadum* – ABV55020.1; *Oreochromis niloticus* – NP001277129.1; *Oryzias latipes* – NP001158348.1; *Oryzias sinensis* – AKG51674.1; *Thunnus orientalis* – BAK20459.1; *Paralichthys olivaceus* – ACO55651.1; *Lateolabrax japonicus* – ABC70398.1; *Pleuronectes platessa* – CAB51618.2; *Larimichthys crocea* – KKF28083.1; *Siganus canaliculatus* – AFH35108.1; *Ctenopharyngodon idella* – ACF70732.1; *Pagrus major* – BAF80459.1; *Tachysurus fulvidraco* – AGX15443.1; *Oncorhynchus mykiss* – NP001184141.1; *Salmo salar* – ACZ62641.1 and *Salmo trutta fario*.

similarity (97.86% and 98.70% respectively) with a previously reported sequence from Atlantic salmon (GenBank ACZ62641.1). Identities with the human PPARγ1 (NCBI NP619726.2), zebrafish (GenBank ABI30002.1) and rainbow trout (GenBank ADN92686.1) were 60.37%, 60.91% and 88.61%, respectively. To address the orthology of the isolated sequence we next undertook phylogenetics. The brown trout sequence robustly groups within the PPARγ teleost clade (Fig. 1.).

Some residues have been suggested to be key in determining the ligand specificity of the ligand pocket in human PPARγ (Nolte et al., 1998). Of these, four hydrophilic residues were proposed to integrate the entry site, D243, R288, E290 and E295. In brown trout, R288, E290 and E295 are conserved, while D243 is replaced by G319. In addition, of the four residues described as important for hydrogen-bonding with the PPAR ligands, H323, H449, K367 and Y473 (Nolte et al., 1998), only K367 and H449 are conserved in this species (H323 and Y473 are replaced by I408 and M557) (Fig. 2.). In the N-terminal (A/B) domain there are eight repeats of the decapeptide motif HSPDRSH(S/F)(C/F/Y)N (Fig. 2).

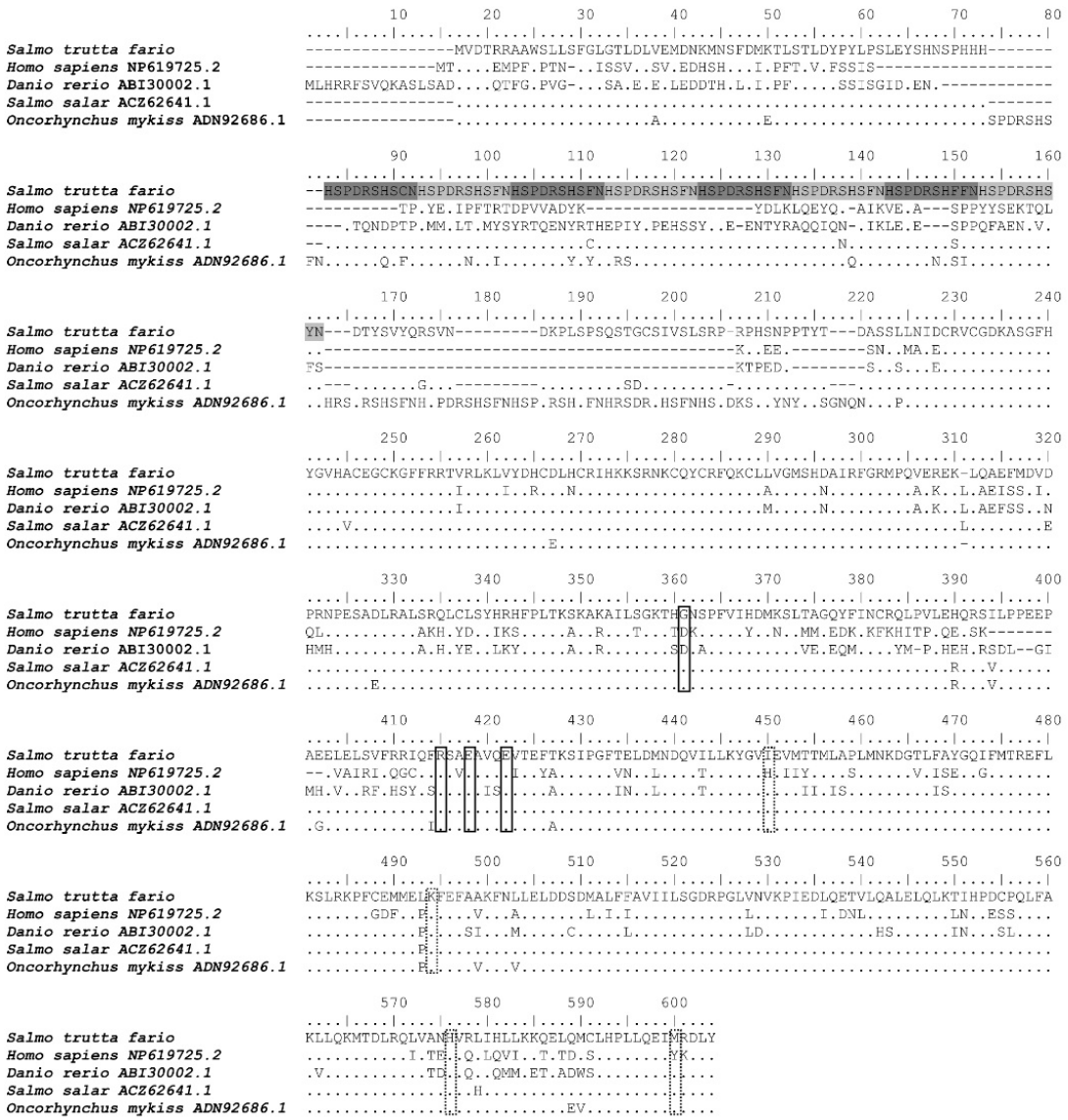

Fig. 2. Comparison of the amino acid sequences of PPARγ protein from *Salmo trutta fario*, *Homo sapiens* (NP619725.2), *Danio rerio* (ABI30002.1), *Salmo salar* (ACZ62641.1) and *Oncorhynchus mykiss* (ADN92686.1). The eight repeats of the decapeptide motif in *Salmo trutta fario* are highlighted in grey. The hydrophilic residues at the ligand entry site and those described as important for hydrogen-bonding with the PPAR ligands are marked with a full or intermittent box, respectively.

3.2. Cell culture viability

At 96 h of exposure, the viabilities ranged from 81.7% to 89.1% in EE2 experiment and 77.8% to 90.9% in the T experiment.

3.3. PPARγ mRNA levels in primary hepatocytes after estrogenic and androgenic inputs

In EE2 experiment there was an increase of 3 fold in the PPARγ mRNA levels after the lowest concentration tested (1 μM), comparing with the solvent control group. Contrarily, at the higher dose (50 μM) the relative mRNA levels decreased significantly in comparison to all other groups, while 10 μM of EE2 did not produce any effect when compared to both controls (Fig. 3). Regarding the T experiment, a significant reduction, of about 13 and 14 folds compared to the solvent control, was observed in the gene expression at the higher doses (10 and 50 μM, respectively) (Fig. 3).

4. Discussion

The PPARγ sequence isolated from brown trout exhibits a great identity (≈98%) with a previously reported sequence from Atlantic salmon (Sundvold et al., 2010). The referred sequence was denominated as PPARγ2 and displays an extra 20 aa, differing from

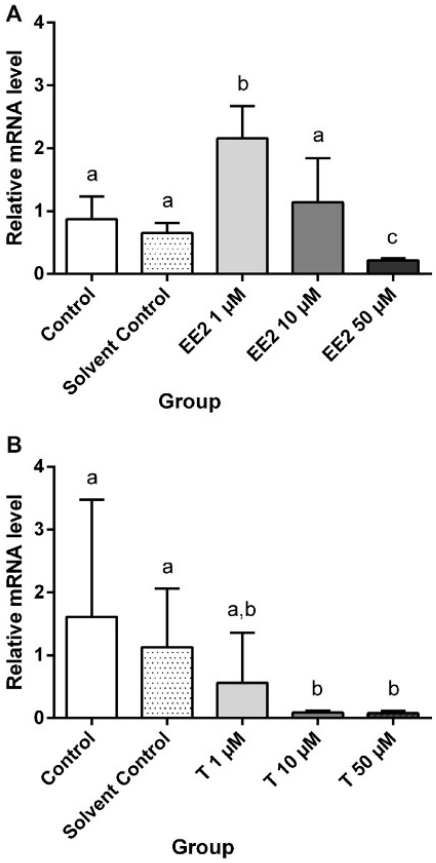

Fig. 3. Effect of (A) EE2 or (B) T in brown trout primary hepatocytes peroxisome proliferator-activated receptor gamma (PPARγ) mRNA levels, after 96 h exposure. Different letters mean significant differences ($p \leq 0.05$) between groups, by Tukey test.

other deposited sequences of the same fish species in the number of copies of a repetitive decapeptide motif. In another Atlantic salmon sequence, it was reported the presence of six repeats of the decapeptide HSPDR(S/N)HS(F/C/Y)N (Andersen et al., 2000), in contrast with the eight replicates found by Sundvold et al. (2010). In our case, brown trout displays eight copies of the unit HSPDRSH(S/F)(F/C/Y)N. Therefore, repetitive sequences seem to be a common point among distinct salmonid lineages, as in rainbow trout (GenBank ADN92686.1). The repeat motif has already been suggested to be involved in cofactor binding, resembling the sequence DHSHSF that occurs once in the A/F-1 region of human PPARγ (Andersen et al., 2000). In the yellow catfish, it has already been pointed the presence of eleven copies of a different repeat motif QE(N/T/I/S)(S/G//N/)Y(R/S), raising the question of potential differences in co-activator selection between fish species (Zheng et al., 2015).

In this study, the putative full PPARγ sequence was isolated from brown trout liver. In other fish species, including plaice and gilthead sea bream (*Sparus aurata*) (Leaver et al., 2005; Maradonna et al., 2015), grass carp (He et al., 2012), Pacific bluefin tuna (*Thunnus orientalis*) (Agawa et al., 2012), and Atlantic salmon (Andersen et al., 2000) the PPARγ transcripts were also found in liver. Interestingly, Sundvold et al. (2010) were not able to detect the PPARγ2 mRNA in the liver, noticing it only in the posterior kidney and spleen. PPARγ is also highly expressed in adipose tissue in several fish species, *e.g.*, plaice and sea bream (Leaver et al., 2005) and yellow catfish (Zheng et al., 2015), although it seems less expressed in others, *e.g.*, Nile tilapia (*Oreochromis niloticus*) (He et al., 2015) and grass carp (He et al., 2012), revealing tissue-species specificities. In 13-month-old Pacific bluefin tuna, PPARγ mRNA levels were found to positively correlate with the lipid content of the various white muscles studied (Agawa et al., 2012), therefore reinforcing the link between the activity of this gene and lipid physiology.

Several studies in human, mouse and rat have reported that sex steroids such as E2, T, and DHT exert an effect on PPARγ expression (Campbell et al., 2003; Jeong and Yoon, 2011; Salehzadeh et al., 2011; Singh et al., 2003). Sex-specific differences on PPARγ gene expression may also exist (Dieudonne et al., 2000; Salehzadeh et al., 2011). It is also well known that the pharmacological effect of pioglitazone, a PPARγ agonist with hypoglycemic action in humans, differs between sexes, with women requiring a smaller treatment dose and experiencing higher side effects (Sato et al., 2013). These gender variations emphasize the role of sexual hormones on the modulation of this gene.

In fish, the interaction between PPARγ and hormones is still a relatively unexplored topic, and data are scarce. In our *in vitro* approach, using primary cultured hepatocytes from juvenile brown trout, the effects of EE2 and T on PPARγ expression were accessed and compared with previous *in vivo* data from the same species (Batista-Pinto et al., 2009). The data obtained herein partially replicate the *in vivo* results, which showed a significant decrease in the PPARγ mRNA levels in the highest estrogenic/androgenic phases of the reproductive cycle. The EE2 concentrations we chose are in the range of estrogen doses used by others to induce, *in vitro*, estrogenic responsive genes, namely ER and vitellogenin (Boyce-Derricott et al., 2009; Riley et al., 2004). The selected T concentrations are also in line with the androgen levels used in other *in vitro* studies with fish (Kortner et al., 2009; Riley et al., 2004).

Despite the cited partial replication of *in vivo vs. in vitro* patterns, the exposure to EE2 revealed a paradoxical effect on PPARγ gene expression. A non-monotonic response was obtained with the different doses of estrogen. The opposite results of lower and higher doses of EE2 on PPARγ gene expression, revealed an apparent hormesis effect. Although this concept is not universally accepted, bidirectional dose responses to natural and xenoestrogens were previously observed in various contexts and organisms (Genovese et al., 2014; Inagaki et al., 2010). Estrogens exert their effects through a diversity of pathways, which make them prone to hormetic responses (Strom et al., 2011). So, herein, the lowest dose (1 μM of EE2) induced an increase on the PPARγ mRNA levels and the opposite effect was obtained at the higher dose (50 μM). In the T experiment, a monotonic decrease of PPARγ mRNA levels was observed after the higher doses (10 and 50 μM).

An increase in PPARγ mRNA expression in response to estrogenic inputs has been already reported, namely in the red gastrocnemius muscle of ovariectomized female rats (Campbell et al., 2003), and in differentiated preadipocytes from epididymal and parametrial tissue of male and ovariectomized female rats (Dieudonne et al., 2000). In mouse 3T3-L1 cells matured into adipocytes, a significant increase in the PPARγ protein levels was also found by western blot after 1 week of exposure to E2 at 10^{-9} M (Sato et al., 2013). Conversely, in white adipose tissue of ovariectomized mice the co-administration of troglitazone (PPARγ agonist) and E2, both at 10 μM, decreased the troglitazone induced upregulation of PPARγ and target genes (Jeong and Yoon, 2011). In fish, there are also conflicting results reporting up or down-regulation of PPARγ after estrogenic inputs. In zebrafish primary hepatocytes, PPARγ mRNA was increased in response to 10 nM of EE2 (Maradonna et al., 2013). In other study with the exact same model there was an increase in PPARγ expression (measured *via*

image analysis as grey level per immunostained cell), in parallel with a decrease in the percentage of PPARγ immunolabeled positive cells, in response to 10 μM of E2 (Ibabe et al., 2005). Also in rainbow trout primary hepatocytes, the PPARγ expression was significantly down-regulated at 30 nM of EE2 (Hultman et al., 2015a). Additionally, other less potent estrogenic compounds, namely phytoestrogens, nonylphenol, 4-*tert*-octylphenol, and bisphenol-A, have been also showing *in vivo* their interferences in PPARγ mRNA expression in distinct fish species (Cleveland and Manor, 2015; Maradonna et al., 2015; Qiu et al., 2016).

The diminishing of the PPARγ mRNA we found, as a result of an androgenic stimulus, is in consensus with other *in vivo and in vitro* studies. In prenatal T-treated sheep, PPARγ mRNAs in liver were significantly reduced in comparison with the control (Nada et al., 2010). Similar trends were obtained *in vitro* after exposure of a human kidney cell line to10 nM of T, in a transcriptional transaction assay (Du et al., 2009). In human prostate cancer cell lines exposed to 0.1–10 nM of DHT, a time and concentration dependent decrease was also observed (Olokpa et al., 2016). Likewise, in a mouse pluripotent mesenchymal cell line a 12 days exposure to T and DHT was able to induce a decrease in PPARγ2 mRNA and protein levels (Singh et al., 2003). DHT also caused a down-regulation of PPARγ expression in mouse 3T3-L1 cells after 2 weeks of exposure to 10^{-10} and 10^{-11} M of DHT (Sato et al., 2013). In fish, an *in vivo* exposure of medaka (*Oryzias latipes*) larvae to the anabolic-androgenic steroid17β-trenbolone led to a down-regulation of two genes with functions in the endocrine system and controlled by the PPARγ signalling pathway (Mizukami-Murata et al., 2015). However, down-regulation of PPARγ by androgens in not always true, as no effects existed on PPARγ2 protein expression in differentiated preadipocytes from male and female rats after T or DHT treatments (Dieudonne et al., 2000).

In theory, the decreases in the PPARγ mRNA levels as a consequence of steroid hormones exposure may be due to the activation of the nuclear receptors of the latter (*i.e.*, ER or AR). Negative feedback relations between nuclear receptors may result from competition for co-activator binding, as it was already demonstrated (Foryst-Ludwig et al., 2008; Lopez et al., 1999). Accordingly, PPARγ shares co-activators with both ER and AR (Foryst-Ludwig et al., 2008; Heinlein et al., 1999; Jeong and Yoon, 2011). In addition, in the case of estrogens it has been suggested that eventually PPARγ and ER may function as synergetic inducers (Sato et al., 2014). However, as illustrated above, the interplay is very complex and conflictual results may arise from estrogenic and androgenic inputs, thus calling for further studies with more conditions and models.

Globally, this study reveals new data regarding the nature of the PPARγ gene and strongly suggests its modulation by female and male sex steroids in brown trout. Nuclear receptors like PPARγ are primary targets of environmental contaminants (Grimaldi et al., 2015), which can have estrogenic and androgenic properties and can mimic or antagonize endogenous hormones (Sonnenschein and Soto, 1998). Tributyltin chloride, for example, was shown to directly interfere with PPARγ in vertebrates, including fish (Grün et al., 2006; Lyssimachou et al., 2015; Pavlikova et al., 2010), inducing adipogenesis and disturbing key regulators of adipogenesis and lipogenic pathways (Grün et al., 2006). Overall, since both estrogens and androgens seem able to interfere with that gene activity, in brown trout and other species, in our understanding PPARγ should be considered as another target gene when assessing the effects of endocrine disrupting chemicals.

Conflict of interest

None.

Acknowledgments

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Chapter 3

Testosterone-induced modulation of peroxisomal morphology and peroxisome-related gene expression in brown trout (*Salmo trutta* f. *fario*) primary hepatocytes

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Testosterone-induced modulation of peroxisomal morphology and peroxisome-related gene expression in brown trout (*Salmo trutta* f. *fario*) primary hepatocytes

Célia Lopes[a,b], Fernanda Malhão[a,b], Cláudia Guimarães[b], Ivone Pinheiro[a,b], José F. Gonçalves[a,c], L. Filipe C. Castro[a,d], Eduardo Rocha[a,b,*], Tânia V. Madureira[a,b]

[a] *Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), U.Porto – University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208, Matosinhos, Portugal*

[b] *Institute of Biomedical Sciences Abel Salazar (ICBAS), U.Porto – University of Porto, Laboratory of Histology and Embryology, Department of Microscopy, Rua Jorge Viterbo Ferreira 228, P 4050-313, Porto, Portugal*

[c] *Institute of Biomedical Sciences Abel Salazar (ICBAS), U.Porto – University of Porto, Department of Aquatic Production, Rua Jorge Viterbo Ferreira 228, P 4050-313, Porto, Portugal*

[d] *Faculty of Sciences (FCUP), U.Porto – University of Porto, Department of Biology, Rua do Campo Alegre, P 4169-007, Porto, Portugal*

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ABSTRACT

Disruption of androgenic signaling has been linked to possible cross-modulation with other hormone-mediated pathways. Therefore, our objective was to explore effects caused by testosterone – T (1, 10 and 50 μM) in peroxisomal signaling of brown trout hepatocytes. To study the underlying paths involved, several co-exposure conditions were tested, with flutamide – F (anti-androgen) and ICI 182,780 – ICI (anti-estrogen). Molecular and morphological approaches were both evaluated. *Peroxisome proliferator-activated receptor alpha* (*PPARα*), *catalase* and *urate oxidase* were the selected targets for gene expression analysis. The *vitellogenin A* gene was also included as a biomarker of estrogenicity. Peroxisome relative volumes were estimated by immunofluorescence, and transmission electron microscopy was used for qualitative morphological control. The single exposures of T caused a significant down-regulation of *urate oxidase* (10 and 50 μM) and a general up-regulation of *vitellogenin*. A significant reduction of peroxisome relative volumes and smaller peroxisome profiles were observed at 50 μM. Co-administration of T and ICI reversed the morphological modifications and *vitellogenin* levels. The simultaneous exposure of T and F caused a significant and concentration-dependent diminishing in *vitellogenin* expression. Together, the findings suggest that in the tested model, T acted *via* both androgen and estrogen receptors to shape the peroxisomal related targets.

1. Introduction

The relevance of natural and synthetic androgens as endocrine disrupting chemicals (EDCs) is still an unexplored subject, compared with the vast amount of studies focusing on estrogenic compounds (Martyniuk and Denslow, 2012; Shilling and Williams, 2000). Despite that, data have shown that androgenic substances exist in the environment (Backe et al., 2011; Hotchkiss et al., 2008) and that their effects have an important and maybe even equivalent environmental significance as their estrogenic counterparts (Huang et al., 2016; Scholz and Mayer, 2008). Therefore, there has been an increasing interest in addressing the toxicological effects of androgens and also their influence in hormonally regulated physiological processes (Ankley et al., 2003; Bogers et al., 2006; Guillette and Gunderson, 2001; Martyniuk and Denslow, 2012; Zheng et al., 2016).

Diverse negative consequences of exposure to androgens are documented. In fish, the most frequent effects are the development of male secondary characteristics in females and male-biased sex ratios (Ankley et al., 2001; Ankley et al., 2003; Huang et al., 2016; Larsson et al., 2000). Other common outcomes are the decrease of fecundity, imbalances in endogenous hormonal levels and histological alterations in the gonads (Ankley et al., 2001; Ankley et al., 2003; Bhatia and Kumar, 2016; Margiotta-Casaluci and Sumpter, 2011; Örn et al., 2006). Another common finding among studies using fish models exposed to androgens has been the modulation, at both gene and protein level, of vitellogenin, a precursor of the egg yolk protein. *In vivo,* fish studies

* Corresponding author at: ICBAS – Institute of Biomedical Sciences Abel Salazar, U.Porto – University of Porto, Laboratory of Histology and Embryology, Department of Microscopy, Rua Jorge Viterbo Ferreira 228, P 4050-313, Porto, Portugal.
E-mail address: erocha@icbas.up.pt (E. Rocha).

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have reported both repression (Ankley et al., 2004; Lazier et al., 1996; Miracle et al., 2006) and induction (Andersen et al., 2006; Ankley et al., 2001; Bhatia and Kumar, 2016). In *in vitro* assays, a large variety of natural and synthetic androgens have also been able to increase the vitellogenin protein and gene levels (Kim et al., 2003; Kwon et al., 2005; Mori et al., 1998; Riley et al., 2004). Natural androgens include testosterone (T), dihydrotestosterone (DHT) and 11-ketotestosterone (11-KT), while synthetics encompass, for example, trenbolone and methyltestosterone (MT) (Hotchkiss et al., 2008). The effects of anti-androgenic substances are also a matter of concern with respect to endocrine disruption (Hotchkiss et al., 2008; Scholz and Mayer, 2008). The latter belong to different classes of chemicals, such as pesticides, fungicides, herbicides, some phthalate plasticizers and drugs like bicutilamide or flutamide (F) (Daxenberger, 2002; Sumpter, 2008). In fish, anti-androgens have been shown to alter some reproduction features, such as courtship behavior and nest building (Bayley et al., 2002; Sebire et al., 2008), reduce fecundity (Jensen et al., 2004; Kang et al., 2006) and induce histological changes, in both male and female gonads (Bhatia and Kumar, 2016; Bhatia et al., 2014a; Bhatia et al., 2014b; Jensen et al., 2004; Kang et al., 2006; Kinnberg and Toft, 2003; Rajakumar et al., 2012).

Peroxisomes are plastic organelles with a high capacity to change their morphology, number and enzyme contents, depending on the physiological demands (Schrader et al., 2012). The peroxisomal functions include the metabolism of hydrogen peroxide and β-oxidation of fatty acids, as well as synthesis of cholesterol, bile acids and plasmalogens (Depreter et al., 2002; Schrader et al., 2012). Peroxisomes are extremely rich in oxidative enzymes, such as catalase, D-amino acid oxidase, acyl-CoA oxidase, and urate oxidase (Depreter et al., 2002; Ram and Waxman, 1994). Peroxisome proliferator-activator receptors (PPARs) play crucial roles mediating the peroxisomal metabolic functions and morphological changes (Arukwe and Mortensen, 2011; Nakajima et al., 2000). Molecular evidence indicates that androgens interfere with PPAR signaling. In human prostate cancer cell lines, mibolerone was shown to down-regulate *PPARα* mRNA levels (Collett et al., 2000) and DHT reduced both *PPARγ* mRNA and protein expressions (Olokpa et al., 2016). In rodents, DHT and T up-regulated *PPARα* mRNA levels in primary mouse hepatocytes (Lin et al., 2008) and in a rat model (Yang et al., 2016), respectively. On the contrary, DHT decreased PPARγ protein expression in mouse adipocytes (Sato et al., 2013). In fish, we recently reported a reduction of *PPARγ* mRNA levels in primary hepatocytes after exposure to T (Lopes et al., 2016). Other study with adult triploid rainbow trout showed no effect on liver *PPAR* (α, β and γ) mRNA levels after supplemented feed with T and DHT (Cleveland and Weber, 2016). In addition, dehydroepiandrosterone (DHEA), an adrenal androgen, induced peroxisome proliferation (Frenkel et al., 1990; Rao et al., 1993) and up-regulated the expression of *PPARα* and related peroxisomal genes, *e.g. acyl-CoA oxidase* and *catalase* in rats (Depreter et al., 2002; Ram and Waxman, 1994).

Sexual dimorphic responses have been reported in PPARα regulated pathways (Djouadi et al., 1998; Dunn et al., 2007; Jalouli et al., 2003; Nakajima et al., 2000). Specifically in fish liver, some studies found no sex differences in PPARα expression levels (Ibabe et al., 2005; Maradonna et al., 2013; Raingeard et al., 2006), while others reported that males have a higher mRNA expression than females (Eide et al., 2014). In an *in vivo* study with brown trout along a reproductive cycle, when the levels of T and estrogens were lower as in early vitellogenesis phase, females expressed higher *PPARα* mRNA than males (Batista-Pinto et al., 2009). These findings suggest an active role of sex hormones, either estrogens or androgens, in mediating peroxisomal signaling. Data from our previous research uncovered estrogenic influences in peroxisomal targets in brown trout (*Salmo trutta* f. *fario*) (Madureira et al., 2015a). Herein, we aimed to expand the preceding studies by investigating the regulating effects of a natural androgen (T) in the expression of some peroxisome-related genes, using primary brown trout hepatocytes as a model. *PPARα* (regulator of hepatic lipid metabolism, including fatty acid uptake, activation and oxidation and triglyceride storage) (Rakhshandehroo et al., 2010) and two peroxisomal enzymes, *catalase* (responsible for detoxifying hydrogen peroxide) (Arukwe and Mortensen, 2011) and *urate oxidase* (catalyzes the conversion of urate to allantoin and hydrogen peroxide) (Moriwaki et al., 1999) were the selected targets. In addition, *vitellogenin A* was also included as a marker of estrogenic signaling. Morphological changes in peroxisomes were also monitored by stereological methods and transmission electron microscopy (TEM). Mixtures of the androgen with both androgen (F) and estrogen receptor (ICI 182,780 – ICI) antagonists were tested to generate an *in vitro* mechanistic model that can offer insights about the hypothesized regulation of peroxisomal signaling by T, exploring both phenotypic and molecular features.

2. Materials and methods

2.1. Animals

Juvenile brown trout (one-year-old) were used in all the experiments, having a mean weight (± standard deviation) of 86.9 (± 15.3) g and a mean total length of 20.5 (± 1.4) cm.

The fish were acquired from a public Aquaculture Station (Torno, Portugal), and acclimated in our animal facility for one month with a photoperiod of 12 h light/12 h dark at ± 16 °C. They were fed daily with dry pellets for salmonids (T-4 Optiline, Skretting) and fasted for 24 h before hepatocytes isolation. Water parameters were tested periodically to ascertain that suitable standard conditions were followed. All the animal procedures were executed in obedience with the Portuguese Decree-Law No. 113/2013 implementing EU Directive No. 2010/63 on animal protection for scientific purposes.

2.2. Primary hepatocyte isolation

The fish were first anesthetized with ethylene glycol monophenyl ether (Merck) at 0.6 mL/L. Excess of circulating blood was removed through puncture of the caudal vein. The liver was then excised and a two-step collagenase perfusion technique was done, as previously detailed (Madureira et al., 2015a). Hepatocytes were obtained by mechanical dissociation and sequentially filtered through two nylon membranes with a mesh size of 200 μm and 50 μm. Finally, cells were centrifuged 3 × 5 min at 160 G and pellets resuspended in Leibovitz's L-15 medium without phenol red (Invitrogen) supplemented with 5% charcoal-stripped fetal bovine serum, 100 μg/mL of streptomycin and 100 U/mL of penicillin (PAA Laboratories GmbH). Cell viability was assessed with the Countess™ Automated Cell Counter (Invitrogen), using the trypan blue exclusion assay. Hepatocytes were plated at 1×10^6 cells/mL, in 500 μL of the above described L15 medium, in 24 well plates (SPL Life Sciences) coated with poly-L-lysine, and cultured at 19 °C (with no extra O_2/CO_2).

2.3. Experimental design

In each experiment, primary hepatocytes from two fish were used. Cells were plated in order to obtain equally distributed wells per fish, for each condition and endpoint. In the present context, each well was considered as the experimental unit (replicate) because each had the same chance of being independently allocated to any of the treatment conditions. Two exposure times were tested: 72 and 96 h, with medium changes every 24 h. Stock solutions were prepared in ethanol p.a. (Merck) and the working solutions in supplemented L15 medium, with a maximum of 0.1% ethanol. These optimized conditions have already been used in few distinct set-ups for the same *in vitro* model (Lopes et al., 2016; Madureira et al., 2016; Madureira et al., 2015a; Madureira et al., 2015b). The viability was measured in two wells per fish/condition at the end of exposures. For catalase immunofluorescence, two

wells per fish/condition were collected at 96 h of exposure. Although wells were maximized considering the isolation yield, only one well per fish/condition was available for processing at each sampling time, for the qualitative electron microscopy morphological control. For the molecular analysis, each condition was assessed in six wells (3 from each fish), at each time of exposure, distributed into three different plates.

At the end of each exposure, the cells were trypsinized using trypsin-EDTA (1x) 0.05%/0.02% in PBS (PAA Laboratories GmbH) and harvested by centrifugation (160 G for 5 min at 4 °C). The cell pellets were then processed for the different purposes.

2.3.1. Experiment I

After 24 h on plate, hepatocytes were exposed to T (CAS 58-22-0, Sigma-Aldrich). Five treatments were tested: control (supplemented L15 medium) (C), solvent control (0.1% ethanol in supplemented L15 medium) (SC) and T at 1, 10 and 50 μM (T1, T10 and T50).

2.3.2. Experiment II

After 24 h on plate, hepatocytes were exposed to F (CAS 13311-84-7, Sigma-Aldrich) and to mixtures of T + F or T + ICI (CAS 129453-61-8, Sigma-Aldrich). The treatments tested were: C (supplemented L15 medium), SC (0.1% ethanol in supplemented L15 medium), F at 10 and 50 μM (F10 and F50), two mixtures of T with F (Mix I T + F - 10:10 μM and Mix II T + F - 10:50 μM) and two mixtures of T with ICI (Mix I T + ICI -10:10 μM and Mix II T + ICI - 10:50 μM).

2.4. Morphological analysis

2.4.1. Catalase immunofluorescence and stereology

Immediately after centrifugation, cell pellets were fixed in a commercial solution of 10% (v/v) neutral buffered formalin (Bio-Optica) for 24 h, placed in histogel (Bio-Optica) and routinely processed for paraffin embedding. Five (2 μm) consecutive sections were obtained in a rotary microtome (Leica RM 2255) and used for indirect immunofluorescence technique. The rabbit anti-catalase antibody (ab87529, Abcam) and a secondary goat anti-rabbit IgG antibody conjugated with CFL 488 (sc-362262, Santa Cruz Biotechnology) allowed the visualization of the immunolabeled structures. The immunofluorescence technique for catalase was fully described before (Madureira et al., 2015b). The immunolabeled slides were visualized and photographed in an Olympus IX71 inverted fluorescent microscope coupled with a digital camera (DP71, Olympus). Photographs were acquired using the 100× immersion objective. Three fields were randomly selected per section (on a total of five), giving 15 photos/animal. For the stereological approach, the photos were analyzed using the Image J software (version 1.47). A digital point grid was superimposed into the photos, allowing the estimation of the relative peroxisome volume in the hepatocyte cytoplasm V_V (peroxisome,cytoplasm), as previously validated for this species (Madureira et al., 2015b). The groups selected for analysis were C, SC and T (1, 10 and 50 μM), in experiment I and C, SC, Mix I T + F, Mix II T + F, Mix I T + ICI and Mix II T + ICI, in experiment II.

2.4.2. Transmission electron microscopy with catalase cytochemistry

Immediately after centrifugation, pellets were fixed in freshly prepared 2.5% glutaraldehyde in sodium cacodylate-hydrochloric acid buffer 0.1 M, pH 7.2, for 1 h at 4 °C. For better visualization of the peroxisomes, pellets were subjected to a catalase cytochemical technique. For this purpose, they were incubated, for 2 h, in a 37 °C water bath, with the catalase enzyme medium, which comprised 2% DAB (3,3′-diaminobenzidine) and 0.12% of hydrogen peroxide in 0.1 M Tris-HCl buffer (pH 8.5). Additionally, a post-fixation step was performed, with a solution of 1.5% potassium ferrocyanide and 1% osmium tetroxide in the aforementioned cacodylate buffer (0.15 M). The pellets were then dehydrated through a graded series of ethanol, followed by propylene oxide baths, before infiltration and embedding in an epoxy mixture. The ultrathin sections, obtained in an ultramicrotome (Leica Reichert Supernova) were then visualized in an electron microscope (JEOL 100CXII) and photographed using a Gatan Orius SC200 CCD camera.

2.5. Gene expression

2.5.1. RNA extraction and cDNA synthesis

Hepatocyte pellets were snap frozen in liquid nitrogen and kept at −80 °C until RNA extraction. Total RNA was extracted using the illustra™ RNAspin Mini Isolation Kit (GE Healthcare), which includes a digestion step with DNase I. The obtained RNA was quantified using the Qubit™ fluorometer (LifeTechnologies), with the Quant-iT RNA BR assay kit (Invitrogen). To verify the quality of the extracted RNA, 2 μL were loaded on a 1% agarose gel stained with GelRed (Biotium). cDNA was synthetized with the iScript™ cDNA Synthesis Kit (Bio-Rad), using 0.25 μg of total RNA to 20 μL of total reaction volume.

2.5.2. qRT-PCR

The relative mRNA expression levels of the target genes were determined by qRT-PCR, using a Bio-Rad IQ5 equipment. A calibration curve was performed for each gene, using a series of known dilutions of liver cDNA. To verify the specificity of the PCR product a melting curve was done at the end of each run (from 55 °C to 95 °C with 0.5 °C increments, 30 s per step). Each plate design comprised duplicates of each sample, and no template controls. For gene expression normalization, two housekeeping genes – *ribosomal protein L8* (*rpl8*) and *β-actin* – were selected. The reaction mixtures (total volume of 20 μL) consisted of 5 μL of diluted (1/10) cDNA, 10 μL of iQ™ SYBR®Green Supermix (Bio-Rad)

Table 1
List of primer sequences and respective annealing temperatures and efficiencies.

Gene	Primer Sequences (5′–3′)	Annealing Temp. (°C)	Efficiency (%)	Reference
Vitellogenin A	F - AACGGTGCTGAATGTCCATAG R - ATTGAGATCCTTGCTCTTGGTC	62.9	99.0	Körner et al. (2008)
Catalase	F - CACTGATGAGGGCAACTGGG R - CTTGAAGTGGAACTTGCAG	58.0	91.4	Batista-Pinto (2007)
Urate oxidase	F - CTTCCGAGACCGCTTCAC R - CATTCTGGACCTTGTTGTAGC	59.0	90.6	Madureira et al. (2015a)
PPARα	F - CGGGTGACAGGGAGGTGGAGGAC R - GGTGAGGATGGTGCGGGCTTTGG	59.0	100.6	Madureira et al. (2015a)
β-actin	F - TCTGGCATCACACCTTCTAC R - TTCTCCCTGTTGGCTTTGG	55.0	96.1	Madureira et al. (2017)
rpl8	F - TCAGCTGAGCTTTCTTGCCAC R - AGGACTGAGCTGTTCATTGCG	59.0	93.8	Körner et al. (2008)

and 0.6 μL (200 nM) of specific primers for each gene (Table 1). Gene expression levels were calculated according to the Pfaffl method of relative quantification (Pfaffl, 2001).

2.6. Statistical analysis

The Past3 (version 3.11) free software (http://folk.uio.no/ohammer/past/) was used for statistical analysis. Graphs were generated in the STATISTICA software (version 12.0, StatSoft Inc.). The normality and homogeneity of variances of all data were checked by using Shapiro-Wilk W and Levene tests, respectively. When needed, data were transformed (logarithm, square root, inverse) to achieve normalization, and the Tukey post-hoc test was used to assess comparisons between groups. When normalization was not attained, the non-parametric Mann-Whitney pairwise test with sequential Bonferroni correction was used for the comparisons. For gene expression, only a couple of differences were observed between the two times of exposure (for *catalase* in experiment I and *vitellogenin A* in experiment II), and results always followed the same trends. For that reason, data were grouped for further analysis. The statistical significance threshold was set at $p < 0.05$.

3. Results

3.1. Cell culture viability

The viabilities ranged from 61% to 91% in experiment I, and between 40% and 95% in experiment II considering all experimental conditions. No statistical differences were observed when comparing all conditions in both experiments.

3.2. Catalase immunofluorescence and stereology

In experiment I, a significant reduction of V_V (peroxisome,-cytoplasm) was evident at the highest T concentration (50 μM), in relation to the SC group (Fig. 1). In experiment II, a significant increase in V_V (peroxisome,cytoplasm) was found between both Mix T + ICI conditions and Mix I T + F (Fig. 1).

3.3. Transmission electron microscopy with catalase cytochemistry

In experiment I, no differences were noted between the C and SC conditions. The highest T concentration (50 μM) showed the presence of smaller peroxisome profiles, in relation to control conditions (Fig. 2). At T 10 μM, the effect was not so evident. In experiment II, the smaller peroxisomes triggered by the T exposure were not observed so frequently in any of the mixture conditions (Fig. 3).

3.4. Gene expression

The mRNA levels obtained after experiment I are shown in Fig. 4. Those of *urate oxidase* were significantly decreased at the higher concentrations of T (10 and 50 μM). As for *vitellogenin A*, a significant increase was observed at all concentrations of androgen tested, in a concentration-dependent manner. The expression of the remaining genes (*PPARα* and *catalase*) was not significantly altered at the tested conditions.

In experiment II, exposure to F interfered with the expression of *catalase*, which did not change in experiment I (Fig. 5). Regarding *urate oxidase* expression, F alone (at 50 μM) and in combination with T caused a significant reduction, just as seen for *catalase* in the same conditions. In both mixtures of T + ICI, the expression of *urate oxidase* was significantly lower than in both controls, but higher than in the conditions containing 50 μM of F (Fig. 5).

The *vitellogenin A* mRNA levels (Fig. 5) were reversed to control levels in the mixture containing T and the higher concentration of ICI (50 μM). In both mixtures with F, a significant increase in relation to C and SC levels was observed, but a concentration-dependent inhibition was clearly noticed with the increasing F concentration, comparing the two mixture conditions (Fig. 5).

4. Discussion

By using *in vitro* approaches with brown trout primary hepatocytes, we previously made advances in deciphering a role of estrogenic inputs in peroxisomes morphology and gene expression alterations in classical peroxisomal pathways (Madureira et al., 2015a). Additionally, the

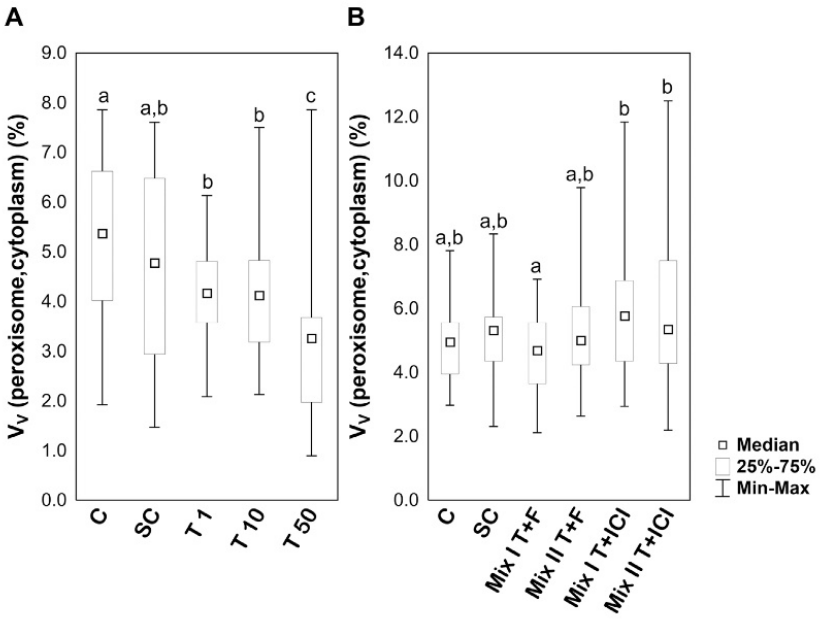

Fig. 1. Brown trout relative volume of peroxisomes in relation to hepatocyte cytoplasm – V_V (peroxisome,cytoplasm) – after catalase immunofluorescence, at 96 h of exposure. A – Experiment I: C – control (L-15 medium); SC – solvent control (0.1% etanol); T1, T10 and T50 – 1, 10 and 50 μM of testosterone. B – Experiment II: C; SC; Mix I T + F – mixture of 10 μM of T and 10 μM of flutamide (F); Mix II T + F – mixture of 10 μM of T and 50 μM of F; Mix I T + ICI – mixture of 10 μM of T and 10 μM of ICI 182,780 (ICI); Mix II T + ICI – mixture of 10 μM of T and 50 μM of ICI. Different letters mean significant differences between groups according to Tukey test (A) or to Mann-Whitney pairwise with sequential Bonferroni correction test (B). n = 4 replicates.

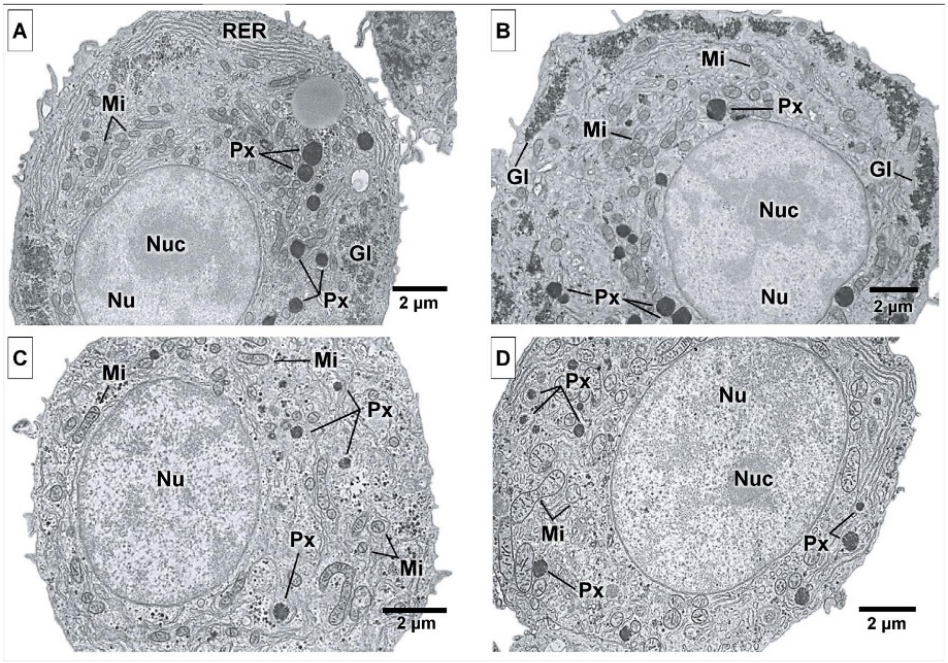

Fig. 2. Brown trout hepatocyte ultrastructure after 96 h of exposure to testosterone (T) in experiment I. Peroxisomes were identified by catalase cytochemistry. A – solvent control (0.1% ethanol); B – 1 µM of T, C – 10 µM of T; D – 50 µM of T. Nu – nucleus, Nuc – nucleolus, Gl – glycogen, Mi – mitochondria, Px – peroxisomes, and RER – rough endoplasmic reticulum.

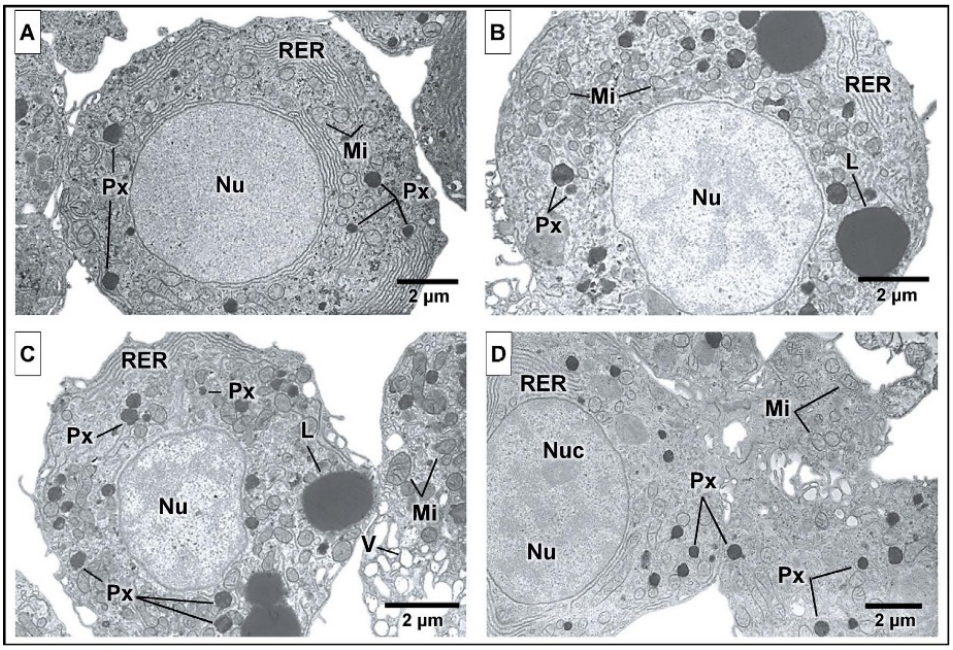

Fig. 3. Brown trout hepatocyte ultrastructure in experiment II, at 72 h of exposure. Peroxisomes were identified by catalase cytochemistry. A – solvent control (0.1% ethanol); B – 50 µM of flutamide (F); C – mixture of 10 µM of testosterone (T) and 50 µM of F; D – mixture of 10 µM of T and 50 µM of ICI 182,780 (ICI). Nu – nucleus, Nuc – nucleolus, Gl – glycogen, Mi – mitochondria, Px – peroxisomes, RER – rough endoplasmic reticulum, L – lipid, and V – vacuole.

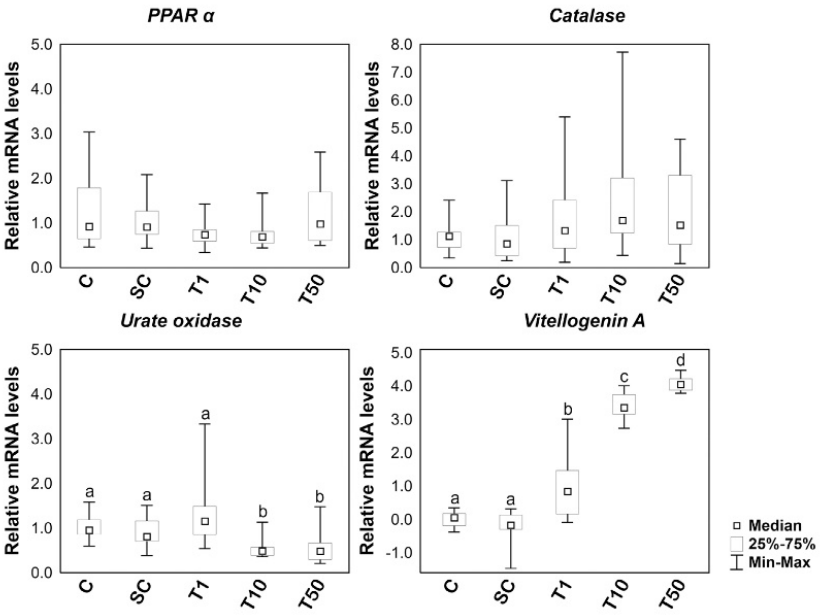

Fig. 4. Brown trout primary hepatocyte relative mRNA levels of the selected target genes in experiment I. The two times of exposure (72 and 96 h) were joined for the statistical analysis. C – control (L-15 medium); SC – solvent control (0.1% ethanol); T1, T10 and T50 – 1, 10 and 50 µM of testosterone. Different letters mean significant differences between groups according to Tukey test or to Mann-Whitney pairwise with sequential Bonferroni correction test (for *vitellogenin A*). *Vitellogenin* mRNA levels are expressed in logarithm (log10). n = 12 replicates.

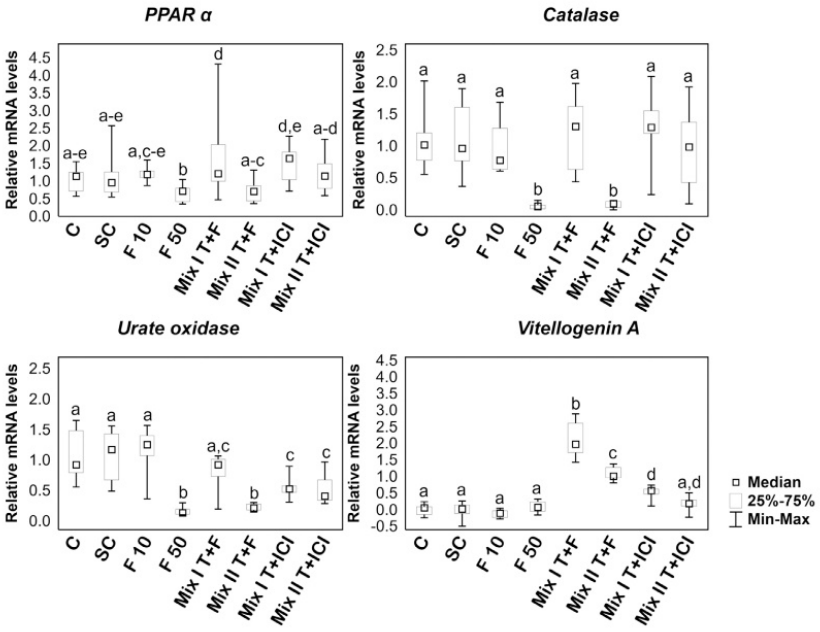

Fig. 5. Brown trout primary hepatocyte relative mRNA levels of the selected target genes in experiment II. The two times of exposure (72 and 96 h) were joined for the statistical analysis. C – control (L-15 medium); SC – solvent control (0.1% ethanol); F10 – 10 µM of flutamide (F); F50 – 50 µm of F; Mix I T + F – mixture of 10 µM of testosterone (T) and 10 µM of F; Mix II T + F – mixture of 10 µM of T and 50 µM of F; Mix I T + ICI – mixture of 10 µM of T and 10 µM of ICI 182,780 (ICI); Mix II T + ICI – mixture of 10 µM of T and 50 µM of ICI. Different letters mean significant differences between groups according to Tukey test or to Mann-Whitney pairwise with sequential Bonferroni correction test (for *vitellogenin A*). *Vitellogenin* mRNA levels are expressed in logarithm (log10). n = 8 to 12 replicates.

knowledge that the expression of *PPARα* is influenced by androgens (Collett et al., 2000; Lin et al., 2008), and that this particular gene has a pivotal action in the modulation of peroxisomal pathways, prompted us to study here, in brown trout hepatocytes, the possible interconnections between androgenic stimulus and peroxisomal signaling, at both morphological and molecular levels. For that purpose, an androgen (T), and antagonists of androgen and estrogen receptors (F and ICI, respectively) were chosen for exposures, by using multiple single and mixture conditions. The concentrations selected were based on the ranges reported by others in fish hepatocyte studies (Kwon et al., 2005; Madureira et al., 2015a; Mori et al., 1998; Riley et al., 2004). The T concentration used in the second experiment (10 µM) corresponds to the lowest level that was able to affect peroxisomal endpoints in the first assay (gene expression and peroxisomes morphology).

In mammals, interferences in the regulation of PPARα by androgens are not linear, as can be inferred by several studies (Collett et al., 2000; Lin et al., 2008; Yang et al., 2016). In a study with primary hepatocytes from mice, DHT treatment resulted in an increase in *PPARα* expression in wild- type, but not in the knockdown model for the androgen receptor, indicating that *PPARα* induction was androgen receptor-

dependent (Lin et al., 2008). In a rat model, castration decreased *PPARα* (both mRNA and protein levels) in ischemic myocardium, an effect that was reversed by T administration. Co-treatment with T and F in the castrated males did not antagonize this effect, which means that T up-regulated PPARα by an androgen-independent pathway (Yang et al., 2016). In a human prostate cell line, the androgen mibolerone caused a down-regulation of *PPARα* mRNA, which was reversed by the anti-androgen Casodex (bicalutamide) (Collett et al., 2000). Diet exposure to the androgen DHEA up-regulated the expression of *PPARα* and peroxisomal genes (*acyl-CoA oxidase*, *catalase* and *peroxin 11α*), in rats. Concomitantly, the same treatment increased the catalase activity and the number of peroxisomes. Peroxisome morphology also changed, since both very small and enlarged organelles were observed (Depreter et al., 2002). In another *in vivo* study, the same compound caused an elevation of acyl-CoA oxidase protein levels, but catalase and urate oxidase stayed stable (Beier et al., 1997). *In vitro*, DHEA 3*β*-sulfate also increased *acyl-CoA oxidase* mRNA levels in rat hepatocytes (Ram and Waxman, 1994). *In vitro*, T increased the catalase activity in rat cerebellar granule cells, *via* an androgen-dependent mechanism (Ahlbom et al., 2001).

Despite the ample evidence of androgenic interferences in PPARα signaling in mammalian models, in fish, to our knowledge, scarce data are available. In rainbow trout, *in vivo* exposure to T and DHT had no effects on the mRNA levels of *PPARα* (Cleveland and Weber, 2016) and DHEA decreased liver catalase activity (Orner et al., 1995). Here, stereological analysis revealed that exposure to androgens changed the quantitative morphology of peroxisomes, reducing the relative volume of these organelles in relation to the cytoplasm, compared to control conditions. In addition, smaller peroxisome profiles were observed after catalase cytochemistry in TEM, supporting that the decrease in relative volume resulted from a reduction in the size of peroxisomes. However, these findings were not accompanied by relative changes in mRNA levels of *PPARα* or *catalase*. On the contrary, the other peroxisomal enzyme, *urate oxidase*, was down-regulated at the higher androgen concentrations (10 and 50 μM). Previously, we also obtained a decrease in the relative volumes of the peroxisomes after estrogenic stimulus, with a concomitant decrease in the mRNA expression levels of *catalase* and *urate oxidase*, which may suggest that different mechanisms are underlaying the regulation of this morphological effect (Madureira et al., 2015a).

We also found *vitellogenin A* mRNA levels to be substantially up-regulated by T. This result highlights a possible interference of T in estrogenic mediated pathways. Increases in *vitellogenin* (mRNA and encoded protein) levels after exposure to androgens have already been reported, both *in vivo* and *in vitro*. Several primary potential mechanisms have been generally proposed to justify this phenomenon: a) aromatization of androgens to estrogens (Hornung et al., 2004; Shilling and Williams, 2000); b) direct binding of androgens to the estrogen receptor (Le Menn et al., 1980; Pelissero et al., 1993); and c) vitellogenin induction by androgens, mediated by the androgen receptor (Kwon et al., 2005; Shilling and Williams, 2000). *In vitro*, a significant induction of vitellogenin was obtained after T and 11-KT at 10 μM in primary rainbow trout (immature female) hepatocytes (Pelissero et al., 1993). The effect was reverted by tamoxifen, a selective estrogen receptor blocker. Also in rainbow trout (immature males), various androgens (T, androsterone, and MT) stimulated *vitellogenin* mRNA expression either at low (2×10^{-9} M) and high (2×10^{-5} or 7.3×10^{-6} M in the case of T) concentrations. This induction was completely blocked by tamoxifen (Mori et al., 1998). More recently, in this species, *vitellogenin* mRNA was increased after incubation of primary hepatocytes with 1000 ng/L of the two trenbolone acetate metabolites, 17β-trenbolone and trendione (Forsgren et al., 2014). In primary hepatocytes obtained from sexually mature female tilapia, T, 17α-MT, and 5α-DHT, at 5×10^{-6} M, were able to increase significantly vitellogenin levels (Kim et al., 2003). In the latter study, tamoxifen was able to reduce the induction caused by DHT, whereas fadrozole (an aromatase inhibitor) failed to suppress the T vitellogenin induction, indicating that conversion of T to estrogens was not the main pathway responsible for the vitellogenin induction. In male individuals, the three androgens were able to potentiate the effect of 17β-estradiol on vitellogenin induction, but had no significant effect in the absence of the estrogen (Kim et al., 2003). In the same species (sexually mature specimens), DHT induced the production of the lipoprotein vitellogenin in female primary hepatocytes at the higher concentration (1000 μM), but had no effect on male hepatocytes (Riley et al., 2004). In immature eel, 17α-MT (in the presence of E2) led to a concentration-dependent synthesis of vitellogenin. The addition of tamoxifen or F significantly reduced vitellogenin levels, with tamoxifen being more effective (Kwon et al., 2005).

Regarding the above *in vitro* data related to the impacts on vitellogenin, there are clear discrepancies in relation to the response obtained, which can be attributed to different experimental designs, the maturation status of individuals and different species sensitiveness (Kim et al., 2003; Örn et al., 2006; Van den Belt et al., 2003). Despite that, the role of androgens in vitellogenin regulation is unquestionable and likely occurs mainly through estrogen receptor-mediated pathways. Our data also show that kind of pattern, since ICI was able to reverse *vitellogenin* induction after T exposure to control levels. In our previous work, also with primary hepatocytes from brown trout juveniles, ICI alone caused a slight, but significant, increase in *vitellogenin* mRNA levels (Madureira et al., 2015a). However, the fact that it was not able to alter estrogen receptor levels and reversed the gene expression effects of estrogenic stimulus, leads us to assume that it has anti-estrogenic specificity in this model at the tested concentrations. Despite this, it should be noticed that ICI could act as anti-androgenic compound in some conditions (Bhattacharyya et al., 2006; Kawashima et al., 2004).

Still regarding *vitellogenin*, here we found a significant diminishing of mRNA levels between the two concentrations of F in combination with the androgen, which denotes a concentration response inhibition, indicating that this result was mediated also *via* androgen receptor. Our data combined with those from Kwon et al. (2005) strongly suggest that, at least in part, the up-regulation of *vitellogenin* (gene and its protein product) after exposure to T happens *via* androgen receptor. Also in female ovariectomized mice, MT changed the uterine weight and morphology through estrogen receptor at low concentrations, and over estrogen and androgen receptors at higher concentrations (Papaconstantinou et al., 2002). Even though aromatization of androgens to estrogens can likely occur *in vivo* (Hornung et al., 2004), it seems less plausible to happen in primary hepatocytes. In fish liver, the expression of aromatases (*CYP19A1 and CYP19A2*) was reported as absent in some studies (Blázquez and Piferrer, 2004; Kim et al., 2003; Nunez and Applebaum, 2006). However, both aromatases have been described by others in fish liver (Ibor et al., 2016; Lynn et al., 2008; Valle et al., 2002). Another intriguing fact is that increase of *vitellogenin* mRNA and protein levels by non-aromatizable androgens like DHT, 11-KT or trenbolone (Ankley et al., 2004; Pelissero et al., 1993; Shilling and Williams, 2000) has been reported (Forsgren et al., 2014; Kim et al., 2003; Pelissero et al., 1993), which further discredits the aromatization mechanism as the one responsible for production of vitellogenin. Interestingly, the binding of androgens to estrogen receptor was demonstrated in both black goby and rainbow trout (Le Menn et al., 1980; Matthews et al., 2000). Methyldihydrotestosterone (MDHT) was also shown to activate an estrogen-inducible reporter in an *in vitro* assay (Bogers et al., 2006). On the contrary, T did not present estrogenic activity in recombinant yeast bioassay expressing rainbow trout estrogen receptor, neither in liver aggregate cells (Le Guevel and Pakdel, 2001).

The anti-androgen F is considered as a model to assess the effects of anti-androgenic signaling (Ankley et al., 2004; Bhatia and Kumar, 2016). Here, exposure of hepatocytes to F resulted in a significant drop of *catalase* and *urate oxidase* mRNA levels at the highest concentration. Also in fathead minnow, F was able to modify the transcription of

numerous genes not related to androgen receptor pathways (Garcia-Reyero et al., 2009).

In our model, F alone did not change *vitellogenin* mRNA levels. However, *in vivo*, F was shown to affect fish vitellogenin protein levels (Bhatia and Kumar, 2016; Bhatia et al., 2014a; Bhatia et al., 2014b; Jensen et al., 2004). Furthermore, the impact of androgenic/anti-androgenic compounds goes beyond the molecular level and can traduce into alterations, for instance, in gonadal differentiation in fish. Androgens have been shown to either accelerate (Bhatia and Kumar, 2016; Stell and Moller, 2017) or delay (Ankley et al., 2001; Zheng et al., 2016) gonadal development, or to produce opposite effects on male and female gonads (Margiotta-Casaluci and Sumpter, 2011). In turn, F affected the maturation of male and female gonads (Bhatia et al., 2014a; Bhatia et al., 2014b; Rajakumar et al., 2012). Moreover, androgenic and anti-androgenic chemicals were shown to reduce fecundity (Ankley et al., 2001; Ankley et al., 2003; Jensen et al., 2004; Kang et al., 2006) and embryo hatching (Jensen et al., 2004). Globally, the described findings anticipate that exposure to such chemicals can result in adverse hindrances on fish reproduction.

In summary, T down-regulated *urate oxidase* expression and up-regulated *vitellogenin A* expression in brown trout primary hepatocytes. Concomitantly, a reduction in peroxisomal relative volumes and smaller peroxisome profiles were observed. Additionally, F was able to modify the expression of some peroxisomal-related genes (*catalase* and *urate oxidase*). Induction of *vitellogenin A* by T was reversed by co-administration of both F and ICI, with ICI being more effective. As to the *urate oxidase* expression levels, ICI did not completely reverse the T effect to basal levels. Morphological reversal of androgenic effects was observed in co-administration conditions. Overall, these data disclose novel interconnections between androgenic signaling, peroxisomal regulation and estrogenic pathways, supporting that the model androgen T worked *via* both androgen and estrogen receptors. Further studies with other androgenic compounds seem crucial at this point to elucidate further the potential of this class of compounds in the modulation of peroxisomal structure and function.

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Chapter 4

Disruption of classical estrogenic targets in brown trout primary hepatocytes by the model androgens testosterone and dihydrotestosterone

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Disruption of classical estrogenic targets in brown trout primary hepatocytes by the model androgens testosterone and dihydrotestosterone

Célia Lopes[a,b], Tânia V. Madureira[a,b,*], José F. Gonçalves[a,c], Eduardo Rocha[a,b]

[a] Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto (U.Porto), Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, P 4450-208 Matosinhos, Portugal
[b] Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Laboratory of Histology and Embryology, Department of Microscopy, Rua Jorge Viterbo Ferreira 228, P 4050-313 Porto, Portugal
[c] Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Department of Aquatic Production, Rua Jorge Viterbo Ferreira 228, P 4050-313 Porto, Portugal

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ABSTRACT

Estrogenic effects triggered by androgens have been previously shown in a few studies. Aromatization and direct binding to estrogen receptors (ERs) are the most proposed mechanisms. For example, previously, a modulation of vitellogenin A (VtgA) by testosterone (T), an aromatizable androgen, was reported in brown trout primary hepatocytes. The effect was reversed by an ER antagonist. In this study, using the same model the disruption caused by T and by the non-aromatizable androgen - dihydrotestosterone (DHT), was assessed in selected estrogenic targets. Hepatocytes were exposed (96 h) to six concentrations of each androgen. The estrogenic targets were VtgA, ERα, ERβ1 and two zona pellucida genes, ZP2.5 and ZP3a.2. The aromatase CYP19a1 gene and the androgen receptor (AR) were also included. Modulation of estrogenic targets was studied by quantitative real-time PCR and immunohistochemistry, using an HScore system. VtgA and ERα were up-regulated by DHT (1, 10, 100 μM) and T (10, 100 μM). In contrast, ERβ1 was down-regulated by DHT (10, 100 μM), and T (100 μM). ZP2.5 mRNA levels were increased by DHT and T (1, 10, 100 μM), while ZP3a.2 was up-regulated by DHT (100 μM) and T (10, 100 μM). Positive correlations were found between VtgA and ERα mRNA levels and ZPs and ERα, after exposure to both androgens. The mRNA levels of CYP19a1 were not changed, while AR expression tended to increase after micromolar DHT exposures. HScores for Vtg and ZPs corroborated the molecular findings. Both androgens triggered estrogen signaling through direct binding to ERs, most probably ERα.

1. Introduction

Fish have reliable biological markers that can reflect hormonal mediated actions (estrogenic or androgenic), and this advantage is one of the reasons for the increasing use of different fish species as experimental models (Scholz and Mayer, 2008; Sumpter and Jobling, 1995). The induction of vitellogenin (Vtg) mRNA or protein, particularly in male or undifferentiated juvenile fish, has been the most widely used biomarker of estrogenicity (Jobling et al., 2004; Van den Belt et al., 2003), while the production of the glycoprotein spiggin by the female stickleback kidney has been recognized as an androgenic biomarker (Jolly et al., 2006; Katsiadaki et al., 2002).

Vtg mRNA and protein are readily inducible by endogenous estrogens (Celius et al., 2000; Scholz et al., 2004; Yost et al., 2014) and by various xenoestrogens (Madureira et al., 2015; Pelissero et al., 1993; Scholz et al., 2004), but zona pellucida proteins (ZPs), the structural glycoproteins that compose the egg envelope of the vertebrate oocyte, have been proposed to be more sensitive than Vtg as estrogen-responsive markers (Arukwe et al., 1997; Celius et al., 2000; Gunnarsson et al., 2007). This hypothesis relates to the fact that the induction of the ZPs (also named zona radiata proteins - ZRPs, vitelline envelope proteins - VEPs or choriogenins - Chgs) (Berg et al., 2004; Berois et al., 2011), precedes the expression of Vtg, both at the mRNA and protein level (Celius et al., 2000; Westerlund et al., 2001). As for Vtg, the liver expression of the ZPs is considered to be physiologically under estradiol (E2) influence, and to be induced by estrogenic compounds (Arukwe et al., 2002; Berg et al., 2004; Hyllner et al., 2001).

Despite the known value of Vtg and ZPs as biomarkers of estrogen exposure, their regulation has been shown to be quite complex in fish. For example, the dynamics of the induction of Vtg and ZPs have been

* Corresponding author at: Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto (U.Porto), Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4450-208 Matosinhos, Portugal.
E-mail address: tvmadureira@icbas.up.pt (T.V. Madureira).

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shown to be influenced by temperature (Anderson et al., 2012; Mackay and Lazier, 1993) and other hormones, including pituitary growth hormone and cortisol (Berg et al., 2004; Kwon et al., 2005). Remarkably, a variety of androgenic compounds have also been shown to modulate the expression of both families of genes/proteins (Bhatia and Kumar, 2016; Brockmeier et al., 2013; Miura et al., 1998; Mori et al., 1998).

There is some evidence of interference of androgenic compounds in classically estrogen-mediated pathways, but little is still known about the mechanisms that mediate those regulations. The estrogenic effects produced by androgenic compounds have been suggested to occur mainly through the (i) binding of androgens to the estrogen receptor (ER) (Le Menn et al., 1980), or (ii) conversion of aromatizable androgens such as testosterone (T) or methyltestosterone (MT) to estrogenic compounds (Hornung et al., 2004; Zerulla et al., 2002).

For the assessment of the mechanism of regulation of specific endocrine processes, *in vitro* testing has been shown as a useful alternative since the controlled environment allows the exclusion of confounding factors that are inherent to *in vivo* studies (Dang, 2014). Previously, we found that the mRNA expression of VtgA was significantly up-regulated by T in brown trout (*Salmo trutta* f. *fario*) primary hepatocytes, and this effect was completely reversed by the ER antagonist ICI 182,780 (ICI), and partially by flutamide, an androgen receptor (AR) antagonist (Lopes et al., 2017). The induction of VtgA by T was also observed in an *in vivo* study with brown trout juveniles, further supporting the androgenic interferences with estrogenic signaling in this fish (Madureira et al., 2018). These data strongly suggest that some androgens can elicit their effects through ERs. Further, a mediation through both ER and AR could be hypothesized given the concentration-dependent inhibition of Vtg levels by flutamide in our previous study (Lopes et al., 2017). Effects mediated by both receptors (ER and AR) at high doses of MT were also noted in other systems, *viz.* the human uterine epithelium (Papaconstantinou et al., 2002).

Based on the above context, this study aimed to explore mechanistically the effects caused by two androgens, T and dihydrotestosterone (DHT), on classical estrogenic targets in brown trout primary hepatocytes. T and DHT are aromatizable and non-aromatizable androgens, respectively, and have been shown to induce Vtg in fish primary hepatocytes (Kim et al., 2003; Lopes et al., 2017) and in fish *in vivo* studies (Bhatia and Kumar, 2016; Madureira et al., 2018). DHT was already detected in fathead minnow plasma at physiological relevant levels (Margiotta-Casaluci et al., 2013), and there is increasing evidence on the role of DHT in fish biology, with special emphasis on reproduction-related processes (Margiotta-Casaluci et al., 2013; Martyniuk et al., 2013).

Thus, this work explored the changes in mRNA levels of a selection of estrogenic target genes - VtgA, ERα, ERβ1, and two zona pellucida genes (zona pellucida glycoprotein 2.5 – ZP2.5 and zona pellucida glycoprotein 3a.2 – ZP3a.2) - assessed after exposure to a range of T and DHT concentrations (from nanomolar to micromolar levels). All these targets had previously been shown to be regulated by estrogens in brown trout (Madureira et al., 2015; Uren Webster et al., 2015). The relative expression of AR was also evaluated as a positive androgenic control. To gain further mechanistic insights, the gene that encodes for the ovarian aromatase CYP19a1 (which converts testosterone to estradiol) was also included as a target gene of interest. This aromatase gene was already shown to be expressed in fish liver (Ibor et al., 2016; Lynn et al., 2008), and to have potential estrogen and androgen response elements in the promoter region (Tong and Chung, 2003). Moreover, CYP19a1 mRNA was found to be up-regulated along with Vtg and ZPs in tilapia liver from contaminated sites of a Nigerian river (Ibor et al., 2016). Here, phenotypic anchoring of changes at the gene level was assessed by immunohistochemistry (IHC). This study contributes to growing data on the underlying mechanisms that drive the regulation of classical estrogenic targets by androgens.

2. Materials and methods

2.1. Animals

Brown trout immature juveniles (one-year-old) were acquired from a state-owned Aquaculture Station (Torno, Portugal), and acclimated in the aquatic animal facilities from ICBAS for two months. Fish were fed daily with dry pellets for salmonids (T-4 Optiline, Skretting), and kept under a 12/12 h dark/light cycle. Water quality parameters, including temperature, dissolved oxygen, pH, hardness, ammonia, and nitrites, were monitored to assure optimal conditions for the fish. The registered minimum and maximum values were as follows: temperature – 16.0–22.9 °C, dissolved oxygen – 70–95%, pH – 6.6–7.9, hardness – 3–5 °dGH, ammonia – 0 mg/L and nitrites – 0.05–1.7 mg/L.

The fish used in the experiments had a mean (standard deviation) weight of 68.2 (21.6) g, and a mean total length of 19.3 (1.6) cm. The animal procedures followed the guidelines for the care and use of animals, in compliance with the Portuguese Decree-Law No. 113/2013 implementing the EU Directive No. 2010/63 on animal protection for scientific purposes.

2.2. Primary hepatocytes isolation

Fish were euthanized with an overdose of ethylene glycol monophenyl ether (Merck), at a minimum of 0.6 mL/L. The liver was then excised and perfused, as previously detailed (Madureira et al., 2015). The final hepatocyte suspension was plated using phenol red-free Leibovitz's L15 (Invitrogen) medium, with 5% of charcoal-stripped fetal bovine serum (Sigma-Aldrich), 100 µg/mL of streptomycin and 100 U/mL of penicillin (Sigma-Aldrich).

The viability of the isolated hepatocytes was measured with the Countess™ Automated Cell Counter (Invitrogen), which is based on the standard trypan blue technique, following the manufacturer's instructions. Then, a total of 5×10^5 cells were plated per well, in 500 µL of L15 medium, in 24 well plates (Orange Scientific) coated with 0.3 mg/mL of poly-L-lysine hydrobromide (Sigma-Aldrich) and cultured at 19 °C (without extra O_2/CO_2).

2.3. Experimental design

Primary hepatocytes were exposed to T – (CAS 58 – 22-0, 17β-hydroxy-4-androsten-3-one, product 86500, Sigma-Aldrich) and DHT – CAS 521 – 18-6, 17β-hydroxy-5α-androstan-3-one, product A8380, Sigma-Aldrich). For each compound, 3 independent experiments were performed. Each experiment was conducted with hepatocytes isolated from a single fish. The set-up was designed in a way each well could be assigned randomly to the different *in vitro* treatments. Wells had the same probability of being assigned to a condition, so they were considered as the experimental unit, in line with theoretical definition (Festing and Altman, 2002). Each experiment consisted of 3 plates, and each plate had 2 wells per condition for the molecular analyses (a single well from each condition per plate was latter randomly assigned for the molecular analysis), and 1 well per condition for morphology. The design aligns with the fact that *in vitro* responses to hormones have been consistent among fish (*e.g.*, Madureira et al., 2015; Lopes et al., 2017).

All experiments included 8 different conditions: control (C) - L15 medium (as described above); solvent control (SC) - 0.1% ethanol in supplemented L15 medium, and six concentrations (1 nM, 10 nM, 100 nM, 1 µM, 10 µM and 100 µM) of the tested androgens (DHT1 to DHT6 and T1 to T6). The androgen concentrations used were selected in order to include the lowest and the highest doses that have already been shown to cause effects on estrogenic targets in fish *in vitro* (Mori et al., 1998; Riley et al., 2004). Estrogenic effects after 17α-ethinylestradiol (EE2) exposure had previously been evaluated for Vtg and ERs in the same experimental model (Madureira et al., 2015).

Stock solutions of T and DHT were prepared in absolute ethanol p.a.

(Merck), and the working solutions in supplemented L15 medium, with a final ethanol concentration of 0.1%. Hepatocytes were exposed 24 h after isolation. The corresponding solutions of each condition were changed every 24 h, and cells were exposed during a total of 96 h, as previously optimized for this model (Madureira et al., 2015).

At the end of exposures, cells were trypsinized, using trypsin/EDTA, 0.05%/0.02% in PBS (Sigma-Aldrich), and cell viability was measured in a total of 3 wells per condition. Hepatocytes were pelleted by centrifugation (160 × g, 5 min at 4 °C), and then processed for the different purposes. For molecular analyses, each well gave a pellet, which was used as a single unit, while for light microscopy 3 wells of the same condition had to be pooled, in other to obtain a workable pellet for the subsequent processes.

2.4. Molecular analysis

2.4.1. RNA extraction and cDNA synthesis

Immediately after centrifugation, hepatocyte pellets were frozen in liquid nitrogen and kept at -80 °C until RNA extraction. A total of 9 pellets (3 per condition from each independent experiment – one per plate) were assigned for the molecular analyses. Total RNA was extracted and purified with the illustra™ RNAspin Mini Isolation Kit (GE Healthcare), following the manufacturer's instructions. The isolated RNA integrity was verified by gel electrophoresis (1% agarose gel), using GelRed nucleic acid stain. The concentration and purity of RNA were checked with the Multiskan Go spectrophotometer (Thermo Scientific), using the µDrop™ Plate. cDNA was synthetized with the iScript™ cDNA Synthesis Kit (Bio-Rad), using 250 ng of total RNA.

2.4.2. Gene expression - qRT-PCR

The expression levels of the selected genes were determined by qRT-PCR, using a CFX Connect™ (Bio-Rad) equipment. Primer sequences and protocol details were published in previous studies with brown trout or Atlantic salmon (Table 1). For ZPs, AR and CYP19 genes a calibration curve, with known dilutions of cDNA, was performed to obtain the efficiencies for each gene in our laboratory conditions. Additionally, the amplification products were subjected to an agarose gel electrophoresis, to confirm the expected size of the product.

Each qRT-PCR plate comprised duplicates of each sample, no template controls, and a calibrator (a mixture of cDNA from randomly selected samples). The reaction mixtures (total volume of 20 µL) consisted of 5 µL of diluted (1/5) cDNA, 10 µL of iQ™ SYBR®Green Supermix (Bio-Rad) and 200 nM of specific primers for each gene. Purity and specificity of the PCR product were assessed at the end of amplification cycles, by melt curve analysis. Expression of the target genes was normalized by the Pfaffl method (Pfaffl, 2001), using the geometric mean of the ribosomal protein L8 (rpl8) and β-actin (β-act) housekeeping genes, already known to be stable after androgenic exposures in the same model (Lopes et al., 2017).

2.5. Morphological analysis

2.5.1. Immunohistochemistry

Pellets were fixed at room temperature in 10% buffered formalin for 24 h, embedded in Histogel™ (Bio-Otica), and placed in 70% ethanol until being routinely processed in a TP1020 automatic tissue processer (Leica Biosystems). Then, paraffin embedding was made in an EG1140C embedding center (Leica Biosystems), and 3 µm sections were obtained in a RM2255 microtome (Leica Biosystems). The conditions selected for IHC were as follows: DHT experiments - C, SC, DHT4, DHT5, DHT6 and T experiments - C, SC, T4, T5, T6. The primary antibodies used for IHC were chosen based on previous publications using the same antibodies, antibody clones or immunization peptides in fish (Arukwe and Røe, 2008; Munchrath and Hofmann, 2010; Tilton et al., 2003; Weber et al., 2018). A total of 4 sections per sample were used for each antibody, one as negative control and 3 sections for immunolabeling.

Sections were deparaffinized, rehydrated, and then antigen retrieval was performed (the method used for each antibody is detailed in Table 2). For antigen retrieval with Tris-EDTA buffer, slides were boiled in a microwave (700 W) for 15 min, and when citrate buffer was used, slides were kept in the pressure cooker for 3 min after the maximum pressure was reached. After each antigen retrieval method, slides were allowed to cool to room temperature (± 20 °C). Endogenous peroxidase activity was quenched by immersion in a 3% hydrogen peroxide solution in methanol for 10 min, and then the IHC staining was performed using the NovoLink™ Polymer Kit (Leica Biosystems), following the manufacturer's instructions. The primary antibodies were incubated overnight, at 4 °C, in a humidified chamber. Antibody and further protocol details are provided in Table 2. Reactions were developed using 3,3′-diaminobenzidine (DAB) as chromogen (provided in the visualization kit), and the nuclei were counterstained for 1 min, with Mayer's hematoxylin (Merck). Slides were dehydrated and mounted with Q Path® Coverquick 2000 (VWR Chemicals). Sections of adult brown trout liver were used as positive controls. For negative controls, the primary antibody was replaced by the antibody dilution buffer (PBS with 5% bovine serum albumin).

The slides were photographed using a BX50 microscope (Olympus), coupled with a DP21 camera (Olympus). A total of 10 photographs of

Table 1
Primer sequences, annealing temperatures (AT) and respective efficiencies (E) used for qRT-PCR.

Gene	Primer sequence (5′-3′)	AT (°C)	E (%)	Reference
VtgA	F – AACGGTGCTGAATGTCCATAG R – ATTGAGATCCTTGCTCTTGGTC	62.9	99.0	(Körner et al., 2008)
ERα	F – GACATGCTCCTGGCCACTGT R – TGGCTTTGAGGCACACAAAC	61.6	91.2	(Körner et al., 2008)
ERβ1	F – TGTGGACCTGTGCCTGTTC R – ACATGAGCCCTAGCATCAGC	66.5	103.3	(Körner et al., 2008)
ZP2.5	F – ATCAATAACCACAGCCACAATG R – ACCAGGGACAGCCAATATG	55.0	99.0	(Uren Webster et al., 2015)
ZP3a.2	F – AACTACACTCCACTTCATC R – CACATCTCCTTCATCTTCA	54.5	101.8	(Uren Webster et al., 2015)
AR	F – ATGCTAGGGAGGATGCCC R – CCATGGGGAACATGTGGT	60.0	101.8	(Mortensen and Arukwe, 2009)
CYP19a1	F – GGGCACTGTCTGATGATGTC R – GGGCTTGAGGAAGAACTCTG	60.0	98.4	(Arukwe et al., 2016)
β-act	F – TCTGGCATCACACCTTCTAC R – TTCTCCCTGTTGGCTTTGG	55.0	96.1	(Madureira et al., 2017)
rpl8	F – TCAGCTGAGCTTTCTTGCCAC R – AGGACTGAGCTGTTCATTGCG	59.0	93.8	(Körner et al., 2008)

Vitellogenin A – VtgA, estrogen receptor alpha – ERα, estrogen receptor beta 1 – ERβ1, zona pellucida glycoprotein 2.5 – ZP2.5, zona pellucida glycoprotein 3a.2 – ZP3a.2, androgen receptor – AR, P450 aromatase A – CYP19a1, beta actin – β-act, ribosomal protein L8 – rpl8.

Table 2
Antibodies and protocols used for immunohistochemistry.

Antibody	Clonality	Immunogen	Antigen retrieval	Dilution	Reference
Vtg					
Bertin Bioreagent Ref: V01409201	Polyclonal	Arctic char vitellogenin purified from plasma of 17-β-estradiol induced fish	Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0)	1:2500	Arukwe, 2008
ZPs					
Bertin Bioreagent Ref: Z03402202	Polyclonal	Purified eggshell proteins from Atlantic salmon	Citrate buffer (0.01 M, 0.05% Tween 20, pH 6.0)	1:3000	Arukwe, 2008
ERα					
Enzo Life Sciences Ref: ALX-803-004	Monoclonal (clone 33)	Peptide from Human ERα VGMMKGGIRKDRRG	Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0)	1:50	Tilton, 2003
ERβ					
Enzo Life Sciences Ref: ALX-210-180	Polyclonal	Peptide from Human ERβ CSPAEDSKSKEGSQNPQSQ	Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0)	1:1000	Munchrath, 2010
AR					
Merck Millipore Ref: 06-680	Polyclonal	Peptide from Human AR MEVQLGLGRVYPRPPSKTYRGC	Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0)	1:1000	Munchrath, 2010
CYP19					
Santa Cruz Biotechnology Ref: sc-374176	Monoclonal (clone E-9)	C-terminus (aa 204-503) of Human CYP19	Citrate buffer (0.01 M, 0.05% Tween 20, pH 6.0)	1:20	Weber, 2018

Vitellogenin – Vtg, Zona pellucida proteins – ZPs, estrogen receptor alpha – ERα, estrogen receptor beta – ERβ, androgen receptor – AR, P450 aromatase – CYP19.

non-overlapping, randomly selected fields, were taken at the immersion (100 ×) objective for each antibody/condition. Peripheral section areas where excluded because the immunostaining tends to be artifactually stronger in these areas. A semiquantitative histological score (HScore) was calculated, considering the abundance and intensity of the cytoplasmic immunostaining and the percentage of non-stained and positively stained cells. The approach followed a protocol originally described for ER and progesterone receptors in human uterine tissue (Lessey et al., 1988). From each photo, hepatocytes were classified as: score 0 = negative, score 1 = weak cytoplasmic staining, score 2 = moderate cytoplasmic staining and score 3 = strong cytoplasmic staining. The classification scores implemented are illustrated in Fig. 1. The final HScore was calculated through the equation HScore = ΣP_i (i + 1), where i are the intensity scores and P is the percentage of cells attributed to each score (Lessey et al., 1988). At least 250 cells were counted for each condition and per antibody.

2.6. Statistical analysis

The Past3 (version 3.19) free software (http://folk.uio.no/ohammer/past/) was used for statistical analysis. Graphs were generated in GraphPad Prism (version 7.00). The normality and homogeneity of variances of all data (viabilities and molecular analyses) were checked by using Shapiro-Wilk W and Levene's tests, respectively. A one-way ANOVA analysis followed by the parametric Tukey test was used for data with normality and equality of variances. When the assumptions were not met, even after data transformation (logarithm, square root, inverse), the Kruskal Wallis ANOVA by ranks followed by the non-parametric Mann-Whitney pairwise test (with sequential Bonferroni correction) was used for the comparisons. For the correlation analysis, as data did not meet the normality and homogeneity assumptions, the Spearman Rank Correlation Coefficient (r_s) was calculated. The strength of association was classified following a proposed guideline (Mukaka, 2012). The *p*-value was set at 0.05 in all the circumstances.

3. Results

3.1. Cell viabilities

Mean (standard deviation) viability of hepatocytes after the isolation was 89 (4)% in the DHT experiments and 90 (3)% in the T

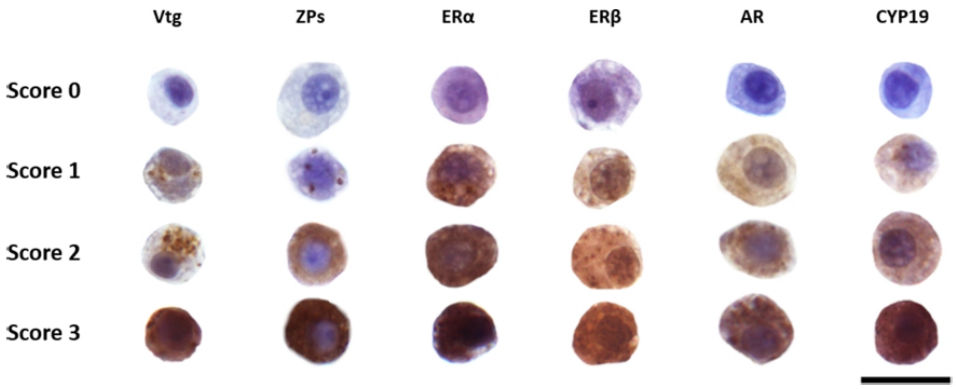

Fig. 1. Immunohistochemistry scores (HScores) considered for the different antibodies. Vitellogenin (Vtg), zona pellucida proteins (ZPs), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), androgen receptor (AR), aromatase (CYP19). Bar = 10 μM.

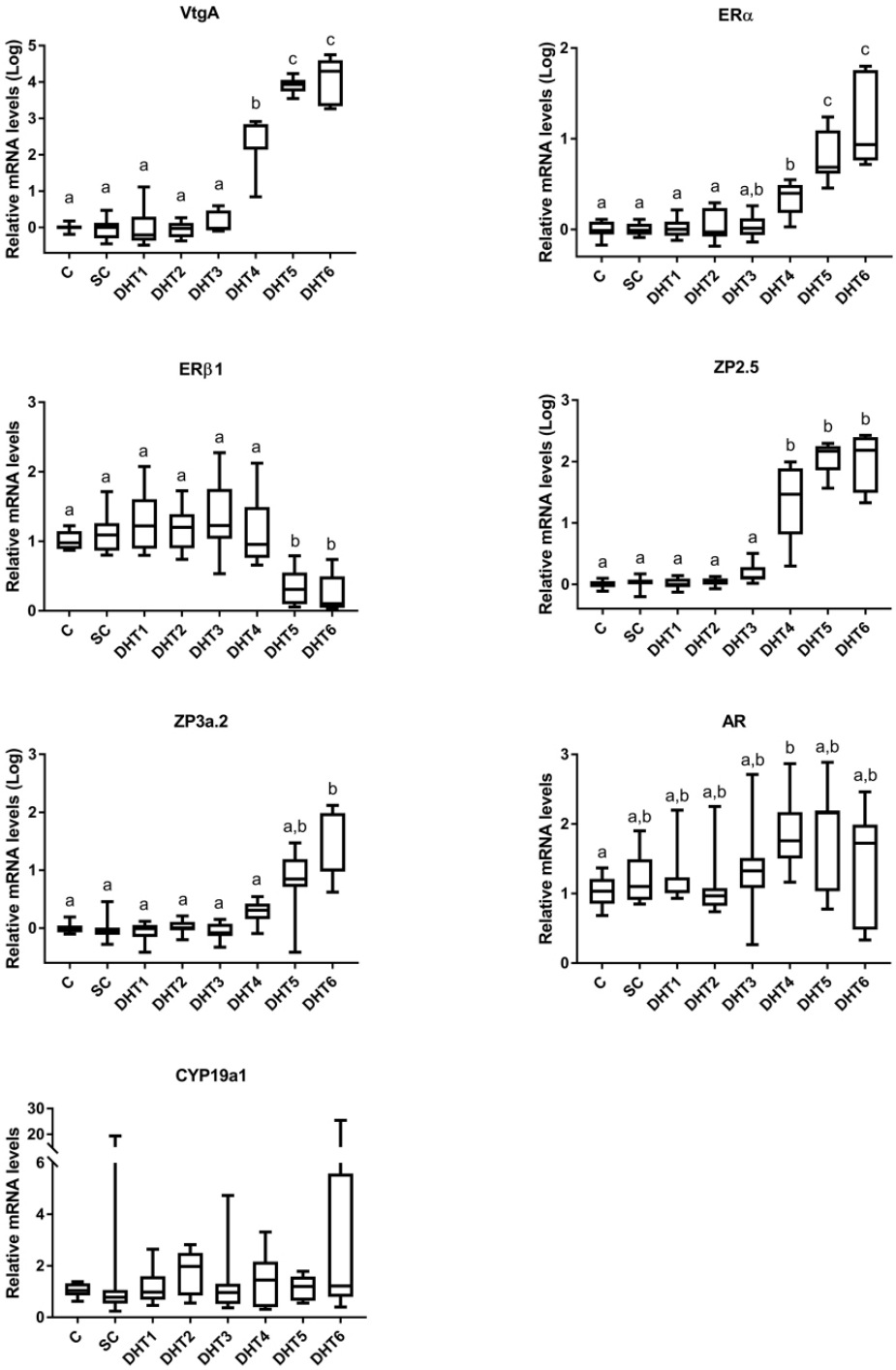

Fig. 2. Relative mRNA levels of the selected target genes after 96 h of exposure to dihydrotestosterone (DHT). C - control (L15 medium); SC - solvent control (0.1% ethanol in L15 medium); DHT1, DHT2, DHT3, DHT4, DHT5 and DHT6 (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM of DHT, respectively). Data correspond to median, maximum, minimum, and interquartile range (Q3-Q1). Different letters mean significant differences between groups according to Mann-Whitney pairwise with sequential Bonferroni correction test. Vitellogenin A (VtgA), ZP glycoprotein 2.5 (ZP2.5), ZP glycoprotein 3a.2 (ZP3a.2) and estrogen receptor alpha (ERα) mRNA levels are expressed in logarithm (log10). n = 9 replicates (3 wells from each independent experiment, total of 3 fish).

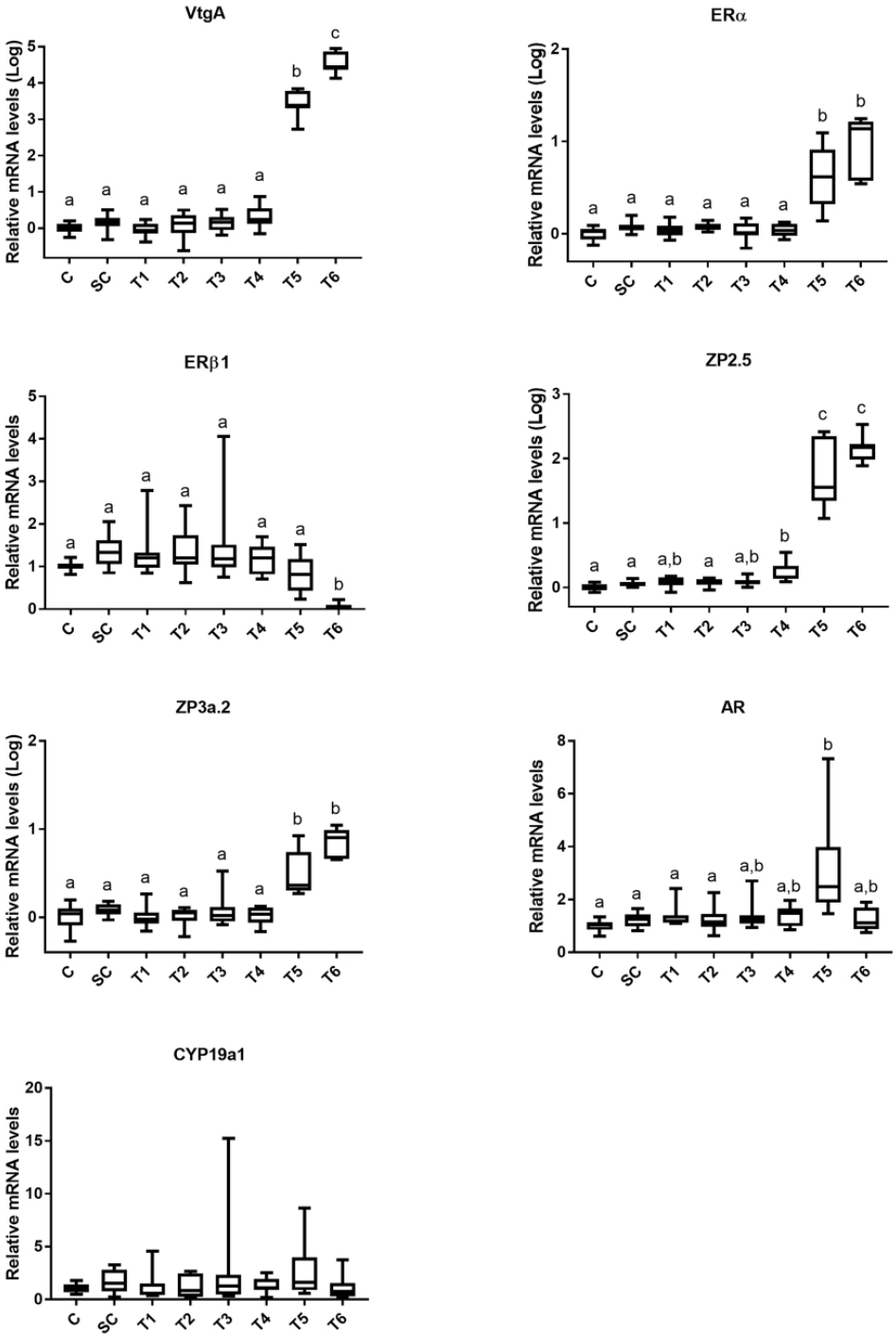

Fig. 3. Relative mRNA levels of the selected target genes after 96 h of exposure to testosterone (T). C - control (L15 medium); SC - solvent control (0.1% ethanol in L15 medium); T1, T2, T3, T4, T5 and T6 (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM of T, respectively). Data correspond to median, maximum, minimum, and interquartile range (Q3-Q1). Different letters mean significant differences between groups according to Mann-Whitney pairwise with sequential Bonferroni correction test. Vitellogenin A (VtgA), ZP glycoprotein 2.5 (ZP2.5), ZP glycoprotein 3a.2 (ZP3a.2) and estrogen receptor alpha (ERα) mRNA levels are expressed in logarithm (log10). n = 9 replicates (3 wells from each independent experiment, total of 3 fish).

experiments. In all experiments, hepatocyte viabilities in C and SC groups after 96 h in culture were always higher than 70%. Regardless of the DHT concentrations, we found no significant differences in cell viability when comparing all conditions with the Mann-Whitney with sequential Bonferroni correction test. However, cells treated with 100 μM of DHT were substantially less aggregated and with non-regular cell limits, resulting in an overall decrease in cellularity and an increase in cellular debris in those wells. Regarding T exposures, the viabilities were between 70 and 97%, with no statistical differences among the tested conditions.

3.2. Molecular analysis

Compared with all other groups, VtgA levels were significantly up-regulated, after exposure to 1, 10, and 100 μM of DHT (Fig. 2), and also after 10 and 100 μM of T (Fig. 3). A similar effect was found for ERα after exposure to DHT and T, at the same concentrations. In contrast, ERβ1 was significantly down-regulated after exposure to 10 and 100 μM of DHT (Fig. 2), and after exposure to 100 μM of T (Fig. 3). A highly significant and positive correlation was found between VtgA and ERα, with $r_s = 0.76$ in both the DHT and T exposures ($p < 0.001$). In contrast, the correlations between VtgA and ERβ1 were moderately negative (-0.52) and low negative (-0.33) for DHT and T, respectively ($p < 0.001$).

ZP2.5 mRNA levels were significantly increased at 1, 10 and 100 μM of DHT (Fig. 2) and T (Fig. 3), compared with the C levels. ZP3a.2 showed a slightly distinct profile, because significantly higher expressions were noted only after exposure to 100 μM of DHT and 10 and 100 μM of T. Highly significant and positive correlations occurred between ZP2.5 and ERα and ZP3a.2 and ERα mRNA levels in the DHT assays ($r_s = 0.73$ and 0.80, respectively) ($p < 0.001$). In the T experiments, the correlation was moderately positive for ZP2.5 and ERα ($r_s = 0.62$) and highly positive ($r_s = 0.78$) between ZP3a.2 and ERα ($p < 0.001$).

AR mRNA levels tended to increase with the concentration of DHT (Fig. 2). When comparing all groups, a significant difference was found only between C and 1 μM of DHT. The exposure to 10 μM of T also induced higher AR relative expression than C levels (Fig. 3).

CYP19a1 gene expression was not altered by the exposure to any of the androgens tested.

3.3. Immunohistochemical analysis

Positive immunolabeling was predominantly observed as brown cytoplasmic staining in the hepatocytes (Figs. 1 and S1 to S4). In the negative controls, no such staining was noted in any of the cell pellets. The translation of the immunostaining into HScore values is given in Fig. 4.

In the livers of female adult brown trout (used as positive control), Vtg was expressed in the hepatocytes with a predominant granular pattern. In the cell pellets, the expression varied from diffuse to granular within the cytoplasm (Figs. 1 and S1). Both patterns co-existed, especially when the immunolabeling was more intense (Fig. 1 and S1D and G). Occasionally, nuclear staining was also noted, which we considered as unspecific antibody binding and thus, excluded for the HScore calculations. Vtg HScores differed from the respective controls, after exposure to 10 and 100 μM of DHT and T (Fig. 4).

ZPs immunolabeling was present as diffuse cytoplasmic staining in the hepatocytes of the positive controls (female adult brown trout livers). In the pellets, the staining was observed either as a diffuse cytoplasmic pattern, granular pattern, or a mixture of both (Fig. 1 and S2). Overall, *in vitro* exposures to DHT produced a significant increase in the expression of the ZPs. For the 1, 10 and 100 μM DHT concentrations, the HScore was significantly higher than that of the controls (Fig. 4). The same was true for T exposures, but a significant increase was evident exclusively after the two highest doses (10 and 100 μM), compared to the other conditions (Fig. 4).

The cytoplasmic immunostaining of ERα in the female trout hepatocytes was diffuse and was additionally evident in the endothelium of the intra-hepatic veins and arteries. In the pelleted hepatocytes there was mainly a diffuse cytoplasmic pattern, although granular staining also occurred (Fig. 1 and S3). The HScores for ERα tended to increase with DHT doses, but they were only significantly different between DHT6 (100 μM) and C (Fig. 4). Exposures to T did not alter ERα HScores (Fig. 4).

In the positive control, ERβ immunolabeling was present in the nuclei and cytoplasm of the hepatocytes and additionally in the cytoplasm of endothelial cells. Nuclear staining was also noted in the hepatocyte pellets and, in addition to diffuse cytoplasmic staining, small cytoplasmic granules were frequently observed (Fig. S4A-C). ERβ HScores seemed to follow an increasing trend after exposure to both androgens, but no significant differences backed this observation.

AR and CYP19 staining were present in the cytoplasm of hepatocytes and endothelial cells of the larger blood vessels of the adult trout liver. In the pellets, the pattern was mostly diffuse in the cytoplasm, with granular clusters often distinguishable (Fig. S4D-F for AR and S4G-I for CYP19). The HScores for both antibodies tended to increase with DHT doses (Fig. 4), which agrees with the qualitative findings (comparisons between Fig. S4D and E for AR; and Fig. S4G and H for CYP19).

4. Discussion

In a previous *in vitro* study, we unveiled new interconnections between androgen signaling and estrogenic pathways in brown trout (Lopes et al., 2017). We found that the expression of VtgA in primary hepatocytes was significantly induced by T, and that the co-treatment with the ER antagonist ICI abolished that effect. In this study, brown trout primary hepatocytes were used to explore the changes in mRNA and protein levels of selected estrogenic targets, after exposure to DHT (non-aromatizable to estrogens) (Martyniuk et al., 2013) and the aromatizable T.

In regards to VtgA and ERα mRNA levels, significant up-regulations after both DHT and T exposures were observed (from 1 μM of DHT and 10 μM of T), suggesting androgen-induction as the causal link for the noted increases in brown trout hepatocytes. On the contrary, the levels of ERβ1 were down-regulated by the highest concentrations of androgens.

In a previous study with juvenile brown trout, the *in vivo* exposure to 50 μg/L of T induced VtgA mRNA levels, with no changes in the ERα and ERβ1 levels (Madureira et al., 2018). Similarly, administration of DHT through diet (2 ppm) to juvenile rainbow trout produced an up-regulation in Vtg1 mRNA, whereas the levels of ERα and ERβ were not changed (Benninghoff and Williams, 2008). For non-salmonid fish, the exposure of juvenile crucian carp to 50 μg/L of MT increased the VtgB transcript levels while ERα and ERβ1 were mainly down-regulated (Zheng et al., 2013). On the contrary, T (1 μg/L) reduced the levels of the VtgA, B and C in female western mosquitofish, along with ERα and ERβ mRNA levels (Huang et al., 2012). Further, in the hermaphrodite fish *Kryptolebias marmoratus,* intraperitoneal injection of MT (0.1–100 μg/g of body weight) resulted in a reduction of Vtg and ERα, with no changes in ERβ mRNA levels, at 7 days post-injection (Park et al., 2013). According to the cited literature examples, no clear pattern had been established to date for the modulation of Vtg or ERs by distinct androgens in fish.

Our present data regarding the induction of Vtg and ER resemble the results from previous *in vitro* fish studies, but after estrogenic stimulation (Madureira et al., 2015; Yadetie et al., 2018). In the referred works, similarly to what happened in this study with T and DHT, VtgA and ERα were up-regulated by EE2 in brown trout primary hepatocytes (Madureira et al., 2015), and in liver slices from Atlantic cod (Yadetie et al., 2018). Highly positive correlations were obtained here between VtgA and ERα mRNA levels. Positive correlations between Vtg induction and ERα mRNA were also found *in vivo*, for instance in juvenile

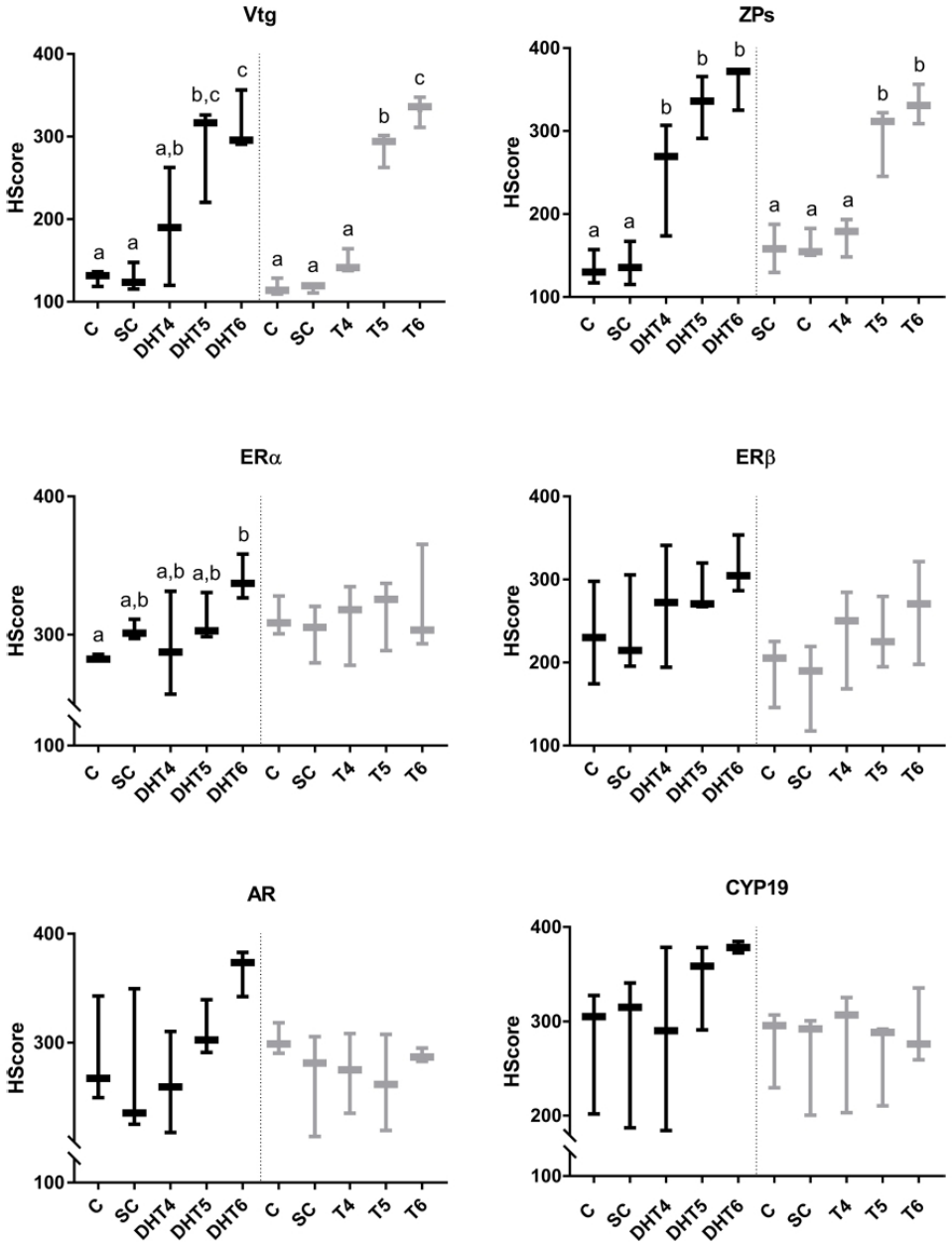

Fig. 4. HScores obtained for the immunohistochemical detection of the selected antibodies after 96 h of exposure to dihydrotestosterone (DHT) and testosterone (T). C - control (L-15 medium); SC - solvent control (0.1% ethanol in L15 medium); DHT4, DHT5 and DHT6 (1, 10 and 100 μM of DHT); T4, T5 and T6 (1, 10 and 100 μM of T). Data correspond to median, maximum, minimum, and interquartile range (Q3-Q1). Different letters mean significant differences between groups, according to the Tukey test. n = 3 replicates, from 3 independent experiments.

Atlantic salmon, after waterborne exposure to the xenoestrogen 4-nonylphenol (Meucci and Arukwe, 2006), juvenile brown trout, after water exposure to EE2 (Körner et al., 2008), and in medaka after the stimulus by various estrogens (Yost et al., 2014). The role that ERβ subtypes may play in the induction of Vtg, mRNA or protein, by estrogenic compounds has been also established in fish, such as goldfish (Nelson and Habibi, 2010), zebrafish (Griffin et al., 2013) and rainbow trout (Leaños-Castañeda and Van Der Kraak, 2007). Contrarily to ERα, ERβ is commonly down-regulated after exposure to estrogens (Madureira et al., 2015; Marlatt et al., 2008; Menuet et al., 2004), which was also observed in our study, after exposure to both androgens.

With regards to Vtg, the results from the morphological analysis agreed with those obtained by the molecular analysis. Thus, the up-regulation of VtgA mRNA levels appeared to have been translated into increased HScores of the Vtg protein that were observed at the higher concentrations of both androgens. The same was partially verified for

the ERs, because the HScores of ERα tended to increase with the concentrations of DHT and T. Interestingly, protein abundance of ERβ also increased (following an opposite pattern than that observed at the mRNA level). It must be noted that the ER antibodies used in the study were synthesized against human proteins. So, even though they cross-reacted with fish receptors (see Section 2.5.1.), they may not be able to distinguish between protein subtypes that do exist in fish. This may explain, at least in part, the differences found at the mRNA and protein levels.

Along with Vtg, ZPs have been also consistently induced by estrogenic compounds (Arukwe et al., 1997; Gunnarsson et al., 2007), and were even more responsive than Vtg to low doses of estrogens (Celius et al., 2000; Uren Webster et al., 2015). Similar to the observations made for Vtg, highly significant and positive correlations were found between ZPs and ERα mRNA expression in Atlantic salmon exposed to nonylphenol (Meucci and Arukwe, 2006), and in medaka after exposure to different estrogenic compounds (Yost et al., 2014). Previously, ZP2.5 and ZP3a.2 were up-regulated in brown trout exposed to E2, with higher induction of ZP2.5 than ZP3a.2 (Uren Webster et al., 2015). Our study also showed an up-regulation of the same genes, with a similar profile (ZP2.5 more responsive than ZP3a.2), but after androgenic stimuli (DHT and T).

Changes in the mRNA levels of ZPs after exposure to androgens have been reported in only a few fish studies, such as in rainbow trout juveniles, where DHT administration caused a significant elevation of a liver ZP gene (Benninghoff and Williams, 2008). Conversely, a down-regulation of hepatic ZP2 and ZP3a.1 was observed after exposure to 1 μg/L of the androgen 17β-trenbolone in western mosquitofish (Brockmeier et al., 2013). In cultured testes from eel, 11-ketotestosterone (11-KT) supressed the mRNA levels of ZP2 and ZP3, while an increase in the mRNA levels of a zona pellucida related protein (zona pellucida protein A domain - ZPA) was observed in Atlantic cod cultured oocytes exposed to T (at both 5 and 10 days of exposure) and 11-KT (only at 5 days of exposure) (Kortner et al., 2008; Miura et al., 1998). *In vivo*, the intraperitoneal injection of Atlantic cod with T resulted in a down-regulation of the ovarian ZPA mRNA levels and another ZP, named egg envelope glycoprotein, while no significant changes were observed after exposure to 11-KT (Kortner et al., 2009). As observed for Vtg, the androgenic interferences in the mRNA levels of ZPs do not follow a standard profile. Once again, our results seem to follow the pattern observed for ZPs induction by estrogenic compounds since moderate to highly positive correlations were found between ZP genes and ERα. As for Vtg, the changes in ZPs HScores fully agreed with the results obtained for the ZP genes in the molecular study, meaning that increased mRNA levels seem to consistently translate to the protein level.

In our study, both AR mRNA and its immunophenotype presented an increasing trend after exposure to micromolar concentrations of DHT, whereas the changes caused by T were mostly insignificant. In mammals, down-regulation of AR after exposure to androgens is a common finding (Kemppainen et al., 1992; Wolf et al., 1993), although an up-regulation has also been reported (Hunter et al., 2018). Fish data regarding AR regulation by androgens are also quite variable. For instance, the liver ARs (ARα and ARβ) of the western mosquitofish were not regulated by T in males (Huang et al., 2013), whereas in females the ARα was suppressed by T (Huang et al., 2012) and the ARβ by 17β-trenbolone (Brockmeier et al., 2013). In contrast, an up-regulation of AR mRNA levels was noted in gonads of juvenile crucian carp exposed to 50 and 100 μg/L of MT (Zheng et al., 2016).

The expression of the aromatase CYP19a1 gene was not altered in our assays by any of the studied androgens, while CYP19 HScores showed an increasing trend at higher doses of DHT. Similarly, in ovaries of Atlantic cod, 11-KT and T did not alter the expression of this gene, though a slight increasing trend was observed after treatment with T (Kortner et al., 2009). Changes in aromatase expression (Mouriec et al., 2009; Zheng et al., 2016) and activity (Hornung et al., 2004) were however found in fish brain and ovary after androgenic exposure. Although we did not measure aromatase activity, the absence of changes in aromatase at the gene expression and protein level suggests that the induction of VtgA and ERα did not occur as a consequence of estrogen synthesis, *via* aromatization of T. In agreement with this idea, in primary hepatocytes isolated from sexual mature Mozambique tilapia, fadrozole (an aromatase inhibitor) failed to reduce Vtg synthesis triggered by T (Kim et al., 2003). Nevertheless, aromatization of MT and T has been shown to occur *in vivo* (Hornung et al., 2004; Zerulla et al., 2002). In fathead minnow, MT conversion into 17α-methylestradiol, was related to increased Vtg plasma levels (Hornung et al., 2004) and fadrozole had an inhibitory effect on Vtg induction by MT (Zerulla et al., 2002). Since aromatase genes are highly expressed in fish brain and gonads (especially ovary) and to a lesser extent in the liver (Cheshenko et al., 2008), the aromatization of androgens to estrogens *in vivo* may play a more relevant role in Vtg production/induction than *in vitro*.

In previous fish studies, some authors have assumed that estrogenic effects produced by DHT may happen *via* conversion of DHT to 3β-diol (5α-androstane-3β, 17β-diol), which is an estrogenic compound (Bhatia and Kumar, 2016; Mouriec et al., 2009). This compound is known to produce its effects through the ERβ (Guerini et al., 2005; Handa et al., 2008). However, the induction of the estrogenic targets observed in our study does not seem to occur by this path, since ERβ mRNA levels were down-regulated after DHT exposures. Further supporting this theory is the fact that 11-KT and 17β-trenbolone, that are both considered not to be convertible to estrogenic compounds, were as well shown to induce Vtg and ZPs (Ankley et al., 2003; Blum et al., 2004; Kortner et al., 2008).

Considering the above discussions, the results point to the induction of Vtg and ZPs by direct binding of androgens to ERs (most probably ERα), rather than by indirect conversion to estrogens. DHT and T modulated all the estrogenic targets in the same direction (up or down-regulation). Both androgens were already shown to bind human and fish ERs (Gaido et al., 1997; Le Dréan et al., 1995; Matthews et al., 2000), even though higher concentrations are required, compared with natural ligands such as E2. This can explain why the effects on the estrogenic targets were only seen at micromolar concentrations of the tested androgens. DHT generally produced a more substantial effect than T. In a yeast assay, DHT was also more potent than T as an agonist to the human ER (Gaido et al., 1997). As well, in primary hepatocytes from Mozambique tilapia females, DHT was more effective than T and MT in the induction of Vtg protein (Kim et al., 2003), further suggesting that the same agonistic differences between androgens for estrogen receptors may be present in fish.

5. Conclusions

Exposure to the aromatizable and non-aromatizable androgens T and DHT, respectively, triggered molecular and phenotypical responses in brown trout hepatocytes similar to those typically elicited by natural and synthetic estrogens. These observations strengthen the notion that androgens can interfere in estrogenic signaling pathways in fish liver, including by direct binding to ERs. Thus, the use of Vtg and ZPs as exclusive estrogenic biomarkers should be carefully considered in fish physiology and toxicology contexts. As to future research perspectives, the ability of androgens to alter the expression of proteins that are essential during the processes of brown trout oogenesis raises the question of the impact of such disruptions at the reproductive levels. In this regard, it is promising to explore the potential of androgens to modulate other metabolic pathways crucial for gonad differentiation and maturation (and regulated by estrogens), such as lipid metabolism.

CRediT authorship contribution statement

Célia Lopes: Conceptualization, Methodology, Investigation,

Formal analysis, Visualization, Writing - original draft. **Tânia V. Madureira:** Conceptualization, Methodology, Validation, Investigation, Writing - review & editing, Supervision. **José F. Gonçalves:** Resources, Writing - review & editing. **Eduardo Rocha:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105586.

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Supplementary Figure Captions

Fig. S1 – Immunohistochemical detection of vitellogenin (Vtg) in brown trout hepatocytes after 96 h of exposure to dihydrotestosterone (DHT) and testosterone (T). Arrowheads point out cells with a mixed diffuse and granular pattern of staining. A - solvent control (0.1% ethanol in L15 medium); B - DHT4 (1 μM of DHT); C - DHT5 (10 μM of DHT); D - DHT6 (100 μM of DHT); E - T4 (1 μM of T); F - T5 (10 μM of T); G - T6 (100 μM of T). n = 3 replicates, from 3 independent experiments.

Fig. S2 – Immunohistochemical detection of zona pellucida proteins (ZPs) in brown trout hepatocytes after 96 h of exposure to dihydrotestosterone (DHT) and testosterone (T). Arrowheads mark cells with a mixed diffuse and granular pattern of staining. A - solvent control (0.1% ethanol in L15 medium); B - DHT4 (1 μM of DHT); C - DHT5 (10 μM of DHT); D - DHT6 (100 μM of DHT); E - T4 (1 μM of T); F - T5 (10 μM of T); G - T6 (100 μM of T). n = 3 replicates, from 3 independent experiments.

Fig. S3 – Immunohistochemical detection of estrogen receptor alpha (ERα) in brown trout hepatocytes after 96 h of exposure to dihydrotestosterone (DHT) and testosterone (T). Arrowheads point cells were the granular pattern of staining is more evident. A - control (L15 medium); B - solvent control (0.1% ethanol in L15 medium); C - DHT6 (100 μM of DHT); D - control (L15 medium); E - solvent control (0.1% ethanol in L15 medium); F - T6 (100 μM of T). n = 3 replicates, from 3 independent experiments.

Fig. S4 – Immunohistochemical detection of estrogen receptor beta (ERβ, A-C), androgen receptor (AR, D-F) and aromatase (CYP19, G-I) in brown trout hepatocytes after 96 h of exposure to dihydrotestosterone (DHT) and testosterone (T). Arrowheads point cells with cytoplasmic granules (A-C) and granular clusters (D-I). A, D and G - solvent control (0.1% ethanol in L15 medium); B, E, H - DHT6 (100 μM of DHT) and C, F and I - T6 (100 μM of T). n = 3 replicates, from 3 independent experiments.

Fig. S1

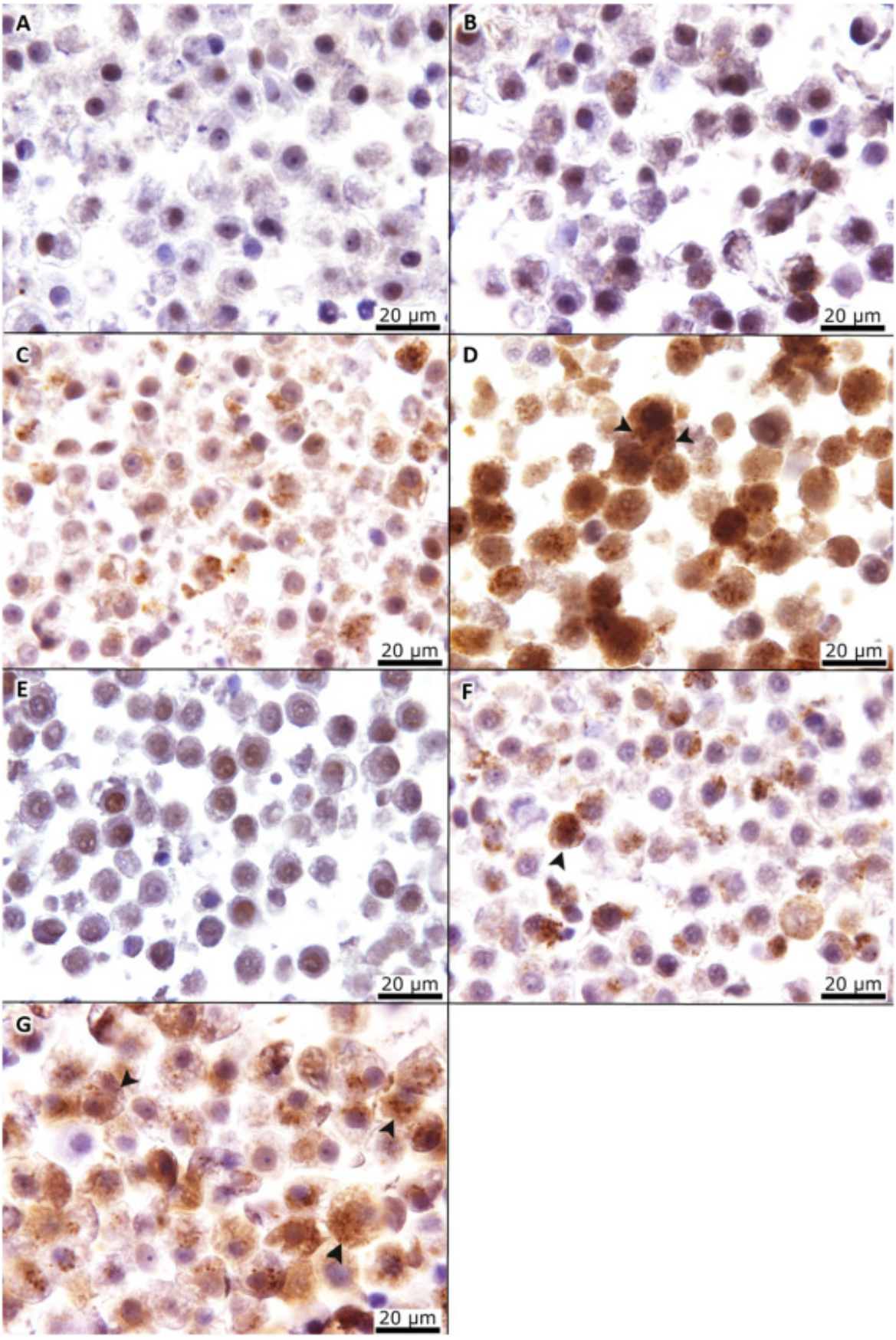


Fig. S2

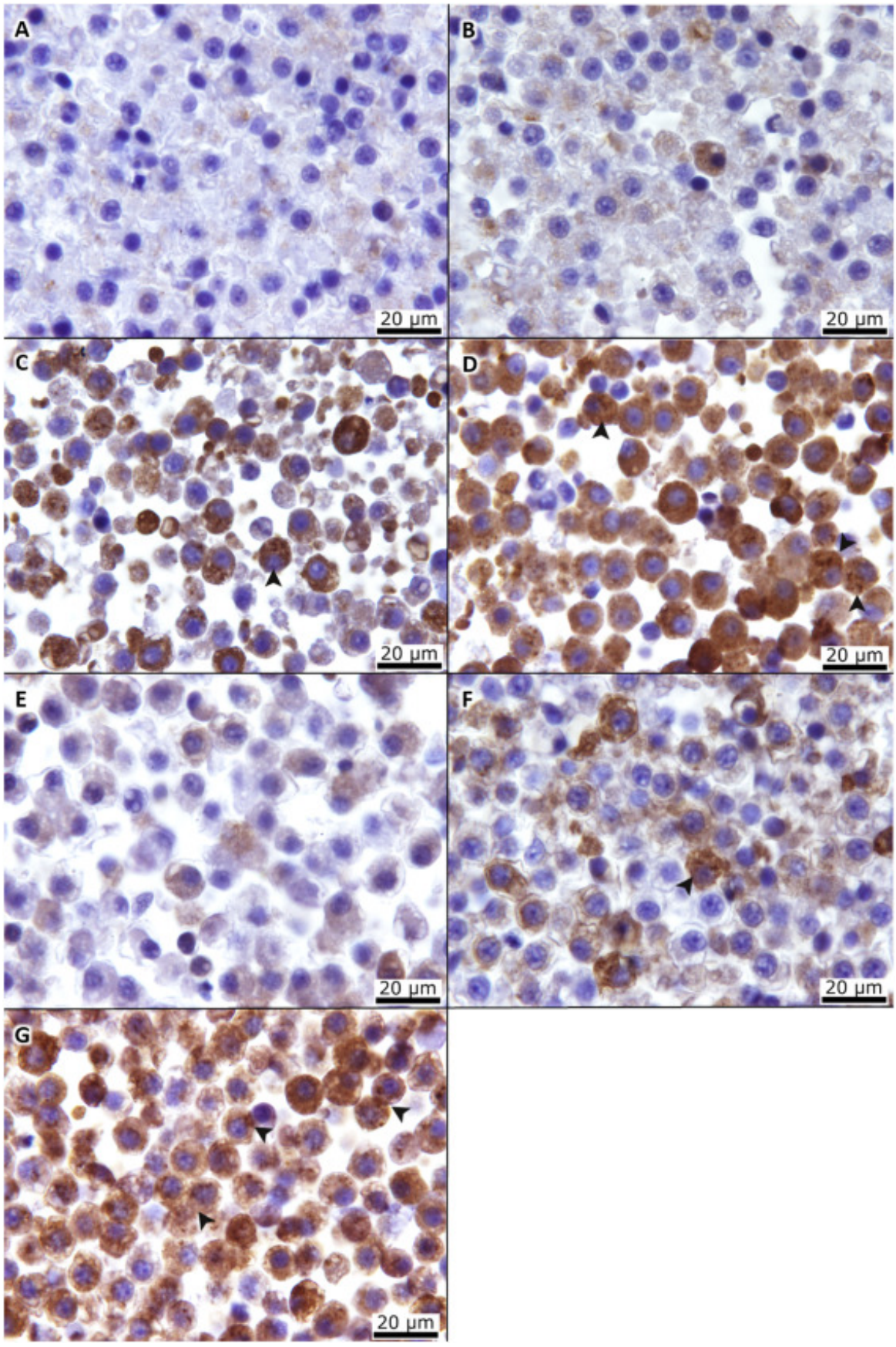


Fig. S3

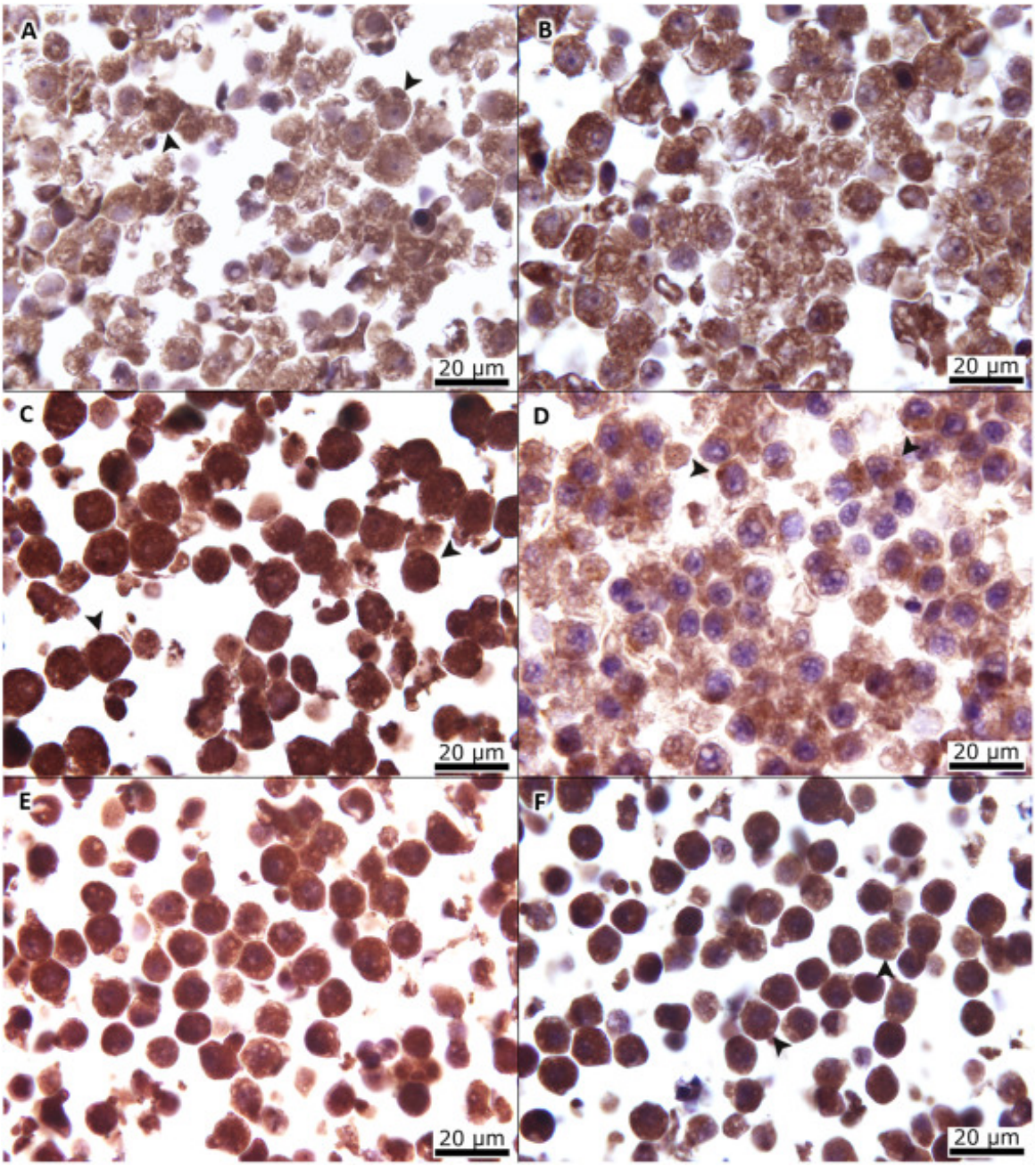
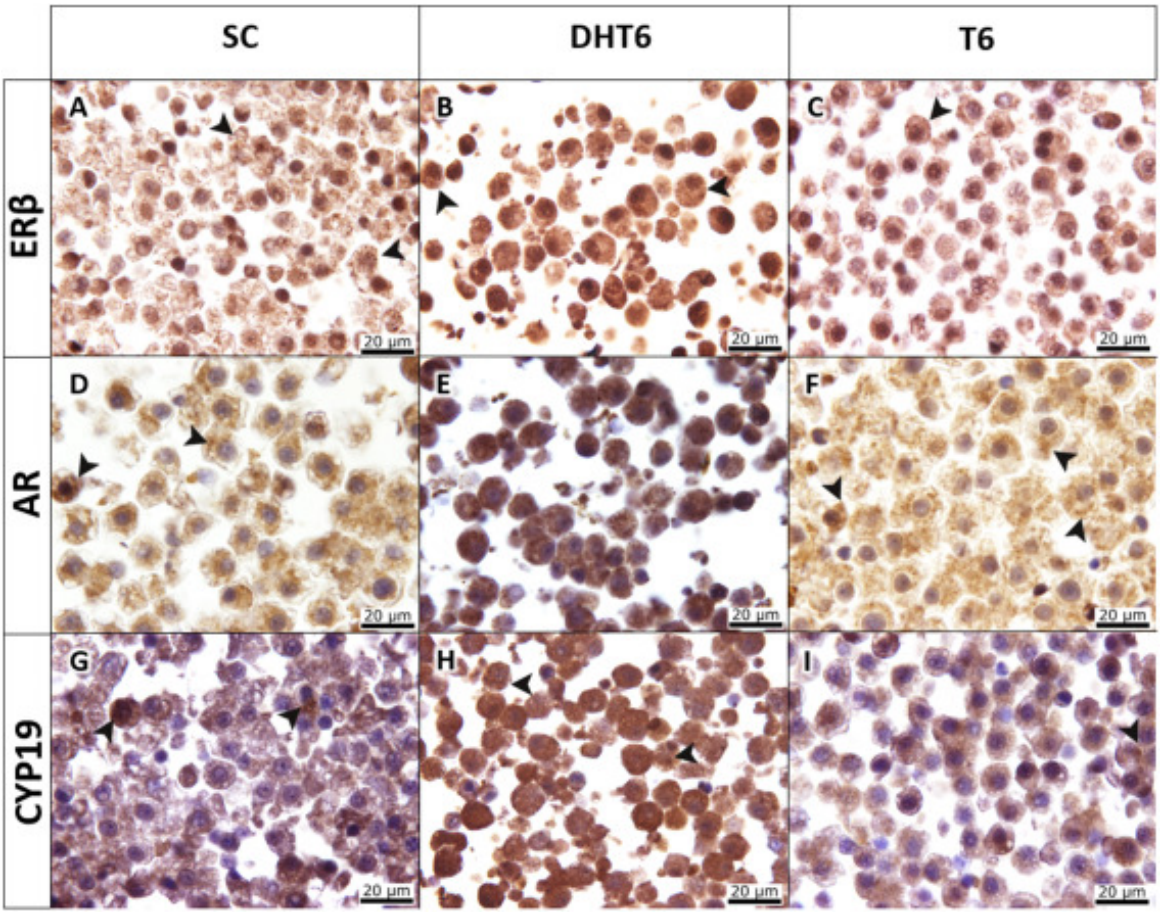


Fig. S4



Chapter 5

Deciphering interferences of testosterone and dihydrotestosterone on lipid metabolism genes using brown trout primary hepatocytes

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Deciphering interferences of testosterone and dihydrotestosterone on lipid metabolism genes using brown trout primary hepatocytes

Abstract

Despite of physiological and toxicological relevance, the potential of androgens to regulate/ disrupt fish lipid metabolism remains a poorly explored topic. Here, brown trout primary hepatocytes were exposed to six concentrations (1 nM to 100 μM) of dihydrotestosterone (DHT) and testosterone (T). The mRNA levels of target genes covering diverse lipid metabolic pathways were quantified. ApoA1, the major component of high-density lipoprotein (HDL), was down-regulated by both androgens, while Fabp1 and StAR were not altered. LPL, which mediates fatty acid up-take, was also not changed. Acsl1, a pivotal enzyme for fatty acid activation, was up-regulated by T and DHT. Lipogenic enzymes FAS and ACC were up-regulated by the highest (100 μM) concentration of T and DHT, respectively. Acox1-3l, rate-limiting enzyme in peroxisomal β-oxidation, was down-regulated by T. PPARγ, regulator of adipogenesis and peroxisomal β-oxidation, was down-regulated by both androgens. Our findings suggest that androgens influence lipid metabolism in fish liver.

Keywords: Lipids, Testosterone, Dihydrotestosterone, Primary hepatocytes, Brown trout.

Abbreviations

11-KT – 11-ketotestosterone; ACC – Acetyl-Coenzyme A carboxylase; Acsl – Long-chain acyl-coenzyme A synthetase; Acox – Acyl-coenzyme A oxidase; Apo – Apolipoprotein; AR – Androgen receptor; ATP – Adenosine triphosphate; β-act – Beta actin; CoA – Coenzyme A; Cpt1 – Carnitine Palmitoyltransferase 1; DHT – Dihydrotestosterone; E2 – Estradiol; ERα – Estrogen receptor alpha; Fabp – Fatty acid binding protein; FAS – Fatty acid synthase; FXR – Farnesoid X receptor; G6PDH – Glucose-6-phosphate dehydrogenase; HDL – High-density lipoprotein; ICDH – Isocitrate dehydrogenase; LPL – Lipoprotein lipase; LXR – Liver X receptor; ME – Malic enzyme; PPAR – Peroxisome proliferator-activated receptor; PUFA – Polyunsaturated fatty acid; rpl8 – Ribosomal protein L8; SREBPs – Sterol regulatory element-binding proteins; StAR – Steroidogenic acute regulatory protein; T – Testosterone; TB – Trenbolone; VLDL – Very low-density lipoprotein; Vtg – Vitellogenin; ZPs – Zona pellucida proteins.

1. Introduction

Fish utilize carbohydrates less efficiently than terrestrial vertebrates, and hence lipids and proteins are the major macronutrients and organic components of the fish body (Leaver et al., 2008; Tocher, 2003). Aside from being an energy resource for key metabolic functions as somatic growth and reproduction, specific lipids, such as PUFA, also control gene transcription from lipid metabolic pathways, including the expression of PPARs (Tocher and Glencross, 2015). PPARs, in turn, regulate the transcription of several enzymes involved in lipid anabolic and catabolic pathways (Karagianni and Talianidis, 2015; McMullen et al., 2014; Wang et al., 2020; Zheng et al., 2013). However, the expression of each gene can be influenced by more than one PPAR subtype (Cunha et al., 2013; Heinäniemi et al., 2007). It is also known that the transcriptional regulatory network of PPARs includes indirect interactions with various signaling pathways, such as AR, ERα, LXR and SREBPs (Karagianni and Talianidis, 2015; McMullen et al., 2014).

The liver has a crucial role in lipid metabolism by controlling energy storage through key pathways such as lipolysis, lipogenesis, lipid transport and oxidation (Ayisi et al., 2018; Birzniece, 2018), which are quite similar in fish and mammals (Tocher and Glencross, 2015; Zheng et al., 2013). The process of lipogenesis includes *de novo* fatty acid synthesis from acetyl-CoA, through the FAS enzymatic complex, and the subsequent synthesis of complex lipids (e.g., triglycerides and phospholipids) by fatty acid esterification with glycerol (Tocher and Glencross, 2015). The catabolic process of fatty acid β-oxidation takes place in mitochondria and peroxisomes, and ultimately results in energy production in the form of ATP (Boukouvala et al., 2004; Tocher and Glencross, 2015).

The lipid metabolism in the liver is hormonally-regulated, under normal physiological and pathological conditions (Kur et al., 2020; Lazo et al., 2015). Low levels of E2 and T have been linked with liver lipid accumulation in mammal models (Birzniece, 2018; Shen and Shi, 2015). However, the presumed protective effect of sex hormones in lipid deposition is not a universal concept, as it has been shown that higher levels of E2 are associated with fatty liver in men and women (Lazo et al., 2015). In the latter study, higher T levels were associated with increased and decreased hepatic lipid contents in women and men, respectively (Lazo et al., 2015). Sex-specific effects of T were also observed in primary hepatocytes from human female donors, where T increased lipogenesis, but had no effect on male hepatocytes (Nasiri et al., 2015). Illustrating the regulatory complexity, the androgen DHT had an opposite effect, decreasing lipogenesis in female hepatocytes (Nasiri et al., 2015). However, when using a human hepatocyte cell line (C3A), DHT and T increased lipogenesis as well as the expression of the lipogenic enzymes ACC and FAS (Nasiri et al., 2015). Male mice with liver targeted AR knockout, and fed a high-fat diet,

developed liver steatosis, increased mRNA levels of ACC, SREBP1c and PPARγ and decreased those of PPARα (Lin et al., 2008). Mice with non-functional AR (Tfm) also had increased liver lipid deposition, and an up-regulation of mRNA and protein expression of FAS and ACC in the liver, an effect that was reversed by T (Kelly et al., 2016; Kelly et al., 2014). Likewise, DHT down-regulated FAS gene expression in orchiectomized rats (Zhang et al., 2013). Therefore, there is evidence of androgenic regulation in lipid synthesis and deposition in mammals, even though the disparity of results between species and models.

In previous works using brown trout (*Salmo trutta* f. *fario*) primary hepatocytes as a model, T had no effect on PPARα mRNA, but decreased the expression of PPARγ and changed the morphology of peroxisomes (Lopes et al., 2016; Lopes et al., 2017). T and DHT also increased Vtg and ZPs expression, which are related to the processes of vitellogenesis and oogenesis in fish (Lopes et al., 2020; Lopes et al., 2017). Considering the existing interplay between fish hormonal levels, gonad maturation and changes in lipid handling (Cleveland and Weber, 2016; Sharpe and MacLatchy, 2007; Singh and Singh, 1990), it is conceivable that androgens (endogenous or exogenous) may also potentially interfere in fish lipidic metabolic pathways. Data from Eastern mosquitofish (*Gambusia holbrooki*) and rainbow trout (*Oncorhynchus mykiss*) transcriptomic studies, after *in vivo* waterborne exposure to 1µg/L of the synthetic androgen (17β)-TB, showed an up-regulation of genes involved in metabolic processes related to lipid binding and fatty acid biosynthesis in the liver (Brockmeier et al., 2013; Hook et al., 2006). The same was noticed in the gonads of male mummichog (*Fundulus heteroclitus*) (Feswick et al., 2014) and female coho salmon (*Oncorhynchus kisutch*) (Monson et al., 2017) after a 21-day water exposure to DHT (5 and 50 µg/L) and a short (1-3 days) exposure to intraperitoneal pellets containing 5 µg of 11-KT, respectively. In the mummichog testis, genes from lipid transport and oxidation pathways were down-regulated by DHT exposure, whereas genes involved in triglyceride biosynthesis were up-regulated (Feswick et al., 2014). In the coho salmon ovary, fatty acid oxidation was also decreased, while genes related to lipid binding, uptake and processing (e.g., LPL, ApoO and Fabp2) were up-regulated (Monson et al., 2017). However, studies have rarely tackled the effects of aromatizable and non-aromatizable androgens on lipid-related targets in fish.

In this study, we used brown trout isolated primary hepatocytes to explore the potential of T and DHT to alter the expression of genes involved in key anabolic and catabolic lipidic pathways, including: 1) PPARγ – regulation of adipogenesis and β-oxidation (Ayisi et al., 2018; Ruyter et al., 1997); 2) ApoA1 and Fabp1 – lipid transport (Ayisi et al., 2018); 3) Acsl1 – fatty acid activation (Li et al., 2010); 4) LPL – lipolysis/fatty acid up-take (Ayisi et al., 2018); 5) StAR – cholesterol transport/metabolism, steroidogenesis and bile acids synthesis (Hall

et al., 2005; Zheng et al., 2016); 6) Acox1-3l – peroxisomal β-oxidation (Madureira et al., 2016; Rakhshandehroo et al., 2010) and 7) ACC and FAS – *de novo* lipogenesis (Ayisi et al., 2018). Some of the selected genes (Acsl1, Acox1-3l, Fabp1, ApoA1, and LPL) contain peroxisome proliferator response elements and are thus recognized as direct targets of PPARs, at least in mammals (Heinäniemi et al., 2007; Rakhshandehroo et al., 2010). A few prior studies with fish presented evidence that the genes ApoA1, LPL, ACC and StAR may be regulated by androgens (Brockmeier et al., 2013; Cleveland and Weber, 2016; Monson et al., 2017; Rolland et al., 2013). Therefore, the present study adds to the minimal knowledge about the regulation of liver lipid metabolism by androgens in fish and expands the existing evidence of interactions between androgens and peroxisomal signaling.

2. Materials and methods

2.1. Animals and hepatocyte culture

One-year-old brown trout juveniles were purchased from a Portuguese aquaculture (Torno, Amarante), and transported to our institute aquatic facilities. During the acclimation period (2 months) fish were maintained under a 12/12 h dark/light cycle and were daily fed once, to satiation, with a pelletized commercial diet (MicroBalance™, T4 Optiline, Skretting). Food was suspended 24 h before the experiments. The fish used had a mean (standard deviation) weight of 60.5 (4.6) g in DHT and 58.0 (7.3) g in T experiments; the total length was 18.3 (1.0) cm in DHT and 18.7 (1.0) cm in T experiments. The procedures with fish followed the guidelines on animal protection for scientific purposes of the Portuguese Decree-Law No. 113/2013 and the EU Directive No. 2010/63.

Fish were killed by an overdose of 2-phenoxyethanol (8.07291, Merck). Then, for primary hepatocytes isolation, the liver was excised and perfused as described elsewhere (Madureira et al., 2016). After separation, hepatocytes were suspended in phenol red-free Leibovitz's L15 medium (21083-027, Alfagene), with 5% of charcoal-stripped fetal bovine serum (F6765, Sigma-Aldrich) and 1% of penicillin-streptomycin solution (P4333, Sigma-Aldrich). Viability was measured by the trypan blue exclusion assay using the Countess™ Automated Cell Counter (Invitrogen), following the manufacturer's instructions. Cells were then plated at a density of $1x10^6$/mL, in previously poly-L-lysine (P2636, Sigma-Aldrich) treated 24 well plates (4430300N, Orange Scientific), cultured at 19 ºC and maintained for 24 h before the exposures.

2.2. Exposure to androgens

Primary hepatocytes were treated with T (86500, Sigma-Aldrich) and DHT (A8380, Sigma-Aldrich). Three independent experiments were made, per compound, each one with cells from a single fish. The experimental conditions included a control, consisting of supplemented culture media (C), a solvent control (SC), 0.1% ethanol in the same culture media, and six concentrations of T or DHT, depending on the experiment, corresponding to1 nM (T1 or DHT1), 10 nM (T2 or DHT2), 100 nM (T3 or DHT3), 1 μM (T4 or DHT4), 10 μM (T5 or DHT5) and 100 μM (T6 or DHT6). The same range of concentrations was previously used to evaluate the effect of androgens on estrogenic targets in brown trout hepatocytes (Lopes et al., 2020).

All individual experiments consisted of three culture plates, and each plate contained two wells to each condition (only one from each plate was later used for the molecular analysis). The different treatments/conditions were randomly assigned to the plate wells so that these can be regarded as the experimental unit *in vitro*, despite it is accepted that opinions on this matter do diverge (Festing and Altman, 2002; Lazic et al., 2018). Exposure solutions were prepared from stock solutions in absolute ethanol (1.00983, Merck), to a final concentration of 0.1% ethanol in the exposure media. After the 96 h of exposure, hepatocytes were detached from the plate wells by trypsinization with 1x trypsin/EDTA solution (59418C, Sigma-Aldrich), and viability was measured in 3 wells per condition. Cells were then pelleted by centrifugation (160 × g, 5 min at 4 ºC), snap-frozen in liquid nitrogen and stored at - 80 ºC until further use.

2.3. Molecular analysis

Per experiment, three independent pellets from each condition, one from each plate (which gives a total of 9 pellets/condition), were randomly assigned for qRT-PCR. Total RNA was extracted with the illustra™ RNAspin Mini Isolation Kit (25-0500-72, GE Healthcare). Quantification and quality assessment of the extracted RNA were made using the Multiskan Go spectrophotometer (Thermo Scientific) with the μDrop™ plate. The iScript™ cDNA Synthesis Kit (1708841, Bio-Rad) was used to synthesize cDNA from 250 ng of total RNA. A CFX Connect™ (Bio-Rad) equipment was used for the qRT-PCRs. The reaction mixtures (20 μL) consisted of 5 μL of diluted (1/5) cDNA, 10 μL of iQ™ SYBR®Green Supermix (1708886, Bio-Rad) or SsoFast™ EvaGreen® Supermix (1725204, Bio-Rad) and 200 nM (for SYBR®Green reactions) or 300 nM (for EvaGreen® reactions) of specific primers for each gene. The cDNA samples were analyzed in duplicate, and no-template controls were included in all qRT-PCR plates. A calibration sample, consisting of a mixture of cDNA from

several experimental conditions was also included in all PCRs. A melt curve was generated in each run, to verify the specificity of the amplified product. Relative mRNA expression of the target genes was normalized by the Pfaffl mathematical model (Pfaffl, 2001). The selected reference genes were rpl8 and β-act (Lopes et al., 2017).

Primer sequences and protocols were adapted from previous studies in brown trout and Atlantic salmon (*Salmo salar*) (Caballero-Solares et al., 2018; Körner et al., 2008; Lopes et al., 2016; Madureira et al., 2016; Madureira et al., 2018; Madureira et al., 2017; Minghetti et al., 2011; Todorčević et al., 2008; Vang et al., 2007). Details are given in Table 1. For the newly optimized genes (FAS, LPL, ACC, and StAR), reaction efficiencies were obtained by the generation of calibration curves with serial dilutions of brown trout liver cDNA. The size of the amplification products was checked by agarose gel (1%) electrophoresis. Because the size of StAR amplicon did not match the previously reported (Vang et al., 2007), the PCR sample was sequenced and confirmed to be the expected product.

Table 1. Primer sequences, annealing temperatures and respective efficiencies used for qRT-PCR.

Gene	Primer Sequence 5'-3'	Annealing Temperature (ºC)	Efficiency (%)	Reference
Acetyl-CoA carboxylase **ACC**	F: TTTTGATGGCGATCTTGACA R: CATCACAATGCCTCGCTCTA	60	102.2#	Caballero-Solares et al., 2018
Acyl-CoA oxidase 1 3l **Acox1–3l**	F: TGTAACAAGGAGCAGTTCG R: TTGCCGTGGTTTCAAGCC	56	96.9*	Madureira et al., 2016
Acyl-CoA long chain synthetase 1 **Acsl1**	F: CGACCAAGCCGCTATCTC R: CCAACAGCCTCCACATCC	55	97.8#	Madureira et al., 2018
Apolipoprotein A1 **ApoA1**	F: ATGAAATTCCTGGCTCTTG R: TACTCTTTGAACTCTGTGTC	55	89.9#	Madureira et al., 2017
Fatty acid binding protein 1 **Fabp1**	F: GTCCGTCACCAACTCCTTC R: GCGTCTCAACCATCTCTCC	57	97.7#	Madureira et al., 2017
Fatty acid synthase **FAS**	F: ACCGCCAAGCTCAGTGTGC R: CAGGCCCCAAAGGAGTAGC	60	95.1#	Minghetti et al., 2011
Lipoprotein lipase **LPL**	F: TGCTGGTAGCGGAGAAAGACAT R: CTGACCACCAGGAAGACACCAT	60	104.1#	Todorčević et al., 2008
Peroxisome proliferator-activated receptor gamma **PPARγ**	F: CGGAATAAGTGCCAGTAC R: GGGTCCACATCCATAAAC	56	98.1*	Lopes et al., 2016
Steroidogenic acute regulatory protein **StAR**	F: AGGATGGATGGACCACTGAG R: GTCTCCCATCTGCTCCATGT	63	104.5#	Vang et al., 2007
Beta actin **β-act**	F: TCTGGCATCACACCTTCTAC R: TTCTCCCTGTTGGCTTTGG	55	96.1# 97.4*	Madureira et al., 2017
Ribosomal protein L8 **rpl8**	F: TCAGCTGAGCTTTCTTGCCAC R: AGGACTGAGCTGTTCATTGCG	59	93.8# 99.0*	Körner et al., 2008

* SsoFast™ EvaGreen® Supermix

2.4. Statistical analysis

The Past3 (version 3.19) software was used for the statistical analysis (Hammer, 2001). Graphs were created in GraphPad Prism (version 7.04). Data were first tested for normality and homogeneity of variances, by the Shapiro-Wilk W and Levene's tests, respectively.

When necessary, data were mathematically transformed to meet the ANOVA assumptions. Statistical differences were assessed by one-way ANOVA, followed by the Tukey's post-hoc test, for comparisons between the different experimental conditions. The *p* value was set at 0.05.

3. Results

3.1. Cell Viabilities

The freshly isolated hepatocytes had mean viability (standard deviation) of 90 (3)%. In the DHT experiments, hepatocytes from the C and CS groups had viabilities of 84 (6) and 83 (10)%, respectively, whereas in the DHT6 group cell viability was 51 (23)%, differing significantly from the controls (Fig. 1). In the T experiments, controls had 89 (9) and 84 (6)% viabilities after 96 h of exposure, and there were no differences in viabilities between conditions (Fig. 1).

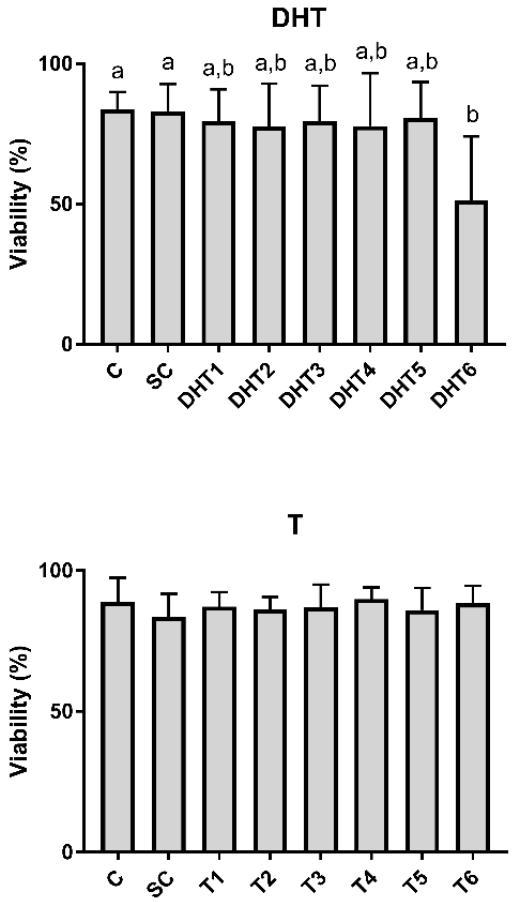

Fig. 1. Hepatocyte viabilities after 96 h of exposure to dihydrotestosterone (DHT) and testosterone (T). C - control (L15 medium); SC - solvent control (0.1% ethanol in L15 medium); DHT1 to DHT6 and T1 to T6 correspond to 1 nM, 10 nM, 100 nM, 1 μM, 10 μM and 100 μM, respectively. Different letters mean significant differences between conditions. n = 9 replicates (3 from each independent experiment).

3.2. Gene expression

The ApoA1 mRNA levels were down-regulated by both androgens, at the highest doses. Both controls differed from the 10 and 100 μM concentrations of DHT (Fig. 2), while only 100 μM of T caused a significant decrease of ApoA1 levels in relation to controls (Fig. 3).

Acsl1 levels were up-regulated by DHT and T, with noticeable increases from 100 nM of DHT and 1 μM of T. Comparing with controls, DHT increased Acsl1 mRNA at 100 nM and higher concentrations (Fig. 2). In contrast, Acsl1 mRNA increased above controls levels only after 10 and 100 μM of T (Fig. 3).

PPARγ was down-regulated by both androgens but with slightly different profiles. While in DHT experiments only the C, 1 nM and 10 nM conditions differed from the 100 μM concentration (Fig. 2), in the T experiments all conditions differed from the 100 μM concentration (Fig. 3).

The Acox1-3I mRNA levels were only changed by exposure to T, with a significant decrease at 100 μM, comparing with the other groups (Fig. 3).

The FAS and ACC genes showed an up-regulation profile after androgen exposures. T and DHT, at 100 μM, significantly increased FAS and ACC mRNA levels, respectively, compared with the controls (Figs. 2, 3).

At last, the LPL, Fabp1 and StAR mRNA levels were not changed by any of the androgens (Figs. 2, 3).

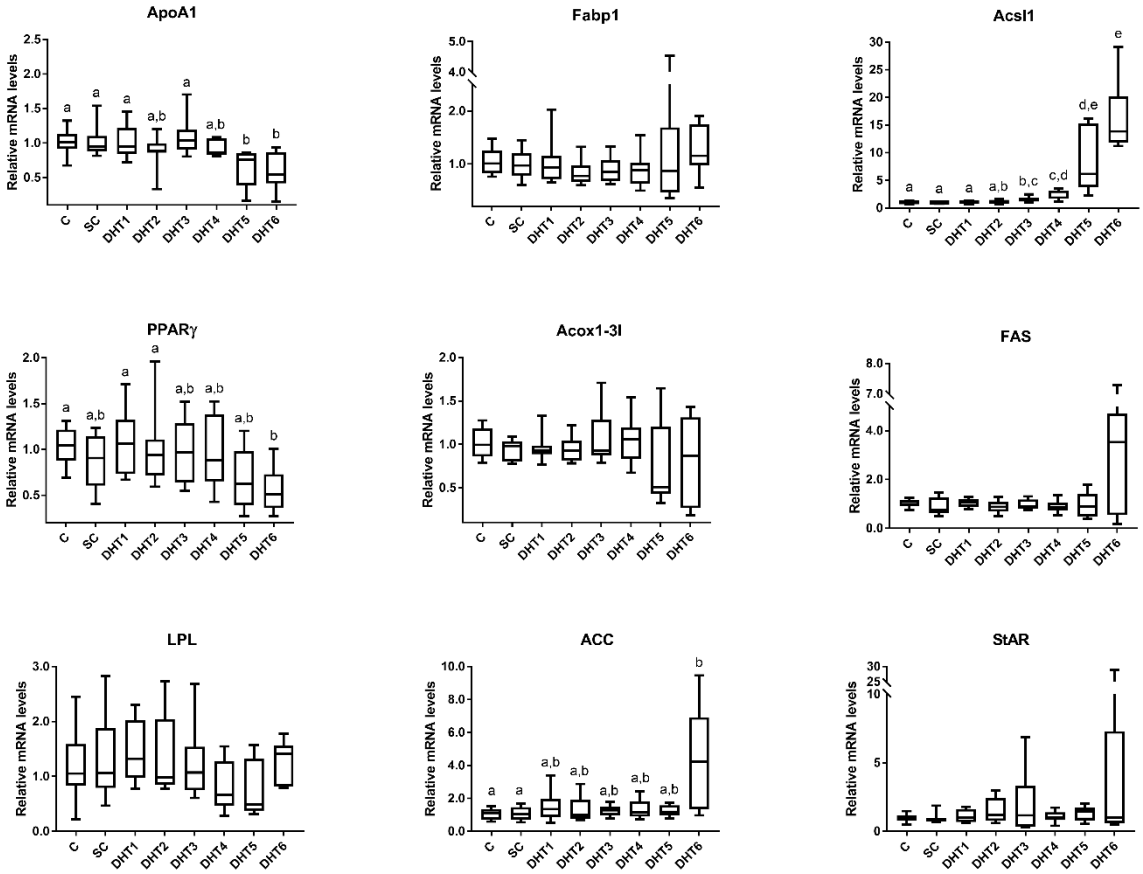

Fig. 2. Primary hepatocyte mRNA levels of the selected target genes after 96 h of exposure to dihydrotestosterone (DHT). C - control (L15 medium); SC - solvent control (0.1% ethanol in L15 medium); DHT1, DHT2, DHT3, DHT4, DHT5 and DHT6 (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM of DHT, respectively). Data correspond to median, maximum, minimum, and interquartile range (Q3-Q1). Different letters mean significant differences between conditions. n = 9 replicates (3 from each independent experiment).

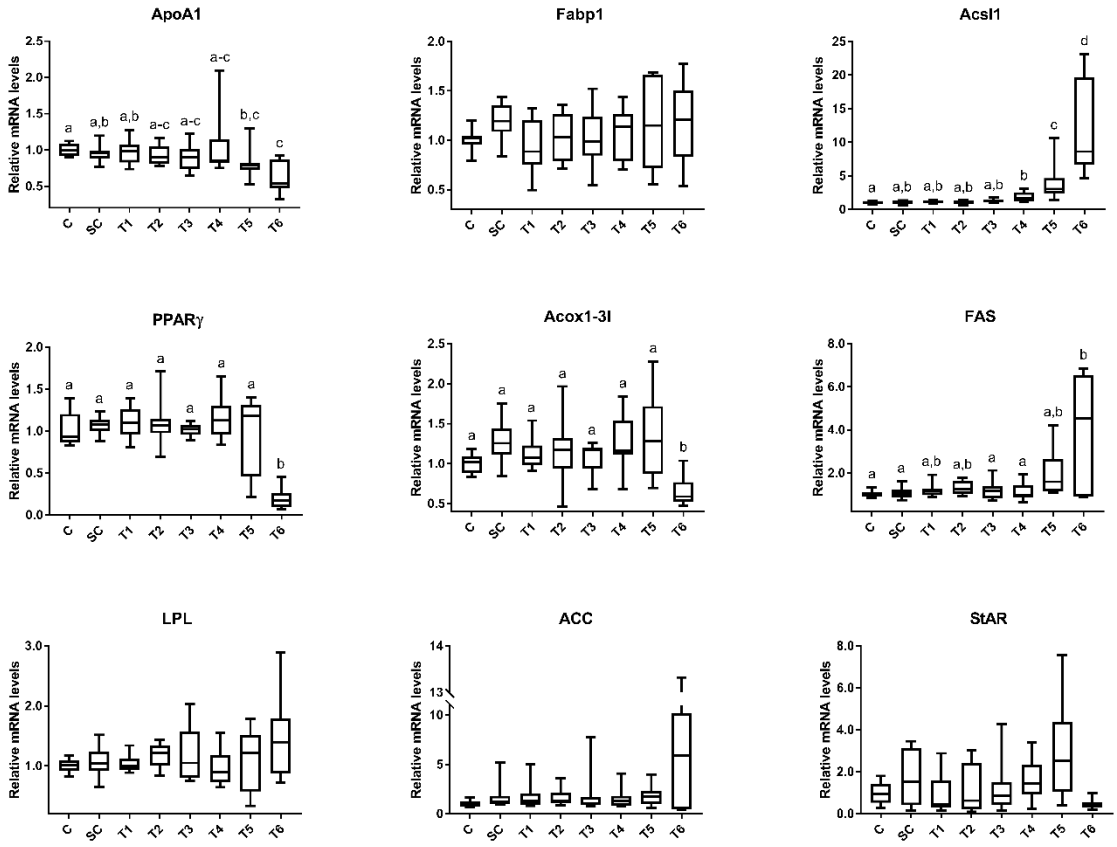

Fig. 3. Primary hepatocyte mRNA levels of the selected target genes after 96 h of exposure to testosterone (T). C - control (L15 medium); SC - solvent control (0.1% ethanol in L15 medium); T1, T2, T3, T4, T5 and T6 (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM of T, respectively). Data correspond to median, maximum, minimum, and interquartile range (Q3-Q1). Different letters mean significant differences between conditions, according to Tukey's test. n = 9 replicates (3 from each independent experiment).

4. Discussion

Previous studies in brown trout hepatocytes revealed that the model androgens DHT and T can disrupt either estrogenic or both estrogenic and peroxisomal signaling, respectively. *In vitro* evidence showed that T decreased the expression of PPARγ (Lopes et al., 2016), while a reduction in the size of peroxisomes was found *in vitro* and *in vivo* (Lopes et al., 2017; Madureira et al., 2018). In primary hepatocytes, both androgens, which are aromatizable (T) and non-aromatizable (DHT) to estrogenic compounds, increased the mRNA and protein expression of Vtg and ZPs, that play pivotal roles in fish oogenesis (Lopes et al., 2020). Considering the crucial role of lipids in fish reproduction, the question of whether the same androgens could also interfere with lipid metabolism was raised here. Thereby, in this

study with brown trout hepatocytes, we assessed the mRNA levels of nine genes selected to include different lipid metabolic pathways.

The genes related to lipid transport were differently regulated by androgens herein. ApoA1 was down-regulated, whereas Fabp1 expression was not changed. The ApoA1 profile follows an opposite pattern to the one observed in livers of female Western mosquitofish and rainbow trout exposed to 1 µg/L (water exposure) of (17β)-TB (Brockmeier et al., 2013; Hook et al., 2006). ApoA1 is the major component of HDL, generally the most abundant lipoprotein in fish plasma (Babin and Vernier, 1989; Tocher, 2003). The functions of ApoA1 are related to reverse trafficking of phospholipids and cholesterol to the liver, cholesterol uptake in the liver to integrate new VLDL particles and cholesterol esterification (Dominiczak and Caslake, 2011; Gordon et al., 2011). In theory, a down-regulation of ApoA1 as an effect of androgenic exposure may ultimately be reflected in HDL and cholesterol dynamics. Accordingly, in humans, the intramuscular administration of T reduced the blood levels of ApoA1 and HDL (Gårevik et al., 2014). As in mammals (Gordon et al., 2011), the ApoA1 functions in fish are also associated with antimicrobial defense and innate immunity (Dietrich et al., 2015; Villarroel et al., 2007). Thus, a down-regulation of ApoA1 after androgenic stimuli could, as well, negatively affect fish immune responses. In agreement, androgens (11-KT, T) impaired innate immunity in chinook salmon *(Oncorhynchus tshawytscha)* (Slater and Schreck, 1993) and three-spined sticklebacks (*Gasterosteus aculeatus*) (Kurtz et al., 2007). Fabp1, involved in the mediation of the intracellular transport of long-chain fatty acids and fatty acyl-CoAs (Leaver et al., 2008; Tocher and Glencross, 2015) was not altered here, after androgenic exposure. Likewise, Fabp1 mRNA levels were not changed by *in vivo* DHT exposure (5 ppm in diet), in the liver of juvenile rainbow trout (Benninghoff and Williams, 2008).

ACC, involved in the first committed step in fatty acid synthesis, the production of malonyl CoA from acetyl-CoA, and FAS, which catalyzes the formation of long-chain fatty acids (Tocher and Glencross, 2015), were up-regulated by the highest doses of T and DHT, respectively. This result differs from the findings in triploid female rainbow trout, where a dietary exposure to T and DHT reduced the levels of ACC and had no effect on FAS expression (Cleveland and Weber, 2016). In contrast, and in line with the up-regulatory trend reported here for ACC and FAS, other lipogenic enzymes (ME, G6PDH, and ICDH) were increased in the liver of Mozambique tilapia (*Oreochromis mossambicus*) after T treatment, *in vivo* and *in vitro* (Sunny et al., 2002). Thus, despite the limited data, androgens seem to have a regulatory role in lipogenic processes in fish liver, as it has been shown in mammals.

Acox1-3l is considered a direct target of PPARα (McMullen et al., 2014; Rakhshandehroo et al., 2010) and catalyzes the first and rate-limiting step in peroxisomal β-oxidation. In our model, Acox1-3l was down-regulated by T but not by DHT, hinting that T may limit the peroxisomal oxidative catabolism of fatty acids. This idea is in agreement with the decreased expression of the genes from the fatty acid oxidation pathways reported in transcriptomic studies, after *in vivo* exposure to 11-KT in coho salmon ovary (Monson et al., 2017). Differing from the results in this study, DHT down-regulated genes from fatty acid oxidation in the mummichog ovary (Feswick et al., 2014). In triploid rainbow trout, however, the hepatic mRNA expression of the beta-oxidation genes Cpt1 and Acox1 was not changed after dietary exposure to T and DHT (Cleveland and Weber, 2016).

PPARγ, a pro-adipogenic nuclear receptor (Tocher, 2003), was down-regulated by both androgens. Previously, in brown trout, the liver PPARγ mRNA levels decreased during gonad maturation (Batista-Pinto et al., 2009), when androgen levels peak in fish (Singh and Singh, 1990; Wallaert and Babin, 1994). So, the results herein reinforce the impression of an inverse relation between hormone levels and mRNA expression of PPARγ in brown trout. This member of the PPAR family has been suggested to regulate fish fatty acid peroxisomal β-oxidation (Ruyter et al., 1997). Thus, the down-regulation of PPARγ may additionally imply an “impairment” of this cellular pathway.

The mRNA levels of LPL, which hydrolyzes triglycerides into free fatty acids (Leaver et al., 2008), were not changed after androgen exposure in this study. Our results parallel with the ones found in triploid rainbow trout liver, after dietary exposure to T and DHT (Cleveland and Weber, 2016). In contrast, the liver LPL mRNA levels were up-regulated by waterborne to E2 in juvenile Nile tilapia (*Oreochromis niloticus*) (Zhang et al., 2020) and down-regulated by dietary exposure to E2 in triploid rainbow trout (Cleveland and Weber, 2016). Despite the scarce data on this topic, fish liver LPL appears to be influenced differently by androgens and estrogens. In the coho salmon, 11-KT exposure (peritoneal implant) increased the ovarian expression of LPL (Monson et al., 2017), which suggests that there may be a role for androgens on the regulation of fish LPL, possibly with tissue specificities.

StAR is an essential enzyme in steroidogenesis, bile acid synthesis and cholesterol metabolism, which facilitates the transport of cholesterol to the inner mitochondrial membrane (Hall et al., 2005; Zheng et al., 2016). Herein, StAR mRNA levels were not altered by any of the androgens. Fish *in vivo* exposures to androgens have mostly produced down-regulation profiles of StAR mRNA in male and female gonads (e.g., Rolland et al., 2013; Zheng et al., 2016) or, in some cases, no effects (Rolland et al., 2013; Rutherford et al., 2015). To our knowledge, there is no evidence of androgenic regulation of StAR expression in fish liver. Further, since it was recognized that the regulation of the gene

expression of StAR is very complex, implying the interaction of a variety of hormones and signaling pathways (Manna et al., 2009), a greater amount of data is needed to have a clearer picture for fish.

The more pronounced effect observed herein was on the expression of the Acsl1 gene, which was up-regulated by both T and DHT, and responded at relatively lower androgen concentrations than the other targets. Acsl1 catalyzes the conversion of fatty acids into their acyl thioesters and its incorporation through lipid synthesis or catabolic (β-oxidation) pathways (Digel et al., 2009; Li et al., 2010; Lopes-Marques et al., 2013). Since the β-oxidation pathway was down-regulated here, at least by T, this increase in the Acsl1 mRNA levels may be indicative of lipogenesis activation after androgenic exposures. In accordance with this notion, in cultured human hepatocytes, Acsl1 overexpression led to increased triglyceride levels, while the opposite effect was obtained after Acsl1 knockdown (Li et al., 2020). Considering our previous studies in primary hepatocytes, where the same androgens induced Vtg and ZPs at gene and protein levels (Lopes et al., 2020; Lopes et al., 2017), we suggest that the stimulation of lipid synthesis could have been triggered in our model, at least partially, to sustain hepatocytic vitellogenin production. In agreement, prior studies in fish reported an increase in liver lipogenic activity during the period of gonad development, which coincides with active vitellogenin synthesis, and high requirement of lipids as an energy source (Sharpe and MacLatchy, 2007; Singh and Singh, 1990).

In line with previous evidence of T interferences on PPAR signaling and peroxisome morphology in brown trout (Lopes et al., 2017; Madureira et al., 2018), here, DHT and T changed the mRNA expression of PPARγ, and both androgens altered the expression of the two direct PPAR targets (Heinäniemi et al., 2007; Rakhshandehroo et al., 2010), ApoA1 and Acsl1. So, this work contributes with new data regarding the androgenic influences on PPAR signaling, calling for further mechanistic studies to determine the mode of action of such interferences.

5. Conclusions

We presented results supporting that both T and DHT can modulate different pathways related to liver anabolic and catabolic lipid metabolism in brown trout. The marked increases in Acsl1 and the stimulation of ACC and FAS suggest that androgens may trigger, or shift, hepatocytic metabolism towards lipogenesis. Peroxisomal β-oxidation followed an opposite trend, with down-regulation of Acox1-3l by T. Additionally, lipoprotein metabolism was affected, with down-regulation of ApoA1 by both androgens. The findings summarized in Fig. 4 reveal the potential of androgens to regulate brown trout lipid homeostasis, which is relevant both in physiological and toxicological contexts. This is a yet unexplored field in fish, and it seems justified at this point to enlarge the number of genes in the portfolio, including other lipidic pathways and other transcription factors known to be master regulators of lipid signaling (e.g., LXR, FXR and SREBPs). Finally, in the sequence of the present and our previous results, where we showed that androgens interfere with brown trout estrogenic signaling, it is logical now to propose studies to address the impact of these disruptions in organ lipid composition and gonadal maturation.

		DHT	T
De novo lipogenesis	FAS	-	↑
De novo lipogenesis	ACC	↑	-
Fatty acid activation	Acsl1	↑	↑
Lipid transport	ApoA1	↓	↓
Adipogenesis	PPARγ	↓	↓
Peroxisomal β-oxidation	Acox1-3l	-	↓
Lipid transport	Fabp1	-	-
Lipid uptake	LPL	-	-
Cholesterol metabolism	StAR	-	-

Fig. 4. Overview of the changes in mRNA levels of target genes and respective lipid metabolic pathways after exposure to dihydrotestosterone (DHT) and testosterone (T).

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Chapter 6

General discussion

General discussion

Peroxisome proliferator-activated receptors (PPARs), as members of the nuclear receptor superfamily, stand out in the regulation of key processes connected with energy production and storage. Among other functions, PPARs participate in the coordination of peroxisome biochemistry and morphology, which may be disrupted, for example, by stimulation of the so-called peroxisome proliferators (Schrader et al., 2016; Wang et al., 2020).

Within this doctoral Thesis, the brown trout (*Salmo trutta* f. *fario*) PPARγ cDNA sequence was cloned (Chapter 2), showing high homology with a salmonid sequence from Atlantic Salmon (*Salmo salar*) (Sundvold et al., 2010) (Fig. 1A). The brown trout protein shared around 98% of identity with the Atlantic salmon sequence, but only about 60% with the zebrafish (*Danio rerio*) and human PPARγ1 sequences. Furthermore, and compared to the human sequence, some amino acids that were described to integrate the ligand-binding site and to be relevant for establishing hydrogen bonds with the thiazolidinedione ligands (Nolte et al., 1998) were not conserved in brown trout, as reported in Atlantic salmon (Andersen et al., 2000). The differences found can change ligand specificity, and thus may explain the non-activation of fish PPARγ by a few screened natural and synthetic mammalian ligands (Kondo et al., 2007; Leaver et al., 2005). Another similarity between brown trout and Atlantic salmon PPARγ sequences was the presence of specific decapeptide motif repeats, which had been identified in Atlantic salmon PPARγ (Andersen et al., 2000). The repetitive motif was present in the A/B domain of the protein and was hypothesized to be related to cofactor binding (Andersen et al., 2000; Sundvold et al., 2010). The existence of repetitive sequences in the A/B domain of PPARγ does not seem to be exclusive to salmonids, since 11 repeats of another decapeptide were identified in yellow catfish (*Pelteobagrus fulvidraco*) (Zheng et al., 2015).

In adult female and male brown trout, the liver PPARγ mRNA decreased in late vitellogenesis and pre-spawning periods, in comparison with the post-spawning season (Batista-Pinto et al., 2009), which raised the question of a possible mediation of PPARγ expression by sex steroids. In the Thesis Chapter 2, brown trout primary hepatocytes were exposed to 1, 10 and 50 μM of 17α-ethinylestradiol (EE2) or testosterone (T). PPARγ mRNA expression changed after EE2 exposure, following a non-monotonic response curve. PPARγ levels increased after the lowest EE2 concentration and decreased after 50 μM of EE2 (Fig. 1A). Negative crosstalk between PPARγ and estrogen receptors (ERs) has been described in distinct models, namely human breast and hepatic carcinoma cell lines (Lin et al., 2013; Wang and Kilgore, 2002). Despite that, estrogens have also been shown to increase PPARγ in specific conditions. For example, in mouse cultured adipocytes, 17β-

estradiol (E2) at 10^{-10} and 10^{-9} M increased PPARγ protein after 1- and 2-weeks treatment (Sato et al., 2013). In skeletal muscle from female Sprague-Dawley rat (*Rattus norvegicus*), treated with subcutaneous implants of E2 for 14 days, PPARγ mRNA (but not protein) was also increased (Campbell et al., 2003). In fish, PPARγ mRNA was up-regulated by 10 nM EE2 in zebrafish primary hepatocytes (Maradonna et al., 2013), but down-regulated by 30 nM of the same compound in juvenile rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes (Hultman et al., 2015). *In vivo*, different effects on liver PPARγ mRNA levels were reported as a consequence of estrogenic exposure. Their increase after waterborne exposure to 200 ng E2/L was reported in male zebrafish (Sun et al., 2019), but decreases were observed after 50 µg/L of EE2 in juvenile brown trout (Madureira et al., 2018), E2 intraperitoneal (IP) injection (5 µg/g body weight) in juvenile rainbow trout (Cleveland and Manor, 2015), and E2 contaminated diet (30 mg/kg diet) in triploid female rainbow trout (Cleveland and Weber, 2016). From the data, there seem to exist different patterns of response between zebrafish and salmonids, which may be linked to different reproductive strategies between fishes.

PPARγ mRNA was down-regulated by T, at 10 and 50 µM (Fig. 1A), as shown in Chapter 2. The results reiterate our initial hypothesis that androgens have a role in the regulation of PPARγ. Down-regulation of PPARγ by androgens was previously noted in a few mammalian studies. Prenatal exposure to T decreased PPARγ mRNA levels in sheep (*Ovis aries*) liver (Nada et al., 2010) and rat uterine tissue (Ferreira et al., 2019). Accordingly, dihydrotestosterone (DHT), at nanomolar concentrations, decreased PPARγ mRNA and protein levels in human prostate cancer cell lines (Olokpa et al., 2016; Olokpa et al., 2017). In fish, androgenic effects on PPARγ expression have been seldom explored, but no alterations in mRNA levels were found *in vivo* after exposure to T, in juvenile brown trout (Madureira et al., 2018), and to T and DHT, in triploid female rainbow trout (Cleveland and Weber, 2016). A common pattern between the two later studies was the little impact obtained at the transcription level after androgenic exposure. Both studies assessed changes on gene targets related to estrogenic and lipid signaling pathways after estrogenic and androgenic stimuli, with more pronounced changes in gene expression after estrogenic exposure. In the rainbow trout study, steroid levels were measured in plasma after the exposures. A marked increase in E2 levels was obtained after E2 treatment, while treatment with DHT and T increased only slightly the respective plasma levels (Cleveland and Weber, 2016), which could explain the overall discrete changes in gene expression after androgen exposure. In the brown trout study, the measured water concentration of T (3.90 µg/L) was quite below the nominal concentration (50 µg/L) (Madureira et al., 2018), standing close to environmental levels. Despite the referred concentration did not impact in PPARγ

expression, it was sufficient to affect estrogenic signaling, by increasing Vtg levels, and the peroxisomal morphology (Madureira et al., 2018).

In Chapter 2, we thus obtained the complete sequence of the brown trout PPARγ and established that both estrogens and androgens can modulate the signaling of this PPAR isoform. Since EE2 modified the morphology of brown trout peroxisomes and modulated the expression of peroxisomal gene targets *in vitro* (Madureira et al., 2015), in Chapter 3 of this Thesis, we proposed to explore if the same interferences were present after androgenic exposure. Hence, primary brown trout hepatocytes were exposed to 1, 10 and 50 μM of T. Smaller peroxisome profiles were observed in transmission electron microscopy (TEM), an effect more evident in the 50 μM concentration (Fig. 1B). The qualitative morphological analysis was confirmed by a quantitative stereological approach, after catalase immunostaining of peroxisomes (Fig. 1B). Comparing with control conditions, the relative volume of peroxisomes, in relation to the hepatocyte cytoplasm, decreased after exposure to 50 μM of T. T also decreased the expression of urate oxidase (Uox), but had no effect on catalase (Cat) and PPARα mRNA levels (Fig. 1B). As an aromatizable androgen, T effects may occur either directly, through androgen receptors, (ARs) or indirectly, via ERs, after conversion into estrogenic compounds. Thus, vitellogenin A (VtgA) was also included as an estrogen target gene. An up-regulation of VtgA mRNA was obtained after exposure to all androgen concentrations (1, 10 and 50 μM) (Fig. 1B). This result prompted us to investigate mechanistically the effects produced by T, at gene level and on peroxisomal morphological parameters. For that purpose, we co-exposed brown trout primary hepatocytes to T (10 μM) and 10 and 50 μM of AR (flutamide – F) or ER (ICI 182,780 – ICI) antagonists. Both antagonists inhibited T effects on peroxisomes morphology (Fig. 1B). VtgA induction by T was completely blocked by ICI, and partially reversed by F, since co-exposure with T caused a dose-dependent inhibition of VtgA (Fig. 1B), denoting a mediation though AR and ER. Similarly to our findings, F inhibited Vtg induction by methyltestosterone (MT) in eel (*Anguilla japonica*), but not as effectively as tamoxifen, an ER blocker (Kwon et al., 2005). A different pattern was obtained here for Uox, since the mRNA levels were still decreased, comparing with controls, in the T + ICI conditions (Fig. 1B), suggesting that different mechanisms trigger the modulations of Uox and Vtg.

Another finding of the study was the interference of F (in single exposures) with Cat and Uox mRNA levels. F, at the highest dose (50 μM), caused a down-regulation of those target genes, comparing with controls. In contrast, we did not note an interference of F in Vtg mRNA levels in absence of androgen. Contrarily to our findings, *in vivo*, F was shown to affect Vtg levels in single exposures, in the Murray rainbowfish and fathead minnow, either increasing or decreasing them (Bhatia et al., 2014a; Bhatia et al., 2014b; Jensen et al.,

2004). F has been shown to bind ERs (Ankley et al., 2003; Le Guevel and Pakdel, 2001) and to increase the mRNA levels of ERα in Murray rainbowfish (*Melanotaenia fluviatilis*) (Bhatia et al., 2014b) and ERβ in fathead minnow (*Pimephales promelas*) (Filby et al., 2007) male fish. F likewise decreased AR mRNA levels in the liver of female fathead minnow (Filby et al., 2007) and male Murray rainbowfish (Bhatia et al., 2014b). The mechanism by which F modulates Vtg *in vivo* is not fully yet understood but is probably related to ERs and/or ARs. Thus, although the results here point to a regulation of peroxisomal targets mainly through ERs, AR signaling contribution cannot be ruled out.

Chapter 4 explored the mechanisms that drive the interferences produced by androgens in estrogenic signaling. In this context, primary hepatocytes were exposed to T and a non-aromatizable androgen (DHT), in nanomolar and micromolar concentrations. Herein, were assessed changes in mRNA levels of classical estrogenic targets (VtgA, ERα, ERβ1, and two zona pellucida proteins (ZPs) – zona pellucida glycoprotein ZP2.5 (ZP2.5) and zona pellucida glycoprotein 3a.2 (ZP3a.2). AR and the ovarian aromatase CYP19a1 were also included. There are different mechanisms which may explain the Vtg induction by androgens, namely: 1) aromatization of androgens, such as T, to estrogenic compounds; 2) direct binding of estrogens to ERs; and 3) Vtg induction through AR. Both androgens up-regulated VtgA, ERα, and ZPs (Fig. 1C), and DHT generally had a greater effect, suggesting that aromatization is not the major mechanism driving androgen induction of Vtg. The fact that the mRNA levels of the aromatase gene were unchanged also supports that view.

Vtg induction by androgens has been described in some *in vitro* fish studies, that used both aromatizable (T, MT) (Mori et al., 1998; Trombley et al., 2015) and non-aromatizable (DHT, 11-ketotestosterone – 11-KT) (Kim and Takemura, 2002; Pelissero et al., 1993) androgens. *In vivo*, Vtg induction has also been reported after exposure to aromatizable (Ankley et al., 2001; Hornung et al., 2004) and non-aromatizable (Bhatia and Kumar, 2016; Le Menn et al., 1980) androgens. An up-regulation of VtgA by T was obtained after water exposure to T in brown trout (Madureira et al., 2018), showing that our *in vitro* model reflects well the *in vivo* modulations of this particular biomarker. Both type of androgens also caused a decrease in the Vtg levels *in vivo*, for example in Japanese medaka (*Oryzias latipes*) (Kang et al., 2008), zebrafish (Örn et al., 2003; Örn et al., 2006) and Eastern and Western mosquitofish (*Gambusia holbrooki* and *G. affinis*) (Brockmeier et al., 2013a). The above data further suggest that aromatization may not be the main mechanism behind androgenic interferences in estrogenic targets. Nevertheless, the *in vivo* effects caused by androgens in estrogenic markers may derive from mixed mechanisms. For example, Vtg induction or repression has been associated either with changes in the levels of endogenous hormones and their receptors (Ankley et al., 2003; Sharpe et al., 2004) or with expression or activity of

steroid-metabolizing enzymes, including the aromatases (Hornung et al., 2004; Zheng et al., 2016). In the fathead minnow, the product of MT aromatization (methylestradiol) was detected in plasma, linking Vtg induction to the aromatization of MT (Hornung et al., 2004).

Herein, we showed in brown trout that the mRNA levels of Vtg and ZPs were moderately to highly correlated to the levels of ERα, which connects this nuclear receptor to Vtg induction by T and DHT. Given the absence of changes in aromatase levels, and the similar results obtained with both androgens, we hypothesize that the Vtg induction is happening through direct binding of the androgens to ERα. In fish studies, the expression of ERα followed different patterns upon androgenic exposure, varying from absence of changes (Benninghoff and Williams, 2008; Madureira et al., 2018; Zerulla et al., 2002), to down-regulation (Huang et al., 2012; Park et al., 2013; Zheng et al., 2016) and, less frequently, to up-regulation (Cleveland and Weber, 2015). On the contrary, ERα is consistently up-regulated after estrogenic exposures, as it occurred herein with androgens.

Here, the mRNA levels of AR tended to increase after micromolar concentrations of DHT, while the 10 μM concentration of T increased AR over control levels. In fish, the expression of liver ARs was assessed in few studies, mainly presenting a down-regulation profile, as seen in Eastern and Western mosquitofish (Brockmeier et al., 2013b; Huang et al., 2012). In mammals, the regulation of AR by androgens may be towards up- or down-regulation (most frequently down-regulation), and in a cell and tissue-selective way (Hunter et al., 2018).

Alterations in the mRNA levels do not always translate to protein level. Thus, we thought to examine if the alterations seen at mRNA levels were replicated phenotypically. We looked for antibodies that had already been used to recognize our targets in fish and optimized them for immunohistochemistry in pellets of hepatocytes. The morphological analysis, using a semi-quantitative scoring system, revealed that Vtg and ZPs were also increased by the micromolar concentrations of both androgens, with similar profiles to the ones seen in the molecular study (Fig. 1C). The results from the other antibodies did not show such close similarity to those obtained with qRT-PCR. It should be noticed that despite the antibodies' cross-specificity, because they were raised against human proteins, they do not distinguish between protein isoforms that exist in fish for ERs, ARs and CYP19. Still, resembling the results from the molecular study, a trend to increased levels of ERα (Fig. 1C) and AR protein levels was also observed after exposure to micromolar concentrations of DHT.

Knowing the potential of androgens to disrupt estrogen signaling, to modify the expression of key molecules in fish reproduction, such as Vtg and ZPs, and PPARs and peroxisomal signaling, the next step was to investigate the potential of androgens to interfere with lipid metabolism. Lipids are essential molecules in fish physiology, representing a major energy

source for key functions as growth and reproduction. In Chapter 5, it was assessed if the same androgens that interfered with estrogenic signaling could modulate the expression of a selection of target genes, included in different lipid metabolic pathways. Previous fish studies reported some of the selected genes to be transcriptionally regulated by androgens, namely lipoprotein lipase (LPL), steroidogenic acute regulatory protein (StAR), acetyl-CoA carboxylase (ACC) and apolipoprotein A1 (ApoA1) (Brockmeier et al., 2013b; Cleveland and Weber, 2016; Hook et al., 2006; Monson et al., 2017; Rolland et al., 2013).

Here, Acsl1 was the most altered target, with up-regulated levels after exposure to both androgens (Fig. 1D). Acsl1 functions are related to fatty acid activation and channeling through synthesis or degradation pathways, but have also been linked to fatty acid up-take and cholesterol metabolism (Li et al., 2010; Singh et al., 2019). The acyl-CoA A oxidase 1 isoform 3l (Acox1-3l), involved in peroxisomal β-oxidation was down-regulated by T and the enzymes from *de novo* lipogenesis, fatty acid synthase (FAS) and ACC, were up-regulated by T and DHT, respectively (Fig. 1D). Thus, these combined results may indicate that androgens can shift lipid metabolism towards lipogenesis in the liver.

Both androgens decreased PPARγ (Fig. 1D), which is considered a pro-adipogenic gene, but has also been linked to fatty acid β-oxidation (Li et al., 2020; Ruyter et al., 1997). In cultured primary human hepatocytes, Acsl1 overexpression led to down-regulation of PPARγ and increased liver triglyceride levels (Li et al., 2020). Curiously, in the same study, the expression of genes related to lipid synthesis, sterol regulatory element binding proteins (SREBPs), ACC, FAS and stearoyl-CoA desaturase 1 (SCD1) were also decreased in hepatocytes overexpressing Acsl1. Since androgenic stimuli increased Vtg mRNA and protein levels in our previous studies (Chapter 2 and Chapter 3), it is tempting to relate the presumed activation of lipogenesis to the synthesis of precursors of the components of Vtg. Accordingly, early studies have reported increased liver lipogenesis during the growth phases of the fish ovary, where Vtg is being synthesized (Sharpe and MacLatchy, 2007; Singh and Singh, 1990).

ApoA1 mRNA levels decreased after androgen exposures (Fig. 1D). ApoA1 plays a fundamental role in cholesterol metabolism and is the major component of high-density lipoprotein (HDL). Down-regulation of this gene may thereafter have direct impacts in HDL and cholesterol dynamics. Interestingly, ApoA1 was up-regulated in liver in two transcriptomic experiments where female Eastern mosquitofish (Brockmeier et al., 2013b) and rainbow trout (Hook et al., 2006) were exposed to trenbolone (TB), with concomitant decreases in Vtg and ZP levels in one of the studies (Brockmeier et al., 2013b). Contrarily, a down-regulation profile of ApoA1 is usually seen in fish exposed to estrogens, as it

happened in brown trout (Madureira et al., 2018) and zebrafish (De Wit et al., 2010; Martyniuk et al., 2007) exposed to EE2. By comparing the results from this Thesis with prior *in vivo* works in juvenile brown trout, the Acsl1, Acox1-3l, PPARγ, and Uox mRNA levels were modulated by T in the same directions previously obtained after exposure to EE2 (Madureira et al., 2018), which constitutes another clue supporting the action of androgens through ERs.

Research focusing on physiological and pathological impacts of androgenic exposure is still scarce compared with the amount of information regarding the effects of estrogenic compounds. Still, androgens are present in the environment and have been measured in water effluents, sometimes at even higher concentrations than estrogens (Chang et al., 2011; Vulliet and Cren-Olivé, 2011). The effects of exposure to androgens have been explored in several fish studies and among the consequences of such exposures are the development of male secondary characteristics in female fish, decreased fecundity and gonadal alterations in both sexes, including intersex (Ankley et al., 2001; Ankley et al., 2003; Bhatia and Kumar, 2016; Margiotta-Casaluci and Sumpter, 2011). However, the role of the different androgens in fish physiology is not completely understood. Plasma levels of T can be higher in female than in male rainbow trout, and may reach greater levels than E2 even in females (Scott et al., 1980; Wallaert and Babin, 1994). It is also known that maximum plasma T levels correlate with maximum liver somatic index in female goldfish, and that this is probably related to the vitellogenesis process (Sharpe and MacLatchy, 2007). Thus, T may have a more relevant role to fish biology than sometimes it is anticipated, and the mechanisms by which this action occurs should be disclosed. It is also known that T can be converted to other steroids, such as E2, 11-KT and DHT, and that all these hormones may play crucial roles in fish physiology. 11-KT, for instance, has been found to be important in the early oocyte development (Forsgren and Young, 2012; Kortner et al., 2009; Monson et al., 2017). DHT, which was for long considered not to be relevant in fish biology, was isolated in plasma of male and female fathead minnow (Margiotta-Casaluci et al., 2013). Several studies suggested a physiological role of DHT in fish, including the regulation of spermatogenesis (García-García et al., 2017; Margiotta-Casaluci and Sumpter, 2011; Martyniuk et al., 2013). As more biological functions of androgens are being disclosed, other new consequences of endocrine disruption by androgenic compounds may still emerge.

In summary, in this Thesis it was found that the expression of PPARγ is influenced by estrogens and androgens, disclosing novel interconnections between peroxisomal and hormonal signaling in brown trout. It was shown that androgens can potentially disrupt liver estrogenic, lipid and peroxisomal signaling. This may be particularly relevant, knowing how much these pathways are correlated and may impact fish physiology. The unveiled

crosstalk between nuclear receptors exposes the complexity of effects that can be obtained in response to chemicals, particularly with hormones and hormone mimics. The results of this Thesis contribute thus to enlarge and strengthen the knowledge on the hormonal impacts, and on the underlying mechanisms that drive those influences, in PPAR, peroxisomal, estrogenic and lipid signaling, and add new information regarding the inter-connections between the distinct pathways. The used *in vitro* model robustly reflected the *in vivo* hormonal modulations and proved its usefulness for mechanistic studies.

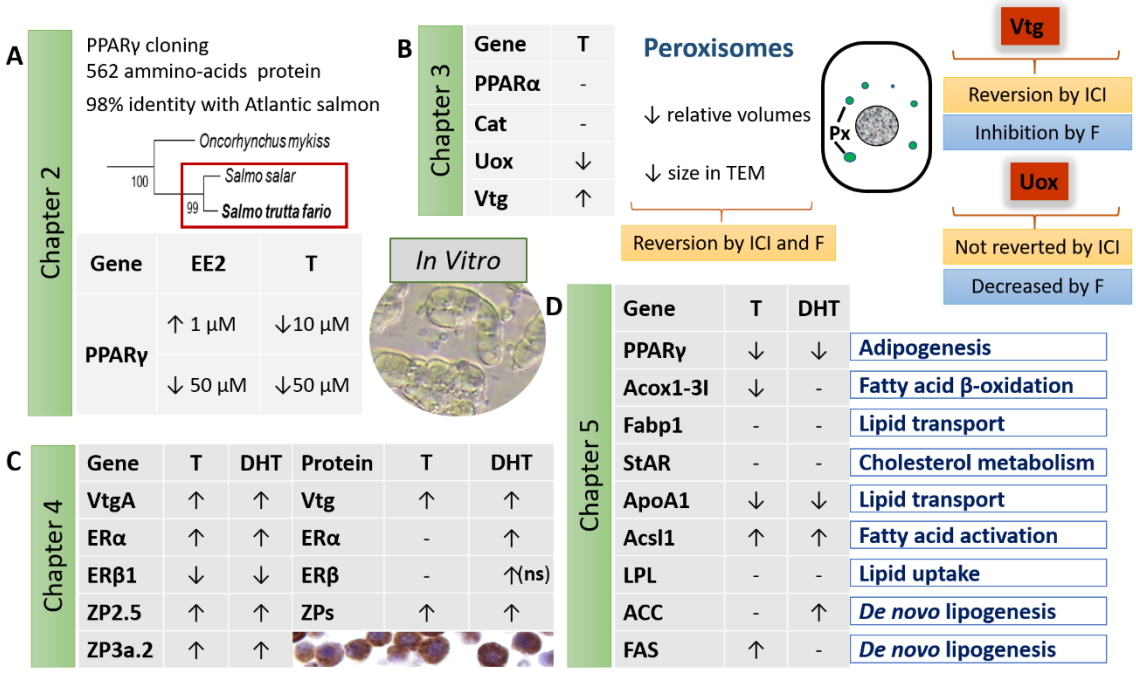

Fig. 1. Summary of the main results of the Thesis (A, Chapter 1; B, Chapter 2; C, Chapter 3; D, Chapter 4). Decrease, ↓; increase, ↑; peroxisome, Px; transmission electron microscopy, TEM; non-significant, ns. 17α-ethinylestradiol, EE2; dihydrotestosterone, DHT; flutamide, F; ICI 182,780, ICI; testosterone, T. Acetyl-coenzyme A carboxylase, ACC; acyl-coenzyme A oxidase 1 3I, Acox1–3I; apolipoprotein A1, ApoA1; catalase, Cat; estrogen receptor alpha, ERα; estrogen receptor β, ERβ; estrogen receptor beta 1, ERβ-1; fatty acid binding protein 1, Fabp1; fatty acid synthase, FAS; lipoprotein lipase, LPL; long-chain acyl-coenzyme A synthetase, Acsl1; peroxisome proliferator-activated receptor alpha, PPARα; peroxisome proliferator-activated receptor gamma, PPARγ; steroidogenic acute regulatory protein, StAR; urate oxidase, Uox; vitellogenin, Vtg; vitellogenin A, VtgA; zona pellucida glycoprotein 2.5, ZP2.5; zona pellucida glycoprotein 3a.2, ZP3a.2 and zona pellucida proteins, ZPs.

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Chapter 7

Concluding remarks and future perspectives

Concluding remarks and future perspectives

Considering the results from this Thesis, adding to those from our previous *in vitro* and *in vivo* studies with brown trout (*Salmo trutta* f. *fario*), we now have increased knowledge on the hormonal (estrogenic and androgenic) regulation of peroxisome proliferator-activated receptor (PPAR) signaling, including in lipid metabolism and peroxisome morphology.

We isolated the brown trout PPARγ cDNA sequence, that presented high similarity with a previously deposited sequence from the closely related species, Atlantic salmon (*Salmo salar*). As hypothesized from the results of PPARs expression along the brown trout reproductive cycle, we concluded that estrogens and androgens could influence the mRNA expression of PPARγ mRNA.

It was likewise disclosed that testosterone (T) can alter the liver peroxisomes morphology, since exposure to T diminished the size of those organelles, and interfered in its enzymatic content, by decreasing the mRNA levels of the peroxisomal enzymes urate oxidase (Uox) and acyl-coenzyme A (CoA) oxidase 1 3I (Acox1-3I). The effects of T on peroxisome morphology were offset with the estrogen receptor (ER) antagonist ICI 182,780 (ICI). In contrast, the androgen receptor (AR) antagonist flutamide (F) interfered with catalase (Cat) and Uox mRNA expressions. Further, T increased the expression of the master estrogenic exposure biomarker, vitellogenin (Vtg), an effect that was abolished by the ICI and partially by F.

Androgenic effects on classical estrogenic targets, Vtg, zona pellucida proteins (ZPs) and ERs, were mechanistically explored using the aromatizable T and a pure, non-aromatizable androgen, dihydrotestosterone (DHT). Both androgens regulated the selected targets in the same direction (up or down-regulation), although the effects of DHT were generally noted after lower concentrations. The mRNA levels of ERα showed moderate to highly positive correlations with the ones of Vtg and ZPs which, in our interpretation, implicates that androgens are acting through ERα to modulate estrogenic signaling.

Finally, we explored the effects of aromatizable and non-aromatizable androgens, T and DHT, on selected gene targets from lipid metabolism pathways. Both androgens up-regulated mRNA levels of long-chain acyl-CoA synthetase (Acsl1), related to fatty acid activation, and down-regulated the expression of apolipoprotein A1 (ApoA1), the major component of high-density lipoprotein (HDL), and PPARγ, a pro-adipogenic PPAR also involved in fatty acid β-oxidation. Additionally, T reduced the expression of Acox1-3I and up-regulated the *de novo* lipogenesis gene fatty acid synthase (FAS). Another enzyme involved in *de novo* lipogenesis, acetyl-CoA carboxylase (ACC), was up-regulated by DHT.

Thus, in a broad perspective, it seems that T and DHT promote a shift in liver metabolism by increasing lipogenesis and decreasing fatty acid β-oxidation.

As to future perspectives, we will take into consideration that PPARγ seems to be particularly prone to endocrine disruption, since it was transcriptionally regulated by xenoestrogens (EE2) and by androgens (T and DHT) in this Thesis. As for PPARα, it was not modulated by T in this Thesis. However, it should be investigated the effects of T and DHT on the expression of the two PPARα isoforms (PPARαBa and PPARαBb) that exist in brown trout, particularly because they are differently regulated by estrogens.

Another topic that deserves further investigation is the relative contributions of AR and ER signaling in the effects of T and DHT (and eventually other androgens in the future) on PPAR and peroxisomal signaling. Since F interfered with two peroxisomal targets in the absence of androgen, other AR antagonists should be tested, and their capacity to antagonize the actions of androgens on the expression of PPARs and downstream genes assessed. This would further disclose the interferences and interconnections between hormonal and PPAR signaling. From a mechanistic perspective, it would be also informative to study the effects of estrogens and androgens mixtures. The additive or antagonistic mixture effects should provide additional information on the actions of androgens through ERs/ARs.

Additionally, it seems logical to explore further the influence of estrogens and androgens on lipid signaling. First, it should be determined the effects of estrogens on the expression of ACC, FAS, lipoprotein lipase – LPL and steroidogenic acute regulatory protein – StAR, differently regulated by T and DHT in this Thesis. Then, the number of target genes should be enlarged to include other master regulators and downstream genes along lipid metabolism. It is also mandatory to measure the lipid content (and composition) under hormone stimulation, to know if the alterations in mRNA levels traduce functionally. This approach would be compared with *in vivo* data (work underway in our team) and strength the utility of the *in vitro* model to study disruptions in lipid metabolism.

The recently optimized three-dimensional (3D) brown trout hepatocyte *in vitro* model, responsive to hormonal stimuli, could be used to explore mechanisms of action, in a cellular environment closer to the *in vivo*. It would allow, for example, time-course studies, by investigating the effects of hormones even in longer exposures, beyond 96 h. Note that we consider the latter time period as the upper limit for a monolayer culture of brown trout hepatocytes, under standard culture medium conditions.

One more challenging broad approach would be to study the influences of hormone (estrogens and androgens) exposures, applying high-throughput sequencing techniques. This kind of analysis would enable to establish more pathways that are governed by these compounds in the brown trout liver, with translational illations to other species.

Finally, and considering the physiological interplay between the fish liver and gonads, it is of relevance to explore, in the latter, the influences of estrogenic and androgenic compounds and the interconnections between hormonal (ER and AR) and PPAR signaling pathways.