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Transcriptomic analysis reveals dose-dependent modes of action of benzo(a)pyrene in polar cod (*Boreogadus saida*)

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Abstract

Polar cod (*Boreogadus saida*) has been used as a model Arctic species for hazard assessment of environmental stressors such as polycyclic aromatic hydrocarbons (PAHs). However, most of the PAH studies using polar cod rely on targeted biomarker-based analysis thus may not adequately address the complexity of the toxic mechanisms of the stressors. The present study was performed to develop a broad-content transcriptomic platform for polar cod and apply it for understanding the toxic mechanisms

of a model PAH, benzo(a)pyrene (BaP). Hepatic transcriptional analysis using a combination of high-density polar cod oligonucleotide microarray and quantitative real-time RT-PCR was conducted to characterize the stress responses in polar cod after 14d repeated dietary exposure to 0.4 (Low) and 20.3 µg/g fish/feeding (High) BaP. Bile metabolic analysis was performed to identify the storage of a key BaP hepatic biotransformation product, 3-hydroxybenzo(a)pyrene (3-OH-BaP). The results clearly showed that 3-OH-BaP was detected in the bile of polar cod after both Low and High BaP exposure. Dose-dependent hepatic stress responses were identified, with Low BaP suppressing genes involved in the defense mechanisms and High BaP inducing genes associated with these pathways. The results suggested that activation of the aryl hydrocarbon receptor signaling, induction of oxidative stress, DNA damage and apoptosis were the common modes of action (MoA) of BaP between polar cod or other vertebrates, whereas induction of protein degradation and disturbance of mitochondrial functions were proposed as novel MoAs. Furthermore, conceptual toxicity pathways were proposed for BaP-mediated effects in Arctic fish. The present study has for the first time reported a transcriptome-wide analysis using a polar cod-specific microarray and suggested novel MoAs of BaP. The analytical tools, bioinformatics solutions and mechanistic knowledge generated by this study may facilitate mechanistically-based hazard assessment of environmental stressors in the Arctic using this important fish as a model species.

1. Introduction

Climate change in combination with anthropogenic activities have brought new challenges to the Arctic ecosystems. Elevated levels of persistent organic pollutants (POPs) and hydrocarbons are found in the

53   Arctic due to river discharges, freshwater run-off from melting sea-ice, oil and gas exploration, and

54   maritime shipping (Macdonald et al., 2005; Harsem et al., 2011; Smith and Stephenson, 2013). Increased

55   contamination may thus pose risk to living organisms in the Arctic. Polar cod (*Boreogadus saida*) is a

56   keystone fish species in the Arctic marine ecosystem due to its abundance, pan-Arctic distribution and

57   central role in the food web (Bradstreet and Cross, 1982; Hop and Gjosaeter, 2013). In the past decade,

58   polar cod has been extensively studied with regards to its sensitivity toward petroleum related

59   contaminants (Geraudie et al., 2014; Bender et al., 2016; Nahrgang et al., 2016; Vieweg et al., 2018)

60   and considered as an Arctic indicator species for environmental monitoring (Nahrgang et al., 2009;

61   Jonsson et al., 2010; Nahrgang et al., 2010a; Nahrgang et al., 2010b).

62   Benzo(a)pyrene (BaP) is a five-ring polycyclic aromatic hydrocarbon (PAH) that has been widely

63   used as a prototypical compound for understanding the effects and modes of action (MoAs) of PAHs

64   (Collins et al., 1991). It is a highly toxic chemical to many organisms and classified as one of the priority

65   pollutants by U.S. Environmental Protection Agency (https://www.epa.gov/eg/toxic-and-priority-

66   pollutants-under-clean-water-act). The toxicity of BaP in mammals and several fish species has been

67   extensively studied, including developmental toxicity, reproductive toxicity, immunotoxicity and

68   carcinogenicity (Carlson et al., 2004; Busquet et al., 2007; Yuen et al., 2007; Seemann et al., 2015; EPA,

69   2017). The main MoAs of BaP are well characterized in model vertebrates, with activation of the aryl

70   hydrocarbon receptor (AhR) leading to genotoxicity being the most commonly recognized MoA. Other

71   known MoAs include modulation of hormone receptor signaling pathways, induction of oxidative stress,

72   DNA damage, apoptosis and immunosuppression (Carlson et al., 2004; EPA, 2017). It has been widely

73   recognized that the compound itself has relatively low toxicity, whereas its primary and secondary

74   metabolites generated by biotransformation are highly genotoxic. Phase I biotransformation of BaP is

75   mediated by cytochrome P450 (CYP) enzymes and produces highly reactive metabolic intermediates,

76   such as 3-hydroxybenzo(a)pyrene (3-OH-BaP) (Zhu et al., 2008; Rey-Salgueiro et al., 2011) and BaP-

77   quinones (BPQs). The 3-OH-BaP metabolite can covalently bind to DNA and protein, and form harmful

78   adducts (Godschalk et al., 2000; Tzekova et al., 2004; Marie-Desvergne et al., 2010), while BPQs

79   undergo one electron redox cycling and subsequently lead to formation of reactive oxygen species (ROS)

80   through Fenton type reactions (Lorentzen and Ts'o, 1977; Flowers et al., 1997). Phase II

81 biotransformation is mediated by epoxide hydrolases which convert the metabolic intermediates of BaP

82 to diol epoxide derivatives, such as benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) (Karle et al.,

83 2004), an ultimate form of BaP metabolite and a potent mutagen which can bind to the exocyclic amino

84 group of purines or guanine nucleobases in DNA and form BPDE-DNA adducts (reviewed in Shimada

85 and Fujii-Kuriyama (2004)). In fish, most of the BaP metabolites are produced in the liver, secreted into

86 the bile, concentrated in the gallbladder, and excreted into the intestine (Ferreira et al., 2006; Zhu et al.,

87 2008; Rey-Salgueiro et al., 2011). The bile concentrations of BaP metabolites thus have been widely

88 used as indicators of BaP exposure and biotransformation in fish (Meador et al., 1995; Moller et al.,

89 2014; Baali et al., 2016; Kammann et al., 2017).

90   Although the mechanistic understanding of BaP biotransformation and toxicity is relatively abundant

91 for several model vertebrates, knowledge is limited for non-model but ecologically important Arctic

92 species. Current approaches for understanding the effects of BaP and other PAHs on Arctic fish are

93 mainly biomarker-based, such as that using the cytochrome P450 genes/enzymes as indicators of AhR

94 activation, and antioxidant enzymes as markers for oxidative stress (Nahrgang et al., 2009; Nahrgang et

95 al., 2010b; Nahrgang et al., 2010c; Vieweg et al., 2017). Although the main MoAs of BaP can be

96 captured using such approaches, the mechanistic knowledge gained is restricted to a few pre-defined

97 toxicological functions being studied. Development and application of broad-content tools such as

98 transcriptomics and other toxicogenomic (OMIC) tools are thus increasingly implemented in un-biased

99 characterization of toxicity mechanisms, identifying relevant MoA and link these to adverse effects

100 relevant for successful survival, development and reproduction of keystone species. Such hypothesis-

101 generating tools can thus be key to developing suitable biomarkers for environmental monitoring

102 purposes, hazard and risk assessment for Artic species.

103   The present study addresses this issue specifically by developing and evaluating the performance of

104 a custom high-density (180,000 features) oligonucleotide microarray by characterizing the hepatic

105 transcriptomic responses in polar cod after dietary exposure to two doses of BaP. The main objectives

106 were to: 1) develop a polar cod transcriptomics (analytical and bioinformatics) platform for

107 ecotoxicological studies; 2) characterize the MoAs of BaP in polar cod based on global transcriptional

108 responses; 3) investigate the biotransformation of BaP in polar cod based on the bile concentrations of

4

109 3-OH-BaP; and 4) propose a set of toxicity pathways relevant to understand the potential hazards of

110 PAHs to Arctic fish.

111

112

2. Materials and Methods

2.1 Field sampling and maintenance

115 Polar cod were caught with a Campelen bottom trawl on board R/V Helmer Hanssen in Svalbard waters

116 (78°N). The samples were transported to the marine biological station of the UiT-The Arctic University

117 of Norway in Kårvika and maintained in 300 L holding tanks with running seawater at 3°C and constant

118 dimmed light until the exposure experiment. During acclimation, polar cod were fed three times weekly

119 ad libidum with frozen *Calanus spp.* (Calanus AS, Tromsø).

120

2.2 Experimental design, feed preparation and exposure

122 The experimental design consisted in force-feeding adult polar cod for 14 days and three times weekly,

123 to 2 doses of dietary BaP (Sigma-Aldrich, St. Louis, USA) and the solvent (acetone) control. The feed

124 preparation consisted in the addition of BaP from a stock solution (10 mg BaP/mL acetone) to thawed

125 *Calanus* spp., to yield final doses of either 0, 10 or 480 µg BaP per g feed (final acetone concentration:

126 50 µL/g feed) for Control, Low and High BaP, respectively. The doses to each fish were chosen to be

127 in the range of those used in the study by Wu et al. (2003) in which alterations of biomarkers at the

128 cellular and physiological levels were identified. In addition, water (200 µL/g feed) was added to the

129 preparation to adjust the consistency of the feed for the force-feeding. All feed preparations were then

130 stirred for 2.5 hours at approximately 50°C to remove acetone. Finally, 1mL Luer-lok™ syringes

131 (Becton, Dickinson and Company, Franklin Lakes, USA) were filled with 0.5 g of the feed and frozen

132 at -80°C until exposure started.

133   Polar cod (n=30, total length 13.2 ± 1.2 cm, total weight 13.2 ± 3.8 g) were weighed and transferred

134 to three experimental tanks (300 L). Fish were force-fed 0.5 g feed, corresponding to 4 ± 1 % body

135 weight and final doses of 0, 0.4 ± 0.16 and 20.3 ± 5.6 µg BaP per g fish. The feeding took maximum 20

136 sec. and each fish was observed for 3 minutes upon feeding to control for regurgitation. Force-feeding

137 was repeated 6 times until final sampling on the 12th of January 2013. Experimental condition

138 (photoperiod and water temperature) were the same as during acclimation. Oxygen levels were daily

139 measured and stayed above 90% saturation.

140 Polar cod were sampled at exposure start (holding tanks, n=10) and after 14 days of exposure (n=10

141 per treatment). Sampling occurred three days following the last feeding event to ensure accumulation of

142 bile. Total length (cm), total, somatic (g, without guts, liver and gonads), gonad and liver weight (g)

143 were recorded. Liver and bile were sampled, snap frozen in liquid nitrogen and stored at -80°C for

144 further analyses. Hepatosomatic index (HSI) and gonadosomatic index (GSI) were determined by the

145 following equations:

146

147 GSI (%) = gonad weight/somatic weight × 100   Eq (1)

148 HSI (%) = liver weight/somatic weight × 100   Eq (2)

149

150 **2.3 Measurement of BaP metabolite**

151 One of the most abundant BaP metabolites, 3-OH-BaP, was measured in the bile of each individual

152 polar cod. Preparation of hydrolysed bile samples was performed as described in Krahn et al. (1992).

153 Briefly, bile (1-20 µL) was mixed with an internal standard (triphenylamine) and diluted with

154 demineralised water (10-50 µL) and hydrolysed with β-glucuronidasearylsulphatase (20 µL, 1 h at

155 37°C). Methanol (75-200 µL) was added and the sample was mixed thoroughly before centrifugation.

156 The supernatant was then transferred to vials and analysed. High pressure liquid chromatography

157 (Waters 2695 Separations Module) was used to separate 3-OH-BaP in a Waters PAH C_{18} column (4.6

158 ×250 mm, 5 µm particle size). The mobile phase consisted of a gradient from 40:60

159 acetonitrile:ammonium acetate (0.05 M, pH 4.1) to 100 % acetonitrile at a flow of 1 mL/min, and the

160 column was heated to 35°C. A 2475 Fluorescence detector measured fluorescence at the optimum for

161 each analyte (excitation/emissions: 380/430). A total of 25 µL extract was injected for each analysis.

162 The results were calculated by use of the internal standard method (Grung et al., 2009). The calibration

163 standards utilized were obtained from Chiron AS, Trondheim, Norway, and were in the range 0.2-200

164 ng/g. Some gallbladder samples were too small to allow the extraction of enough material and ensure

165 data quality. In this case, samples were removed from the dataset. The final number of samples analysed

166 per treatment is presented in Table 1.

167

168 **2.4 Transcriptional analyses**

169 A combination of optimizing RNA isolation procedures, performing RNA sequencing of pooled RNA

170 for de novo (multi-tissue) transcriptome assembly, developing an oligo nucleotide array (microarray)

171 for global transcriptomics analysis and identifying suitable biomarker genes for qPCR and developing

172 a suitable bioinformatics pipeline, were performed to provide a suite of tools for rapid, cost-efficient,

173 and reliable characterization of transcriptional responses in polar cod.

174

175 **2.4.1 RNA isolation**

176 Total RNA was extracted from 20-30 mg frozen liver tissues from each individual fish using RNeasy

177 Plus Mini Kit (Qiagen, Hilden, Germany), as previously described (Song et al., 2014). The RNA yield

178 (>2 µg) and purity (260/280 >1.8, 260/230>2) was measured using Nanodrop® spectrophotometer ND-

179 1000 (Nanodrop Technologies, Wilminton, Delaware, USA). The RNA integrity (RIN>8) was assessed

180 using Agilent Bioanalyzer RNA 6000 Nano chips (Agilent technologies, Santa Clara, California, USA).

181

182 **2.4.2 RNA sequencing and microarray design**

183 RNA from various tissues (muscle, liver, heart, gills, brain, and spleen) were sampled from a separate

184 fish group, snap frozen in liquid N2, subjected to RNA extraction and RNA quality control as previously

185 described (see 2.4.1). Pooled RNA from different tissues were subjected to poly(A)(+) mRNA

186 enrichment by Oligo(dT), RNA fragmentation, cDNA synthesis by reverse transcriptase using random

187   hexamer, cDNA size selection, PCR amplification and RNA deep sequencing by Illumina HiSeq 2000

188   by Beijing Genome Institute (BGI, Hong Kong, China). De novo transcriptome assembly of resulting

189   68 million raw reads yielded 82,900 consensus sequences (Unigenes) that were separated into 21,463

190   distinct clusters (nt size>300 nucleotides) and 61,437 singletons (nt size>200 nucleotides). A total of

191   53,812 Unigenes were successfully mapped to other fish species and multiple functional categories

192   annotated into COG and GO classifications. Where possible, protein coding regions (CDS) were

193   predicted by blasting the sequences against protein databases (BlastX and ESTScan) and RNA sequence

194   translated into an amino acid sequences. Of the total transcripts obtained, 82,000 sequences with

195   annotations (approx. 50,000 CDS) yielded high-quality probes that were randomly positioned on a

196   180,000 feature, 60-mere oligo nucleotide array (in duplicate) using Agilent Earray

197   (https://earray.chem.agilent.com/earray). The resulting custom microarray was manufactured by Agilent.

198   The detailed descriptions of the RNA sequencing, annotation and microarray design can be found in

199   Appendix A.

200

201   2.4.3 Microarray analysis

202   Microarray gene expression analysis was conducted using 50 ng hepatic total RNA (n=4) according to

203   the "Agilent One-Color Microarray-Based Gene Expression Analysis (v6.5)" protocol, as previously

204   described (Song et al., 2016). The raw data was extracted using the Feature Extraction software v10.7

205   (Agilent), and corrected for background signal, flagged for low quality/missing features, normalized

206   (quantile method) and log2 transformed using GeneSpring GX v11.0 (Agilent). One-way analysis of

207   variance (ANOVA) followed by Tukey posthoc test was used to determine differentially expressed

208   genes (DEGs) by comparing the exposed groups to the control. The Benjamin Hochberg (BH) multiple

209   testing correction was applied to the statistical analysis to eliminate false positives (adjusted p-value

210   <0.05).

211   To understand the functions of the DEGs, gene ontology (GO) functional enrichment analysis was

212   performed using the Cytoscape v2.8 application Bingo v2.4. A hypergeometric test in combination with

213   BH multiple testing correction was used to identify overrepresented GO functions (adjusted p-value

214 <0.05). The polar cod DEGs were further mapped to the orthologs of model organisms, including human

215 (*Homo sapiens*), mouse (*Mus musculus*), common rat (*Rattus norvegicus*), zebrafish (*Danio rerio*) and

216 Atlantic cod (*Gadus morhua*) using a two-pass BLAST approach implemented in Inparanoid 7 (Ostlund

217 et al., 2010), as previously described (Song et al., 2014). The mapped ortholog DEGs were used for gene

218 network and pathway analyses (Fisher's exact test, p-value <0.05) for species such as humans, mouse

219 and rat, as well as supported orthology functionality for species such as zebrafish in Ingenuity Pathway

220 Analysis (IPA, Ingenuity®Systems, www.ingenuity.com). No multiple testing correction was applied to

221 the ortholog-based functional analyses to avoid loss of significant biological information. The enriched

222 pathways were anchored to existing knowledge on the MoAs of BaP in fish and mammals to avoid false-

223 positive conclusions. Venn diagram analysis was performed using Venny

224 (bioinfogp.cnb.csic.es/tools/venny/).

225

226 **2.4.4 Quantitative real-time RT-PCR**

227 To measure a selection of biomarker gene responses and validate the microarray results, quantitative

228 real-time reverse transcription polymerase chain reaction (qPCR) was conducted as previously described.

229 The qPCR assay was run using the BioRad CFX384 platform, as previously described (Song et al.,

230 2016). Briefly, 2 μg hepatic total RNA (identical as used for microarray analysis) was reversely

231 transcribed to complementary DNA (cDNA) using the High Capacity cDNA Archive Kit (Applied

232 Biosystems, Foster City, California, USA). The cDNA template (n=5) was then amplified in technical

233 duplicates using the PerfeCTa® SYBR® Green FastMix® (Quanta BioSciencesTM, Gaithersburg, MD,

234 USA) in combination with 400 nM forward/reverse primers (Invitrogen™,Carlsbad, USA) in a 20 μL

235 reaction. The primers were designed using Primer3 v0.4.0 (frodo.wi.mit.edu/primer3), purchased from

236 Invitrogen™ (Carlsbad, USA) and optimized for annealing temperatures (Table A1). Standard curves

237 were generated for each gene using pooled cDNA from all test samples to calculate amplification

238 efficiencies (90%-105%). Non-template controls (NTCs) and no-reverse-transcriptase controls (NRCs)

239 were also included in the qPCR reactions for quality assurance. The relative expression of target genes

240 were calculated based on the quantification cycle (Cq) values using the Pfaffl Method (Pfaffl, 2001) and

normalized to the geometric mean expression of three housekeeping genes using the $\Delta\Delta Cq$ method (Vandesompele et al., 2002). The relative expression of target genes in the exposed groups were further normalized to that in the control group to calculate fold changes (FC). Prior to statistical analysis using Graphpad Prism v5.0 (Graphpad Software, Inc., San Diego, CA, USA), the normalized data was checked for outliers using Grubb's test (Grubbs, 1950). One-way ANOVA in combination with Tukey posthoc test was used to determine the statistical difference between the exposure groups when the assumptions of normality and equal variance were met. Otherwise, Kruskal-Wallis non-parametric test followed by a Dunn's post-hoc test was used.

3. Results

3.1 Exposure and chemistry

Polar cod were exposed to 0 (Control), 0.4 ± 0.16 (Low) and 20.3 ± 5.6 µg (High) BaP per g fish, respectively. The concentrations of BaP metabolites (1.54 ± 0.96 and 120.3 ± 64.70 µg BaP per g bile in Low and High groups, respectively) measured in bile after 2 weeks of exposure were in accordance with the ingested doses (Table 1).

The sizes of the exposed fish were similar across the groups and in gonadal maturing stages with high GSI, except for one female in the high treatment (2.1%). Length, weight, GSI, and HSI were not significantly different between treatments and gender. The sex ratio in the experimental tanks was unbalanced, with majority of males in the Control and High BaP groups.

Table 1. Overview of benzo(a)pyrene (BaP) doses in prepared feed and received by polar cod, concentration of 3-OH-BaP metabolites in bile as well as morphometric (total length, weight, sex ratio, GSI, and HSI) of analyzed samples (n=10). Data is presented as mean and standard deviation. LOD is below limits of analytical detection. Numbers in parentheses are n samples when different from 10.

Exposure duration (week)	Treatment	N	BaP dose in feed	BaP dose in fish per feeding		3-OH-BaP	Total length	Total weight	Sex ratio	GSI	HSI
			µg/g	µg/ fish	µg/ g fish	µg/ g bile	cm	g	Male:Female	%	%

|											
0	Control	10				<LOD (6)	12.5 ± 1.3	11.1 ± 3.1	8:2	16.4 ± 6.1	6.6 ± 1.8
2	Control	10	0	0	0	<LOD (8)	12.9 ± 0.8	12.7 ± 2.4	9:1	18.9 ± 5.2	7.1 ± 2.3
2	Low	10	10	5	0.4 ± 0.16	1.54 ± 0.96 (7)	13.4 ± 1.4	14.2 ± 4.9	5:5	17.0 ± 7.7	6.9 ± 2.2
2	High	10	480	240	20.3 ± 5.6	120.3 ± 64.70 (7)	13.2 ± 1.3	12.7 ± 1.3	7:3	14.9 ± 4.8	7.6 ± 1.8

265

3.2 Transcriptomic responses

3.2.1 Differentially expressed genes

The microarray analysis identified a total of 747 (246 up- and 501 down-regulated) and 2190 (1453 up-
and 737 down-regulated) DEGs after dietary exposure to Low and High BaP, respectively (Appendix B,
Table A1). Among these, 232 DEGs were commonly regulated by Low and High BaP, whereas the
majority was dose-specific (Figure 1).
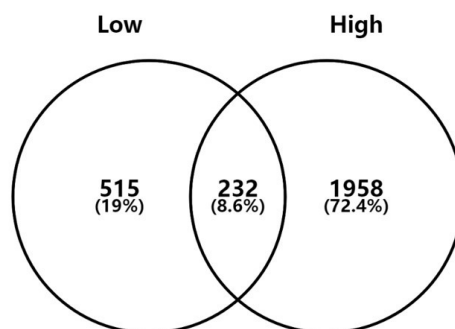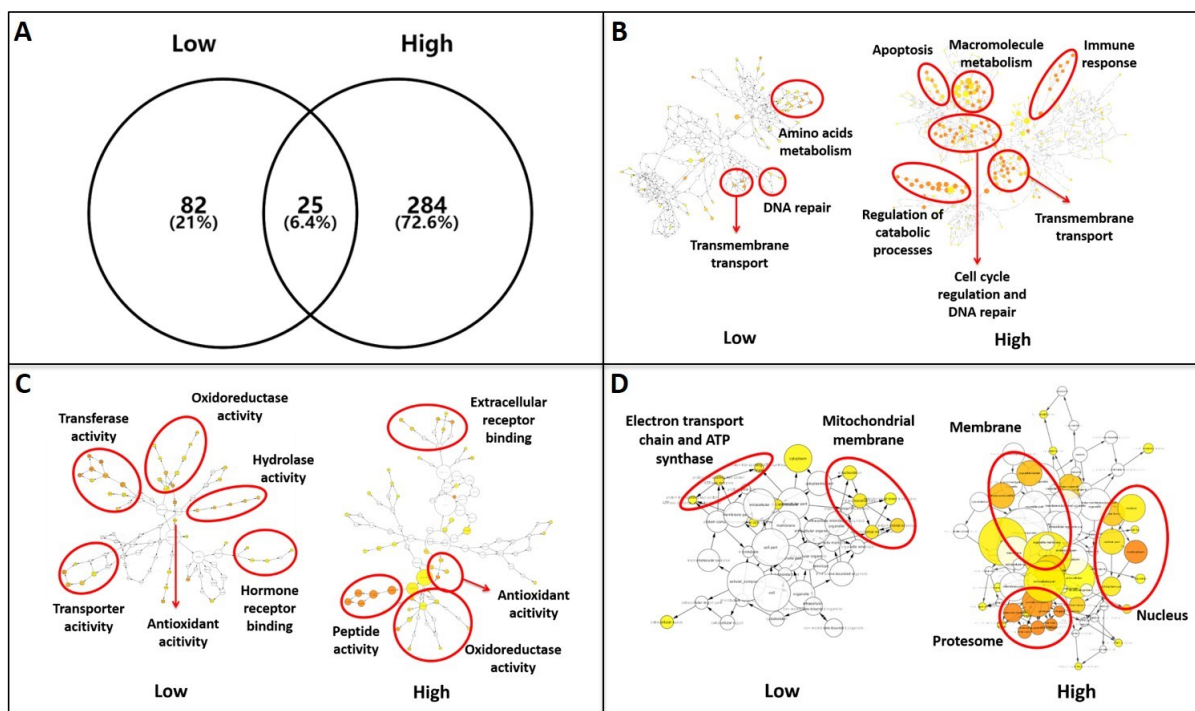

Figure 1. Differentially expressed genes (DEGs) in the liver (n=4) of polar cod (*Boreogadus saida*) after 14d repeated dietary exposure to 0.4
(Low) and 20.3 ug/g fish (High) benzo(a)pyrene (BaP).

3.2.2 Gene ontology enrichment

Gene ontology analysis showed that a total 107 and 309 GO terms were overrepresented after exposure
to Low and High BaP, respectively (Appendix B, Table A2). Twenty-five GO functions were commonly
affected by both Low and High dose BaP, whereas the majority was dose-specific (Figure 2A). Exposure
to Low BaP mainly affected DEGs involved in biological processes such as transmembrane transport,

DNA repair and amino acids metabolism, whereas exposure to High BaP affected DEGs associated with
apoptosis, macromolecule metabolism, immune response, regulation of catabolic processes, cell cycle
regulation and DNA repair and transmembrane transport (Figure 2B). In terms of molecular functions,
Low BaP caused transcriptional responses associated with transferase activity, oxidoreductase activity,
hydrolase activity, transporter activity, antioxidant activity and hormone receptor binding processes,
whereas High BaP exposure led to differential gene expression linked to extracellular receptor binding,
peptide activity, antioxidant activity and oxidoreductase activity (Figure 2C). Low BaP regulated genes
mainly involved in cellular components such as electron transport chain and ATP synthase and
mitochondrial membrane, whereas High BaP affected genes involved in the cell membrane, nucleus and
proteosome (Figure 2D). Several GO terms such as oxidoreductase activity, cell redox homeostasis and
antioxidant activity were identified to be commonly regulated by both Low and High BaP.

Figure 2. Overrepresented gene ontology (GO) functions that were affected in the liver (n=4) of polar cod (*Boreogadus saida*) after 14d repeated
dietary exposure to 0.4 (Low) and 20.3 ug/g fish (High) benzo(a)pyrene (BaP). A: Venn diagram analysis of common and unique GO functions;
B: Overrepresented biological processes; C: Overrepresented molecular functions; D: Overrepresented cellular components.

3.2.3 Pathway analysis

The ortholog mapping showed that 41.8% (Low) and 32.6% (High) of the polar cod DEGs had orthologs in the model organisms used in IPA (Appendix B, Table A3). Enrichment analysis using ortholog DEGs showed that Low BaP uniquely affected DEGs related to 2 toxicity endpoints such as CAR/RXR activation, High BaP uniquely affected DEGs related to 23 toxicity endpoints such as AhR signaling, mitochondrial membrane potential, oxidative stress responses, lipid metabolism and liver damage (Appendix B, Table A4). Both Low and High BaP commonly affected DEGs involved in mitochondrial dysfunction (Figure 3).
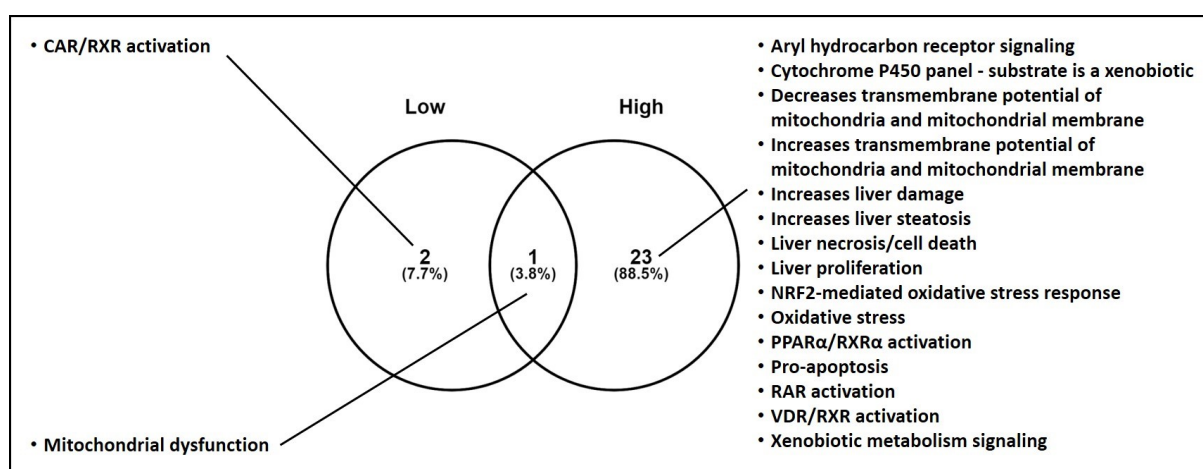
Figure 3. A selection of toxicity lists that were enriched by differentially expressed genes in the liver (n=4) of polar cod (*Boreogadus saida*) after 14d repeated dietary exposure to 0.4 (Low) and 20.3 ug/g fish (High) benzo(a)pyrene (BaP).

The ortholog DEGs were further mapped to the curated mammalian canonical pathways in IPA to get more mechanistic insight into the toxicity of BaP. The results clearly showed that the majority of the enriched canonical pathways were dose-specific, with Low BaP uniquely affected 7 pathways mainly related to immune response and endocrine regulation, High BaP uniquely affected 60 pathways mainly related to biotransformation, oxidative stress response, apoptosis, protein degradation, lipid metabolism, immune response and endocrine regulation (Appendix B, Figure A5). Both Low and High BaP exposure commonly affected 3 pathways mainly related to mitochondrial dysfunction. A selection of toxicologically relevant canonical pathways and associated DEGs is listed in Table 2.

Table 2. A selection of toxicologically relevant canonical pathways that were affected in the liver (n=4) of polar cod (*Boreogadus saida*) after 14d repeated dietary exposure to 0.4 (Low) and 20.3 ug/g fish (High) benzo(a)pyrene (BaP). The full gene names can be found in Appendix B (Table A3). ↑: up-reguation; ↓: down-regulation.

| Functional category	Canonical Pathways	p-value	Pathway coverage	Supporting differentially expressed genes
Low				
Oxidative phosphorylation/Apoptosis	Mitochondrial dysfunction	5.75E-05	5%	ndufa10↓, atp5f1↓, psenen↓, atp5l↓, ndufa6↓, glrx2↓, atp5a1↓, cox6c↓, atp5g3↓
Immune response	Lipid antigen presentation by CD1	2.29E-02	8%	ap2a2↓, ap2m1↓
Endocrine regulation	Estrogen receptor signaling	2.95E-02	3%	g6pc3↓, med10↓, polr2g↓, taf11↓
High				
Biotransformation	Xenobiotic metabolism signaling	2.14E-04	5%	mapk14↓, abcc2↑, cyp1a1↑, map3k12↓, cyp3a4↑, camk1d↓, prkcd↑, ugt2b15↑, hs6st2↑, gstp1↑, map3k4↑, hsp90aa1↑, keap1↑, cyp1b1↑, rbx1↑
Biotransformation	Aryl hydrocarbon receptor signaling	4.37E-03	5%	faslg↓, nfix↓, cyp1a1↑, ccna2↓, gstp1↑, hsp90aa1↑, nedd8↑, cyp1b1↑
Antioxidant defence	NRF2-mediated oxidative stress response	1.58E-04	6%	prkcd↑, mapk14↑, abcc2↑, usp14↑, cbr1↑, txn↑, sod1↑, gstp1↑, prdx1↑, keap1↑, rbx1↑, dnajb14↑
Antioxidant defence	Glutathione redox reactions II	7.94E-03	25%	glrx↑, txndc12↑
Antioxidant defence	Thioredoxin pathway	4.07E-04	33%	txn↑, txnrd2↑, txnrd3↑
Antioxidant defence	Antioxidant action of vitamin C	8.91E-04	7%	mapk14↑, glrx↓, txn↑, plce1↓, plcl2↓, txnrd2↑, pla2g3↑, txnrd3↑
Apoptosis	Apoptosis signaling	4.90E-03	7%	capn3↑, faslg↓, capn1↑, casp9↑, casp3↑, sptan1↑
Apoptosis	Death receptor signaling	5.75E-03	6%	tiparp↑, cflar↑, faslg↓, casp9↑, casp3↑, sptan1↑
Oxidative phosphorylation/Apoptosis	Mitochondrial dysfunction	1.23E-04	6%	ndufb6↑, cyb5a↑, psenen↑, glrx2↑, casp9↑, atp5a1↑, casp3↑, txnrd2↑, prdx3↑, fis1↑, cox6c↑, atp5g3↑
Protein degradation/Post-translational modification	Protein ubiquitination pathway	2.00E-21	13%	psmb1↑, psma2↑, psmb3↑, psmc4↑, psmb5↑, psmb6↑, psmd7↑, psmd10↑, psmd12↑, psmd14↑, dnajc2↑, hspa4l↑, usp5↑, hsp90aa1↑, dnajb14↑, usp14↑, ube2v1↑, uchl3↑, psmd13↑, rbx1↑, usp33

Lipid metabolism	PPARα/RXRα activation	4.47E-02	4%	mapk14↑, ap2a2↓, lpl↓, adipor2↓, plce1↓, plcl2↓, hsp90aa1↑
Immune response	Antigen presentation pathway	2.82E-02	8%	psmb6↑, hla-doa↑, psmb5↑
Endocrine regulation	Androgen signaling	3.63E-02	4%	shbg↑, prkcd↑, gnb5↑, polr2j↑, polr2g↑, hsp90aa1↑

324

3.3 Biomarker gene responses

No significant difference in expression was found for the reference genes tested between Low and High BaP. The qPCR results clearly showed that for the 18 genes representative of different toxicological functions, the transcriptional responses were in general consistent between that measured using qPCR and microarray (Figure 4).

330
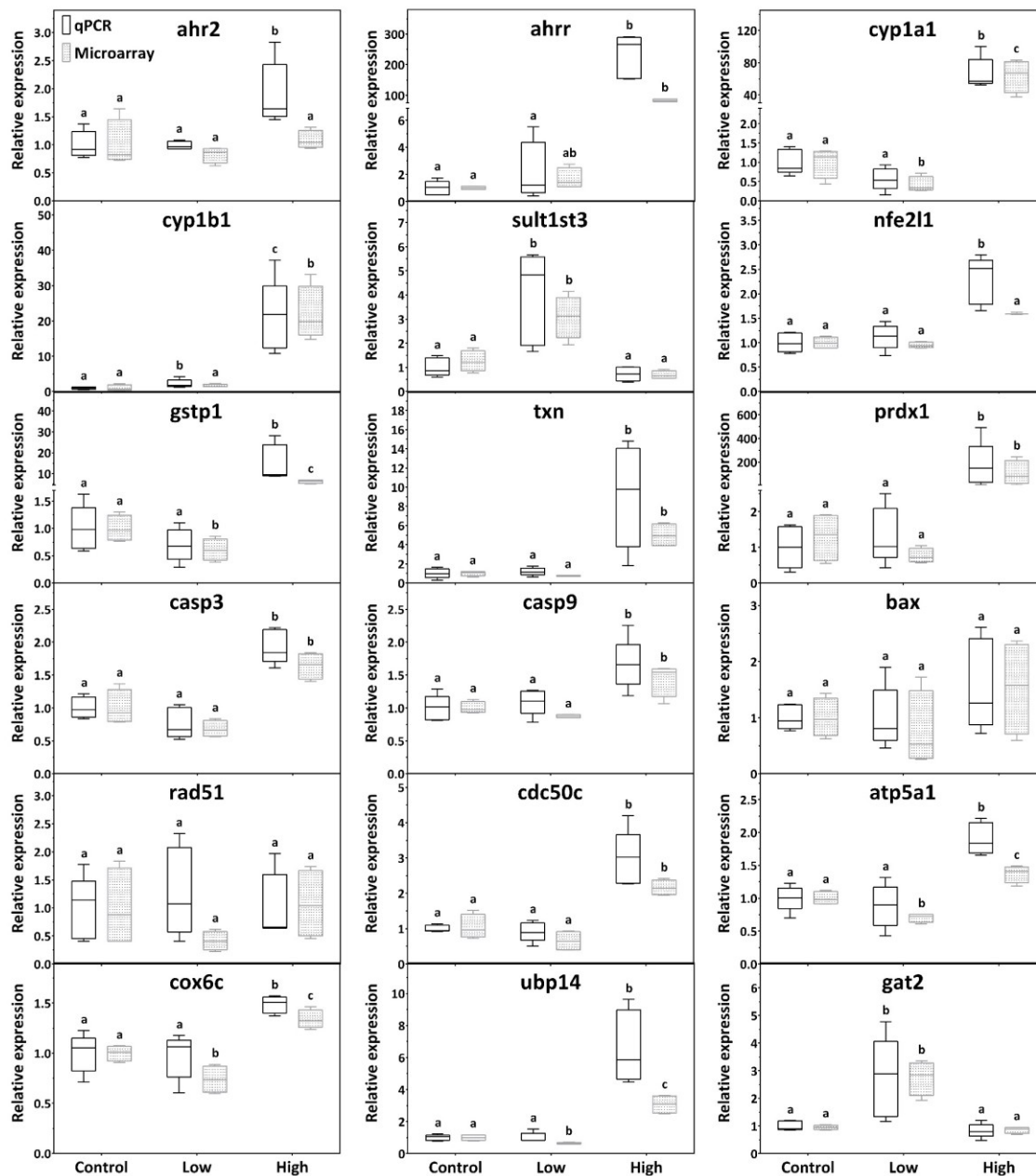
Figure 4. Biomarker gene responses in the liver (n=5) of polar cod (*Boreogadus saida*) after 14d repeated dietary exposure to 0.4 (Low) and 20.3 ug/g fish (High) benzo(a)pyrene (BaP), measured by quantitative real-time RT-PCR and microarray. Ahr2: aryl hydrocarbon receptor 2; ahrr: aryl-hydrocarbon receptor repressor; cyp1a1: cytochrome P450 family 1 subfamily A member 1; cyp1b1: cytochrome P450 family 1 subfamily B member 1; nfe2l1: nuclear factor erythroid 2-like 1; gstp1: glutathione S-transferase P; txn: thioredoxin; prdx1: peroxiredoxin 1; casp3: caspase 3; casp9: caspase 9; bax: apoptosis regulator BAX; rad51: RAD51 recombinase; cdc50c: cell cycle control protein 50C; atp5a1: ATP synthase F1 subunit alpha; cox6c: cytochrome c oxidase subunit Vic; ubp14: ubiquitin carboxyl-terminal hydrolase 14; sult1st3: sulfotransferase family 1 cytosolic sulfotransferase 3; gat2: solute carrier family 6 member 13. a: not significantly different from the corresponding control (either qPCR or microarray); b: significantly different from the corresponding control (either qPCR or microarray); c: significantly different from Low BaP treatment (either qPCR or microarray).

340

4. Discussion

As an Arctic keystone species, polar cod is an indicator species for ecosystem health in the Arctic, notably in relation to aquatic pollution. The present study thus used polar cod as a representative Arctic fish species to study the molecular responses and to characterize the toxic MoAs of the model PAH, BaP. Benzo(a)pyrene is an extensively studied toxicant in vertebrates and is considered a model compound for PAHs due to its well-characterized MoA and well-documented adverse effects in temperate fish (EPA, 2017). The levels of BaP in the feed used in the present study were chosen to mimic that found in mussels from contaminated areas (Olenycz et al., 2015) and were in the range of that used in other dietary exposure studies with different fish species (Wu et al., 2003; Au et al., 2004; Costa et al., 2011). However, these levels are in the high end of those likely encountered in many Arctic species such as polar cod, as these Arctic fish species feed on pelagic zooplankton from relatively pristine environments. Nevertheless, the stress responses to BaP and a range of other pollutants at the molecular level are poorly characterized in this Arctic fish, and effort to characterize the global transcriptional responses to this pyrogenic PAH is thus the first in its kind. Although an earlier effort has been made to characterize the transcriptomic response in polar cod after crude oil exposure and under elevated temperature, the analytical tool used in this study was indeed an Atlantic cod (*Gadus morhua*) microarray, and the results obtained were based on cross-hybridization between the two fish species (Andersen et al., 2015). The novel polar cod microarray used in this study was developed based on the polar cod-specific sequences, and displayed good reproducibility across biological replicates and coverage of genes in the stress-responsive transcriptome of polar cod. Although a number of DEGs were excluded in the functional analysis due to limitations in ortholog mapping (e.g. peroxisome proliferator-activated receptor gamma/ppar-g), the results generated provided substantial knowledge on known and novel toxicity mechanisms of BaP which will be discussed in detail below.

4.1 Aryl hydrocarbon receptor signaling and biotransformation

17

It is well known that PAHs such as BaP can bind to the AhR and trigger phase I and phase II biotransformation in fish (Hahn, 1998; Karchner et al., 2005). Biotransformation, in many cases, is beneficial for detoxification and rapid elimination of xenobiotic substances. However, evidence from multiple species has demonstrated that several BaP metabolites such as 3-OH-BaP, BPQs and BPDE are more toxic than the parent compound and are directly associated with the genotoxicity of BaP (Lorentzen and Ts'o, 1977; Flowers et al., 1997; Godschalk et al., 2000; Tzekova et al., 2004; Marie-Desvergne et al., 2010). In the present study, the polar cod ahr2 gene and its transcriptional regulator, AhR repressor (ahrr) were up-regulated by exposure to High BaP treatment, indicating activation of the AhR signaling pathway. The ahr2 gene, encoding for the ligand-activated transcription factor involved in regulation of biological responses to PAHs, polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated diphenylsulfides (Roy et al., 2018; Zhang et al., 2018), is involved in a number of toxicity pathways associated with xenobiotic metabolism, mitochondrial dysfunction, cardiovascular abnormality, hepatic injury and DNA methylation inhibition in fish (Du et al., 2017; Knecht et al., 2017; Roy et al., 2018). The ahrr gene encodes a protein, which competes with the binding site of the AhR and suppresses the receptor signaling through a negative feedback mechanism (Evans et al., 2008). The induction of ahrr in this study may indicate demand for compensatory response to hyperactivation of the AhR by BaP. In addition, functional analyses in the present study suggest that besides activation of the AhR, the AhR-mediated xenobiotic metabolism pathways were activated. Differentially expressed genes involved in phase I biotransformation, such as cytochrome p450 1a1 (cyp1a1), cyp1b1, and phase II biotransformation, such as glutathione s-transferase p (gstp1) (Sarasquete and Segner, 2000; Schlenk et al., 2008) were up-regulated by exposure to High BaP. Induction of cyp1a1 and gst has previously been observed in polar cod after exposure to 6.6-378 μg/kg (16h-7d) BaP (Nahrgang et al., 2009) and petroleum related mixtures (Nahrgang et al., 2010c; Vieweg et al., 2018). Genes involved in the AhR signaling and biotransformation pathways, such as cyp1a, cyp1b1 and gst were also induced in zebrafish larvae after 96h exposure to 42±1.9 mg/l BaP (Fang et al., 2015). Induction of cyp1b1 has also been reported in zebrafish (*Danio rerio*) after 24h injection with 1 mg/kg BaP (Bugiak and Weber, 2009) and after 96h waterborne exposure to 50 μg/L BaP (Corrales et al., 2014), in rainbow trout (*Oncorhynchus mykiss*) after 14d injection with 100 mg/kg

395 BaP (Phalen et al., 2017), and channel catfish (*Ictalurus punctatus*) after 4d injection with 20 mg/kg

396 BaP (Willett et al., 2006). In addition to up-regulation of genes involved in the biotransformation

397 processes, increase in the bile 3-OH-BaP concentration suggest that biotransformation was indeed

398 causing potentially toxic metabolites as observed in other fish species (Rey-Salgueiro et al., 2011;

399 Moller et al., 2014). On the contrary, no genes related to the AhR signaling were differentially expressed

400 in polar cod after exposure to Low BaP in this study, albeit detectable concentration of 3-OH-BaP was

401 identified in the bile after 14d exposure. Moreover, sulfotransferase family 1 cytosolic sulfotransferase

402 3 (sult1st3), a gene involved in the CAR/RXR-mediated phase II biotransformation of PAH metabolic

403 intermediates (Falany and Wilborn, 1994; Glatt, 2000; Meland et al., 2011), was up-regulated by Low

404 BaP. These findings taken together suggested that either a dose threshold was required for BaP to induce

405 AhR-mediated biotransformation in this Arctic species as seen for dioxin-like compounds (Hailey et al.,

406 2005), or an alternative xenobiotic metabolic pathway was induced to transform BaP at this low

407 exposure dose.

408

409 **4.2 Oxidative stress**

410 Induction of oxidative stress is considered a major MoA of BaP. Biotransformation of BaP by CYP

411 isozymes and peroxidases may generate highly reactive intermediates, such as BPQs which may produce

412 ROS through participation in the one electron redox cycling and induce oxidative stress (Lorentzen and

413 Ts'o, 1977; Flowers et al., 1997). Induction of antioxidant (AOX) biomarkers as indication of oxidative

414 stress has been documented in a number of fish species after exposure to BaP (Nahrgang et al., 2009;

415 Curtis et al., 2011; Palanikumar et al., 2012). Results from the present study also suggest that both Low

416 and High BaP exposure modulated oxidative stress responses in polar cod, however, through different

417 mechanisms. Exposure to Low BaP affected DEGs involved in several GO functions related to oxidative

418 stress responses, such as AOX activity, thioredoxin-disulfide reductase activity and cellular redox

419 homeostasis. Supporting DEGs, such as thioredoxin domain-containing protein 12 (txndc12) and

420 thioredoxin reductase 3 (txnrd3) were mainly related to the thioredoxin-mediated AOX defense (Arner

421 and Holmgren, 2000) and found to be down-regulated after exposure to Low BaP, possibly indicating

422 reduced demand for antioxidant (AOX) enzymes. It is not clear how the AOX defense mechanism in

423 polar cod was suppressed by Low BaP. However, reduction in AOX enzymes, such as glutathione

424 peroxidase (gpx) and catalase (cat), has been associated with decreased endogenous ROS production as

425 a result of reduced metabolic activities in fish (Janssens et al., 2000). It is therefore possible that in

426 response to Low BaP exposure, the metabolic (and AOX) levels were reduced to avoid massive

427 production of endogenous ROS. In contrast to Low BaP, exposure to High BaP induced multiple types

428 of AOX pathways in polar cod, such as NRF2-mediated oxidative stress response (transcriptional

429 regulation of AOX), glutathione redox reaction 2 (enzymatic AOX), thioredoxin pathway (enzymatic

430 AOX) and antioxidant action of vitamin C (non-enzymatic AOX), thus indicating BaP-induced

431 oxidative stress. Supporting DEGs such as nuclear factor erythroid 2-like 1 (nfe2l1), a sensor of

432 oxidative stress and master transcription regulator of AOX (Kaspar et al., 2009), and kelch-like ech-

433 associated protein 1 (keap1), the dimerization partner of nfe2 (Kobayashi and Yamamoto, 2005), were

434 up-regulated, indicating the initiation of upstream transcriptional regulation in response to oxidative

435 stress. Downstream AOX DEGs widely used as oxidative stress biomarkers, such as superoxide

436 dismutase (sod1), thioredoxin (txn), and peroxiredoxin 1 (prdx1) (Arner and Holmgren, 2000;

437 Valavanidis et al., 2006; Birben et al., 2012) were also up-regulated, indicating demand for increased

438 AOX capacity. These transcriptional responses collectively suggested that exposure to High BaP caused

439 excessive ROS production which likely overwhelmed the AOX cellular defense. This is confirmed by

440 up-regulation of AOX genes such as sod, cat and gpx in Polar cod after exposure to 6.6-378 µg/kg (16h-

441 4d) BaP (Nahrgang et al., 2009) and BaP-containing petroleum products (Nahrgang et al., 2010b;

442 Nahrgang et al., 2010c; Vieweg et al., 2017). Induction of key genes involved in the NRF2-mediated

443 oxidative stress response pathway, such as nuclear factor erythroid 2-like 2b (nfe2l2b), and glutathione-

444 mediated detoxification pathway, such as gst has been documented in zebrafish larvae after 96h

445 waterborne exposure to 42 ± 1.9 mg/l BaP (Fang et al., 2015).

446

447 **4.3 DNA damage**

448 DNA damage is considered another major MoA of BaP. Metabolism of BaP can lead to the generation

449 genotoxic metabolites, such as BPDE (Karle et al., 2004) which can directly distort the structure of DNA

450 by forming harmful BPDE-DNA adducts (Kucab et al., 2015; Long et al., 2016), thus causing

451 genotoxicity in fish (Nishimoto and Varanasi, 1985; Smolarek et al., 1987; Dolcetti et al., 2002). Other

452 reactive intermediates, such as 3-OH-BaP (Zhu et al., 2008; Rey-Salgueiro et al., 2011) has been

453 strongly correlated with formation of DNA and haemoglobin adducts in mammals (Godschalk et al.,

454 2000; Tzekova et al., 2004; Marie-Desvergne et al., 2010). In addition, metabolic intermediates such as

455 BPQs may also indirectly cause DNA damage through ROS (Regoli et al., 2003; Srut et al., 2015).

456 Results from the present study clearly showed that the bile 3-OH-BaP was detected in polar cod in a

457 dose-dependent manner, indicating an increased potential for formation of DNA adducts and associated

458 DNA damage in the fish. Transcriptomic analysis further revealed that exposure to both Low and High

459 BaP affected DEGs caused molecular responses related to DNA damage in polar cod, however, in

460 different manners. Exposure to Low BaP mainly caused down-regulation of DEGs involved in DNA

461 ligation during base-excision repair, such as high-mobility group protein B2 (hmgb2b), a key gene

462 involved in DNA transcription, recombination, and repair in fish (Moleri et al., 2011). Similar to the

463 down-regulation of AOX genes found in this study, the suppression of DNA repair genes may likely

464 indicate reduced demand for DNA damage responses, possibly due to the suppression of oxidative stress

465 as a result of reduced metabolic activities and ROS formation. On the contrary, exposure to High BaP

466 led to up-regulation of DEGs mainly involved in the cell division regulation, such as cell division

467 control protein 42 (cdc42-cell cycle control), cdc50c (cell cycle control) (Langerak and Russell, 2011),

468 and DNA excision repair, such as UV excision repair protein RAD23 homolog A (rad23a-nucleotide

469 excision repair) (Dantuma et al., 2009). Surprisingly, no genes associated with DNA double-strand break

470 (DSB) repair, such as RAD51 recombinase (rad51), were differentially expressed, possibly suggesting

471 that the BaP-induced DNA damage did not sufficiently cause DSB in this study. Benzo(a)pyrene-

472 mediated DNA damage has been documented in polar cod after chronic (4-week) exposure to BaP-

473 containing crude oil (Nahrgang et al., 2010b), and in several other fish species, such as zebrafish (Srut

474 et al., 2015), Chinese rare minnow (*Gobiocypris Rarus*) (Yuan et al., 2017), European eel (*Anguilla*

475 *anguilla*) (Nigro et al., 2002) and the Atlantic Killifish (*Fundulus heteroclitus*) (Wills et al., 2010).

4.4 Mitochondrial dysfunction

One of the novel findings was that exposure to BaP modulated genes involved in the mitochondrial electron transport chain (ETC) functions in a dose-dependent manner. Exposure to PAHs is known to cause mitochondrial dysfunction in higher vertebrates (Zhu et al., 1995; Li et al., 2003; Ko et al., 2004; Xia et al., 2004; Bansal et al., 2014). However, the mitochondrial toxicity of PAHs has not been well studied in fish. It is widely known that the mitochondrial ETC is a key component of the oxidative phosphorylation (OXPHOS) machinery and vital for ATP synthesis, antioxidant defense and apoptosis (Richter et al., 1996; Brookes et al., 2002; Orrenius, 2007; Hoye et al., 2008). Results from the present study clearly showed that both Low and High BaP affected the same targets (i.e. protein complex I, IV, V) in the mitochondrial ETC in polar cod, however, by different mechanisms. Exposure to Low BaP uniformly suppressed DEGs involved in the ETC, such as ndufa6 and ndufa10 (ETC complex I), cox6c (complex IV), atp5a1, atp5f1, atp5l and atp5g3 (complex V), likely due to the demand for reduced production of endogenous ROS by mitochondrial OXPHOS (Murphy, 2009). On the contrary, exposure to High BaP uniformly up-regulated DEGs in the ETC, such as ndufb6 (complex I), cox6c (complex IV), atp5a1 and atp5g3 (complex V). The toxicity mechanisms of BaP in the mitochondria of polar cod has not been well studied and is also poorly characterized in other vertebrates (Venkatraman et al., 2008; Du et al., 2015). However, since the mitochondrial ETC is a major source of endogenous ROS (Murphy, 2009), the dose-dependent effects observed in this study may likely be due to that polar cod suppressed the ETC activities to reduce ROS formation under Low BaP stress, whereas elevated the ETC activity due to higher demand for ATP-dependent defense mechanisms (Song et al., 2016; Blajszczak and Bonini, 2017) under High BaP stress. It is also possible that exposure to High BaP impaired the functions of ETC complexes by oxidative damages, which in turn facilitated the production of ROS in the mitochondria due to abnormal redox reactions (Blajszczak and Bonini, 2017). Moreover, studies on the mammalian models suggest that AhR may interact with the mitochondrial ETC, especially protein complex V and cause mitochondrial ETC dysfunction (Tappenden et al., 2011; Hwang et al., 2016). In fish, only one recent study has reported increased complex I and complex IV activities, and proton leak

503 on the mitochondrial membrane in the Atlantic killifish (*Fundulus heteroclitus*) after 24h exposure to

504 50 mg/kg BaP (Du et al., 2015), which partially supports the current findings.

505

506 **4.5 Apoptosis**

507 Apoptotic cell death is normally activated as a consequence of oxidative stress, DNA damage, or

508 mitochondrial dysfunction to eliminate damaged cells and avoid mutation (Elmore, 2007). Exposure to

509 BaP is known to induce apoptosis as an MoA in various fish species, such as tilapia (*Oreochromis*

510 *niloticus*) (Holladay et al., 1998), Chinese rare minnow (Yuan et al., 2017) and zebrafish (Gao et al.,

511 2015). The present transcriptional analysis also showed that two key DEGs involved in the regulation

512 of apoptosis, caspase 9 (casp9, initiator of apoptosis) and caspase 3 (cap3, the executor of apoptosis)

513 (Takle and Andersen, 2007) were up-regulated by exposure to High BaP (but not Low BaP), indicating

514 potential activation of caspase-dependent apoptotic signaling. It is however not clear which type of

515 apoptotic signaling pathway (i.e. intrinsic/mitochondrial or extrinsic/death receptor pathway) was

516 involved in the activation of the caspases, as none of the upstream genes in the intrinsic apoptotic

517 signaling, such as apoptosis regulator BAX (intrinsic apoptosis activator) or B-cell lymphoma 2 (Bcl-2,

518 intrinsic apoptosis inhibitor) were differentially expressed, whereas several DEGs involved in the

519 extrinsic apoptotic signaling, such as Fas ligand (faslg), and CASP8 and FADD like apoptosis regulator

520 (cflar) (Jin and El-Deiry, 2005) were found to be suppressed by exposure to High BaP. Although

521 previous studies suggest that exposure to BaP may up-regulate caspases through activation of the

522 intrinsic apoptotic signaling both *in vivo* (Zha et al., 2017) and *in vitro* (Kobayashi and Yamamoto, 2005;

523 Santacroce et al., 2015), the detailed mechanisms involved in the activation of caspase-dependent

524 apoptotic signaling by BaP still remain to be better characterized in polar cod.

525

526 **4.6 Protein degradation**

527 Protein degradation is usually accompanied with oxidation of macromolecules and apoptosis to

528 eliminate damaged proteins or regulatory proteins involved in a variety of toxicological processes

529   (Hershko and Ciechanover, 1998; Aiken et al., 2011; Ulrich, 2012). Stress-induced protein degradation

530   has been extensively studied in mammals under disease situations (Lecker et al., 2006; Reinstein and

531   Ciechanover, 2006). However, this type of stress response has only been occasionally reported in

532   wildlife. Another novel finding in the present study was that both Low and High BaP exposure affected

533   DEGs involved in protein degradation in polar cod, however, in different manners. Exposure to Low

534   BaP caused down-regulation of DEGs related to proteasome activities, such as proteasome subunit alpha

535   type 1 (psma1), 4 (psma4) and 7 (psma7). The mechanism underlying this suppressive regulation is

536   unclear, albeit a recent study on zebrafish also showed that a gene involved in protein ubiquitination,

537   ubiquitin carboxy-terminal hydrolase L1 (uchl1), was down-regulated after 230d exposure to 5 and 50

538   nmol/L BaP (Gao et al., 2015). In contrast, exposure to High BaP consistently up-regulated DEGs

539   involved in the protein ubiquitination pathway, such as ubiquitin carboxyl-terminal hydrolase 5 (usp5),

540   14 (usp14), ubiquitin-conjugating enzyme E2 variant 1 (ube2v1), ubiquitin carboxyl-terminal hydrolase

541   isozyme L3 (uchl3), heat shock protein 90 alpha (hsp90aa1) and ten psma genes. These genes play

542   important roles in different steps of protein ubiquitination, such as ubiquitin-protein conjugation and

543   proteasomal degradation of target protein (Hershko and Ciechanover, 1998; Flick and Kaiser, 2012).

544   The induction of protein ubiquitination genes clearly indicated that exposure to High BaP activated the

545   protein degradation machinery in polar cod.

546

547   **4.7   Other potential mechanisms**

548   *Hormone receptor signaling*

549   Benzo(a)pyrene is proposed to act as an endocrine disruptor in several organisms (Tian et al., 2013;

550   Kennedy and Smyth, 2015; Wen and Pan, 2015; Regnault et al., 2016). The present study showed that

551   exposure to Low BaP consistently suppressed DEGs involved in the estrogen receptor (ER) signaling

552   pathway, such as glucose-6-phosphatase catalytic subunit 3 (g6pc3), mediator complex subunit 10

553   (med10), RNA polymerase II subunit G (polr2g) and TATA-box binding protein associated factor 11

554   (taf11). These DEGs, however, were not the core regulator genes (e.g. ERα or ERβ) in the ER signaling,

555   but more generally involved in RNA polymerase II-mediated transcription regulation (Salgado et al.,

2004; Meka et al., 2005; Robinson et al., 2005; Just et al., 2016). It was therefore not entirely clear whether the ER signaling in polar cod was inhibited by exposure to Low BaP, albeit the previous studies on cross-talks between the AhR and ER pathways suggest that activated AhR may inhibit ER activity through various mechanisms in mammals (Matthews and Gustafsson, 2006; Helle et al., 2016) and fish (Bemanian et al., 2004; Yan et al., 2012; Hultman et al., 2015). In comparison with Low BaP, exposure to High BaP up-regulated DEGs involved in the androgen signaling pathway, such as sex hormone binding globulin (shbg), protein kinase C delta (prkcd), G protein subunit beta 5 (gnb5), heat shock protein 90 alpha family class A member 1 (hsp90aa1), polr2g and polr2j. Genes such as shbg, prkcd and gnb5 are involved in the nongenomic actions of androgens (Foradori et al., 2008; Bobe et al., 2010), whereas polr2g and polr2j are generally involved in RNA polymerase II-mediated transcription regulation (Meka et al., 2005). The hsp90aa1 gene is also involved in various biological processes, such as protein stabilization, protein degradation, hypoxic response and regulation of androgen receptor (AR) signaling (Roberts et al., 2010; De Leon et al., 2011). The evidence taken together suggests that the androgen signaling pathway in polar cod may be a target for BaP exposure, albeit the mechanism does not involve modulating the expression of AR itself. This assumption was contradictory to the previous findings from the mammalian studies where AhR agonists such as BaP are usually anti-androgenic (Kizu et al., 2003; Okamura et al., 2004), and thus suggest more in-depth studies to clarify the role of BaP or its metabolites on androgen signaling in polar cod.

Immune functions

A number of studies suggest that BaP can modulate immune functions in vertebrates (reviewed in EPA (2017)). In this study, exposure to Low BaP in general down-regulated DEGs involved in lipid antigen presentation, such as adaptor related protein complex 2 alpha 2 subunit (ap2a2) and adaptor related protein complex 2 mu 1 subunit (ap2m1). Benzo(a)pyrene has been shown to inhibit cluster of differentiation 1 (CD1) protein-mediated lipid antigen presentation in human dendritic cells (Sharma et al., 2017). In fish, however, this inhibitory effect has not been well documented. On the contrary, exposure to High BaP up-regulated DEGs involved in the antigen presentation pathway, such as major histocompatibility complex class II DO alpha (hla-doa) and two protein degradation associated psmb

584   genes (psmb5 and psmb6), possibly indicating demand for activation of humoral immunity (Myers et

585   al., 1987).

586

587   *Lipid homeostasis*

588   Benzo(a)pyrene has been shown to affect lipid homeostasis in mammals (Layeghkhavidaki et al., 2014;

589   Wang et al., 2015; Hu et al., 2016), possibly through cross-talks between the AhR signaling and

590   peroxisome proliferator-activated receptor (PPAR) pathway (Shaban et al., 2005; Borland et al., 2014).

591   The PPAR signaling pathway is considered the central regulator of lipid metabolism in vertebrates (Ferre,

592   2004). Perturbations to lipid metabolism by PAH-containing petroleum compounds have also been

593   documented in several fish studies (Bilbao et al., 2010; Adeogun et al., 2016; Xu et al., 2016; Cocci et

594   al., 2017; Vieweg et al., 2018). Results from the present study showed that exposure to High BaP in

595   general down-regulated DEGs involved in the PPARα signaling pathway, such as lipoprotein lipase (lpl,

596   hydrolysis of triglycerides in lipoproteins), adiponectin receptor 2 (adipor2, fatty acid oxidation),

597   phospholipase C epsilon 1 (plce1, hydrolysis of polyphosphoinositides), phospholipase C like 2 (plcl2,

598   hydrolysis of the phospholipids) which are key for lipid metabolism. On the contrary, pparγ was found

599   to be highly up-regulated by exposure to High BaP. This discrepancy in transcriptional regulation may

600   attribute to the slightly different roles of PPAR isoforms in the maintenance of lipid homeostasis

601   (Lamichane et al., 2018). It has been suggested that PPARα and PPARβ/δ promote energy dissipation,

602   whereas PPARγ promotes energy storage in mammals (Dubois et al., 2017). The up-regulation of pparγ

603   in polar cod may likely indicate demand for lipid synthesis, possibly due to elevated activity of the

604   mitochondrial energetic machinery which consumes fatty acids as energy sources (Nsiah-Sefaa and

605   McKenzie, 2016) and/or increased degradation of damaged lipids as a consequence of oxidative stress

606   induced by BaP metabolism. A recent study also suggested reduced lipid metabolism in polar cod after

607   dietary exposure to crude oil, as indicated by the down-regulation of cytochrome P4507A1 (cyp7a1), a

608   gene involved in cholesterol metabolism (Vieweg et al., 2018).

609

610   **4.8 Conceptual toxicity pathway network**

On the basis of the hepatic transcriptomic responses in polar cod, a conceptual toxicity pathway network is proposed for understanding of the dose-dependent toxicity mechanisms of BaP in this Arctic fish (Figure 5). After exposure to the low dietary doses of BaP (0.4 ug/g), low level of reactive metabolites may be formed through biotransformation of BaP and potentially disturb the cellular redox homeostasis by increasing the production of endogenous ROS. To maintain the homeostasis and avoid oxidative stress, adaptive responses (or compensatory mechanisms, e.g. negative feedback loop) may be induced to suppress the mitochondrial ETC activity, a major source of endogenous ROS formation (Figure 5-A). This may consequently reduce the demand for defense mechanisms such as AOX defense and DNA repair. It is also possible that the BaP metabolites directly disrupt the mitochondrial ETC thus causing reduction in ATP synthesis.

Exposure to High BaP activates the AhR-mediated biotransformation, thus generating reactive metabolites of BaP (Figure 5-B). These metabolites may disturb the redox reactions and produce excessive ROS, thus inducing oxidative stress and activating the AOX defense. Oxidative damage to the DNA may activate DNA repair, protein degradation and apoptosis to protect the cells and avoid mutation. However, since the repair mechanisms usually require energy, the mitochondrial ETC activity is elevated to produce more ATP, and simultaneously, more endogenous ROS. When the oxidative damages overwhelm the repair mechanisms, adverse effects at higher level of biological organization may be induced.

The present work demonstrate how transcriptional approaches can be used to characterize the MoA of pollutants, and how functional responses such as bile metabolites can support weight of evidence considerations using molecular and physiological data. The current approach is as such explorative as pointing out potential toxicity pathways that can be triggered by stressors, and the logical continuance of such work is to assess whether these perturbations at the molecular and physiological scale transplant to adverse (phenotypical) effects that can be associated with reduction of fish health and successful recruitment to natural populations of polar cod.
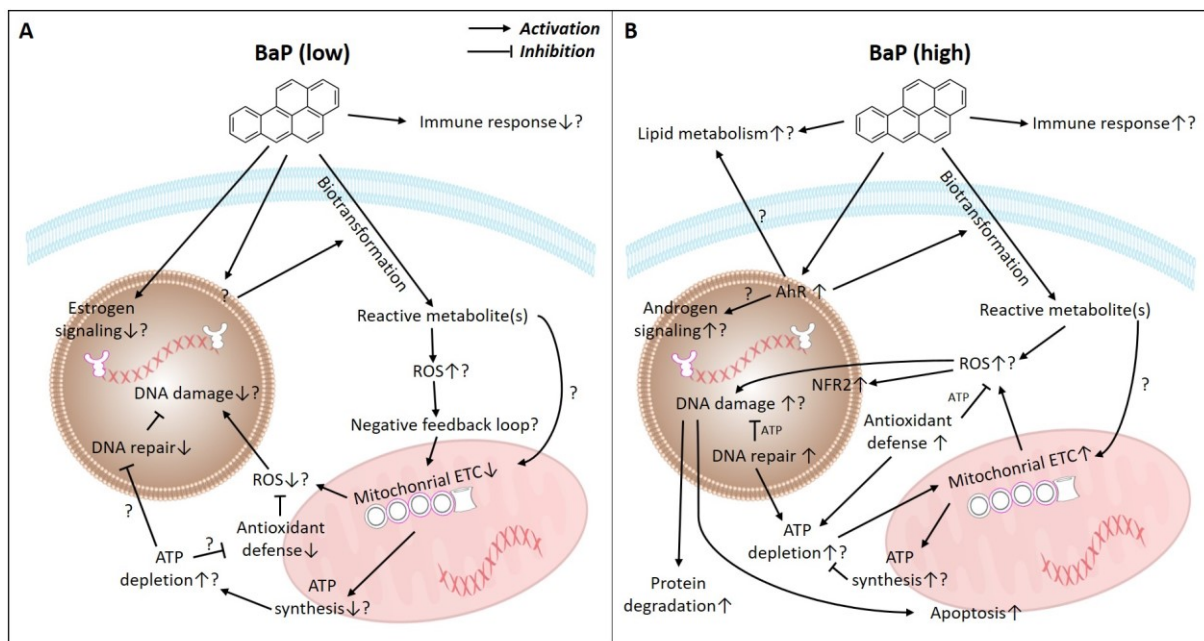
Figure 5. Proposed network of hepatic toxicity pathways in Arctic fish after exposure to Low (A) and High (B) levels of benzo(a)pyrene (BaP).

ROS: Reactive oxygen species; ETC: Electron transport chain: AhR: Aryl hydrocarbon receptor. ↑ : Increase; ↓ : Decrease.

5. Conclusions

The present study developed and applied a transcriptomics platform (microarray and qPCR) for understanding the effects and MoAs of BaP on a key Arctic fish, polar cod. The global transcriptional analysis in combination with targeted metabolic identification suggest that BaP and its biotransformation product 3-OH-BaP affected polar cod in a dose-dependent manner, potentially through induction of oxidative stress, DNA damage and apoptosis as the main MoAs, similarly to that reported for other vertebrates. Novel MoAs in polar cod such as disturbance of mitochondrial ETC and induction of protein degradation were also proposed. Although similar biological targets were identified for both Low and High BaP exposure, the detailed toxicity mechanisms contributing to the MoAs of this compound were dissimilar, with Low BaP in general suppressing DEGs involved in the defense pathways and High BaP mainly inducing DEGs in the compensatory mechanisms. Moreover, dose-dependent responses related to disturbance of hormone receptor signaling, perturbation to immune functions and disruption of lipid homeostasis were also characterized and suggested as additional MoAs

655   of BaP in polar cod. The present study reported the first transcriptomic analysis in polar cod. The tools

656   and knowledge generated may thus serve as a foundation for future mechanistically-based and

657   phenotypically-anchored impact assessment of environmental pollutants in the Arctic using this

658   important fish as a forecaster species.

659

660

Acknowledgement

667

668

Appendix A. Microarray design

Appendix B. Microarray data

671

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References

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