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17α-Ethinylestradiol and atorvastatin effects in liver and blood-related parameters of juvenile brown trout

Tiago Lourenço

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Dissertation for applying to the degree of Master in Marine Sciences – Marine Resources, Specialization in Marine Biology and Ecology as submitted to the ICBAS – School of Medicine and Biomedical Sciences of the University of Porto.

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I would like to dedicate this work to my grandfather. No one has ever taught me more about overcoming obstacles, and he is nothing if not the biggest believer in my capabilities. I can only wish to make him proud.

Declaration of Honour

I declare that this dissertation work is my authorship and has not been previously used in another course or curricular unit from this or another institution. References to other authors (statements, ideas, thoughts) scrupulously respect the attribution rules and are duly indicated in the text and in the bibliographic references following the referencing rules. I am aware that the practice of plagiarism and self-plagiarism constitutes an academic offence.

Porto, 12th of June 2023

Tiago Maria Ribeiro da Cunha Quaresma Lourenço

Scientific Outputs Derived from the Dissertation Project

Lourenço T, Madureira TV, Rocha MJ, Gonçalves, JF, Rocha E (2022) Effects of atorvastatin and 17α-ethinylestradiol on blood and liver lipids contents in brown trout juveniles. 30th CECE & 9th ISFE Joint Conference of the European Society for Comparative Endocrinology and the International Society for Fish Endocrinology. 4-8 September. Faro, Portugal. Oral communication.

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Abstract

Salmonids are a widely studied fish group with undeniable socioeconomic importance for human societies and play a crucial environmental role within their ecosystems. These fish have been widely used as model organisms in various research areas, including aquatic toxicology, due to their sensitivity to contaminants, particularly juveniles. Hypolipidemic compounds, such as statins and estrogenic hormones (or their mimics), can cause dyslipidemia in humans and other vertebrates, in the latter due to the similarity in the regulation pathways of lipid metabolism. However, the impacts of statins on fish remain largely unstudied. Thus, experimental fish models of dyslipidemia have a great physiological, toxicological, and ecological interest.

In this context, the present dissertation set out to evaluate the effects of statins and their interaction with a common xenoestrogen by examining the in vivo effects in juvenile brown trout (approximately 1-year-old) of exposure to atorvastatin (ATV – 0.3 µg/g), 17α-ethinylestradiol (EE2 - 2 µg/g) and a mixture of both chemicals (MIX – 0.3 µg/g ATV plus 2 µg/g EE2). Control and solvent control groups were established and corresponded to fish injected with a saline solution (0.7% NaCl) and a saline solution fortified with solvent (0.7% NaCl, 0.1% DMSO and 0.9% ethanol), respectively. For each exposure condition, fish (n = 10) were injected with 4 µl/g, two times a week for two weeks (totalling 4 injections per fish).

Endpoints included biometric parameters, blood lipid biochemistry (cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, very low-density lipoproteins (VLDL)), hepatic lipid droplets quantification from histological sections through software ImageJ, after using osmium tetroxide post-fixation, gonadal maturation grading from histological sections and hepatic mRNA expression of estrogenic targets vitellogenin A (VtgA) and estrogen receptor alpha (ERα) and lipidic targets acetyl-CoA carboxylase (ACC), acyl-CoA long chain synthetase 1 (Acsl1), apolipoprotein AI (ApoAI), acyl-CoA oxidase 1 3I (Acox1-3I), acyl-CoA oxidase 3 (Acox3), fatty acid binding protein 1 (Fabp1), fatty acid synthase (FAS), hydroxy-3-methylglutaryl Co-A reductase (HMGCoAR), lipoprotein lipase (LPL), peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor alpha Ba (PPARαBa), peroxisome proliferator-activated receptor alpha Bb (PPARαBb), peroxisome proliferator-activated receptor gamma (PPARγ) and steroidogenic acute regulatory protein (StAR) measured by real-time qPCR.

Among biometric measurements, only the liver weight and the hepatosomatic index increased after exposure to EE2 and with MIX. Cholesterol, LDL, VLDL and triglycerides were significantly lower in ATV exposed fish, whilst triglycerides and VLDL increased after

EE2 treatment, and HDL diminished in all experimental groups (ATV, EE2 and MIX). Lipid quantification in osmicated liver tissues was significantly higher in the EE2 and MIX groups, and the osmicated liver fragments from fish exposed to ATV, EE2 and MIX showed heterogeneity of lipid droplets distribution and size. None of the chemicals nor their combination caused alterations in gonadal histology.

Additionally, ERα and VtgA were upregulated by EE2 and MIX. Classic statin target HMGCoAR was significantly upregulated by EE2 and ATV. Acsl1 and FAS also experienced upregulation after exposure to EE2, whilst Fabp1 demonstrated the inverse pattern, being downregulated in the EE2 group. ATV caused the upregulation of ApoAI, whilst EE2 and MIX downregulated this target. PPARγ was downregulated by ATV and MIX, and all three treatments caused downregulation for Acox1-3I and Acox 3.

In summary, in vivo, exposure of brown trout juveniles through intramuscular injection to ATV, EE2, and a mixture of both chemicals led to lipid metabolism alterations. The changes in blood lipid profiles and hepatic lipid droplet content and distribution differed between the ATV and EE2 groups, with EE2 causing an increase in lipid deposition and ATV leading to a reduction. In MIX, ATV and EE2 counteracted each other. The same pattern of antagonism between ATV and EE2 is also observed at the molecular level, where ATV seemed to have an opposite or null effect compared to EE2. In the MIX group, ATV appeared to have an antiestrogenic effect.

Resumo

Os salmonídeos são um grupo de peixes muito estudados com inegável importância socioeconómica para as sociedades humanas e que desempenham um papel crucial nos seus ecossistemas. Estes peixes são usados como organismos modelo em várias áreas de estudo, incluindo toxicologia aquática, devido à sua sensibilidade a poluentes aquáticos, particularmente no caso de juvenis. Compostos hipolipidémicos, como as estatinas, bem como hormonas estrogénicas são capazes de causar dislipidemia tanto em humanos como em peixes devido à sua semelhança nas vias regulatórias do metabolismo lipídico, contudo, os impactos de estatinas em peixes encontram-se largamente por estudar. Posto isto, modelos experimentais de dislipidemia em peixes possuem grande interesse fisiológico, toxicológico e ecológico.

Neste contexto, a presente dissertação comprometeu-se a avaliar os efeitos das estatinas e a sua interação com outros poluentes ambientais através da análise dos efeitos da exposição *in vivo* de truta fario juvenil (aproximadamente com 1 ano) a: atorvastatina (ATV – 0.3 µg/g), 17α-etinilestradiol (EE2 – 2 µg/g) e uma mistura dos dois químicos (MIX – 0.3 µg/g ATV mais 2 µg/g EE2). Os grupos de controlo e controlo solvente corresponderam a peixes injetados com solução salina (0.7% NaCl) e peixes injetados com solução salina fortificada com solvente (0.7% NaCl, 0.1% DMSO e 0.9% etanol), respetivamente. Para cada condição de exposição, os peixes (n = 10) foram injetados com 4 µl/g duas vezes por semana durante 15 dias, correspondendo a 4 injeções por peixe.

Os parâmetros analisados incluiram medições biométricas, bioquímica dos lípidos do sangue (colesterol, triglicerídeos, colesterol HDL, colesterol LDL e VLDL), quantificação com ImageJ de gotículas lipídicas hepáticas em secções histológicas de fígado previamente pós-fixado em tetróxido de ósmio, classificação da maturação das gónadas a partir de cortes histológicos e avaliação de expressão de mRNA hepático de genes estrogénicos (VtgA e ERα) e genes lipídicos (ACC, Acsl1, ApoAI, Acox1-3l, Acox3, Fabp1, FAS, HMGCoAR, LPL, PPARα, PPARαBa, PPARαBb, PPARγ e StAR) medido por RT-qPCR.

De todos os parâmetros biométricos, só o peso do fígado e o índice hepatossomático aumentaram após exposição ao EE2 e com o MIX. O colesterol, LDL, VLDL e triglicerídeos diminuíram após tratamento com ATV enquanto o EE2 aumentou os triglicerídeos e VLDL e o HDL diminuiu em todos os grupos experimentais (ATV, EE2, MIX). A quantificação de lípidos em tecidos osmificados foi significativamente mais elevada no EE2 e MIX e os fragmentos de fígado osmificados de peixes expostos a ATV, EE2 e MIX apresentavam heterogeneidade de tamanho e distribuição das gotículas lipídicas. Nenhum

dos químicos ou a sua combinação foram capazes de causar alterações na histologia das gónadas.

Adicionalmente, o ERα e VtgA foram mais expressos no EE2 e no MIX. O alvo clássico das estatinas, a HMGCoAR, foi significativamente mais expresso no EE2 e na ATV. A Acsl1 e a FAS também demonstraram regulação positiva após exposição ao EE2, enquanto que a Fabp1 teve o padrão inverso, sendo menos expressa no EE2. A ATV causou regulação positiva da ApoAI enquanto que o EE2 e o MIX tiveram o efeito oposto na expressão deste gene. No caso do PPARγ, o MIX também causou uma redução da expressão. A exposição a todos os três tratamentos causou redução de expressão da Acox1-3l e Acox3 relativamente aos controlos.

Em suma, a exposição *in vivo* de juvenis de truta fario a ATV, EE2 e à sua mistura através de injeções intramusculares é capaz de induzir modificações no metabolismo lipídico. As alterações nos perfis lipídicos do sangue e do conteúdo e distribuição de lípidos hepáticos contrastam entre a ATV e o MIX, geralmente com aumento no EE2 e redução na ATV. No MIX, a ATV e o EE2 mostraram padrões contraditórios. O mesmo padrão de antagonismo entre ATV e EE2 também foi observável a nível molecular, onde a ATV tinha um efeito oposto ou nulo relativamente ao EE2 na expressão dos vários genes. No MIX, a ATV parecia ter um efeito antiestrogénico.

TABLE OF CONTENTS

List of abbreviations

11KT – 11-ketosterone

E2 – 17β-estradiol

EE2 – 17α-ethinylestradiol

Acetyl-CoA – Acetyl coenzyme A

ACC – Acetyl coenzyme A carboxilase

Acsl – Acetyl coenzyme A synthetase

Acsl1 – Acetyl coenzyme A long chain synthetase 1

Acox – Acetyl coenzyme A oxidase

Acox1-3I – Acetyl coenzyme A oxidase 1 - 3I

Acox3 – Acetyl coenzyme A oxidase 3

ATP – Adenosine triphosphate

ANOVA – Analysis of variance

AR – Androgen receptor

ApoAI – Apolipoprotein A I

ATV – Atorvastatin

BPA – Bisphenol A

b-act – β-actin

CA – Clofibric acid

cDNA – Complementary deoxyribonucleic acid

P450scc – Cytochrome P450 side chain cleavage enzyme

DNA – Deoxyribonucleic acid

DMSO – Dimethyl sulfoxide

DHA – Docosahexaenoic acid

EPA – Eicosapentaenoic acid

Ef-1α – Elongation factor 1α

EDC – Endocrine-disrupting chemical

E1 – Estrone

E3 – Estriol

ER – Estrogen receptor

Erα – Estrogen receptor α

ERE – Estrogen responsive element

EDTA – Ethylenediamenetetraacetic acid

EU – European Union

FAS – Fatty acid synthase

Fabp – Fatty acid binding protein

Fabp1 – Fatty acid binding protein 1

FSH – Follicule-stimulating hormone

FAO – Food and Agriculture Organization

Gapdh – Glyceraldehyde-3-phosphate dehydrogenase

GSI – Gonadosomatic index

HIS – Hepatosomatic index

HDL – High density lipoproteins

HMGCoA – Hydroxy-3-methylglutaryl coenzyme A

HMGCoAR – Hydroxy-3-methylglutaryl coenzyme A reductase

HPG – Hypothalamic-pituitary-gonadal axis

ICBAS – Institute of Biomedical Sciences Abel Salazar

LDC – Lipid-disrupting compound

LPL – Lipoprotein lipase

LXR – Liver receptor X

LC-PUFA – Long chain polyunsaturated fatty acid

LDL – Low density lipoproteins

LH – Luteinizing hormone

Malonyl-CoA – Malonyl coenzyme A

MIX – Mixture

n-3 PUFA – Omega 3 polyunsaturated fatty acid

OECD – Organization for Economic Cooperation and Development

PPAR – Peroxisome proliferator-activated receptor

PPARα – Peroxisome proliferator-activated receptor α

PPARαBa – Peroxisome proliferator-activated receptor α Ba

PPARαBb – Peroxisome proliferator-activated receptor α Bb

PPARβ – Peroxisome proliferator-activated receptor β

PPARγ - Peroxisome proliferator-activated receptor γ

qRT-PCR – Quantitative real-time polymerase chain reaction

RXR – Retinoid receptor X

RNA – Ribonucleic acid

Rpl8 – Ribosomal protein L8

mRNA – Messenger ribonucleic acid

sGnRH1 – Salmonid gonadotropin releasing hormone 1

sGnRH2 – Salmonid gonadotropin releasing hormone 2

SC – Solvent control

StAR – Steroidogenic acute regulatory protein

SREBP – Sterol regulatory element-binding protein

T – Testosterone

UK – United Kingdom

USD – United States dollars

USA – United States of America

VtgA – Vitellogenin A

VLC-PUFA – Very long chain polyunsaturated fatty acid

VLDL – Very low-density lipoproteins

Chapter 1. Introduction

1. Salmonid's ecological and socio-economical importance

1.1. Ecology of the *Salmonidae* family

Fish is an extremely vast group of animals that comprehend over half of the known vertebrates and exhibit unparalleled diversity in terms of morphology, physiology, behaviour and demography due to their evolution over 500 million years (Nelson et al., 2016). The taxonomical classification and clade nomenclature of fish is mainly based on the animal's morphology; however, molecular studies have been used to study the relationship between species and, in some cases, thrown the clades' relative position into question (Bone and Moore, 2008).

Salmonidae is the only family belonging to the *Salmoniformes* order and is divided into three subfamilies: *Salmoninae*, *Coregoninae* and *Thymallinae* (Nelson et al., 2016). Salmon and trout species are all in the *Salmoninae* subfamily (Nelson et al., 2016), with trout species restricted to animals from the *Oncorhynchus*, *Salmo* and *Salvelinus* genera (Wenger et al., 2011). Up to 240 fish species can be included in the *Salmonidae* family, all endemic to the northern hemisphere (Collares-Pereira et al., 2021); however, they have been introduced in Oceania, South America and South Africa (FAO, 2020).

Morphologically, all salmonids are characterized by possessing a streamlined body, an adipose fin close to their forked tail, large mouths, and cycloid scales (Collares-Pereira et al., 2021). Salmonids also share the fact that all of them spawn in gravel nests in freshwater environments and reproduce through external fertilization (Buschmann and Muñoz, 2019). Apart from this, they can vary greatly in size, or life history, such as the number of migrations or the times they reproduced (Wilson, 1997).

Salmonids hold essential ecological functions in their ecosystems. Their carcasses feed many animals living near river basins, such as ursids or mustelids, and anadromous species bring in high amounts of nutrients, such as nitrogen and phosphorous, from the oceans, contributing up to 30% of the nitrogen in the food chain (Hilderbrand et al., 2004). Their role as invasive species cannot be underestimated. In some cases, invasive trout species have entirely replaced the native populations, like the brown trout (*Salmo trutta*) did to the brook trout (*Salvelinus fontinalis*) in Scandinavia (Wenger et al., 2011) and caused many other problems in areas like Japan (Kitano, 2004).

Salmonids are currently threatened by several factors, such as the rise in temperatures, given they are cold-water fish (Isaak et al., 2010) or alterations in water flow regimes in rivers (Milner et al., 2012), which can be altered by rain frequency and other

climacteric events (Wenger et al., 2011). Anthropogenic actions, such as dams and water extraction (Warren et al., 2015), can also affect salmonid survival at various life stages (Nislow and Armstrong, 2012).

1.2. Socio-economical role of salmonids

Fish have provided an essential source of protein since the start of fishing and aquaculture (Tacon and Metian, 2013). Between 2000 and 2019, global production from fisheries and aquaculture grew by 41%, reaching 178 million tons of seafood products by the end of that period, valued at over 400 billion USD (FAO, 2021). By 2013 approximately 82% of all fish captured by fishing operations were from saltwater (Tacon and Metian, 2013), but the aquaculture of freshwater and anadromous species has been rapidly rising, going up 137% between 2000 and 2019 (FAO, 2021).

Farmed salmonid species belong almost exclusively to the *Salmo* and *Oncorhynchus* genus, and the largest producers of salmonids worldwide are Norway and Chile, although there are other significant producers such as the UK, Iran and Turkey (Buschmann and Muñoz, 2019). The Atlantic salmon is by far the most farmed salmonid in the world, followed by the rainbow trout (*Oncorhynchus mykiss*) and then the Coho salmon (*Oncorhynchus kisutch*) in 3rd place (Buschmann and Muñoz et al., 2019) with the main countries responsible for each of their production represented in Figure 1. Even regions that do not lead the global production of salmonids follow the trend of their farming, with rainbow trout (*Oncorhynchus mykiss*) being the most-reared species in the EU in 2018, accounting for 17% of the total production (Eurostat, 2020).

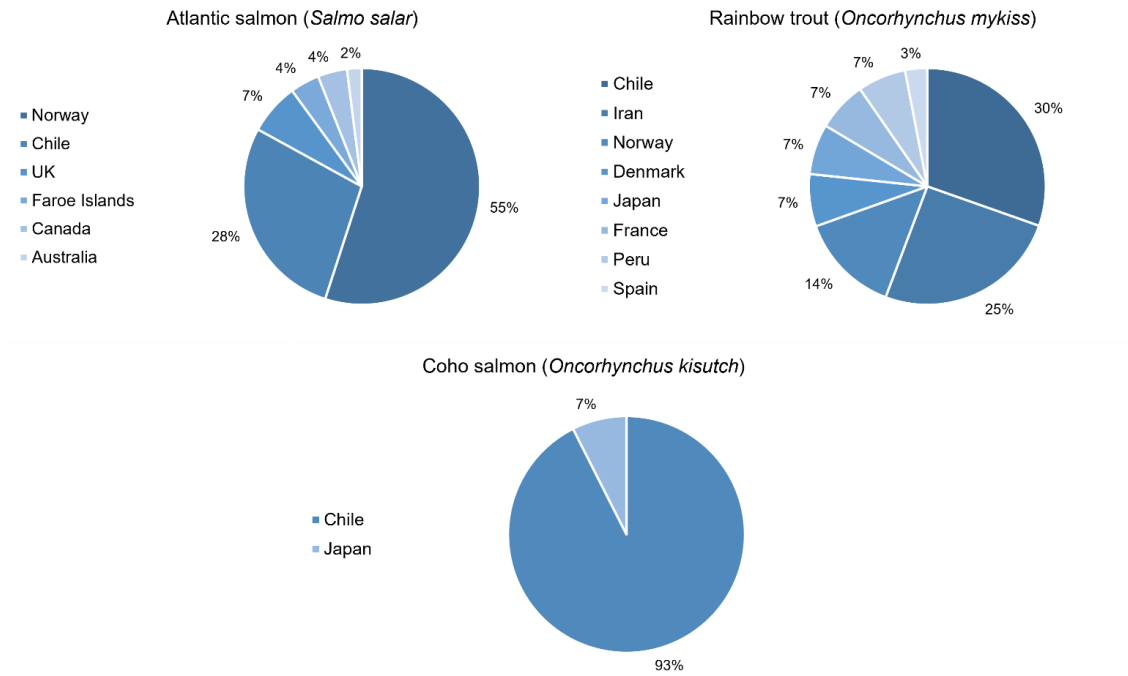


Figure 1. Share of the aquaculture production of the three main farmed salmonid species by country (Data from Buschmann and Muñoz, 2019).

In 2017, fish consumption stood at 20.3 kg per capita and in 2018, approximately 88% of all captured fish in the world was used for direct human consumption (FAO, 2018a). Fish possess a very high nutritional value, being particularly good sources of amino acids like taurine and choline, essential micronutrients such as phosphorous, vitamins and omega 3 polyunsaturated fatty acids (n-3 PUFAs) (Tilami and Samples, 2018). Traditionally, n-3 PUFAs have been the most sought-after nutrient in fish, given that fish oil is very n-3 PUFA rich, particularly among salmonids (Lund, 2013). Despite this, the dietary uptake of n-3 PUFAs in western diets remains sub-optimal (Raatz et al., 2013). The ingestion of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two of the most prominent n-3 PUFAs in fish (Figure 2), has been associated with reduced risk of coronary disease and the ingestion of two weekly 180 g portions of salmon has increased EPA and DHA levels to optimal for their action (Raatz et al., 2013). EPA, DHA, and other n-3 PUFAs, have been linked to reduced risk of certain cancers (Usydus et al., 2011) and immunosuppressant properties (Tilami & Samples, 2018), as well as in early fetal neurological development and the immunologic maturation in early life stages (Urwin et al., 2012).

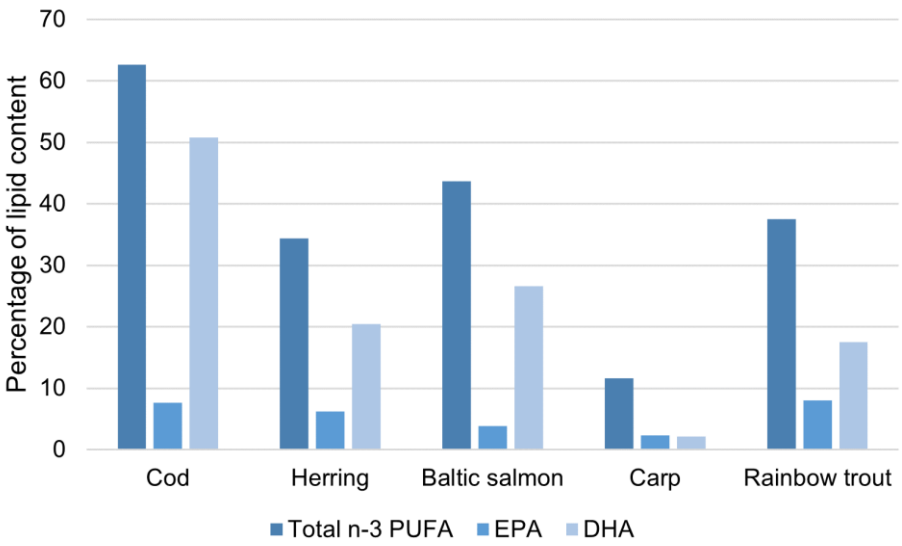


Figure 2. Percentage of n-3 PUFAs from the extracted lipid content of cod (*Gadus morhua callaries*), herring (*Clupea harengus membras*), Baltic salmon (*Salmo salar*), carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) sold in the Polish market (Data from Usydus et al., 2011).

1.3. Brown trout

The brown trout (*Salmo trutta*) is a widely dispersed salmonid that endemically colonizes Europe (Santos et al., 2021). It can be either anadromous or strictly freshwater based, known as sea trout or brown trout, respectively (Collares-Pereira et al., 2021). In freshwater river basins, brown trout inhabits highly oxygenated, cold water with moderately high flow in the upper portions of rivers preying on invertebrates and smaller fish (Collares-Pereira et al., 2021). Brown trout have a lot of different phenotypes and can vary greatly among populations (Santos et al., 2021). Freshwater brown trout usually have greenish-brown backs with black or reddish spots and a light-yellow ventral area (Figure 3), and mature males develop an elongated lower jaw during mating season that stops them from closing their mouths fully (Collares-Pereira et al., 2021).

Figure 3. Brown trout (*Salmo trutta*) juveline. Photo by Tiago Lourenço.

This species of trout spawns in the Fall, with its juveniles usually more affected by the Winter floods (Wenger et al., 2011). They spawn in gravel nests in the riverbed, and the fry are territorial from the moment of eclosion, setting up micro-territories where they hunt, a trend that remains throughout their development and adulthood (Collares-Pereira et al., 2021). They reach sexual maturity between 2 to 3 years (Collares-Pereira et al., 2021).

Brown trout has a very high socioeconomic value, mainly for sport fishing (Collares-Pereira et al., 2021), but also for commercial fishing and aquaculture. In 2018, over 4000 tonnes of brown trout were caught, and over 27,000 tonnes were farmed worldwide (FAO, 2018). Ecologically this species is also very significant, with a bigger focus nowadays on its role as an invasive species (Kitano, 2004; Wenger et al., 2011) (Figure 4) and as a sentinel species for aquatic pollution due to its low tolerance to poor water quality (Santos et al., 2021).

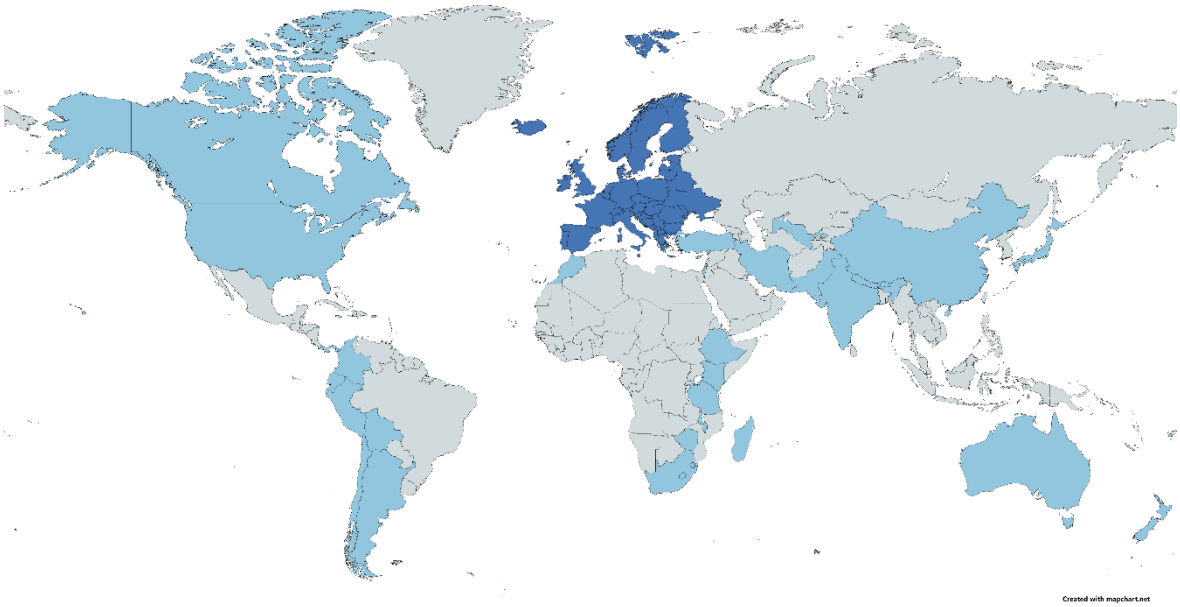


Figure 4. Brown trout (*Salmo trutta*) endemic distribution (dark blue) and known introductions (light blue) (Data from Collares-Pereira et al., 2021 and FAO, 2020; chart made with mapchat.net).

2. Lipid and fatty acid metabolism in fish

Lipids are among the most important and diverse groups of biomolecules and possess a wide range of physiological functions (Al-Habsi et al., 2018). Dietary lipids in fish are usually either incorporated in cellular membranes, stored in adipose tissue, or oxidized to produce energy (Tocher, 2003). Moreover, for most physiological activities in fish, lipid and fatty acid oxidation is the predominant source of energy (Tocher, 2003). In this vein, salmonids and other freshwater fish balance fatty acid oxidation and storage (Menoyo et al., 2003).

Lipogenesis is the endogenous process of *de novo* synthesis of fatty acids from acetyl coenzyme A (acetyl-CoA) (Tocher, 2003), happening in fish essentially the same way as it does in mammals (Sheridan et al., 1994). The process begins with the oxidation of acetyl-CoA into malonyl coenzyme A (malonyl-CoA) catalyzed by the acetyl-CoA carboxylase (ACC) (Leaver et al., 2008). In the cytosol, the malonyl-CoA is assembled by the fatty acid synthetase (FAS) into fatty acids with either 16 or 18 carbons (Tocher, 2003). Dietary fatty acids inhibit the expression of FAS mRNA and thus reduce the lipogenesis rate (Peng et al., 2017).

Most lipogenesis happens in the liver (Tocher, 2003) (Figure 5), meaning that the fatty acids generated must be transported to other tissues. Fatty acids are transported in the circulatory system mainly as triglycerides or lipoproteins (Leaver et al., 2008). Their subsequent uptake from the bloodstream to the tissues occurs through active transport mediated by the fatty acid transport protein in the case of un-esterized fatty acids (Karagianni and Talianidis, 2015) or with the intervention of the lipoprotein lipase (LPL) (Leaver et al., 2008). The LPL is a glycoproteic enzyme located in the endothelium of blood vessels and in other tissues, such as adipocytes and hepatocytes, and it hydrolyzes triglycerides from circulating lipoproteins into free fatty acids (Albalat et al., 2006). Once inside the cell, the fatty acid binding protein (Fabp) accepts those acids, shuttling them between the cell membrane and the oxidative organelles (Leaver et al., 2008).

The catabolism of fatty acids by β-oxidation is the main source of energy for many fish, and it happens in the mitochondria and peroxisomes in a multi-enzymatic process that involves the cleavage of acetyl-CoA and the donation of electrons to the transport chain to synthesize adenosine triphosphate (ATP) (Tocher, 2003; Karagiannis and Talianidis, 2015). The production of acetyl-CoA in the cells from the fatty acids is controlled by isoforms of the acetyl-CoA synthetase (Acsl), and so the rate-limiting step of fatty acid β-oxidation is governed by these enzymes (Leaver et al., 2008; Li et al., 2010). Although the reactions in the mitochondrial and peroxisomal β-oxidation are very similar, the fact that different

enzymes catalyze them means that peroxisomal β-oxidation loses nearly half of the produced energy in the form of heat when compared to the mitochondrial β-oxidation (Leaver et al., 2008). The amount of β-oxidation done by either peroxisome or mitochondria varies from tissue to tissue, but in fish, the liver shows the preponderance of peroxisomal β-oxidation (Nanton et al., 2003).

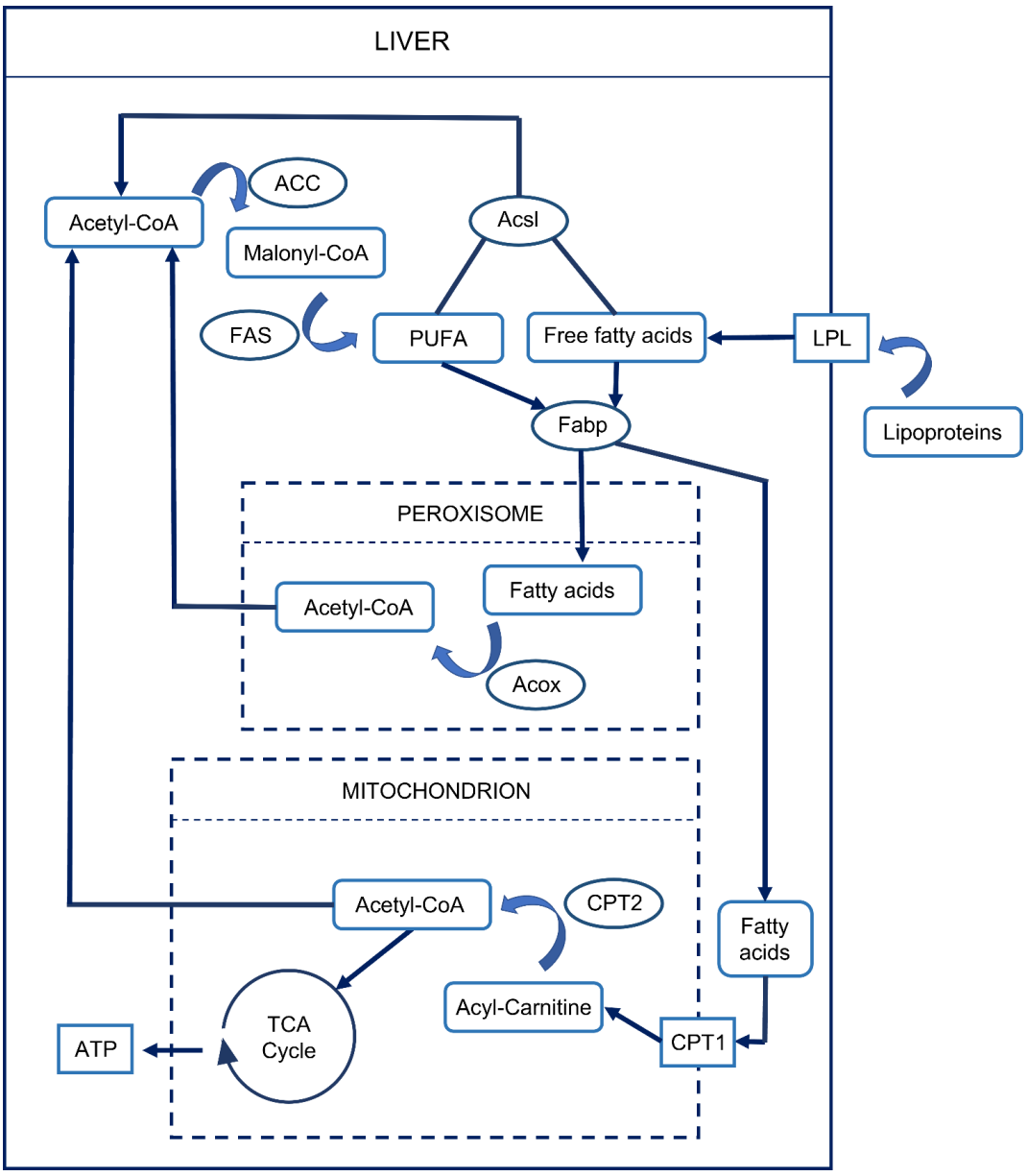


Figure 5. Simplified overview of the lipid metabolism in the liver of salmonids. ACC – Acetyl-coenzyme A carboxylase; Acetyl-CoA – Acetyl-coenzyme A; Acox – Acetyl coenzyme A oxidase; Acsl – Acetyl coenzyme A synthetase; ATP – Adenosine tri-phosphate; CPT1 – Carnitine palmitoyl transferase 1; CPT2 – Carnitine palmitoyl transferase 2; Fabp – Fatty acid binding protein; FAS – Fatty acid synthase; LPL – Lipoprotein lipase; Malonyl-CoA – Malonyl coenzyme A; TCA Cycle – Tricarboxylic acid cycle (Data from Tocher, 2003 and Leaver et al., 2008).

2.1. Control mechanisms of lipid metabolism in fish

All lipid and fatty acid metabolism processes in fish are co-regulated by hormones and transcription factors (Karagiannis and Talianidis, 2015). According to the same authors, the main hormones controlling lipid metabolism include insulin, glucagon, growth hormone and leptin. The hormones can stimulate either anabolic or catabolic pathways. For example, insulin has anabolic functions in fish and promotes lipogenesis (Nelson and Sheridan, 2006) and lipid storage in rainbow trout by stimulating LPL activity in adipose tissue (Leaver et al., 2008). By contrast, glucagon has a catabolic effect in rainbow trout and coho salmon, stimulating lipolysis in the liver (Leaver et al., 2008).

There is a small network of hepatic transcription factors that regulate the lipid metabolism in the liver, which includes sterol regulatory element-binding protein (SREBPs), liver receptor X (LXR), retinoid receptor X (RXR) and peroxisome proliferator-activated receptors (PPARs) (Karagiannis and Talanidis, 2015). The SREBPs and LXR are crucial regulators of mammalian fatty acid metabolism and, in the same vein, have been shown to have their expression regulated by exposure to EPA and DHA in Atlantic salmon hepatocytes (Minghetti et al., 2011), which further evidenced that fatty acids can regulate the activity of various transcription factors related to their metabolism in fish (Leaver et al., 2008). PPARs are involved in the β-oxidation of fatty acids and can also be regulated by their presence, although RXR ligands can also activate PPARs given that these two transcription factors act by forming a heterodimer (Leaver et al., 2008).

PPARs are ligand-activated transcription factors that belong to a superfamily of nuclear receptors and have a very similar structure to the receptors of steroid hormones, although their natural ligands are lipid-derived substances (Lopes et al., 2016). There are three isotopes of PPARs with tissue-specific distribution and varying functions: PPARα, PPARβ and PPARγ (Baptista-Pinto et al., 2009). PPARγ is the most abundant of all other PPARs being up to 30 times more present in adipose tissue than the rest, and it mainly affects the transcription of genes associated with lipogenesis, whilst PPARα and PPARβ are associated with the regulation of peroxisomal β-oxidation (Manor et al., 2015). In some model organisms, namely in rat and mouse, PPARα has been associated with exacerbated proliferation of peroxisomes when exposed to exogenous substances that can act as ligands, like hypolipidemic pharmaceuticals (Corton et al., 2000). However, exposure of brown trout (*Salmo trutta*) hepatocytes to clofibrate did not evoke peroxisome proliferation or changes in the expression of PPARαBb and PPARαBa (Madureira et al., 2017a). On the other hand, administration of clofibric acid and bezafibrate to Atlantic salmon (*Salmo salar*)

hepatocytes has caused increased expression of PPARγ and activity of the Acox enzymes (Ruyter et al., 1997).

2.2. Interactions between reproduction and lipid metabolism in fish

The reproductive endocrinology of teleosts occurs along the hypothalamic-pituitary-gonadal (HPG) axis through a series of hormonal cascades (Frisch, 2004). These hormonal cascades culminate in the release of gonadotropins into the bloodstream, eventually reaching their receptors in the gonads, stimulating the production of sex steroids (Yaron and Levave-Sivan, 2011) (Figure 6). Sex steroids are the sexual hormones of fish and the main excretion product of the gonads and are synthesized from cholesterol in a series of reactions catalyzed by enzymes from the cytochrome P450 family (Piferrer, 2011). This means cholesterol movement through membranes with the help of the steroidogenic acute regulatory (StAR) protein is one of the limiting factors in sex steroid synthesis (Arukwe et al., 2016). Androgens are the primary male sex steroids due to their masculinizing effect and presence in higher levels in males (Piferrer, 2011), whilst estrogens are the main female steroidal hormones with a wide range of functions and physiological processes, most related to reproduction (Lamm et al., 2015). The most significant endogenous estrogen is 17β-estradiol (E2) (Atteke et al., 2003; Martyniuk et al., 2006) and the predominant male sex steroid in teleost fish, 11-Ketotestosterone (11KT) (Fricsh, 2004); however, significant levels of testosterone (T) were also found in salmonid plasma (Melo et al., 2015).

Sex steroids exert physiological effects through downstream signaling after binding to specific receptors (Thibaut and Porte, 2004). They are known to regulate lipid homeostasis in mice and humans by activating estrogen receptors (ERs) and androgen receptors (ARs) in the liver using classic heterodimer bonds with estrogen and androgen response elements, respectively (Faulds et al., 2012; Shen and Shi, 2015). Modulation of lipidic pathways by sex steroids also happens in fish. One example is vitellogenesis, where E2 activates ERs in the liver, resulting in the expression of the vitellogenin A (VtgA) gene and subsequent synthesis of vitellogenin, the precursor of egg yolk proteins which is later imprisoned by the oocytes (Jamazaldeh et al., 2012). This process requires the uptake of lipids to the oocytes that originate mainly from plasma very low density lipoproteins (VLDLs) (Lubzens et al., 2010), which LPL metabolizes outside the oocyte before entering the cells (Reading et al., 2018).

Exposure of fish to different sex steroids has also caused lipidic and sex-specific responses. Dietary exposure of zebrafish (*Danio rerio*) to atorvastatin at 0.53 μg/g caused upregulation of PPARα in females but not in males as well as a reduction of triglyceride

levels in females but not in males (Al-Habsi et al., 2016). A 30-day exposure of rainbow trout (*Oncorhynchus mykiss*) to T or E2-treated diets saw both sex steroids down-regulate ACC mRNA expression and E2 alone decreasing LPL and PPARγ mRNA (Cleveland and Weber, 2016).

Lipid metabolism regulation by sex steroids has also been proposed through the cross-talk mechanisms between the PPARs signalling pathways, for example, in brown trout (*Salmo trutta*) (Lopes et al., 2021). The regulation of PPAR expression by sex steroids happens naturally in brown trout, where different stages of the reproductive cycle showed sex-specific alterations in PPARα liver expression, with the highest expression in early vitellogenic females with higher E2 levels and the males showing no significant changes across the reproductive cycle (Batista-Pinto et al., 2009). In vivo, modulation of PPAR pathways by sex steroids in fish can also be experimentally induced. Intraperitoneal injections of E2 at 5 µg/g in rainbow trout (*Oncorhynchus mykiss*) caused downregulation of PPARα (Cleveland and Manor, 2015), whilst the waterborne exposure of brown trout (*Salmo trutta*) to 17α-ethinylestradiol (EE2) at 50 µL/L led to upregulation of PPARα and downregulation of PPARγ in the liver (Madureira et al., 2018). Dietary exposure of rainbow trout to E2 also induced downregulation of PPARγ expression (Cleveland and Weber, 2016). Other xenobiotics can also induce sex-specific modulation of PPARs, with female zebrafish (*Danio rerio*) fed atorvastatin-treated diets showing increased expression of PPARα and the males having no significant changes (Al-Habsi et al., 2016).

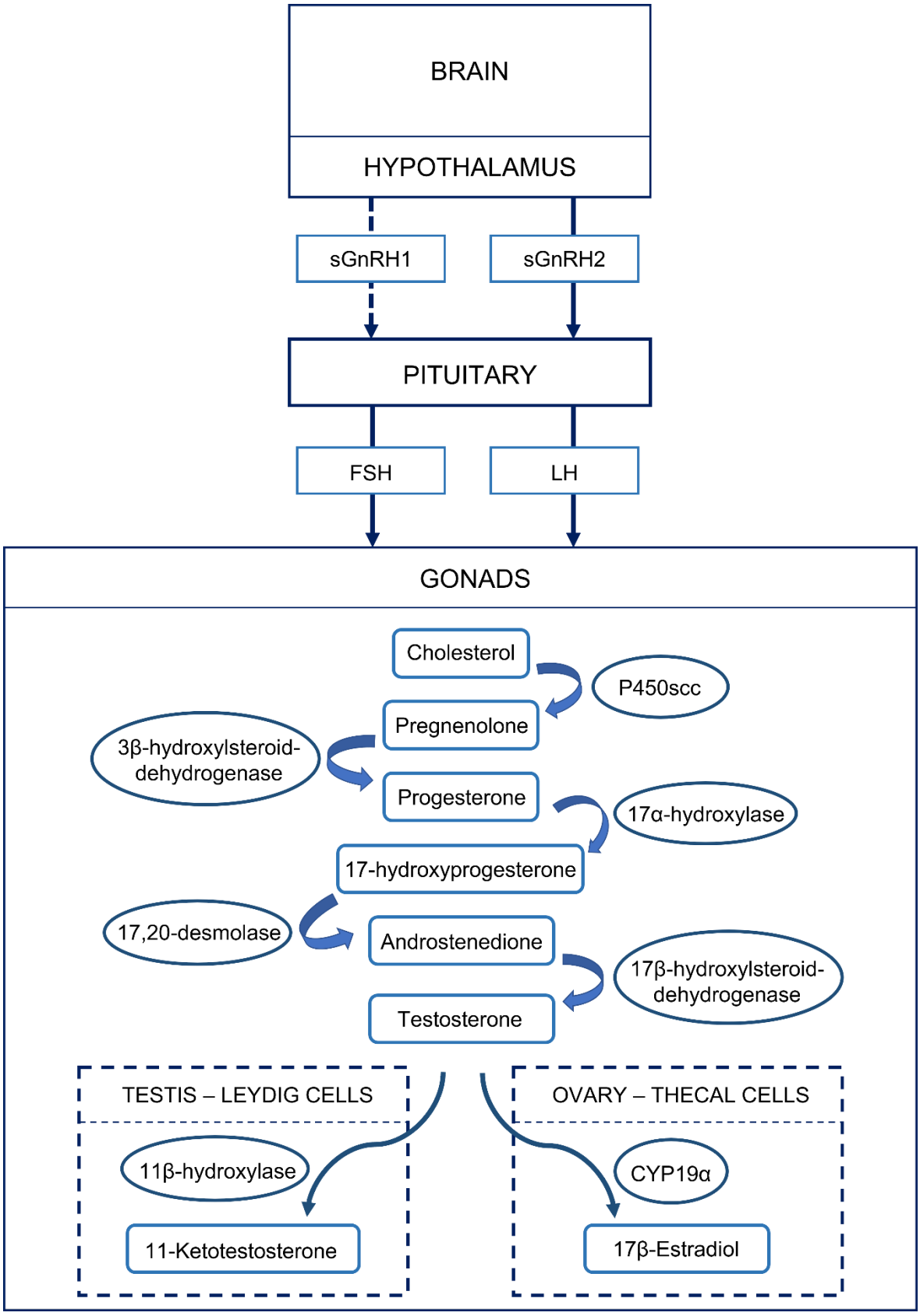


Figure 6. Androgenic and estrogenic synthesis pathways along the HPG axis of salmonids (Data from Martyniuk et al., 2006 and Piferrer, 2011).

3. Endocrine-disrupting and hypolipidemic chemicals and fish

3.1. Estrogenic endocrine-disrupting chemicals

The rapid development of human populations has created an ever-growing pressure on aquatic environments, with uncountable pollutants contributing to their degradation (Weber and Sciuba, 2019). Among them are endocrine-disrupting chemicals (EDCs), commonly defined as environmental pollutants capable of modulating and interfering with the normal function of the endocrine system of an animal, its progeny or a population (Mills and Chichester, 2005; Blewett et al., 2014). EDCs are known to have three fundamental mechanisms of toxicological action: i) disruption of the hormonal action by mimicking either agonists or antagonists of the endogenous hormone; ii) disruption of the synthesis, transport and metabolism of endogenous hormones; iii) disruption of the production and function of hormone receptors (Barreiros et al., 2016). Some defend that EDCs do not have a specific threshold below which they do not produce adverse effects – they are always biologically active, and any concentration can be harmful (Norris and Hobbs, 2006).

One of the most important classes of EDCs is the estrogenic EDCs, which include synthetic estrogens present in medication, natural estrogens excreted by humans like estrone or estradiol, phytoestrogens such as genistein, polychlorinated biphenyls like BPA and organochlorinated pesticides (Segner et al., 2006; Hanson et al., 2017). These chemicals reach water matrices through agricultural runoffs, industrial and urban effluents, or pharmaceutical degradation (Wise et al., 2011). Xenoestrogen concentrations in surface water are usually low, within the ng/L range, but even at such low levels, they are very potent and capable of causing disruptions (Czarny et al., 2017).

In fish, estrogenic EDCs cause mainly reproductive disruptions, such as gonadal function alterations, reduced sperm counts, feminization of male fish (Czarny et al., 2017), intersex conditions (Wise et al., 2011), abnormal gonadal development, sex steroid level alterations and reduced egg fecundity (Osachoff et al., 2013). Perhaps the most common effect of estrogenic EDCs is the induction of vitellogenin synthesis in male and sexually immature juvenile fish, so much so that it has become the primary biomarker of exposure to xenoestrogens (Osachoff et al., 2013). This ability of estrogenic substances to interfere with gene expressions is also widely demonstrated by their ability to modulate ER and zona pellucida protein (ZP) genes (Hultman et al., 2015).

Salmonids are affected by xenoestrogens, much like other fish. For instance, phytoestrogens like genistein have been shown to induce VtgA production by male rainbow trout (*Oncorhynchus mykiss*) and activate ERs (Cleveland and Manor, 2015). Nonylphenol has also promoted VtgA synthesis in male and juvenile rainbow trout (*Oncorhynchus*

mykiss) and increased the E2 and T levels in males and females, respectively (Naderi et al., 2015). E2 has also been linked with the dysregulation of the physiological and metabolic processes involved in growth (Cleveland and Manor, 2015).

3.1.1. 17α-Ethinylestradiol

The increase of estrogenic activity in water bodies worldwide is an increasing concern that can be mainly traced back to both natural and synthetic estrogenic hormones in the water (Garriz et al., 2017). Natural hormones like estrone (E1), 17β-estradiol (E2), estriol (E3) and synthetic hormones such as 17α-ethinylestradiol (EE2) have been found in surface waters, with E1 usually the most abundant (Czarny et al., 2017). E2 is the main estrogen of vertebrates, including fish, but it has powerful endocrine-disrupting capabilities and is mainly introduced in surface waters through human urine discharged by sewers and water treatment plants (Li and Yang, 2010; Wise et al., 2011). On average, men and women excrete 1.6 and 2.5 μg of E2 per day, respectively (Li and Yang, 2010), and this estrogenic hormone has been found in concentrations as high as 631 ng/L (Garriz et al., 2017). However, perhaps the most relevant estrogenic EDC is EE2 (Wise et al., 2011). The latter is a synthetic hormone derived from E2 (Figure 7) (Barreiros et al., 2016), and it is used in most birth control pills and hormonal replacement therapy (Czarny et al., 2017; Real et al., 2020). EE2 has a very high bioavailability and is more resistant to metabolization (Real et al., 2020), which, combined with its potency, gives it a very high potential for endocrine disruption. The strength of estrogenic EDCs is usually measured relatively to E2, which has a potency of 1 (Wise et al., 2011). According to Wise et al. (2011), E1 has a relative potency of 0.2, and E3 is the least potent, with a relative potency of 0.024. EE2 is by far the most dangerous estrogenic hormone, with a relative potency of 2. By 2011, it was estimated that 32.8% of women in the USA and 58.4% of women in Europe used oral contraceptives (Liu, 2011). Barreiros et al. (2016) estimated that if 17% of women worldwide use oral contraceptives regularly, that will equate to 4.4 kg of EE2 being introduced yearly into the environment per million inhabitants.

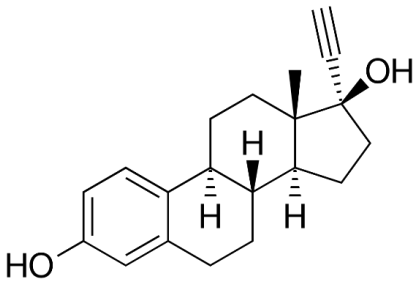


Figure 7. Molecular structure of 17α-ethinylestradiol (EE2).

As recently as 2019, EE2 was found in concentrations of 24 ng/L and 4.7 ng/L in a bay in China and a river mouth in the USA, respectively (Zhang et al., 2019). Concentrations of up to 300 ng/l were also reported in a creek in Argentina (Garriz et al., 2017). The half-life of estrogenic hormones in the water is about 10 days before degradation; however, they have concerning levels of bioaccumulation in food chains due to their lipophilic nature and can easily bind to sediments, extending their lifetimes in water (Czarny et al., 2017). Indeed, EE2 has already been found in sediments a few kilometres from industrial effluents bound with particulate organic matter from sewers (Braga et al., 2005).

Salmonids are more sensitive to sexual modulation by estrogenic inputs than other fish, like cyprinids (Bjerregaard et al., 2008). That increased sensitivity, combined with the fact that some salmonid species – including brown trout – are very territorial and thus reluctant to abandon a certain river area (Bjerregaard et al., 2006), makes these fish particularly vulnerable to the effects of EE2. This hormone has been shown to cause intersex in wild brown trout living near water treatment plant effluents (Korner et al., 2008), as well as inducing increased expression of VtgA and ERα and higher hepatosomatic indices in brown trout exposed in vivo (Madureira et al., 2018).

3.2. Hypolipidemic chemicals

Human and veterinary pharmaceuticals frequently contaminate aquatic ecosystems, causing significant environmental impacts worldwide (Ortúzar et al., 2022). Hypolipidemic drugs comprehend a wide range of chemicals with differing mechanisms of action, including bile acid sequestrates, ezetimibe, nicotinic acid derivates, fibrates and statins (Kopin & Lowenstein, 2017), with the last two being the most prescribed worldwide (Tete et al., 2020). Their widespread consumption and subsequent excretion ultimately lead to their occurrence in aquatic environments where they threaten non-target organisms (Mezzelani and Regoli, 2022), aggravated by incomplete degradation in water treatment plants (Santos et al., 2016).

Both statins and fibrates (and their respective metabolites) can disrupt the normal lipid physiology of aquatic animals, and in that sense, they can be named lipid-disrupting compounds (LDC) or simply lipid disruptors (Martínez et al., 2019). Their detection levels in surface waters are above the theoretical threshold from which they can cause toxicity in fish (Tete et al., 2020). Clofibric acid (CA) is one of the main metabolites of fibrates, which can disrupt fatty acid metabolism due to its interaction with the PPARα signalling. CA has been frequently quantified in surface waters due to its high mobility and persistence (Sovadinová et al., 2014). Statins like simvastatin, atorvastatin and rosuvastatin are currently the most

prescribed hypolipidemic drugs, and their input on aquatic environments is very significant in water bodies closer to densely inhabited areas, like the Baltic Sea (Sulaiman et al., 2015; Falfushynska et al., 2019). Statins are frequently found in surface waters at concentrations in the ng/L range (Tete et al., 2020) and can persist for up to 430 days (Sulaiman et al., 2015). Their bioaccumulation ability and possible noxious effects on non-target organisms remain understudied (Mezzelani and Regoli, 2022).

The first step of cholesterol synthesis in humans is the conversion of hydroxy-3-methylglutaryl coenzyme A (HMGCoA) into mevalonic acid in a reaction catalyzed by the HMG-CoA reductase (HMGCoAR) (Sulaiman et al., 2015). Statins are potent competitive inhibitors of HMGCoAR, which reduces cholesterol synthesis and circulating levels while increasing the number of low-density lipoprotein receptors in the hepatocytes, which causes LDL uptake from the bloodstream (Al-Habsi et al., 2018). Given that the HMGCoAR molecular structure is highly preserved on the evolutionary scale, the effects caused in fish could be similar to those of humans (Falfushynska et al., 2019).

3.2.1. Atorvastatin

Atorvastatin (ATV) (Figure 8) was released by Pfizer in 1997 and is currently one of the most used statins, reaching 5.3 billion USD worth of sales by 2010 (Al-Habsi et al., 2018). This statin is a synthetic inhibitor of HMGCoAR and, when administered in doses of 10-20 mg/day, lowers cholesterol, LDL and triglycerides levels (Wang et al., 2021). It has greater effects on cholesterol levels when compared to other drugs in the statin family due to its prolonged inhibition period (Malhotra and Goa, 2001). Once it enters the system, ATV is rapidly metabolised into three inactive and two active metabolites: 2-hydroxy-atorvastatin and 4-hydroxy-atorvastatin (Sulaiman et al., 2015). Despite its rapid metabolisation, ATV has a bioavailability of only 12% in humans and unlike other statins, ATV is excreted primarily through the faeces and not the urine, with up to 95% of the excretion products being expelled in faeces and the rest in urine (Wang et al., 2021).

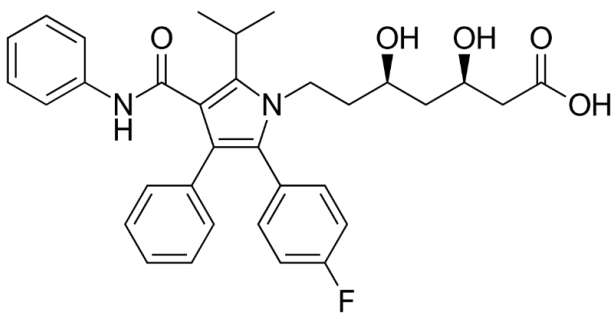


Figure 8. Molecular structure of atorvastatin (ATV).

ATV and its metabolites resist the barriers commonly employed by water treatment plants (Falfushynska et al., 2019). The only truly effective method of removal from the water is a very expensive solution that involves activated carbon (Ali et al., 2019). This immunity to sewage treatment, combined with its widespread use and low absorption in the human body, means that ATV has found its way into many water matrices being found in concentrations up to 439 ng/L in sewage effluents (Falfushynska et al., 2019) and 42 ng/L in surface waters in Spain (Tete et al., 2020).

Water contamination with ATV has been linked with human pathologies, including hepatic and sexual disruptions, diabetes, myopathy, memory loss and rhabdomyolysis (Ali et al., 2019). Examples of disruptive fish effects caused by ATV exposures have been reported. Prolonged in vivo waterborne exposure of a gobiid fish (*Mugilogobius abei*) to AVT at 0.5 to 50 µg/L caused the accumulation of reactive oxygen species and histopathological injuries (Wang et al., 2021). Zebrafish (*Danio rerio*) exposed to ATV at 53 µg/g of food exhibited reduced triglyceride and cholesterol whole-body levels and upregulation of PPARα and PPARγ (Al-Habsi et al., 2016). In vitro exposure of rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes to ATV also upregulated the PPARα, PPARγ and CYP3A27 gene transcripts, suggesting influences in lipid metabolism and xenobiotic biotransformation (Al-Habsi et al., 2018).

4. Dissertation objectives

Salmonids are often used as sentinel species of aquatic pollution and endocrine disruption, and their juveniles are highly susceptible to EDCs, namely estrogenic EDCs. The study of in vivo effects of LDCs – particularly statins – in salmonids is extremely scarce, with only two published studies to our knowledge, both using rainbow trout (*Oncorhynchus mykiss*) as a model organism (Estey et al., 2008; Ellesat et al., 2012). When including other teleosts, only two other works with zebrafish (*Danio rerio*) (Al-Habsi et al., 2016) and a gobiid (*Mugilogobius abei*) (Wang et al., 2021) have been published regarding the effects of statins in vivo*,* showing how unexplored this line of research remains.

In order to address the gap in knowledge of the hypolipidemic effects of ATV in fish, and the possible interactions with other known environmental pollutants, brown trout juveniles were chosen as the model organism to investigate the effects of ATV, EE2 and a mixture of both chemicals in vivo. With this in mind, this dissertation set out the following objectives:

(i) Assessing the capacity of ATV, EE2 and their combination to cause dyslipidemia in brown trout by analyzing blood biochemistry, examining hepatical structural changes linked with lipidic deposition and evaluating the expression of hepatic molecular targets, all directly or indirectly related to lipid homeostasis;

(ii) Investigate the possibility of ATV altering the gonadal development of juvenile brown trout, either alone or in combination with EE2, in the scope of a sub-acute exposure**;**

(iii) Create an in vivo model using juvenile brown trout exposed via intramuscular injection to be used to test known or candidate hypolipidemic substances.

Chapter 2. Materials and Methods

1. Fish acclimatization and housing

Fish acclimatization was performed in two distinct phases, which are described below. In mid-November 2021, 182 juvenile brown trout (*Salmo trutta fario*) of both sexes arrived at the Institute of Biomedical Sciences Abel Salazar's (ICBAS) aquatic animal facilities from a government aquaculture facility (Torno, Amarante, Portugal). Upon arrival, the fish were on average ± standard deviation 15 ± 0.1 cm in length and 30 ± 0.05 g in weight. The animals were randomly distributed in groups of 12 to 14 individuals across 15 opaque fibreglass tanks that held approximately 100 L of water. They integrated an in-house recirculatory aquaculture system with powerful filtration and a continuous water flow. The room had a photoperiod of 12 h light : 12 h dark. From this moment, the first acclimatization period began and lasted for 28 days, as previously recommended for this species (Madureira et al., 2019). During this first stage of acclimatization, fish were fed once a day (Trout Plus 4, AquaSoja).

Oxygen saturation of 89.5 ± 3.8 % in the water was assured by an air pump system that released air through submerged air stones. Room temperature climatization kept the water temperature (16.6 ± 0.4 °C) stable. Both dissolved oxygen levels (mg/L) and water temperature (ºC) were measured daily throughout the first 28-day period using an oximeter (DO210, VWR). The water levels of nitrite (NO_2^-, mg/L), ammonia (NH_3, mg/L), ammonium (NH_4, mg/L) and pH were measured twice a week using testing kits (Prodactest NO_2; Prodactest NH_3/NH_4) and a pH meter (pH530, WTW), respectively. At each time, 6 random tanks were selected.

After the first 28-day stage of acclimatization, fish were maintained in 12 tanks under an individualized recirculating water system (second stage of acclimatization) equipped with an external canister filter (Eheim 350 Experience) (Figure 9). This scheme avoided cross-contaminating the experimental groups via parental or metabolites excreted by the fish. Half of the water was replaced every other day to guarantee maximum quality. The substitution was done with aspiration and water pumping at a low flow to avoid inducing stress on the fish.

Figure 9. Closed water system tanks fitted with Eheim 350 Experience external canister filters. Photo by Tiago Lourenço.

The photoperiod, water temperature, and oxygenation and feeding conditions of the initial acclimatisation were maintained in the second phase. Nitrites, ammonia, ammonium, and pH were measured twice a week in half of the tanks, randomly selecting sampled tanks.

The assay and fish handling were supervised and made by personnel accredited by the Portuguese Directorate-General for Food and Veterinary. The experiment planning and procedures followed the legal requirements (Decree Laws 113/2013 and 1/2019) and the European directive on the protection of animals used for scientific purposes (2010/63/EU).

2. Exposure and sampling

2.1. Experimental design and exposure injections

Five experimental groups were defined: i) control (C); ii) solvent control (SC); iii) 17α-ethinylestradiol (EE2); iv) atorvastatin (ATV); and v) mixture (MIX). Fish from each experimental group were exposed to different solutions: C - saline solution (NaCl) at 0.7%; SC - saline solution (0.7%) plus dimethyl sulfoxide (DMSO) at 0.1% and ethanol at 0.9%; EE2 - 17α-ethinylestradiol ($C_{20}H_{24}O_2$, Sigma-Aldrich®) at 2 µg/g; ATV - atorvastatin ($2C_{33}H_{34}FN_2O_5Ca$, LGC Ltd.) at 0.3 µg/g; and MIX 17α-ethinylestradiol plus atorvastatin at

2 µg/g and 0.3 µg/g, respectively (Table 1). The EE2 and ATV concentrations were defined according to the compounds' solubility and literature reports of in vivo exposures of fish to EE2 and ATV (Cleveland and Manor, 2015; Al-Habsi et al., 2016).

Table 1. Composition of the injection solutions used for exposures for each experimental group (C – Control, SC – Solvent Control, EE2 – 17α-Ethinylestradiol, ATV – Atorvastatin, MIX – Mixture).

Group	NaCl (g)	DMSO (µL)	Ethanol (µL)	EE2 (mg)	ATV (mg)	H_2O (mL)
C	0.7	-	-	-	-	100
SC	0.7	20	230	-	-	100
EE2	0.7	20	230	12.5	-	25
ATV	0.7	20	230	-	2	25
MIX	0.7	20	230	12.5	2	25

Solution injections were freshly prepared before each injection. The saline solution was prepared first, followed by adding 17α-etthinylestradiol, atorvastatin or both to DMSO, then ethanol and finally the dissolved chemicals in the corresponding volume of saline solution.

A closed system tank was randomly assigned to each of the experimental groups. Every tank was stocked with 10 fish weighing 30 ± 10.9 g and measuring 12 ± 1.4 cm, on average ± standard deviation. During the exposure period, the water parameters were measured similarly and with the same periodicity as during the second acclimatization stage (see *1. Fish acclimatization and housing*). Except for the sampling day, the animals were fed daily.

The exposure period lasted 14 days (Appendix 1), and the animals were exposed to the test compounds via intramuscular injections. Therefore, each fish was an independent experimental unit. The injections were administered every third day, thus amounting to 4 injections per animal. This gap between each injection allowed to stagger the injections in each group and thus do all the exposures simultaneously (Appendix 1) as well as providing the animals with enough time to recover from the stress of the injections. Furthermore, this systematic exposure method ensured that the animals were consistently exposed to the different chemicals throughout the exposure period. The injection solutions (Table 1) were prepared using autoclaved water before injections and administered at 4 µL/g of body weight (Madsen et al., 2004), thus ensuring that every fish was exposed to the same dose.

In the defined injection protocol (Figure 10), animals were collected from a tank one at a time, placed in a container with 10 L of water and anaesthetized with 250 µL/L of ethylene monophenyl ether ($C_6H_5OCH_2CH_2OH$, Merk KGaA). Each fish was kept in the anaesthesia solution until it reached stage III, plane 3 of deep narcosis (Zahl et al., 2012), corresponding to a loss of balance, responsiveness to touch and breathing patterns near absent. It took, on average, 7 minutes for fish to reach that stage. Then, the fish was placed on a tray with a wet cloth soaked in tank water to keep them moist, and its head was covered to reduce exposure to light stimuli. The animal was quickly weighed with a digital balance (572, Kern), and the injection volume was calculated for the specific weight of the animal. The injection was performed with a 0.5 mL insulin syringe (BD Micro-Fine™) above the lateral line and immediately behind the dorsal fin; the fish showed no reaction. To prevent injury, the side where the fish were injected alternated between each injection and the injection site was monitored before and after the procedure for any possible lesions. After, the fish was placed in a recovery tank with aerated water. The recovery was monitored closely until the animal regained its full swimming patterns, at which point it was returned to the housing tank.

Figure 10. Exposure setup for intramuscular injections. Photo by Tiago Lourenço.

2.2. Sampling

The sampling occurred on the 15th day after the start of exposures (Appendix 1).

Fish were euthanized with glycol monophenyl ether at a concentration of 1 mL/L. Then, fish were weighed and measured for total and standard lengths, and blood was immediately collected from the caudal vein with 1 mL syringes (BD Plastipak^{TM}). The total blood was placed into K3-EDTA coated 1 mL tubes (Geriner Bio-One Vacuette$^{®}$).

After, the fish was dissected (Figure 11), and the liver and gonads were sampled and weighed. Then, the liver was sectioned into slices of approximately 2 mm of thickness and used for molecular analysis, hepatic lipid quantification and histology. The liver slices for histology and lipid quantification, as well as the gonads, were stored in formaldehyde at 4%. Liver slices for molecular analysis were snap-frozen in liquid nitrogen and stored at -80 ^{o}C.

Figure 11. Dissected juvenile brown trout. Photo by Tiago Lourenço.

Hepatosomatic index (HSI), gonadosomatic index (GSI) and Fulton's condition factor (K) were calculated using the following formulas: HSI = liver weight (g) / fish weight (g) x 100; GSI = gonad weight (g) / fish weight (g) x 100; K = 100 x fish weight (g) / (fish total length)^3 (cm).

3. Blood lipid levels

The selected blood lipid levels were measured by a combination of direct and indirect methods using the Cobas b101 analyzer (Roche Diagnostics) (Figure 12 A). A total of 20 µL of K3-EDTA anticoagulated whole blood was loaded into a Lipid Panel Test disc (Roche Diagnostics) (Figure 12 B), which automatically determines the total cholesterol, high-density lipoproteins (HDL), triglycerides and low-density lipoproteins (LDL), all in mg/dL.

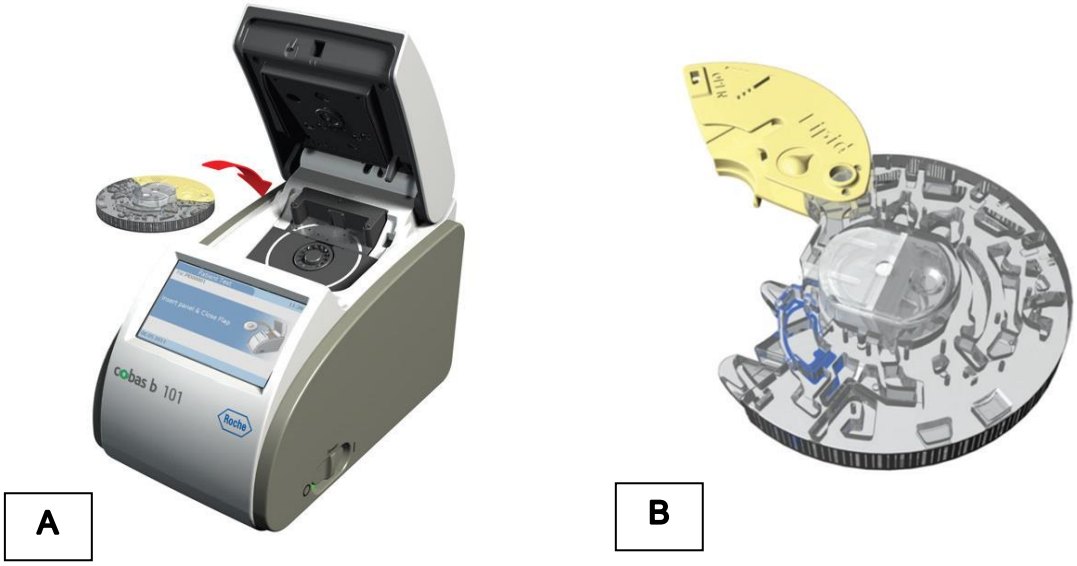


Figure 12. (A) Cobas b101 portable blood analyser. (B) Lipid disc. Images retrieved from the Cobas b101 operating manual.

Cholesterol levels were determined directly through an enzymatic reaction. Firstly, the erythrocytes were separated by centrifugation, and then cholesterol esterase was applied to the plasma, separating the cholesterol and the fatty acids. The cholesterol dehydrogenase and diaphorase were then applied to the plasma, originating NAD^+ and formazan dye. The concentration of formazan dye was measured by absorbance at 460 nm and is directly proportional to the cholesterol concentration.

The HDL concentration was determined in the same manner as the cholesterol but with the addition of phosphotungstic acid as a precipitation factor that precipitates all other lipidic components other than the HDL after the cholesterol esterase acts. The concentration was also measured by reading the formazan dye absorbance at 460 nm.

Triglyceride concentration was also measured through an enzymatic reaction. The triglycerides were hydrolyzed into glycerol and fatty acids by the lipoprotein lipase, followed by hydrolyzation of the glycerol by the glycerol dehydrogenase, which produces NADH. At this point, the diaphorase reacted with the NADH-originating NAD^+ and formazan dye. The formazan dye concentration was directly proportional to that of the triglycerides and measured at 460 nm.

The LDL levels were measured indirectly by the Cobas b101 equipment according to the following formula: LDL = total cholesterol − HDL − (triglycerides ÷ 5). The HDL/LDL, LDL/HDL, total cholesterol/HDL and triglycerides/HDL were calculated using data from the Cobas b101 analyses. The following formula was applied to obtain the very low-density

lipoproteins (VLDL) in mg/dL: VLDL = triglycerides ÷ 5 (Sharpe and MacLatchy, 2007). Non-HDL cholesterol was calculated by subtracting HDL levels from total cholesterol.

4. Hepatic lipid quantification

4.1. Osmium tetroxide post-fixation

Hepatic lipid droplets were quantified in one liver fragment (2 mm), representing approximately 16% of the liver. The fragment was firstly fixed for 24 hours in formaldehyde at 4% and then post-fixated with osmium tetroxide at 2% for lipid staining (Gates et al., 2016).

In the post-fixation protocol with osmium – or osmification – the liver fragment was first removed from the formaldehyde and delicately rinsed in running water for approximately 1 minute. After, it was placed in a flask containing a mixture of 1 mL of potassium dichromate at 5% ($K_2Cr_2O^-$, BDH Chemicals Ltd.) and 1 mL of osmium tetroxide at 2% that had previously been diluted in cacodylate buffer at 0.1 M ($(CH_3)_2AsO_2Na$, BDH Chemicals Ltd.) from the stock osmium tetroxide solution at 4% (OsO_4, Agar Scientific). Once placed in the osmicating solution, the fragment was agitated for 8 hours. In the end, fragments were repeatedly rinsed in running water every 10 minutes for 1 hour. In between rinses, the fragments remained in water on the agitation plate. Fragments were processed in an automated tissue processor (Leica TP1020 v10.1) (Appendix 2), embedded in paraffin and included in paraffin blocks using a modular tissue embedding centre (Leica EG1140).

The blocks of osmicated liver fragments were sectioned at 3 µm thick slices retrieved from random points on the block. Two sections per block were placed into silane-coated microscope slides (VWR) and left to dry in a hoven with air recirculation at 37 ºC for 48 hours (Gates et al., 2016). Then, the sections were carefully pressed with a soft cloth to enhance the adhesion. The deparaffinized sections were coverslipped with mounting medium (DPX) without staining. The latter approach aimed to improve the contrast between the black osmicated lipids and the remaining tissue.

4.2. Lipid content quantification

Per fish, 12 systematically sampled fields of view exhibiting liver parenchyma were photographed using the ×40 objective. The lipid droplet content was quantified in the digital photographs using the ImageJ software (version 1.53). The images were first converted to 32-bit files, thus making them black and white. Next, a threshold was applied to the images

and manually adjusted to correspond only to the black-stained lipids droplets. The threshold allowed the software to quantify the selected black areas as a percentage of the total image area. The result per animal was the average of the 12 measurements. The relative area of the lipid droplets in all the randomly sampled fields estimates the relative volumes of the droplets in the animal's liver (Marcos et al., 2012): V_V (lipid droplets, liver parenchyma).

5. Liver mRNA expression

5.1. RNA extraction and cDNA synthesis

Liver RNA extraction was performed using the illustra™ RNAspin Mini Isolation Kit (GE Healthcare). A fragment of liver weighing between 14 and 20 mg was homogenized in 350 µL of lysis buffer and 3.5 µL of β-mercaptoethanol using a rotor-stator (Ultraturrax® IKA T10). Next, the homogenized tissue was submitted to distinct protocol steps according to manufacturer recommendations, as detailed in Appendix 3. An agarose gel at 1% stained with 1 µL of GelRed was made for all RNA samples. RNA samples were quantified by loading 2 µL of each sample into the µDrop microplate reader in the Multiskan Go equipment (Thermo Scientific). The quantification and the 260/280 nm and 260/230 nm ratios were obtained using the SkanIt software (version 4.1). The samples were considered pure if the ratios were between 1.8 and 2.2.

The cDNA synthesis from 1 µg of total RNA was done using the iScript cDNA synthesis kit (BioRad) for a total volume of 20 µL. The reaction took place in a Tgradient PCR thermal cycler (Biometra) and followed a three-step protocol: i) 5 minutes at 25 ºC; ii) 20 minutes at 46 ºC; iii) 1 minute at 95 ºC. By the end of the protocol, the samples were diluted with DNA/RNA-free water (1:5) and stored at -4 ºC.

5.2. Quantitative real-time polymerase chain reaction (qRT-PCR)

The qRT-PCR was done using a Real-Time PCR Detection System (CFX Connect, BioRad). Each qRT-PCR reaction had a total volume of 20 µL, which included 5 µL of cDNA sample diluted 1:5, 10 µL of either SsoFastTM EvaGreen Supermix or iQ SYBR Green and 300 nM of each primer plus 3.8 µL of nuclease-free water for the EvaGreen reactions genes or 200 nM of each primer plus 4.2 µL of water for the SYBR Green reactions. Each qRT-PCR plate (Appendix 4) included all samples in duplicate and a duplicate of no template controls (NTC) to help detect contaminations. In each run, a melting curve was generated

from 55 ºC to 95 ºC with 0.5 ºC increments every 30 seconds to ensure the specificity of the amplified products.

The Pfaffl method (Pfaffl, 2001) was used for relative quantification, evaluating the expression of target genes through the following expression ratio: Ratio = $(E_{target})^{\Delta CTtarget\ (control - sample)}$ / $(E_{ref})^{\Delta CTref\ (control - sample)}$, where E_{target} is the amplification efficiency of the target gene, E_{ref} is the amplification efficiency of the reference gene, ΔC_T target (control – sample) is the difference between the C_T of the target gene in the control, and the sample and ΔC_T ref (control – sample) is the difference of the C_T of the reference gene between control and sample. A multiple reference gene approach was used to reduce bias (Urbatzka et al., 2013). The geometric mean of two reference genes – rpl8 and gapdh – was used for gene normalization. The two reference genes were selected from four candidate genes (b-act – β-actin, ef-1α – elongation factor 1α, rpl8 and gapdh) according to the stability value of the several combinations (Pfaffl et al., 2004). All primer sequences, annealing temperatures and efficiencies are listed in Table 2.

Table 2. Gene list used for qRT-PCR, with primer sequences, annealing temperatures and amplification efficiencies.

Gene Name	Abbreviation	Primer Forward (5'-3')	Primer Reverse (5'-3')	Annealing Temperature (ºC)	Amplification Efficiency (%)	Reference
Acetyl-CoA carboxylase	ACC	TTTTGATGGCGATCTTGACA	CATCACAATGCCTCGCTCTA	60.0	102.2#	Caballero-Solares et al. (2018)
Acyl-CoA long chain synthetase 1	Acsl1	CGACCAAGCCGCTATCTC	CCAACAGCCTCCACATCC	55.0	97.8#	Madureira et al. (2018)
Acyl-CoA oxidase 1 3I	Acox1-3I	TGTAACAAGGAGCAGTTCG	TTGCCGTGGTTTCAAGCC	56.0	96.9*	Madureira et al. (2016)
Acyl-CoA oxidase 3	Acox3	GGGAAGACGGCTACACACG	CAACAATTACTCCTGGCATCGC	55.0	105.3*	Madureira et al. (2016)
Apolipoprotein AI	ApoAI	ATGAAATTCCTGGCTCTT	TACTCTTTGAACTCTGTGTC	55.0	89.9#	Madureira et al. (2017)
Estrogen receptor alpha	ERα	GACATGCTCCTGGCCACTGT	TGGCTTTGAGGCACACAAAC	61.6	91.2#	Körner et al. (2008)
Fatty acid binding protein 1	Fabp1	GTCCGTCACCAACTCCTTC	GCGTCTCAACCATCTCTCC	57.0	97.7#	Madureira et al. (2017)
Fatty acid synthase	FAS	ACCGCCAAGCTCAGTGTGC	CAGGCCCCAAAGGAGTAGC	60.0	95.1#	Minghetti et al. (2011)
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	CCACCTATGTAGTTGAGTC	ACCTTGAGGGAGTTATCG	55.0	92.8# or 100.6*	Madureira et al. (2017)
Hydroxy-3-methylglutaryl Co-A reductase	HMGCoAR	CCTTCAGCCATGAACTGGAT	TCCTGTCCACAGGCAATGTA	57.0	94.2#	Minghetti et al. (2011)
Lipoprotein lipase	LPL	TGCTGGTAGCGGAGAAAGACAT	CTGACCACCAGGAAGACACCAT	60.0	104.1#	Todorčević et al. (2008)
Peroxisome proliferator-activated receptor alpha	PPARα	CGGGTGACAGGGAGGTGGAGGAC	GGTGAGGATGGTGCGGGCTTTGG	59.0	100.6#	Madureira et al. (2015)
Peroxisome proliferator-activated receptor alpha Ba	PPARαBa	ATCCACTACTCCCACAGG	GTCTAAACCCAGCCAAATAC	55.0	106.7#	Madureira et al. (2017)
Peroxisome proliferator-activated receptor alpha Bb	PPARαBb	GAGTCTCCTGTCCTATCC	AGTTCTGCTGTTCTTTCAC	55.0	99.3#	Madureira et al. (2017)
Peroxisome proliferator-activated receptor gamma	PPARγ	CGGAATAAGTGCCAGTAC	GGGTCCACATCCATAAAC	56.0	98.1*	Lopes et al. (2016)
Ribosomal protein L8	Rpl8	TCAGCTGAGCTTTCTTGCCAC	AGGACTGAGCTGTTCATTGCG	59.0	93.8# or 99.0*	Körner et al. (2008)
Steroidogenic acute regulatory protein	StAR	AGGATGGATGGACCACTGAG	GTCTCCCATCTGCTCCATGT	63.0	104.5#	Vang et al. (2007)
Vitellogenin A	VtgA	AACGGTGCTGAATGTCCATAG	ATTGAGATCCTTGCTCTTGGTC	62.9	99.0#	Körner et al. (2008)

Amplification efficiencies determined with iQ SYBR Green Supermix.

* Amplification efficiencies determined with SsoFastTM EvaGreen Supermix.

6. Gonad histology

After fixation, the gonads were changed to ethanol at 70% until processing in an automated tissue processor following the same 8-step protocol described in *4.1. Osmium tetroxide post-fixation*. Gonads were embedded in paraffin, and the blocks were sectioned longitudinally at 5 µm. Two serial sections were taken from each animal and placed on silane-coated microscope slides (VWR) before being dried at 60 ºC for 1 hour. Sections were stained with hematoxylin (Hematoxylin 7211, Thermo Scientific) and eosin (Merck KGaA) (H&E) (Appendix 5) and mounted with DPX (Sigma-Aldrich).

The gonadal histology was qualitatively evaluated under an optical microscope (Olympus BX50) to infer the developmental stage of each gonad following the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (Johnson et al., 2009). This guide classified the development stages of testis and ovaries in the fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*) as model organisms (Table 3) and was used here since it is adaptable to evaluate the developmental stage of the juvenile brown trout gonads.

Table 3. Criteria of classification of each developmental stage of testes and ovaries for fathead minnow and zebrafish (Johnson et al., 2019).

	Criteria	
Developmental stage	**Ovaries**	**Testes**
Juvenile	Gonad exclusively composed of oogonia; sexing may be difficult.	Gonad exclusively composed of spermatogonia; sexing may be difficult.
Stage 0	Undeveloped gonad with only immature phases from oogonia to perinucleolar oocytes and no cortical alveoli.	Undeveloped gonad with only immature phases of spermatogonia to spermatids and absence of spermatozoa.
Stage 1	Early vitellogenic gonad where the vast majority are pre-vitellogenic follicles that range from perinucleolar to cortical alveolar.	Early spermatogenic gonad where immature phases are still predominant, but spermatozoa may become visible.
Stage 2	Mid-development gonad with at least half the observed follicles being early to mid vitellogenic.	Mid-spermatogenic gonad with approximately equal proportions of spermatocytes, spermatids and spermatozoa and a germinal epithelium thinner than in stage 1.
Stage 3	Late development gonad with the majority of follicles late vitellogenic.	Late spermatogenic gonad where the germinal epithelium is thinner than in stage 2 and mature sperm predominates.
Stage 4	Hydrated gonad with the majority of the follicles late vitellogenic to mature/spawning follicles and of bigger size when compared to stage 3.	Spent gonad composed mainly of loose connective tissue and remainders of sperm.
Stage 5	Post-ovulatory gonad with predominately spent follicles and thecal remains.	

7. Statistical analysis

Statistical analysis used the software PAST 4.3 (Hammer et al., 2001). One-way analysis of variance (ANOVA) was used to compare blood lipids, hepatic lipid quantification, and biometric parameters between the different exposure groups, followed by Tukey's pairwise post-hoc test. Data were first tested for normality with the Shapiro-Wilk test, and the homogeneity of variances was also verified with Levene's test for equal variances. For data that did not follow the assumption even after transformations (cholesterol, triglycerides, HDL, triglycerides/HDL and hepatic lipid quantification data), the non-parametric Kruskal-Wallis H (one-way ANOVA on ranks) test was used. If significant, the post-hoc test was the Mann-Whitney U, with sequential Bonferroni correction. The significance level was 0.05.

Chapter 3. Results

1. Biometric data

Fish biometric data from all experimental groups are summarized in Table 4. All biometric parameters were analyzed with a one-way ANOVA complemented by Tukey's pairwise post-hoc test. The mean for total weight varied from a maximum of 66.08 g in the EE2 group and a minimum of 42.74 g in the ATV group, but no significant differences were recorded between groups ($p > 0.05$). The minimum mean of the total length was also recorded in the ATV group at 16.68 cm, while the maximum average length was obtained in the EE2 group, with 18.38 cm, again with no significant differences between groups ($p > 0.05$). Mean K values varied between 1.04 (EE2 group) and 1.12 (C group) without significant differences ($p > 0.05$). Gonadal weight had its lowest mean value in the SC, ATV and MIX groups (0.12 g) and the highest in the C and EE2 groups (0.15 g), whilst mean GSI values varied between 0.20 and 0.24 in the SC and ATV groups, respectively, with neither the gonadal weight nor GSI varying significantly between groups.

The mean liver weight was the highest in the EE2 group (2.17 g) and lowest in the ATV group (0.70 g), with Tukey's post-hoc test revealing that the liver weight in the EE2 group was significantly larger when compared to all the other experimental groups ($p < 0.05$) (Table 4). HSI had its highest mean value in the EE2 group (3.30) and lowest in the SC group (1.39), and here the Tukey's test also disclosed significant differences between the EE2 group and all other experimental groups ($p < 0.05$) and between the MIX and all other experimental groups ($p < 0.05$) (Table 4).

Table 4. Biometric data from the juvenile brown trout across the five experimental groups (Control (C), Solvent Control (SC), 17α-Ethinylestradiol (EE2), Atorvastatin (ATV), Mixture (MIX).

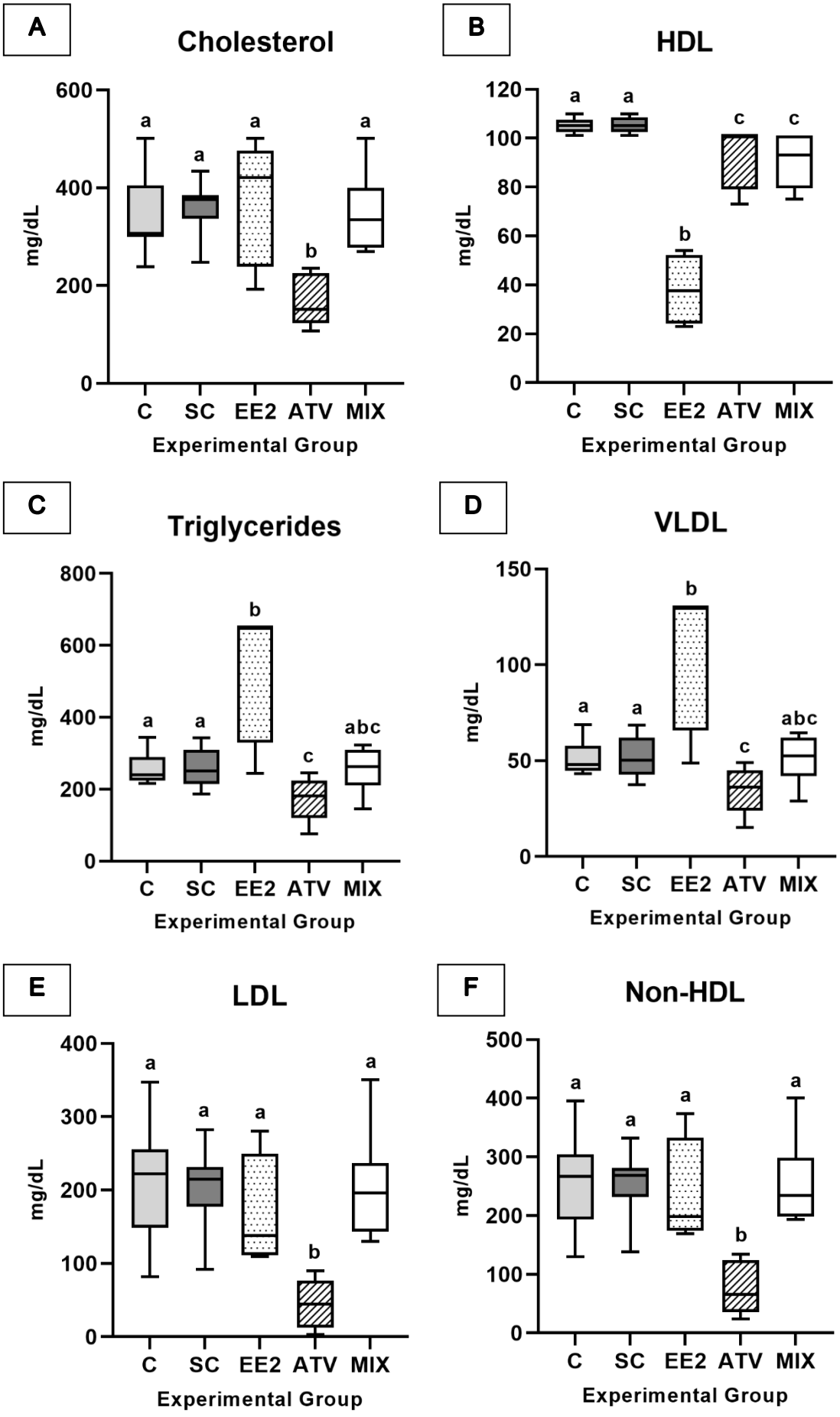
Experimental Group	Total Weight (g)	Total Length (cm)	Fulton's Condition Factor (K)	Liver Weight (g)	Hepatosomatic index (HSI) (%)	Gonad Weight (g)	Gonadosomatic index (GSI) (%)
C	65.56 (14.30) 41.31-83.33	17.92 (1.26) 15.3-19.5	1.12 (0.09) 0.98-1.29	0.93 (0.17)[a] 0.66-1.11	1.43 (0.09)**[a]** 1.28-1.60	0.15 (0.06) 0.06-0.26	0.23 (0.08) 0.07-0.33
SC	62.08 (15.06) 40.74-91.29	17.68 (1.36) 15.5-20.0	1.11 (0.11) 0.87-1.27	0.85 (0.18)[a] 0.53-1.06	1.39 (0.21)**[a]** 1.03-1.78	0.12 (0.03) 0.05-0.15	0.20 (0.07) 0.07-0.32
EE2	66.08 (18.78) 47.99-114.15	18.38 (1.33) 16.5-21.5	1.04 (0.12) 0.90-1.29	2.17 (0.58)[b] 1.47-3.54	3.30 (0.34)**[b]** 2.62-3.97	0.15 (0.04) 0.07-0.19	0.23 (0.06) 0.15-0.31
ATV	42.74 (9.28) 27.42-60.69	16.68 (1.05) 14.0-18.0	1.06 (0.07) 0.98-1.17	0.70 (0.17)[a] 0.34-0.99	1.42 (0.26)**[a]** 1.03-1.83	0.12 (0.03) 0.07-0.17	0.24 (0.06) 0.17-0.35
MIX	50.27 (10.42) 36.75-74.67	16.81 (1.23) 15.3-19.5	1.05 (0.06) 0.93-1.14	0.93 (0.25)[a] 0.61-1.44	1.85 (0.29)**[c]** 1.30-2.43	0.12 (0.06) 0.04-0.26	0.23 (0.08) 0.11-0.35

Data are expressed as mean and SD (standard deviation) with minimum-maximum values for each group; n = 9 animals / group. According to the Tukey test, lower-case letters represent significant differences ($p < 0.05$) among experimental groups.

2. Blood lipid contents

Blood lipid levels are summarized in Figure 13. Total cholesterol varied between 107 mg/dL (ATV) and 500 mg/dL (C, EE2 and MIX). A significant decrease in cholesterol levels was noted in the ATV group ($p < 0.004$) compared to all other groups (Figure 13 A). HDL levels ranged from a minimum of 23 mg/dL (EE2) up to over 100 mg/dL (C and SC), having significantly lower values in the EE2, ATV and MIX groups ($p < 0.05$) when compared to the controls, with EE2 significantly more decreased than in the ATV and MIX groups, that showed very similar variation (Figure 13 B). Triglycerides ranged from 76 mg/dL (ATV) to over 650 mg/dL (EE2) and significantly increased in the EE2 group ($p < 0.006$) compared to both controls and the ATV group. A significant decrease in triglycerides was observed in the ATV group ($p < 0.007$) when compared to both controls and the EE2 group (Figure 13 C). The VLDL concentrations varied between 15.2 mg/dL (ATV) and 130.2 mg/dL (EE2), and the differences between experimental groups followed the same pattern as the triglycerides (Figure 13 D). LDL levels varied between 3 mg/dL (ATV) to 350 mg/dL (EE2) and evidenced a significant decrease in the ATV group ($p < 0.03$) when compared to the other groups (Figure 13 E). The non-HDL cholesterol showed the same pattern as the LDL, with the ATV group demonstrating a significant reduction ($p < 0.004$) in comparison to the rest of the groups (Figure 13 F).

The cholesterol/HDL ratio showed a similar pattern to that observed for the triglycerides and VLDL, with a significant increase in the EE2 group ($p < 0.001$) and a significant decrease in the ATV group ($p < 0.001$) when compared to C, SC and MIX groups (Figure 13 G). The LDL/HDL ratio also had a significant increase in the EE2 group ($p < 0.001$) and a decrease in the ATV group ($p < 0.001$) when compared to both controls and the MIX (Figure 13 H). Conversely, the HDL/LDL ratio showed the opposite pattern to the LDL/HDL (Figure 13 I). Lastly, for the triglycerides/HDL ratio a significant reduction in the MIX group ($p < 0.003$) compared to the other groups (Figure 13 J).


A
Cholesterol
mg/dL
600
400
200
0
a
a
a
b
a
C
SC
EE2
ATV
MIX
Experimental Group
B
HDL
mg/dL
120
100
80
60
40
20
0
a
a
b
c
c
C
SC
EE2
ATV
MIX
Experimental Group
C
Triglycerides
mg/dL
800
600
400
200
0
a
a
b
c
abc
C
SC
EE2
ATV
MIX
Experimental Group
D
VLDL
mg/dL
150
100
50
0
a
a
b
c
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C
SC
EE2
ATV
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Experimental Group
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a
a
b
a
C
SC
EE2
ATV
MIX
Experimental Group
F
Non-HDL
mg/dL
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EE2
ATV
MIX
Experimental Group

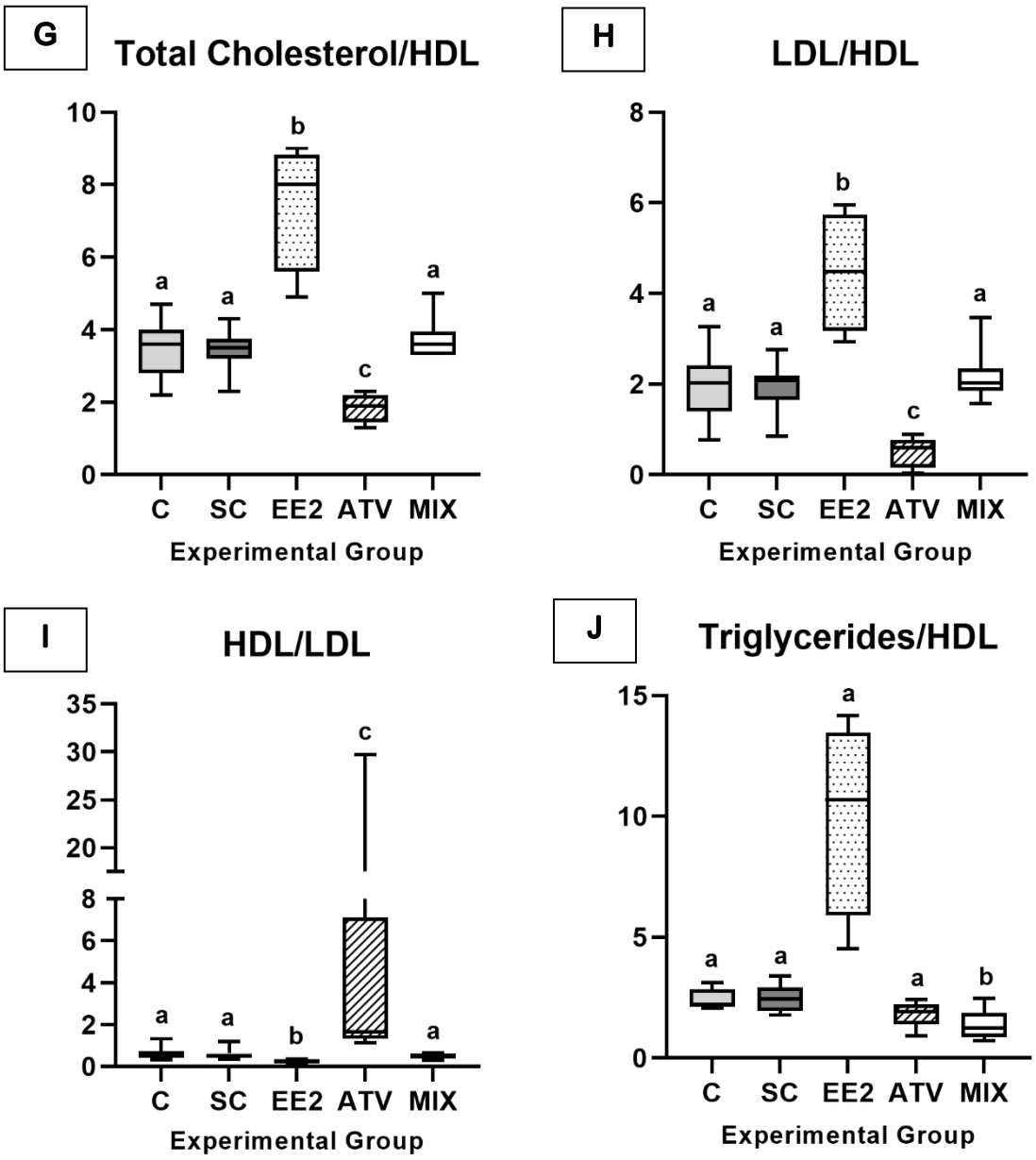


Figure 13. Blood lipid content from all five experimental groups (C – Control, SC – Solvent Control, EE2 – 17α-Ethinylestradiol, ATV – Atorvastatin, and MIX – Mixture). (A). Cholesterol; (B). High-density lipoproteins (HDL); (C). Triglycerides; (D). Very low-density lipoproteins (VLDL); (E). Low-density lipoproteins (LDL); (F). Non-HDL cholesterol; (G). Total cholesterol/HDL ratio; (H). LDL/HDL ratio; (I). HDL/LDL ratio; (J). Triglycerides/HDL ratio. Data are expressed as the minimum, first quartile, median, third quartile and maximum for each parameter. Lowercase letters indicate significant differences ($p < 0.05$) between groups.

3. Hepatic lipid content

The results obtained after hepatic lipid quantification are shown in Table 5. The relative volume of the lipid droplets in the C and SC groups varied between 0.004% to 0.007% and 0.003% to 0.042%, respectively (Table 4). In the EE2 group, the lipid levels ranged from 0.383% up to 17.640%, showing a significant increase ($p < 0.005$) in comparison to both controls, ATV and MIX groups (Table 5). In the ATV group, lipid content varied from a minimum of 0.004% and a maximum of 8.610%, whilst in the MIX group, lipid levels ranged from 0.010% to 1.729%. Both ATV and MIX groups significantly differ from both the controls and EE2 groups ($p < 0.01$) (Table 5).

Table 5. Minimum, maximum, and median (%) for the V_V (lipid droplets, liver parenchyma).

Group	Minimum	Maximum	Median
C	0.004	0.007	0.006[a]
SC	0.003	0.042	0.007[a]
EE2	0.383	17.640	1.362[b]
ATV	0.004	8.610	0.018[c]
MIX	0.010	1.729	0.038[c]

Data from all five experimental groups (C – Control, SC – Solvent Control, EE2 – 17α-Ethinylestradiol, ATV – Atorvastatin, and MIX – Mixture) with n = 9 fish per group. According to the Mann-Whitney test, lowercase letters represent significant differences ($p < 0.05$) among experimental groups.

The qualitative analyses of the distinct histological sections illustrate the results obtained for quantifying lipids in the different groups. The C and SC sections were almost identical, with nearly no droplets detectable, and those that could be seen had a very sparse distribution and size (Figure 14). In the EE2 group, the lipid droplets were the largest observed in any experimental group. These lipid droplets were also extremely abundant and had a homogeneous distribution across the tissue samples (Figure 15 A). An apparent reduction in lipid droplet size and abundance was detected in the ATV group compared to the EE2, although the droplets were still more prominent and abundant than in the controls (Figure 15 B). The lipid droplets distribution in the ATV group was relatively sparse, arranged into small clusters, with larger areas of tissue with little to no lipidic deposition. In the MIX group, the abundance and distribution of lipid droplets were between those of the EE2 and ATV groups, with a size closer to that of the EE2 group but an abundance and distribution similar to those of the ATV group (Figure 16).

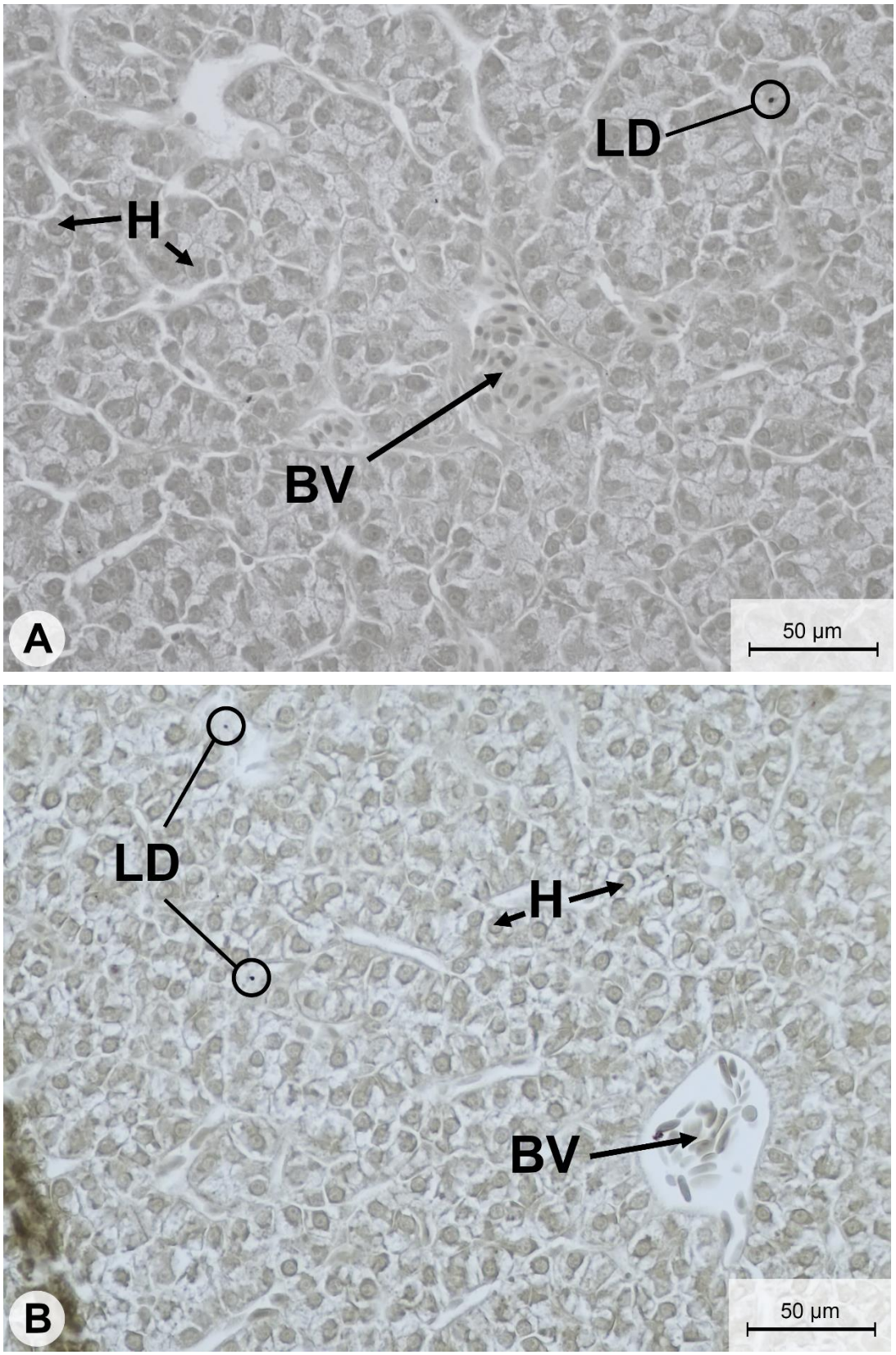


Figure 14. Histological sections of juvenile brown trout osmicated liver from the Control (A) and Solvent Control (B) groups. The area is filled with hepatocytes (H) with roundish nuclei, blood vessels (vein; BV), and circles showing lipid droplets (LD).

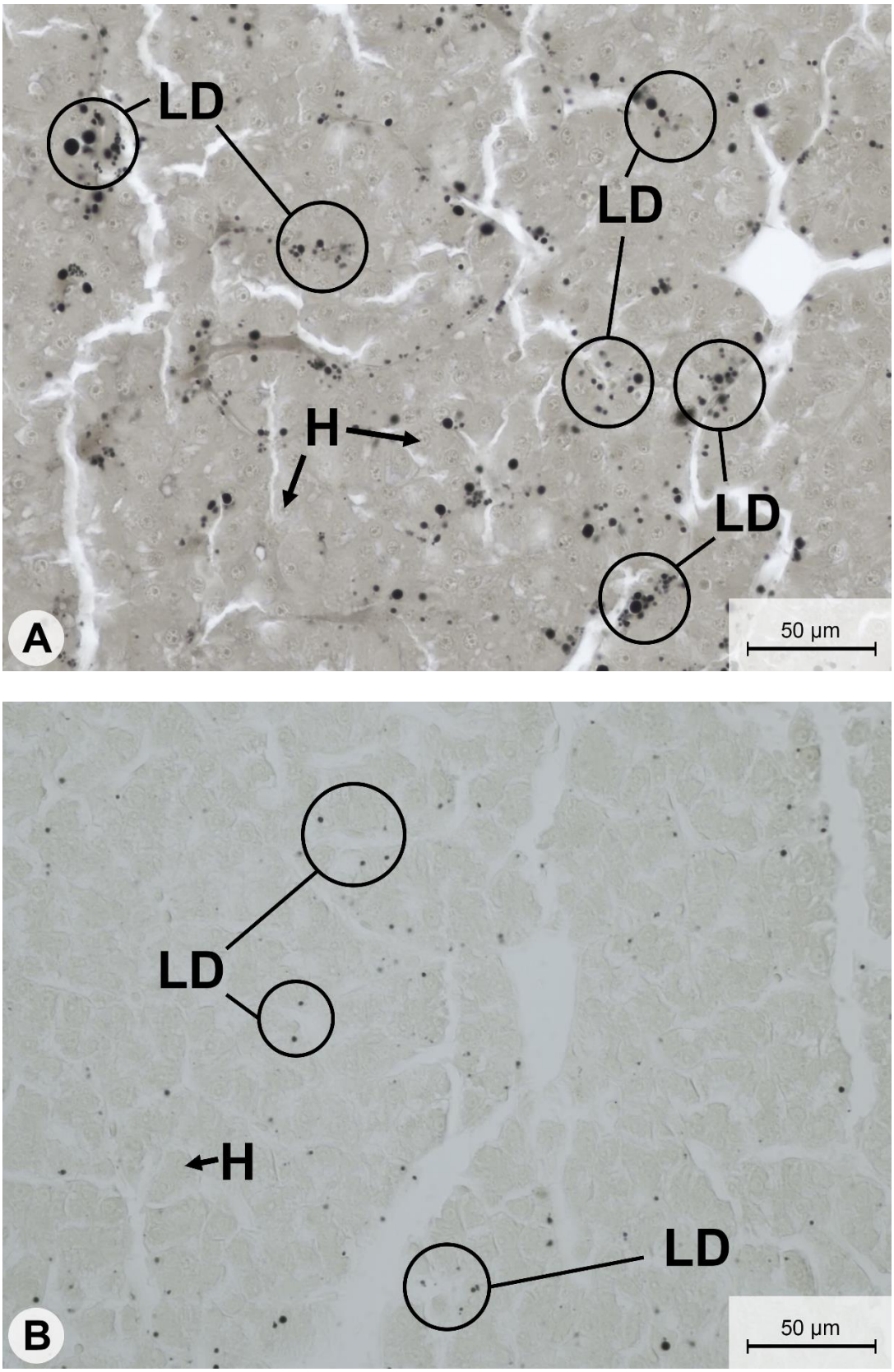


Figure 15. (A) Histological section of juvenile brown trout osmicated liver from the 17α-Ethinylestradiol group with hepatocytes (H) and multiple lipid droplets (LD) spread across the whole section. (B) Histological section of osmicated liver from the Atorvastatin groups showing hepatocytes (H) with scarcer and smaller lipid droplets (LD).

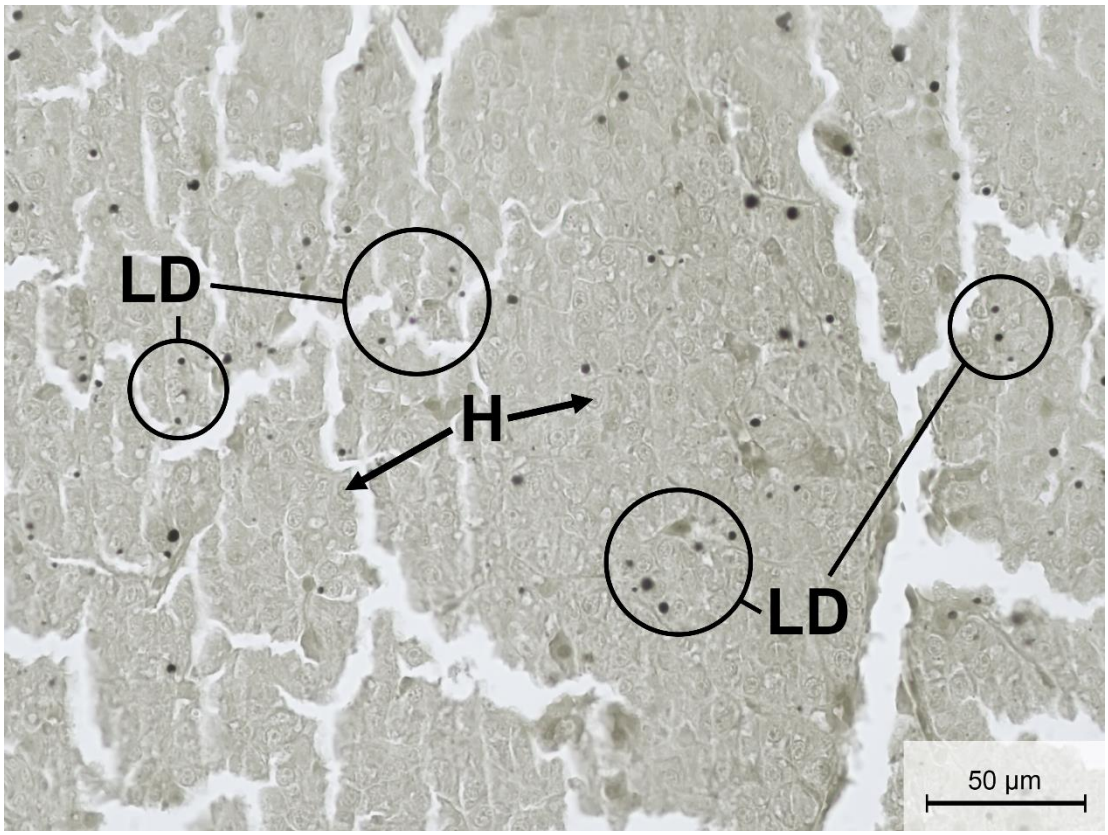


Figure 16. Histological sections of juvenile brown trout osmicated liver from the Mixture group. The area contains hepatocytes (H) with frequent lipid droplets (LD).

4. Liver mRNA expression

ERα and VtgA mRNA levels were up-regulated in the EE2 group, over 10-fold for ERα (Figure 17 A) and 35,000-fold for VtgA (Figure 17 B) compared to the control groups. The MIX group also revealed a significant increase in mRNA levels of both genes compared to controls and ATV groups. The exposure to ATV did not cause significant changes in the expression of both estrogenic targets.

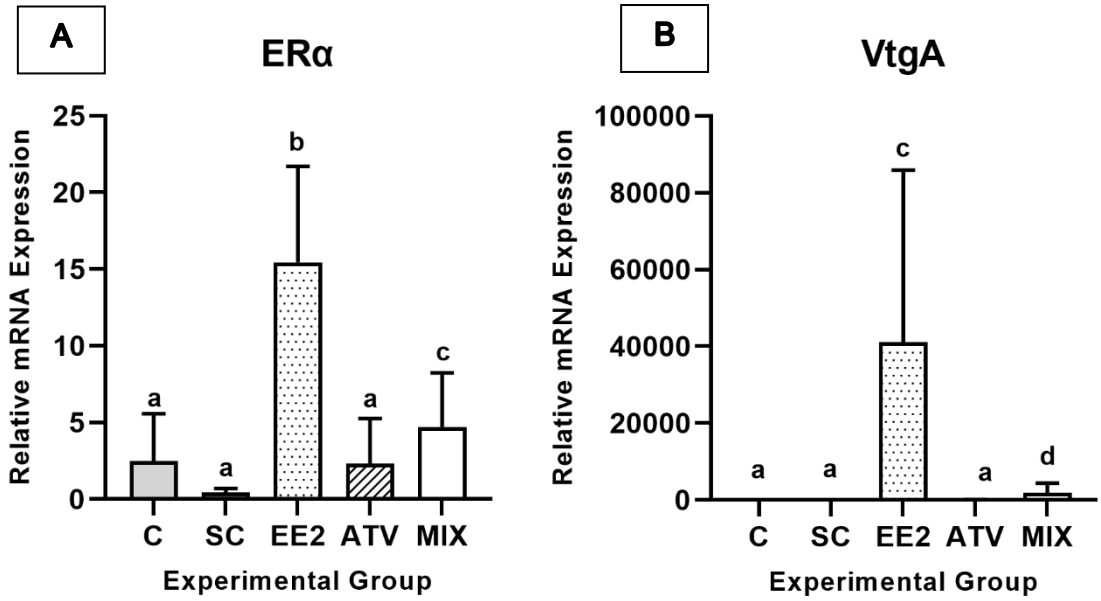
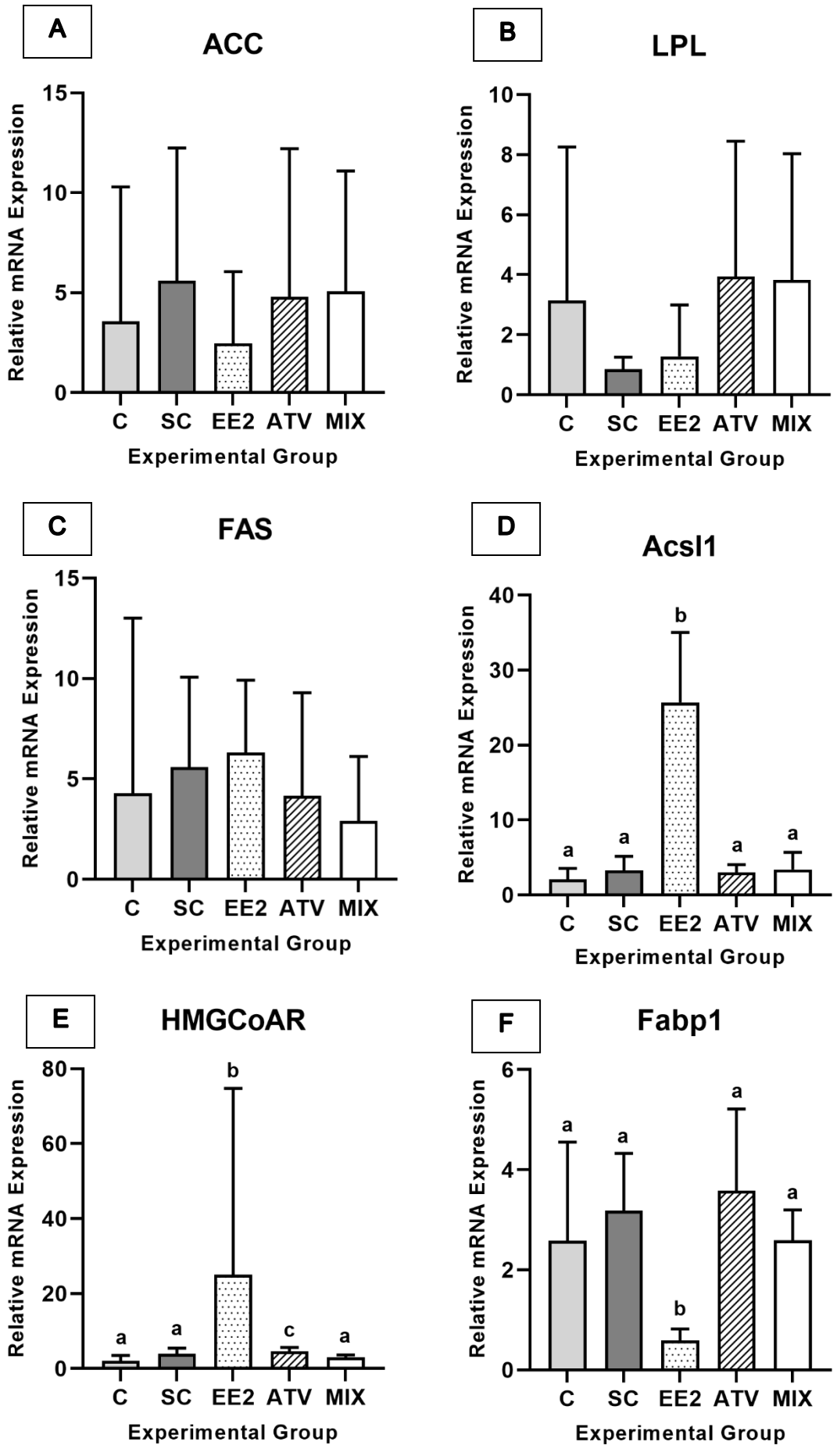


Figure 17. Relative mRNA expression of the estrogen receptor alpha (Erα) (A) and vitellogenin A (VtgA) (B) genes across all experimental groups (C – Control, SC – Solvent Control, EE2 - 17α-Ethinylestradiol, ATV – Atorvastatin, MIX – Mixture). Data are expressed as mean and standard deviation. Lowercase letters indicate significant differences ($p < 0.05$) between groups.

The mRNA levels of ACC, LPL and FAS did not change in response to treatments (Figure 18 A, B and C). On the contrary, Acsl1 and HMGCoAR mRNA expression significantly increased in the EE2 group compared to the other groups (Figure 18 D and E). Further, the HMGCoAR mRNA levels were also significantly up-regulated by ATV compared with both controls and MIX groups. Fabp1 mRNA levels were significantly down-regulated by EE2 (Figure 18 F). EE2 and MIX groups also caused a significant decrease in ApoAI mRNA levels compared to controls, while an increase was noted after exposure to ATV (Figure 18 G). As to the StAR mRNA, no significant differences between groups were observed (Figure 18 H).


A
ACC
Relative mRNA Expression
15
10
5
0
C SC EE2 ATV MIX
Experimental Group
B
LPL
Relative mRNA Expression
10
8
6
4
2
0
C SC EE2 ATV MIX
Experimental Group
C
FAS
Relative mRNA Expression
15
10
5
0
C SC EE2 ATV MIX
Experimental Group
D
Acsl1
Relative mRNA Expression
40
30
20
10
0
a a b a a
C SC EE2 ATV MIX
Experimental Group
E
HMGCoAR
Relative mRNA Expression
80
60
40
20
0
a a b c a
C SC EE2 ATV MIX
Experimental Group
F
Fabp1
Relative mRNA Expression
6
4
2
0
a a b a a
C SC EE2 ATV MIX
Experimental Group

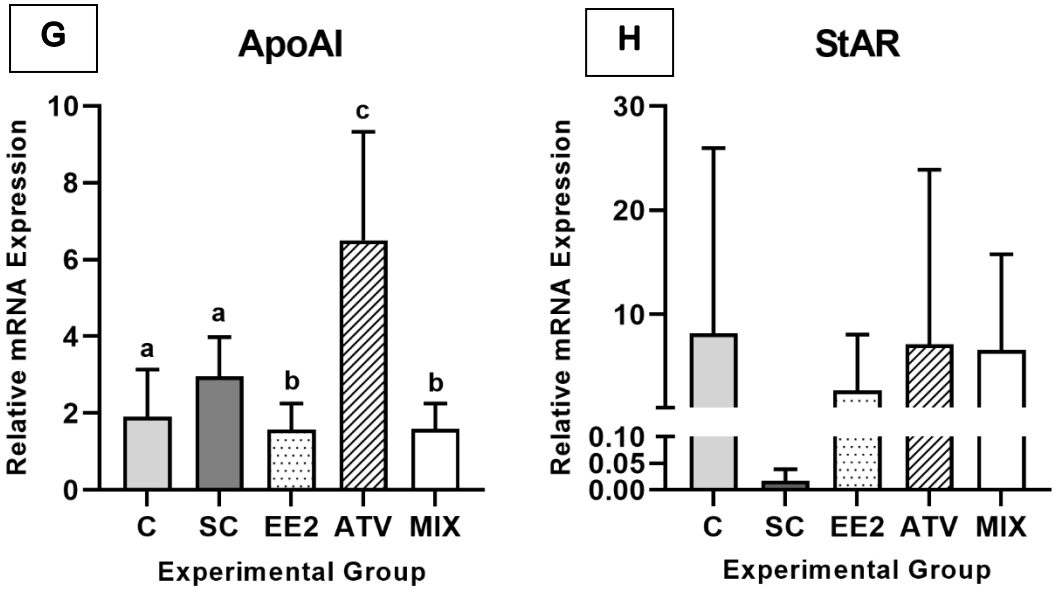


Figure 18. Relative mRNA expression of the acetyl-CoA carboxylase (ACC) (A), lipoprotein lipase (LPL) (B), Acyl-CoA long chain synthetase 1 (Acsl1) (C), fatty acid synthase (FAS) (D), hydroxy-3-methylglutaryl Co-A reductase (HMGCoAR) (E), fatty acid binding protein 1 (Fabp1) (F), apolipoprotein AI (ApoAI) (G) and steroidogenic acute regulatory protein (StAR) (H) genes across all experimental groups (C – Control, SC – Solvent Control, EE2 - 17α-Ethinylestradiol, ATV – Atorvastatin, MIX – Mixture). Data expressed as mean and standard deviation. Lowercase letters indicate significant differences ($p < 0.05$) between groups.

The Acox1-3I and Acox3 mRNA levels were significantly down-regulated by EE2 and ATV, and EE2, ATV and MIX, respectively. Acox1-3I mRNA levels had no significant differences between the MIX and ATV groups (Figure 19 A), whereas Acox 3 mRNA showed a further decrease in MIX group when compared to EE2 and ATV (Figure 19 B). As for PPARα mRNA levels, no significant differences were observed between groups (Figure 19 C). PPARαBa and PPARαBb had similar behaviour to PPARα (Figure 19 D and E). Lastly, PPARγ mRNA levels were significantly down-regulated by ATV and MIX in comparison to C, SC and EE2 groups (Figure 19 F).

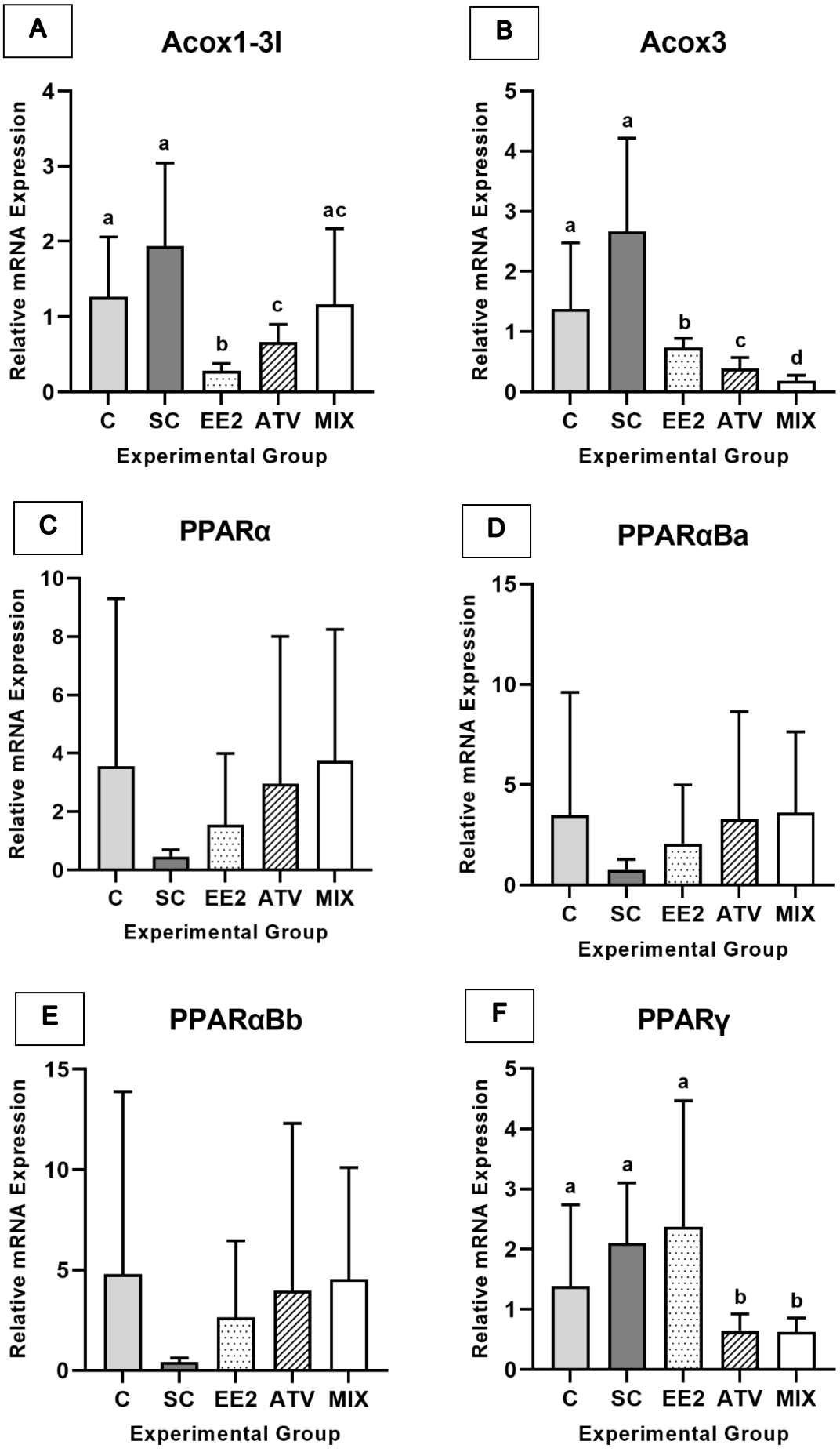

A
Acox1-3l
Relative mRNA Expression
0
1
2
3
4
a
a
b
c
ac
C
SC
EE2
ATV
MIX
Experimental Group
B
Acox3
Relative mRNA Expression
0
1
2
3
4
5
a
a
b
c
d
C
SC
EE2
ATV
MIX
Experimental Group
C
PPARα
Relative mRNA Expression
0
2
4
6
8
10
C
SC
EE2
ATV
MIX
Experimental Group
D
PPARαBa
Relative mRNA Expression
0
5
10
15
C
SC
EE2
ATV
MIX
Experimental Group
E
PPARαBb
Relative mRNA Expression
0
5
10
15
C
SC
EE2
ATV
MIX
Experimental Group
F
PPARγ
Relative mRNA Expression
0
1
2
3
4
5
a
a
a
b
b
C
SC
EE2
ATV
MIX
Experimental Group

Figure 19. Relative mRNA expression of the acyl-CoA oxidase 1 3I (Acox1-3I) (A), acyl-CoA oxidase 3 (Acox3) (B), peroxisome proliferator-activated receptor alpha (PPARα) (C), peroxisome proliferator-activated receptor alpha Ba (PPARαBa) (D), peroxisome proliferator-activated receptor alpha Bb (PPARαBb) (E) and peroxisome proliferator-activated receptor gamma (PPARγ) (F) genes across all experimental groups (C – Control, SC – Solvent Control, EE2 - 17α-Ethinylestradiol, ATV – Atorvastatin, MIX – Mixture). Data expressed as mean and standard deviation. Lowercase letters indicate significant differences ($p < 0.05$).

5. Gonad maturity

Only stage 0 and stage 1 of gonadal development were observed in both males and females. No significant differences in the maturation stages were observed between the experimental groups, as shown in Table 6.

Table 6. Sex and grading of gonad maturity of every animal from each experimental group.

Group		Animals								
C	Sex	M	F	F	M	M	F	F	M	F
	Grading	0	0	1	0	0	0	0	0	0
SC	Sex	M	M	F	F	M	M	M	M	F
	Grading	0	0	0	0	0	0	0	0	0
EE2	Sex	M	F	F	M	F	M	M	F	M
	Grading	0	0	0	0	1	0	0	0	0
ATV	Sex	F	M	M	F	F	M	F	F	F
	Grading	0	0	0	0	0	0	0	0	0
MIX	Sex	F	F	M	M	F	F	F	F	M
	Grading	0	1	0	0	0	0	0	0	0

Gonadal maturation stages in the five experimental groups (C – Control, SC – Solvent Control, EE2 - 17α-Ethinylestradiol, ATV – Atorvastatin, MIX – Mixture); n = 9 animals / group. Sex presented as M – Male and F – Female. Grading presented as undeveloped (stage 0) – 0 and early vitellogenic/spermatogenic (stage 1) – 1.

The sex of each individual was confirmed by histological analysis accounting for a total of 21 males and 24 females. The histological grading showed that all males were graded as undeveloped (stage 0), with the testes being almost filled with spermatogonia, although even spermatids could occasionally appear (Figure 20). As for females, 87.5% were graded as undeveloped (stage 0) when only perinucleolar were visible despite showing a high prevalence (Figure 21 A), and 12.5% were graded as early vitellogenic (stage 1) when they showed cortical alveolar oocytes (Figure 21 B).

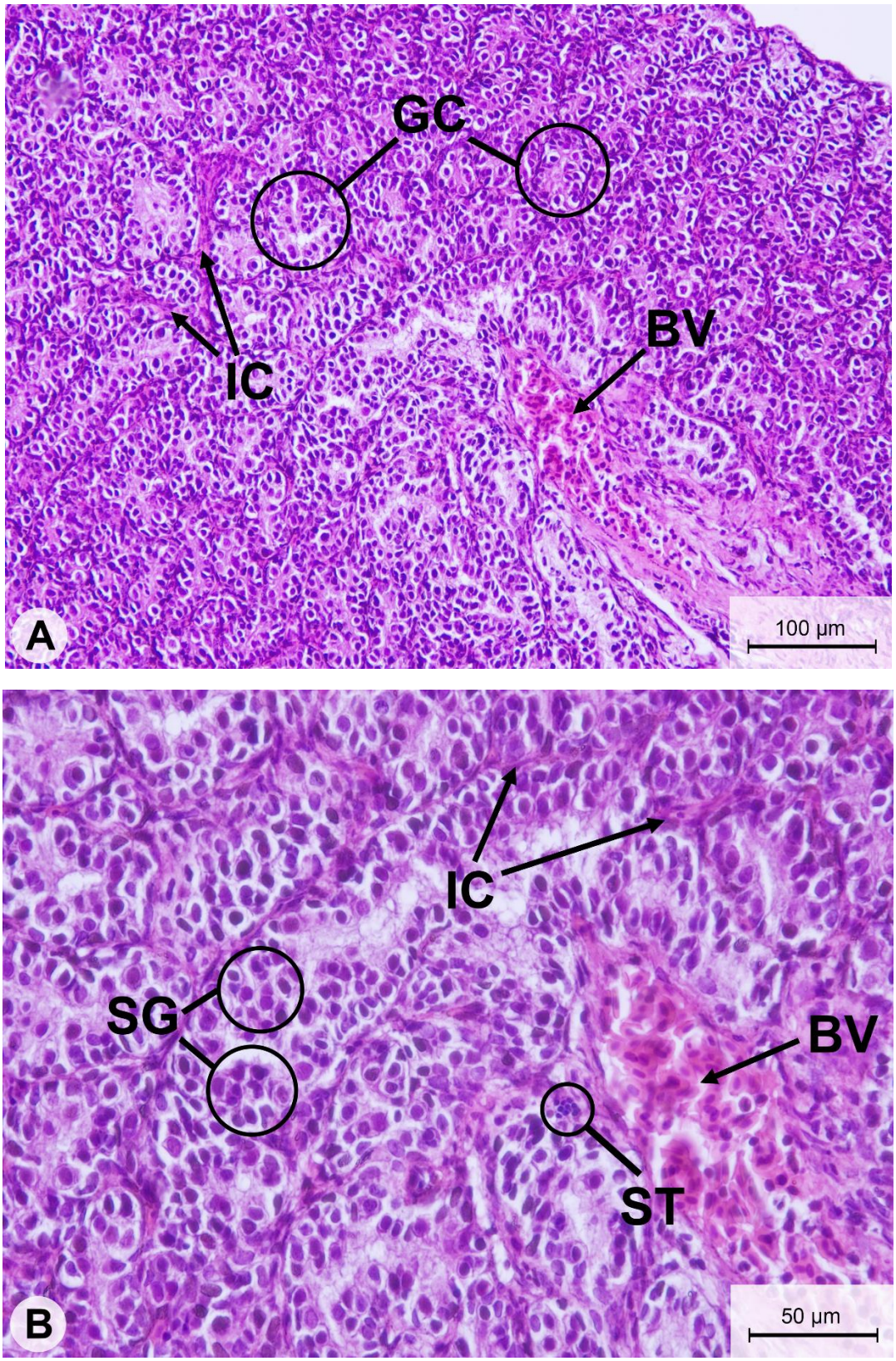


Figure 20. Testes of juvenile brown trout graded as undeveloped. (A) General view at x20 magnification. (B) Detail of central zone of image (A) at x40 magnification. The seminiferous tubules of the germinal compartment (GC) are compact, i.e., with no visible luminal space and filled with germinal cells, mostly type A and B spermatogonia (SG). Foci of other germinal cells, such as spermatids (ST), could be spotted. BV – blood vessel (vein); IC – Interstitial compartment.

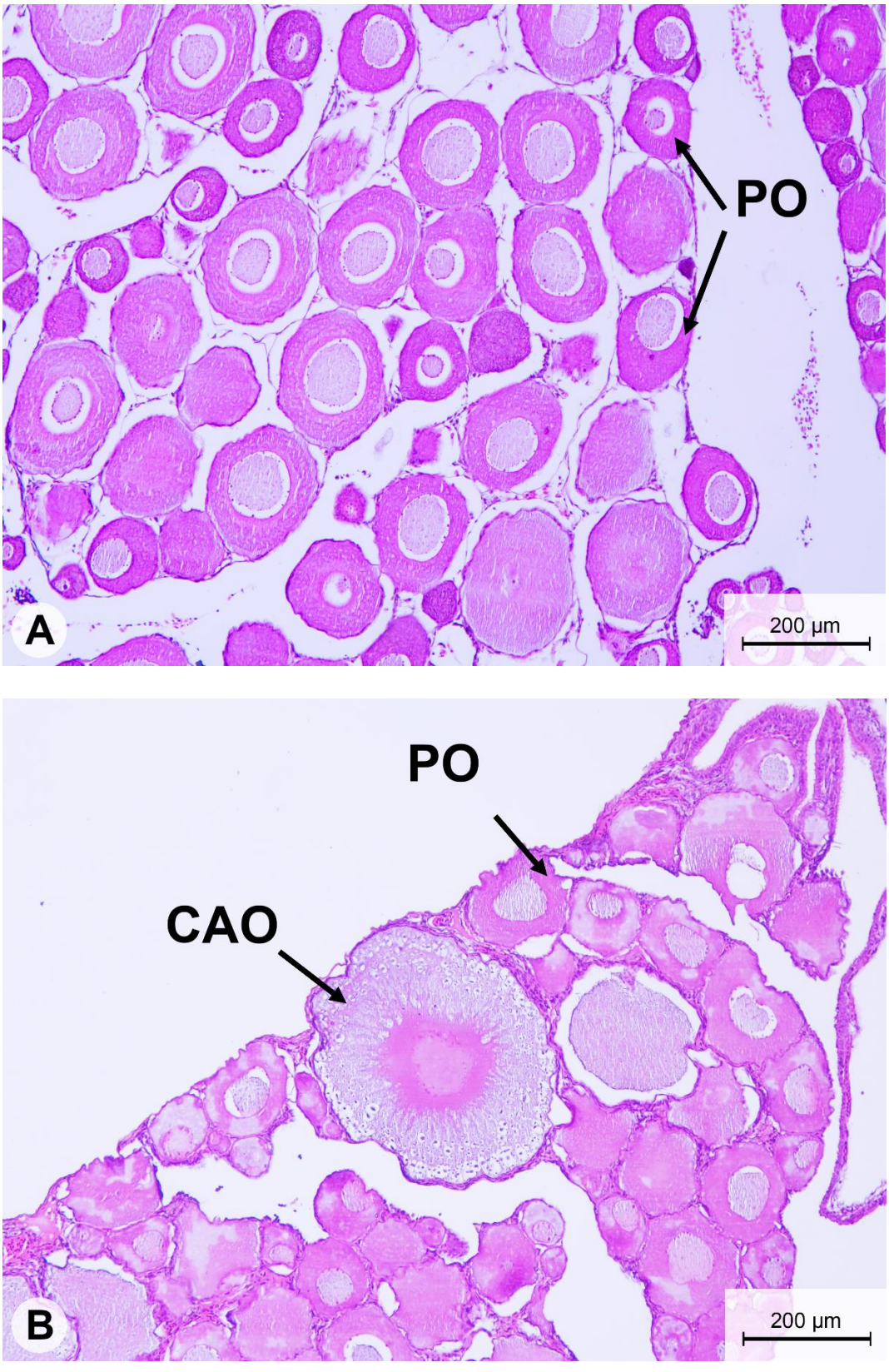


Figure 21. (A) Ovary of juvenile brown trout graded as undeveloped (stage 0) at x10 magnification. Section comprising mainly of perinucleolar oocytes (PO). (B) Ovary of juvenile brown trout graded as early vitellogenic (stage 1); the illustrative section is filled with perinucleolar oocytes and has one cortical alveolar oocyte (CAO).

Chapter 4. Discussion and Conclusions

Despite the emergence of hypolipidemic chemicals in water matrices worldwide (Falfushynska et al., 2019; Tete et al., 2020), their effects and consequences for fish and other aquatic animals remain misunderstood, as well as the way they interact with other absorbed environmental pollutants. Salmonids have immense socio-economical and environmental importance (see 1. Introduction). However, the impacts that statins have on the lipid metabolism of these fish were seldom studied, with only two published studies to our knowledge, where female rainbow trout (*Oncorhynchus mykiss*) were exposed for up to 24 hours to cerivastatin at 1.4 and 11.2 ng/g via intraperitoneal injection (Estey et al., 2008) and an in vitro work where primary rainbow trout hepatocytes were exposed to atorvastatin at 45 ng/mL for 3 to 6 hours (Al-Habsi et al., 2018). Given the massive worldwide use of atorvastatin (Falfushynska et al., 2019; Tete et al., 2020), understanding its impacts on salmonids and how they might be translated to other organisms is paramount. In this line, Falfushynska et al. (2019) proposed that due to the evolutionary conservation of HMGCoAR, the effects of atorvastatin in fish might be similar to those in humans. This fact opens the chance to explore salmonids as experimental models to test chemicals with the potential to modulate HMGCoAR.

Synthetic estrogenic hormones, particularly EE2, are environmental pollutants with quite a considerable occurrence and prevalence in water matrices (Garriz et al., 2017; Czarny et al., 2017), and their disruptive impacts on salmonids and other fish are well documented (Körner et al., 2008; Madureira et al., 2018). The choice of EE2 as a positive estrogenic control in this study is justified by the fact that many of their toxicological effects are well characterized, and their cross-talk influences over lipid metabolism pathways in fish have also been fairly well documented (Madureira et al., 2015; Madureira et al., 2018). Furthermore, the combined effects of statins and estrogens on fish are poorly studied, giving the use of EE2 in combination with ATV further relevance.

1. Blood lipid contents

The blood lipid profiles in the C and SC groups aligned with those recorded for the same species (Madureira et al., 2018) and from Caspian brown trout (Kenari et al., 2011). Exposure to ATV at 0.53 µg/g caused a reduction in blood levels of total cholesterol, which was consistent with its effects on adult zebrafish (*Danio rerio*) after a 30-day in vivo food exposure (Al-Habsi et al., 2016). Fish from the ATV group also had decreased levels of blood lipoproteins, namely HDL, LDL and VLDL. ATV is expected to trigger an increased abundance of LDL receptors (LDLRs) in the liver (Al-Habsi et al., 2018), leading to liver

uptake of LDL from the blood and lowering its plasmatic concentration. Intraperitoneal injection of juvenile female rainbow trout (*Oncorhynchus mykiss*) with gemfibrozil, a hypolipidemic fibrate, at 100 mg/kg for 15 days also caused a decrease in the blood levels of HDL, LDL and VLDL (Prindiville et al., 2011), which, according to the authors, can negatively impact energy obtention from lipids to vital functions such as swimming and be highly disruptive in migratory fish – such as salmonids. In the present study, triglyceride concentration in the blood also decreased due to the effects of ATV, as also reported in zebrafish (*Danio rerio*) exposed to the same compound via food intake at 0.53 µg/g for 30 days (Al-Habsi et al., 2016). This reduction in triglyceride levels might be related to the increase of LPL expression that ATV may induce in some cells, as previously suggested in vitro when L6 skeletal muscle cells were exposed to 0.1 to 10 µmol/L for 24 or 48 hours (Ohira et al., 2012), which should lead to triglyceride uptake.

In contrast to the ATV effects, the juvenile brown trout (*Salmo trutta*) exposed to EE2 displayed higher circulating levels of triglycerides and VLDL, in line with prior in vivo water exposure of juveniles of the same species to EE2 at 50 µg/L (Madureira et al., 2018) and 7 days in vivo exposure of hybrid tilapia to E2 at 10 to 50 mg/kg via injection (Zhang et al., 2019). EE2 promotes triglyceride transport to the piscine liver to be used in the process of vitellogenesis (Reading et al., 2018), and whilst in the bloodstream, triglycerides are mainly transported by VLDLs (Wallaert and Babin, 1992), thus offering a possible explanation for the observed effects. Furthermore, EE2 caused a pronounced decrease in HDL blood levels, as seen in experiments with rats subjected to a single dose of orally administered EE2 at 330 µg/g (Yamaguchi et al., 2016). This HDL decrease caused by EE2 was even greater than that caused by ATV.

Interestingly, the animals from the MIX group were very closely related to the ones from the controls regarding their lipidic blood content. The MIX group only differed from the controls in the TGL/HDL ratio and for the HDL levels, which decreased in a very similar pattern to that observed for ATV, but not as pronounced as for EE2. The lack of major alterations in the MIX group suggests that EE2 and ATV can act antagonistically.

2. Hepatic lipid deposition and biometric parameters

Both control groups had tiny and scarce lipid droplets after the staining with osmium tetroxide, which agrees with the lipid droplet descriptions in juvenile Atlantic salmon (*Salmo salar*) hepatocytes (Robertson and Bradley, 1992). In contrast, the livers of EE2-exposed brown trout (*Salmo trutta*) here had the highest amount of lipid droplet deposition, like what occurred in adult hybrid tilapia injected with E2 (Zhang et al., 2019) and after waterborne

exposure of juvenile brown trout (*Salmo trutta*) to EE2 at 50 µg/L (Madureira et al., 2018). However, wild adult female Ohrid trout (*Salmo letnica*) have been shown to have seasonal changes in the lipid content within the hepatocytes, with these having increased levels in pre-vitellogenic stages (Jordanova et al., 2016), a time when endogenous steroids are low, but following the spawning and pos-spawning peaks of E2 (Jordanova et al., 2018).

ATV exposure also caused an increase in lipid droplets in the hepatocytes, but not as severe as the one caused by estrogenic inputs. The mechanism of lipid uptake into the liver from the bloodstream should be a consequence of ATV treatment which induces LDLR expression, as it has been demonstrated in zebrafish (*Danio rerio*) exposed in vivo to food containing 0.53 µg/g of ATV (Al-Habsi et al., 2016) and in vitro with primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 45 ng/mL of ATV.

As for the MIX group, the amount of lipidic deposition in the liver was significantly lower than that of the EE2 group but still greater than that observed in the ATV-treated fish. As for the blood lipid contents, ATV seems able to antagonize somewhat the effects of EE2 also in which concerns the load of lipid droplets in hepatocytes.

In parallel with larger amounts of lipid deposition, the livers of EE2 and MIX-treated fish displayed heavier weights than any other experimental group, as well as a paler colour and soft consistency. The animals in these two groups also showed higher HSI. These results were previously observed in juvenile brown trout after in vivo waterborne exposure to EE2 (Madureira et al., 2018), but the similar effects registered in the MIX group suggest that EE2 more strongly modulates the effects on liver biometry than ATV. Moreover, regarding HSI, ATV once more acted in a way that reduced the effect of EE2. No other biometric parameters showed alterations caused by EE2, ATV or their combination, at least at this of the brown trout life cycle stage.

3. Liver mRNA expression

3.1. Estrogenic targets

The estrogenic targets VtgA and ERα were included in the gene portfolio as positive controls of EE2 exposure, as it is well described in the literature that estrogens induce the expression of VtgA and ERα in fish, including in brown trout, both in vitro and in vivo (Madureira et al., 2015; Madureira et al., 2018). In addition, the known cross-talk mechanisms that regulate lipid metabolism and estrogenic signalling pathways also justify the study of possible interactions between EE2 and ATV at the molecular level.

Exposure of juvenile brown trout to EE2 caused an increase of VtgA mRNA expression in the liver, as well as a significant up-regulation of ERα mRNA. Both estrogenic effects were previously demonstrated in brown trout in vitro and in vivo waterborne EE2 exposures (Körner et al., 2008; Madureira et al., 2015; Madureira et al., 2018). The study of the effects of hypolipidemic compounds in the expression of VtgA and ERα in fish liver remains largely unexplored, and data is mainly focused on fibrate effects (Mezzelani and Regoli, 2022). For instance, clofibrate did not affect VtgA expression in juvenile brown trout after in vivo exposure (Madureira et al., 2018), which was also the case with ATV exposure in the present study. As for ERα, we found no data for fish exposed to statins. However, exposure of ovariectomized mice to simvastatin at 5 to 20 mg/kg through food had contradictory outcomes, either causing an up-regulation (Menze et al., 2021) or a down-regulation of ERα expression as a consequence (Li et al., 2011). However, here ATV did not show estrogenic or antiestrogenic activity. Despite that, when in combination with EE2, ATV did demonstrate a potential antiestrogenic effect on the expression of both ERα and VtgA because significant increases in the expression of both genes were noted in the MIX group, but less intense than in the EE2 group. The mechanism by which this antiestrogenic action took place is yet to be understood. In the same vein as the current data, Sovadinová et al. (2014) reported antiestrogenic effects from the combination of clofibric acid and E2 at $2x10^{-4}$ g/L and $2.7x10^{-7}$ g/L, respectively, on the expression of VtgA in rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes, proposing that fibrates may either cause disruption in the stability of VtgA mRNA or stimulate other transcription factors, such as PPARα, which will connect to estrogen-responsive elements (EREs) and diminish the estrogenic response. However, AVT differs from clofibric acid in what respects the mechanisms of action, at least concerning the hypolipidemic effects. A mechanism may pass by interference in lipid bioavailability needed for building VtgA, a lipophosphoprotein.

3.2. Lipid metabolism genes

ACC, the enzyme responsible for kickstarting lipogenesis, did not show significant alterations in its hepatic expression in any of the experimental groups. Previous reports of ACC response to estrogens in rainbow trout (*Oncorhynchus mykiss*) are not consensual, with a one-month in vivo diet exposure to E2 at 30 mg/kg causing up-regulation (Cleveland and Weber, 2016) and intraperitoneal injection of the same estrogen at 5 µg/g causing down-regulation after 24 hours (Cleveland and Manor, 2015). The in vivo diet exposure of mice to ATV at 0.1% w/w for 3 days and at 30 mg/kg during 8 weeks caused an up-regulation of ACC expression (Roglans et al., 2002; Ji et al., 2011), suggesting that statins

have a different effect over ACC depending on the context and eventually species. The next step of lipogenesis is catalyzed by FAS, which was not significantly altered by EE2 in this study, which is in agreement with E2 waterborne and dietary exposure at various concentrations of rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*) showed no alterations in FAS liver expression (Cleveland and Weber, 2016; Zhang et al., 2020). In our study, ATV did not affect the expression of FAS mRNA. We could find articles on the effects of statins on liver FAS expression in other fish. Nevertheless, mice fed ATV-treated diets or administered ATV via oral gavage showed an up-regulation of FAS expression (Roglans et al., 2002; Ji et al., 2011; Patel et al., 2021). In contrast, no changes in FAS expression after statin exposure were found in mice after exposure to rosuvastatin at 0.01% w/w in the food for 4 weeks (Orime et al., 2016). Here, the expression of FAS in the MIX group did not change as in the ATV group, reinforcing an antagonistic interaction between EE2 and ATV and an antiestrogenic effect of ATV.

Acsl1 and Fabp1 mRNA levels were up- and down-regulated by EE2 exposure, as previously observed in brown trout juveniles exposed to the same compound (Madureira et al., 2018). Acsl1 up-regulation suggests that free fatty acids are being converted into acetyl-CoA and thus being used for lipid synthesis (Li et al., 2010), which would make sense given the high levels of blood lipids and FAS expression. Fabp1 had an inverse response pattern being downregulated by EE2. Our results are in line with those found after dietary exposure of rainbow trout (*Oncorhynchus mykiss*) and waterborne exposure of Nile tilapia (*Oreochromis niloticus*) to E2 (Cleveland and Weber, 2016; Zhang et al., 2020). At last, in our experiment, ATV did not cause Acsl1 and Fabp1 mRNA changes, which is in accordance with evidence described in mice treated with ATV by oral gavage (Patel et al., 2021).

The mRNA levels of LPL were not significantly altered in this study EE2 group, as previously noted in hybrid tilapia after E2 injection (Zhang et al., 2019). However, E2 caused down-regulation of LPL expression in rainbow trout (*Oncorhynchus mykiss*) liver, interpreted as a response to increased vitellogenin output, to prevent LPL from removing triacylglycerols from lipoproteins, such as vitellogenin (Cleveland and Weber, 2016). Herein, ATV treatment also failed to cause alterations in LPL expression, with the same outcome registered after in vitro exposure of mice pre-adipocytes to pitavastatin at 100 ng/mL for 8 days (Ishihara et al., 2010). The MIX exposure also failed to cause significant alterations in LPL expression. Similarly, StAR mRNA levels showed no significant alterations in response to any treatment, despite a reduction in StAR expression in fathead minnow (*Pimephales promelas*) having been demonstrated after waterborne exposure to EE2 at 10 ng/L for 21 days (Filby et al., 2007).

Statins act as competitive inhibitors of HMGCoAR, and Estey et al. (2008) have previously demonstrated that intraperitoneal injection of rainbow trout (*Oncorhynchus mykiss*) with cerivastatin at 1.4 or 11.2 ng/g reduces HMGCoAR enzymatic activity. However, studies in fish and mice reported higher HMGCoAR mRNA levels after ATV exposure (Al-Habsi et al., 2018; Roglans et al., 2002; Ji et al., 2011), which corroborates our data. This phenomenon was explained for zebrafish (*Danio rerio*) after dietary exposure to ATV, where the reduction of cholesterol led to an up-regulation of HMGCoAR mRNA, interpreted as an attempt to increase cholesterol biosynthesis and restore original levels (Al-Habsi et al., 2016). Interestingly, EE2 caused an increase in HMGCoAR expression much greater than that of ATV, although there is no evidence of estrogenic modulation of HMGCoAR expression in fish to our knowledge. This increase was not observed in the MIX group, where none of the effects of either EE2 or ATV manifested itself, which seems to suggest further an antagonistic effect of AVT on the EE2 action. In our assay, exposure to EE2 reduced ApoAI mRNA levels, in accordance with the results reported in rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes exposed to EE2 at 0.03 to 30 nM/L for 48h (Hultman et al., 2015). By contrast, exposure to ATV induced higher levels of ApoAI expression, in line with what was reported in human HepG2 cells after exposure to ATV at 1 to 50 μM and rosuvastatin at 0.25 to 5 μM for 48 hours (Maejima et al., 2004; Qin et al., 2008). Fish treated with the combination of EE2 and ATV followed a nearly identical pattern found after EE2 exposure, suggesting that the estrogenic effect is prevalent concerning ApoAI expression.

3.2.1. Peroxisomal β-oxidation genes

Previous studies in brown trout demonstrated that PPARα expression across the reproductive cycle is conversely related to estrogen levels (Baptista-Pinto et al., 2009). However, in vivo, waterborne exposure of brown trout (*Salmo trutta*) to EE2 caused upregulation of PPARα (Madureira et al., 2018). In the present study, PPARα mRNA expression was unaltered by treatment with EE2. ATV treatment produced a similar response to EE2 by not altering the expression of the peroxisome proliferator's expression, contrarily to what was achieved after dietary exposure to zebrafish (*Danio rerio*), which upregulated PPARα mRNA (Al-Habsi et al., 2016). In accordance with what happened in the EE2 and ATV groups, the MIX group showed no significant alterations in PPARα mRNA expression. Both isoforms of PPARα, the PPARαBa and PPARαBb genes, did not show any significant alterations, despite having previously been upregulated and downregulated by EE2, respectively, in the same species, but after in vitro exposures (Madureira et al., 2017b).

Another peroxisome proliferator analyzed here was PPARγ. Treatment with EE2 in isolation did not cause alterations to PPARγ, which goes against previous results where juvenile rainbow trout (*Oncorhynchus mykiss*) injected or diet-fed E2 and juvenile brown trout (*Salmo trutta*) exposed to EE2 in the water showed downregulation of PPARγ (Cleveland and Manor, 2015; Cleveland and Weber, 2016; Madureira et al., 2018). Also, in contrast with the literature, ATV caused downregulation of PPARγ, whereas the previous reports of the effects of this statin on zebrafish (*Danio rerio*) after dietary exposure indicated upregulation of PPARγ (Al-Habsi et al., 2016). Here, brown trout treated with the EE2 plus AVT followed a similar pattern to ATV alone, suggesting a stronger modulation of PPARγ by statins rather than estrogens.

The mRNA of Acox1-3I showed reduced expression, as it had previously done in juvenile brown trout (*Salmo trutta*) under a 28-day waterborne exposure (Madureira et al., 2018). The effects of ATV over Acox1-3I in fish have not yet been extensively studied, but the dietary exposure of juvenile mice to atorvastatin at 15 mg/kg has been shown to upregulate Acox levels in a process thought to be related to PPARα upregulation (Park et al., 2016). ATV downregulated Acox1-3I mRNA here, perhaps due to a lack of PPARα stimulation. Fish from the MIX group exhibited Acox1-3I mRNA expression higher than that of the EE2-treated animals but sat between the levels of ATV and the controls, again pointing to a prevalent effect of ATV in peroxisomal pathways.

Lastly, Acox3 was downregulated by all treatments, although in a more pronounced manner in the ATV and MIX groups. Waterborne exposure of juvenile brown trout (*Salmo trutta*) to EE2 at 50 µg/L had earlier downregulated Acox3, accompanied by downregulation of PPARγ (Madureira et al., 2018). Regulation of Acox3 expression by PPARγ might be why the expression of Acox3 mRNA was reduced by ATV and the mixture of chemicals, given that PPARγ mRNA had also been significantly less expressed in those treatment groups.

4. Gonadal development

The gonads of male and female fish were all classified in the first two development stages without significant differences between groups.

Three females exposed to EE2, either in isolation or in combination with ATV, scarcely showed cortical alveolar oocytes, with the same pattern being observed in one female from the control group. Thus, a significant influence of EE2 in gonad maturation under these experimental conditions is an unlikely scenario. Furthermore, male gonads exhibited no histological signs of feminization, which estrogens have been known to cause

in fish after continuous exposure (van Aerle et al., 2002), thus suggesting no feminization effects on the gonads.

Exposure to ATV did not cause any visible alteration in gonadal histology. However, its capability to disrupt lipid metabolism from this early stage warns that throughout the development and later during reproduction, ATV has potential to impact the fish gonads.

5. Conclusions and perspectives

EE2, ATV and their mixture were able to alter the blood lipid profile. Nevertheless, the changes noted to the circulating lipids were mostly contradictory between ATV and EE2, with the MIX causing estrogenic, hypolipidemic or intermediate responses, depending on the specific lipid class measured. EE2 tended to increase the levels of lipids in the blood, particularly triglycerides and VLDL, which are important and closely related to reproductive processes. In contrast, ATV generally diminished the concentrations of circulating lipids and cholesterol according to its reported medical effects. All three treatments could also disrupt the lipid deposition process in the liver by increasing the content (relative volume) of lipid droplets in hepatocytes, particularly EE2. On a molecular scale, both ATV and EE2 were able to modulate the expression of various lipidic targets similarly to what had been earlier demonstrated in other in vivo tests with brown trout (*Salmo trutta*) and zebrafish (*Danio rerio*). However, the effects caused by those two chemicals were usually contradictory. The MIX also regulated mRNA expression of lipid targets in the liver, and the effects caused by this treatment generally supported antagonistic interaction between ATV and EE2.

This collection of outcomes demonstrated that ATV, EE2 and their combination are all capable of inducing dyslipidemia in brown trout, with ATV causing hypolipidemia and EE2 leading to hyperlipidemia, whilst their joint administration effects varying between lipid-lowering or increasing effects. Interestingly, the results also showed that ATV, alone or in combination with EE2, was not able to alter the gonadal development of juvenile brown trout (*Salmo trutta*) in a sub-acute exposure via intramuscular injection, which was also the case for EE2 alone.

The lack of mortality during the exposure was a promising indicator for the viability of using juvenile brown trout exposed via intramuscular injection as an in vivo model for testing hypolipidemic substances. Furthermore, the effects caused by the ATV treatment were not only noticeable but also in line with the results of previous in vivo testing with other fish and mammalian models. This gives confidence that the brown trout (*Salmo trutta*) model created is suitable for testing already known or candidate hypolipidemic substances,

although there is room for improvement. One exciting option is first to cause hyperlipidemia by EE2 and then test a hypothetically hypolipidemic molecule against a reference drug.

Despite being able to cause dyslipidemia with all three experimental treatments, our in vivo test model outputs with juvenile brown trout were not capable of fully discerning the mechanisms by which ATV and EE2 interacted to produce the effects of the MIX group, nor was it able to cause alterations to the development of the gonads. Moreover, even with the clear demonstration of hypolipidemic effects by treatment with ATV, the study of the impacts of statins on fish is still in its infancy, and mechanisms remain unexplained. Therefore, future studies should focus on refining the model so that it can further explain the physiological mechanisms by which statins can affect fish, particularly brown trout (*Salmo trutta*). Those works could benefit from evaluating a more comprehensive range of statins in comparison to ATV as well as experimenting with different exposure periods or adding extra endpoints, such as plasma and liver fatty acid profiles, the addition of SREBP1, SREBP2 and LDLR to the genetic portfolio and measure the enzymatic activity of targets, such as HMGCoAR.

References

Albalat, A., Sánchez-Gurmaches, J., Gutiérrez, J., Navarro, I. (2006). Regulation of lipoprotein lipase activity in rainbow trout (*Oncorhynchus mykiss*) tissues. *General and Comparative Endocrinology*, *146*(3), 226-235.

Al-Habsi, A. A., Massarsky, A., & Moon, T. W. (2016). Exposure to gemfibrozil and atorvastatin affects cholesterol metabolism and steroid production in zebrafish (*Danio rerio*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *199*, 87-96.

Al-Habsi, A. A., Massarsky, A., Moon, T. W. (2018). Atorvastatin alters gene expression and cholesterol synthesis in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *224*, 262-269.

Ali, I., Alharbi, O. M., ALOthman, Z. A., Alwarthan, A., Al-Mohaimeed, A. M. (2019). Preparation of a carboxymethylcellulose-iron composite for uptake of atorvastatin in water. *International Journal of Biological Macromolecules*, *132*, 244-253.

Arukwe, A., Carteny, C. C., Moder, M., Bonini, A., Maubach, M. A., Eggen, T. (2016). Differential modulation of neuro- and interrenal steroidogenesis of juvenile salmon by the organophosphates - tris(2-butoxyethyl)- and tris(2-chloroethyl) phosphate. *Environmental Research*, 148, 63-71.

Atteke, C., Vetillard, A., Fostier, A., Garnier, D. H., Jego, P., Bailhache, T. (2003). Effects of progesterone and estradiol on the reproductive axis in immature diploid and triploid rainbow trout. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 134(4), 693-705.

Batista-Pinto, C., Rocha, E., Castro, L. F. C., Rodrigues, P., Lobo-da-Cunha, A. (2009). Seasonal and gender variation of peroxisome proliferator activated receptors expression in brown trout liver. *General and Comparative Endocrinology*, *161*(1), 146-152.

Barreiros, L., Queiroz, J. F., Magalhães, L. M., Silva, A. M. T., Segundo, M. (2016). Analysis of 17-β-estradiol and 17-α-ethinylestradiol in biological and environmental matrices – A review. *Microchemical Journal*, 126, 243-262.

Bjerregaard, L. B., Madsen, A. H., Korsgaard, B., Bjerregaard, P. (2006). Gonad histology and vitellogenin concentrations in brown trout (*Salmo trutta*) from Danish streams impacted by sewage effluent. *Ecotoxicology*, *15*(3), 315-327.

Bjerregaard, P., Hansen, P. R., Larsen, K. J., Erratico, C., Korsgaard, B. and Holbech, H. (2008). Vitellogenin as a biomarker for estrogenic effects in brown trout, *Salmo trutta*: Laboratory and field investigations. *Environmental Toxicology and Chemistry*, 27(11), 2387-2396.

Blewett, T. A., Chow, T. L., MacLatchy, D. L., Wood, C. M. (2014). A species comparison of 17-α-ethinylestradiol uptake and tissue-specific distribution in six teleost fish. *Comparative Biochemistry and Physiology*, 161, 33-40.

Bone, Q., Moore, R. (2008). Biology of Fishes (Third Edition). Taylor & Francis, London, UK. 5-29.

Braga, O., Smythe, G. A., Schäfer, A. I., Feitz, A. J. (2005). Steroid estrogens in ocean sediments. *Chemosphere*, 61, 827-833.

Buschmann, A. H., J. L. P. Muñoz. (2019). Challenges for Future Salmonid Farming. Encyclopedia of Ocean Sciences (Third Edition). J. K. Cochran, H. J. Bokuniewicz and P. L. Yager. Oxford, Academic Press: 313-319.

Caballero-Solares, A., Xue, X., Parrish, C. C., Foroutani, M. B., Taylor, R. G., & Rise, M. L. (2018). Changes in the liver transcriptome of farmed Atlantic salmon (*Salmo salar*) fed experimental diets based on terrestrial alternatives to fish meal and fish oil. *BMC Genomics*, *19*, 1-26.

Cleveland, B. M., & Manor, M. L. (2015). Effects of phytoestrogens on growth-related and lipogenic genes in rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *170*, 28-37.

Cleveland, B. M., & Weber, G. M. (2016). Effects of steroid treatment on growth, nutrient partitioning, and expression of genes related to growth and nutrient metabolism in adult triploid rainbow trout (*Oncorhynchus mykiss*). *Domestic Animal Endocrinology*, *56*, 1-12.

Collares-Pereira, M. J., Alves, M. J., Ribeiro, F., Domingos, I., Almeida, P. R., da Costa, L., Gante, H., Filipe, A. F., Aboim, M. A., Rodrigues, P. M., Magalhães, M. F. (2021). Guia dos Peixes de Água Doce e Migradores de Portugal Continental. Edições Afrontamento, Porto, Portugal.

Corton, J. C., Anderson, S. P., Stauber, A. (2000). Central role of peroxisome proliferator-activated receptors in the actions of peroxisome proliferators. *Annual Review of Pharmacology and Toxicology*, *40*, 491.

Czarny, K., Szczukocki, D., Krawczyk, B., Zieliński, M., Miękoś, E., Gadzała-Kopciuch, R. (2017). The impact of estrogens on aquatic organisms and methods for their determination. *Critical Reviews in Environmental Science and Technology*, 47, 909-963.

Ellesat, K. S., Holth, T. F., Wojewodzic, M. W., Hylland, K. (2012). Atorvastatin up-regulate toxicologically relevant genes in rainbow trout gills. *Ecotoxicology*, *21*(7), 1841-1856.

Estey, C., Chen, X., & Moon, T. W. (2008). 3-Hydroxy-3-methylglutaryl coenzyme A reductase in rainbow trout: Effects of fasting and statin drugs on activities and mRNA transcripts. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *147*(3), 386-398.

Falfushynska, H., Sokolov, E. P., Haider, F., Oppermann, C., Kragl, U., Ruth, W., Stock, M., Glufke, S., Winkel, E. J., Sokolova, I. M. (2019). Effects of a common pharmaceutical, atorvastatin, on energy metabolism and detoxification mechanisms of a marine bivalve *Mytilus edulis*. *Aquatic Toxicology*, *208*, 47-61.

FAO. (2021). Statistical Yearbook: World Food and Agriculture. Food and Agriculture Organization of the United Nations. Rome, Italy.

Faulds, M. H., Zhao, C., Dahlman-Wright, K., Gustafsson, J. A. (2012). The diversity of sex steroid action: regulation of metabolism by estrogen signaling. *Journal of Endocrinology*, *212*, 3-12.

Filby, A. L., Thorpe, K. L., Maack, G., & Tyler, C. R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen-and estrogen-induced feminization in fish. *Aquatic Toxicology*, *81*(2), 219-231.

Frisch, A. (2004). Sex-change and gonadal steroids in sequentially-hermaphroditic teleost fish. *Reviews in Fish Biology and Fisheries*, 14, 481-499.

Garriz, Á., del Fresno, P. S., Miranda, L. A. (2017). Exposure to E2 and EE2 environmental concentrations affect different components of the brain-pituitary-gonadal axis in pejerrey fish (*Odontesthes bonariensis*). *Ecotoxicology and Environmental Safety*, 144, 45-53.

Gates, L., Adler, R. R., Elangbam, C. S. (2016). Osmium tetroxide post-fixation and periodic acid-Schiff dual-staining technique to demonstrate intracellular lipid and glycogen in the mouse liver section–a novel method for co-visualization of intracellular contents in paraffin-embedded tissue. *Journal of Histotechnology*, *39*(1), 2-7.

Godwin, J. (2011). In: Farrell, A. P. (Ed.), Socially controlled sex change in fishes. Encyclopedia of Fish Physiology. Academic Press, London, pp. 662-669.

Hammer, Ø., Harper, D.A.T., Ryan, P.D. 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, 4(1), 9.

Hanson, A. M., Ickstadt, A. T., Marquart, D. J., Kittilson, J. D., Sheridan, M. A. (2017). Environmental estrogens inhibit mRNA and functional expression of growth hormone receptors as well as growth hormone signaling pathways in vitro in rainbow trout (*Oncorhynchus mykiss*). *General and Comparative Endocrinology*, *246*, 120-128.

Hilderbrand, G. V., Farley, S. D., Schwartz, C. C., Robbins, C. T. (2004). Importance of Salmon to Wildlife: Implications for Integrated Management. *Ursus*, 15(1), 1-9.

Holmlund, C. M., Hammer, M. (1999). Ecosystem services generated by fish populations. *Ecological Economics*, 29(2), 253-268.

Hook, S. E., Nagler, J. J., Cavileer, T., Verducci, J., Liu, Y., Hayton, W., Schultz, I. R. (2011). Relationships between the transcriptome and physiological indicators of reproduction in female rainbow trout over an annual cycle. *Environmental Toxicology and Chemistry*, *30*(2), 309-318.

Hultman, M. T., Song, Y., Tollefsen, K. E. (2015). 17α-Ethinylestradiol (EE2) effect on global gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Aquatic Toxicology*, *169*, 90-104.

Isaak, D. J., Luce, C. H., Rieman, B. E., Nagel, D. E., Peterson, E. E., Horan, D. L., Parkes, S., Chandler, G. L. (2010). Effects of climate change and wildfire on stream temperatures and salmonid thermal habitat in a mountain river network. *Ecological Applications*, 20(5), 1350-1371.

Jamalzadeh, H., Hajirezaee, S., Nazeri, S., Khara, H., Mirrasuli, E. (2012). Identification of follicular vitellogenesis stage by monitoring of plasma calcium and estradiol-17β concentrations in the cultured Caspian brown trout, *Salmo trutta caspius* Kessler, 1877. *Biologia*, *67*(4), 796-799.

Ji, G., Zhao, X., Leng, L., Liu, P., & Jiang, Z. (2011). Comparison of dietary control and atorvastatin on high fat diet induced hepatic steatosis and hyperlipidemia in rats. *Lipids in Health and Disease*, *10*, 1-10.

Johnson, R., Wolf, J., Braunbeck, T. (2009). OECD guidance document for the diagonosis of endocrine-related histopathology of fish gonads. Organisation for Economic Co-operation and Development.

Jordanova, M., Rebok, K., Malhão, F., Rocha, M. J., & Rocha, E. (2016). Seasonal changes in hepatocytic lipid droplets, glycogen deposits, and rough endoplasmic reticulum along the natural breeding cycle of female Ohrid trout (*Salmo letnica* Kar.) — A semiquantitative ultrastructural study. *Microscopy Research and Technique*, *79*(8), 700-706.

Jordanova, M., Rebok, K., Rocha, M. J., & Rocha, E. (2018). Histochemical Evaluation of Iron Content in the Liver of Wild Female Ohrid Trout (*Salmo Letnica* Kar.) along the Breeding Cycle. *Contributions, Section of Natural, Mathematical and Biotechnical Sciences*, *39*(2), 129-132.

Karagianni, P., Talianidis, I. (2015). Transcription factor networks regulating hepatic fatty acid metabolism. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1851*(1), 2-8.

Kenari, A. A., Mozanzadeh, M. T., & Pourgholam, R. (2011). Effects of total fish oil replacement to vegetable oils at two dietary lipid levels on the growth, body composition, haemato-immunological and serum biochemical parameters in caspian brown trout (*Salmo trutta caspius* Kessler, 1877). *Aquaculture Research*, *42*(8), 1131-1144.

Kitano, S. (2004). Ecological impacts of rainbow, brown and brook trout in Japanese inland waters. *Global Environmental Research*, 8(1), 41-50.

Korner, O., Kohno, S., Schonenberger, R., Suter, M.J.F., Knauer, K., Guillette, L.J., Burkhardt-Holm, P. (2008). Water temperature and concomitant waterborne ethinylestradiol exposure affects the vitellogenin expression in juvenile brown trout (*Salmo trutta*). *Aquatic Toxicology*, 90(3), 188-196.

Lamm, M. S., Liu, H., Gemmell, N. J., Godwin, J. R. (2015). The need for speed: Neuroendocrine regulation of socially-controlled sex change. *Integrative and Comparative Biology*, 55, 307-322.

Leaver, M. J., Bautista, J. M., Björnsson, B. T., Jönsson, E., Krey, G., Tocher, D. R., Torstensen, B. E. (2008). Towards fish lipid nutrigenomics: Current state and prospects for fin-fish aquaculture. *Reviews in Fisheries Science*, 16(1), 73-94.

Li, L. O., Klett, E. L., Coleman, R. A. (2010). Acyl-CoA synthesis, lipid metabolism and lipotoxicity. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1801*(3), 246-251.

Li, Q., Yang, G. (2010). Influence of coexisting contaminants on the sorption of 17β-estradiol by marine sediments. *Journal of Environmental Science and Health*, 45, 1815-1822.

Li, X., Song, Q. S., Wang, J. Y., Leng, H. J., Chen, Z. Q., Liu, Z. J., Dang, G. T., & Song, C. L. (2011). Simvastatin induces estrogen receptor-alpha expression in bone, restores bone loss, and decreases ERα expression and uterine wet weight in ovariectomized rats. *Journal of Bone and Mineral Metabolism*, *29*, 396-403.

Liu, Z., Ogejo, J. A., Pruden, A., Knowlton, K. F. (2011). Occurrence, fate and removal of synthetic oral contraceptives (SOCs) in the natural environment: A review. *Science of the Total Environment*, 409, 5149-5161.

Lopes, C., Malhão, F., Guimarães, C., Pinheiro, I., Gonçalves, J. F., Castro, L. F. C., Rocha, E., Madureira, T. V. (2016). 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.

Lopes, C., Rocha, E., Pereira, I. L., Madureira, T. V. (2021). Deciphering influences of testosterone and dihydrotestosterone on lipid metabolism genes using brown trout primary hepatocytes. *Aquatic Toxicology*, *235*, 105819.

Lund, E. K. (2013). Health benefits of seafood; Is it just the fatty acids?. *Food Chemistry*, 140(3), 413-420.

Madsen, S. S., Skovbølling, S., Nielsen, C., Korsgaard, B. (2004). 17-β Estradiol and 4-nonylphenol delay smolt development and downstream migration in Atlantic salmon, *Salmo salar*. *Aquatic Toxicology*, *68*(2), 109-120.

Madureira, T. V., Malhão, F., Pinheiro, I., Lopes, C., Ferreira, N., Urbatzka, R., Castro, L. F. C., Rocha, E. (2015). Estrogenic and anti-estrogenic influences in cultured brown trout hepatocytes: Focus on the expression of some estrogen and peroxisomal related genes and linked phenotypic anchors. *Aquatic Toxicology*, *169*, 133-142.

Madureira, T. V., Castro, L. F. C., Rocha, E. (2016). Acyl-coenzyme A oxidases 1 and 3 in brown trout (*Salmo trutta f. fario*): can peroxisomal fatty acid β-oxidation be regulated by estrogen signaling? *Fish Physiology and Biochemistry*, *42*(1), 389-401.

Madureira, T. V., Pinheiro, I., Malhão, F., Lopes, C., Urbatzka, R., Castro, L. F. C., & Rocha, E. (2017a). Cross-interference of two model peroxisome proliferators in peroxisomal

and estrogenic pathways in brown trout hepatocytes. *Aquatic Toxicology*, *187*, 153-162.

Madureira, T. V., Pinheiro, I., de Paula Freire, R., Rocha, E., Castro, L. F., & Urbatzka, R. (2017b). Genome specific PPARαB duplicates in salmonids and insights into estrogenic regulation in brown trout. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *208*, 94-101.

Madureira, T. V., Malhão, F., Simões, T., Pinheiro, I., Lopes, C., Gonçalves, J. F., Urbatzka, R., Castro, L. F. C., Lemos, M. F. L., Rocha, E. (2018). Sex-steroids and hypolipidemic chemicals impacts on brown trout lipid and peroxisome signaling—Molecular, biochemical and morphological insights. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *212*, 1-17.

Madureira, T. V., Costa, J. L., Malhão, F., Lopes, C., Gonçalves, J. F., Rocha, E. (2019). Design of a multi-parametric profile for assessing the acclimation period of juvenile brown trout after an acute transport to new housing environment. *Applied Animal Behaviour Science*, *219*, 104835.

Maejima, T., Yamazaki, H., Aoki, T., Tamaki, T., Sato, F., Kitahara, M., & Saito, Y. (2004). Effect of pitavastatin on apolipoprotein AI production in HepG2 cell. *Biochemical and Biophysical Research Communications*, *324*(2), 835-839.

Malhotra, H. S., Goa, K. L. (2001). Atorvastatin. *Drugs*, *61*(12), 1835-1881.

Marcos, R., Monteiro, R. A., & Rocha, E. (2012). The use of design-based stereology to evaluate volumes and numbers in the liver: A review with practical guidelines. *Journal of anatomy*, *220*(4), 303-317.

Martínez, R., Navarro-Martín, L., Luccarelli, C., Codina, A. E., Raldúa, D., Barata, C., Tauler, R., & Piña, B. (2019). Unravelling the mechanisms of PFOS toxicity by combining morphological and transcriptomic analyses in zebrafish embryos. *Science of the Total Environment*, *674*, 462-471.

Martyniuk, C. J., Gallant, N. S., Marlatt, V. L., Wiens, S. C., Woodhouse, A. J., Trudeau, V. L. (2006). In: Reinecke, M., Zaccone, G., Kapoor, B. G. (Ed.), Current perspectives on 17β-estradiol (E2) action and nuclear estrogen receptors (ER) in teleost fish. Science Publishers, Enfield, New Hampshire, pp. 625-663.

Melo, M. C., van Dijk, P., Andersson, E., Nilsen, T. O., Fjelldal, P. G., Male, R., Nijenhuis, W., Bogerd, J., de Franca, L. R., Taranger, G. L., Schulz, R. W. (2015). Androgens

directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar)*. *General and Comparative Endocrinology*, 211, 52-61.

Menoyo, D., Lopez-Bote, C. J., Bautista, J. M., Obach, A. (2003). Growth, digestibility and fatty acid utilization in large Atlantic salmon (*Salmo salar*) fed varying levels of n-3 and saturated fatty acids. *Aquaculture*, *225*(1-4), 295-307.

Menze, E. T., Ezzat, H., Shawky, S., Sami, M., Selim, E. H., Ahmed, S., Maged, N., Nadeem, N., Eldash, S., & Michel, H. E. (2021). Simvastatin mitigates depressive-like behavior in ovariectomized rats: Possible role of NLRP3 inflammasome and estrogen receptors' modulation. *International Immunopharmacology*, *95*, 107582.

Mezzelani, M., & Regoli, F. (2022). The biological effects of pharmaceuticals in the marine environment. *Annual Review of Marine Science*, *14*, 105-128.

Mills, L. J., Chichester, C. (2005). Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of The Total Environment*, 343(1), 1-34.

Milner, N. J., Cowx, I. G., Whelan, K. F. (2012). Salmonids and flows: A perspective on the state of the science and its application. *Fisheries Management and Ecology*, *19*(6), 445-450.

Minghetti, M., Leaver, M. J., Tocher, D. R. (2011). Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar L.*) established cell line, SHK-1. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1811*(3), 194-202.

Naderi, M., Zargham, D., Asadi, A., Bashti, T., Kamayi, K. (2015). Short-term responses of selected endocrine parameters in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to 4-nonylphenol. *Toxicology and Industrial Health*, *31*(12), 1218-1228.

Nagler, J. J., Cavileer, T. D., Verducci, J. S., Schultz, I. R., Hook, S. E., Hayton, W. L. (2012). Estrogen receptor mRNA expression patterns in the liver and ovary of female rainbow trout over a complete reproductive cycle. *General and Comparative Endocrinology*, *178*(3), 556-561.

Nanton, D. A., Lall, S. P., Ross, N. W., McNiven, M. A. (2003). Effect of dietary lipid level on fatty acid β-oxidation and lipid composition in various tissues of haddock, Melanogrammus aeglefinus L. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *135*(1), 95-108.

Nelson, J. S., Grande, T. C., Wilson, M. V. H. (2016). Fishes of the World (Fifth Edition). John Wiley & Sons. New Jersey, USA. 244-248.

Nelson, L. E., Sheridan, M. A. (2006). Gastroenteropancreatic hormones and metabolism in fish. *General and Comparative Endocrinology*, *148*(2), 116-124.

Nislow, K. H., Armstrong, J. D. (2012). Towards a life-history-based management framework for the effects of flow on juvenile salmonids in streams and rivers. *Fisheries Management and Ecology*, 19(6), 451-463.

Norris, D. O., Hobbs, S. L. (2006). In: Reinecke, M., Zaccone, G., Kapoor, B. G. (Ed.), The HPA axis and functions of corticosteroids in fishes. Science Publishers, Enfield, New Hampshire, pp. 721-765.

Ohira, M., Endo, K., Saiki, A., Miyashita, Y., Terai, K., Murano, T., Watanabe, F., Tatsuno, I., & Shirai, K. (2012). Atorvastatin and pitavastatin enhance lipoprotein lipase production in L6 skeletal muscle cells through activation of adenosine monophosphate-activated protein kinase. *Metabolism*, *61*(10), 1452-1460.

Orime, K., Shirakawa, J., Togashi, Y., Tajima, K., Inoue, H., Nagashima, Y., & Terauchi, Y. (2016). Lipid-lowering agents inhibit hepatic steatosis in a non-alcoholic steatohepatitis-derived hepatocellular carcinoma mouse model. *European Journal of Pharmacology*, *772*, 22-32.

Ortúzar, M., Esterhuizen, M., Olicón-Hernández, D. R., González-López, J., & Aranda, E. (2022). Pharmaceutical pollution in aquatic environments: A concise review of environmental impacts and bioremediation systems. *Frontiers in Microbiology*, *13*.

Osachoff, H. L., Shelley, L. K., Furtula, V., Van Aggelen, G. C., Kennedy, C. J. (2013). Induction and recovery of estrogenic effects after short-term 17β-estradiol exposure in juvenile rainbow trout (*Oncorhynchus mykiss*). *Archives of Environmental Contamination and Toxicology*, *65*(2), 276-285.

Park, H. S., Jang, J. E., Ko, M. S., Woo, S. H., Kim, B. J., Kim, H. S., Park, H. S., Park, I. S., Koh, E. H., & Lee, K. U. (2016). Statins increase mitochondrial and peroxisomal fatty acid oxidation in the liver and prevent non-alcoholic steatohepatitis in mice. *Diabetes & Metabolism Journal*, *40*(5), 376-385.

Patel, O., Muller, C. J., Joubert, E., Rosenkranz, B., Louw, J., & Awortwe, C. (2021). Therapeutic effects of an aspalathin-rich green rooibos extract, pioglitazone and atorvastatin combination therapy in diabetic db/db mice. *PloS one*, *16*(5).

Piferrer, F. (2011). In: Farrell, A. P. (Ed.), Endocrine sex control strategies for the feminization of teleost fish. Encyclopedia of Fish Physiology. Academic Press, London, pp. 229-281.

Peng, S., Shi, Z., Gao, Q., Zhang, C., Wang, J. (2017). Dietary n-3 LC-PUFA s affect lipoprotein lipase (LPL) and fatty acid synthase (FAS) activities and mRNA expression during vitellogenesis and ovarian fatty acid composition of female silver pomfret (*Pampus argenteus*) broodstock. *Aquaculture Nutrition*, *23*(4), 692-701.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, *29*(9), 45-51.

Pfaffl, M. W., Tichopad, A., Prgomet, C., Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology Letters*, *26*(6), 509-515.

Prindiville, J. S., Mennigen, J. A., Zamora, J. M., Moon, T. W., & Weber, J. M. (2011). The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout. *Toxicology and Applied Pharmacology*, *251*(3), 201-208.

Qin, S., Koga, T., Ganji, S. H., Kamanna, V. S., & Kashyap, M. L. (2008). Rosuvastatin selectively stimulates apolipoprotein AI but not apolipoprotein A-II synthesis in Hep G2 cells. *Metabolism*, *57*(7), 973-979.

Raatz, S. K., Rosenberger, T. A., Johnson, L. K., Wolters, W. W., Burr, G. S., Picklo, M. J. (2013). Dose-dependent consumption of farmed Atlantic Salmon (*Salmo salar*) increases plasma phospholipid n-3 fatty acids differentially. *Journal of the Academy of Nutrition and Dietetics*, 113(2), 282-287.

Reading, B. J., Andersen, L. K., Ryu, Y. W., Mushirobira, Y., Todo, T., & Hiramatsu, N. (2018). Oogenesis and egg quality in finfish: yolk formation and other factors influencing female fertility. *Fishes*, *3*(4), 45.

Real, M. V., Rocha, M. J., Gonçalves, J. F., Rocha, E. (2020). Histology and design-based estimation of hepatocellularity and volumes of hepatocytes in control and ethynylestradiol exposed males of platyfish (*Xiphophorus maculatus*). *Tissue and Cell*, *63*, 101327.

Robertson, J. C., & Bradley, T. M. (1992). Liver ultrastructure of juvenile Atlantic salmon (*Salmo salar*). *Journal of Morphology*, *211*(1), 41-54.

Roglans, N., Verd, J. C., Peris, C., Alegret, M., Vázquez, M., Adzet, T., Díaz, C., Hernández, G., Laguna, J. C., & Sánchez, R. M. (2002). High doses of atorvastatin and simvastatin induce key enzymes involved in VLDL production. *Lipids*, *37*(5), 445-454.

Ruyter, B., Andersen, Ø., Dehli, A., Farrants, A. K. Ö., Gjøen, T., Thomassen, M. S. (1997). Peroxisome proliferator activated receptors in Atlantic salmon (*Salmo salar*): effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, *1348*(3), 331-338.

Santos, M. M., Ruivo, R., Lopes-Marques, M., Torres, T., Carmen, B., Castro, L. F. C., Neuparth, T. (2016). Statins: An undesirable class of aquatic contaminants? *Aquatic Toxicology*, *174*, 1-9.

Santos, D., Rocha, E., Malhão, F., Lopes, C., Gonçalves, J. F., Madureira, T. V. (2021). Multi-parametric portfolio to assess the fitness and gonadal maturation in four key reproductive phases of brown trout. *Animals*, *11*(5), 1290.

Segner, H., Eppler, E., Reinecke, M. (2006). In: Reinecke, M., Zaccone, G., Kapoor, B. G. (Ed.), The impact of environmental hormonally active substances on the endocrine and immune systems of fish. Science Publishers, Enfield, New Hampshire, pp. 809-865.

Sharpe, R. L., MacLatchy, D. L. (2007). Lipid dynamics in goldfish (*Carassius auratus*) during a period of gonadal recrudescence: Effects of β-sitosterol and 17β-estradiol exposure. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *145*(4), 507-517.

Shen, M., Shi, H. (2015). Sex hormones and their receptors regulate liver energy homeostasis. *International Journal of Endocrinology*, *2015*.

Sheridan, M. A. (1994). Regulation of lipid metabolism in poikilothermic vertebrates. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, *107*(4), 495-508.

Sovadinová, I., Liedtke, A., Schirmer, K. (2014). Effects of clofibric acid alone and in combination with 17β-estradiol on mRNA abundance in primary hepatocytes isolated from rainbow trout. *Toxicology In Vitro*, *28*(6), 1106-1116.

Sulaiman, S., Khamis, M., Nir, S., Lelario, F., Scrano, L., Bufo, S. A., Mecca, G., Karaman, R. (2015). Stability and removal of atorvastatin, rosuvastatin and simvastatin from wastewater. *Environmental Technology*, *36*(24), 3232-3242.

Tacon, A. G. J., Metian, M. (2013). Fish matters: Importance of aquatic foods in human nutrition and global food supply. *Reviews in Fisheries Science,* 21(1), 22-38.

Tete, V. S., Nyoni, H., Mamba, B. B., Msagati, T. A. (2020). Occurrence and spatial distribution of statins, fibrates and their metabolites in aquatic environments. *Arabian Journal of Chemistry*, *13*(2), 4358-4373.

Thibaut, R., Porte, C. (2004). Effects of endocrine disrupters on sex steroid synthesis and metabolism pathways in fish. *Journal of Steroid Biochemistry and Molecular Biology*, 92(5), 485-494.

Tilami, S. K., Sampels, S. (2018). Nutritional value of fish: Lipids, proteins, vitamins, and minerals. *Reviews in Fisheries Science & Aquaculture*, 26(2), 243-253.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews in Fisheries Science*, *11*(2), 107-184.

Todorčević, M., Vegusdal, A., Gjøen, T., Sundvold, H., Torstensen, B. E., Kjær, M. A., & Ruyter, B. (2008). Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; effects of n-3 and n-9 fatty acids. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1781*(6-7), 326-335.

Urbatzka, R., Galante-Oliveira, S., Rocha, E., Castro, L. F. C., Cunha, I. (2013). Normalization strategies for gene expression studies by real-time PCR in a marine fish species, *Scophthalmus maximus*. *Marine Genomics*, *10*, 17-25.

Urwin, H. J., Miles, E. A., Noakes, P. S., Kremmyda, L. S., Vlachava, M., Diaper, N. D., Perez-Cano, F. J., Godfrey, K. M., Calder, P. C., Yagoobs, P. (2012). Salmon consumption during pregnancy alters fatty acid composition and secretory IgA concentration in human breast milk. *Journal of Nutrition*, 142(8), 1603-1610.

Usydus, Z., Szlinder-Richert, J., Adamczyk, M., & Szatkowska, U. (2011). Marine and farmed fish in the Polish market: Comparison of the nutritional value. *Food Chemistry*, *126*(1), 78-84.

van Aerle, R., Pounds, N., Hutchinson, T. H., Maddix, S., & Tyler, C. R. (2002). Window of sensitivity for the estrogenic effects of ethinylestradiol in early life-stages of fathead minnow, *Pimephales promelas*. *Ecotoxicology*, *11*, 423-434.

Vang, S. H., Kortner, T. M., & Arukwe, A. (2007). Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450 scc) as molecular and cellular targets for 17α-ethynylestradiol in salmon previtellogenic oocytes. *Chemical Research in Toxicology*, *20*(12), 1811-1819.

Wallaert, C., & Babin, P. J. (1992). Effects of 17β-estradiol and starvation on trout plasma lipoproteins. *Lipids*, *27*(12), 1032-1041.

Wang, Y., Wang, C., Xie, M., Tang, T., Wang, Z., Nie, X. (2021). Atorvastatin causes oxidative stress and alteration of lipid metabolism in estuarine goby *Mugilogobius abei*. *Environmental Pollution*, *289*, 117879.

Warren, M., Dunbar, M. J., Smith, C. (2015). River flow as a determinant of salmonid distribution and abundance: A review. *Environmental Biology of Fishes*, 98(6), 1695-1717.

Weber, H., & Sciubba, J. D. (2019). The effect of population growth on the environment: Evidence from European regions. *European Journal of Population*, *35*, 379-402.

Wenger, S. J., Isaak, D. J., Luce, C. H., Neville, H. M., Fausch, K. D., Dunham, J. B., Dauwalter, D. C., Young, M. K., Elsner, M. M., Rieman, B. E., Hamlet, A. F., Williams, J. E. (2011). Flow regime, temperature, and biotic interactions drive differential declines of trout species under climate change. *Proceedings of the National Academy of Sciences*, *108*(34), 14175-14180.

Willson, M. F. (1997). Variation in salmonid life histories: Patterns and perspectives (Vol. 498). US Department of Agriculture, Pacific Northwest Research Station. Pages?

Wise, A., O'Brien, K., Woodruff, T. (2011). Are oral contraceptives a significant contributor to the estrogenicity of drinking water? *Environmental Science and Technology*, 45, 51-60.

Yamaguchi, K., Ishii, M., Maeda, N., Iwano, H., & Yokota, H. (2016). Suppression of liver Apo E secretion leads to HDL/cholesterol immaturity in rats administered ethinylestradiol. *FEBS Open Bio*, *6*(9), 928-936.

Yaron, Z., Levave-Sivan, B. (2011). In: Farrell, A. P. (Ed.), The diversity of fish reproduction: An introduction. Encyclopedia of Fish Physiology. Academic Press, London, pp. 1500-1508.

Zahl, I. H., Samuelsen, O., Kiessling, A. (2012). Anaesthesia of farmed fish: Implications for welfare. *Fish Physiology and Biochemistry*, *38*(1), 201-218.

Zhang, Z., Wang, J., Pan, Z., Zhang, Y., Zhang, X., Tian, H., Wang, W., Ru, S. (2019). Distribution of vitellogenin in Japanese flounder (*Paralichthys olivaceus*) for biomarker analysis of marine environmental estrogens. *Aquatic Toxicology*, 216, 105321.

Zhang, X., Han, Z., Zhong, H., Yin, Q., Xiao, J., Wang, F., Zhou, Y., & Luo, Y. (2019). Regulation of triglyceride synthesis by estradiol in the livers of hybrid tilapia (Oreochromis niloticus♀× O. aureus♂). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *238*, 110335.

Zhang, X., Zhong, H., Han, Z., Tang, Z., Xiao, J., Guo, Z., Wang, F., Luo, Y., & Zhou, Y. (2020). Effects of waterborne exposure to 17β-estradiol on hepatic lipid metabolism genes in tilapia (*Oreochromis niloticus*). *Aquaculture Reports*, *17*, 100382.

Appendix 1

Calendarization of Exposure Injections and Samplings

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Morning		SC	C	EE2	ATV	MIX	
Afternoon					SC	C	EE2

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Morning		SC	C	EE2	ATV	MIX	
Afternoon	ATV	MIX			SC	C	EE2

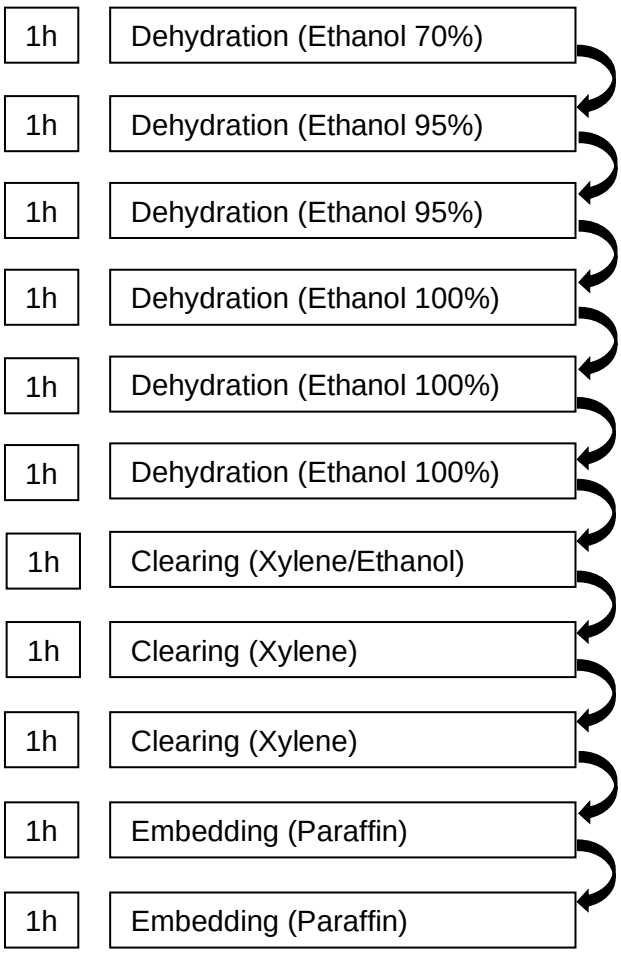
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Morning		Sampling SC	Sampling C	Sampling EE2	Sampling ATV	Sampling MIX	
Afternoon	ATV	MIX					

Experimental groups:

- **C**: Control
- **SC**: Solvent Control
- **EE2**: 17α-Ethinylestradiol
- **ATV**: Atorvastatin
- **MIX**: 17α-Ethinylestradiol + Atorvastatin

Appendix 2

Tissue Processing in the Leica TP1020 v10.1



Material used:

- **Xylene**: VWR International.
- **Ethanol**: Manuel Vieira e Cª (Irmão) Sucurs, LDA.
- **Liquid Paraffin**: Epredia.

Appendix 3

RNA Extraction Protocol for the illustra™ RNAspin Mini Isolation Kit

I – Tissue Homoenization

- Weight 14 to 20 mg of frozen tissue.
- Add 350 µL of lysis solution and 3.5 µL of β-mercaptoethanol.
- Disrupt with rotor-stator until no tissue pieces are visible.

II – Filtration

- Transfer the lysate to the RNAspin Mini Filter.
- Centrifuge at 11000 g for 1 minute.

III – Adjust RNA binding condition

- Transfer the filtrated lysate to a nuclease-free microcentrifuge tube.
- Add 350 µL of ethanol 70%.
- Vortex the tube twice for 5 seconds to mix.

IV – RNA binding

- Pipet lysete doing an up-and-down 2 to 3 times.
- Transfer the lysate to the RNAspin Mini Column.
- Centrifuge at 8000 g for 30 segonds.
- Discard the flowthrough and transfer the column to a new collection tube.

V – Desalting

- Apply 350 µL of desalting buffer to the column.
- Centrifuge at 11000 g for 1 minute.
- Discard the flowthrough.

VI – DNA digestion

- Mix 90 µL of DNAse reaction buffer and 10 µL of DNAse I in a nuclease-free microcentirufge tube.
- Apply 95 µL of the DNAse I reaction mixture to the center of the silica membrane in the column.
- Room temperature for 15 minutes.

VII – <u>Wash and dry</u>

- Apply 200 µL of wash buffer I to the column.
- Centrifuge at 11000 g for 1 minute and discard the flowthrough.
- Apply 600 µL of wash buffer I to the column.
- Centrifuge at 11000 g for 1 minute and discard the flowthrough.
- Apply 250 µL of wash buffer II to the column.
- Centrifuge at 11000 g for 2 minutes to fully dry the membrane and discard the flowthrough.

VIII – <u>Elution</u>

- Transfer the column to a nuclease-free microcentrifuge tube.
- Apply 30 µL of RNAse-free water to the center of the silica membrane in the column.
- Centrifuge at 11000g for 1 minute.
- Pipete the flowthrough and reapply it to the center of the silica membrane in the column.
- Centrifuge at 11000 g for 1 minute.
- Discard the column and store the microcentrifuge tube at -80ºC.

Appendix 4

qRT-PCR Plate Layout

NTC	**4** (C4)	**8** (C8)	**12** (CS3)	**16** (CS8)	**20** (E3)	**24** (E7)	**28** (AT1)	**32** (AT5)	**36** (AT10)	**40** (M4)	**44** (M8)
NTC	**4** (C4)	**8** (C8)	**12** (CS3)	**16** (CS8)	**20** (E3)	**24** (E7)	**28** (AT1)	**32** (AT5)	**36** (AT10)	**40** (M4)	**44** (M8)
1 (C1)	**5** (C5)	**9** (C10)	**13** (CS4)	**17** (CS9)	**21** (E4)	**25** (E8)	**29** (AT2)	**33** (AT7)	**37** (M1)	**41** (M5)	**45** (M9)
1 (C1)	**5** (C5)	**9** (C10)	**13** (CS4)	**17** (CS9)	**21** (E4)	**25** (E8)	**29** (AT2)	**33** (AT7)	**37** (M1)	**41** (M5)	**45** (M9)
2 (C2)	**6** (C6)	**10** (CS1)	**14** (CS6)	**18** (CS10)	**22** (E5)	**26** (E9)	**30** (AT3)	**34** (AT8)	**38** (M2)	**42** (M6)	
2 (C2)	**6** (C6)	**10** (CS1)	**14** (CS6)	**18** (CS10)	**22** (E5)	**26** (E9)	**30** (AT3)	**34** (AT8)	**38** (M2)	**42** (M6)	
3 (C3)	**7** (C7)	**11** (CS2)	**15** (CS7)	**19** (E2)	**23** (E6)	**27** (E10)	**31** (AT4)	**35** (AT9)	**39** (M3)	**43** (M7)	
3 (C3)	**7** (C7)	**11** (CS2)	**15** (CS7)	**19** (E2)	**23** (E6)	**27** (E10)	**31** (AT4)	**35** (AT9)	**39** (M3)	**43** (M7)	

NTC: No template control

n = 9 animals/ group

In bold are the sample number corresponding to each well, and between brackets is the animal to which the sample corresponds. C – Control; SC – Solvent Control; E – 17α-Ethinylestradiol; AT – Atorvastatin; M – Mixture.

Appendix 5

Hematoxylin and Eosin Staining Protocol

I – <u>Deparaffinization</u>

- Xylene for 10 minutes.
- Xylene for 10 minutes

II – <u>Hydration</u>

- Ethanol 100% for 5 minutes.
- Ethanol 95% for 5 minutes.
- Ethanol 70% for 5 minutes.
- Running water for 5 minutes

III – <u>Staining</u>

- Mayer's hematoxylin for 2 minutes.
- Running water for 5 minutes.
- Eosin for 5 minutes.
- Wash in running water.

IV – <u>Dehydration</u>

- Ethanol 100% I.
- thanol 100% II.
- Ethanol 100% III.

V – <u>Diaphanization</u>

- Xylene I.
- Xylene II.

Material used:

- **Xylene**: VWR International.
- **Ethanol**: Manuel Vieira e Cª (Irmão) Sucurs, LDA.
- **Hematoxylin**: Hematoxylin 7211, Thermo Scientific.
- **Eosin**: Merck KGaA.