17α-Ethinylestradiol *(EE2)* effect on global gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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Abstract

The potential impact of endocrine disrupting chemicals (EDCs) in the aquatic environment has driven the development of screening assays to evaluate the estrogenic properties of chemicals and their effects on aquatic organisms such as fish. However, obtaining full concentration-response relationships in animal (*in vivo*) exposure studies are laborious, costly and unethical, hence a need for developing feasible alternative (non-animal) methods. Use of *in vitro* bioassays such as primary fish hepatocytes, which retain many of the native properties of the liver, has been proposed for *in vitro* screening of estrogen receptor (ER) agonists and antagonists. The aim of present study was to characterize the molecular Mode of Action (MoA) of the ER agonist

17α-ethinylestradiol (EE2) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. A custom designed salmonid 60,000-feature (60k) oligonucleotide microarray was used to characterize the potential MoAs after 48h exposure to EE2. The microarray analysis revealed several concentration-dependent gene expression alterations including classical estrogen sensitive biomarker gene expression (e.g. *estrogen receptor α, vitellogenin, zona radiata*). Gene ontology (GO) analysis displayed transcriptional changes suggesting interference of cellular growth, fatty acid and lipid metabolism potentially mediated through the estrogen receptor (ER), which were proposed to be associated with modulation of genes involved in endocrine function and reproduction. Pathway analysis supported the identified GOs and revealed modulation of additional genes associated with apoptosis and cholesterol biosynthesis. Differentially expressed genes (DEGs) related to impaired lipid metabolism (e.g. *peroxisome proliferator-activated receptor α and γ*), growth (e.g. *insulin growth factor protein 1*), phase I and II biotransformation (e.g. *cytochrome P450 1A, sulfotransferase, UDP-glucuronosyltransferase and glutathione S-transferase*) provided additional insight into the MoA of EE2 in primary fish hepatocytes. Results from the present study suggest that biotransformation, estrogen receptor-mediated responses, lipid homeostasis, growth and cancer/apoptosis in primary fish hepatocytes may be altered after short-term exposure to ER-agonists such as EE2. In many cases the observed changes were similar to those reported for estrogen-exposed fish *in vivo*. In conclusion, global transcriptional analysis demonstrated that EE2 affected a number of toxicologically relevant pathways associated with an estrogenic MoA in the rainbow trout hepatocytes.

Key words: *Oncorhynchus mykiss*, primary hepatocytes, EDCs, microarray, gene expression, estrogen receptor

1 Background

Compounds that modulate the endocrine system and cause adverse effects causally related to these changes are known as endocrine disrupting chemicals (EDCs). These chemicals may enter the environment through anthropogenic activities such as effluents of sewage treatment plants, industrial processes and agricultural run-off (Sumpter, 2005). During the past decade, increasing awareness of the adverse effects of EDCs in wildlife and human has given rise to the implementation of stricter legislations in international regulatory organizations worldwide (Hecker and Hollert, 2011). Adverse effects such as impaired reproduction in fish, reproductive disorders and various cancer types (e.g. breast and ovary cancer) in human and other mammals have been associated with EDCs such as 17α-ethinylestradiol (EE2), dichlorodiphenyl-trichloroethane (DDT) and bisphenol A (BPA) (Benninghoff and Williams, 2008; Purdom et al., 1994; Soto and Sonnenschein, 2010; vom Saal et al., 2007). Characterization of a chemical's mode of action (MoA) involving interference with specific molecular, cellular and biochemical changes, behavioral alterations and adverse effects are often laborious and expensive due to extensive use of animals (Aardema and MacGregor, 2002). Although the use of single biomarker screening approaches has facilitated the understanding of MoAs of EDCs, the knowledge obtained from such biomarker studies is still limited, as the response of a single endpoint may not always represent complex biological responses at higher levels of organization.

In recent years, the development of broad-content screening approaches such as transcriptomics has made it possible to characterize the global gene expression changes after exposure to single EDCs and mixture of these in different *in vitro* and *in vivo* experimental models (Finne et al., 2007; McHale et al., 2010; Wang et al., 2010; Yang et al., 2007). Deciphering complex molecular interactions of chemicals using transcriptomic studies has enabled detailed studies on the *in vivo* responses of (xeno)estrogens in common laboratory species such as zebrafish

(*Danio rerio*), fatheaded minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), but also non-model species such as coho salmon (*Oncorhynchus kisutch*) (Harding et al., 2013; Hook et al., 2008; Levi et al., 2009; Villeneuve et al., 2011; Wang et al., 2010). Many of these studies involve the analysis of estrogenic responses, which typically involve the binding and activation of the estrogen receptor (ER) and of genes containing estrogen response elements (ERE) to initiate multi-organ endocrine responses in fish. The ER signaling pathway may further regulate the expression of several classical estrogenic biomarker genes such as the *egg-yolk precursor protein vitellogenin* (*vtg*), *egg envelope proteins zona pellucida* and *zona radiata protein* (*zrp*) (Arukwe et al., 1997; Sumpter and Jobling, 1995). The endocrine modulatory effects of EDCs have been extensively studied *in vivo,* including the characterization of MoA associated with hormone binding, lipid and cholesterol metabolism and steroidogenesis, immune function and ion homeostasis in fish (Colli-Dula et al., 2014; Flores-Valverde et al., 2010; Hook et al., 2007; Kausch et al., 2008; Levi et al., 2009; Wit et al., 2010). Various estrogen-mediated responses have also been observed in biomarker studies in various piscine *in vitro* (non-animal) bioassays, and consequently led to the proposal of using these experimental models as screening assays for environmental estrogens and antiestrogens (Björkblom et al., 2008; Hultman et al., 2015; Kordes et al., 2002; Navas and Segner, 2006; Rankouhi et al., 2004; Tollefsen et al., 2003).

Use of alternative approaches provided by *in vitro* methods have offered rapid screening methods and facilitated better understanding of the MoA of chemicals whilst implementing the 3R`s (refinement, reduction and replacement) into toxicological testing (National research council (NRC), 2007). *In vitro* hepatic models such as primary hepatocytes have demonstrated to be advantageous proxies for the assessment of *in vivo* bioactivity as the cells retain many of the native hepatic functions including biotransformation, detoxification and ER-mediated responses (Flouriot et al., 1993; Pedersen and Hill, 2000; Pesonen and Andersson, 1997; Segner

and Cravedi, 2000). The hepatocytes have previously demonstrated their potential for toxicological screening of cellular toxicity, endocrine disruption (ED) and bioaccumulation in various assay formats including suspension, monolayer and 3-dimensional spheroid cultures (Baron et al., 2012; Hultman et al., 2015; Mingoia et al., 2010; Smeets et al., 1999; Tollefsen et al., 2008a). Despite the broad applicability of such assays, thorough characterization of the MoAs and concentration-dependent global changes of gene and protein expression are generally lacking, and effort to provide this for EDCs is highly warranted.

The objectives of this study were 1) to characterize the molecular MoAs of the ER agonist - 17α-Ethinylestradiol (EE2) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after a short-term (48h) *in vitro* exposure; 2) to determine the concentration-dependent transcriptional changes occurring, and 3) to evaluate the potential of primary hepatocytes to predict *in vivo* hepatic responses in fish.

2 Material and methods

2.1 Chemicals

17α-Ethinylestradiol (EE2, ≥98%, CAS 57-63-6) and sodium bicarbonate (CAS 144-55-8) were purchased from Sigma–Aldrich (St. Louis, MI, US). The test chemical was dissolved in dimethylsulfoxide (DMSO) and stored in the dark at -20ºC until use.

2.2 Fish

Sexually immature rainbow trout (200-500g) from the same fish stock were obtained from the Valdres rakfisk AB hatchery (Valdres, Norway) and reared at the Department of Biology, University of Oslo (Norway) for a minimum of 4 weeks prior to the start of the studies. The

fish were maintained in tap water at 6±2 ºC, pH 6.6, 100% air saturation and light regime of 12h light/12h dark. Rainbow trout were fed daily with commercial pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body weight.

2.3 Cell culture and exposure

A total of four donor fish were collected (January-July, 2012) and terminated by cephalic concussion, followed by immediate dissection to expose the abdominal cavity. Only juvenile fish (with no visual gonads) were used in a 2-step hepatic cell isolation procedure as described by Tollefsen et al. (2003) and modified for studies on gene expression by Hultman et al. (2015). The viability of primary hepatocytes (>80%) was assessed using a Bürker counting chamber and trypan blue:cell suspension (2:1). The cell suspension was diluted to 500 000 cells/ml in serum-free L-15 medium with phenol-red containing amphotericin (0.25 g/ml), L-glutamine (0.29 mg/ml), streptomycin (100 g/l), penicillin (100 Units/ml) and $NaHCO_3$ (4.5 mM), seeded in 24- well Primaria^{TM} microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) with a density of 625 000 cells/well and incubated in ambient atmosphere at 15ºC. After 24 hours of acclimation to the test wells, 50% of the medium was removed from the cells and replaced with media spiked with EE2 (0.03, 0.3, 3 and 30 nmol/L (nM)) or solvent control (0.1% DMSO) in triplicate. The chemical exposure concentration in the medium at the start of the experiment was verified by ultra-performance liquid-chromatography tandem mass spectrometer (UPLC-MS) analysis on derivatized EE2 and d3 labeled estradiol (d2-E2) and described in detail by Hultman et al. (2015). The measured concentrations ranged between 75% and 93% of the nominal concentrations (Hultman et al., 2015). After 48h exposure, the test medium was removed and the cells were sampled and lysed with RNeasy lysis buffer (Qiagen GmbH, Hilden, Germany) and stored at -80ºC for later RNA isolation and gene expression analysis.

2.4 Gene expression analysis

2.4.1 Microarray analysis

A high-density (60,000-feature) custom salmonid oligonucleotide microarray (Agilent Technologies, City, Country; GEO accession number: GPL18864) was used to study the global transcriptional changes in the rainbow trout hepatocytes. The performance of the array was thoroughly evaluated for different salmonid species and pollutants (Song et al., 2012). Prior to microarray hybridization, the frozen primary hepatocytes were subjected to RNA extraction using Qiagen RNeasy Plus mini kit with on-column DNAs treatment (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications to accommodate high RNA purity and yield. The modifications included extended incubation time of RNA membrane-bound washing buffer (2-3 minutes) and increased centrifugation time and speed (35 sec, 9700 g), with a final removal of excess fluid from the O-ring prior to RNA elution. Protocol modifications were performed in order to remove excess guanine salts and avoid the need for further clean-up/purification of the samples. The RNA concentration was measured spectrophotometrically (Spectrophotometer ND 1000, Nanodrop technologies Inc., Wilmington, USA) with quality cut-off criteria as follows: 280/260 ratios of >2.0 and 260/230 ratios of >1.8. The RNA integrity was assessed using Agilent Bioanalyzer RNA 6000 nano series kit (Agilent technologies, Santa Clara, CA, USA), with samples obtaining RIN value >8.0 passing the quality cut-off criteria (Fleige and Pfaffl, 2006). Technical replicates of RNA were pooled and the four biological replicates (individual batches of cells from different fish) were used in the subsequent analysis. The biological replicates were subsequently subjected to cDNA synthesis (input: 50 ng total RNA), linear amplification, cRNA synthesis and Cyanine-3 (Cy-3) labelling strictly performed according to Agilent's "One-color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) protocol, Version 6.5 May 2010". The Cy-3 labelled samples were hybridized on the microarray, followed by washing and scanning

in a high resolution microarray scanner (Agilent technologies, USA) at 3 μm resolution and scanning area of 61×21.6mm.

2.4.2 Quantitative Real time PCR (qPCR)

Verification of differentially expressed genes (Table 1) was performed by quantitative real-time polymerase chain reaction (qPCR) essentially as described by Hultman et al. (2015). Synthesis of cDNA was performed by reverse transcription of total RNA (0.5-1μg) using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosences Inc., Gaithersburg, USA) according to the manufacturer's instructions. The primer optimization was performed with a CFX-384 thermal cycler (Bio-Rad laboratories Inc., USA) using a 5-step dilution series (2.5-50 ng/reaction) with pooled template cDNA. SYBR®Green Supermix (Quanta Biosences Inc., Gaithersburg, USA) was used in the qPCR amplification reaction, where technical triplicates of 10 ng template/reaction were used in the final mastermix reaction (20 μl/reaction). The primers were obtained from previously published papers (Table 1) and were purchased from Eurofins MWG synthesis GmbH (Ebersberg, Germany). Primers were optimized for concentrations and annealing temperature in order to yield an amplification efficiency of 90-110%. The qPCR protocol was performed as following, Cycle 1: 95ºC for 3 min, Cycle 2-40: 95ºC for 20 sec, followed by the specific primer annealing temperature for 20 sec and 72ºC for 20 sec. All primers run had a none-template control (NTC) and a no-reverse transcriptase control (NRT) to exclude any contamination of primer or presence of genomic DNA in the mastermix or the RNA sample that may influence the qPCR analysis. The qPCR analysis reported no amplification of either NRT or NTC for any of the primers used. In the end of the qPCR protocol a melt curve analysis was performed, verifying that no primer-dimer formation or unspecifically amplified products were formed in the samples. Accepted threshold cycle (Cq)-value of NTC was set to be either non-detectable (N/A) or Cq value >30 with minimum 7 cycles in between template replicate and NTC. The expression of elongation factor 1α (EF1α) was relatively stable in all

treatments and was therefore used as a reference gene (Table 1). Data normalization was performed using the Pfaffl method (Pfaffl, 2001).

2.5 Statistics and bioinformatics

2.5.1 Microarray

Scanned microarray images were quality assessed and extracted using Agilent Feature Extraction software v10.7 (Agilent Technologies). The raw data were subjected to normalization, including correction for background signal, flagged for missing and low quality features, followed by statistical analysis using GeneSpring GX v12.6 (Agilent Technologies). Determination of differentially expressed genes (DEGs) was performed using one-way analysis of variance (ANOVA), followed by Tukey`s post hoc test ($p<0.05$). Statistics were not corrected for Benjamin and Hochberg (BH) false discovery rate (FDR) correction to avoid potential loss of biologically relevant data (Villeneuve et al., 2011). The Venn diagrams were generated using Venny (Oliveros, 2007) and the Gene ontology (GO) enrichment analysis was performed in Cytoscape v2.8 (Smoot et al., 2011) using the application Bingo v.2.4 (Maere et al., 2005). Pathway and protein-protein interaction network analyses were performed using Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) on basis of identification of mammalian orthologs by the standalone Inparanoid 4.1 algorithm (Ostlund et al., 2010) implementing BLAST 2.2.27+ binaries from the NCBI expert-curated mammalian-based databases Ensembl, Entrez Gene, RefSeq, GenBank, UniProt/Swiss-Prot Accession, GenPrept and UniGene.

2.5.2 qrtPCR analysis

Prior to statistical analyses, the qPCR data was normalized against a reference gene followed by the Grubb's outlier test (Burns et al., 2005) to exclude clearly erroneous values (10 out of 140 values removed). The statistical analyses were performed in Graphpad Prism v5.04

(Graphpad Software, Inc., San Diego, CA, USA), applying a one-way ANOVA test, followed by Tukey`s post hoc test. The significant level was set to $p<0.05$ for all statistical tests.

3 Results

The qPCR analysis reported no amplification of either NRT or NTC for any of the primers used.

3.1 Global transcriptional changes

A total of 1098 differentially expressed gene (DEG) transcripts (707 up-regulated and 391 down-regulated) were identified to be regulated in the EE2 exposed cells when compared to the control. Concentration-dependent transcriptional changes were further determined using Tukey HSD post hoc test. A total of 66 (up-regulated: 27, down-regulated: 39), 114 (up-regulated: 84, down-regulated: 30), 468 (up-regulated: 301, down-regulated: 167) and 992 (up-regulated: 695, down-regulated: 297) genes were identified to be differentially expressed after exposure to 0.03, 0.3, 3 and 30 nM EE2, respectively (Fig. 1). The Venn diagram analysis identified 3 up-regulated DEGs being commonly regulated across all treatments (i.e. *transposable element Tc1 transposase* (*tca1)*, *diaphanous homolog 2* (*diap2)* and *uncharacterized protein*) and 2 down-regulated (i.e. *dedicator of cytokinesis protein 9* (*dok9)* and *protein naked cuticle homolog 2-B* (*nkd2b)*). The cells exposed to 3 and 30 nM EE2 had a high number of common DEGs being regulated (246 DEGs, 179 up- and 67 down-regulated). Both total number (Fig. 1) and the expression of the DEGs were found to be concentration-dependent and the complete list of DEGs can be found in supplementary table A.

3.2 Gene Ontology-based functional enrichment analysis

The biological roles of the DEGs were first characterized by functional Gene Ontology (GO) analysis and grouped into molecular functions, biological processes and cellular components (Table 2). Assigning the biological roles for the gene products naturally supposes that they are the same in rainbow trout hepatocytes as in mammals, which the GO is based on. The total numbers of overrepresented GO biological processes and molecular functions were found to be affected in a concentration-dependent manner between 0.3-30 nM EE2. Increased enrichment of DEGs related to functional categories such as lipid and fatty acid metabolism was observed between 0.3-3 nM EE2, whereas biological processes related to reproduction and the regulation of the endocrine system were mainly observed between 3-30 nM EE2. A complete list of GO terms associated with the DEGs can be found in supplementary table B.

3.3 Ortholog-based functional enrichment analysis

The DEGs were further mapped to their mammalian orthologs to better understand their biological roles using well-curated mammalian databases, assuming that the functions of the gene products are conserved between mammals and fish. A total of 54.3% (0.03 nM), 52% (0.3 nM), 57.4% (3 nM) and 58.5% (30 nM) of the rainbow trout DEGs were successfully mapped to mammalian orthologs (see suppl. table B for a complete list of mapped orthologs). The gene network, toxicity and canonical pathway analysis revealed a concentration-dependent increase of enriched gene/protein networks and pathways.

3.3.1 Gene networks

The list of successfully mapped orthologs was subjected to protein-protein interaction (PPI)-based gene network analysis to obtain a better overview of the functional processes associated with the DEGs identified (Table 3.). The gene networks of 0.03 nM EE2 were mainly related to cell signaling, molecular transport and nucleic acid metabolism, whereas 0.3 nM EE2

regulated DEGs associated with cellular function and maintenance, small molecule biochemistry and cellular development. The 3 nM and 30 nM EE2 exposure predominantly resulted in transcriptional changes associated with hematological disease, hereditary disorder, carbohydrate metabolism and post-translational modification, lipid metabolism and alterations of small molecule biochemistry in the cells, respectively.

3.3.2 Toxicity pathways

The toxicity pathway analysis was performed to better understand the potential MoAs of EE2 based on the mapping of DEGs to well-characterized mammalian toxicity pathways. No toxicity pathways were identified to be commonly affected across all EE2 concentrations (Fig. 2). Exposure to 0.03 nM EE2 predominantly altered the expression of DEGs associated with toxicity pathways related to organismal injury and abnormalities. One toxicity pathway (negative acute phase response proteins) was found to be affected by 0.3 nM EE2, whereas 3 nM EE2 led to alterations of a number of DEGs associated with lipid metabolism, endocrine system development and function, as well as cellular growth and proliferation. A majority of the identified DEGs in the 30 nM EE2-exposed hepatocytes were related to processes such as cell death and survival, cardiovascular disease, cellular growth and proliferation. The complete list of toxicity pathways is provided in supplementary table C.

3.3.3 Canonical pathways

Canonical pathway analysis was performed to obtain more detailed knowledge on the potential MoAs of EE2. The number of toxicologically relevant canonical pathways increased in an apparent concentration-dependent manner (Table 4). A total of 10, 5, 28 and 36 pathways were uniquely affected by 0.03, 0.3, 3 and 30 nM EE2, respectively. Two canonical pathways (CCR3 Signaling in Eosinophils and Protein Kinase A Signaling) were commonly affected at all EE2 concentrations (Supplementary table D). The cells exposed to 3 nM and 30 nM EE2 responded by modulating DEGs involved in cell growth and development, immune response, intracellular

and second messenger signaling, apoptosis and metabolic pathways. Exposure to 0.03 nM EE2 led to the enrichment of DEGs in pathways linked to intracellular and second messenger signaling (e.g. glucocorticoid receptor- and insulin receptor signaling), whereas DEGs regulated by 0.3 nM EE2 were enriched in the apoptotic signaling pathway. In the cells exposed to 3 nM EE2, gene transcripts related to cell growth and development (e.g. Growth hormone (GH) and Insulin growth factor-1 (IGF-1) signaling), apoptosis (e.g. death receptor signaling), intracellular and second messenger signaling (e.g. Glucocorticoid receptor and Calcium signaling) and nuclear receptor signaling (e.g. Pregnane X receptor/Retinoic X receptor (PXR/RXR) activation) were mainly affected. Primary hepatocytes exposed to the highest (30 nM) concentration of EE2 exhibited enrichment of DEGs associated with regulation of cell growth and development (e.g. Corticotropin-Releasing Hormone Signaling), neurotransmitters and other signaling pathways (e.g. Gonadotropin-releasing hormone (GNRH) signaling), nuclear receptor signaling (e.g. Peroxisome proliferator-activated receptor (PPAR) signaling)) and disease-specific pathways (e.g. molecular mechanism of cancer). A detailed list of the canonical pathways can be found in supplementary table D.

3.4 Quantitative real-time polymerase chain reaction verification

Verification of the microarray results was performed on a selection of DEGs involved in key biological processes such as ER regulation (*esr1, vtg1*), biotransformation (*cytochrome P450 1A* (*cyp1a*)), lipid metabolism/homeostasis (*pparγ*) and cellular growth (*growth hormone receptor 1* (*ghr-1*), *IGF binding factor 1* (*igfbp-1*)). The selected genes were consistently expressed in a concentration-dependent manner in coherence with the microarray data, although some differences in the magnitude of expression were observed. In addition, *pparα* was verified as regulated using qPCR, despite not being identified as significantly altered on the microarray. Among the genes analyzed, four (*esr1, vtg1*, *igfbp-1* and *pparα*) were verified to be significantly regulated when compared to the solvent control (Fig. 3 and 4). An apparent concentration-

dependent regulation was also observed in the remaining genes, and found to be in general agreement with the microarray data.

4 Discussion

Exposure to ER-agonists such as EE2 has been reported to affect a number of estrogen sensitive genes and their proteins product in both *in vivo* and *in vitro* fish models (Arukwe et al., 1997; Finne et al., 2007; Hultman et al., 2015; Sumpter and Jobling, 1995). The majority of available studies with EDCs have been focused on a few responses associated with the endocrine functions in fish, thus contributing to the development and evaluation of a number of estrogen sensitive biomarkers suitable for laboratory and field-based studies (Arukwe et al., 1997; Folmar et al., 2000; Harries et al., 1997; Purdom et al., 1994; Sumpter and Jobling, 1995). Although this approach has been highly successful, most of these studies have limited their approaches to the MoA characterization using single or small suites of biomarkers, which only provide a snapshot of the biological responses to EDCs. The present study has implemented a broad-content transcriptomic (microarray) analysis to provide an unbiased characterization of the complex and concentration-dependent cellular transcriptional responses in rainbow trout hepatocytes after exposure to the ER-agonist EE2. The exposure duration (48h), and bioassay protocol have previously been identified as optimal for determination of Vtg gene and protein expression in rainbow trout hepatocytes exposed to non-cytotoxic concentrations of EE2 (Hultman et al., 2015). Dimethylsulfoxide (0.1%) was used as the solvent control in these studies as it has been shown to not affect Vtg gene and protein expression (Hultman et al., 2015).

The present study identified clear concentration–dependent responses in DEGs (Fig. 1,3 and 4), GOs (Table 2) and pathways (Fig. 2; Table 3) relevant for a number of potential toxic MoAs

(Fig. 5 and 6A-D). The lowest concentration of EE2 (0.03 nM) affected a limited number of DEGs (66) mainly associated with cellular signaling, cellular transport, and biotransformation, whereas 0.3 nM EE2 modulated about twice as many DEGs (114) that were related to cellular metabolism of fatty acids and lipids, cellular development, and apoptosis. Ten times higher concentrations of EE2 (3 nM) led to regulation of 466 DEGs predominantly associated with cellular processes such as growth, development, metabolism, apoptosis, but also to nuclear receptor signaling, endocrine regulation, reproductive functions, immune functions and cancer development. The highest concentration of EE2 (30nM) affected a high number of DEGs (992), whereof many were associated with similar functions as those observed for 3 nM, but modulation of DEGs associated with cell cycle regulation neuro-signaling were also observed. Overall, the lowest concentration of EE2 (0.03 nM) was considered to only marginally affect toxicologically-relevant processes, whereas a number of processes relevant for endocrine disruption in fish was identified at higher EE2 concentrations (Fig. 2) and discussed in detail below.

4.1 Estrogen receptor signaling

Estrogens bind to and activate the ER and ER-mediated signaling pathways, which may regulate sexual development and reproduction in fish. The genomic ER signaling pathway is well characterized and includes homodimerization of the ligand and ER heat shock protein complex, translocation of the dimer into the nucleus, and recruitment of co-regulators to the ERE-promoter region leading to induced or suppressed transcription of downstream genes (Fig. 3 and 5). Exposure of the primary rainbow trout hepatocytes to EE2 in the present study resulted in a concentration-dependent up-regulation of genes encoding ERα (*esr1*), transcriptional regulators (e.g. *nuclear receptor coactivator 4* (*ncoa4*), *nuclear receptor subfamily group*

member 1 (*nr2f1*), *nuclear receptor subfamily group member 2* (*nr0b2*)), and genes encoding downstream reproductive-relevant hepatic proteins such a vitellogenin (*vtg1)* and zona radiata proteins (*zrp3 and zrp4*) (Fig. 3 and 6 B-D) whereof several have previously been reported to respond to estrogens (Colli-Dula et al., 2014; Doyle et al., 2013; Hoffmann et al., 2006; Mortensen and Arukwe, 2007). Many of these genes (i.e. *esr1, vtg and zrp*) have been proposed as biomarkers for estrogenicity in fish (Arukwe et al., 1997; Heppell et al., 1995; MacKay et al., 1996; Sumpter and Jobling, 1995), and the present microarray and qPCR analysis (Suppl. Table A, fig. 3) confirmed that they were highly responsive to estrogens at low concentrations *in vitro* (NOEC of 0.03-0.3 nM EE2). Other estrogen responsive genes such as *follistatin* (*fst*), *fatty acid binding protein 3* (*fabp3*) and *nitric oxide synthase interacting protein* (*nosip*) were also regulated in the primary hepatocytes. Although the roles of these genes were not studied in detail herein, they have previously been reported to be regulated in the liver of fish after exposure to E2 and EE2 (Gunnarsson et al., 2007; Levi et al., 2009).

Interestingly, the microarray analysis revealed that *nr0b2* (also known as *the small heterodimer partner, SHP*), the product of which is a potential transcriptional repressor of *esr1* and downstream genes (Ehrlund and Treuter, 2012), was significantly up-regulated after exposure to 30 nM EE2. Although controversy still exists with regard to the actual role of *nr0b2* in fish (Park et al., 2007), up-regulation of *nr0b2* may provide a potential explanation for the slight reduction in transcriptional activation observed for *esr1* and *vtg1* after exposure to the highest EE2 concentration (Fig. 3). Although the repression of the vtg gene and protein expression at high estrogen concentrations have been amply demonstrated elsewhere (Hultman et al., 2015; Petersen and Tollefsen, 2011; Rankouhi et al., 2004), further investigations will be required to properly elucidate the role of *nr0b2* in piscine ER signaling.

Enrichment of hepatic DEGs in additional estrogen and ER-mediated pathways were identified to be associated with several reproductive processes involving calcium (e.g. oocyte formation),

GNRH (e.g. hormone regulation) and androgen signaling (Table 4) that is relevant also for other organs. These observations agree well with responses to estrogens in fish tissues such as the pituary (Harding et al., 2013). The GO-based functional analysis further identified calcium signaling being affected which is involved in various key regulatory processes in the cell such as cell death (Pretorius and Bornman, 2005), molecular transport, reproduction, GNRH signaling and hormone signaling (Harding et al., 2013; Jobin and Chang, 1992).

4.2 Biotransformation

Biotransformation is a key step in the detoxification in organisms and important for the reduction of intracellular concentrations of both xenobiotics and compounds of endogenous origin (Newman, 2009). Several genes involved in the biotransformation of (xeno)estrogens were affected in the hepatocytes at low (0.03 nM), intermediate (0.3 and 3 nM) and high (30 nM) concentrations of EE2. The present study identified differential modulation of genes involved in phase I (*cyp1a* and *comt)*, phase II biotransformation (*gst, sult6b1, ugt1b5, and ugt2a1*) and phase III-multidrug transport (resistance) (*abc* and *abcb11b).* The induction of *ugt2a1* (Suppl. table A) was consistent with increased hepatic glucurononyl conjugation of hydroxylated substrates from phase I biotransformation as previously described for endogenous steroids and xenobiotic compounds in fish (Gao et al., 2014). Subsequent induction of the bile salt pump *abc* and *abcb11b,* which are involved in hepatic cellular efflux transport of xenobiotics (Luckenbach et al., 2014) and reported to be induced by EE2 (Finne et al., 2007), confirm that EE2 was also actively excreted from the hepatocytes. Down-regulation of other phase II biotransformation genes such as *gst, sult6b1, and ugt1b5*, suggest that these pathways play less important roles in the biotransformation of estrogens in fish (Kurogi et al., 2013;

Mortensen and Arukwe, 2007; Skillman et al., 2006; Solé et al., 2000; Sovadinová et al., 2014; Wang et al., 2014).

Interestingly, the *cyp1a* gene, which is normally induced by exposure to xenobiotics in fish (Schlenk et al., 2008), was suppressed in a concentration-dependent manner in both the microarray and qPCR analysis (Fig. 3). Down-regulation of *cyp1a* has previously been associated with estrogen exposure and proposed to be the result of a uni- or bi-directional negative crosstalk between the upstream nuclear receptors ER and aryl hydrocarbon receptor (AhR) (Bemanian et al., 2004; Gräns et al., 2010). The present microarray study shows indication of such cross-talk as up-regulation of the ERα-recruited squelching nuclear factor 1 (*nf1b*) may reduce AhR mediated *cyp1a* expression (Ricci et al., 1999). However the *ahr* gene transcript, assumed to be central in the AhR-ER cross-talk (Bemanian et al., 2004; Matthews et al., 2005; Ohtake et al., 2003; Safe and Wormke, 2003) was not significantly expressed. Although this lack of positive verification may have been due to high inter-replicate variation, additional studies to decipher the role of AhR in modulating the ER activity in fish may be warranted. This seems also to be the case for the CYP P450 isoform 2M1 (CYP2M1), a major regulator of lauric acid hydroxylation, which has been reported to be suppressed by estrogenic compounds (Sovadinová et al., 2014). However, this gene was not identified to be differentially regulated in the present study. Nevertheless, the present results clearly demonstrated that primary hepatocytes retain many of their native detoxification properties and agree with previous suggestions elsewhere (Finne et al., 2007; Hultman et al., 2015; Segner, 1998; Segner and Cravedi, 2000).

4.3 Lipid and cholesterol homeostasis

Disruption of steroidogenesis and lipid metabolism in fish exposed to estrogens may lead to accumulation of lipids, impairment of hormone, glucose and cholesterol homeostasis in various

organs (Koren et al., 1982; Tocher, 2003), affecting vitellogenesis and normal oocyte formation and development (Doyle et al., 2013; Levi et al., 2009; Luckenbach et al., 2008). Steroid, lipid (e.g. apolipoprotein and high density lipoprotein (HDL) cholesterol) and fatty acid homeostasis (metabolism, biosynthesis, uptake, and transport) is mainly regulated in the liver by *pparα* (Lee et al., 2003; Tocher, 2003), which in the present study was transcriptionally suppressed by EE2 in a concentration-dependent manner (Fig. 4). Estrogens such as E2 and EE2 are not PPAR-ligands themselves, but may indirectly interact with PPARs through crosstalk with the ER (Keller et al., 1995). This receptor crosstalk has been proposed to be involved in competitive binding of shared transcriptional co-regulators upon estrogen exposure (Wang and Kilgore, 2002) in addition to incomplete PPAR binding to the ERE resulting in reduced transcriptional activation of PPAR (Keller et al., 1995), as indicated in the present work. Another abundantly expressed PPAR in the fish liver is the *pparγ,* the product of which is responsible for peroxisomal *β*-oxidation of fatty acids in fish (Ruyter et al., 1997), and may therefore aid the uptake and to some extent also metabolism of lipids in the liver. The present study identified that *pparγ* was significantly suppressed only at the highest EE2 concentration (30 nM) (Fig. 3), hence suggesting that *pparγ* is less susceptible to EE2-induced modulation than *pparα* in the hepatocytes. Nevertheless, suppression of *pparα* and *pparγ* in primary hepatocytes exposed to estrogens is suggestive of chemical interference with key regulators of lipid metabolism in fish hepatocytes.

Impaired *pparα* expression may also affect transcriptional activation of *apolipoprotein A-I* (*apoa1*) and *A-II* (*apoa2*) in fish (Tocher, 2003), transcripts which the present study did not identify as significantly altered. These apolipoproteins have a major role in the uptake and transport of lipids across the cellular membrane and into the blood, where they function as co-regulators in transport, lipoprotein uptake, lipid metabolism or act as inhibitors of catabolic pathways (Erkelens, 1989; Kingsbury and Bondy, 2003). In fish, apolipoproteins consist of

various classes including apolipoprotein a (apoa) and apolipoprotein e (apoe) which regulates the incorporation of lipids and lipoproteins in the oocyte (Luckenbach et al., 2008). Suppression of *apo1a* and *apoe* in liver tissue has previously been associated with exposure to E2 and EE2 (Hoffmann et al., 2006; Martyniuk et al., 2007; Wit et al., 2010), which ultimately may modify uptake and transport of cholesterol and lipids during vitellogenesis and oocyte formation (Doyle et al., 2013; Hoffmann et al., 2006). None of these genes were identified as significantly regulated in the microarray analysis in the present study, an observation in line with previous transcriptional studies in primary hepatocytes (Finne et al., 2007; Sovadinová et al., 2014) and whole fish (Doyle et al., 2013; Levi et al., 2009) exposed to EE2 and E2. The lack of modulation of *apo1* and *apoe* may suggest that lipid transport and uptake is maintained during vitellogenesis, hence allowing normal oocyte development (Doyle et al., 2013), which has previously been demonstrated to be compromised in fish after EE2 exposure (Schäfers et al., 2007).

Interestingly, genes associated with increasing biosynthesis and cellular uptake of cholesterol such as *apof* (also known as *lipid transfer inhibitor protein (ltip)*), *angiopoietin-related protein 3, 7-dehydrocholesterol reductase* (*dhcr*), *low-density lipoprotein receptor* (*ldlr*) and *neutral cholesterol ester hydrolase 1* (*nceh1)* were all up-regulated in the EE2-exposed hepatocytes. These genes are associated with increased cholesterol biosynthesis and transport during vitellogenesis in fish (Hoffmann et al., 2006; Kersten, 2005; Levi et al., 2009) and may be modified through ERα-mediated interference by negative feedback regulation of cholesterol biosynthesis (Wang et al., 2006).

Overall, present study found xenoestrogen-mediated molecular changes associated with lipid and cholesterol homeostasis which previously has been proposed to affect fish through

disruption of sex steroid biosynthesis (Levi et al., 2009) and potentially cause delayed sexual maturation and subsequent reproductive success in fish (Schäfers et al., 2007).

4.4 Cellular growth and development

Impaired cellular growth and development in juvenile and adult fish has previously been associated with exposure to low concentrations of estrogenic compounds (Ashfield et al., 1998; Schäfers et al., 2007; Shved et al., 2008). The present study identified concentration-dependent modulation of genes associated with negative cell growth regulation (e.g. *ghr-1, igfbp-1, myostatin*) (Table 4, Fig. 3, 6C and D, Suppl. table B, E), whereof several genes have been reported regulated in EE2 exposed fish elsewhere (Martyniuk et al., 2007; Shved et al., 2008). Impaired cell and organism growth has previously been correlated with disruption of steroid homeostasis and reproduction in fish, potentially due to energetic constraints introduced by simultaneously occurring energy demanding processes such as reproduction, growth (Davis et al., 2008) and detoxification of xenoestrogens (Schäfers et al., 2007). Although not studied in detail, this may also be applicable to the primary hepatocytes studied herein as they likely have limited energy/lipid storage capacity and lack the ability to compensate large energy losses by mobilization of nutrients from other tissues. However, additional explanations such as nuclear receptor cross-talk between the ER and GH/IGF-1 systems leading to suppression of *ghr, igfbp-1* genes has been proposed to explain estrogen-induced impairment of growth during sexual differentiation in fish (Davis et al., 2008; Nelson and Habibi, 2013; Shved et al., 2008). The findings of the present *in vitro* study indicate that primary fish hepatocytes retain the molecular regulatory networks specifically associated with IGF-1 and GH signaling, and may thus provide valuable insight into how these processes are affected after exposure to xenoestrogens in fish.

4.5 Cancer and other signaling pathways

Estrogens exert carcinogenic effects in mammals by ER signaling and transduction pathways associated with inhibition of apoptosis and increased cell proliferation (Pearce and Jordan, 2004; Yager and Davidson, 2006). Evidence of estrogen-induced liver carcinogenesis and perturbation of cell cycle regulation has been demonstrated in E2-exposed fish and been proposed to result from interference with putative genes associated with cell cycle regulation such as G1-S and S-G2 phase transition (Lam et al., 2011), both being mechanisms that regulate mitosis and cellular growth. The present study identified that several affected pathways and GOs were associated with suppression of cell death (caspase activity, death receptor and apoptosis signaling) supported by the suppression (*direct inhibitor-of-apoptosis protein-binding protein with low pI* (*Diablo*), *tumor necrosis factor ligand superfamily member 6* (*faslg*)) and induction (*X-linked inhibitor of apoptosis* (*xiap*)) of putative DEGs, which further have been associated with a number of cancer types (Reed, 2003). Interestingly, the present study identified enrichment of several additional pathways associated with cancer (i.e. breast cancer regulation by stathmin1, molecular mechanism of cancer and estrogen-dependent breast cancer) and regulation of DEGs (*reticulon 1/3* (*rtn1/3*), *pim-1/3 oncogene* (*pim1/3*) and *breast cancer metastasis-suppressor 1-like* (*brms1*)), whereof several have been reported modulated in fish exposed to estrogens (Colli-Dula et al., 2014; Harding et al., 2013; Levi et al., 2009). However, estrogens have also been proposed to induce oxidative DNA damage (Lam et al., 2011) through production of reactive metabolites that subsequently may cause mutagenic, carcinogenic and genotoxic effects (Roy et al., 2007; Russo et al., 2003). Interestingly, putative DEGs involved in DNA damage such as *breast cancer 1* (*brca1*) were suppressed. This seems to contradict the behavior of *brca1* in E2-exposed fish (Lam et al., 2011). The induction of *brca1* is primarily mediated by estrogens binding to and activating the ER, but recent studies have suggested that recruitment of unliganded AhR to the proximal *brca1* transcriptional binding domain is required to potentiate its expression (Hockings et al., 2006). Lack of AhR

regulation (Suppl. table A and D) after exposure to EE2 in this study suggested that AhR may not respond in a similar manner as that seen *in vivo*. However, the decreased transcription of the genes encoding the death receptor (*faslg*) and proteins involved in apoptosis signaling (*Diablo* and *xiap*) may suggest that EE2 is associated with estrogen-mediated inhibition of apoptosis and induced cell proliferation in primary hepatocytes as observed *in vivo* (Harding et al., 2013; Lam et al., 2011; Levi et al., 2009).

4.6 Primary hepatocytes as a screening model for estrogen mimics

Rainbow trout hepatocytes have been suggested to be a rapid and high-throughput assay for the identification of estrogenic and antiestrogenic properties of chemicals (Hultman et al., 2015; Navas and Segner, 2000; Smeets et al., 1999; Tollefsen et al., 2008b), and recently demonstrated to yield highly reproducible responses to estrogenic biomarkers such as Vtg expression both at the gene and protein level (Hultman et al., 2015). The present study demonstrated that the rainbow trout hepatocyte bioassay, in combination with the global transcriptional analysis, may provide better understanding of the MoAs of EE2. The main advantages of applying this approach to rainbow trout hepatocytes are: low amount of biological material required for the analysis, rapid and unbiased MoA characterization and low biological variation. The primary hepatocytes displayed well-characterized biomarker gene responses, which was generally in agreement with that occurring *in vivo* in fish after exposure to E2 and EE2. This applies in particular to the *in vivo* expression of DEGs associated with estrogen receptor signaling (e.g. *esr1, vtg1, zrp*) , biotransformation (e.g. *cyp1a, abc11b*), lipid and cholesterol metabolism (*e.g. dhcr,fabp3, ldlr*), growth (e.g.*igfbp-1*, *ghr*), and cancer/apoptosis (*rtn1/3, pim1/3, xiap, diablo*), which were conserved and responsive in the rainbow trout hepatocyte model. The observed *in vitro* transcriptional changes increased in number of DEGs identified and their responses to EE2, a finding also seen when analyzing

functional (GO) enrichment and pathway analysis. Interestingly, the sensitivity of the hepatocytes to well-known biomarkers such as *vtg, esr1* and *cyp1a* also seemed to be in accordance with that observed in *in vivo* exposure studies with 0.87-125 ng/L EE2 (Doyle et al., 2013; Gunnarsson et al., 2007; Hoffmann et al., 2006; Martyniuk et al., 2007; Skillman et al., 2006). The *in vitro* to *in vivo* extrapolation performed followed principles demonstrated by Skillman et al. (2006) by using measured liver cell concentrations of EE2 in the hepatocyte assay (see Hultman et al., 2015). Using a fixed water:liver EE2 accumulation ratio derived from a 48h *in vivo* exposure study to 125 ng/L EE2 (Skillman et al., 2006), the *in vitro* Lowest Observed Effect Concentration (LOEC = 0.3 nM, fig. 3) were estimated to correspond to an external water exposure concentration of 6.4-9.6 ng/L in the present study. These external exposure concentrations has been demonstrated not only to be in the range of sensitivity observed *in vivo* (Gunnarsson et al., 2007; Martyniuk et al., 2007), but also considered to be environmentally relevant (Larsson et al., 1999).

Although molecular methods such as transcriptomics have greatly improved our ability to perform global response assessments, transcriptional regulation will not account for posttranscriptional modifications leading to alterations of downstream molecular events such protein expression, cellular signaling, and metabolic activity (Schirmer et al., 2010). However, close correlation of Vtg gene and protein expression were observed in the same experimental models (Hultman et al., 2015), and suggest that certain transcriptional responses are descriptive for the activity occurring at the functional level. As a thorough evaluation of the predictability of transcriptional changes to other levels of organization was not conducted in the present study, it is recommended that such effort is undertaken in the future characterization of MoAs of EE2 and other xenoestrogens. The use of primary hepatocytes also has some limitations for larger scale implementation in MoA assessment of EDCs as the bioassay is restricted to hepatic responses and does not necessarily reflect MoAs relevant for other organs (e.g. oocyte

formation) and adverse effects (e.g. feminization) at the whole organism level. However, this may also be advantageous as the primary hepatocytes reflect key molecular events in the liver and can thus be studied without influences from other organs involved in the hypothalamus-pituary-gonad axis. Overall, rainbow trout hepatocytes seem to represent a feasible complement to *in vivo* testing as displaying many central hepatic MoA relevant for endocrine disruption, and in particular the response to ER agonists.

5 Conclusion

The present study showed that the primary rainbow trout hepatocyte bioassay can be a suitable and responsive *in vitro* model when using a broad content transcriptomic analysis to characterize the effects of the ER-agonist 17α-ethinylestradiol (EE2). The potential toxic mechanisms of EE2 in the primary hepatocytes were successfully characterized and found to affect gene expression related to biotransformation, lipid metabolism and ER signaling and ER-mediated cellular responses. The potential of using primary hepatocytes for assessment of estrogen mimics was supported by the clear concentration–dependent enrichment of functional categories (GO), pathways and DEGs associated with the well-known MoAs of EE2 (e.g. induction of estrogen signaling, suppression of biotransformation and growth, disruption of lipid homeostasis, and cancer) *in vivo*. Furthermore, well-characterized estrogen-responsive biomarker genes (e.g. *esr1, vtg, zrp*) were identified to be differentially expressed in accordance with several previous *in vitro* and *in vivo* estrogen studies. The primary hepatocyte model is thus proposed to represent a promising complement to *in vivo* testing of EDCs, and in particular for estrogen mimics that affect central liver functions associated with endocrine regulation and reproductive processes in fish.

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Availability of supporting data

The raw data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) repository, accession number: GSE68335 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68335. Other data sets supporting the results of this article are included in attached excel sheet.

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Figures

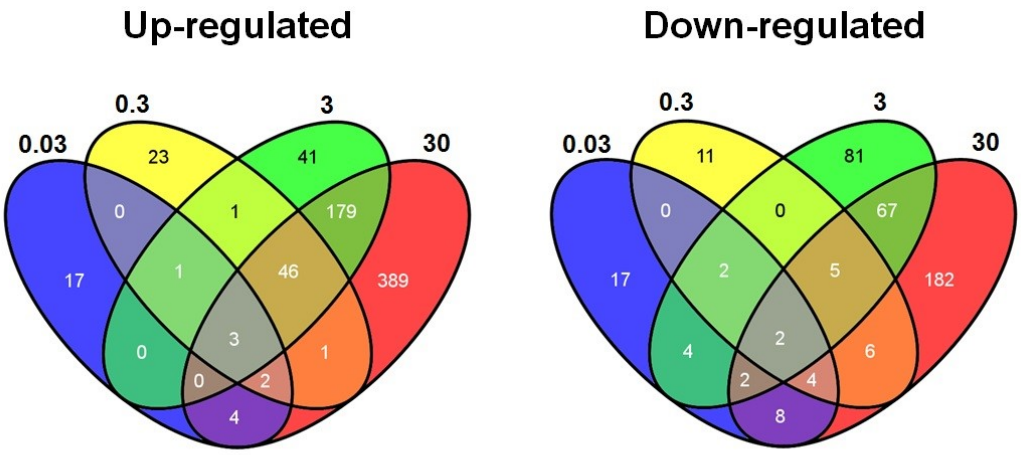

Figure 1. Differentially (up- and down-) expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 0.03, 0.3, 3 and 30 nmol/L 17α-ethynylestradiol (EE2) for 48h.

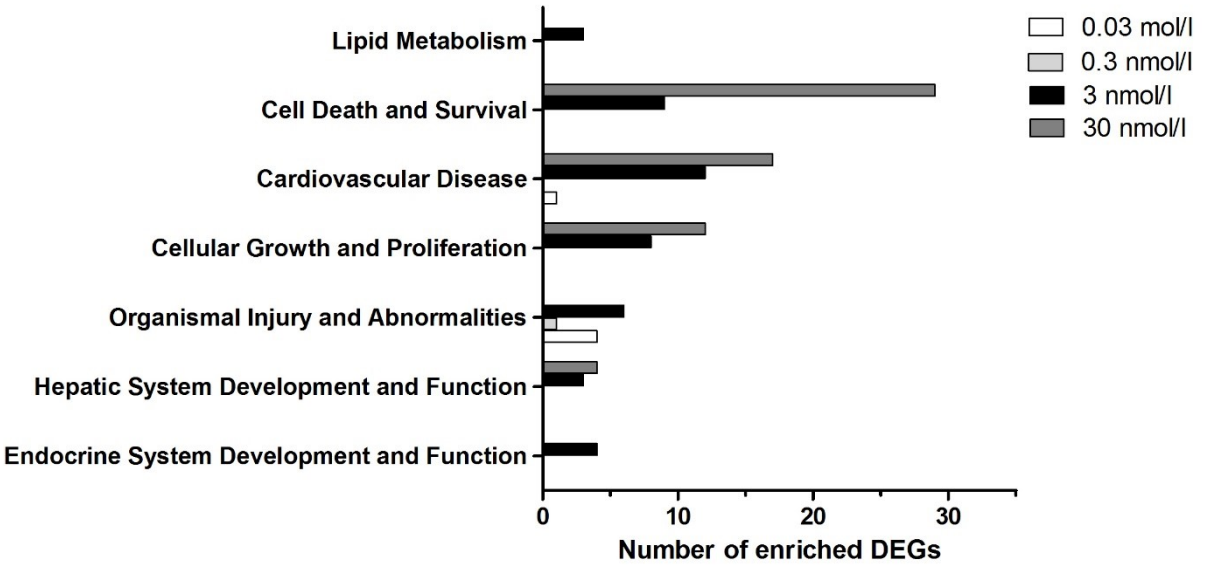

Figure 2. Toxicity pathways associated with the modulation of differently expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethynylestradiol (EE2) for 48h. All chemical concentrations having significant enrichment of relevant toxicity pathways are in the figure represented by individual bars.

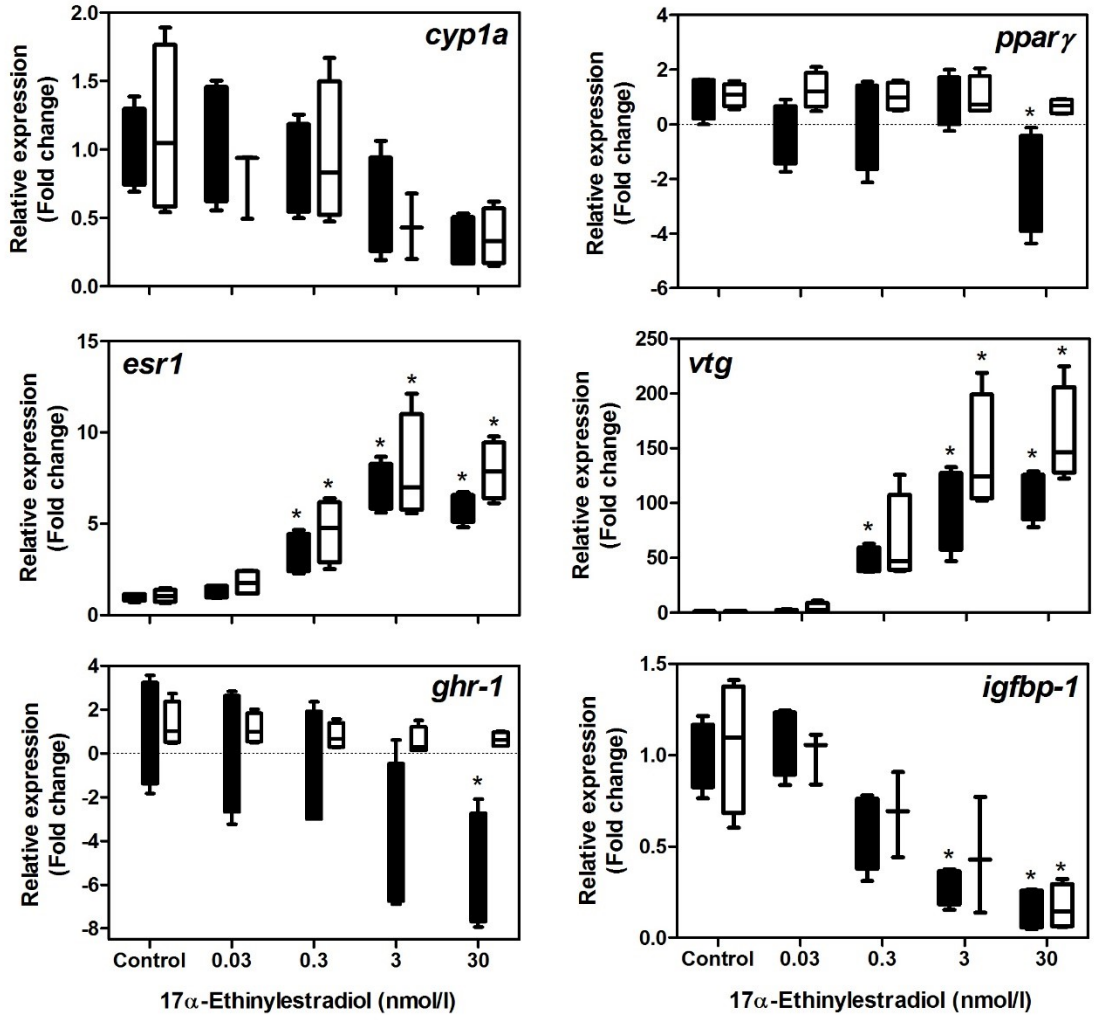

Figure 3. A comparison of gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethynylestradiol (EE2) for 48h. Data (Mean ± SEM) depicts quantitative real time polymerase chain reaction, qPCR (open boxplots, n=3-4) and microarray (filled boxplots, n=4). *Denotes genes being significantly different ($p<0.05$) from the solvent control. Abbrevations: cyp1a1 – cytochrome P450 1a1; esr1 – estrogen receptor 1; fst - follistatin; ghr-1 – growth hormone receptor 1; igfbp-1 – insulin growth factor binding protein 1; pparα – peroxisome proliferator-activated receptor α; pparγ – peroxisome proliferator-activated receptor γ; vtg1 – vitellogenin 1.

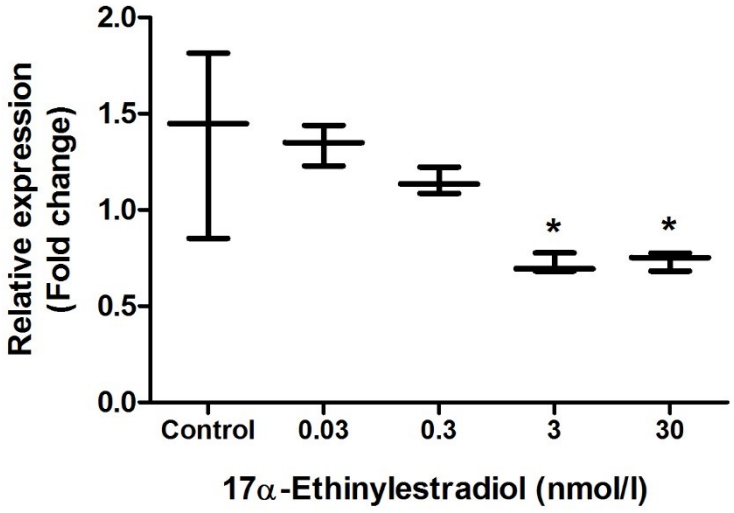

Figure 4. Gene expression of proliferator-activated receptor α (pparα) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethynylestradiol (EE2) for 48h. Data (Mean ± SEM) depicts quantitative real time polymerase chain reaction, qPCR (open boxplots, n=3). *Denotes the gene being significantly different ($p<0.05$) from the solvent control.

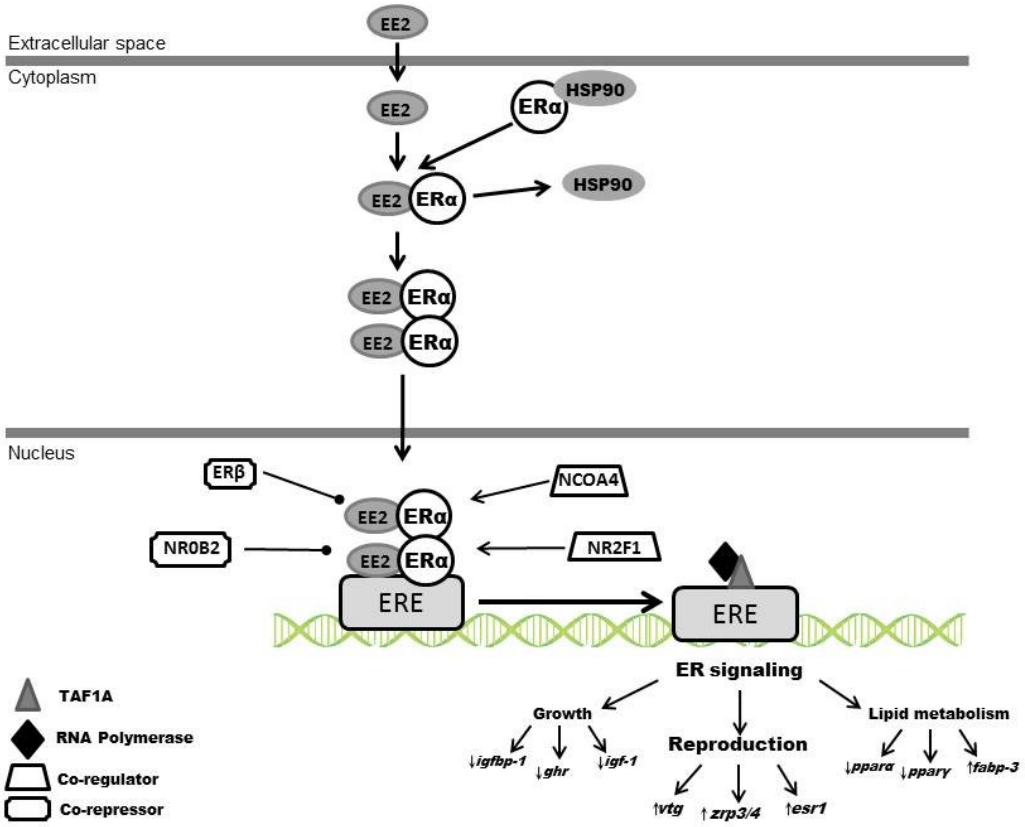

Figure 5. Estrogen receptor (ER) signaling pathway in fish based on differential gene expression in primary rainbow trout hepatocytes exposed to 17α-ethynylestradiol (EE2) for 48h. Abbrevations: HSP90 - heat shocking protein 90; ERE – Estrogen responsive element; NCOA4 - nuclear receptor coactivator 4, NR2F1- nuclear receptor subfamily group member 1; NR0B2 - nuclear receptor subfamily group member 2 ; TAF1A – TATA box-binding protein-associated factor RNA polymerase I subunit A; IGFBP-1 – insulin growth factor binding protein 1; IGF-1 – insulin growth factor 1; GHR – growth hormone receptor; VTG – vitellogenin; ZRP3/4 – zona radiata protein 3/4; *PPARα/γ* – peroxisome proliferator-activated receptor α/γ, FABP-3 – fatty acid binding protein 3. The pathway is modified from Lanzino et al., (2005), Petit et al., (1999) and Sanyal et al., (2002).

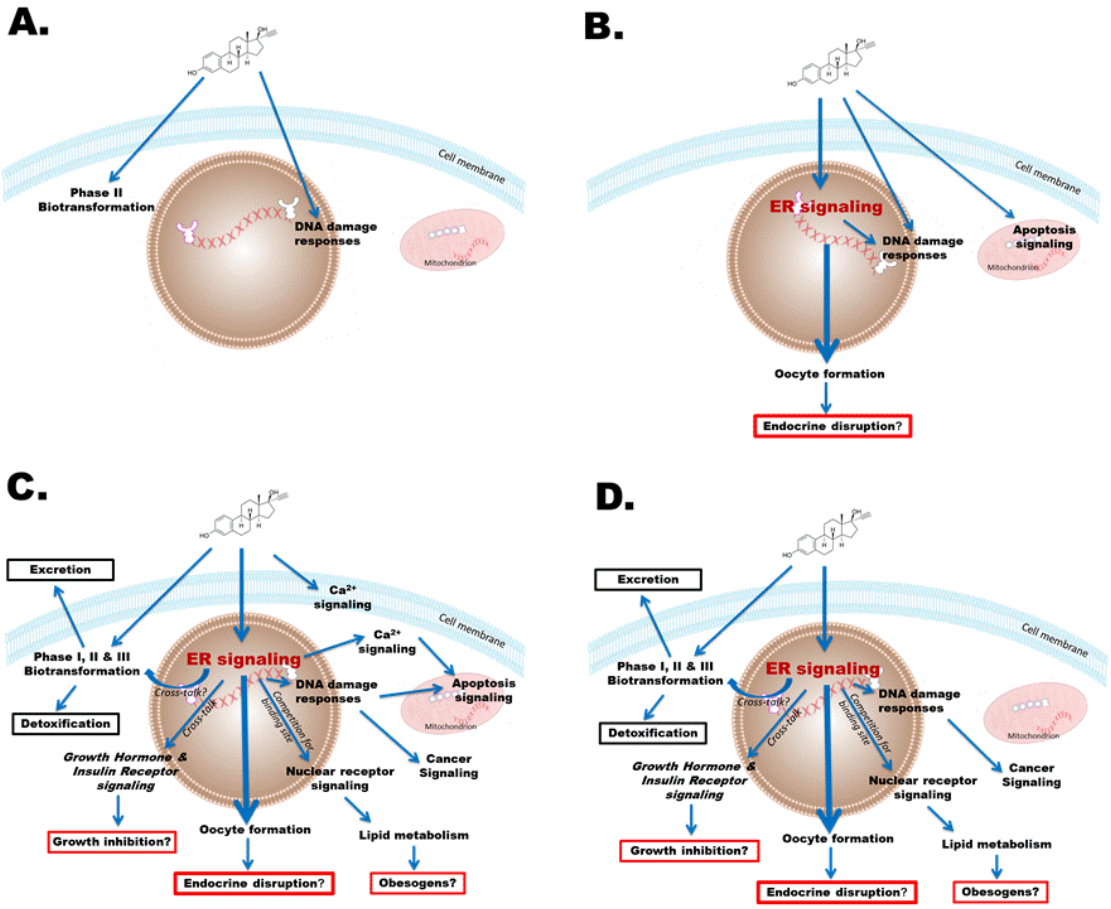

Figure 6. Concentration-dependent modulation of toxicity (canonical) pathways and supporting differently expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 0.03 (A), 0.3 (B), 3 (C) and 30 (D) nmol/L 17α-ethynylestradiol (EE2) for 48 hours.

Tables

Table 1. Genes, primer sequences, accession numbers and analysis protocol used for the qPCR analysis.

Target gene		Primer sequence	Amplicon size	Annealing temperature	Primer conc. (nmol/L)	Efficiency (%)	Acc. no	Reference
Ef1α	*-forward*	5'-AGCGCAATCAGCCTGAGAGGTA-3'	NR	62°C	600	107.5	NM_001141909.1[1)]	Gabillard et al., 2006
	-reverse	5'-GCTGGACAAGCTGAAGGCTGAG-3'			600			
PPARα	*-forward*	5'-CTGGAGCTGGATGACAGTGA-3'	195	55°C	500	95.0	AY494835	Cruz-Garcia et al., 2002
	-reverse	5'-GGCAAGTTTTTGCAGCAGAT-3'			500			
PPARγ	*-forward*	5'-GACGGCGGGTCAGTACTTTA-3'	171	60°C	700	94.2	AY356399.1	Cruz-Garcia et al., 2002
	-reverse	5'-ATGCTCTTGGCGAACTCTGT-3'			700			
ERα	*-forward*	5'-CCCTGCTGGTGACAGAGAGAA-3'	NR	61°C	270	99.9	AJ242741	Nagler et al., 2007
	-reverse	5'-ATCCTCCACCACCATTGAGACT-3'			620			
Vtg	*-forward*	5'-GAGCTAAGGTCCGCACAATTG-3'	NR	61.4°C	700	110	X92804	Celius et al., 2000
	-reverse	5'-GGGAAACAGGGAAAGCTTCAA-3'			700			
GHR-1	*-forward*	5'-CGTCCTCATCCTTCCAGTTTTA-3'	NR	62°C	500	97.0	NM_001124535.1[1)]	Gabillard et al 2006
	-reverse	5'-GTTCTGTGAGGTTCTGGAAAAC-3'			500			

IGFBP-1	*-forward*	5'-AGTTCACCAACTTCTACCTACC-3'	NR	62°C	700	107.2	NM_001124561.1[1)]	Gabillard et al 2006
	-reverse	5'-GACGACTCACACTGCTTGGC-3'			700			
CYP1A	*-forward*	5'-TCCTGCCGTTCACCATCCCACACTGCAC-3'	NR	57°C	700	90.5	U62797.1	Gräns et al., 2010
	-reverse	5'-AGGATGGCCAAGAAGAGGTAGACCTC-3'	NR		700			

Footnotes

1) Acc. No. was not provided by reference.

Abbreviations: nM – nmol/L; ef1α – Elongation factor 1 α; *pparα* – Peroxisome proliferator-activated receptor α; *pparγ* - Peroxisome proliferator-activated receptor γ; esr1 – Estrogen receptor 1/α; vtg – Vitellogenin; ghr-1 – Growth hormone 1; igfbp-1 - Insulin growth factor protein 1; cyp1a – Cytochrome P450 1A

Table 2. Common Gene Ontology (GO) processes over-represented in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48h exposure to 17α-ethinylestradiol (EE2). The exposure to 0.03 nmol/l did not yield any significant enrichment of GOs.

nmol/l	GO-ID	GO namespace	*P*-value (<0.05)	No. of DEGs	Total features on array	GO Term	Functional category
0.3	GO:0019216	P	$2.69E^{-02}$	5	270	Regulation of lipid metabolic process	Lipid and fatty acid metabolism
	GO:0019395	P	$2.69E^{-02}$	4	154	Fatty acid oxidation	
	GO:0034440	P	$2.69E^{-02}$	4	154	Lipid oxidation	
	GO:0046320	P	$3.65E^{-02}$	3	74	Regulation of fatty acid oxidation	
	GO:0043154	P	$4.15E^{-03}$	4	46	Negative regulation of caspase activity	Programmed cell death
	GO:0043028	F	$3.65E^{-02}$	3	74	Caspase regulator activity:Regulation of caspase activity	
	GO:0043027	F	$1.28E^{-02}$	3	35	Caspase inhibitor activity: Regulation of caspase activity	
3	GO:0060397	P	$2.57E^{-02}$	3	13	JAK-STAT cascade involved in growth hormone signaling pathway	Cell Growth and development
	GO:0046543	P	$4.19E^{-03}$	3	5	Development of secondary female sexual characteristics	Reproduction
	GO:0032504	P	$1.72E^{-02}$	21	838	Multicellular organism reproduction	
	GO:0048609	P	$1.72E^{-02}$	21	838	Reproductive process in a multicellular organism	
	GO:0000003	P	$2.24E^{-02}$	28	1344	Reproduction	
	GO:0022414	P	$2.76E^{-02}$	27	1330	Reproductive process	
	GO:0045136	P	$3.22E^{-02}$	3	15	Development of secondary sexual characteristics	
	GO:0046544	P	$2.57E^{-02}$	2	3	Development of secondary male sexual characteristics	

	GO:0019395	P	$2.18E^{-02}$	8	154	Fatty acid oxidation	Lipid and fatty acid metabolism
	GO:0034440	P	$2.18E^{-02}$	8	154	Lipid oxidation	
	GO:0010876	P	$2.18E^{-02}$	14	446	Lipid localization	
	GO:0006635	P	$2.57E^{-02}$	6	89	Fatty acid beta-oxidation	
	GO:0006631	P	$2.76E^{-02}$	15	539	Fatty acid metabolic process	
	GO:0015645	F	$2.57E^{-02}$	5	55	Fatty acid ligase activity	
	GO:0035257	F	$3.67E^{-02}$	8	182	Nuclear hormone receptor binding: Hormon receptor binding	Regulation of endocrine system
	GO:0005102	F	$3.94E^{-02}$	31	1661	Receptor binding: Hormon receptor binding	
	GO:0035258	F	$6.38E^{-03}$	7	83	Steroid hormone receptor binding: Hormon receptor binding	
	GO:0050681	F	$2.57E^{-02}$	5	59	Androgen receptor binding: Hormon receptor binding	
30	GO:0040008	P	$3.97E^{-02}$	18	753	Regulation of growth	Cell Growth and development
	GO:0040008	P	$3.79E^{-05}$	40	753	Regulation of growth	
	GO:0040007	P	$6.67E^{-05}$	56	1294	Growth	
	GO:0016049	P	$2.25E^{-03}$	29	576	Cell growth	
	GO:0001558	P	$2.37E^{-03}$	24	435	Regulation of cell growth	
	GO:0035264	P	$7.27E^{-03}$	13	171	Multicellular organism growth	
	GO:0045454	P	$1.51E^{-02}$	12	164	Cell redox homeostasis	
	GO:0030308	P	$2.79E^{-02}$	13	208	Negative regulation of cell growth	
	GO:0045926	P	$3.11E^{-02}$	14	239	Negative regulation of growth	
	GO:0007275	P	$4.21E^{-02}$	191	7536	Multicellular organismal development	
	GO:0060397	P	$1.16E^{-02}$	4	13	JAK-STAT cascade involved in growth hormone signaling pathway	

GO:0000003	P	$2.25E^{-03}$	52	1344	Reproduction	Reproduction
GO:0032504	P	$2.37E^{-03}$	37	838	Multicellular organism reproduction	
GO:0048609	P	$2.37E^{-03}$	37	838	Reproductive process in a multicellular organism	
GO:0022414	P	$4.91E^{-03}$	50	1330	Reproductive process	
GO:0046543	P	$9.60E^{-03}$	3	5	Development of secondary female sexual characteristics	
GO:0019953	P	$2.53E^{-02}$	30	737	Sexual reproduction	
GO:0032355	P	$3.63E^{-02}$	8	91	Response to estradiol stimulus	Regulation of endocrine system
GO:0042562	F	$4.19E^{-02}$	7	73	Hormone binding	

Abbreviations

P - biological process; F - molecular function; DEGs – differently expressed genes

Table 3. Top gene networks (score>10, supporting genes >10) and supporting differently expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48h exposure to 17α-ethinylestradiol (EE2).

EE2 Conc. (nmol/L)	Top network function	Score	DEGs
0.03	Cell Signaling, Molecular Transport, Nucleic Acid Metabolism	80	34
0.3	Cellular Function and Maintenance, Small Molecule Biochemistry, Cellular Development	109	46
3	Hematological Disease, Hereditary Disorder, Carbohydrate Metabolism	172	98
	Cellular Assembly and Organization, Cellular Function and Maintenance, Lipid Metabolism	100	65
	Cellular Development, Cellular Growth and Proliferation, Cell Cycle	91	63
	Cell Death and Survival, Post-Translational Modification, Cardiac Necrosis/Cell Death	11	14
30	Post-Translational Modification, Lipid Metabolism, Small Molecule Biochemistry	121	89
	Embryonic Development, Organ Development, Organismal Development	104	81
	Cellular Assembly and Organization, Cellular Function and Maintenance, Cellular Movement	99	80
	Post-Translational Modification, Cell Signaling, Hereditary Disorder	85	70
	Amino Acid Metabolism, Small Molecule Biochemistry, Connective Tissue Disorders	82	69
	Cell Cycle, Tissue Morphology, Cancer	63	58
	Cell Morphology, Cellular Function and Maintenance, Cell Cycle	63	59

Score – Numerical value used to rank networks according to their degree of relevance to the Network Eligible molecules in your dataset according to Ingenuity pathway analysis (definition by IPA).

DEGs – Differently expressed genes

Table 4. A selection of relevant canonical pathways and supporting differentially expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48h exposure to 17α-ethinylestradiol (EE2). Arrows indicates ↑ up- and ↓ down-regulation of DEGs. Full description of gene symbols is found in supplementary table A.

EE2 conc. (nmol/L)	Apical toxicological category	Ingenuity Canonical pathway	P-value[a]	Ratio	Supporting DEGs
0.03	Intracellular and Second-Messenger Signaling	Glucocorticoid Receptor Signaling	$1.35E^{-2}$	$1.00E^{-2}$	JAK1↓,POU2F1↓,NPPA↓
		Insulin Receptor Signaling	$2.69E^{-2}$	$1.34E^{-2}$	JAK1↓, PPP1CB↑
0.3	Apoptosis	Apoptosis Signaling	$2.09E^{-2}$	$2.00E^{-2}$	ROCK1↑,XIAP↑
3	Apoptosis	Death Receptor Signaling	$3.16E^{-2}$	$4.41E^{-02}$	DIABLO↓,XIAP↑,FASLG↓
		Apoptosis Signaling	$2.04E^{-2}$	$4.00E^{-02}$	ROCK1↑,DIABLO↓,XIAP↑,FASLG↓
	Disease-Specific Pathways	Estrogen-Dependent Breast Cancer Signaling	$3.89E^{-2}$	$4.11E^{-2}$	STAT5A↑, STAT5B↑, Creb5↑
	Immune Response	IL-6 Signaling	$4.90E^{-2}$	$3.23E^{-2}$	GRB2↑, CRP↑, STAT3↓, CEBPB↓
	Intracellular and Second-Messenger Signaling	Calcium Signaling	$2.04E^{-2}$	$2.76E^{-2}$	MYL2↑, RYR3↑, HDAC10↓, ASPH↑, CABIN1↑, Creb5↑
		Glucocorticoid Receptor Signaling	$1.26E^{-2}$	$2.68E^{-2}$	STAT5A↑, POU2F1↓, GRB2↑, STAT3↓, CEBPB↓, NPPA↓, STAT5B↑, POLR2I↓
		Phospholipase C Signaling	$6.76E^{-3}$	$3.02E^{-2}$	MYL2↑, GRB2↑, GNB2L1↑, HDAC10↓, GNG3↓, PLA2G12B↑, RHOF↓, Creb5↑
	Nuclear Receptor Signaling	PXR/RXR Activation	$4.47E^{-2}$	$3.02E^{-2}$	CPT1A↑,ABCB11↑,IGFBP1↓
		TR/RXR Activation	$1.82E^{-2}$	$3.67E^{-2}$	TSHB↑, NCOA4↑, PFKP↑, TBL1XR1↓
		Androgen Signaling	$4.37E^{-2}$	$2.76E^{-2}$	GNB2L1↑, NCOA4↑, GNG3↓, POLR2I↓

		LPS/IL-1 Mediated Inhibition of RXR Function	$4.79E^{-2}$	$2.45E^{-2}$	CPT1A↑, ACSL5↑, ABCB11↑, SLC35A2↑, FABP3↑, ACOX3↑
	Metabolic Pathways	Thyroid Hormone Biosynthesis	$3.47E^{-2}$	$1.43E^{-2}$	IYD↓
30	Disease-Specific Pathways	Molecular Mechanisms of Cancer	$2.75E^{-2}$	$3.87E^{-2}$	MAP2K4↓, RAPGEF1↑, JAK1↓, GRB2↑, XIAP↑, CDC25B↑, PTK2↓, GNAI2↑, LAMTOR3↓, PRKCD↓, ARHGEF11↑, BMP6↑, RHOF↓, DIABLO↓, PTCH2↑
	Cell Growth and development	Corticotropin Releasing Hormone Signaling	$2.4E^{-2}$	$4.83E^{-2}$	GNAI2↑, PRKCD↓, NPR1↓, JUND↑, OPN1SW↑, PTCH2↑, Creb5↑
	Neurotransmitters and Other Nervous System Signaling	GNRH Signaling	$5.62E^{-3}$	$5.88E^{-2}$	MAP2K4↓, PTK2↓, GNAI2↑, GRB2↑, PRKCD↓, MAP3K8↑, OPN1SW↑, MAP3K2↑, Creb5↑
	Nuclear receptor signaling	PPAR Signaling	$3.16E^{-2}$	$5.61E^{-2}$	IL33↑,NR2F1↑,STAT5A↑,NR0B2↑,GRB2↑,STAT5B↑
	Intracellular and Second-Messenger Signaling	Insulin Receptor Signaling			EIF2B1↑, GRB2↑, JAK1↓, PPP1CB↑, PPP1R7↓, PPP1R3C↑, RAPGEF1↑
	Metabolic Pathway	CDP-diacylglycerol Biosynthesis I	$5.75E^{-3}$	$1.11E^{-1}$	GPAM↑, CDS1↓, CDS2↓
	Commonly regulated between 3 and 30nM[c]				
	Immune Response	CXCR4 Signaling	$5.50E^{-3}$	$5.75E^{-2}$	MAP2K4↓, PTK2↓, ROCK1↑, GNAI2↑, MYL2↑, PRKCD↓, ARHGEF11↑, GNG3↓, RHOF↓, OPN1SW↑
		CCR3 Signaling in Eosinophils	$2.63E^{-2}$	$5.22E^{-2}$	ROCK1↑, GNAI2↑, PRKCD↓, PPP1CB↑, GNG3↓, PLA2G12B↑, OPN1SW↑
	Intracellular and Second-	Tec Kinase Signaling	$6.03E^{-4}$	$6.52E^{-2}$	MAP2K4↓, PTK2↓, GNAI2↑, STAT5A↑, JAK1↓, PRKCD↓, STAT3↓, GNG3↓, STAT1↑, STAT5B↑, RHOF↓, ITK↓

Messenger Signaling	Protein Kinase A Signaling	$1.74E^{-2}$	$4.16E^{-2}$	PTPRG↓, MYL2↑, PPP1R3C↑, PPP1CB↑, GNG3↓, PTPN5↑, Creb5↑, CDC25B↑, PTK2↓, ROCK1↑, GNAI2↑, ADD3↑, FLNC↑, PPP1R7↓, PRKCD↓, PTCH2↑, OPN1SW↑
Neurotransmitters and Other Nervous System Signaling	Neurotrophin/TRK Signaling	$2.82E^{-2}$	$6.58E^{-2}$	MAP2K4↓, GRB2↑, NTRK1↑, MAP2K5↑, Creb5↑
Cell Growth and development	Oncostatin M Signaling	$1.82E^{-4}$	$1.71E^{-1}$	STAT5A↑, JAK1↓, GRB2↑, STAT3↓,STAT1↑, STAT5B↑
	Growth Hormone Signaling	$7.76E^{-3}$	$7.69E^{-2}$	STAT5A↑, GHR↓, PRKCD↓, STAT3↓, STAT1↑, STAT5B↑
	IGF-1 Signaling	$3.63E^{-2}$	$5.61E^{-2}$	PTK2↓, JAK1↓, GRB2↑, STAT3↓, IGFBP1↓, CYR61↑
Disease-Specific Pathways	Breast Cancer Regulation by Stathmin1	$3.98E^{-4}$	$6.54E^{-2}$	GRB2↑, PPP1R3C↑, TUBB2A↑, PPP1CB↑, GNG3↓, ROCK1↑, GNAI2↑, PPP2CB↑, PPP1R7↓, PRKCD↓, PPM1L↑, ARHGEF11↑, TUBA3E↑, OPN1SW↑

[a] P-value is defined to $p < 0.05$
[b] Ratio is calculated as the number of regulated molecules in a given pathway that meet cutoff criteria, divided by total number of molecules that make up that pathway.
[c] Represents 30nM EE2