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Xenoestrogenic Modulation of Sex-Steroid Related Targets in Brown Trout Primary Hepatocyte Spheroids

Rodrigo Franco Alves

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Porto, 25 de Outubro de 2022

Abstract

Toxicological disruption and mechanistic physiological studies can be tested using *in vitro* fish cultures, either cell lines or primary cells. Fish primary hepatocytes have been successfully used for investigating metabolism, bioaccumulation, and biotransformation pathways. Hepatocyte two-dimensional (2D) in vitro systems do not retain tissue architecture and can only be maintained for a short period in culture. Contrarily, three-dimensional (3D) systems can reflect in vivo conditions because intercellular contacts, cell-matrix interactions, and re-establishment of cell polarity are restored. Nevertheless, the application of 3D systems in fish studies is still very limited.

Recently, in the host laboratory, it was developed a spheroid model obtained from juvenile brown trout (*Salmo trutta*) primary hepatocytes. By using this model, the main aims of this Thesis were: 1) to functionally characterize the hepatocyte spheroids along different days in culture; and 2) to use this 3D model to evaluate the effects caused by a classic estrogenic compound (17α-ethinylestradiol - EE2), at distinct concentrations (including environmental concentrations).

To accomplish the objectives, an optimization assay was first carried out to define better the maturity period of hepatocyte spheroids, both morphological and functionally. Thus, hepatocyte spheroids from juvenile brown trout were evaluated in terms of cell viability (lactate dehydrogenase – LDH and resazurin assays), biometry (equivalent diameter, area, and sphericity), histomorphology (Hematoxylin & Eosin - H&E and Periodic Acid Schiff - PAS followed by PAS with diastase) and molecular analyses. The mRNA levels of a selection of metabolic (Cytochrome P450 1A - CYP1A, cytochrome P450 3A27 - CYP3A27, glutathione S-transferase - GST, UDP-glycosyltransferase - UGT, catalase - Cat), efflux transporters (bile salt export pump - BSEP), multidrug resistance protein 1 - MDR1, multidrug resistance-associated protein 2 - MRP2) and estrogenic target (vitellogenin A - VtgA, estrogenic receptor α - ERα and zona pellucida glycoprotein 2.5 - ZP 2.5) genes, were evaluated at distinct days in culture and compared to whole liver samples, as a way to verify the functional maturity of spheroids. Overall, morphological and functional maturity of spheroids was achieved after the 12th day in culture.

In the second part of this study, the exposure of hepatocyte spheroids was performed for 6 days (from the 12th to 18th day in culture) to EE2 (1 ng/L, 10 ng/L, 50 ng/L or 100 ng/L).

There were no alterations in cell viability, biometry, and morphology of EE2 exposed spheroids. mRNA levels were unchanged except for VtgA, which was significantly up-regulated after the highest concentration of EE2.

The present data showed the potential of juvenile brown trout primary hepatocytes spheroids as an experimental in vitro model to evaluate hepatic disruptive effects of estrogenic compounds. The model is an alternative to decrease in vivo testing.

Resumo

Disrupção toxicológica e estudos mecanicistas fisiológicos podem ser testados usando culturas de peixes *in vitro*, sejam linhas celulares ou células primárias. Hepatócitos primários de peixes têm sido usados com sucesso para investigar vias de metabolismo, bioacumulação e biotransformação. Os sistemas bidimensionais (2D) de hepatócitos *in vitro* não retêm a arquitetura do tecido e só podem ser mantidos por um curto período em cultura. Contrariamente, os sistemas tridimensionais (3D) conseguem refletir as condições *in vivo* porque há restauro dos contatos intercelulares, interações célula-matriz e restabelecimento da polaridade celular. No entanto, a aplicação de sistemas 3D em estudos de peixes ainda é muito limitada.

Recentemente, no laboratório anfitrião foi desenvolvido um modelo esferoide obtido a partir de hepatócitos primários de truta castanha (*Salmo trutta*). Ao utilizar este modelo, os principais objetivos desta Tese foram: 1) caracterizar funcionalmente os esferóides de hepatócitos ao longo de diferentes dias de cultura; e 2) utilizar este modelo 3D para avaliar os efeitos causados por um composto estrogénico clássico (17α-etinilestradiol - EE2), em concentrações distintas (incluindo concentrações ambientais).

Para atingir os objetivos, primeiramente foi realizado um ensaio de otimização para melhor definir o período de maturidade dos esferoides de hepatócitos, tanto morfológica quanto funcionalmente. Assim, esferoides de hepatócitos de truta castanha juvenis foram avaliados em termos de viabilidade celular (ensaios de lactatodesidrogenase – LDH e resazurina), biometria (diâmetro equivalente, área e esfericidade), histomorfologia (Hematoxilina & Eosina - H&E e Ácido Periódico de Schiff - PAS seguido de PAS com diastase) e análises moleculares. Os níveis de mRNA de uma seleção de genes metabólicos (citocromo P450 1A - CYP1A, citocromo P450 3A27 - CYP3A27, glutationa S-transferase - GST, UDP-glicosiltransferase - UGT, catalase - Cat), transportadores de efluxo (bomba de exportação de sais biliares - BSEP), proteína de resistência a múltiplas drogas 1 - MDR1, proteína associada à resistência a múltiplas drogas 2 - MRP2) e alvos estrogénicos (vitelogenina A - VtgA, receptor estrogénico α - ERα e glicoproteína Zona pelúcida 2.5 - ZP 2.5), foram avaliados em dias distintos em cultura e comparados a amostras de fígado inteiro, como forma de verificar a maturidade funcional dos esferoides. No geral, a maturidade morfológica e funcional dos esferoides foi alcançada após o 12º dia em cultura.

Na segunda parte deste estudo, foi realizada a exposição de esferoides de hepatócitos por 6 dias (do 12º ao 18º dia em cultura) ao EE2 (1 ng/L, 10 ng/L, 50 ng/L ou 100 ng/L). Não houve alterações em termos de viabilidade celular, biometria e morfologia dos esferoides expostos a EE2. Os níveis de mRNA permaneceram inalterados, exceto para VtgA, que foram significativamente aumentados após a maior concentração de EE2.

Os presentes dados mostraram o potencial de esferoides de hepatócitos primários de juvenis de truta castanha como um modelo experimental *in vitro* para avaliar os efeitos disruptivos hepáticos de compostos estrogénicos. O modelo é uma alternativa para diminuir os testes *in vivo*.

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Chapter 1. Introduction

1.1 Environmental Contamination – General Considerations

Atmospheric, terrestrial and/or aquatic contamination can cause global disruptions on the normal ecosystem conditions. Currently, a large diversity of contaminants is recognized, namely polycyclic aromatic hydrocarbons, pesticides and pharmaceuticals (Saaristo et al., 2019, Liu et al., 2019). Specifically, the anticonception products represent a class of pollutants, for which a wide range of negative impacts in the aquatic biota have been described (Coricovac et al., 2018). These hormonal compounds are eliminated (parental, conjugated, or metabolized forms) by urine, and remain accumulated in waters and sediments, since they are not totally removed during the wastewater treatment process (Goldzieher and Brody, 1990, Müller et al., 2021). In addition to human and pets excretion, livestock animals also contribute to the increase of the environmental levels of hormones, since these compounds are used in hormonal therapy, promoting rapid growth and preventing several diseases (Kidd et al., 2007, Saaristo et al., 2019, Liang et al., 2017). According to Ojoghoro et al. (2021), there is still a need to invest more effort to identify the main sources of steroid hormones entering the aquatic ecosystems.

Hormones are natural or synthetic substances with a large range of functions, namely in the control of growth and development, reproduction and regulation of metabolism. Hormones are endocrine disrupting chemicals (EDCs) that can interfere with the function of the endocrine system, promoting several morphological and functional alterations in aquatic organisms leading to several disruptions in aquatic populations, including the collapse of a population, e.g. due the feminization of males and changes in fecundity by estrogenic compounds (Mathew et al., 2017, Cram et al., 2019).

1.1.1 Impacts of Endocrine Disrupting Chemicals in the Liver - In Vivo Examples

EDCs own the capacity to affect directly or indirectly biologic pathways, causing physiological and behavioral effects in exposed organisms (Ojoghoro et al., 2021). Several studies have been carried out to better understand the major liver effects caused by EDCs.

According to Voisin et al. (2019) exposure of mangrove rivulus (*Kryptolebias marmoratus*) to 4 ng/L or 120 ng/L of EE2 for 28 days caused an increase of some

differentially abundant proteins, such as proteases and proteins involved in the stress response. However, proteins involved in lipid and steroid metabolism, carbohydrate and energy metabolism were down-regulated after exposure to 4 ng/L. A continuous exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) over 21 days to Luppe River contaminated sediment with nonylphenol (maximum concentration of 22.1 mg/kg) and estrone (maximum concentration of 10.0 µg/kg), showed an increase abundance of transcripts in the liver, namely related to the induction of transmembrane protein 163-like (involved in the tolerance to metal ions), vitelline envelope protein α transcripts, CYP1A and Vtg (only for fish exposed to the 100 % Luppe sediment treatment) (Müller et al., 2021). Further, Marlatt et al. (2014) reported that Vtg was highly inducible in the liver of rainbow trout swim-up fry after 1 µg/L of 17β-estradiol (E2). It was also found by the same authors that exposures in the early stages of fish life affected growth-related genes.

1.1.2 A classic EDC – 17α-ethinylestradiol – Fish Liver Effects

EE2 is a semi-synthetic steroid, derived from estrone (Arsova-Sarafinovska et al., 2006, PubChem, 2022).

As a way to better characterize the EE2 potential fish liver impacts,, several studies have been carried out using *in vivo* and *in vitro* systems.

Thus, according to Nagler et al. (2010), Vtg synthesis in the liver of adult rainbow trout is regulated by intracellular ERs, since after an intra-arterial injection to 0.1 mg/kg of EE2 or 1.0 mg/kg of EE2-PEP (EE2 conjugated to a peptide and therefore cannot cross the cell membrane), only the non-conjugated EE2 stimulated Vtg synthesis. The induction of Vtg in male fish by EE2 was also studied with intra-arterial doses (0.001, 0.1, 1.0 and 10.0 mg/kg) in male rainbow trout. EE2 induced the Vtg synthesis (similar for all doses) with maximum levels within 7–9 days after injection (Schultz et al., 2001). A continuous water exposure to 125 ng/L of EE2 for up to 61 days caused a 10-fold increase in ERα mRNA levels after 48 h compared to the control group (Skillman et al., 2006). The ERα mRNA levels also significantly increased in liver of male rainbow trout after intra-peritoneal injections (0, 1.5, 15 or 150 µg) with E2 or an equimolar amount of EE2 (Boyce-Derricott et al., 2009).

Hultman et al. (2015b) showed that primary rainbow trout hepatocytes exposured to 0.03, 0.3, 3 and 30 nM of EE2 over 48 h had an increase expression of ERα, Vtg1, zona radiata proteins, but only for concentrations above 0.3 nM. Another study using 0.001 to 300 nM of EE2 over 96 h, suggested that primary hepatocytes from rainbow trout had an increase of Vtg expression, with the increase of concentrations, but just for the doses higher than 0.01 nM (Hultman et al., 2015a). Several concentrations of EE2 (0.01, 0.1 and 1 μM) were used for testing the impacts in primary hepatocytes from Atlantic salmon (*Salmo salar*), demonstrating a decrease of CYP1A expression and a increase of CYP3A, UGT, GST expression (Mortensen et al., 2007). A similar decreasing pattern of CYP1A expression was reported by Finne et al. (2007) after exposure of primary rainbow trout hepatocytes to 10 nM of EE2 for 24 h. An increase of VtgA and BSEP mRNA levels was also reported in this study.

1.2 Brown Trout (*Salmo trutta*) – Origin and Use in Ecotoxicology Studies

Brown trout (*Salmo trutta*) is endemic from Europe, north-western Africa and western Asia. It is the most widely distributed salmonid species in the world. Over the last years, the specie has been introduced in several ecosystems on most continents, except Antarctica (Couso-Pérez et al., 2019). This specie is polymorphic and can be found in freshwater or saltwater. Thus, Salmo trutta species present different migratory life histories and can be distinguishable between resident (river-resident, lake-resident) and migratory fish (Bernaś and Wąs-Barcz, 2020). Nevertheless, both are used by commercial purposes and recreational fishery, representing a huge economic importance (Couso-Pérez et al., 2019). It has been observed a decrease in wild trout populations, in part due to the increase of anthropogenic pressure. To counteract this, several methods to improve spawning have been developed with the main goal to increase the number of animals (Schubert et al., 2008).

Some field studies showed the negative impacts of xenobiotics in brown trout. Carrola et al. (2009) evidenced liver lesions in brown trout from contaminated harvest sites, namely Fraga-do-Borralheiro, Peliteira-Tinhela, Reboredo and Murça (along the Tinhela River, Portugal). Results showed a significantly increase in the hepatosomatic index, a decrease of condition factor and liver changes/lesions, such as bile duct hyperplasia, small and basophilic hepatocytes, hepatocellular necrosis, hepatocellular vacuolization and others several changes for trout captured at the contaminated sites when compared to the control group.

Furthermore, according to Bjerregaard et al. (2008), 166 brown trout (< 2 years) caught in the field had Vtg synthesis induced by exposure to estrogens in the environment. The same study showed that synthetic estrogens resulted in higher vitellogenin liver concentrations comparatively to natural estrogens.

In a laboratory context, an *in vivo* exposure (adult brown trout) to E2, estrone and 4-nonylphenol mixture (14.0 ng/l for estrone, 2.1 ng/l for E2 and 111.0 ng/l for 4-nonylphenol) during 150 days caused a plasma Vtg induction (Schubert et al., 2008). Uptake and biomarker responses in juvenile brown trout were also studied using 3- or 6-h pulses of concentrations up to 370 ng/L of E2. Results showed that a single 6 h pulse at the referred concentration induced both ERα and Vtg mRNA levels (Knudsen et al., 2011).

1.3 In Vitro Cultures

1.3.1 Bi-Dimensional (2D) versus Tri-Dimensional (3D) Cultures

Due to ethical reasons, a reduction number of animals involving scientific experiments have been the main objective over the last few years. In fact, the use of innovative and alternative methods in laboratory research, mainly in the field of toxicology, has been developed (Jaroch et al., 2018).

Initially developed in 1959, the "3R" policy - replace, reduce and refine - in order to sustain the handling of animals in research (reduce), minimize the suffering and stress of animals (refine) and use/adapt new research methods, where the results obtained have the same scientific value (replace), has proportionate this reduction (Russell and Burch, 1959).

Cell cultures have been allowing the control of environmental variables, enabling a greater reproducibility of results and assays on large scales. They are used as resources in the development of new medications, identification of several diseases targets, in toxicological tests (Souza et al., 2018).

2D cultures or also known as traditional monolayer cell cultures are a well-established methodology, but flat surfaces represent a disadvantage in comparison to 3D cell culture types, due to the lack of cellular structural architecture. Consequently, there is a loss of cell-cell and cell-matrix interactions. In addition, several other limitations have been also pointed, as the deficiency of mechanical/biochemical signals present in the *in vivo* environment, the

existence of non-physiological microenvironments and the rapid proliferation and cell differentiation (Souza et al., 2018, Shah et al., 2018). All these negative characteristics contribute for a gradual replacement by other *in vitro* models that have emerged and are closer *in vivo* models, as the 3D cultures. This *in vitro* system own a greater similarity to the *in vivo* in part due to the maintenance of polarity, more heterogeneous cell-cell interactions and better behavior and typologies/morphologies (Hultman et al., 2019, Pingitore et al., 2019).

The development of spheroids as a 3D model used in hepatic toxicology have been considered an interesting research alternative. In fact, spheroids can be used in longer exposures than 2D systems (Suurmond et al., 2019). Despite that, 3D culture models have been largely explored in human and rat studies.

1.3.2 3D Mammals Hepatocyte Cultures

The main advantages of using hepatocyte 3D cultures are related to the maintenance of hepatocyte function, that are associated to cell-cell interactions, transport of large macromolecules and metabolic activities, which according to Lee et al. (2010), are an indispensable prerequisite for these models. For 3D mammal hepatocyte systems, the methodology is now very well established. Primary human hepatocytes (PHH) in 3D spheroid configuration (gold standard in vitro model system) present closely analogy to human liver function. PHH are able to keep higher viability, functionality and stability for longer periods in culture, secreting and maintain levels of essential proteins, such as albumin and urea (Bell et al., 2016, Tostões et al., 2012, Kozyra et al., 2018). In addition, cellular polarity and functional bile ducts formation are also present in these aggregates (Bell et al., 2016, Messner et al., 2018). PHH spheroids also exhibited a stable phenotype over 5 weeks in culture (Messner et al., 2018).

Several new techniques have been tested as a way to improve these systems, such as the implementation of a continuous flow in 3D cultures, to facilitate the active transport of nutrients, metabolites, and oxygen in spheroids (Bachmann et al., 2015). 3D microfluidic cultivation systems have also been developed, such as multi-compartment hollow-fiber bioreactors, where hepatocyte spheroids of rats and humans maintained enzymatic activity and gene expression of several CYP450 family genes, transporters, and phase II enzymes,

over 3 weeks (Bachmann et al., 2015). The encapsulation of rat hepatocytes into alginate beads protects the cells from shear stress allowing CYP inducibility, albumin and urea secretion and maintenance of hepatocyte functionality over one month (Miranda et al., 2010). A multi-chamber modular bioreactor (MCmB, applied to human hepatocytes led to an up-regulation of CYP1A1 (among other CYP450 family genes), UGT, MDR1, MRP2 and several phase II enzymes. Finally, a up-regulation of CYP3A4, CYP2A1 in human hepatocytes was observed with the use of HepaChip® (Bachmann et al., 2015). The evolution of these models has allowed the study of several hepatic human diseases. A human hepatic 3D spheroid system was used to study steatosis and insulin resistance (Kozyra et al., 2018). The PHH spheroids were also used to assess liver diseases and drug-induced liver injury (DILI) (Bell et al., 2016). Spheroids were cultured for a period of at least 5 weeks, maintaining their morphology, viability, functionality and a stable phenotype. The same model was used to study steatosis and cholestasis, by using chlorpromazine and cyclosporine A as disease inducers (Bell et al., 2016).

1.3.3 3D Fish Hepatocyte Cultures

The use of 3D cultures in fish has been increasing, but exposure studies are very limited because models are still being characterized in terms of morphological and functional characteristics (Uchea et al., 2013, Baron et al., 2017).

According to Baron et al. (2012) the spheroids' maturity progress includes an immature (1–5 days) and a mature stage (more than 6 days), according to size and shape. Contrarily, Flouriot et al. (1993) reported that at 8 days post-isolation, spheroids presented variable sizes and shapes without a stable structure. Further, Lammel et al. (2019) also stated that the spheroid size and shape variability can affect cell functionality (Lammel et al., 2019). Taking this point into account, some authors have proposed that the best way to evaluated spheroid formation is to consider not only the morphological maturation, but also the functional and biochemical (protein, glucose, albumin and lactate dehydrogenase) characteristics of spheroids (Baron et al., 2012, Flouriot et al., 1993). However, the time spheroids reach structural stability seems not to correspond precisely to the point spheroids are functionally mature. In fact, according to Uchea et al. (2015) gene expression stabilization

(functional maturity) occur at a later stage than morphological maturation, which was reached at 6 to 8 days.

The ideal culture conditions for the formation of fish liver spheroids have been tested. Flouriot et al. (1993) reported that aggregation and spheroid stability was improved for at least one month using Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) supplemented with Ultroser G serum substitute (Ultroser SF). Furthermore, DMEM supplemented with serum replacement 3 was also reported by Uchea et al. (2015) as the medium that allowed to maintain primary rainbow trout hepatocyte spheroids in culture for 30 days. Baron et al. (2012) successfully used Leibovitz's L-15 medium (L-15) with 10 % fetal bovine serum (FBS) for culturing primary rainbow trout hepatocyte spheroids.

An important condition for hepatocyte spheroid formation seems to be the orbital shaking, that influences the cell aggregation, size and sphericity of spheroids over time (Baron et al., 2012, Lammel et al., 2019). The orbital shaking method is the most commonly used for obtaining hepatocyte fish spheroids, but other methodologies, such as the magnetic levitation and hanging drop have been implemented (Alves et al., 2022). A higher rotation speed (70 rpm rotation) was used by Baron et al. (2012) to culture primary rainbow trout hepatocyte spheroids, while Lammel et al. (2019) selected lower rotations (45 rpm and 50 rpm) as the best conditions to maintain rainbow trout liver cell line (RTL-W1) spheroids.

Spheroids' size is also an important parameter to consider. Electron paramagnetic resonance oximetry indicates that spheroids over 200 µm showed the presence of hypoxic cells and a necrotic/apoptotic core, affecting the spheroids functionality (Langan et al., 2016).

There are few studies that use hepatocyte spheroids to test exposure effects to distinct compounds. Exposure of rainbow trout liver spheroids to pyrene at a concentration of 25 nM for 30 h, showed an efficient biotransformation and elimination of the compound. Only mature spheroids, with more than 8 days, were used for exposure, since they were metabolically more active than immature (5–7d post-isolation) spheroids (Hultman et al., 2019). Furthermore, according to Rodd et al. (2017) an exposure to benzo-alpha-pyrene for 24 h at concentrations from 1 nM to 5 µM, caused changes in hepatocyte architecture and cell death. The effects of a 24 h exposure to E2 (1 µM) using rainbow trout hepatocyte

spheroids was published by Flouriot et al. (1993), evidencing an increase of ER and Vtg mRNA levels from 1 to 8 days in culture. The mRNA levels were still detectable at 30 days. Another study evaluated the exposure of rainbow trout hepatocyte spheroids to β-naphthoflavone at 0.36 µM, reporting an increase of EROD activity on day 30, but not on day 5 (Cravedi et al., 1996). Overall, data showed that fish hepatic spheroids respond to distinct inductors and retain metabolic capacity (Hultman et al., 2019, Baron et al., 2017).

1.4 Background and Aims

Aquatic contamination has been causing negative impacts on a wide range of organisms (Saaristo et al., 2019, Coricovac et al., 2018). In vivo and in vitro models are largely used for studying the morphological, mechanistical and functional alterations, that can be consequences of exposure to distinct contaminants. Over the last few years, a reduction of the number of animals used in scientific experiments has been a major objective of the 3R's policy (Replacement, Reduction, and Refinement). New replacement models emerged, such as the 3D cultures. The spherical multicellular aggregates (spheroids) in mammals have close responses to in vivo models (Alves et al., 2022). Despite that, 3D models are little explored in fish, as recently reviewed within the context of this Thesis (Alves et al., 2022).

Recently, in the host laboratory of this Thesis, it was established a new 3D in vitro model of brown trout (*Salmo trutta*) primary hepatocyte spheroids. Within that project the main goal was to define the culture conditions and to assess a biometric and morphological characterization of the model. Morphological maturity was achieved around the 12th day post-isolation, and spheroids remained viable until 30 days in culture. The model was also tested in terms of androgenic stimuli after exposures to 5α-dihydrotestosterone (5α-DHT), at 10 and 100 µM, which altered the mRNA levels of some specific lipidic target genes (Acox1-3I, PPARγ, Acsl1 and Fabp1) (Pereira et al., 2022).

Based on the described background knowledge, the present Thesis had two main purposes. The first aim was to functionally characterize the brown trout hepatocyte spheroids along distinct time points until day 25 in culture and to compare the 3D model with whole liver samples from the same individuals. Further, biometric, and morphological analyses will also complement spheroid’s characterization. Although it is known from the group's previous study

that morphological stabilization occurs around 12 days in culture, functional stabilization may occur at a later period, as suggested by others (Uchea et al., 2015). Thus, the morphological and functional maturity period will be proposed here as a step-forward to better characterize this 3D model for future ecotoxicological applications. A selection of different target genes involved in xenobiotic metabolism, efflux transport, and estrogenic pathways will be tested to assess the functional maturity of spheroids. Targets involved in xenobiotic metabolism were CYP1A, a liver microsomal cytochrome P-450 monooxygenase capable of biotransforming xenobiotics (Dar et al., 2020); CYP3A27, involved in the oxidation of distinct compounds and endogenous steroid hormones (Lee and Buhler, 2002); GST, that catalyzes the conjugation of electrophilic substrates to glutathione (Klusek et al., 2014); UGT, responsible for conjugates one or two glucuronic acid molecules to the carboxyl groups (Sugatani, 2013) and catalase (Cat) that catalyzes the conversion of hydrogen peroxide to water and oxygen (Goyal and Basak, 2010). The MDR1 (an ATP-dependent transmembrane transporter important in the detoxification of tissues from xenobiotics and toxins) (Bossennec et al., 2018), MRP2 (a phase II ATP-binding cassette transporter important in detoxification and chemoprotection through the transport of a wide range of lipophilic compounds) (Jedlitschky et al., 2006) and BSEP (an ATP-dependent protein responsible for the secretion of bile salts into the bile canaliculi of hepatocytes) (Thompson and Strautnieks, 2001) were chosen as efflux transport genes. Finally, VtgA, described as a well-known estrogenic biomarker (Lopes et al., 2020, Müller et al., 2021, Flouriot et al., 1993); ERα, a nuclear receptor activated by estrogenic hormones (Flouriot et al., 1993, Lopes et al., 2020) and ZP2.5, an inducible target by estrogens (Lopes et al., 2020) were selected as estrogenic target genes.

The second aim of this Thesis was to test the applicability of brown trout hepatocyte spheroids in response to a classic EDC (EE2) over 6 days of exposure to four concentrations (1 ng/L, 10 ng/L, 50 ng/L, 100 ng/L). Viability, biometric and morphological analyses will be done to characterize the exposed spheroids. mRNA levels of VtgA, CYP1A, GST, UGT and BSEP will be assessed to infer the EE2 effects.

2 Chapter 2. Materials and Methods

2.1 Fish Provenance and Maintenance

Brown trout juveniles were acquired at Torno Aquaculture (Amarante, Portugal). Acclimation occurred during at least 3 weeks, in 150 L tanks, under a continuous water recirculation system, at ICBAS-UP aquatic facilities. A photoperiod of 12 h light/12 h dark was maintained, and animals were fed daily (T4 Optiline, Skretting), except on the day prior to hepatocyte isolation procedure. Water quality was monitored at least once a week, obtaining the following values [mean (± standard deviation)]: ammonium and ammonia – 0.00 (± 0.00) mg/L, nitrates – 39.24 (± 5.3) mg/L, nitrites – 0.06 (± 0.03) mg/L, oxygen (O_2) – 88.41 (± 2.78) %, pH – 8.10 (± 0.23) and temperature – 19.22 (± 1.07) °C. Oxygen and temperature values were obtained with a specific probe (VWR® DO210).

2.2 Isolation Procedures

2.2.1 Stock Solutions, Buffers and Culture Medium Preparation

Five stock solutions were prepared, following the protocol described in Appendix 1. All buffers were sterilized by filtration, on the isolation day, using 0.2 µm membranes (Whatman™ ME24).

The Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F-12) (GE Healthcare Life Sciences) with 10% fetal bovine serum (FBS), 15 mM of 2-[4-(2-hydroxyethyl)1-piperazinyl]-ethanesulfonic acid (HEPES) and 10 mL/L of antibiotic/antimycotic solution (Appendix 1) was prepared and sterilized by filtration and stored on the fridge (≈ 4º C). The selection of this medium was based on previous experiments with brown trout hepatocyte spheroids (Pereira et al., 2022).

2.2.2 Isolation of Hepatocytes from Brown Trout

Fish were euthanized with an overdose of 0.6 mL/L of ethylene glycol monophenyl ether (Merck). All procedures involving animal handling followed the Portuguese Decree-Law No. 113/2013 implementing EU Directive No. 2010/63 on animal protection for scientific purposes. Hepatocytes were obtained through a two-step collagenase perfusion technique (Madureira et al., 2015).

Fish were weighed (KERN 572) and measured (standard and total lengths) and blood was collected through the caudal vein with an insulin syringe (Pic solution®).

The liver was collected and weighed (KERN 572), and a small sample of tissue was snap-frozen in liquid nitrogen, and stored at -80º C.

Collagenase perfusion started with the first buffer (Hank´s type buffer), by using a 24G needle plastic cannula (Abbocath) in the hepatic vein. The second buffer with collagenase at 0.05% allowed tissue disaggregation by collagen enzymatic digestion.

Mechanic disruption of the liver was achieved with the third buffer. The obtained cell suspension was filtrated with nylon membranes (200 µm and 50 µm). Then, centrifugation steps at 160 g for 5 min at 4 ºC (Eppendorf model 5804 R) were used for obtaining and washing the cell pellet. Cell viability was measured with an automatic cell counter (Invitrogen™, Countess™ Automated Cell Counter), using a 1:1 dilution of cell suspension and trypan blue 0.4 % (Invitrogen™).

2.3 Three-Dimensional (3D) Cultures

2.3.1 Optimization Assays

Primary hepatocytes were isolated from 5 independent fish (5 experiments). Biometrical parameters and liver weigh are detailed in Table 1.

Table 1. Biometrical parameters of juvenile brown trout used for optimization assays.

Parameters	Mean±standard deviation
Weigh (g)	50.15±13.11
Standard length (cm)	15.4±1.4
Total length (cm)	17.1±1.8
Liver weigh (g)	0.73±0.24

Cells were plated in non-treated 6 well-plates (Falcon®) at a cell density of 0.5x106 cells/mL. Each well had a total volume of 3 mL. Three replicates (three plates) were used per experiment, whenever sufficient cells were obtained. Cells were incubated at 18 ºC, without additional supply of O_2/CO_2 and at constant agitation (~100 rpm) (IKA® MTS 2/4 digital microtiter shaker). Spheroids were maintained during a maximum of 25 days in culture.

However, for two experiments, it was not possible to collect spheroids in the last day of sampling (25 days).

Medium change was performed on alternate days by removing 1.5 mL of old media, with insulin syringes (Pic solution®) and adding 1.5 mL of fresh one. Sampling was done at 8th, 12th, 16th, 20th and 25th days post-isolation. At each sampling day, spheroids were collected for viability, biometry, morphological and molecular analyses. The plate design for the optimization assays is shown in Appendix 2.

2.3.2 EE_2 Exposure Assays

Primary hepatocytes were isolated from 3 independent fish (3 experiments). Fish biometric parameters are presented in Table 2.

Table 2. Biometric parameters of juvenile brown trout used for exposure assays.

Parameters	Mean±standard deviation
Weigh (g)	82.32±12.31
Standard length (cm)	17.8±0.8
Total length (cm)	20.0±0.5
Liver weigh (g)	1.38±0.36

The initial cell culture conditions and spheroid maintenance until the 12th day post-isolation were the same as described for the optimization assays. Four replicates (four plates) were used per experiment and one well/plate corresponded to one of the following exposure conditions: control – C (DMEM/F-12 with 15 mM HEPES, 10 mL/L of antibiotic/antimycotic solution and charcoal FBS 10%, v/v), solvent control – SC (0.1% ethanol in supplemented DMEM/F-12 medium), and four EE2 concentrations – 1 ng/L (1), 10 ng/L (10), 50 ng/L (50) and 100 ng/L (100) in supplemented DMEM/F-12 medium. The layout of each plate was randomly assigned. The plates design for exposure assays is shown in Appendix 2.

The exposures started at 12th day post-isolation until the 18th day, in the same 6-well plates where the cells were plated initially, following the exposures layout shown in Appendix 2. Along this period, medium changes were performed on alternate days (14th and 16th days), by removing the maximum volume and adding 3 mL of the corresponding medium.

At the 18th day post-isolations spheroids were sampled for the same analyses described in the optimization assays.

2.4 Viability Assays

2.4.1 Lactate Dehydrogenase (LDH) Assay

LDH is a cytosolic enzyme that can be found in all cells. Under cytotoxicity conditions, the plasmatic membrane is damage (apoptosis, necrosis and other forms of cell damage) leading to the release of cytoplasmic enzymes, such as LDH. Free LDH will catalyze the dehydrogenation of lactate to pyruvate, reducing NAD to NADH. Then, NADH reduces a tetrazolium salt forming an orange formazan dye. The amount of formazan dye produced is proportional to the amount of LDH released into the medium. (Kumar et al., 2018).

The LDH Cytotoxicity WST Assay kit (Enzo Life Sciences, ENZ-KIT157) was used for measuring LDH activity in cell culture supernatant in 96-well microplates (non-tissue cultured-treated plate by Falcon®).

For each optimization and exposure assay the background controls (n = 12 and n = 8, respectively) were performed by adding 100 µL of the corresponding fresh culture medium to each well. Samples included 100 µL of supernatant medium from each well (after centrifugation at 1500 rpm for 5 min). A total of 12 (n = 4 supernatant medium samples taken from each well) and 8 samples (n = 2 supernatant medium samples taken from each well) were measured for each optimization and exposure assays. Then, 100 µL of working solution was added to all wells and plates were incubated for 30 min at room temperature and protected from light. Reactions were stopped by adding 50 µL of stop solution and the absorbance measured at 490 nm in a Multiskan GO device (Thermo Fisher).

The same protocol was used for exposure assays (Apendix 3).

All absorbances were adjusted by the respective background subtraction in both assays. Absorbances were plotted over time/condition.

2.4.2 Resazurin Assay

The principle of resazurin assay is based on the intracellular reduction of resazurin (blue-colored solution with no intrinsic fluorescence) into resorufin (fluorescent, pink-colored

product). After excitation, resorufin emits a quantifiable fluorescent signal (Präbst et al., 2017).

For each optimization experiment, at the sampling days, a total of 12 spheroids (4 spheroids from each plate) were collected and transferred individually to 96 well-plates (non-tissue cultured-treated plate by Falcon®) (Appendix 3 – sample wells). Then, 90 µL of fresh culture medium was added to each well. A stock solution of 2.2 mM of resazurin (Cayman Chemical Company) was prepared in sterilized PBS (1x) and working solutions of 0.44 mM were prepared by dilution. Each well had a final concentration of 44 µM of resazurin (10 µM of resazurin working solution), as previously described (Erikstein et al., 2010). Blank wells (without spheroids) were included by adding the same amount of medium, and resazurin in the wells (Appendix 3). The plates were protected from light and incubated at 18 ºC for 3 and 6 h, at constant agitation (~100 rpm). A microplate Synergy™ HTX multimode reader, with the software Gen5 (Biotek, Agilent) was used for fluorescent quantification at 550 nm and 588 nm, excitation and emission lengths respectively. RFU values for each sample were adjusted by blank subtraction. RFU values obtained at each sampling day were plotted over time.

2.4.2.1 AlamarBlue™ HS Cell Viability Reagent Assay

For each exposure assay, at the 18th day, 8 spheroids/condition (2 spheroids from each plate) were assigned individually to 96 well-plates (non-tissue cultured-treated plate by Falcon®) (Appendix 3). A total of 90 µL of the exposure medium was transferred to the corresponding spheroids. Then, 10 µL of the AlamarBlue™ HS Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific, A50101) was added to all wells. For each exposure condition, blanks (n = 8 wells) were performed using the corresponding medium without spheroids. The plates were protected from light and incubated at 18 ºC for 4 and 24 h, at constant agitation (~100 rpm). Fluorescence was read at 550 nm and 588 nm (excitation and emission lengths, respectively), using a microplate Synergy™ HTX multimode reader. Fluorescence values for each sample were adjusted by blank subtraction and viability percentage was calculated by applying the following formula:

$$\text{Viability (\%)} = \frac{\boldsymbol{RFU\ exposed}}{\boldsymbol{RFU\ control}} \times \mathbf{100}$$

2.5 Spheroids Biometric Parameter Analysis

Spheroids were photographed using an Olympus U-TV1X-2 digital camera coupled to an Olympus CKX41 light microscope, under a 10x objective lens at all sampling days (8th, 12th, 16th, 20th and 25th days post-isolation) for the optimization assay and at 12th, 16th and 18th days for the exposure assay.

Photos were acquired in a TIFF format and then changed for “TIFF-tiff” through ImageJ software. Imagens (n = 30 spheroids per time or condition) were analyzed using AnaSP software (Piccinini, 2015) to obtain the equivalent diameter, area and sphericity of spheroids.

2.6 Light Microscopy – Morphological Analysis

For the optimization assay, 6 spheroids were sampled at each day. Spheroids were individually transferred to 1.5 mL eppendorf tubes (Merck). Fixation was carried out using 500 µL of 10% w/v formaldehyde (Thermo Scientific), at room temperature. After 24 h, formaldehyde was changed to ethanol 70%. Then, spheroids were embedded using Richard-Allan Scientific HistoGel (Thermo Scientific). A 12 h histological procedure (ethanol 70% (1 h), ethanol 90% (1 h), ethanol 96% (1 h), ethanol 99.9% (1 h), ethanol 99.9%/xylene (1 h), xylene (1 h), xylene/paraffin (1 h) and paraffin (2 h)) was performed using an automatic processor (Leica TP 1020). Posteriorly, spheroids were embedded in paraffin (Thermo Scientific Histoplast), using a modular tissue embedding Leica EG 1140 C. Blocks were obtained and sectioned at 3 µm in a fully automated rotatory microtome (RM2255 Leica Biosystems). For optimization assays, 3 spheroids / sampling time were sectioned.

Paraffin sections (5 sections/slide) were stained using routine staining (Appendix 4), Hematoxylin & Eosin (H&E), for general tissue structure assessment. Sections were observed and photographed with a digital camera (Olympus EP50) coupled at light microscope.

For exposure assays, 2 spheroids/condition per plate were collected (total of 8 spheroids/condition) at 18th day post-isolation from each independent fish. Histological process was similar to the described previously. Three spheroids per condition were

sectioned (5 sections/slide) and stained with H&E, and Periodic Acid- Schiff (PAS) with and without digestion with α-amylase (Sigma-Aldrich). Staining protocol is detailed in Appendix 5.

2.7 Molecular Biology

2.7.1 RNA Extraction, Quantification and cDNA Synthesis

The spheroids collected for molecular analyses were pooled/fish. The pools of spheroids were centrifuged at 1500 rpm for 5 min and the pellets were snap-frozen in liquid nitrogen and stored at -80ºC.

An illustra™ RNAspin Mini RNA isolation Kit (GE Healthcare) was used for total RNA extraction, which includes a DNase I treatment step.

RNA purity and quantification was obtained using a µDrop™ Plate (Thermo Scientific) in a Multiskan GO equipment (Thermo Fisher), using a SkanIt Microplate Reader software (Thermo Scientific). The λ 260/280 nm ratio was always (mean ± standard deviation) 2.20 ± 0.1. Agarose gel with GelRed (Biotium) staining was performed. Images were acquired using a ChemiDoc XRS$^+$ (BioRad) with the ImageLab data analysis software (BioRad).

cDNA synthesis was performed using a iScript™ Reverse Transcription Supermix kit for a total volume of 20 µL using 300 ng of total RNA, fowling the respective protocol.

2.7.2 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using a CFX Connect real-time PCR detection system, with a CFX Manager software (Bio-Rad). SYBR Green reactions had a total volume of 20 µL, which included 10 µL of iQ™ SYBR® Green Supermix (Bio-Rad), 5 µL of cDNA (diluted 1:5), 200 nM of each primer and the appropriate volume of water. The cDNA samples were analyzed in duplicate and no-template controls were also included. In each run, the product specificity was checked by performing a melt curve. The Pfaffl method was used for relative quantification (Pfaffl, 2001). Reference gene normalization was made with the geometric mean of ef1a (elongation factor-1 alpha) and β-actin for optimization assays and rpl8 (ribosomal protein L8) and gapdh (glyceraldehyde-3-phosphate dehydrogenase) for exposure assays. The selection of the most stable reference genes was done with the NormFinder algorithm (Andersen et al., 2004). The

target genes in the optimization assays were: CYP1A, CYP3A27, GST, UGT, cat, BSEP, MDR1, MRP2, VtgA, ERα and ZP 2.5. For the exposure assay, target genes included: CYP1A, GST, UGT, BSEP and VtgA. All primer sequences and efficiencies are detailed in Appendix 6. For the new optimized genes (CYP1A, CYP3A27, GST, BSEP, MDR1 and MRP2), calibration curves were obtained using serial dilutions of brown trout liver cDNA (Appendix 7).

2.8 Statistical Analyses

Past 3 software, version 3.25 was used for statistical analyses. All graphs presented were generated with GraphPad Prism 8. Normality and homogeneity of data were assessed by Shapiro-Wilk and Levene's tests, respectively. Subsequently, one-way analysis of variance (ANOVA) was performed, and Tuckey's pairwise comparisons test was used. When assumptions were not met, even after data transformation, the Mann-Withney test with sequential Bonferroni corrections was used. Significant differences where considered when p value < 0.05.

3 Chapter 3. Results

3.1 Optimization Assay of Spheroids from Brown Trout Primary Hepatocytes

3.1.1 Biometric Parameters

The biometric parameters of spheroids cultured over 25 days are represented in Figure 1.

The spheroids´ area significantly increased until the 16th day in culture (highest median area value of 27739 μm^2) and after that it was almost stabilize until the 25th day.

The diameter increased significantly until the 12th day. The lowest median value was obtained at day 8 (132 µm), while at 12th, 16th and 20th days median values were 187 µm, 191 µm and 193 µm, respectively.

For sphericity no major differences were noted up to day 20 in culture. Values varied between 0.95 and 0.96 during that period. At the 25th day, sphericity significantly decreased comparing to the other days.

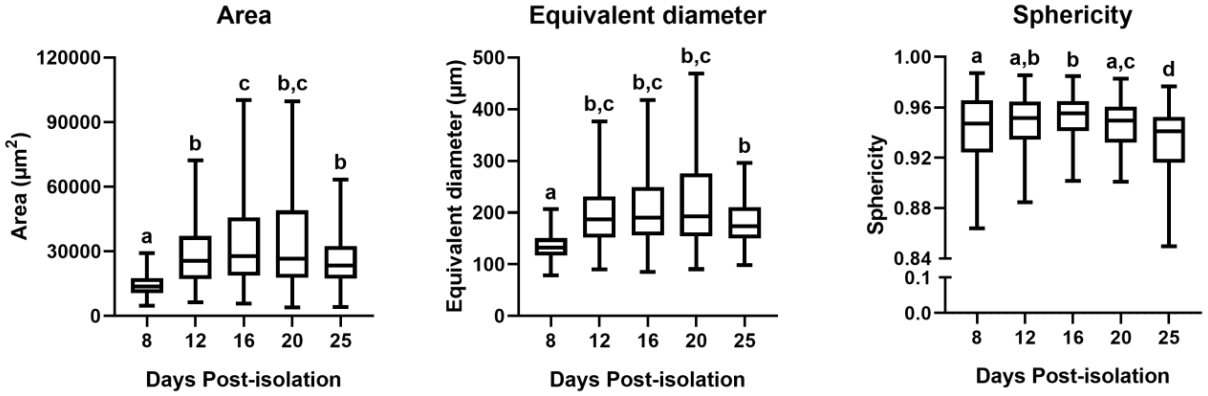


Figure 1. Biometric parameters of brown trout hepatocyte spheroids over 25 days in culture. Values were obtained by performing manual segmentation of photographs using the AnaSP software (n= ± 30 spheroids for each day). Data correspond to median, minimum and maximum values. Significant differences ($p < 0.05$) between days are represented by different letters, according to Mann-Whitney with Bonferroni sequential corrections.

3.1.2 Viability Assessment

LDH and resazurin data are represented in Figure 2.

For LDH, the highest median absorbance was obtained at 8th day and the lowest at 25th day. From 16th to 20th day the absorbances were stable.

In general, for resazurin, the RFU values did not vary significantly between the days in culture, neither using 3 nor 6 h of incubation.

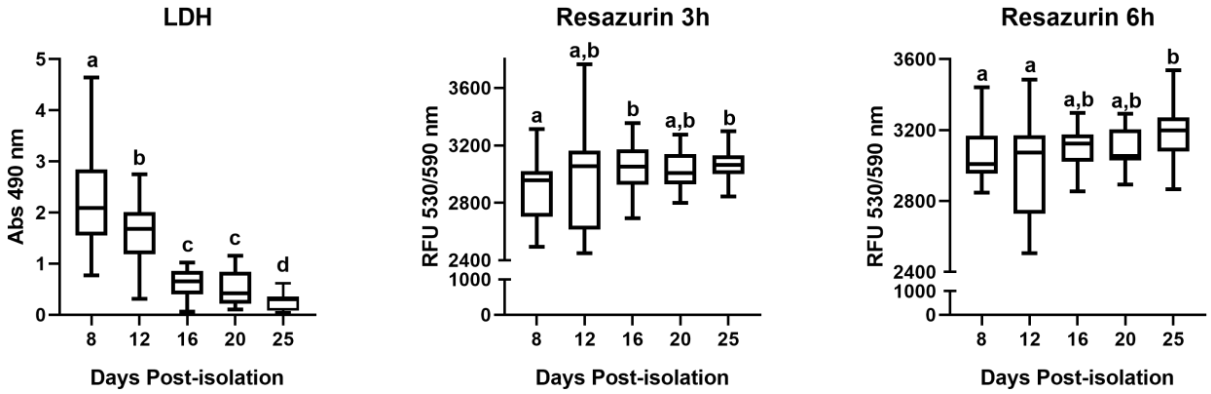


Figure 2. LDH (n= 12 well for each day) and resazurin data at distinct days in culture (n= 12 spheroids for each day). Data correspond to median, minimum and maximum values. Significant differences ($p < 0.05$) between days are represented by different letters, according to Mann-Whitney with Bonferroni sequential corrections. Abs 490 nm: Absorbance at 490 nm.

3.1.3 Morphology – Qualitative Analysis

The brown trout hepatocyte spheroids showed a spherical/elliptical shape in all time-points and increased size and compactness along the days in culture (Figure 3). Also, spheroids have a well-defined external limit on the periphery from day 8. Hepatocytes presented a defined cellular membrane and an intact nucleus. Several cytoplasmic vacuoles were noted along the days in culture.

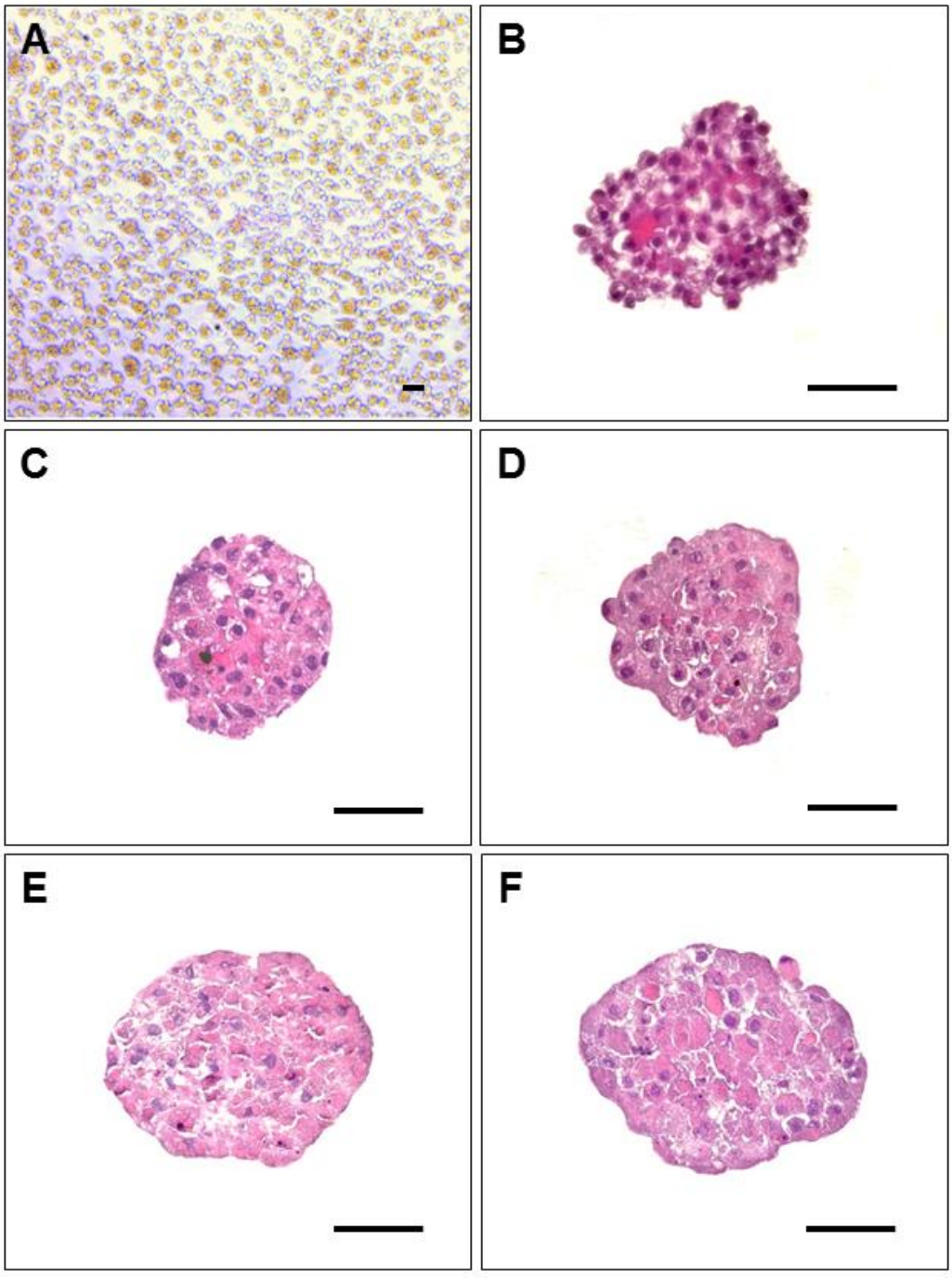


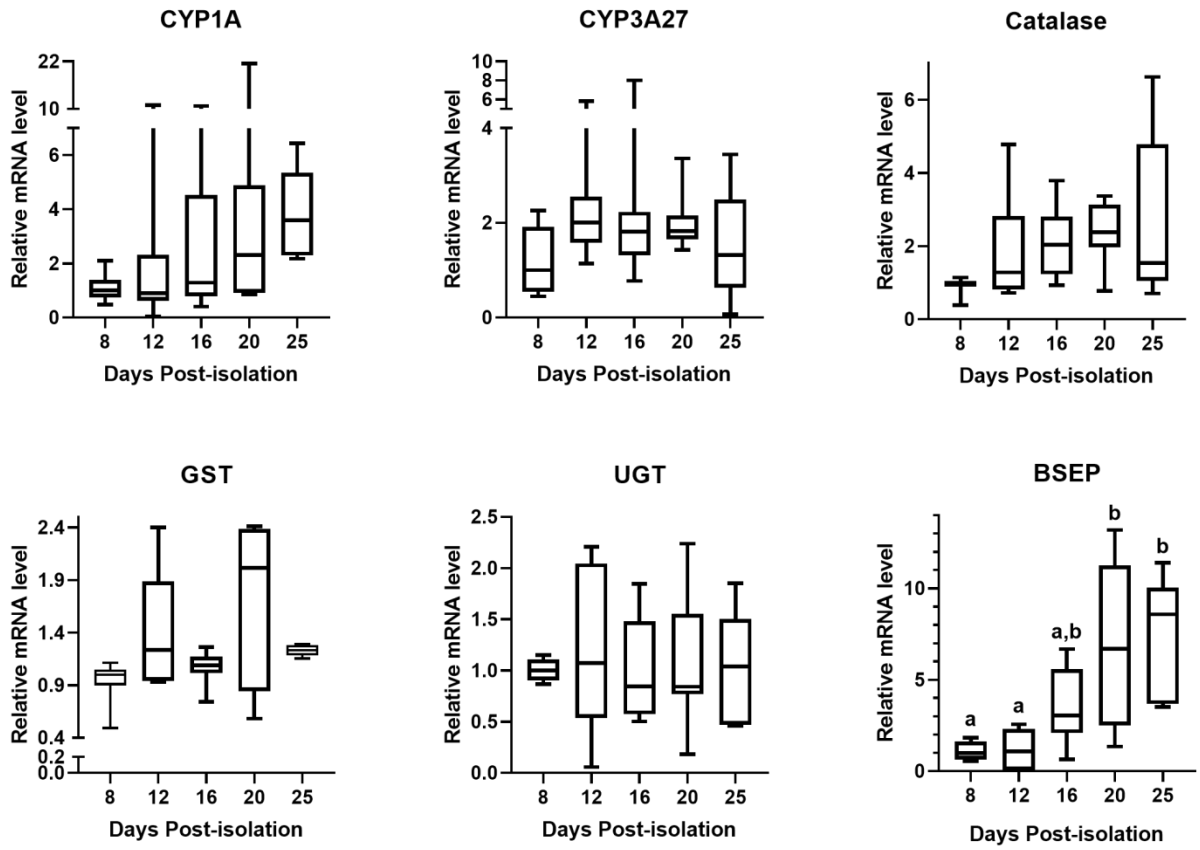
Figure 3. Freshly isolated hepatocytes and H&E histological sections of brown trout hepatic spheroids along 25 days in culture. A) Isolation day; B) 8th day; C) 12th day; D) 16th day; E) 20th day and F) 25th day. Scale bar= 50 μm.

3.1.4 Gene Expression

No significant differences were noted in the mRNA levels of CYP1A, CYP3A27, catalase, UGT and GST (Figure 4).

The MDR1 and MRP2 mRNA levels were also stable over the 25 days in culture (Figure 4). Contrarily, at 20th and 25th days the BSEP mRNA levels were significantly higher than the ones obtained at 8th and 12th days.

ZP2.5 and VtgA mRNA levels were stable between the 12th and 20th day (Figure 4). ZP2.5 mRNA levels were significantly up-regulated at 25th day comparing with the 8th day, while VtgA mRNA levels were down-regulated at 25th *versus* the 8th day (Figure 4). The ERα mRNA levels also showed a stability period between the 12th and 20th day, but significantly higher levels were found at the 20th and 25th days comparing with the 8th day (Figure 4).



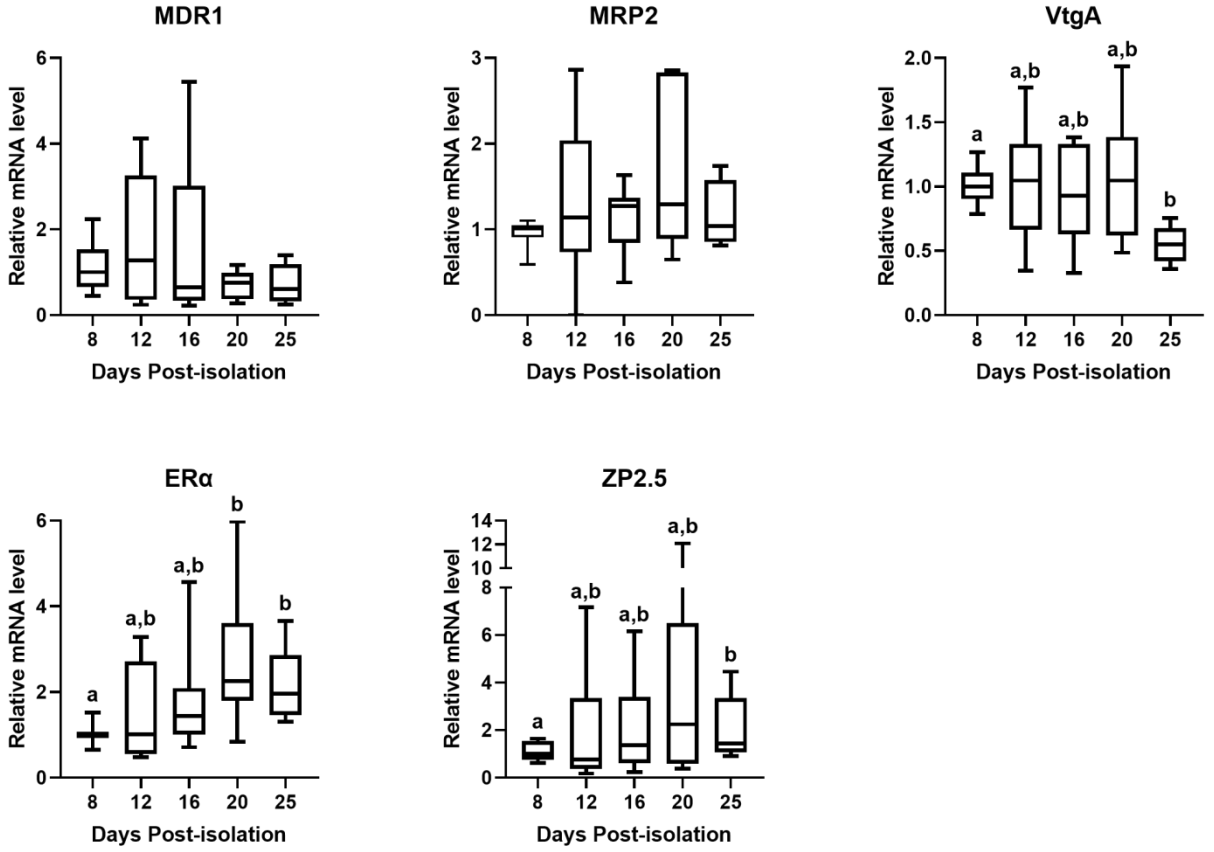


Figure 4. Relative mRNA levels of metabolization (CYP1A, CYP3A27, Cat, GST and UGT), efflux transporter (BSEP, MDR1 and MRP2) and estrogenic (VtgA, ERα and ZP2.5) target genes in primary hepatocyte spheroids from brown trout juveniles during 25 days in culture. n= 37 samples. Data correspond to median, minimum and maximum values. Significant differences ($p < 0.05$) between days are represented by different letters, according to Mann-Whitney with Bonferroni sequential corrections. CYP1A: Cytochrome P450 1A; CYP3A27: Cytochrome P450 3A27; Cat: Catalase; GST: Glutathione S-transferase; UGT: UDP-glycosyltransferase; BSEP: Bile salt export pump; MDR1: Multidrug Resistance Protein 1; MRP2: Multidrug resistance-associated protein 2; VtgA: Vitellogenin A; ERα: Estrogen receptor alpha; ZP2.5: Zona pellucida glycoprotein 2.5.

3.1.5 Expression Levels in Whole Liver versus Hepatocyte Spheroids of Brown Trout

Expression levels of metabolization (Catalase, CYP1A, CYP3A27, GST and UGT), efflux transporters (MDR1, BSEP and MRP2) and estrogenic target genes (ZP2.5, ERα and VtgA)

were compared between spheroid cultures (8, 12, 16, 20 and 25 days post isolation) and whole liver samples (Figure 5).

In general, results suggest lower expression levels for the spheroid in comparison to the whole liver. However, some exceptions were pointed out, such as GST and VtgA, which showed higher levels of expression mainly at the first times of culture.

A similar pattern between BSEP and CYP1A was found, since these two genes were the only ones that maintained expressions until 25th day in culture, and showed the highest expression on that day.

Overall, for most genes, spheroids presented the closest pattern to *in vivo* expressions from 8th day to 20th day, except BSEP and CYP1A that was after 12th days until the last culture day.

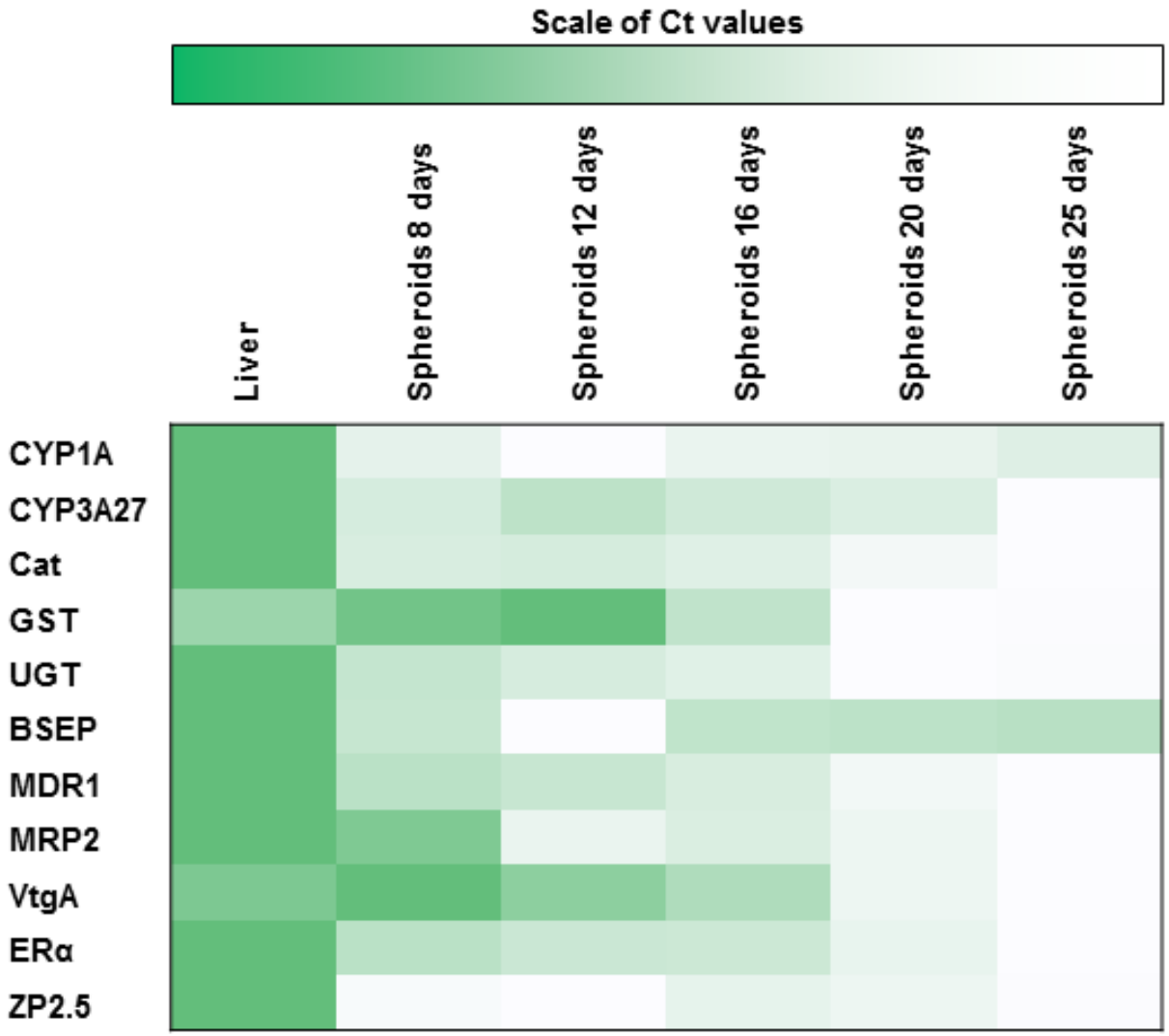


Figure 5. Comparison of expression levels of xenobiotic metabolization, efflux transport and estrogenic target genes in hepatocyte spheroids of brown trout juveniles over 25 days in culture and whole liver samples. The highest Ct value (lowest level of expression) is shown in white and the lowest Ct value (highest level of expression) is shown in green. CYP1A: Cytochrome P450 1A; CYP3A27: Cytochrome P450 3A27; Cat: Catalase; GST: Glutathione S-transferase; UGT: UDP-glycosyltransferase; BSEP: Bile salt export pump; MDR1: Multidrug Resistance Protein 1; MRP2: Multidrug resistance-associated protein 2; VtgA: Vitellogenin A; ERα: Estrogen receptor alpha; ZP2.5: Zona pellucida glycoprotein 2.5.

CHAPTER 3. RESULTS

3.2 Exposure Assay of Spheroids from Brown Trout Primary Hepatocytes

3.2.1 Biometric Parameters

Biometric results of spheroids obtained from brown trout primary hepatocytes and exposed to four different concentrations of EE2 (1 ng/L, 10 ng/L, 50 ng/L and 100 ng/L) are presented in Figure 6.

For all parameters (area, equivalent diameter and sphericity), results suggest no impact of EE2 exposure.

However, in general, occasional significant differences were pointed in some groups, when compared the last two days of exposure, as shown in Figure 6.

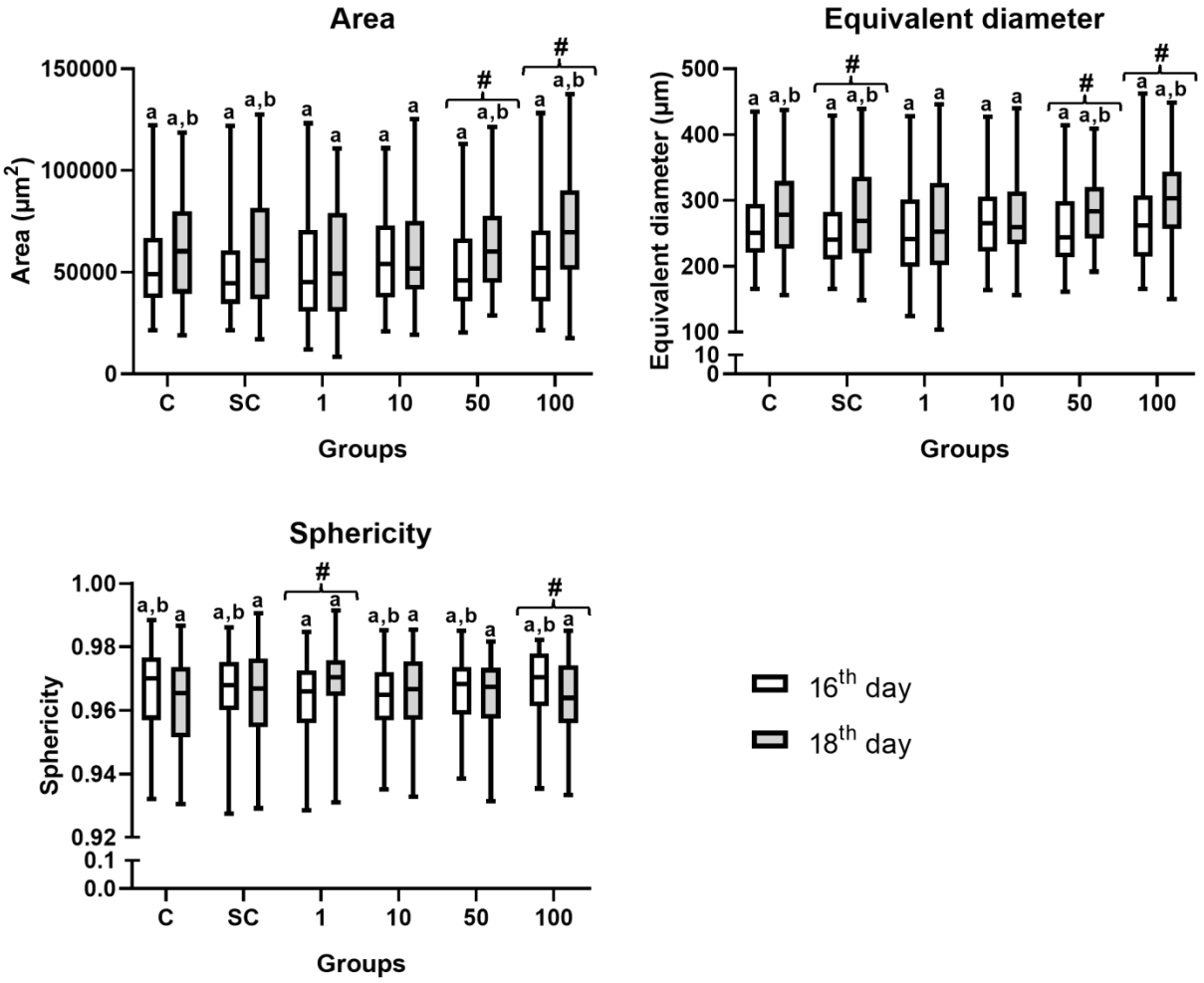


Figure 6. Biometric parameters of brown trout hepatocyte spheroids after 6 days exposures to: C: control (supplemented DMEM/F-12 medium), SC: solvent control (0.1% ethanol in supplemented DMEM/F-12 medium), 1: 1 ng/L of EE2 in supplemented DMEM/F-12 medium, 10: 10 ng/L of EE2 in supplemented DMEM/F-12 medium; 50: 50 ng/L of EE2 in supplemented DMEM/F-12 medium and 100: 100 ng/L of EE2 in supplemented DMEM/F-12 medium. Values were obtained by performing manual segmentation of photographs using the AnaSP software (n= ± 30 spheroids per condition/each sampling day). Data correspond to median and range. Significant differences ($p < 0.05$) between groups are represented by different letters according to Mann-Whitney with Bonferroni sequential corrections. #: significant differences ($p < 0.05$) between sampling times (16^{th} and 18^{th} days) according to Mann-Whitney with Bonferroni sequential corrections.

3.2.2 Viability Assessment

The LDH and resazurin (at both 4 and 24 h) assays did not evidence significant differences between exposure groups, as shown in Figure 7.

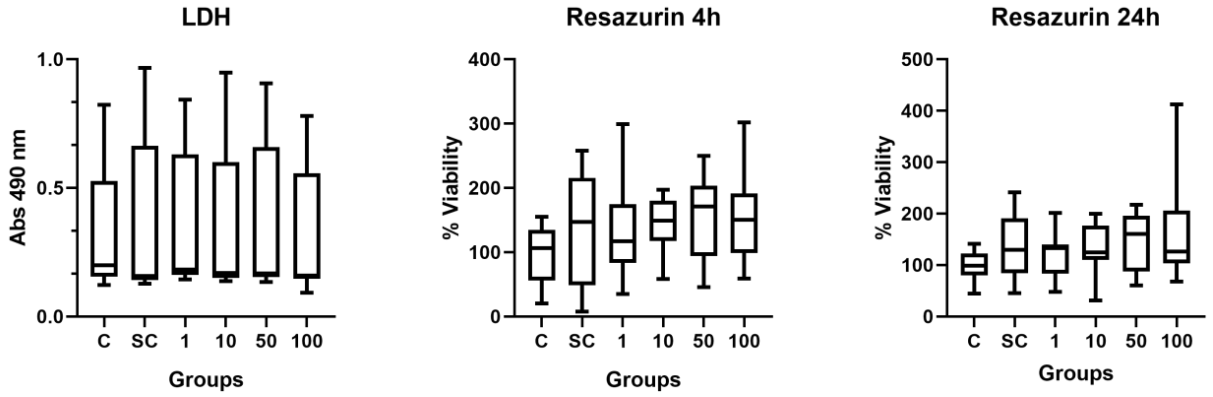


Figure 7. LDH (n= 8 well for condition/ each sampling day) and resazurin (n= 8 spheroids for for condition/ each sampling day) data of brown trout primary hepatocyte spheroids after 6 days of exposures to: C: control (supplemented DMEM/F-12 medium), SC: solvent control (0.1% ethanol in supplemented DMEM/F-12 medium), 1: 1 ng/L of EE2 in supplemented DMEM/F-12 medium, 10: 10 ng/L of EE2 in supplemented DMEM/F-12 medium; 50: 50 ng/L of EE2 in supplemented DMEM/F-12 medium and 100: 100 ng/L of EE2 in supplemented DMEM/F-12 medium. Data correspond to median, minimum and maximum. Abs 490 nm: Absorbance at 490 nm.

3.2.3 Morphology – Qualitative Analysis

No evident alterations were noted in the general structure of spheroids and hepatocytes between exposures (Figure 8).

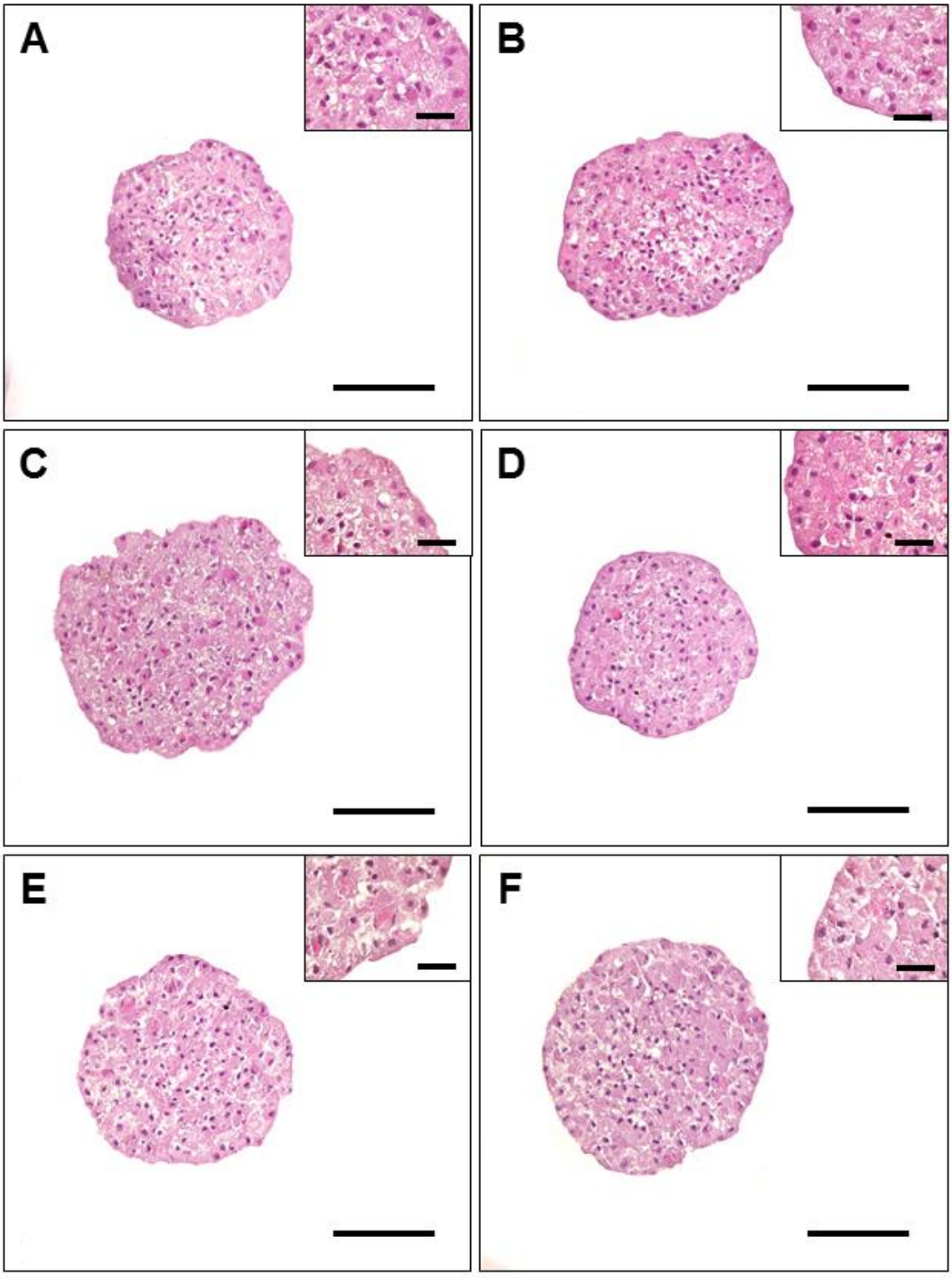
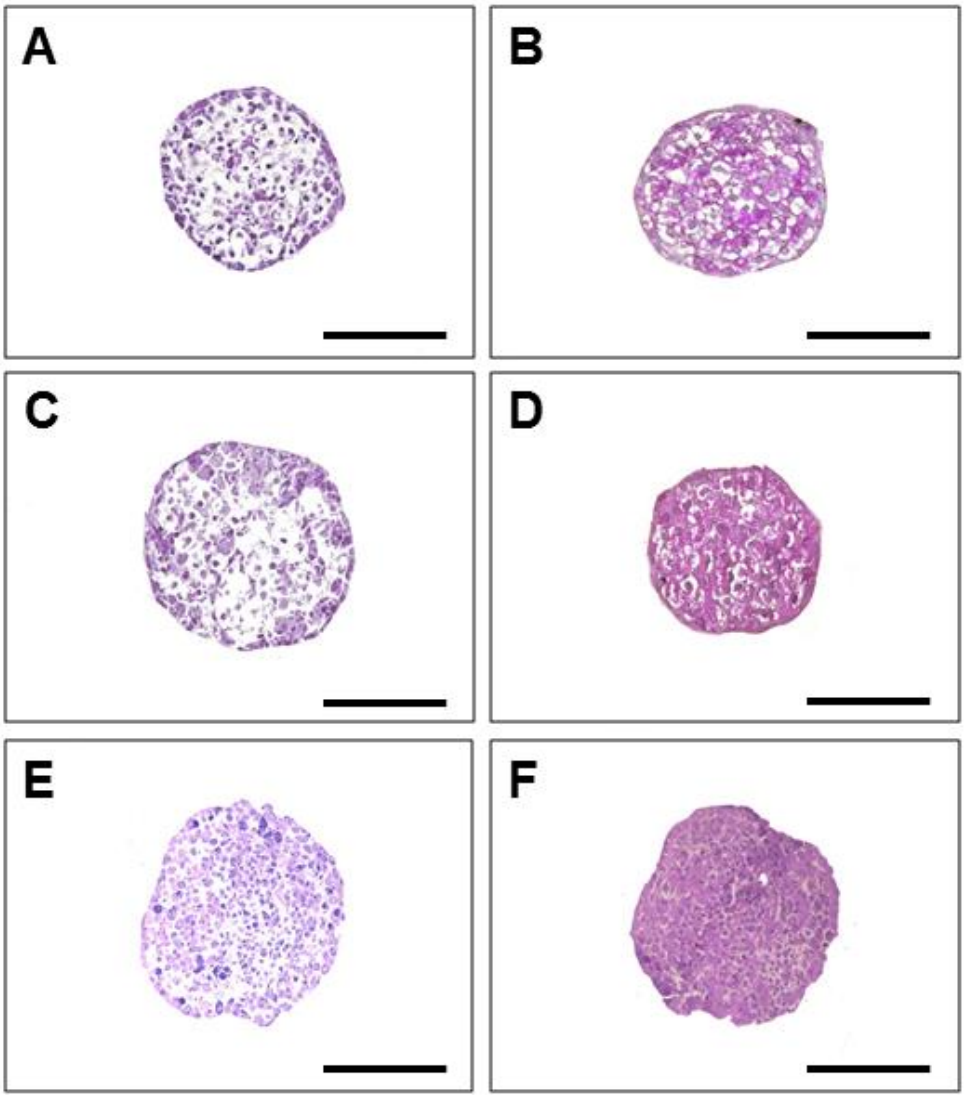

A
B
C
D
E
F

Figure 8. Histological sections of 18 day-old brown trout spheroid exposed to EE2 and stained with H&E. A) Control; B) Solvent control; C) 1 ng/L of EE2; D) 10 ng/L of EE2; E) 50 ng/L of EE2 and F) 100 ng/L of EE2. Scale bar 20x = 100 µm. Scale bar 100x = 27 µm.

PAS and PAS with α-amylase allowed the identification of a disperse localization of glycogen in spheroids (Figure 9). Control spheroids presented a close pattern of glycogen content to the ones exposed to lower concentrations of EE2 (1 and 10 ng/L) (Figures 9A-D *versus* E-H). Changes in staining intensities suggested a decrease of glycogen content, with the increase of EE2 concentrations (Figure 9I-L).



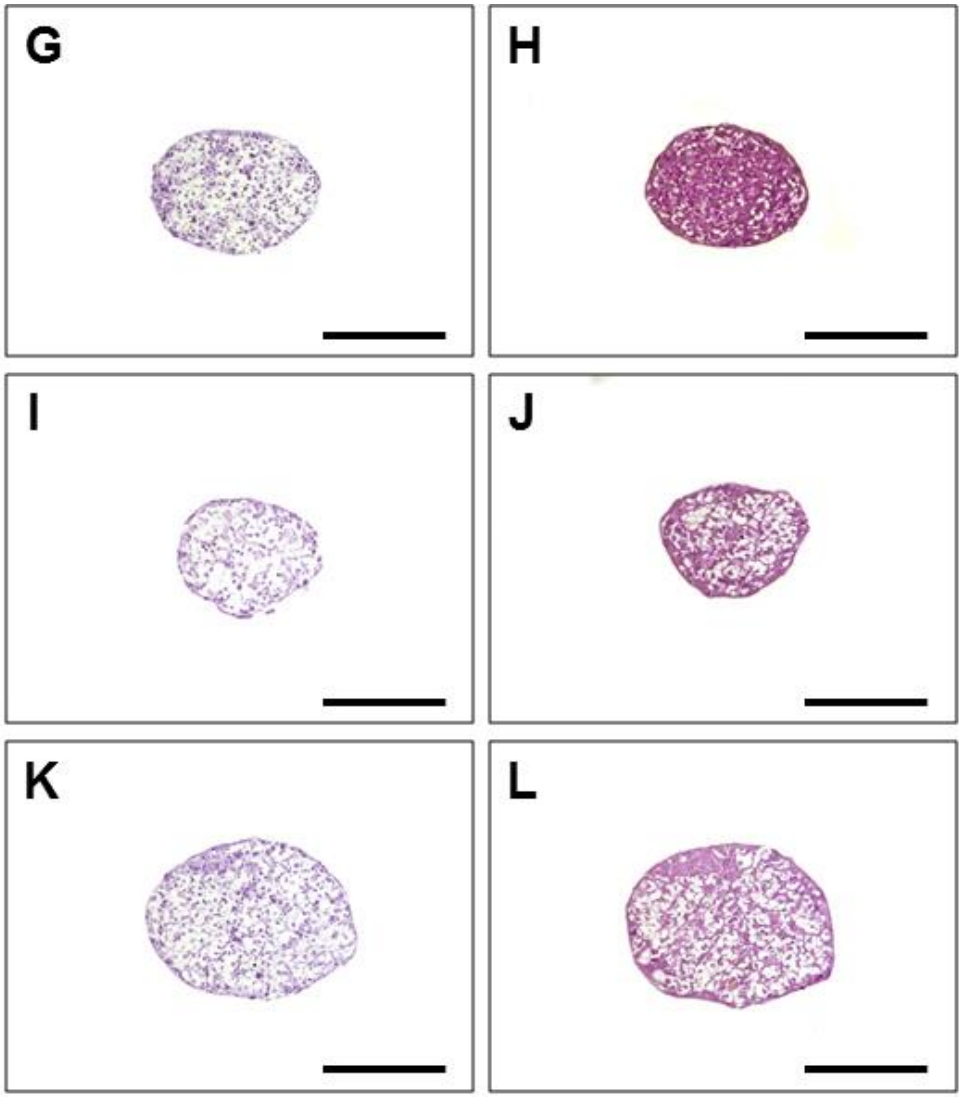


Figure 9. Histological sections of 18 day-old brown trout spheroid exposed to EE2 after 6 days and stained with PAS (B, D, F, H, J and L) and PAS with α-amylase (A, C, E, G, I and K). A and B - Control; C and D - Solvent control; E and F - 1 ng/L of EE2; G and H - 10 ng/L of EE2; I and J - 50 ng/L of EE2 and K and L - 100 ng/L of EE2. Scale bar= 100 µm.

3.2.4 Gene Expression

mRNA levels of xenobiotic metabolism (CYP1A, GST and UGT) and efflux transport (BSEP) genes were not altered by EE2 (Figure 10).

VtgA mRNA levels were significantly higher after exposure to 100 ng/L of EE2 comparing with the remaining conditions (Figure 10). Lower doses (1, 10 and 50 ng/L) of EE2 did not caused significative impacts on VtgA mRNA levels, but a dose effect concentration was observed.

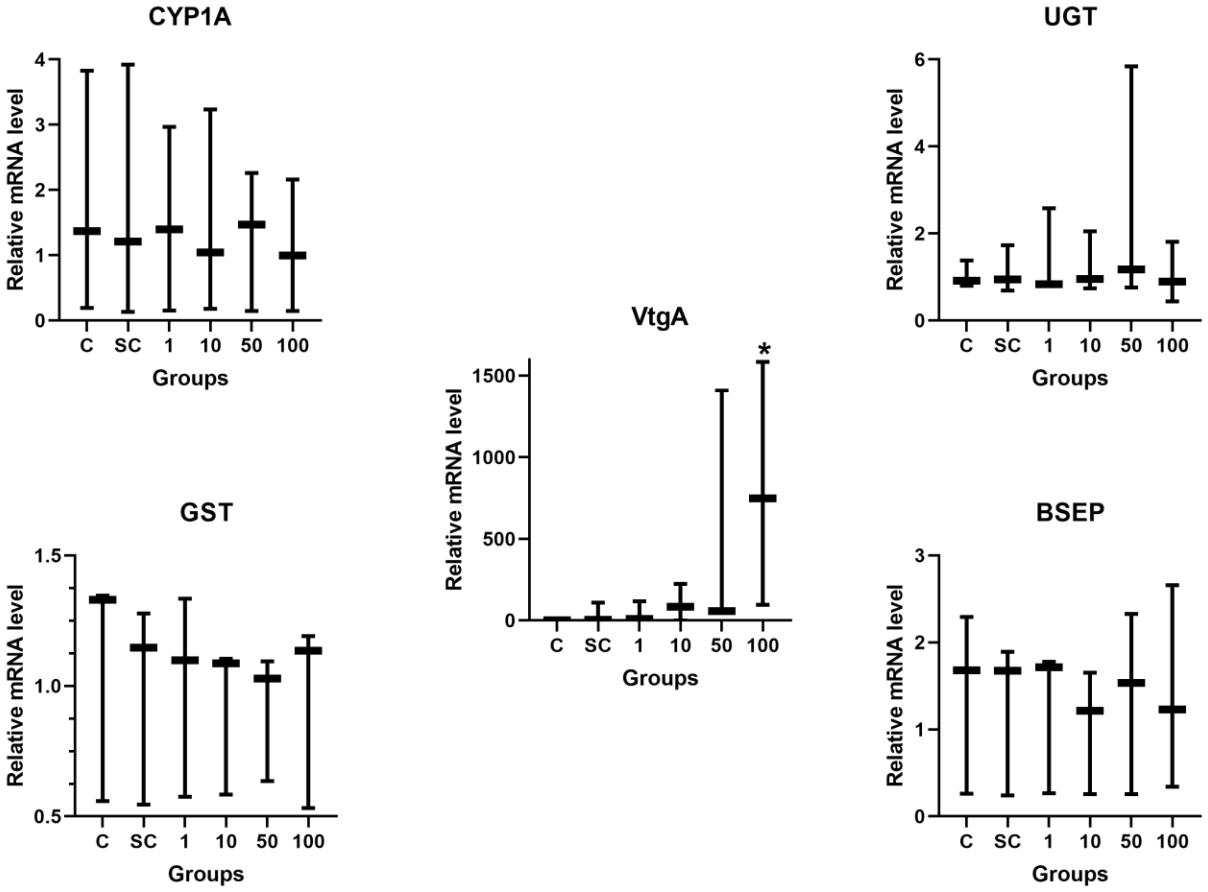


Figure 10. Relative mRNA levels of xenobiotic metabolism (CYP1A, GST and UGT), efflux transport (BSEP) and estrogenic target (VtgA) genes of brown trout hepatocyte spheroids after 6 days exposure to: C: control (supplemented DMEM/F-12 medium), SC: solvent control (0.1% ethanol in supplemented DMEM/F-12 medium), 1: 1 ng/L of EE2 in supplemented DMEM/F-12 medium, 10: 10 ng/L of EE2 in supplemented DMEM/F-12 medium; 50: 50 ng/L of EE2 in supplemented DMEM/F-12 medium and 100: 100 ng/L of EE2 in supplemented DMEM/F-12 medium. Data correspond to median, minimum and maximum. CYP1A: Cytochrome P450 1A; BSEP: Bile salt export pump; GST: Glutathione S-transferase; UGT:

UDP-glycosyltransferase; VtgA: Vitellogenin A. * Significant differences ($p < 0.05$) in comparison to control group, according to Tukey´s test.

4 Chapter 4. Discussion

4.1 Biometric and Morphologic Characterization of Hepatocyte Spheroids from Juvenile Brown Trout over Optimization Assays´

Fish spheroids characterization over the culture days has been done by cell viability, functionality and morphological analyses.

After 24 h to 48 h in culture, primary hepatocytes started to aggregate forming heterogeneous agglomerates of irregular size and shape. Thus, despite the enzymatic/mechanical dissociation during the isolation procedure, hepatocytes were able to restore cell-cell interactions, as previously described (Baron et al., 2012, Abu-Absi et al., 2002).

At 10th day post-isolation, spheroids became more compact, and it was difficult to distinguish individual hepatocytes. Furthermore, the size of spheroids increased along the time in culture and their shapes became more elliptical/spherical with well-defined limits. According to Baron et al. (2012) and Uchea et al. (2015), all these characteristics can be considered markers´ of morphological maturity of spheroids. Even so, it is known that spheroids size continues to increase even after 10 days (Baron et al., 2012), which corroborates the results found in this study. The morphological stabilization period was found to be between the 12th and the 20th day. At 25th day it was noted a decrease in the spheroid's sphericity, which is probably related with the cluster formation between spheroids. Lammel et al. (2019) interrupted the rotation when the spheroids are formed to avoid clusters formation. Results showed that the procedure delayed the formation of large agglomerates, but did not avoid their formation (Lammel et al., 2019). Changes in the sphericity at the 25th day, were also related with an increase cell disaggregation that was noticed at this time. Area and equivalent diameters deceased, but without significant differences. Cell disaggregation reported may be related with a possible alteration in cell-cell interaction or even in the extracellular matrix content, thus compromising the longevity of spheroids in culture.

The H&E staining of spheroids at the distinct time-points corroborate the biometric characterization of spheroids along the 25 days in culture. It has been reported, namely in mammals that larger spheroids (diameters of 500 µm) showed a necrotic/ apoptotic core due

to the limited diffusion of oxygen and nutrients to the inner part of spheroid (Zanoni et al., 2016). In this study, spheroids had a median diameter of 190 µm (between 12th to 20th day), and it was not observed in any case necrotic/apoptotic areas. In fact, resazurin assay was used to assess viability at different sampling times, and no changes were observed. However, it should be noted that in the resazurin test, we found that smaller spheroids led to sensitivity problems. Thus, it is recommended to use for this protocol a high number of spheroids or a higher sensitivity assay (as used in exposure assays). LDH assay was used to measure the LDH released to the culture media, which occurs after damage caused in cell plasmatic membranes (Bains and Kennedy, 2004, Baron et al., 2012). Here, a significant decrease in the LDH levels from 8th day up to 25th day was noted. This suggests that mature spheroids were able to retain membrane integrity, avoiding the LDH leakage. In accordance, it was already reported that mature spheroids can retain seven-fold higher amounts of intracellular LDH in comparison to immature spheroids (Baron et al., 2012). Further, it is reported that LDH leakage is a great indicator of cell membrane integrity, very useful to elucidate the viable state of spheroids

4.2 Functional Characterization of Hepatocyte Spheroids from Juvenile Brown Trout over Optimization Assays´

The number of studies that evaluate the functional capacity of hepatocyte spheroids in fish is very limited, as recently reviewed (Alves et al., 2022). In this study, hepatocyte spheroids from brown trout were characterized in terms of mRNA expression levels of xenobiotic metabolization, efflux transport and estrogenic target genes along different times in culture. No significant differences were noted in the mRNA levels of xenobiotic metabolization genes (CYP1A, CYP3A27, Cat, UGT and GST). Despite that, for CYP1A mRNA levels higher levels of expression were observed in mature spheroids. The same pattern was previously noted by Uchea et al. (2015). Overall, the expression levels of xenobiotic metabolization genes from the 8 days onwards increase (although with no significant differences). In fact, this increase could be explained by the fact that early-formed spheroids presented a period of biochemical and functional instability, which then stabilizes with the days in culture. Similar facts were reported for rat hepatocyte spheroids (Ma et al., 2003) and rainbow trout hepatic spheroids (Uchea et al., 2015).

CHAPTER 4. DISCUSSION

Regarding the expression of efflux transport genes, the MDR1 and MRP2 mRNA levels remained stable over culture time. Stable expression levels of efflux transporters´, in trout cell lines (Boaru et al., 2006, Loncar et al., 2010), appear to be related to differentiation status, that can be lost along the isolation procedure (Uchea et al., 2015). According to Boaru et al. (2006), lower levels of organic anion transporter polypeptide (OATP) expression, responsible for the uptake of microcystin-LR, were noted in a rainbow trout cell line (RTL-W1). Freshly isolated hepatocytes showed higher levels of expression of OATP, presenting several damages in subcellular structures, thus indicating the importance of the differentiation status on the OATP expression.

Contrarily, BSEP was the efflux transporter that evidenced an increase of mRNA levels over time and showed the greatest expression of all transporters. In rainbow trout liver, Zaja et al. (2008) reported that BSEP levels were 750 and 114 folds higher than MDR1 and MRP2, respectively. Higher expression of BSEP compared to other efflux transporters was also found by Uchea et al. (2015). Further, the canalicular structure for bile release was evidenced in fish (Uchea et al., 2015) and rat (Abu-Absi et al., 2002) mature spheroids, suggesting that spheroids retain BSEP expression and efflux functions. In hepatocyte-derived cell lines these structures were not observed (Uchea et al., 2015, Abu-Absi et al., 2002), and the functional activity of BSEP was also not identified (Fischer et al., 2011).

Finally, estrogenic target genes (ZP2.5, ERα and VtgA) were stable between the 12th and the 20th day. This stability was also reported during at least one month in rainbow trout hepatocyte spheroids (Flouriot et al., 1995).

4.3 Comparison of Whole Liver Samples versus Hepatocyte Spheroids

Overall, all genes were more expressed in the whole liver samples than in hepatocyte spheroids. Despite that, close similarities were found between whole liver and hepatocyte spheroids, except for ZP2.5 gene. The differences found between the two models can be explained by the absence of parenchymal and non-parenchymal cell types in the spheroids. In fact, it was reported that co-culture systems had an increased drug metabolism capability (Van Berkel and Van Tol, 1979, Cross and Bayliss, 2000, Uchea et al., 2015). Further, non-

parenchymal cells are also important for gene regulation (Lester et al., 1993, Hampton et al., 1989, Altin and Bygrave, 1988).

4.4 Biometric and Morphologic Characterization of Hepatocyte Spheroids from Juvenile Brown Trout over Exposure Assays´

The exposure of brown trout primary hepatocytes spheroids to EE2 did not influenced the biometry of 18-day-old spheroids (area, equivalent diameter and sphericity). Thus, the EE2 concentration range did not affect the tridimensional structure of spheroids (cell-cell interactions). The histomorphology of spheroids showed compact spheroids in all conditions, with intact hepatocytes. H&E staining suggested the presence of cytoplasmic vacuoles in all conditions, but this number tended to decrease with the increase of EE2 concentration. The presence of cytoplasmic vacuoles was also observed in 3D cultures by Rodd et al. (2017), using PLHC-1 cell lines. Some of those vacuoles can correspond to glycogen deposits as it was confirmed by using PAS with α-amylase reaction. In fact, similar results evidencing glycogen content storage were described using PLHC-1 cell lines (Rodd et al., 2017) and also human cells (Vosough et al., 2013).

Cell viability of brown trout primary hepatocytes was also not affected by EE2 exposures. Therefore, results suggested that hepatocytes from all conditions were metabolically competent.

4.5 Functional Characterization of Hepatocyte Spheroids from Juvenile Brown Trout over Exposure Assays´

A selection of target genes was used to test the functional activity of hepatocyte spheroids after exposures to EE2. The selection included three xenobiotic metabolization genes (CYP1A, UGT and GST), one efflux transporter (BSEP) and one estrogenic target gene (VtgA). These genes showed stable mRNA level profiles during the 12^{th} and 20^{th} day in culture, which included the exposure period. Further, according to Hultman et al. (2015b) a concentration-dependent down-regulation was pointed in the CYP1A gene using RT-qPCR and microarray analysis in primary rainbow trout hepatocytes after 48 h of exposure to four concentrations (8.83, 88.3, 883.2 and 8832 ng/L) of EE2. On the other hand, an exposure of a primary culture of salmon hepatocytes to three concentrations (0.01, 0.1 and 1 μM)/(2964.03, 29640.3, 296403 ng/L) of EE2 over 48 h showed a concentration-dependent

increase of CYP1A mRNA levels (Mortensen et al., 2007). Over the same experiment, the remaining two metabolization genes (GST and UGT) were also analyzed, showing a similar pattern in comparison to CYP1A mRNA levels (Mortensen et al., 2007). The BSEP mRNA levels were up-regulated after rainbow trout primary hepatocytes exposure to 10 nM (2964 ng/L) of EE2 over 24 h (Finne et al., 2007). Finally, the VtgA expression levels were inducted in male rainbow trout primary hepatocyte spheroids by 1 μM (272400 ng/L) of 17β-estradiol (E2) over 24 h, with maximum stimulatory effect of E2 at 8th day (Flouriot et al., 1993). Furthermore, a concentration-dependent up-regulation for VtgA gene was also reported in primary rainbow trout hepatocytes after 48 h of exposure to 0.03, 0.3, 3 and 30 nM (8.83, 88.3, 883.2, and 8832 ng/L) of EE2 (Hultman et al., 2015a). Thus, according to the presented facts, all selected genes showed sensibility to exposure to EE2. However, is evident a lack of available information in terms of exposures using environmental concentrations of estrogenic compounds in 3D systems in fish.

Here, brown trout hepatic spheroids exposed to EE2 doses did not show significant alterations in the expression patterns of metabolism, and transport-related genes at the range of concentrations tested. However, according to Mortensen et al. (2007), all metabolization genes here analyzed suffered an up-regulation for higher concentrations 2964.03, 29640.3, 296403 ng/L) of EE2, after primary hepatocytes from Atlantic Salmon were exposed over 48 h. Exposure to lower concentrations (8.83, 88.3, 883.2, and 8832 ng/L) of EE2 caused a concentration-dependent down-regulation of the CYP1A gene in primary rainbow trout hepatocytes after 48 h of exposure

The VtgA mRNA levels were significantly higher after exposure to EE2 at 100 ng/L, compared with the other groups. Despite that, results showed some variability because both males and females were included. Females had higher levels of endogenous estrogens and, consequently increased expression of estrogenic targets, comparatively to males. In fish, Vtg is produced after estrogenic stimuli (Pelissero et al., 1993), but the question here was if the tested doses would cause an induction effect on hepatic spheroids. In fact, an estrogenic induction of Vtg was also reported by Flouriot et al. (1993), showing that short exposure to 1 μM (272400 ng/L) of E2 over 24 h led to an increase of Vtg mRNA levels (from 1 to 8 days) in hepatic spheroids from male rainbow trout. Moreover, Vtg mRNA levels were still detectable

at 21 days, showing that hormonal stimulation is effective after a long period post-exposure in hepatic spheroids. According to Park et al. (2022), this increase in expression can result from vitellogenin synthesis in mature spheroids. Another study reported that rainbow trout primary hepatocytes spheroids exposed to EE2 at concentrations of 1, 10, 100, and 1000 nM (296.403, 2964.03, 29640.3, and 296403 ng/L) showed Vtg induction, but just for the three higher concentrations. However, 1 nM (296.403 ng/L) produced a measurable response (Pelissero et al., 1993). Continuous water exposure to 125 ng/L of EE2 over 61 days led to an increase in Vtg mRNA levels (Skillman et al., 2006). Furthermore, intra-arterial injections in male rainbow trout also induced Vtg synthesis, with a similar pattern for all doses (0.001, 0.1, 1.0, and 10.0 mg/kg) of EE2 (Schultz et al., 2001).

5 Chapter 5. Conclusion

In summary, this Master Thesis proved that it is possible to culture viable hepatocyte spheroids from brown trout primary hepatocytes for 25 days. The biometric, morphological and functional stabilization of spheroids was primarily obtained between 12 and 20 days. Thus, this period should be considered the functional and morphological maturity range for spheroids since the morphological and functional stability is regarded as a good criterion for differentiation maintenance in 3D cultures.

The hepatocyte spheroids showed lower expression levels of xenobiotic metabolization, efflux transport, and estrogenic target genes compared to the whole liver samples. Despite that, close similarities between both models were found for some targets, namely VtgA.

3D exposures to four concentrations of EE2 (1 ng/L, 10 ng/L, 50 ng/L, and 100 ng/L) did not impact spheroids' biometric and morphologic features. At the functional level, EE2 doses did not cause impacts on the expression patterns of metabolism and transport-related genes. Contrarily, VtgA mRNA levels were significantly induced by 100 ng/L of EE2, but concentration-dependent up-regulation was noted, mainly for 10 and 50 ng/L of EE2.

It has been proved that this 3D model responds to EE2 and has the potential to test estrogenic effects for longer exposures than 2D models, which opens new opportunities in the ecotoxicological field.

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Appendix 1: Preparation of Stock Solutions, Buffers and Culture Medium

Stock solutions per fish:

Stock 1: Dissolve 8.0 g of NaCl (Merck KGaA) and 0.4 g of KCl (Merck KGaA) in 100 mL of distilled water.

Stock 2: Dissolve 0.358 g of Na_2HPO_4 (Merck KGaA) and 0.6 g of KH_2PO_4 (Merck KGaA) in 100 mL of distilled water.

Stock 3: Dissolve 0.72 g of $CaCl_2$ (Merck KGaA) in 50 mL of distilled water.

Stock 4: Dissolve 1.23 g of $MgSO_4 \times 7H_2O$ (Merck KGaA) in 5 mL of distilled water.

Stock 5: Dissolve 0.35 g $NaHCO_3$ (Merck KGaA) in 10 mL of distilled water.

NaCl: Sodium chloride; KCl: Potassium chloride; Na_2HPO_4: Sodium phosphate dibasic; KH_2PO_4: Potassium phosphate monobasic; $CaCl_2$: Calcium chloride; $MgSO_4 \times 7H_2O$: Magnesium sulphate heptahydrate; $NaHCO_3$: Sodium bicarbonate.

Isolation buffers per fish:

Buffer 1 (50 mL): Mix 5 mL of stock1 + 0.5 mL of stock 2 + 8 mL of distilled water + 0.5 mL of stock 4 + 0.5 mL of stock 5 + 10 mM of EDTA*(Sigma-Aldrich).

*10 mM EDTA preparation: 35 mL distilled water + 0.146 g EDTA. Solution prepared one day before hepatocyte isolation procedure, and added on the isolation day, followed by adjustment of the buffer's pH to 7.9 – 8.0.

EDTA: Ethylenediamine tetraacetic acid.

Buffer 2 (30 mL): Mix 3 mL of stock 1 + 0.3 mL of stock 2 + 0.3 mL of stock 3 + 25.8 mL of distilled water + 0.3 mL of stock 4 + 0.3 mL of stock 5 + 0.05% of collagenase** (Sigma-Aldrich). pH adjusted to 7.2 – 7.4.

**Preparation of 0.05% collagenase: 15 mg of collagenase (Sigma-Aldrich) were weighted, storage at -20 ºC and added only on the moment of its use.

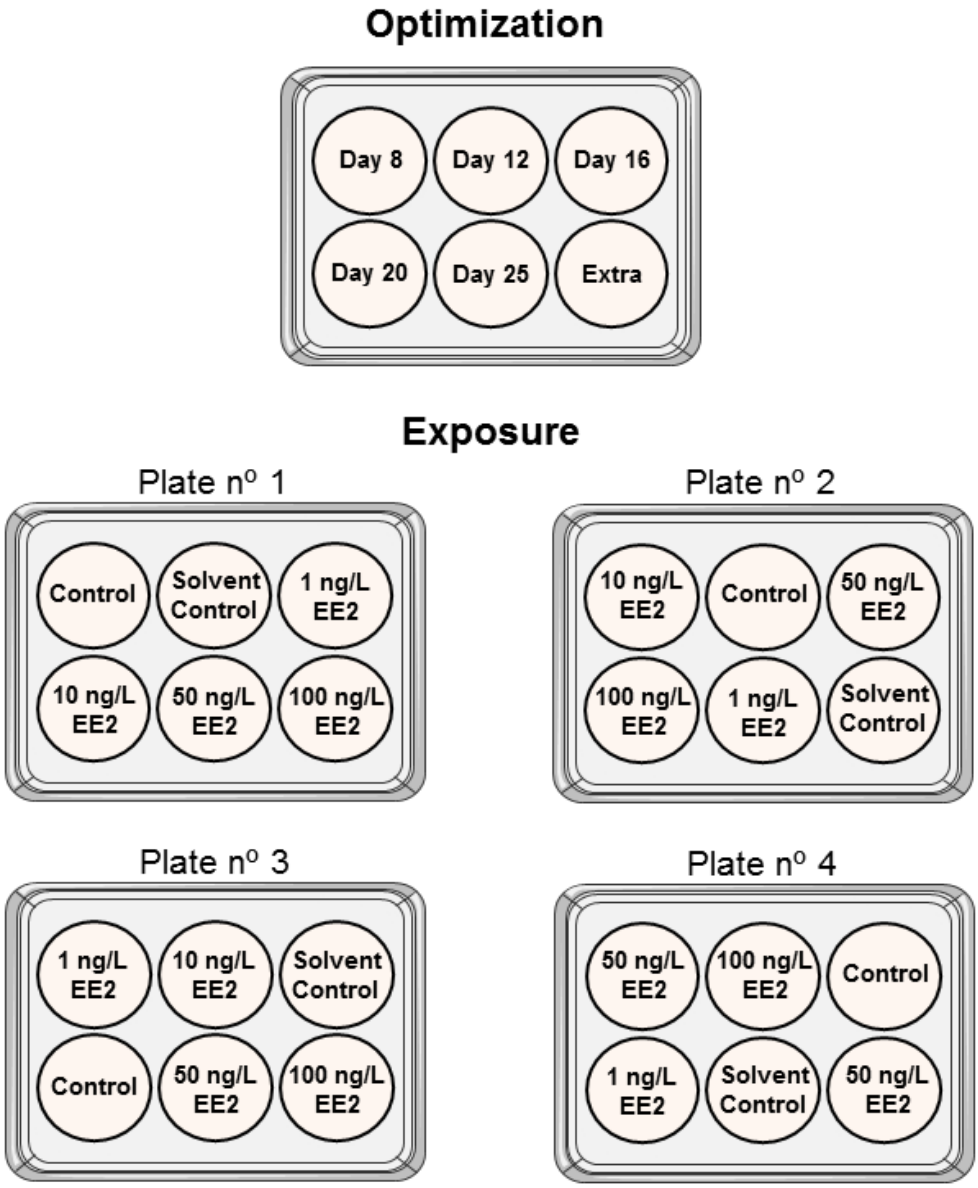
Buffer 3 (200 mL): Mix 20 mL of stock 1 + 2 mL of stock 2 + 2 mL of stock 3 + 172 mL of distilled water + 2 mL of stock 4 + 2 mL of stock 5. The pH was adjusted to 7.2 – 7.4.

Culture medium preparation

Per fish were prepared 500 mL of DMEM-F12 culture medium containing: 5 mL of antibiotic/ Antimicotic solution (Sigma-Aldrich) + 50 mL FBS + 445 mL of DMEM-F12 + 1,952 g of HEPES.

FBS: Fetal bovine serum; **DMEM-F12:** Dulbecco's Modified Eagle Medium/nutrient mixture F-12; **HEPES:** 2-[4-(2-hydroxyethyl)1-piperazinyl]-ethanesulfonic acid

Appendix 2: Plates Design for Optimization/ Exposure Assays

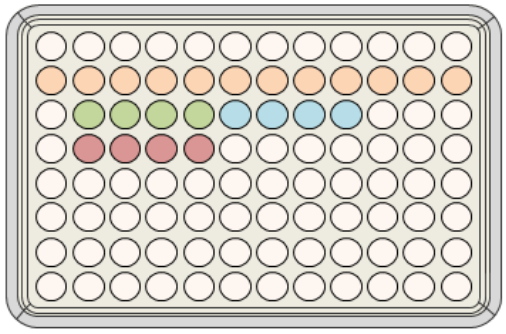


Appendix 3: Plate Layout of LDH and Resazurin Assays

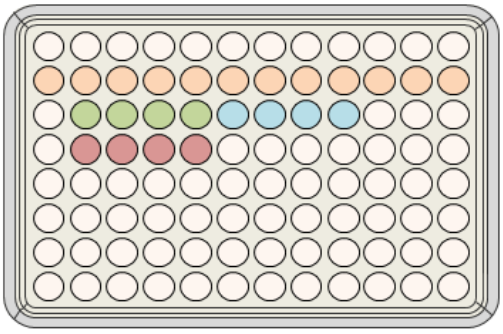
LDH assay

Resazurin assay

Optimization

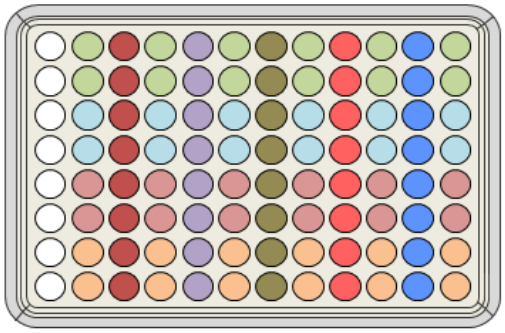


- Background control (100 µL of fresh medium)
- Low control (100 µL of culture medium supernatant - Plate n°1)
- Low control (100 µL of culture medium supernatant - Plate n°2)
- Low control (100 µL of culture medium supernatant - Plate n°3)

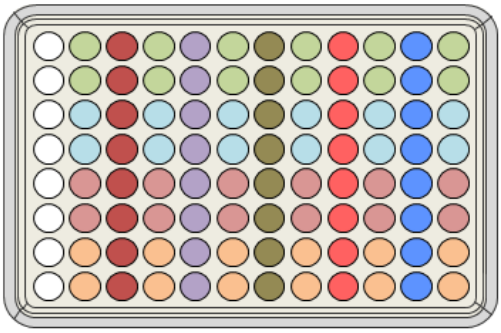


- Blanks (without spheroid)
- Sample wells (added 1 spheroid from Plate n°1)
- Sample wells (added 1 spheroid from Plate n°2)
- Sample wells (added 1 spheroid from Plate n°3)

Exposure



- Background Control (100 µL of fresh medium)
- Background Solvent control (100 µL of fresh medium)
- Background 1 ng/L of EE2 (100 µL of fresh medium)
- Background 10 ng/L of EE2 (100 µL of fresh medium)
- Background 50 ng/L of EE2 (100 µL of fresh medium)
- Background 100 ng/L of EE2 (100 µL of fresh medium)
- Low control (100 µL of specific culture medium supernatant - Plate n°1)
- Low control (100 µL of specific culture medium supernatant - Plate n°2)
- Low control (100 µL of specific culture medium supernatant - Plate n°3)
- Low control (100 µL of specific culture medium supernatant - Plate n°3)



- Blanks Control (100 µL of fresh medium)
- Blanks Solvent control (100 µL of fresh medium)
- Blanks 1 ng/L of EE2 (100 µL of fresh medium)
- Blanks 10 ng/L of EE2 (100 µL of fresh medium)
- Blanks 50 ng/L of EE2 (100 µL of fresh medium)
- Blanks 100 ng/L of EE2 (100 µL of fresh medium)
- Specific condition sample wells (added 1 spheroid from Plate n°1)
- Specific condition sample wells (added 1 spheroid from Plate n°2)
- Specific condition sample wells (added 1 spheroid from Plate n°3)
- Specific condition sample wells (added 1 spheroid from Plate n°4)

Appendix 4: Hematoxylin & Eosin (H&E) Staining Protocol

1- Deparaffinization using xylene (VWR Chemicals) during 10 min (2 x);
2- Hydration in a graded series of ethanol (100%; 95%; 70%) for 5 min each;
3- Washing in running tap water (5 min);
4- Staining nuclei with commercial Mayer's Hematoxylin (Merck) for 2 min;
5- Washing in running tap water (5 min);
6- Staining cytoplasm with aqueous 1% Eosin (Bio-Optica) for 2 - 4 min;
7- Washing in tap water;
8- Dehydration in ethanol 99.9% (3x);
9- Passing in xylene for 2 min each;
10- Mount with Q Path® Coverquick 2000 media (VWR Chemicals).

Appendix 5: Periodic Acid Schiff (PAS) with Alfa-Amylase Protocol

1- Deparaffinization using xylene (VWR Chemicals) during 10 min (2 x);
2- Hydration in a graded series of ethanol (100%; 95%; 70%) for 5 min each;
3- Alfa-amylase 0.5% (Sigma-Aldrich) digestion for 30 min at 37 ºC;
4- Washing in running tap water (5 min);
5- Oxidize sections with Periodic Acid Solution 0.5% (ThermoFischer Scientific) for 15 min;
6- Washing in running tap water (5 min);
7- Passing through distilled water;
8- Schiff's Reagent (ThermoFischer Scientific) for 20 min;
9- Washing in running tap water (5 min);
10- Staining nuclei with commercial Mayer's Hematoxylin (Merck) for 1 min;
11- Washing in running tap water (5 min);
12- Dehydration in ethanol 99.9% (3x);
13- Xylene for 2 min each;
14- Mount with Q Path® Coverquick 2000 media (VWR Chemicals).

Appendix 6: Primer Sequences, Annealing Temperature (AT) and Efficiency (E) of Respective Metabolic/ Transport/ Estrogenic and Reference Genes Used for RT-qPCR.

Gene	Primers sequences	AT (ºC)	E (%)	References
CYP1A	F: 5′-GAT GTC AGT GGC AGC TTT GA-3′ R: 5′-TCC TGG TCA TCA TGG CTG TA-3′	60.0	99.9	(Uchea et al., 2015)
CYP3A27	F: 5′-GAC GGT GGA GAT CAA CG-3′ R: 5′-GAG GAT CTC GAC CAT GG-3′	60.0	96.2	(Uchea et al., 2015)
UGT	F: 5′-ATA AGG ACC GTC CCA TCG AG-3′ R: 5′-ATC CAG TTG AGG TCG TGA GC-3′	60.0	100.8	(Uchea et al., 2015)
Cat	F: 5´-CACTGATGAGGGCAACTGGG-3′ R: 5´-CTTGAAGTGGAACTTGCAG-3′	58.0	91.4	(Madureira et al., 2017)
GST	F: 5´- AGCTGCTCCCAGCTGATCC-3′ R: 5´- CAAACCACGGCCACATCATGTAATC-3′	60.0	92.5	(De Anna et al., 2021)
MDR1	F: 5´- ACGTGCGCTCCCTGAACGTG-3′ R: 5´- GCGTTGGCCTCCCTAGCAGC-3′	60.0	103.6	(De Anna et al., 2021)
BSEP	F: 5′-CCG ACC AGG GCA AAG TGA TT-3′ R: 5′-CAG AAT GGG CTC CTG GGA TAC-3′	60.0	93.5	(Uchea et al., 2015)
MRP2	F: 5′-CCA TTC TGT TCG CTG TCT CA-3′ R: 5′-CTC GTA GCA GGG TCT GGA AG-3′	60.0	98.5	(Uchea et al., 2015)
VtgA	F: 5′-AACGGTGCTGAATGTCCATAG-3′ R: 5′-ATTGAGATCCTTGCTCTTGGTC-3′	62.9	99.0	(Lopes et al., 2020)
ERα	F: 5′-GACATGCTCCTGGCCACTGT-3′ R: 5′-TGGCTTTGAGGCACACAAAC-3′	61.6	91.2	(Lopes et al., 2020)
ZP 2.5	F: 5′-ATCAATAACCACAGCCACAATG-3′ R: 5′-ACCAGGGACAGCCAATATG-3′	55.0	99.0	(Lopes et al., 2020)
Gapdh	F: 5′-CCTCTGTGTTGGAATCAATG-3′ R: 5′-AGACCTCACCGTTGTAAC-3′	55.0	92.8	(Madureira et al., 2019)
rpl8	F: 5′-TCAGCTGAGCTTTCTTGCCAC-3′ R: 5′-AGGACTGAGCTGTTCATTGCG-3′	59.0	93.8	(Lopes et al., 2020)
β-actin	F: 5′-TCTGGCATCACACCTTCTAC-3′ R: 5′-TTCTCCCTGTTGGCTTTGG-3′	55.0	96.1	(Lopes et al., 2020)
Ef1a	F: 5′-TGCCACACTGCTCACATC-3′ R: 5′-TCTCCAGACTTCAGGAACTTG-3′	55.0	109,0	(Madureira et al., 2018)

BSEP: Bile salt export pump; **Cat:** Catalase; **CYP1A:** Cytochrome P450 1A; **CYP3A27:** Cytochrome P450 3A27; **Ef1a:** Elongation factor-1 alpha; **ERα:** Estrogenic receptor α; **Gapdh:** Glyceraldehyde-3-Phosphate Dehydrogenase; **GST:** Glutathione S-transferase; **MDR1:** Multidrug Resistance Protein 1; **MRP2:** Multidrug resistance-associated protein 2; **rpl8:** Ribosomal protein l8; UGT: UDP-glycosyltransferase; **VtgA:** Vitellogenin A; **ZP 2.5:** Zona pellucida glycoprotein 2.5.

Appendix 7: Temperature Gradient/ Calibration Curves- Primer Optimization

Gradient temperature

Dillution 1:5

Stock I- 5 µL cDNA + 20 µL H_2O nuclease free

STANDARD I- 5 µL stock I + 20 µL H_2O nuclease free

STANDARD I- 5 µL standard I + 20 µL H_2O nuclease free

STANDARD III- 20 µL standard II + 80 µL H_2O nuclease free

MIXTURE

Component	Volume/reaction	Total Volume
SYBR Green	10 µL	220 µL
Primer F	0.4 µL [200 nM]	8.8 µL
Primer R	0.4 µL [200 nM]	8.8 µL
H_2O	4.2 µL	92.4 µL

Calibration curves

STANDARD I- 5 µL cDNA + 20 µL H_2O nuclease free

STANDARD II- 5 µL standard I + 20 µL H_2O nuclease free

STANDARD III- 5 µL standard II + 20 µL H_2O nuclease free

STANDARD IV- 5 µL standard III + 20 µL H_2O nuclease free

STANDARD V- 5 µL stock IV + 20 µL H_2O nuclease free

STANDARD VI- 5 µL standard V + 20 µL H_2O nuclease free

STANDARD VII- 5 µL standard VI + 20 µL H_2O nuclease free

Mixture

Component	Volume/reaction	Total Volume
SYBR Green	10 µL	180 µL
Primer F	0.4 µL [200 nM]	7.2 µL
Primer R	0.4 µL [200 nM]	7.2 µL
H_2O	4.2 µL	75.6 µL