Evaluation of the sensitivity, responsiveness and reproducibility of primary rainbow trout hepatocytes vitellogenin expression as a screening assay for estrogen mimics.

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Abstract

Vitellogenin (Vtg) induction in primary fish hepatocytes have been proposed as an *in vitro* screening assay for ER agonists and antagonists, but have not yet been used extensively as a high-throughput screening tool due to poor reproducibility, sensitivity and overall feasibility. The present work have evaluated the role of seasonal variation, normalization, optimal culture and assay conditions on the sensitivity, responsiveness and reproducibility of *in vitro* vtg gene and protein production in rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes using the xenoestrogen 17α-ethynylestradiol (EE2) as a test chemical. The results show that primary hepatocytes display a relatively high individual and seasonal variation in both Vtg gene and protein induction potential, although less variance was observed in assay sensitivity. Data normalization of assay response to maximum (3 nM EE2) and minimum (DMSO) Vtg production dramatically reduced this variance and led to improved assay reproducibility. A time-dependent response was observed both for gene and protein expression, reaching

maximum Vtg induction after 96h of exposure, although reproducible concentration response curves for both Vtg mRNA and protein could be obtained already after 48h. A need for chemical re-exposure of the hepatocytes was identified to be important for sustaining exposure concentrations in extended studies (>48h), whereas different plate formats (96, 24 or 6 wells) did not affect the bioassay performance. In conclusion, standardization of hepatocyte bioassay and test conditions as well as data-normalization procedures are proposed to be instrumental for more consistent and comparable results in future use of this *in vitro* assay.

Key words: Oncorhynchus mykiss, primary hepatocytes, EDCs, vitellogenin mRNA, protein

1 Introduction

The impact of endocrine disrupting chemicals (EDCs) on aquatic wildlife has been an issue of environmental concern for many years (Purdom et al., 1994). Endocrine disruptors, such as estrogen mimics, are known to interfere with the endocrine system through activation of the estrogen receptor (ER) and cellular responses associated with normal sexual maturation and differentiation in fish (Hook et al., 2007). In recent years the potential challenge of environmental EDCs have been acknowledged and various international organizations (e.g. OECD, US-EPA, Japanese Environmental Agency) have proposed regulatory frameworks and test approaches to assess the potential hazard and risk of EDCs. The recent proposal of an OECD conceptual framework (CF) for testing and assessing EDCs has proposed to categorize different assays into 5 levels ranging from computational efforts (CF1) and *in vitro* screening (CF2) to *in vivo* testing (CF 3-5) based on their ability to address both mode of action (MoA) and adverse endpoints of regulatory concern (OECD, 2010). The OECD conceptual framework for ED testing and assessment proposes *in vitro* assays as a tool to prioritizing and characterize EDCs MoA, but suggest performing *in vivo* (CF level 4-5) studies for assessment of adversity

and in-depth characterization of the MoA (OECD, 2010). However, it's expected that the international effort to address EDCs will also increase the demand for toxicological testing (Hecker and Hollert, 2011). Implementation of the European Union chemical legislation REACH is estimated alone to generate a need for hazard assessment of over 30,000 single chemicals for various toxic properties, potentially requiring the use of millions of test animals (ECHA newsletter; Rovida and Hartung, 2009). Although the true number of chemicals that may require *in vivo* testing in REACH may still be unknown, the potential demand for an increase in regulatory testing clearly call for larger implementation of the 3R`s (Reduction, Refinement and Replacement) in animal testing and further development and evaluation of alternative (non-animal) methods for EDC testing.

In vitro methods such as mammalian continuous cell lines and transgenic organisms (bacteria, yeast, and cells) have been proposed as suitable high-throughput screening (HTS) assays for EDCs at the OECD CF2 level (OECD, 2010). Development of *in vitro* methods for non-mammalian species such as fish has also been proposed, but only the fish embryo toxicity test have been validated for regulatory purposes (OECD, 2012b). Nevertheless, induction of the estrogenic biomarker vitellogenin (Vtg) in fish hepatocytes is along with estrogen receptor /androgen receptor (ER/AR) binding affinity-, aromatase and steroidogenesis assays identified as potential candidates for inclusion as OECD CF level 2 assays (OECD, 2010). Primary cultures of fish hepatocytes retain many of the innate properties of the liver such as biotransformation activity, detoxification and ER mediated responses (Pedersen and Hill, 2000; Segner and Cravedi, 2000). Thus, vitellogenin induction in primary cultures of fish hepatocytes have successfully been implemented in screening of the ER agonistic and antagonistic properties of single chemicals, complex synthetic mixtures and environmental extracts (Kim and Takemura, 2003; Petersen and Tollefsen, 2011; Tollefsen et al., 2003; Tollefsen et al., 2008b). The success of these *in vitro* bioassay have been ascribed a well-defined endocrine

MoA in oviparous fish, involving xenoestrogen binding to and activation of the ER (Mortensen and Arukwe, 2006; Petersen and Tollefsen, 2012; Tollefsen, 2002), transcription of Vtg mRNA (Mortensen and Arukwe, 2006; Scholz et al., 2004; Smeets et al., 1999) and subsequent translation into the functional protein (Tollefsen, 2002), that can be monitored and quantitated by a variety of different methods. Although clearly showing a screening potential, the feasibility of using these assays in EDC screening has been questioned due to low reproducibility, variable sensitivity and overall feasibility as high-throughput screening tools (Navas and Segner, 2006; Scholz et al., 2013). This applies in particular to the inter-species variability in Vtg gene and protein response often observed when exposed to the same xenoestrogen (Rankouhi et al., 2004). The physiology of the donor fish has been proposed to be a major contributor to differences in estrogen sensitivity, where mature individuals induce Vtg at a higher magnitude of response than juveniles (Navas and Segner, 2006). The differences in reproducibility and sensitivity among cell batches and species may also be caused by differences in fish strains and cell isolation procedures within and amongst labs (Navas and Segner, 2006; Rankouhi et al., 2004). Further, cell culture and bioassay conditions has been shown to also affect the assay performance and analytical determination of Vtg (Kim and Takemura, 2003; Pawlowski et al., 2000; Tollefsen et al., 2003), thus illustrating the importance of assay protocol optimization and harmonization. As primary hepatocytes have been demonstrated to be a highly versatile multi-endpoint experimental model (Farmen et al., 2010; Finne et al., 2007; Petersen and Tollefsen, 2011; Tollefsen et al., 2008a; Tollefsen et al., 2006), thorough evaluation of assay performance and suggestions for improvements to facilitate larger implementation in EDC screening is therefore highly warranted.

The aim of the present study was to evaluate the feasibility of primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes as a potential screening assay for ER agonists. This was achieved by evaluating the role of seasonal variation in the Vtg (gene and protein) sensitivity

and responsiveness (e.g. induction potential), optimal culture and assay conditions (exposure time and well format), and the role of data normalization procedures using the model xenoestrogen 17α-ethynylestradiol (EE2). Chemical analysis of internal cell concentrations and media was additionally determined to assess the role of chemical depletion on bioassay performance. In addition, species-species and inter-laboratory differences in estrogen sensitivity were compared to identify key factors affecting the bioassay performance.

2 Material and methods

2.1 Chemicals

All solvents and materials used in this study were obtained from the following sources: 17α-ethynylestradiol (EE2, ≥98%, CAS 57-63-6) and d2 labelled estradiol (d2-E2, CAS 53866-33-4) (>99%), sodium bicarbonate (CAS 144-55-8), sodium carbonate (CAS 497-19-8) , ammonium acetate (CAS 631-61-8), dansyl chloride (CAS 605-65-2) and copper sulfate ($CuSO_4 \cdot 5H_2O$, CAS 7758-99- 8) were all purchased from Sigma–Aldrich (St. Louis, MI, US). Methanol (MeOH), acetone and toluene were of HPLC grade or analytical-reagent grade and obtained from Merck KGaA (Darmstadt, Germany). Dansylation buffer (pH 9.0) was prepared by dissolving 0.5g of sodium carbonate and 4.2g sodium bicarbonate in 200mL of HPLC grade water. All test chemicals (with the exception of copper sulfate which was prepared freshly in cell media) were dissolved in dimethylsulfoxide (DMSO) and stored at -20ºC in the dark until use.

2.2 Rainbow trout

Juvenile rainbow trout (200-500g) from the same fish stock were obtained from the Valdres rakfisk BA hatchery (Valdres, Norway) and kept at the Department of Biosciences, University of Oslo for a minimum of 4 weeks prior to the start of the studies. The fish were kept at 6±2 ºC, pH 6.6, 100% oxygen saturation under a 12h light/12h dark photo cycle. The fish were fed daily

with commercial pellets (Skretting, Stavanger, Norway) that corresponded to approximately 0.5% of total body weight.

2.3 Hepatocyte isolation

Fish was collected from their rearing tanks in the period January-July (1-3 fish/sampling), terminated by a blow to the head and the abdominal cavity exposed by dissection. Only juvenile fish (with no visual gonads) were used in the 2-step hepatic cell isolation procedure described by Tollefsen et al. (2003). The *in situ* perfusion of the liver (5 ml/min, 10–15 min, 4ºC) was performed using a calcium free perfusion buffer (4.8mM KCl, 1.2mM $MgSO_4$, 122mM NaCl, 11mM Na_2HPO_4,3.3mM NaH_2HPO_4, 3.7mM $NaHCO_3$, 4 ◦C) with 26 µM EGTA to remove the blood from the liver and to disrupt cell-cell interactions. Digestion of connective tissues and dislodgement of cells was performed by perfusion (5 ml/min, 10–15 min, 35-40ºC) with a calcium and EGTA free buffer containing collagenase (0.3 mg/ml) and $CaCl_2$ (1.5 mM). The liver cells were thereafter transferred to a sterile glass beaker containing ice-cold calcium free perfusion buffer with 0.1% BSA, then homogenized by successive filtering through a 250µm and 100µm nylon mesh and centrifuged (500 rpm for 4, 3 and 3 min, 4ºC) before being resuspended in a serum-free L-15 medium containing amphotericin (0.25 µg/ml), streptomycin (100 µg/l), penicillin (100 Units/ml), L-glutamine (0.29 mg/ml) and NaHCO3 (4.5 mM). A final filtration through a 100µm nylon mesh was performed prior to assessing the cell viability (>80%) using a Bürker counting chamber and trypan blue:cell suspension (2:1). The cell suspension was thereafter diluted to 500 000 cells/ml, seeded in 6- (6 ml), 24- (1.25 ml) or 96-well (200 µl) Primaria^{TM} microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) and incubated in ambient atmosphere at 15ºC. This temperature has previously been observed to yield the most sensitive concentration-response relationships for Vtg in rainbow trout hepatocytes (Tollefsen et al., 2008b).

2.3.1 In vitro exposure

After 24 hours of acclimation of the cells in the wells, 50% of the media was removed and replaced with cell media spiked with the test chemical EE2 (0.001-300nM), positive control for cytotoxicity (0.078-10mM $CuS0_4$) and solvent control (0.1% DMSO) in triplicate. The cells were subsequently re-exposed after 48h of exposure to compensate for any depletion of the chemical from the medium. At the end of exposure, 100µl of cell media was sampled from each well and transferred to individual Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed (Nunc, Roskilde, Denmark) and frozen for subsequent Vtg protein analysis by enzyme-linked immunosorbent assay (ELISA). The cells were analyzed for cytotoxicity (96 well format) and total RNA was isolated (24 well plate format) for subsequent gene expression analysis. Sampling for Vtg protein and gene expression was performed after 24, 48, 72 and 96 hours exposure and all samples were frozen at -80ºC prior to analysis.

2.4 Cytotoxicity

Cytotoxicity was determined by measuring metabolic activity and cell membrane integrity, using Alamar Blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) according to the method described by Tollefsen et al. (2008a). The cell media was removed from each well and replaced with 100µl of Tris buffer (50 mM, pH 7.5) containing 5% AB and 4µM CFDA-AM, followed by incubation in the dark on an orbital shaker set to 100 rpm for 30 minutes (room temperature). Fluorescence was measured at excitation- emission pairs of 530-590nm (AB) and 485-530nm (CFDA-AM) by a Victor V^3 multilabel counter (Perkin Elmer, Waltham, MA, USA). The viability data was expressed as relative cytotoxicity, where the data was normalized between solvent control (100% viability) and 0.1M $CuSO_4$ (0% viability).

2.5 Capture enzyme-linked immunosorbent assay (Capture ELISA)

Vitellogenin was measured by a capture ELISA previously described by Tollefsen et al. (2003). In brief, frozen microtiter plates with samples were thawed at 4ºC, 100µl of positive control

(plasma samples with 3-3000ng/ml Vtg) was applied to the control wells and the plates were incubated in the dark overnight (16h). The capture ELISA was carried out using mono-clonal mouse anti-salmon antibodies Vtg (BN-5, 1:6000x diluted in PBS with 1% BSA, Bioscense Laboratories, Bergen, Norway) and the second antibody goat anti-mouse IgG (1:6000x diluted in PBS with 1% BSA, Bio-Rad, Herculeas, CA, USA) followed by addition of HRP enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) to start the color development. The plates were thereafter incubated in the dark at room temperature. The color development was stopped after 15-20 min with 50µl 1M H_2SO_4 and the plates were measured at 450nm using a Thermomax microplate reader (Molecular Devices, USA) within 20 minutes after H_2SO_4 addition. The relative Vtg expression was calculated as percentage of maximum vtg induction (3 nM EE2) at 96 hours of exposure or at individual exposure durations of 24, 48, 72 hours (3-30nM EE2) by normalizing against the solvent control, plotted and fitted to a sigmoidal concentration-response curve with a variable slope (Eq. 1).

$$log(X) = -\frac{log\frac{Top - Bottom}{Y - Bottom} - 1}{Hill\ Slope} + logEC50 \qquad \textbf{Eq. 1}$$

In Eq.1 the bottom value represents the minimum Vtg induction (solvent control) and the top value represents the maximum Vtg induction, fixed at 0 and 100 respectively. The hill slope represents the steepness of the curve, X value represents the fitted relative Vtg response and Y is the experimental Vtg response obtained from the Vtg ELISA analysis.

2.6 RNA isolation and quality assessment

Total RNA was isolated using Qiagen RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions. Further concentration and purification of resulting RNA were performed with the ZYMO ZR-96 RNA Clean & Concentrator™ (Zymo Research, The Epigenetic company, USA). RNA concentration was measured spectrophotometrically (Spectrophotometer ND 1000, Nanodrop technologies Inc., Wilmington, USA) using the following quality cut-off criteria: 280/260 ratios of >2.0 and 260/230 ratios of >1.6. The RNA integrity of the samples was checked using Agilent BioAnalyzer RNA 6000 nano series kit (Agilent technologies, USA). All samples had RIN values >8, indicative of high RNA integrity (Fleige and Pfaffl, 2006).

2.6.1 Quantitiative Real time PCR (qPCR)

Quantitiative polymerase chain reaction (qPCR) was performed for the target gene Vtg and the reference gene ubiquitin (ubiq). Total RNA (0.5-1 µg) was reverse transcribed to cDNA using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosences Inc., Gaithersburg, USA) according to the manufacturer's instructions. Primer pair optimization was performed using a 5-step dilutions series (5-100 ng) in a 96 well plate format on a SFX-96 thermal cycler (Bio-Rad laboratories Inc., USA). The fluorescent dye SYBR®Green Supermix (Quanta Biosences Inc., Gaithersburg, USA) was used in the amplification reaction, where duplicates of 10 ng sample cDNA/well was pipetted in a final reaction volume of 20 µl/well. The vitellogenin primer pair was obtained from Celius et al. (2000) and the ubiquitin primer sequences were designed with Beacon designer™ and both were produced by Eurofins MWG synthesis GmbH (Ebersberg, Germany). The genes accession numbers and optimized primer assays are presented in table 1. All corresponding primers had a non-template control (NTC) to exclude any contamination of primer in the amplification reaction. A melting curve was determined for each primer to confirm specific amplification of each sample. Accepted threshold cycle (Cq) value of NTC was set to

be either non-detectable (N/A) or Cq value >30, ensuring a non-significant amount of quantified primer product. Ubiquitin was identified as a stable gene over all the treatments and used as a reference gene in normalization of gene signals by the ΔC_T ($2^{-\Delta\Delta Ct}$) method (Real-Time PCR application guide, Bio-Rad) (Table 1).

2.7 EE2 chemical analysis

Two mL of cell medium from a 24-well cell culture plate was transferred to a 10 mL glass reaction vial and 10 ng of internal standard (d2-E2) was added before the solution was extracted with 1.5 mL toluene by shaking for 5 min. The phases were allowed to separate and a 1 ml aliquot of the toluene layer was transferred to a 1.5 ml HPLC vial. Similarly, 10 ng of internal standard (d2-E2) was added to a 450 uL cell suspension which was extracted with 0.8 mL of toluene and a 0.5 ml aliquot of the toluene layer was transferred to a 1.5 ml HPLC vial. The toluene was evaporated to dryness under nitrogen. Derivatization of EE2 and d2-E2 was performed according to a previously published method by Fox et al. (2011). The dried sample extract was resuspended in 200 uL of 250 mM sodium carbonate/sodium bicarbonate buffer and 200 uL of 1 mg/mL dansyl chloride in acetone. After mixing, the solution was incubated for 30 min at 65 °C. Following, 50 uL of MeOH was added and the sample analyzed directly on UPLC-MS using a Waters BEH C8 (1.7 μm, 100 × 2.1 mm) with a Waters Acquity UPLC module (Waters Micromass, Manchester, UK). Analyte separation was achieved by linear gradient elution, starting from MeOH–water containing 2.6 mM ammonium acetate 20:80 v/v, rising to 98% MeOH over 9 min, held for 3 min, then switched back to the start-eluent. The UPLC system was coupled to a Waters Premier XE triple quadrupole mass spectrometer operating with an electrospray ionization (ESI) interface. Typical ESI parameters were a spray voltage of 2.5 kV, desolvation temperature at 400 °C, source temperature at 100 °C and cone gas and desolvation gas at 50 and 800 L N_2/hour, respectively. The mass spectrometer was operated in MS/MS mode with argon as collision cell gas. Ionization and MS/MS collision

energy settings were optimized while continuously infusing (syringe pump) 100 ng/mL of the derivatized standards at 5 µL/min. Detection of the dansyl derivatized analytes was performed by multiple reaction monitoring (MRM) in positive ionization mode; EE2 dansyl 530.2>171.1 and d2E2 dansyl 508.2>171.1.

2.8 Data analysis and statistics

Graphic design and statistical analysis were performed with Graphpad Prism Version 5.04 software (GraphPad Software Inc., San Diego, CA, USA). The mean Vtg protein and mRNA expression with standard error of the mean (SEM) were fitted a concentration-response curve by non-linear regression. All data were log-transformed to fulfill criteria`s of normality and equal variances among groups. All data was tested with Bartlett`s test for equal variances followed by statistical analysis ($p<0.05$) using the t-test for pairwise comparison or analysis of variance (ANOVA) with Dunnett`s post-hoc test for dose-response curves.

3 Results

The reproducibility, sensitivity and responsiveness of Vtg gene and protein expression in the rainbow trout primary hepatocytes were assessed by evaluating the effect of seasonal variation, assay conditions (exposure time, and well format) and data normalization procedures. Verification of EE2 exposure concentration was performed to determine the role of cellular biotransformation and chemical loss of EE2 at different exposure concentrations.

3.1 Cytotoxicity

No cytotoxicity was observed in cells exposed to EE2 at any concentration or duration of exposure (24-96h) when compared to the solvent control (0.1% DMSO) and the cell media alone (data not shown).

3.2 Vitellogenin gene and protein expression

The solvent control (DMSO) was found not to produce a significant increase in Vtg gene or protein response at any exposure time when compared to the response of the cell media alone (data not shown).

3.2.1 Seasonal variation

An inter-individual and seasonal (January-July) variation was observed in both Vtg protein and hepatic mRNA expression (Fig. 1). The inter-assay variability in Vtg mRNA and protein expression had no apparent coherence with season. The Vtg protein expression was described by concentration response curves (CRCs) with $r^2 > 0.7$ at 96h of exposure (Fig 1). When compared (Fig. 2), the sensitivity of the protein response measured as the No Observed Effect Concentration (NOEC) varied 3-fold (0.03-0.1nM) and apparent maximum Vtg expression varied 2.4 fold (8.4-19.9 fold change from control). A larger inter-assay variation was observed in Vtg mRNA induction, where the NOEC varied 33 fold (0.003-0.1nM) while the responsiveness was highly variable (74-10 000 fold). A 6-23% reduction in Vtg mRNA and protein expression from the maximum levels was observed at concentrations above 30nM EE2 (data not shown), but were considered being outside of the applicability range of the assay and therefore omitted from further analysis.

3.2.2 Effect of exposure duration

Evaluation of exposure duration was performed to assess whether it had an effect on Vtg gene and protein expression by exposing the cells for 24, 48, 72 and 96 hours to EE2 with re-exposure after 48 hours. A clear increase in Vtg protein expression was observed after 24h of exposure, although the data did not yield a high quality CRC due to low level of induction and high variation in the Vtg response (Fig. 3). Production of the Vtg protein increased with the duration of exposure, illustrated by a high quality CRC in the range 0.03-10 nM from 48 h exposure. Maximum Vtg protein expression was obtained after 96 hours of exposure to EE2 and an

apparent increase in assay responsiveness was observed with prolonged exposure time (Fig. 3 and Table 2). A significant induction of the hepatic Vtg mRNA expression was observed already after 24 hours exposure to 1nM EE2 (Fig. 3), yielding a high quality CRC in the concentration range 0.03-10 nM EE2. The data show an apparent increase in assay sensitivity (24h EC10: 0.0743nM, EC50: 0.923nM; 96h EC10: 0.0285nM, EC50: 0.381nM) with prolonged duration of exposure (Table 2), although these differences were not identified as being significantly different. Determination of the coefficient of variation (CV, %) for Vtg mRNA at 1nM EE2 (Fig. 3), showed an irregular decrease that were not associated with the exposure duration (e.i. 48% at 24h, 39% at 48h, 14% at 72h and 27% at 96h), which in protein expression was less variable with prolonged exposure (e.i. 77% at 24h, 41% at 48h, 17% at 72h and 8% at 96h). A deviation from ideal concentration-dependent response was observed as a reduction in Vtg expression for both protein and gene expression at concentrations in the range from 10-30 nM EE2 (results not shown).

When normalized against the maximum Vtg response at 24, 48, 72 and 96 hours exposure to EE2, the data yielded high quality CRCs in both protein and mRNA expression in the concentration range 0.01-10 nM (Fig. 4 and Table 2). Vitellogenin protein and mRNA was expressed in a consistent manner between 48-96 hours (protein) and 24-96 hours (mRNA) (Fig. 4) with similar EC-values at 24-72 hours, but displaying an apparent increase in Vtg responsiveness over time (Table 2). An apparent temporal increase in sensitivity, measured as a reduction in the EC10, was observed for both protein and mRNA expression (Table 2). The cells exposed to EE2 for 24 hours did not produce a consistent Vtg protein expression and was thus omitted from the analysis. Vitellogenin protein expression varied considerably after 24 hours of exposure despite normalization, but was greatly improved and comparable from 48-96 hours of exposure. A large degree of consistency was found between the shape of the CRCs (Fig. 4) and ECs for protein and gene expression after 96h exposure (Table 2).

3.2.3 Effect of plate format

The effect of well size exhibiting different volume/total surface ratios on induction of Vtg protein production was assessed by exposing the cells to EE2 for 48 and 96 hours in 96, 24, and 6 well plates. No significant differences in sensitivity or responsiveness were observed in the Vtg protein induction (non-normalized data, results not shown) or the Vtg CRCs (normalized data, Fig. 5) for the different plate formats after 48- and 96 hours exposure.

3.3 Chemical depletion of EE2 in the bioassay

Verification of EE2 exposure concentration was performed in the 24-well format. When exposed to 3nM EE2 in a time dependent (0-48h) manner, cells were found to contain 70.7 times higher concentration of EE2 than in the media within an hour of exposure (Fig. 6A). The EE2 concentration in the cells was fairly stable for 8 hours, before decreasing over time. Concentrations of EE2 in the media decreased already from the start of the exposures and throughout the 48 hour exposure period. When primary hepatocytes were exposed to 3 and 30 nM EE2 for 0 and 48 hours (Fig. 6B), measured EE2 concentrations in media were identified at 0 hours to be 74 and 93% of nominal EE2 concentration, albeit as little as 4 and 9% of nominal EE2 concentrations could be measured in media after 48hours exposure (Fig. 6B).

4 Discussion

Knowledge about the potential impact of endocrine disrupting chemicals (EDCs) on humans and the environment has increased substantially the last 20 years with the establishment of international EDC screening programs and regulatory guidelines (Hecker and Hollert, 2011). As the regulations for EDCs develop, a higher number of chemicals will require regulatory testing and tiered approaches using alternative (*in silico* and *in vitro)* methods to characterize EDCs are likely to surface as alternatives to resource-demanding and ethically challenging *in vivo* tests. Various international organizations are working towards a larger implementation of the 3R`s into integrated Approaches for Testing and Assessments (IATAs) and these initiatives

often involve using *in silico* methods such as (quantitative) structure activity relationship, structural alerts and category formation, and *in vitro* assays such as estrogen receptor (ER)- and androgen receptor (AR)-binding assay (OECD, 2012a; US-EPA, 2009; US-EPA, 2011). Well-characterized *in vitro* methods such as the primary fish hepatocyte assay has shown to be a promising tool in the screening of estrogenic and anti-estrogenic chemicals, complex mixtures and environmental monitoring as they retain many of their native metabolic and biochemical properties (Petersen and Tollefsen, 2012, 2011; Segner and Cravedi, 2000; Tollefsen et al., 2006; Tollefsen and Nilsen, 2008). However, large variations in sensitivity, responsiveness and reproducibility have been reported among laboratories and species (Navas and Segner, 2006) and thus proposed to limit the applicability of this assay within regulatory settings (Combes et al., 2006). Use of laboratory-specific strains of fish and lack of standardized guidelines, have made direct comparison between data and assays challenging. The present paper evaluated key properties to the primary hepatocyte assay's performance, such as quantification of the Vtg response at the protein and mRNA level, role of seasonal variation, assay conditions (e.g. well plate format) and data normalization procedures to identify the assay`s applicability as a potential screening assay for ER-agonists.

4.1 Seasonal variation

It's well documented that *in vivo* Vtg production varies throughout the season and maturation status of the fish (Bon et al., 1997; Larsson et al., 2002). This has led to the use of either male or juvenile fish in *in vivo* and *in vitro* studies for EDCs (Bickley et al., 2009; Tollefsen et al., 2003). Use of juvenile or male fish as performed in the present and a number of studies elsewhere is thus expected to reduce a large source of variability, increase sensitivity towards estrogens, improve assay performance and allow inter-assay comparability (Bickley et al., 2009; Navas and Segner, 2006). Despite such improvements, considerable variability in the maximal Vtg response (i.e the fold induction) at the protein and mRNA level was observed after

exposing primary hepatocytes from rainbow trout to EE2 in the present study. This level of variance in Vtg response has previously been reported in hepatocytes from bream (*Abramis brama*) and common carp (*Cyprinus carpio*) (Bickley et al., 2009; Rankouhi et al., 2004) and proposed being associated with a number of factors such as seasonal differences in the host fish physiology, rearing temperature and photoperiod, and *in vitro* culture conditions (Clark et al., 2005; Pawlowski et al., 2000). Changes in the basal activity of Vtg has previously been associated both with the seasonal changes in rearing temperatures by affecting the liver`s estrogen sensitivity and capacity to regulate ER and Vtg transcription, translation and post translation events downstream binding and activation of estrogen responsive elements (EREs) (MacKay et al., 1996; Mackay and Lazier, 1993). Elevated temperatures have also been demonstrated to affect the general cellular metabolism and specifically increase the expression of Vtg and ER mRNA both *in vitro* and *in vivo* (MacKay et al., 1996; Mackay and Lazier, 1993; Pawlowski et al., 2000). Other cellular processes such as biotransformation involving the aryl hydrocarbon receptor (AhR) and CYP1A may directly or indirectly be affected by changes in ER activity (Gräns et al., 2010; Mortensen and Arukwe, 2007) as well affect the transcriptional activation of the ER (Klinge et al., 1999) by potential unidirectional cross-talk (Bemanian et al., 2004). Besides temperature, maturation status of the donor fish has been proposed to affect the estrogen response in fish hepatocytes (Bickley et al., 2009; Smeets et al., 1999). Changes in Vtg expression and concentrations of nuclear and cytosolic ER with a factor up to 4 have been reported, although no correlation between the amount of natural estrogen E2 and induced nuclear and cytosolic ER could be determined (Smith and Thomas, 1991). Furthermore, the activity of the CYP-family is also reported to be closely associated with the reproductive cycle and seasonal maturation of fish (Koivusaari et al., 1984; Larsen et al., 1992), potentially affecting the biotransformation of the test compound. Despite suggestions of a number of factors that may affect regulation of circulating steroids and their biological activity (Clark et

al., 2005; Pawlowski et al., 2000), performance of the current study under a static photoperiod (*in vivo* rearing: 12h light/12h dark, *in vitro* culturing: in the dark), minor variations in temperature (*in vivo* rearing: 6±2 ºC, *in vitro* culturing: 15±0.5 ºC) and use of juvenile fish for all cell isolations have likely minimized any differences in responsiveness between different cell batches. It is therefore suggested that individual differences between cell batches are difficult to minimize further and any improvements in the assay performance will have to be performed through data normalization procedures (Bickley et al., 2009; Smeets et al., 1999).

4.2 Data normalization

Variation in production of Vtg has been proposed to be reduced by normalizing either to basal Vtg production calculating relative fold induction or to fit the responses within a minimum (basal) to maximal response relations to derive relative potencies (Bickley et al., 2009; Navas and Segner, 2006; Rankouhi et al., 2004). The Vtg production may also be reported as total quantity of protein or amount of RNA (ng)/well (Navas and Segner, 2006), but such normalization procedures will not minimize any data variations arising from different studies, although clearly improve the ability to perform absolute quantification. The strategy of the current exposure and analysis approach were developed to enhance sample throughput capacity (24 or 96 well format) and rapid detection by capture ELISA (protein detection) and/or qPCR (mRNA detection), and data normalization was best served by the relative potency approach. Implementation of this normalization procedure clearly improved the quality of the CRCs, minimized the inter-assay (e.g. cell batch) variation, and increased the reproducibility of both gene and the protein data considerably. Furthermore, the data normalization greatly improved the previously reported inter-cell batch variation, yielding high-quality CRCs for both Vtg mRNA and protein for different exposure durations. The variability in the 95% confidence interval of EC50 was greatly improved at 96 hours when data was normalized (non-normalized: 50%, normalized: 31%), further increased the reproducibility and reliability of the primary

hepatocyte assay. The variation in Vtg protein expression between replicates ranged from 25% to 6 % when data were normalized at 96 hours, which was consistent with previous studies with primary salmon hepatocytes (Tollefsen et al., 2003). Although the current study used between four and seven donor fish, a minimum of four assays were normally found to sufficient to obtain high quality CRCs for both Vtg mRNA and protein expression.

4.3 Bioassay-specific factors

4.3.1 Effect of exposure duration

Regulation of Vtg gene and protein production is a sensitive and time-dependent translation process from the molecular (gene) to the subcellular (protein) response. Hepatocytes exposed to EE2 for 24-96 hours expressed high-quality CRCs for Vtg mRNA already after 24 hours exposure when normalized using relative expression as performed for fish hepatocytes elsewhere (Bickley et al., 2009; Finne et al., 2007). In contrast, clear induction and high-quality CRCs for Vtg protein expression was first observed after 48 hours exposure. This is in compliance with exposure studies with Atlantic salmon hepatocytes exposed to E2 (Tollefsen et al., 2003), and likely reflect the time delay between mRNA transcription, translation and subsequent protein synthesis (Scholz et al., 2004) and transport of the protein to the exterior of the hepatocyte. Although gene expression was initiated earlier than 24 hours, consistent induction of both Vtg mRNA and protein production may require as much as 48 hours of exposure to produce high-quality CRCs (Bickley et al., 2009; Gagné and Blaise, 1998; Scholz et al., 2004; Tollefsen et al., 2003). Prolonging the exposure duration to 72 and 96 hours improved the magnitude of both the mRNA and protein Vtg response (Table 2). Optimal exposure time was identified as 96 hours whereupon both the Vtg protein and mRNA expression showed an apparent increase in the assay sensitivity, responsiveness and reduced CV, consistent with previous studies performed on primary salmon hepatocytes exposed to E2 (Tollefsen et al., 2003). However extending the duration from 48 to 96 hours of exposure, required chemical

re-exposure as the concentration of EE2 in the wells was reduced to less than 50% after 24 hours of exposure and almost depleted within 48 hours (Fig. 6A). Such rapid depletion has previously been observed for E2 and accredited high biotransformation activity in rainbow trout hepatocytes and liver preparations (Miller et al., 1999; Schmieder et al., 2004). These observations are in agreement with observations that E2 require re-exposure after 48 hours to maintain reproducible CRCs (results not shown). The observed relationship between nominal and measured concentration of EE2 obtained herein suggests a concentration-independent depletion of EE2 (Fig. 6B), thus confirming that the hepatocyte are highly metabolically capable (Pedersen and Hill, 2000; Segner and Cravedi, 2000). Despite the clear advantages of having metabolically active cells to mimic the natural biotransformation and thus potentially detect both estrogenic mother compounds and their metabolites (Nillos et al., 2010; Pedersen and Hill, 2000; Segner and Cravedi, 2000), the rapid depletion occurring may challenge accurate calculation of effective concentration or potencies for metabolically susceptible EDCs (Lindholst et al., 2003). Although routine measurement of exposure concentrations in *in vitro* bioassays are clearly a complicating factor that will limit sample throughput, lack of consistency between *in vitro* potency and *in vivo* toxicity (Tollefsen et al., 2008a) may require analytical or computational corrections to adjust for chemical depletion in bioassays such as primary hepatocytes.

4.3.2 Plate format

The different plate formats used in chemical exposures often reflects the samples need for the down-stream endpoint analysis. Determination of viability and high-throughput use of various biochemical probes are often conducted in the 96 well plate formats (Farmen et al., 2010; Tollefsen et al., 2008b), whereas sublethal effects such as gene expression may require either 24 or 6 well formats to ensure sufficient biological material for analysis using most cDNA construction protocols (Finne et al., 2007). Use of different plate formats with different volume

to surface ratios may thus introduce confounding bioassay factors such as differences in sorption to and possible interaction with the plastic surface (Schreiber et al., 2008). Such confounding factors may potentially affect the freely dissolved concentration of hydrophobic chemicals (log Kow >3) in the bioassay and result in overestimation of the chemicals actual concentration in the assay (Brown et al., 2001; Mayer et al., 1999; Riedl and Altenburger, 2007). Despite potential for such confounding factors, no significant difference in Vtg protein production was observed in assays run in 6, 24 or 96 well formats with different total surface-volume ratio after 48 and 96 hours of exposure. Although this holds true for EE2, larger discrepancies may be expected for chemicals that are more hydrophobic and volatile than EE2.

4.4 Bioassay performance

Exposure to EE2 for 24-96h caused a sensitive, responsive and reproducible induction of Vtg gene and protein production similar to that observed for primary salmonid hepatocytes (Olsen et al., 2005; Tollefsen et al., 2003). However, deviation from ideal CRCs for both Vtg protein and mRNA expression at higher concentrations of EE2 (10-30nM) in the primary rainbow trout hepatocytes suggest that EE2 cause complex cellular responses that are not accounted for by pure receptor-mediated responses alone. This has been reported in rainbow trout, bream and carp hepatocytes previously, although the rationale for such discrepancy is currently not understood (Petersen and Tollefsen, 2011; Rankouhi et al., 2004). Unpublished global transcriptional analysis using materials generated in the same bioassay as used herein show a 14% lower expression of ERα at 30nM compared to the second highest concentration (3nM), thus suggesting that part of the reduction in Vtg observed is mediated through regulation of the activity of the ER (results not shown). Phase II biotransformation enzymes, involved in the steroid homeostasis (UDP-glucuronosyl transferase) and phase III biotransformation (ABC-family transporters), involved in steroid transport were also induced at this concentration, thus may potentially lead to a reduction in the cellular level of EE2 and consequently also ER and

Vtg mRNA expression. Other factors such as concentration-dependent ER desensitization, receptor competition, receptor down regulation and /or negative endocrine feedback loops, receptor cross-talk and induction of sex steroid-binding proteins (SBPs) that limit cellular access of EE2 (Foucher et al., 1991; Gräns et al., 2010; Nagel et al., 1998; Nagel et al., 1997; Vandenberg et al., 2012) are additional explanations to the relative reduction in the Vtg response at high EE2 concentrations.

4.5 Inter-laboratory and inter-species differences

Large variances in estrogen sensitivity (LOEC: 10^{-12} to 10^{-7} M) and Vtg responsiveness have previously been reported in primary hepatocytes among different laboratories and donor species (Supplementary table A) (Navas and Segner, 2006). In an attempt to address this issue, a comparison of relevant *in vitro* bioassays (primary hepatocytes, liver slices, liver homogenate) was performed as to identify potentially contributing factors to the observed intra- and inter species bioassay`s estrogen sensitivity (Suppl. Table A). The ER-sensitivity has previously been suggested as one of the contributing factors to the inter-species discrepancies of *in vitro* Vtg responses (Rankouhi et al., 2004). However when comparing ER relative binding affinity (ER-RBA) for EE2 in four different cold and warm water species it differed no more than 10 fold (Table 3), which is in compliance with previous inter-species comparisons of RBA for fish ERs (Denny et al., 2005). Somewhat higher differences (<200-fold) were observed when comparing the Lowest Observed Effect Concentration (LOEC) for *in vivo* Vtg-induction in the same species (Table 4, Suppl. Table B). Interestingly, the LOECs for rainbow trout *in vitro* and *in vivo* Vtg-induction differed with no more than 2-19 fold for E2, which was within the range of variation (0-19.5 fold change) of the LOECs for *in vivo* Vtg-induction in cold water species alone (Table 3 and 4). However, orders of magnitude differences (2-367 000 fold) were observed when comparing the *in vitro* Vtg- sensitivity in primary hepatocytes from cold- and warm-water species (Table 3), thus suggesting that factors related to the *in vitro* bioassays were

the main reason for the large discrepancy. Bioassay factors such as cell incubation temperature, cell density, culture plate formats and especially media supplements (e.g. fetal bovine serum, Ultroser SF) varied greatly within and among the bioassays based on warm-water species. Such *in vitro* culturing factors have previously been demonstrated to significantly affect Vtg induction, as well as metabolism and bioavailability of the exposure chemicals (Pawlowski et al., 2000; Tollefsen et al., 2003). Additionally, the selection of Vtg gene transcripts (eg.*Vtg 1,Vtg 3*) used in the gene expression analysis may contribute to the observed discrepancies as they may have different estrogen responsiveness (Martyniuk et al., 2007). The present comparison suggests that inter-laboratory factors and bioassay conditions (eg. incubation temperature, media supplements, cell density etc.) are the largest contributors to the observed Vtg variability of sensitivity and responsiveness among and within species. In additional to these confounding bioassay factors, different analytical approaches for determining Vtg and normalization procedures are applied across laboratories, making the results difficult to compare (Navas and Segner, 2006). It is evident that there is still a need for intra-species protocol standardization of rearing temperatures, cell isolation procedures, bioassay conditions, analytical approaches and data normalization procedures to reduce inter-assay and inter-laboratory variability.

4.6 Future directions for use of fish hepatocytes

The present work has demonstrated that rainbow trout primary hepatocyte`s are sensitive, reproducible and responsive when exposed to the estrogen agonist EE2. The primary hepatocytes variable capacity to induce Vtg gene and protein could not fully be associated with either seasonal or variable inter-cell batch endocrine regulation. Although differences in incubation temperature and inter-cell batch variation might have affected the Vtg response, this variation was dramatically reduced by data normalization. The present paper also briefly addressed the inter-lab and inter-species variability in Vtg sensitivity and concluded that there is a need for larger standardization of the primary hepatocyte culture and exposure protocols.

Future assay standardization should consider use of serum-free exposure media, fixed intra-species incubation temperatures, standardized bioassay cell density and verification of chemical exposure concentrations to account for depletion of chemicals in the bioassays. Chemical bioavailability of hydrophobic chemicals has proven particularly challenging due to various bioassay factors (e.g. chemical depletion, well format, wall sorption) in which partition-driven administration dosing may alleviate some of the problems (Booij et al., 2011; Kwon et al., 2009; Riedl and Altenburg, 2007; Schreiber et al., 2008; Smith et al., 2009). Development of continuous liver cell lines or use of cryopreserved primary hepatocytes, which retain properties of freshly isolated hepatocytes (Markell et al., 2014; Mingoia et al., 2010), may additionally reduce inter-lab and inter-assay variance to a level that accommodate suitable high-throughput screening formats (Bols et al., 2005; Petersen and Tollefsen, 2012, 2011; Tollefsen et al., 2008b; Tollefsen et al., 2003).

5 Conclusion

The present study has shown primary rainbow trout hepatocytes to be a sensitive, reproducible and responsive *in vitro* model. The confounding effect of inter-individual-, inter-assay- and seasonal variations in Vtg protein expression and mRNA induction were reduced when data normalization procedures were implemented. Standardization of protocols for cell culture conditions, exposure procedures, chemical verification and data normalization has the potential to reduce inter-assay and inter-laboratory variance in use of primary hepatocytes for screening of ER-agonists. Implementation of more harmonized efforts in bioassay testing with primary hepatocytes should thus be expected to increase the assay`s potential as an experimental model. The present proposal for optimization and standardization of protocols will hopefully facilitate improvement of the assay`s robustness, reproducibility, sensitivity and render this assay more applicable to lower tier regulatory testing strategies (e.g an OECD CF2 assay).

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