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Transcriptome responses in polar cod (*Boreogadus saida*) liver slice culture exposed to benzo[*a*]pyrene and ethynylestradiol: insights into anti-estrogenic effects

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ABSTRACT

Polar cod (*Boreogadus saida*) is a key species in the arctic marine ecosystem vulnerable to effects of pollution, particularly from petroleum related activities. To facilitate studying the effects of those pollutants, we adapted a precision-cut liver slice culture protocol for this species. Using this system on board a research vessel, we studied gene expression in liver slice after exposure to the polycyclic aromatic hydrocarbon (PAH) benzo[*a*]pyrene (BaP), ethynylestradiol (EE2), and their mixtures, to map their molecular targets and examine possible anti-estrogenic effects of BaP. The exposure experiments were performed with BaP alone (0.1, 1, and 10 μM) or in combination with low concentrations of EE2 (5 nM) to mimic physiological estradiol levels in early vitellogenic female fish. Transcriptome analysis (RNA-seq) was performed after 72 h exposure in culture to map the genes and cellular pathways affected. The results provide a view of global transcriptome responses to BaP and EE2, which resulted in enrichment of many pathways such as the aryl hydrocarbon (Ahr) and estrogen receptor pathways. In the mixture exposure, BaP resulted in anti-estrogenic effects, shown by attenuation of EE2 activated transcription of many estrogen target genes. The results from this *ex vivo* experiment suggest that pollutants that activate the Ahr pathway such as the PAH compound BaP can result in anti-estrogenic effects that may lead to endocrine disruption in polar cod.

1. Introduction

Arctic marine fish species are under stress from climate change-induced impacts in the Arctic marine environment (Dahlke et al., 2018; Huserbråten et al., 2019). As a result of the receding ice edge, anthropogenic activities, including petroleum exploration, maritime shipping and mining industries have expanded further north, thereby increasing the risk of pollution. The Arctic marine environments are susceptible to polycyclic aromatic hydrocarbon (PAH) pollution from sources such as atmospheric transport and local petroleum activities (Balmer et al., 2019; Beyer et al., 2020). In addition to petrogenic PAHs, the expanding petroleum activities in the Barents Sea introduce substanital amounts of produced water that contains a range of polluting chemicals including estrogenic compounds (Beyer et al., 2020). It is thus imperative to study toxicological responses and mechanisms involved in cold-water marine fish such as polar cod under exposure to such compounds.

Polar cod (*Boreogadus saida*) is a key species in the Arctic food web linking lower trophic levels and higher predators such as fish, seabirds, and marine mammals (Hop and Gjøsæter, 2013). Polar cod has become a focus of ecotoxicological investigations in the Arctic marine ecosystem, particularly with regard to potential effects of crude oil pollution on somatic growth, reproduction, and lipid homeostasis (Bender et al., 2016; Nahrgang et al., 2019; Nahrgang et al., 2010a; Vieweg et al., 2018). While experimental studies on its early life stages have shown a high sensitivity to exposure to petroleum related compounds (Laurel et al., 2019; Nahrgang et al., 2019), subtle physiological alterations were also observed in adult fish (Bender et al., 2016; Nahrgang et al., 2019;

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Vieweg et al., 2017). Furthermore, our recent data suggest that exposure of mature fish to crude oil water soluble fractions (WSF) can result in dysregulation of reproductively important genes and disruption of spawning patterns in polar cod (Strople et al., in preparation). The mechanisms underlying these effects could be multiple (Segner, 2011) and may involve endocrine disruption by PAHs that activate the aryl hydrocarbon receptor (Ahr) signaling pathway.

The Ahr and estrogen receptor (ER) pathways are important in molecular responses of a large group of environmental pollutants such as petroleum-related PAH compounds and endocrine disruptors. Ahr-activating environmental pollutants such as the PAH BaP can interfere with the estrogen receptor signaling through anti-estrogenic mechanisms (Goksøyr, 2006; Matthews and Gustafsson, 2006; Safe and Wormke, 2003). In polar cod exposed to BaP or crude oil WSF, transcriptome responses show activation of biotransformation processes through the Ahr pathway (Andersen et al., 2015; Song et al., 2019). However, the effect of Ahr pathway activation on estrogen signaling in polar cod remains poorly understood. It is also important to investigate transcriptome responses to estrogenic compounds in polar cod and develop molecular biomarkers to monitor endocrine disruptors found in complex mixtures of produced water released from offshore petroleum installations (Beyer et al., 2020; Geraudie et al., 2014; Meier et al., 2007; Meier et al., 2010). To our knowledge, global transcriptome responses to estrogenic compounds have not been investigated before in polar cod.

To reduce the number of experimental animals and to allow for efficient and high throughput assays, *in vitro* and *ex vivo* assays are encouraged. Such methods may also be applied in field studies such as research cruises to study toxicological responses of fish that are not routinely cultured in the laboratory, for example, deep-sea fish species (Lemaire et al., 2012). Many toxicological processes such as molecular mechanisms of Ahr and estrogen signaling pathways can be studied *ex vivo* by precision-cut liver slice (PCLS) culture methods where both Ahr and ER pathways can be activated (Eide et al., 2014; Yadetie et al., 2018). For this study, the fish liver slice culture methods developed for Atlantic cod and polar cod (Aranguren-Abadía, 2015; Eide et al., 2014) were further modified and improved for use on a research cruise to the Barents Sea with polar cod. The *ex vivo* methods combined with transcriptome assays can help in understanding molecular mechanisms involved in responses to pollutants in fish species such as polar cod. This study aimed to map the global transcriptome responses to the PAH compound BaP and the pharmaceutical estrogen ethynylestradiol (EE2) and to investigate possible anti-estrogenic effects of BaP in mixture exposure experiments using liver slice culture in polar cod.

2. Materials and methods

2.1. Polar cod sampling

The experiments were performed on the Norwegian Nansen Legacy project (arvenetternansen.com) cruise to the Northern Barents Sea in August 2019. Polar cod were collected by trawling (78.7°N, 34.0°E) (Fig. S1) and kept in a 500 L tank with running seawater at the ambient seawater temperature outdoor on the deck of the cruise ship in the Northern Barents Sea. Female polar cod (total 6 fish) with average body and liver weight (Mean ± SD) of 35.8 ± 7.8 and 3.2 ± 1.2 g, respectively, were used in this experiment (Table S1).

2.2. Chemicals and reagents

Unless specified otherwise, all chemicals including benzo[*a*]pyrene (BaP) (CAS No: 50–32-8), 17α-ethynylestradiol (EE2) (CAS No: 57–63-6), DMSO (CAS No: 67–68-5), TRI Reagent, and PCR primers were purchased from Sigma-Aldrich (Sigma-Aldrich, Oslo, Norway).

2.3. Preparation and culture of PCLS

Polar cod PCLS preparation was performed by slightly modifying protocols previously described for Atlantic cod (Eide et al., 2014; Yadetie et al., 2018) and polar cod (Aranguren-Abadía, 2015). The major modification was that coring and agarose gel embedding was avoided and the whole liver tissue was used for slicing. This has enabled more efficient slicing of the small liver of polar cod. Briefly, fish was killed by a blow to the head and the whole liver was carefully removed and rinsed in ice-cold PCLS buffer containing NaCl (122 mM), KCl (4.8 mM), $MgSO_4$ (1.2 mM), Na_2HPO_4 (11 mM), and $NaHCO_3$ (3.7 mM), pH 8.4. The whole intact liver was fixed to the specimen plate using Loctite® Super Glue and mounted on Leica vibrating blade microtome VT1200 (Leica, Wetzlar, Germany) brought on board the ship. Slices (250 μm thick) were cut at a speed of 0.9 mm/s and amplitude 3 and transferred to a petri dish with ice-cold culture medium (Leibowitz-15 medium, Life Technologies™ Gibco®, Paisley, UK) supplemented with 10% charcoal-stripped and heat-inactivated fetal bovine serum and 1% penicillin–streptomycin–amphotericin (10,000 U/mL potassium penicillin, 10,000 μg/mL streptomycin and 25 μg/mL amphotericin B, Sigma-Aldrich). The 250 μm thick slices were cut into smaller (about 4 × 4 mm) pieces using a sterile razor blade and subsequently pre-incubated at 6 °C for 2 h in the culture medium before exposure.

2.4. Experimental design for PCLS exposure studies

Liver slices from a total of six female fish (biological replicates $n = 6$) were distributed in different treatment groups and cultured in multi-well plates in a *matched pairs* design as shown in Fig. S2. Thus, each group contained slices from each of the six fish used. The slices were treated with DMSO, low, medium, and high concentrations of BaP (0, 0.1, 1, and 10 μM, respectively). This set-up was replicated in the presence of 5 nM EE2 to simulate effects of BaP at physiological plasma levels of estradiol during early vitellogenesis in the female polar cod (Bender et al., 2016) (Fig. S2). After 2 h of pre-incubation, the polar cod liver slices (4–8 slices per well) were cultured in 12-well plates (Costar, Corning, New York, USA) in 2 mL of the culture medium containing either DMSO solvent control, BaP (0.1, 1 and 10 μM), 5 nM EE2, and BaP (0.1, 1 and 10 μM) + 5 nM EE2, in an incubator at 6 °C with horizontal shaking (50 rpm) for 72 h. The concentration of DMSO solvent in each group was 0.2%. After 72 h in culture, slices were collected and snap-frozen in liquid nitrogen, and both the media (for LDH and vitellogenin assay) and slices were stored at −80 °C until further processing.

2.5. RNA extraction

Total RNA was isolated from frozen slices using TRI Reagent according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). RNA concentration and quality were assessed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All the RNA samples had RNA integrity number (RIN) values >9.

2.6. RNA sequencing

RNA samples from DMSO control, 10 μM BaP, 5 nM EE2, 5 nM EE2+ BaP 10 μM treated groups ($n = 5$ per group) were submitted to Novogene (Cambridge, UK) for sequencing. The sequencing was performed using the Ilumina NovaSeq 6000 system to generate 20 million 150 bp paired-end reads per sample.

2.7. Analysis of RNA-Seq data

RNA-seq analysis was performed essentially as described before (Yadetie et al., 2018), following the RNA-seq analysis workflow RASflow

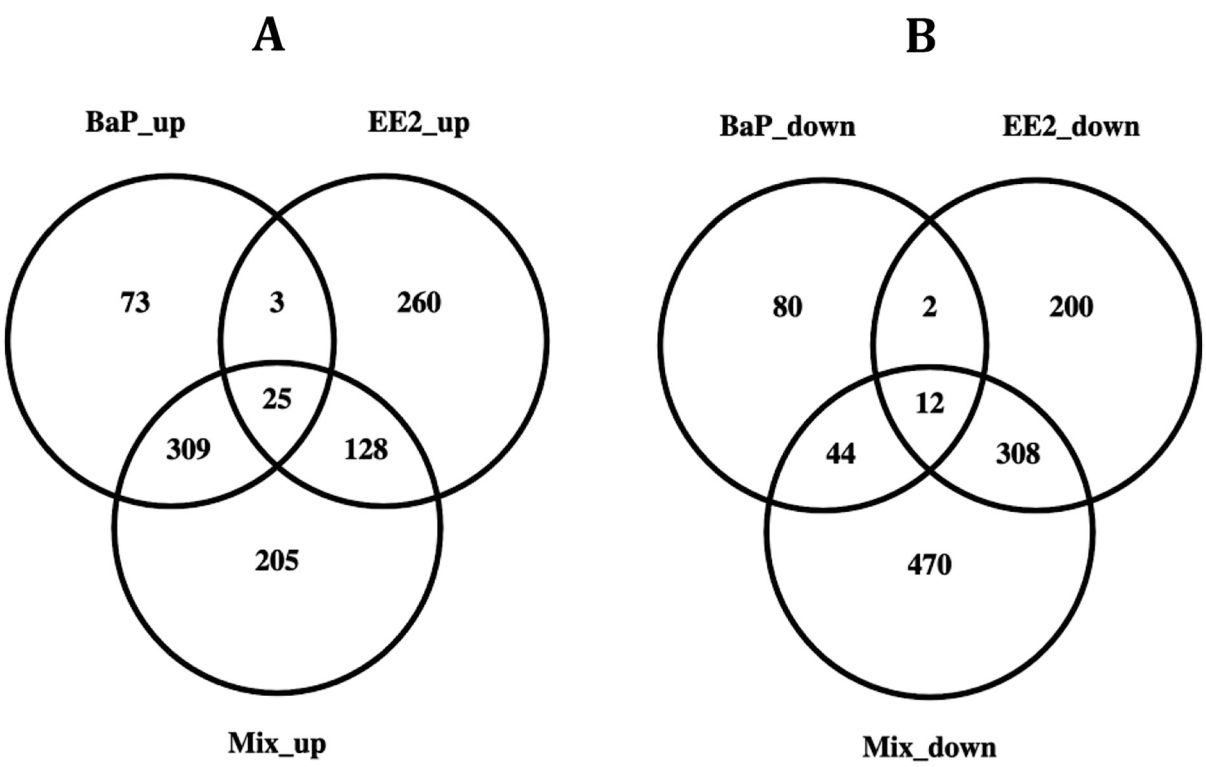

Fig. 1. Venn diagram showing the numbers and overlap of up-regulated (A) and down-regulated (B) genes in EE2, BaP and BaP and EE2 mixture (Mix) groups.

(Zhang and Jonassen, 2020), using the Atlantic cod reference genome in the Ensembl database (www.ensembl.org). The well-annotated genome of Atlantic cod was used as a reference genome for mapping and quantification of the polar cod transcriptome since the two genomes are highly similar. At a 1.5 fold-change (FC, 1.5 for up-regulated and 0.67 for down-regulated), and False Discovery Rate (FDR) < 0.05 cutoff, the analysis resulted in 548, 938, 1501 differentially expressed genes (DEGs) (compared to DMSO control) in BaP, EE2, and BaP + EE2 treated slices, respectively. The RNA-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-10509.

2.8. Pathway and network enrichment analyses

Human or zebrafish orthologs of the cod genes (considered orthologs of polar cod genes) were retrieved from the Ensembl database as previously described (Yadetie et al., 2018). Pathway and network analyses were performed using Enrichr (Kuleshov et al., 2016), the STRING protein-protein interaction network database and analysis tools (Szklarczyk et al., 2015) and the Cytoscape network visualization and analysis tools with Cluego application (Bindea et al., 2009; Shannon et al., 2003). An FDR of < 0.05 was used for significant pathway and enrichment analysis.

2.9. Hierarchical clustering

For hierarchical clustering of RNA-seq data (log2-transformed normalized counts per million reads mapped) of liver slice samples of the treatment groups DMSO control, 10 μM BaP, 5 nM EE2, and 5 nM EE2 + 10 μM BaP ($n = 5$ per group) were used (Euclidian metric, complete linkage) in Qlucore Omics Explorer. Significant genes (FDR q-value <0.05) in paired-test Multi Group Comparison (Qlucore Omics Explorer) were used to generate a heatmap showing expression profiles.

2.10. Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) assays were performed as described previously (Yadetie et al., 2018). Briefly, cDNA was prepared from 1.0 μg of each total RNA sample in 20 μL reactions and 5 μL of a 1:10 dilution and was used as template in a 20 μL PCR reaction. qPCR was performed using BioRad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The reaction conditions were as follows: an initial incubation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing, and elongation at 60 °C for 30 s. The qPCR primers are described before (Yadetie et al., 2018). The beta-actin (*actb*) housekeeping gene was used for normalization. For relative quantification and comparison of mRNA levels, fold-changes of expression in treated and control samples were calculated according to the ΔΔCq method (Schmittgen and Livak, 2008).

2.11. Western blot analysis

Western blot analysis of vitellogenin (Vtg) in spent liver slice culture media was performed using polyclonal antibody against Atlantic cod Vtg (*Gadus morhua*), according to the manufacturer's protocols (Biosense Laboratories AS, Bergen, Norway). Vtg level in culture medium loaded (15 uL/well) was quantitated by densitometric analysis of western blots using Image Lab Software (Bio-Rad Laboratories). Band intensity for each sample was then normalized with slice weight (mg) in the corresponding well and Vtg levels expressed in fold-changes compared to DMSO control group ($n = 4$ per group).

2.12. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) assay in the spent media was performed using the Cytotoxicity Detection Kit (Roche Molecular Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. Briefly, after centrifugation (500 *g* for 5 min at 4 °C) to remove debris, for each sample, 50 μl growth medium supernatant (in

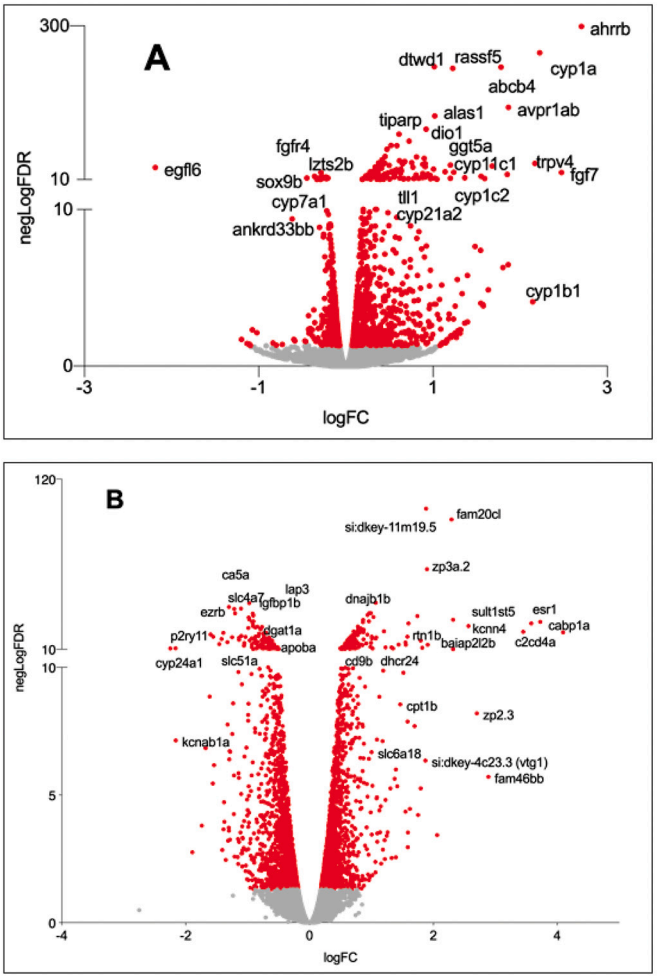

Fig. 2. Volcano plot visualization of differentially expressed genes in BaP (A) and EE2 (B) treated polar cod liver slices. Log2 fold-changes (log2FC) and negative logarithm values of FDR (negLogFDR) represent the horizontal and vertical axes, respectively. Genes with FDR <0.05 are depicted in red dots (down-regulated, on the left and up-regulated on the right. Gene symbols (based on zebrafish orthologs) are shown for some top DEGs. For complete list and details of DEG in BaP and EE2 treated slices, see Table S2A and Table S2B, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

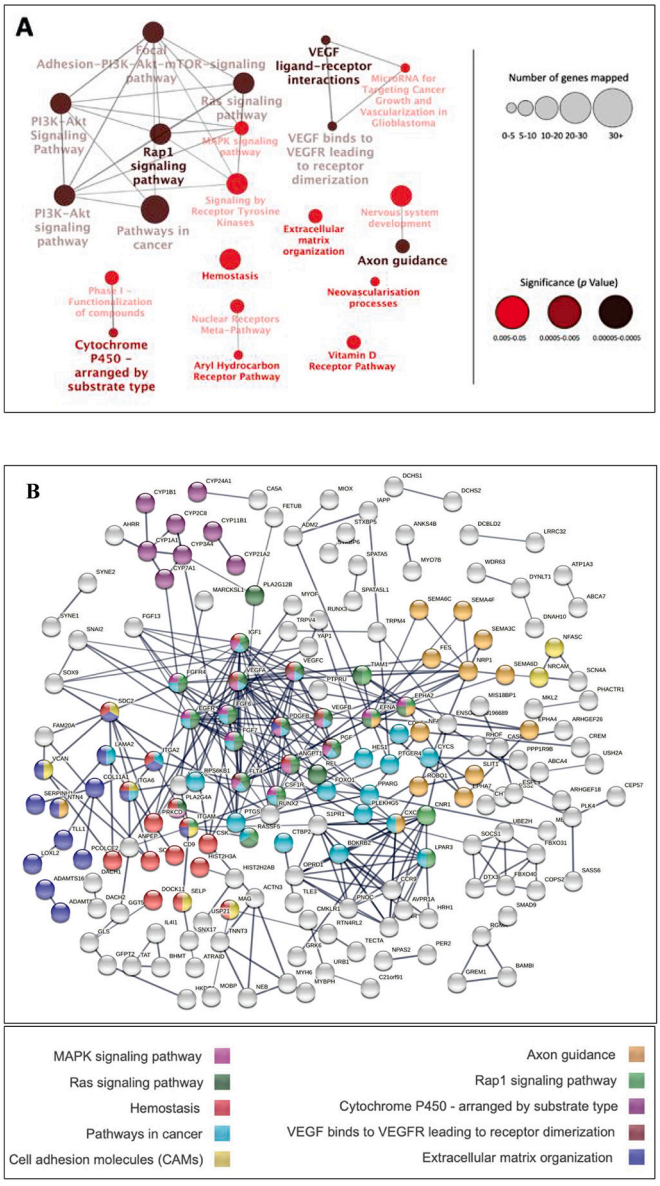

Fig. 3. STRING interaction network of DEGs in BaP treated liver slices. Networks based on significantly enriched (adjusted p value <0.05) Wikipathways, KEGG and Reactome pathways visualized in Cytoscape using Cluego application (A) and a high confidence STRING interaction network of DEGs highlighting significantly enriched KEGG and Reactome pathways indicated by color codes (bottom panel) that correspond to the color of the populating genes (B). Disconnected nodes were removed in the network for better visualization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

triplicates) was mixed with 50 μl of substrate solution in a 96-well plate and incubated at room temperature for 10 min. Absorbance was measured in EnSpire Multimode Plate Reader (Perkin Elmer, Waltham, USA) at wavelengths 490 nm and 690 nm for colored substrate and reference, respectively.

2.13. Statistical analysis

Statistical analysis of qPCR and Western blot data was performed with GraphPad Prism Software version 7 (GraphPad Prism, La Jolla, CA, USA). After the Shapiro-Wilk test for normality, a one-way ANOVA followed by Dunnett's test (for normally distributed) or Friedman test followed by Dunn's test (non-normally distributed) was used. Paired t-test (for normally distributed) or Wilcoxon matched pairs test (for non-normally distributed) was used for two-group comparisons. Data are reported as mean ± standard deviation (SD), and $p < 0.05$ was considered significant. Hierarchical clustering analysis of RNA-seq data was performed using Qlucore Omics Explorer version 3.6 (Qlucore AB, Lund, Sweden). Multi-group (ANOVA) paired test was performed in Qlucore Omics Explorer.

3. Results

3.1. Transcriptome responses to BaP, EE2, and BaP + EE2 treatment

3.1.1. Differentially expressed genes

In total, 548, 938 and 1501 DEGs were identified in BaP, EE2, and mixture (BaP and EE2) treated slices, respectively (Table S2A—C). About 85% and 80% of the DEGs could be matched with zebrafish and human orthologs, respectively. The Differentially expressed genes (DEGs) are shown in Venn diagram separated into up-regulated (Fig. 1A) and down-regulated genes (Fig. 1B). About 82% of the up-regulated genes in the BaP treated group were also up-regulated in the mixture group, whereas only 37% of the up-regulated genes in the EE2 treated

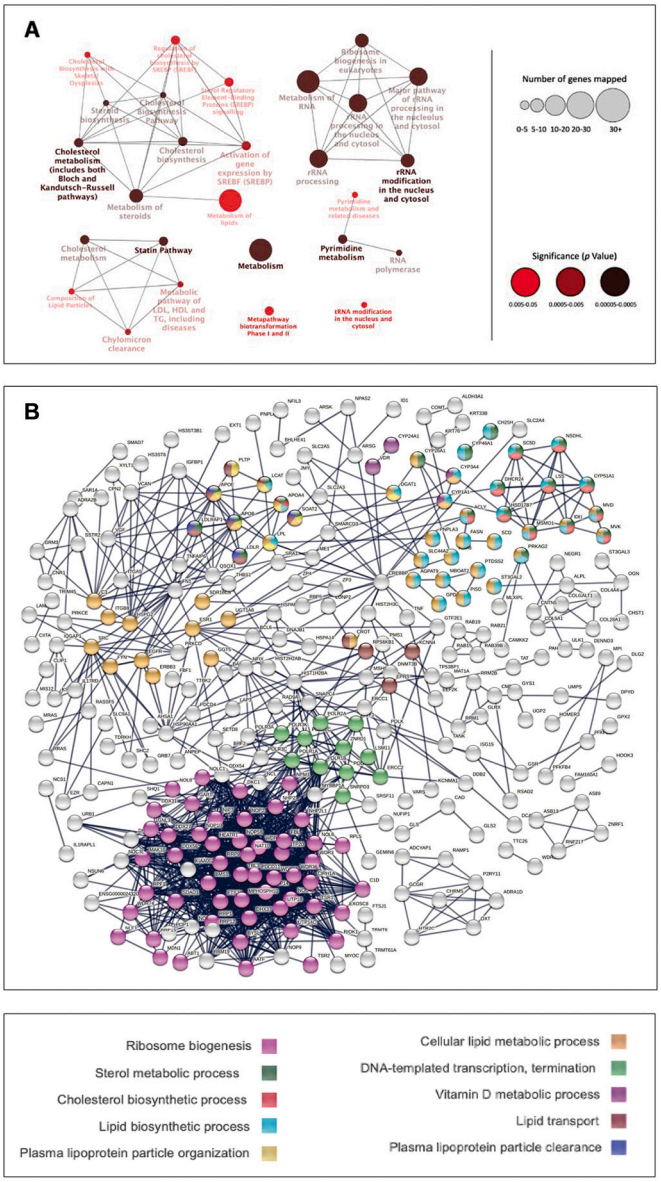

Fig. 4. STRING interaction network of DEGs in EE2 treated liver slices. Networks based on significantly enriched (adjusted p value <0.05) Wikipathways, KEGG and Reactome pathways visualized in Cytoscape using Cluego application (A) and a high confidence STRING interaction network of DEGs highlighting the main significantly enriched GO biological processes indicated by color codes (bottom panel) that correspond to the color of the populating genes (B). Disconnected nodes were removed in the network for better visualization. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

group were up-regulated in the mixture group (Fig. 1A). The corresponding proportions of down-regulated genes in BaP and EE2 shared with the mixture group are 41% and 61%, respectively (B).

3.1.2. Genes and pathways affected in BaP treated liver slices

Among the top up-regulated genes in BaP treated liver slices are the Ahr repressor (*ahrrb*), many cytochrome P450 (cyp) genes (*cyp1a*, *cyp1b1* and *cyp1c2*, *cyp2y3*, *cyp21a2*, *cyp11c1*), *fgf7*, *trpv4*, *avpr1ab* and *abcb4* (Fig. 2A, Table S2A). All the cyp genes in the DEG list were up-regulated except *cyp24a1* and *cyp7a1*, which were down-regulated (Table S2A).

Pathway and network analysis of DEGs in BaP treated slices resulted in enrichment of pathways mainly related to Ahr and nuclear receptors, as well as signal transduction pathways such as the Ras-MAPK signaling (Fig. 3A and B, Table S3). Fig. 3A shows a summary of significantly enriched networks and pathways as determined using the Cluego application in Cytoscape (Bindea et al., 2009; Shannon et al., 2003). Fig. 3B shows a STRING interaction network of DEGs where the genes populating the top enriched KEGG and Reactome pathways are highlighted. In addition to Ahr and nuclear receptor pathway genes, many genes in cancer related and various cellular signaling pathways were enriched. The most significant and gene-rich pathway was *Pathways in cancer* and it interacted with the significantly enriched cellular signaling pathways with many shared DEGs (Fig. 3A and B, Table S3).

3.1.3. Genes and pathways affected in EE2 treated liver slices

The top genes differentially expressed in EE2 treated PCLS include estrogen receptor 1 (*esr1*), 4 vitellogenin (vtg) genes (*vtg1-1* and *vtg3*), eggshell zona pellucida (zp) genes (*zp2.3* and *zp3a.2*), and *fam20cl* (Fig. 2B, Table S2B). Pathway and network analyses revealed that pathways related to translation, transcription, lipid metabolism, and reproduction were among the top affected in EE2 treated liver slices (Fig. 4A and B, Table S4). Most genes related to rRNA processing, transcription, ribosome biogenesis, and reproduction (vitellogenesis related) were up-regulated, whereas genes populating the lipid metabolism pathways such as plasma lipoprotein assembly, remodeling, and clearance were down-regulated (Fig. 4B, Table S4). All genes populating the *Cholesterol Biosynthesis*, *Eukaryotic Transcription Initiation*, *RNA polymerase* and *Ribosome biogenesis in eukaryotes* pathways were up-regulated (Fig. 4B, Table S4). All genes in the pathways *Cholesterol metabolism* (*e.g.*, apolipoprotein genes), *Composition of Lipid Particles, Satin pathway,* and *Vitamin A and Carotenoid Metabolism* (except CYP26A1) were down-regulated (Fig. 4B, Table S4).

3.1.4. Hierarchical cluster analysis

Distinct gene expression profiles can be seen in the different treatment groups as depicted in the heatmap (Fig. 5). The genes were clustered into three major groups of genes up-regulated in EE2 treated group but attenuated in the co-treatment with BaP (A), genes equally up-regulated in BaP treated and BaP + EE2 treated groups (B), and genes down-regulated in EE2 treated and BaP + EE2 treated groups (C). Notably, some of the top up-regulated genes in EE2 treatment groups in cluster A such as *esr1*, *zp3a.2* and *fam20cl* were down-regulated, or their induction was attenuated in the BaP + EE2 treated group (Fig. 5, cluster A). In contrast, EE2 had no significant effect on top BaP induced genes (Fig. 5, cluster B). Fig. 5, cluster C, shows genes down-regulated in both EE2 and co-treatment groups, with no significant effect of BaP.

3.2. qPCR assay

Since RNA-seq was performed only for the high dose groups, dose-response trends in the expression of selected biomarker genes from the list of DEGs (*cyp1a*, *esr1,* and *vtg1*) were assessed by qPCR. The genes *esr1* and *vtg1* were induced by EE2 in a dose-dependent manner and induction appeared to be attenuated by the high concentrations of BaP in the mixture (BaP and EE2) treatment groups (Fig. 6A and B). In particular, the *vtg1* gene that was significantly induced by EE2-alone treatment, was no longer significant in the co-treatment with the highest concentration of BaP (Fig. 6B). The *cyp1a* gene was induced dose-dependently to similar levels in BaP and mixture (BaP and EE2) treated groups, but not significantly affected by EE2 in the co-treatments (Fig. 6C). Similar changes were observed in both qPCR and the RNA-seq data (Fig. 6A-C, Table S2A—C), except a small but significant increase of *cyp1a* mRNA in the EE2-alone group detected by RNA-seq (Table S2B). The corresponding *cyp1a* levels in the EE2-alone group detected by qPCR also showed a slight but not significant increase (Fig. 6C).

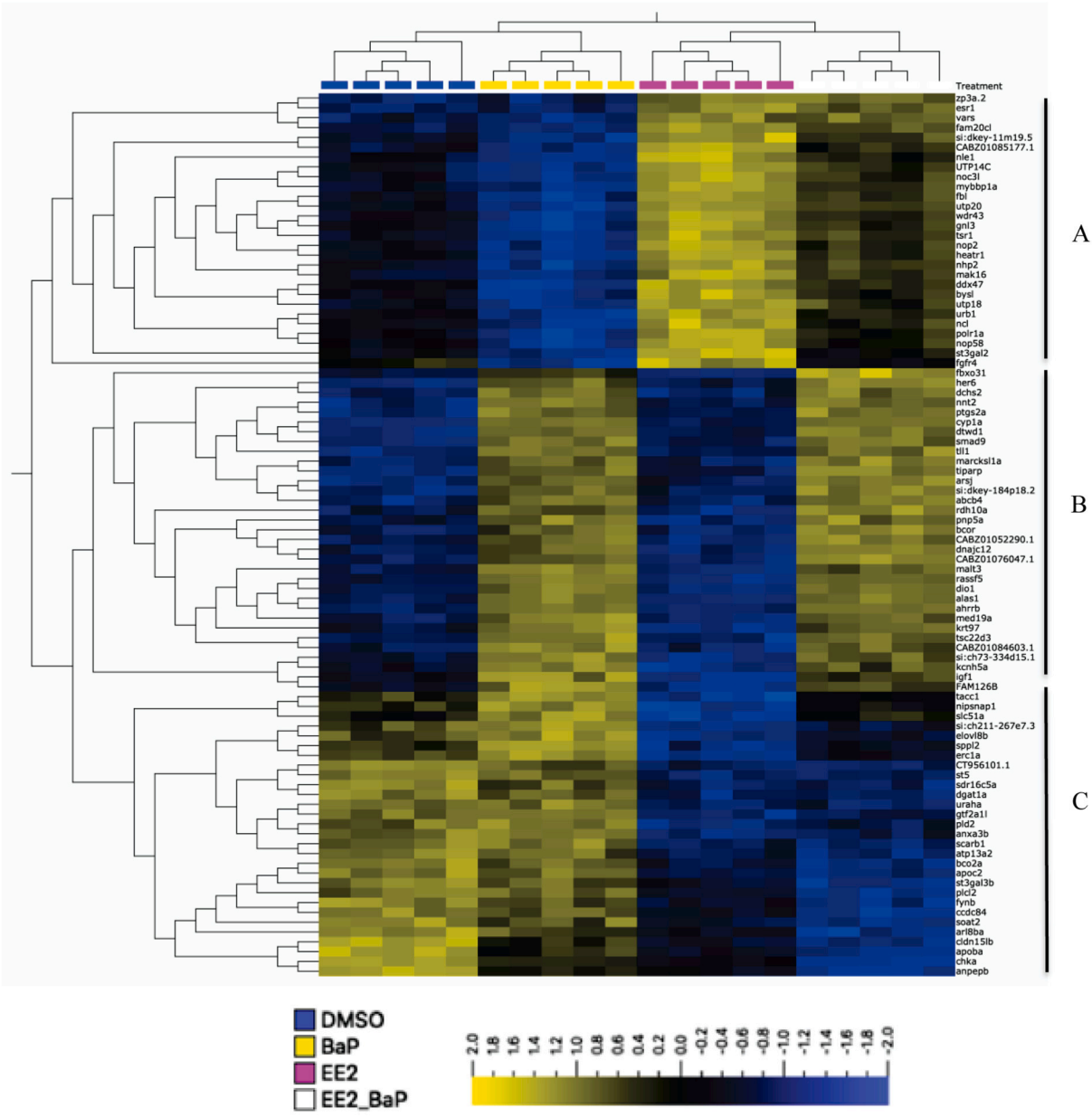

Fig. 5. Heatmap of the top differentially expressed genes. Hierarchical clustering was performed based on log2-transformed normalized count values of differentially expressed genes (FDR q-value <0.05) in Multi Group Comparison (Qlucore Omics Explorer). The color code of the different groups (DMSO control, BaP (10 uM), EE2 (5 nM) and EE2_BaP (5 nM EE2 + 10 uM BaP) are shown in the legend (bottom left). The vertical lines on the right (A-C) indicate the major distinct expression profiles. The color scale bar (bottom right) indicates relative expression, with yellow and blue ends representing the highest and lowest levels, respectively. Gene symbols are based on zebrafish orthologs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Vitellogenin Western blot

In order to evaluate the effects of the treatments on the levels of vitellogenin protein in the liver slice culture media, Western blot analysis was performed using an anti-Atlantic cod Vtg polyclonal antibody (Fig. 7A). There was a significant increase ($p < 0.05$, paired t-test) in Vtg in EE2 treated slices compared to DMSO control (Fig. 7B), similar to the transcriptome assay (Fig. 6B, Table S2B). The figure also shows a non-significant decrease in Vtg levels in the EE2 + BaP treatment group compared to EE2 alone, similar to decreases in the transcripts of many estrogen responsive genes determined by RNA-seq and qPCR assay (Fig. 5, Fig. 6A and B).

3.4. LDH assay

The LDH assay for cytotoxicity showed that there was no significant cytotoxicity in any of the BaP, EE2, and mixture treatment groups (Fig. S3).

4. Discussion

In this study, we investigated the endocrine-disrupting potential of a model PAH compound targeting the Ahr signaling pathway by mapping the transcriptome profile of polar cod PCLS exposed to BaP and EE2. We successfully adapted and improved PCLS preparation protocols developed for Atlantic cod (Eide et al., 2014) and polar cod (Aranguren-Abadía, 2015) making them more efficient and suitable for use in small fish species such as polar cod on board a research vessel. Transcriptome

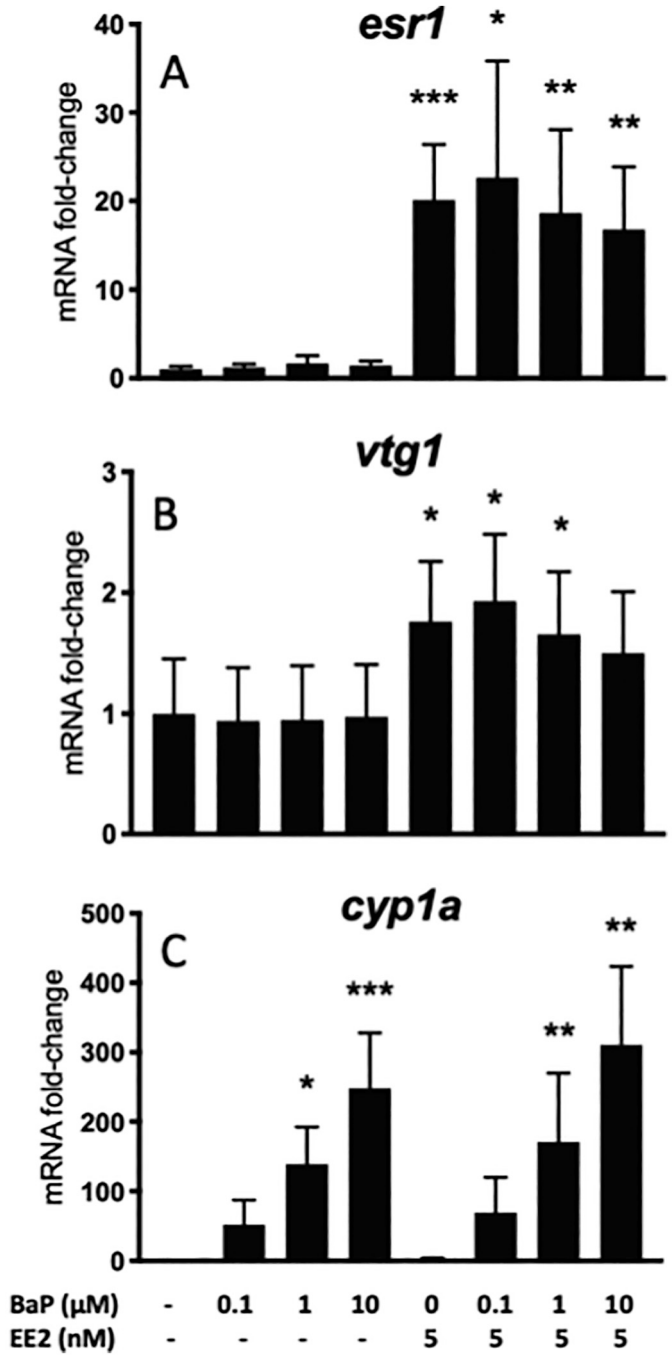

Fig. 6. qPCR analysis of selected genes differentially expressed in polar cod liver slices. Data show fold-changes in mRNA levels of the indicated genes *esr1* (A), *vtg1* (B), and *cyp1a* (C) in liver slice culture treated with different concentrations of BaP, EE2, and mixtures of BaP and EE2 compared to DMSO control. RNA samples from liver slices of 6 fish used for RNA-seq experiment were used ($n = 6$ small slices per treatment group). Significant changes (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) indicate comparison with DMSO control. Data points represent the mean ± SD of fold-changes compared to DMSO control.

analysis showed anti-estrogenic effects of BaP in PCLS exposed to the mixture of BaP and EE2. BaP exposure in polar cod liver slices resulted in large number DEGs, with Ahr pathway genes at the top of the list. BaP also affected other processes including cellular signal transduction pathways. BaP activates Ahr, a ligand-activated transcription factor that regulates the expression of numerous genes involved in xenobiotic metabolism and exerts cross-talk with many cellular signaling pathways affecting diverse processes such as cell proliferation, immune responses, embryonic development, endocrine responses and carcinogenesis (Denison et al., 2011; Jenny et al., 2009; Schiering et al., 2017; Wincent et al., 2015). Pathways related to xenobiotic metabolism, carcinogenesis, cellular signal transduction and lipid metabolism were among the top affected in BaP treated polar cod liver slices, similar to transcriptome changes observed in response to BaP in the human HepG2 cell-line (van Delft et al., 2012). Thus, the diverse pathways affected in BaP treated liver slices are consistent with the wide range of effects of the Ahr pathway. A limited set of studies have characterized transcriptomic responses in polar cod to Ahr pathway activating compounds. Early studies have investigated responses to oil compounds in polar cod using selected biomarkers such as Phase I and II enzyme activities, biliary PAH metabolites, and antioxidant enzymes (Nahrgang et al., 2009; Nahrgang et al., 2010b; Vieweg et al., 2017). Recent *in vivo* studies have also analyzed high throughput transcriptome responses of crude oil and BaP (Andersen et al., 2015; Song et al., 2019). The top affected genes and pathways in the present study are related to Ahr signaling pathway (*e.g. cyp1a*, *cyp1b1*, *cyp1c2*, and *ahrrb*), similar to the *in vivo* studies in polar cod (Andersen et al., 2015; Song et al., 2019) and to PCLS exposure studies in the related species Atlantic cod (Yadetie et al., 2018). The three cyp1 genes and the *ahrr* gene up-regulated here are known to be inducible by Ahr ligands (Goldstone et al., 2009; Jenny et al., 2009; Karchner et al., 2002). Notably, the *fgf7* gene that was activated in polar cod exposed to crude oil (Andersen et al., 2015) was also among the top up-regulated genes in the liver slices exposed to BaP, further suggesting its Ahr dependent transcription.

EE2 treatment resulted in differential expression of several ER signaling pathway genes in the polar cod liver, including biomarker egg protein vitellogenin and *zona pellucida protein* genes (Arukwe et al., 1997; Sumpter and Jobling, 1995). The gene coding for the liver estrogen receptor 1 (Esr1), often used as a biomarker of estrogenic compounds in fish (Yadetie et al., 1999), was also strongly up-regulated. In response to EE2 treatment, a similar gene expression profile was obtained as in Atlantic cod (Yadetie et al., 2018), the most common estrogen responsive genes *esr1*, eggshell protein *zona pellucida* (zp2.3 and zp3a.2) and vitellogenin (*vtg1-1*) dominating the top list of differentially expressed genes. At the protein level too, similar induction of Vtg by EE2 and apparent attenuation by BaP in the mixture group were observed in Western blot analysis. Interestingly, *esr1* transcript had the highest fold-change compared to the egg protein genes in the current study (Table S2B). A potential reason for this difference may be related to a time-dependent difference in mRNA levels of the *esr1* and the egg protein genes (*zps* and *vtgs*), with the former reaching peak levels earlier (Yadetie et al., 1999).

In many fish species, estrogens modulate genes coding for Esr1, Vtgs, and Zps that represent the hallmark of estrogen-activated transcription in fish liver, as well as genes and pathways related to translation, transcription, and lipid metabolism (Feswick et al., 2017; Hultman et al., 2015; Levi et al., 2009; Yadetie et al., 2018). Similarly, these genes and pathways were affected in EE2 treated polar cod liver slices (Fig. 2B, Table S2B): Most genes related to rRNA processing, transcription, and translation were up-regulated (Fig. 4B, Table S4). This appears consistent with the increases in general cellular processes such as transcription and translation during estrogen-stimulated vitellogenesis in fish liver (reviewed in Arukwe and Goksøyr, 2003; Lubzens et al., 2010). During the estradiol stimulated process, large amounts of the phosphoglycolipoprotein vitellogenin are synthesized, transporting lipids, fat-soluble vitamins, and other nutrients to the growing oocytes. Notably, apolipoprotein genes, populating pathways related to plasma lipoprotein assembly, remodeling, and clearance, were down-regulated in a coordinated manner in the EE2 treated liver slices (Fig. 4B, Table S4). Similarly, genes coding for proteins that constitute mammalian lipid particles and fat-soluble vitamins were mostly down-regulated. Fish apolipoproteins, which are evolutionarily related to vitellogenins, are involved in intracellular and extracellular transfer of lipids and their

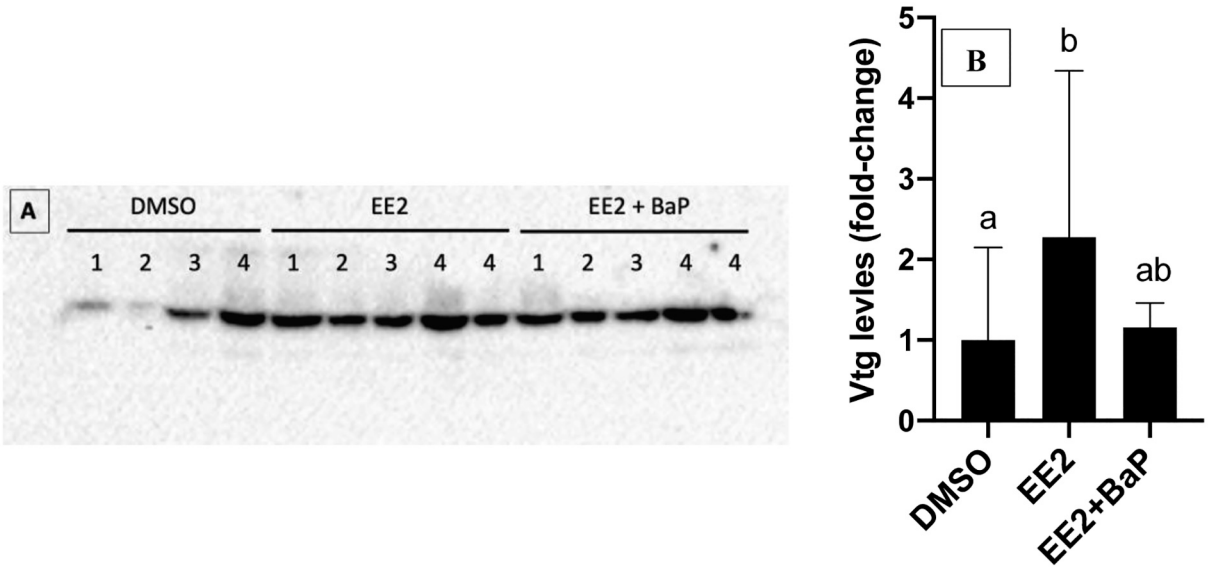

Fig. 7. Vtg levels in polar cod liver slice culture media.
A Western blot showing levels of Vtg protein (about 200 kDa) detected in liver slice culture media from individual fish (1–4) treated with DMSO, EE2, and BaP + EE2 (A). Relative quantification of Vtg levels per mg liver slice (fold-changes) as determined from Western blot (in A) densitometric analysis (B). Vtg levels were determined in the culture medium from each well from the treatment groups of DMSO control, EE2 (5 nM), and EE2 + BaP (5 nM EE2 + 10 μM BaP). Data points represent the mean ± SD of fold-changes compared to DMSO control ($n = 4$ per group). Different letters at the top of the bars indicate statistically significant differences ($p < 0.05$).

synthesis is affected by several factors including diet and season (Babin et al., 1999; Babin and Vernier, 1989). The effect of EE2 on transcription of genes for apolipoproteins is likely related to their roles in major lipid mobilization processes taking place during vitellogenesis. Consistent with our results here, some apolipoproteins were reported to be down-regulated in estrogen-treated zebrafish liver (Levi et al., 2009; Martyniuk et al., 2007). Many studies have reported estrogen modulation of liver egg protein genes (*vtgs* and *zps*), as well as genes involved in pathways such as lipid metabolism, transcription, and translation in other fish species (reviewed in Martyniuk et al., 2020). To our knowledge, this is the first study of global gene expression in response to estrogens in the polar cod liver tissue and some of the top estrogen-responsive genes identified can be used as biomarkers of exposure to estrogenic compounds in polar cod.

To explore possible anti-estrogenic effects of Ahr activating pollutants during vitellogenesis when important estrogen pathway genes are activated, we performed co-exposure of liver slices with BaP and EE2, that activate the Ahr and estrogen signaling pathways, respectively. Low EE2 concentration (5 nM or 1.5 ng/ml) used was within the range of plasma levels of estradiol during early vitellogenesis in the female polar cod (Bender et al., 2016). BaP resulted in a marked attenuation of EE2-activated gene expression, consistent with the known anti-estrogenic effects of Ahr activators (Goksøyr, 2006; Matthews and Gustafsson, 2006). In fish, many studies have shown that Ahr ligands can have anti-estrogenic effects and decrease the expression of estrogen receptor-regulated vitellogenesis related genes such as *esr1*, vitellogenin, and eggshell protein genes in the liver (Bemanian et al., 2004; Bugel et al., 2013; Gräns et al., 2010; Hultman et al., 2017; Navas and Segner, 2000; Smeets et al., 1999). Anti-estrogenic potency of Ahr-ligands is known to correlate with their potencies to activate Ahr (Smeets et al., 1999). Mechanisms of anti-estrogenic effects by ligand-activated Ahr may involve degradation of the estrogen receptor protein, interference with binding of the estrogen receptor to promoter elements of target genes, competition for transcriptional co-activators and depletion of endogenous estrogens (Matthews and Gustafsson, 2006; Ohtake et al., 2011). In another polar cod exposure experiment, crude oil WSF also resulted in down-regulation of liver estrogen-regulated genes such as *esr1* and *vtg1* (Strople et al., in preparation), similar to the anti-estrogenic effects of BaP observed in the current experiment. Furthermore, similar down-regulation of liver estrogenic responsive genes such as vitellogenin and eggshell protein genes have been observed also in fish affected by the Deepwater Horizon oil spill, but the consequences on the fish reproduction were not evaluated (Garcia et al., 2012; Whitehead et al., 2012). Although the long-term physiological consequences of anti-estrogenic effects of pollutants in wild fish are unknown, a study chronically exposing zebrafish (*Danio rerio*) to TCDD suggested that anti-estrogenic effects can lead to reduced plasma estradiol levels and interfere with ovarian development (Heiden et al., 2006). Taken together, these reports and our results here suggest possible consequences of Ahr activating pollutants on fish reproduction and warrant further studies.

5. Conclusions

We have demonstrated the successful implementation of an *ex vivo* liver slice culture method in a field laboratory (on board research vessel) to study transcriptome responses to pollutants. Using this system, we have mapped transcriptional responses to BaP and EE2. Furthermore, we have shown the anti-estrogenic effects of activated Ahr pathway on the liver estrogen regulated genes in polar cod. This study suggests that pollutants such as crude oil components which can activate the Ahr pathway can potentially lead to reproductive disturbances in polar cod.

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Declaration of Competing Interest

AG is a major shareholder in Biosense Laboratories AS, supplier of anti-Vtg polyclonal antibody.

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Appendix A. Supplementary data

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