Susceptibility of polar cod (Boreogadus saida) to a model carcinogen

Adélaïde Lerebours, Svetlana Murzina, You Song, Knut Erik Tollefsen, Maura Benedetti, Francesco Regoli, Jeanette M Rotchell, Jasmine Nahrgang

Susceptibility of polar cod (*Boreogadus saida*) to a model carcinogen

Adélaïde Lerebours[1,2,]*, [3]Svetlana Murzina, [4]You Song, [4]Knut Erik Tollefsen, [5]Maura Benedetti, [5]Francesco Regoli, [6]Jeanette M. Rotchell and [7]Jasmine Nahrgang

[1]School of Biological Sciences, University of Portsmouth, Portsmouth, PO1 2DY, United Kingdom

[2]UMR CNRS LIENSs, Littoral Environnement et Sociétés, Université de La Rochelle, La Rochelle, 17 000, France

[3]Institute of Biology of the Karelian Research Centre of the Russian Academy of Sciences (IB KarRC RAS) 185910 Petrozavodsk, Russia

[4]Norwegian Institute for Water Research (NIVA), Section of Ecotoxicology and Risk Assessment, Gaustadalléen 21, N-0349 Oslo, Norway

[5] Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, 60131, Italy

[6]Department of Biological and Marine Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, United Kingdom

[7]Department of Arctic and Marine Biology, Faculty of Biosciences, Fisheries and Economics UiT The Arctic University of Norway, 9037 Tromsø, Norway

Keywords: Carcinogenesis, polar cod, benzo(a)pyrene, cancer genes, DNA repair genes

* Corresponding author: Adélaïde Lerebours, phone number: +33 (0) 5 46 50 76 23, email: adelaide.lerebours@univ-lr.fr

Abstract

Studies that aim to characterise the susceptibility of the ecologically relevant and non-model fish polar cod (*Boreogadus saida*) to model carcinogens are required. Polar cod were exposed under laboratory conditions for six months to control, 0.03 µg BaP/ g fish/ week and 0.3 µg BaP/ g fish/ week dietary benzo(a)pyrene (BaP), a reference carcinogen. The concentrations of the 3-OH-BaP bile metabolite and transcriptional responses of genes involved in DNA adduct recognition (*xpc*), helicase activity (*xpd*), DNA repair (*xpf*, *rad51*) and tumour suppression (*tp53*) were assessed after 0, 1, 3 and 6 months of exposure, alongside body condition indexes (gonadosomatic index, hepatosomatic index and condition factor). Micronuclei and nuclear abnormalities in blood and spleen, and liver histopathological endpoints were assessed at the end of the experiment.

Fish grew steadily over the whole experiment and no mortality was recorded. The concentrations of 3-OH-BaP increased significantly after 1 month of exposure to the highest BaP concentration and after 6 months of exposure to all BaP concentrations showing the biotransformation of the mother compound. Nevertheless, no significant induction of gene transcripts involved in DNA damage repair or tumour suppression were observed at the selected sampling times. These results together with the absence of chromosomal damage in blood and spleen cells, the subtle increase in nuclear abnormalities observed in spleen cells and the low occurrence of foci of cellular alteration suggested that the exposure was below the threshold of observable effects. Taken together, the results showed that polar cod was not susceptible to carcinogenesis using the BaP exposure regime employed herein.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a very large group of ubiquitous organic compounds that can originate from petrogenic, pyrogenic, biogenic and diagenic sources. A number of PAHs are identified as having carcinogenic properties and have been associated to an increased incidence of liver tumours in flatfish in highly polluted environments (Malins *et al.*, 1985; Myers *et al.*, 1991; Harshbarger and Clark, 1990; Vogelbein *et al.*, 1990; Baumann and Harshbarger, 1998). This pathology has been used to monitor the effects of exposure to PAHs and the health of marine ecosystem since the 1980s (Malins *et al.*, 1985; Veethaak and Ap Rheinallt, 1992) and its assessment recommended by the International Council for Exploration of the Sea (ICES) and the Oslo and Paris Convention (OSPAR) Joint Assessments and Monitoring Programme (JAMP) (Lyons *et al.*, 2010).

Benzo(a)pyrene (BaP) is a well-known pyrogenic carcinogen in a plethora of animals such as marine mammals (Acevedo-Whitehouse *et al.*, 2018; Poirier *et al.*, 2019), fish (Wang *et al.*, 2010; Wills *et al.*, 2010) and mice (Kasala *et al.*, 2015; Chen *et al.*, 2019). The reference oral dose below which no effect is expected is 3.10^{-4} µg BaP /g per day, based on animal and human studies (reviewed in EPA/635/R-17/003). The metabolites generated by endogenous metabolism (biotransformation) are highly genotoxic. Phase I biotransformation of BaP is mediated by cytochrome P450 (CYP) enzymes and produces highly reactive metabolic intermediates such as diol-epoxide, dihydrodiol and 3-hydroxybenzo(a)pyrene (3-OH-BaP) (Karle *et al*., 2004; Zhu *et al.*, 2008; Rey-Salgueiro *et al.*, 2011). Those metabolites form DNA adducts that interfere with DNA repair and replication (Phillips and Arlt, 2007). This represents a critical event in the initiation of tumorigenesis, potentially leading to mutations within specific regions of DNA, such as proto-oncogenes and tumour suppressor genes (Rotchell *et al.*, 2001, Du Corbier *et al.*, 2005, Lerebours *et al.*, 2014, 2016). The carcinogenicity of BaP has been well studied in several temperate fish species where specific

DNA adducts are used as markers for exposure and potential genotoxic effects. Exposure to BaP specifically caused DNA adducts in fish such as pale chub (*Zacco platypus*) (Lee et *al.*, 2014) and killifish species (*Fundulus grandis* and *F. similis*) (Willett *et al.*, 1995; Rose *et al.*, 2000, 2001). Moreover, BaP exposure was associated with neoplastic lesions in brown bullhead (*Ameiurus nebulosus*) (Ploch *et al.*, 1998), English sole (*Parophrys vetulus*) (Reichert *et al.*, 1998) and rainbow trout (Hendricks *et al*., 1985). PAH-induced lesions have also recently been suggested in marine mammals such as harbour porpoises (*Phocoena phocoena*) (Acevedo-Whitehouse *et al.*, 2018) and beluga whales (Poirier *et al.*, 2019). Pollution induced cancer affects many aquatic species and represents a growing concern for aquatic wildlife (for a review see Baines *et al*., 2021).

Tumourigenesis is a progressive process characterised by different stages for which the underlying molecular steps and the role of environmental exposure are not always well-known. Nonetheless, liver tumourigenesis has been well studied in flatfish (Stentiford *et al.*, 2010; Lerebours *et al.*, 2013; 2014; 2017) and in the model fish Japanese medaka (*Oryzias latipes*) (Rotchell *et al.*, 2001) and zebrafish (*Danio rerio*) (Li *et al.*, 2017; 2019). DNA repair mechanisms have been associated with tumour formation perturbing several steps of the nucleotide excision repair (NER) pathway, which recognises and repairs DNA adducts induced by numerous environmental mutagens, including PAHs (Gillet and Scharer, 2006; Rastogi *et al.*, 2010). While such mechanisms involved in the development of tumours in certain fish species are well characterised, a substantial knowledge gap exists for non-model and ecologically important species inhabiting remote regions in particular. The Arctic is currently experiencing a rapid decline in sea ice (Kumar *et al*., 2021) that may lead to a significant increase in marine shipping (Ho *et al*., 2010), oil and gas exploration and operation (Elias, 2018), and tourism (Meier *et al*., 2014) and associated release of potential carcinogenic contaminants (Elias, 2018). The polar cod (*Boreogadus saida*) is a keystone fish species in the arctic marine ecosystem due to its abundance, distribution and central role in the food web (Welch *et al.,* 1992). Polar cod has been considered a model fish for arctic ecotoxicology studies (Jonsson *et al*., 2010; Nahrgang *et al*., 2009, 2010a,b,c). The toxicity of petroleum compounds on the physiology of polar cod has been well studied (Geraudie *et al*., 2014; Bender *et al*., 2016; Nahrgang *et al*., 2016; Vieweg *et al*., 2018; Nahrgang *et al*., 2019) but the tumourigenic potential of a potent carcinogen remains unknown in that species. A few studies however have reported a potential susceptibility to carcinogenic contaminants including BaP. The hepatic metabolism of BaP is particularly efficient in polar cod and a significant increase of covalently bound reactive intermediates of BaP in the bile of fish has been found after dietary exposure to BaP (Ingebrigtsen *et al*., 2000; Bakke *et al*., 2016). These reactive intermediates were found to induce the formation of DNA adducts in the liver of that species (Aas *et al*., 2003). This genotoxic effect can in turn result in cellular abnormalities and cancer initiation. Finally, a recent study showed that expression of genes involved in DNA repair and cell cycle regulation processes was modified in liver of polar cod dietary exposed to BaP (Song *et al*., 2019).

In order to evaluate the susceptibility of polar cod to a carcinogenic compound, adult specimens were exposed under laboratory conditions for six months to control, 0.03 µg BaP/ g fish/ week and 0.3 µg BaP/ g fish/ week dietary BaP. Selected body condition indexes, bile metabolite concentrations and transcriptional responses of genes involved in DNA adduct recognition (*xpc*), helicase activity (*xpd*), DNA repair (*xpf*, *rad51*) and tumour suppression (*tp53*) were assessed after 0, 1, 3 and 6 months of exposure. Blood and spleen micronuclei, nuclear abnormalities and liver histopathological endpoints were assessed at the end of the experiment.

The sampling times were selected because carcinogenesis is a long-term process. They were comparable to the exposure durations used in several studies interested in carcinogenesis in European eel (Nogueira *et al.*, 2006), brown bullhead and channel catfish (Ploch *et al.*, 1985) rainbow trout (Hendricks et al., 1985, Black et al., 1985) and coho salmon (Black *et al.*, 1985) exposed to BaP. The BaP doses selected were lower than the concentrations frequently used in previous studies. They were 10 and 100 times lower than the concentration of 3 µg BaP/g of fish /week (Colli-Dula *et al.*, 2018) that induced a decrease of body indexes in Nile tilapia after one month of exposure. In addition, our highest concentration was 4 times lower than the lowest concentration used in the study of Song *et al.*, (2019) that found gene expression changes in polar cod after two weeks of exposure, a twelve times shorter exposure duration.

Methods

Fish collection and exposure

Adult polar cod (4 years old) were collected along the west coast of the Svalbard archipelago (Norway) onboard RV Helmer Hanssen in January 2014 using a Campelen bottom trawl (at 200m depth) and a fish-lift (Holst and McDonald, 2000). At the Tromsø aquaculture research station (Havbrukstasjon i Tromsø), fish were kept in 3000 L acclimation tank under a natural light and temperature (1.5 - 3 °C) regime of 79°N (based on mooring data in Wallace *et al*., 2010). During this period, fish were fed until satiation with thawed *Calanus sp.* copepods (Calanus AS, Tromsø). Ninety fish were selected based on similar length (15 ± 1 cm) and weight (25 ± 7 g) for the experiment (June 2014).

Polar cod were dietarily exposed to 0, 0.03 and 0.3 µg BaP per gram fish per week, for 6 months (2^{nd} of July 2014 to 31^{st} January 2015). The experiment was conducted in compliance with the policies of the Norwegian animal welfare authorities (application ID 6571). Briefly, a BaP (Sigma Aldrich, St. Louis, USA) solution in acetone was mixed with *Calanus* spp (Calanus AS) to yield 0.5 or 5 µg BaP per g feed or acetone alone (acetone control). The acetone was volatilized by constant stirring on a magnetic stirrer for 2.5 hours at 30 °C. Small pellets were then created with the addition of 0.5 mL gelatin per g feed. Fish were fed pellets corresponding to 4% of their body wet weight (bw) 5 days a week. On the first, third and fifth day of a week, fish were exposed to dietary BaP or a solvent control by receiving the 2% bw exposed feed (or solvent control) and 2% bw of unexposed feed (no BaP, no acetone). Feeding was done by distributing the pellets to the surface of the tank. Thus, feeding hierarchies may have occurred resulting in some intra-tank individual exposure variations. On the remaining 2 days of a week, all fish were fed 4% bw of unexposed feed. The amount of food given to each tank was adjusted at each sampling point to account for both growth and sampling of specimens. With this feeding regime, the fish nominally received an average of 0, 0.03 and 0.3 µg BaP per gram of fish per week. After 1 (2nd August), 3 (3rd October) and 6 (31th of January) months, 10 fish per condition were anaesthetized and killed by a sharp blow to the head. Total body weight (g) and fork length (cm) were measured and the presence of parasites recorded. Liver and gonads were removed and weighed. Bile was snap frozen in liquid nitrogen and stored at -80°C until 3-OH BaP metabolite determination. A liver section was snap frozen in liquid nitrogen and stored at -80°C for molecular analyses. During the final sampling (6 months of exposure), a standardized liver cross-section was fixed for 24 hrs in neutral buffered formaldehyde (4%) before being transferred to 70% ethanol for subsequent histological assessment. Blood and spleen samples were preserved in Carnoy solution (3 methanol: 1 acetic acid) and stored at +4°C for subsequent identification of nuclear abnormalities and micronuclei. Finally, somatic weight (g) was determined as weight of eviscerated fish. Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as follows:

GSI = (gonad weight/somatic weight) x 100

HSI = (liver weight/ somatic weight) x 100.

3-OH-benzo[a]pyrene measurement

Biliary 3-OH-benzo[a]pyrene metabolite concentration was determined after 1, 3 and 6 months of exposure following the procedure detailed in Song *et al.,* (2018). Preparation of hydrolysed bile samples was performed as described in Krahn *et al.*, (1992). Briefly, bile (1–20 μL) was mixed with an internal standard (triphenylamine) and diluted with demineralised water (10–50 μL) and hydrolysed with β-glucuronidasearylsulphatase (20 μL, 1 h at 37 °C). Methanol (75–200 μL) was added and the sample was mixed thoroughly before centrifugation. The supernatant was then transferred to vials and analysed. High pressure liquid chromatography (Waters 2695 Separations Module) was used to separate 3-OH-BaP in a Waters PAH C_{18} column (4.6 × 250 mm, 5 μm particle size). The mobile phase consisted of a gradient from 40:60 acetonitrile:ammonium acetate (0.05 M, pH 4.1) to 100% acetonitrile at a flow rate of 1 mL/min, and the column was heated to 35 °C. A 2475 fluorescence detector measured fluorescence at the optimum for each analyte (excitation/emissions: 380/430). A total of 25 μL extract was injected for each analysis. The results were calculated by use of the internal standard method (Grung *et al*., 2009). The calibration standards utilized were obtained from Chiron AS, Trondheim, Norway, and were in the range 0.2–200 ng/g. Values below the limit of detection were considered as equal to 0 ng/g in the analyses.

Histopathological analyses

Livers were processed in a vacuum infiltration processor (Shandon Citadel 1000) using standard histological protocols (Feist *et al*., 2004). The tissues were embedded in paraffin using an STP-120 spin tissue processor (Thermo Fisher Scientific, USA). Sections of 4 µm thickness were cut using a microtome HM 450 (Thermo Fisher Scientific, USA) and subsequently stained with haematoxylin and eosin (H&E). The liver sections were examined for microscopic pre-tumour and tumour lesions according to BEQUALM and ICES criteria (Feist *et al.*, 2004). The pre-tumour lesions sought were the vacuolated, basophilic and eosinophilic foci of cellular alteration (FCA). Tumour lesions were the benign hepatocellular adenoma and the malignant hepatocellular carcinoma (HCC). Lesions associated to nuclear and cellular polymorphism, cell death, inflammation and regeneration were also examined. A total of 5, 6 and 4 fish were assessed from control, low and high exposure condition, respectively.

Micronucleus test and nuclear abnormalities

The micronuclei and nuclear abnormalities frequencies were measured in blood and spleen of polar cod tissues fixed in Carnoy's solution; subsequently separated cells were dispersed on glass slides, and stained with the fluorescent dye 4′,6-diamidino- 2-phenylindole at 100 ng/ mL. For each experimental condition, a range of 6 to 8 fish were investigated, and for each specimen 2000 cells with preserved cytoplasm were scored to assess the presence of micronuclei and nuclear abnormalities. Micronuclei are defined as round structures, smaller than 1/3 of the main nucleus diameter, on the same optical plan and clearly separated from nucleus; Nuclear abnormalities include (i) binucleated: cell with two nuclei, (ii) notch nuclei: looks like nucleus but do not have nuclear materials, (iii) nuclear bud: evagination of bud-like structure from the nucleus, and (iv) blebbed nuclei: small euchromatin evagination of the nuclear membrane (Gorbi *et al*., 2009; Islam *et al*., 2021).

Gene expression analyses

Following 1, 3 and 6 months of exposure, a cross section of each liver, next to the one dedicated to histological analyses at 6 months was used for gene transcriptional response analyses. Total RNAs were extracted using the High Pure RNA Tissue kit (Roche Diagnostics Ltd, West Sussex, U.K.) according to the supplier's instructions which included a DNase treatment. RNA quality (integrity of 18S and 28S ribosomal bands) was evaluated by electrophoresis on a 1% agarose-formaldehyde gel. RNA purity was assessed by measuring the ratios of absorbance: A_{260}/A_{280} and A_{260}/A_{230} using a spectrophotometer (NanoDrop, ThermoFisher). All samples were of high purity (ratios' values > 2.1).

First strand cDNAs were synthesized from 1 µg of total RNA using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Stockport, U. K.) using random hexamer primers and according to the supplier's instructions. Putative coding sequences (Figure S1) were identified by nucleotide and protein BLAST searches on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequence homologies across fish species on the EMBL-EBI platform (https://www.ebi.ac.uk/Tools/msa/clustalo/). The contigs produced in the study of Song *et al.*, (2019) were also used. Primer pairs and FAM™-TAMRA™ dye probes used to amplify the target sequences were designed using the Prime Express software (Applied Biosystem) (Table 1). Ten ng of the reverse transcribed product measured by a qubit fluorometer (Thermo Fisher Scientific) was used as a template for subsequent polymerase chain reaction (PCR) in a 20 µL final volume using 1x of TaqMan® Fast Advanced Master Mix (Life technologies, Paisley, U.K.), 900 nM primers and 250 nM probe (final concentrations) according to the supplier's protocol. PCR reactions were performed in the Applied Biosystems™ ViiA™ 7 Real-Time PCR System using the following programme: one cycle at 95°C for 20 s and 40 amplification cycles at 95°C for 3 s and 60°C for 30 s. Primer efficiencies were determined by 10 times dilution series of the cDNA template and were about 100%. The optimal normalization gene was selected by testing the expressions of 3 reference genes (*β tubulin*, *hprt1* and *28S*) on all the samples using the NormFinder algorithm. The expression of the *β tubulin* gene displayed the highest stability. The melting curves were carefully checked after each qPCR run. The gene expression was calculated according to the delta delta Ct method.

Statistical analyses

Statistical analyses were performed using R (version 3.1.2). The effect of the BaP exposure concentration and time of exposure were assessed on all the biological parameters measured using 1-way ANOVA. When the normality of the residuals was not verified by the Shapiro-Wilk test, the non-parametric Kruskal-Wallis tests were used. Post-hoc comparisons were performed using the least-square mean test for parametric test and the Wilcoxon rank test for non-parametric test. The α error was adjusted using the Bonferroni correction for each post-hoc test.

Results

Over the course of the six-month exposure, all specimens grew significantly in weight and underwent gonadal maturation with mean GSI ranging from 1.5 ± 0.7 to 20.8 ± 3.9 % (Table 2). Endoparasites were commonly found across all treatments and sampling times. Nematodes on the liver surface were the most common parasites with a frequency of occurrence of 26%. Parasites of the phylum Platyhelminthes were less common (7%). No mortality was observed.

The dietary BaP exposure of polar cod led to a dose-dependent production of biliary 3-OH BaP metabolites for the low (0.03 µg BaP/g of fish/week) and high (0.3 µg BaP/g of fish/week) BaP exposure conditions after 1 and 6 months of exposure (Figure 1). The concentrations of bile 3-OH BaP ranged from 20 to 40 ng/g of bile for the low exposure condition and were approximately 10 times higher, from 132 to 390 ng/g of bile, for the highest exposure condition.

The transcriptional responses related to DNA adduct recognition (*xpc*), helicase activity (*xpd*), DNA repair (*xpf*, *rad51*) and tumour suppression (*tp53*) were not significantly changed by any BaP dietary exposures as compared to controls ($p > 0.05$) (Figure 2).

The number of micronuclei recorded in the blood and spleen of polar cod ($p > 0.05$) exposed to BaP did not significantly vary as compared to control (Figure 3 A, B). Nuclear abnormalities in polar cod spleen were significantly increased in the high dose group ($p = 0.03$), while close to significant in the low dose group ($p = 0.057$) (Figure 3 C, D). No significant nuclear abnormalities were observed after BaP exposure in blood cells ($p > 0.05$).

Histopathological analyses revealed one basophilic focus of cellular alteration in liver of two individuals exposed to the low exposure condition after six months of exposure (Figure S2). No tumour-related lesions were observed in livers of control and highly exposed individuals.

Discussion

The present study showed that polar cod grew steadily through the entire experiments for all exposure conditions (0, 0.03 and 0.3 µg BaP/g of fish/ week). These results were expected under chronic low dose exposure scenarios and are consistent with similar results obtained in polar cod exposed to higher dietary BaP concentrations (1.2 and 60.9 µg BaP/ g of fish/ week) but for a shorter period of two weeks (Song *et al.*, 2019). In Nile tilapia exposed to intraperitoneal injections of 3 µg BaP/g of fish/ week for 4 weeks, K, GSI and GSI were slightly decreased (Colli-Dula *et al.*, 2018). This suggests that there is a dose and time dependent threshold above which exposure to BaP induce significant body condition indexes changes. The fish species and the mode of BaP administration (injected intraperitoneally versus dietary) may also be important factors to consider.

The bile concentration of 3-OH-BaP has been used as an indicator of BaP exposure and biotransformation in many fish species including polar cod (Baake *et al*., 2016; Baali *et al*., 2016; Kammann *et al*., 2017; Song *et al*., 2019). Indeed, previous studies led on polar cod exposed to either PAHs or crude oil have shown a very high correlation between bile metabolites of PAHs and both cyp1a mRNA expression and EROD activity (Bakke *et al*., 2016, Bender *et al*., 2016, Vieweg *et al*., 2018, Nahrgang *et al*., 2019, Song *et al*., 2019). The increase in biliary 3-OH-BaP metabolite concentrations after 1 and 6 months exposure supported that of a similar, albeit shorter, exposure study (Song *et al*., 2019). When exposed to a four times higher exposure dose (1.2 µg BaP/ g of fish/ week) than the highest dose used in the present study, a 3-OH-BaP concentration of 800 ng/ g of bile was found, which was two to six times higher than the metabolite concentration range identified in our study. The reactive BaP intermediates have been found to accumulate and covalently bind DNA in the biliary system of polar cod one month after exposure to a single dietary concentration (Baake *et al*., 2016) equivalent to the cumulative dose received in the high BaP exposure condition during the first month of our study. Those reactive BaP metabolites covalently bind to biological molecules such nucleic acids and form DNA adducts that can lead to tumour formation. For instance, higher levels of BaP-7,8-diol metabolites and DNA binding activity were found in bile of English sole (*Parophrys vetulus*) a fish species more sensitive to carcinogenesis than the more resistant starry flounder (*Platichthys stellatus*) (Varanasi *et al.,* 1986). In polar cod dietarily exposed to higher BaP concentrations (from 5 µg BaP/ g of fish in a single injection) DNA adducts were found (Aas *et al.*, 2003), revealing an increased risk of liver tumour formation later on. Indeed, 50% of rainbow trout displayed pre-tumour (basophilic FCA) and tumour (HCC) liver lesions after six months of exposure to a similar dose injected intraperitoneally (Hendricks *et al*., 1985). In their study, 25 % of the trouts displayed similar liver lesions after twelve months of dietary exposure to a high dose of BaP (estimated to 1-2 mg BaP/ g fish/ week) (Hendricks *et al*., 1985). In the present study, the potential genotoxic damage generated by the BaP metabolites produced did not cause significant tumour lesions. This could be the result of several factors potentially in combination, including low dose, low exposure duration and effective DNA repair mechanisms.

The DNA repair system and cell cycle regulators can prevent DNA adducts and the onset of tumorous events. In the present study, the transcriptional response of genes involved in the nucleotide excision repair (NER) process (*xpc*, *xpd*, *xpf*), DNA double strand breaks repair (*rad51*) and cell division regulation (*tp53*) did not vary significantly after 1, 3, and 6 months of exposure to both BaP dietary concentrations. The exposure levels of BaP may have been too low to cause significant accumulation of cell damage and trigger a significant gene transcriptional response. Interestingly, a dose-specific transcriptional response of some genes has been observed in liver of polar cod dietary exposed to BaP (Song *et al*., 2019). For instance, some genes involved in apoptosis (*bax* and *casp9*), a process that eliminates damaged cells and prevent the proliferation of abnormal cells in tumour formation, were upregulated in polar cod exposed to the high exposure level (60.9 µg BaP/ g of fish/ week). The expression levels of those genes were not modified in fish exposed to the low exposure dose (1.2 µg BaP/ g of fish/week) suggesting a threshold above which gene transcription is modified (Song *et al*., 2019). The basal gene expression level may also be sufficient to repair DNA and/or delay the cell cycle to maintain the genetic integrity. Moreover, the DNA repair gene measured in our study, *rad51*, may not be involved in the repair of specific DNA damage induced. Similarly to the results herein, this gene was not differentially expressed in liver of polar cod dietary exposed to BaP (Song *et al*., 2019). *Rad51* is involved in the repair of DNA double strand breaks, which belong to a different pathway than the NER. The mechanism of DNA damage induced by BaP exposure is more likely to involve DNA adducts than double strand breaks. Other genes involved in DNA repair processes and control of cell cycle have been found induced at higher exposure regimes. For example, the gene encoding for the growth arrest and DNA damage inducible beta gene (*gadd45b*) was induced in the liver of the tropical fish, Nile Tilapia (*Oreochromis niloticus*) after one month of exposure to 3 µg BaP/ g of fish/week (Colli-Dula *et al.,* 2018). In polar cod exposed to 1.2

and 60.9 µg BaP/g of fish/ week, genes involved in the excision DNA repair process (such as *hmgb2b* and *rad23a*) were differentially expressed (Song *et al.*, 2019). Shorter timepoints may have also been necessary to observe a gene expression modulation as an early response to stressors. In the liver of polar cod, transcriptional responses of genes involved in DNA damage repair were changed after two weeks of dietary exposure to BaP (Song *et al.*, 2019). Some studies using a reference genotoxic compound showed that DNA damage was rapidly repaired with increased transcription of DNA repair genes such as *rad51* in zebrafish larvae, as early as 6 hours (Reinardy *et al*. 2013). The addition of early sampling times seems relevant to include in future studies.

Other biological processes such as detoxification mechanisms could have prevented polar cod from the genotoxic effects of BaP exposure. Activation of detoxification events could explain the resistance of polar cod to BaP exposure and the absence of liver tumours in the present study. Variation in the expression of genes and proteins belonging to the cytochrome P450 family involved in phase I of BaP detoxification process has been well described in liver of fish (Nahrgang *et al.*, 2009; Lee *et al.*, 2014; Colli-Dula *et al.*, 2018). Interestingly, *cyp1a1* and *cyp1b1* genes were upregulated in liver of polar cod following dietary exposure to 60.9 µg BaP/g of fish/ week but were not differentially expressed after exposure to a lower dose of BaP (1.2 µg BaP/g of fish/ week) (Song *et al.*, 2019). This suggests a dose threshold for activating the detoxification mechanisms during a chronic exposure. Activation of genes and proteins involved in phase II detoxification process has been also described in liver of fish exposed to BaP (Nahgang *et al.*, 2009). For instance, *gstA1* gene expression was modified in the liver of Nile tilapia exposed to 3 µg BaP/ g of fish/ week (Colli-Dula *et al.*, 2018). Interestingly, GST activity was higher in starry flounder, a tumour resistant species, than in English sole, a tumour sensitive species, after exposure to a BaP dose that induced carcinogenesis (Varanasi *et al.*, 1987). Finally, phase III detoxification process based on active efflux of chemicals by ATP-binding cassette (ABC) transporters could be involved in BaP elimination. For example, a rainbow trout ABCG2 transporter was found to interact with BaP (Zaja *et al*., 2016).

In accordance with relatively low biliary BaP metabolite concentrations observed throughout the exposure and limited responses of genes involved in DNA damage identification and repair, no effect of dietary BaP on the micronuclei and nuclear abnormalities was recorded in the present study except in spleen where nuclear abnormalities increased in the high dose group. Micronuclei are formed during the anaphase stage of the cell division. They are considered as a reliable index of chromosomal breakage, chromosomal loss and cellular spindle malfunction (Bolognesi and Hayashi, 2011). Additionally, micronuclei constitute an irreversible form of genotoxic damage compared to DNA strand breaks and their induction are regulated by a large number of experimental carcinogens, including chlorinated hydrocarbons, benzidine, aflatoxins, methylcholanthrene, and common carcinogenic pollutants, such as PAHs, heavy metals, and pesticides (Bolognesi and Hayashi, 2011). Many research studies reported the increased in micronuclei frequency in erythrocytes of different fish species exposed to PAHs (Shirmohammadi *et al*., 2018).

Contrary to micronuclei, nuclear abnormalities origin has not been clearly explained; some suggest that nuclear abnormalities can be a primary response, prior to the micronuclei formation, highlighting their relevance in the evaluation of genotoxic damage (Bolognesi and Hayashi, 2011; Seriani *et al*., 2011). An increase of erythrocytic nuclear abnormalities and strand breaks was observed in eels (*Anguilla anguilla* L.) and juvenile sea bass (*Dicentrarchus labrax*) exposed to a range of 0.3 to 2.7 µM of BaP and naphthalene (Maria *et al*., 2002; Teles *et al*., 2003; Gravato and Santos, 2002), while on the contrary, lower concentrations of BaP (0.1 µM) did not affect DNA integrity (Nogueira *et al*., 2006). The induction of micronuclei and other nuclear abnormalities were also caused by crude oil exposure in turbot (*Scophthalmus maximus*) and Atlantic cod (*Gadus morua*) (Baršienė *et al*., 2004; 2006). The exposure duration and levels are extremely important in determining micronuclei and nuclear abnormalities formation; long-term chemical exposures can cause genetic changes and consequently physiological alterations or pathologies including cancer development (Depledge and Hopkin, 1995). In a study led on the European flounder, *Platichthy flesus*, Köhler and Ellesat, (2008), first suggested that nuclear anomalies inside liver lesions of hepatocellular cancers were correlated with micronuclei frequencies in fish blood and that the histopathological grading of cancers from preneoplastic, benign to malignant types was clearly associated with micronuclei increase.

The present study showed that polar cod were consistently exposed to dietary BaP through the entire experiment and biotransformed the mother compound to intermediate metabolites. However, this exposure did not lead to significant changes in the transcription of selected genes, nor in chromosomal alterations and significant tissue lesions. Some early responses to stress may have occurred prior to the first sampling time point at one month of exposure, and basal expression of genes or potentially activated compensatory mechanisms may have been sufficient to control the damage caused by the reactive metabolites. Moreover, protective mechanisms such as detoxification and apoptosis could have prevented the cells from the accumulation of cell damage caused by the reactive metabolites. Therefore, we deduce that the BaP exposure concentrations were below the threshold of observable effects. As a whole, our results showed that polar cod exposed to 0.03 and 0.3 µg BaP/ g fish/ week was not sensitive to the model carcinogen and liver carcinogenesis. The present work encourages the addition of earlier sampling points and indicators of detoxification mechanisms in future studies.

References

Aas E, Liewenborg B, Grøsvik BE, Camus L, Jonsson G, Børseth JF, Balk L. 2003. DNA adduct levels in fish from pristine areas are not detectable or low when analysed using the nuclease P1 version of the ^{32}P-postlabelling technique. Biomarkers, 8: 445-460.

Acevedo-Whitehouse K, Cole KJ, Philips DH, Jepson PD, Deaville R, Arlt VM. 2018. Hepatic DNA Damage in Harbour Porpoises (*Phocoena phocoena*) Stranded along the English and Welsh Coastlines. Environmental and Molecular Mutagenesis, 59: 613-624.

Baali A, Kammann U, Hanel R, El Qoraychy I, Yahyaoui A. 2016. Bile metabolites of polycyclic aromatic hydrocarbons (PAHs) in three species of fish from Morocco. Environmental Sciences Europe. 28, 25.

Baines C, Lerebours A, Thomas F, Fort J, Kreitsberg R, Gentes S, Meitern R, Saks L, Ujvari B, Giraudeau M, Stepp T. 2021. Linking pollution and cancer in aquatic environments: A review. Environment International 149: 106391.

Bakke MJ, Nahrgang J, Ingebrigtsen K. 2016. Comparative absorption and tissue distribution of ^{14}C-benzo(a)pyrene and ^{14}C-phenanthrene in the polar cod (*Boreogadus saida*) following oral administration. Polar biology, 39: 165–1173.

Baršienė J, Dedonytė V, Rybakovas A, Andreikėnaitė L, Andersen OK. 2006. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquatic Toxicology, 78: S99-S104

Baršienė J, Lazutka J, Šyvokienė J, Dedonytė V, Rybakovas A Bagdonas E, Bjornstad A, Andersen OK. 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and North Seas. Environmental Toxicology, 19: 365-371.

Baumann, PC, Harshbarger JC. 1998. Long term trends in liver neoplasm epizootics of brown bullhead in the Black River, Ohio. Environmental Monitoring and Assessment 53: 213-223.

Bender ML, Frantzen M, Vieweg I, Falk-Petersen IB, Johnsen HK, Rudolfsen G, Tollefsen KE, Dubourg P, Nahrgang J. 2016. Effects of chronic dietary petroleum exposure on reproductive development in polar cod (*Boreogadus saida*). Aquatic Toxicology, 180: 196–208.

Black JJ, Maccubbin AE and Martin M. 1985. A Reliable, Efficient, Microinjection Apparatus and Methodology for the In Vivo Exposure of Rainbow Trout and Salmon Embryos to Chemical Carcinogen. Journal of the National Cancer Institute, 75: 1123-8.

Bolognesi C, Hayashi M. 2011. Micronucleus assay in aquatic animals. Mutagenesis, 26: 205–213.

Chen W, Zhang H and Liu Y. 2019. Anti-Inflammatory and Apoptotic Signaling Effect of Fucoxanthin on Benzo(A)Pyrene-Induced Lung Cancer in Mice. Journal of Environmental Pathology, Toxicology and Oncology, 38: 239-251.

Colli-Dula RC, Fang X, Moraga-Amador D, Albornoz-Abud N, Zamora-Bustillos R, Conesa A, Zapata-Perez O, Moreno D, Hernandez-Nuñez E. 2018. Transcriptome analysis reveals novel insights into the response of low-dose benzo(a)pyrene exposure in male tilapia. Aquatic Toxicology, 162-173.

Depledge M.H., Hopkin S.P. 1995. Methods to assess effects on brackish, estuarine, and near-coastal water organisms. Wiley (Ed.), SCOPE 53-Methods to Assess the Effects of Chemicals on Ecosystems, the Scientific Committee On Problems of the Environment (SCOPE).

Du Corbier, FA, Stentiford GD, Lyons BP, Rotchell JM. 2005. Isolation of the retinoblastoma cDNA from the marine flatfish dab (*Limanda limanda*) and evidence of mutational alterations in liver tumors. Environmental Science and Technology, 39: 9785-9790.

Elias, 2018. Conservation issues: Tundra ecosystems. Encyclopedia of the Anthropocene, 2, 2018,165-175

EPA/635/R-17/003Fa. Toxicological Review of Benzo[a]pyrene. 2017. U.S. Environmental Protection Agency Washington, DC.

Feist SW. 2004. Biological effects of contaminants: use of liver pathology of the European flatfish dab *(Limanda limanda L.)* and flounder *(Platichthys flesus L.)* for monitoring (International Council for the Exploration of the Sea, Copenhagen).

Geraudie P, Nahrgang J, Forget-Leray J, Minier C, Camus L. 2014. In vivo effects of en vironmental concentrations of produced water on the reproductive function of polar cod (*Boreogadus saida*). Journal of Toxicology and Environmental Health A, 77: 557–573.

Gillet LCJ and Scharer OD. 2006. Molecular mechanisms of mammalian global genome nucleotide excision repair. Chemical Reviews, 106: 253−276.

Gorbi S., Benedetti M., Virno Lamberti C., Pisanelli B., Moltedo G., Regoli F. 2009. Biological effects of diethylene glycol (DEG) and produced waters (PWs) released from offshore activities: A multi-biomarker approach with the sea bass *Dicentrarchus labrax*. Environmental Pollution, 157: 3166-3173.

Gravato C, Santos MA. 2002. Juvenile sea bass liver P450, EROD induction, and erythrocytic genotoxic responses to PAH and PAH-like compounds. Ecotoxicology and Environmental Safety, 51: 115-127

Harshbarger JC, Clark JB. 1990. Epizootiology of neoplasms in bony fish of North America. Science of the Total Environment, 94: 1-32.

Hendricks JD, Meyers TR, Shelton DW, Casteel JL, Bailey GS. 1985. Hepatocarcinogenicity of benzo[a]pyrene to rainbow trout by dietary exposure and intraperitoneal injection. Journal of national cancer institute, 74 : 839-851.

Ho, J. 2010. The implications of Arctic sea ice decline on shipping. Marine Policy, 34 : 713–715.

Holst, JC, McDonald, A. 2000. Fish-lift: a device for sampling live fish with trawls. Fish. Research, 48 : 87–91.

Ingebrigtsen K, Christiansen JS, Lindhe Ö, Brandt I. 2000. Disposition and cellular binding of ^{3}H-benzo(a)pyrene at subzero temperatures: studies in an aglomerular arctic teleost fish - the polar cod (*Boreogadus saida*). Polar Biology, 23: 503-509.

Islam MJ, Slater MJ, Thiele R, Kunzmann A. 2021. Influence of extreme ambient cold stress on growth, hematological, antioxidants, and immune responses in European seabass, *Dicentrarchus labrax* acclimatized at different salinities. Ecological Indicators, 122: 107280.

Jonsson H, Sundt RC, Aas E, Sanni S. 2010. The Arctic is no longer put on ice: evalua- tion of polar cod (*Boreogadus saida*) as a monitoring species of oil pollution in cold waters. Marine Pollution Bulletin, 60: 390–395.

Kammann U, Akcha F, Budzinski H, Burgeot T, Gubbins MJ, Lang T, Le Menach K, Vethaak AD, Hylland K. 2017. PAH metabolites in fish bile: from the seine estuary to Iceland. Marine Environmental Research, 124: 41–45.

Karle IL, Yagi H, Sayer JM, and Donald MJ. 2004. Crystal and molecular structure of a benzo[a]pyrene 7,8-diol 9,10-epoxide N2-deoxyguanosine adduct: Absolute configuration and conformation. Proceedings of the National Academy of Sciences of the United States of America, 6: 1433–1438**.**

Kasala ER, Bodduluru LN, Barua CC, Sriram CS, Gogoi R. 2015. Benzo(a)pyrene induced lung cancer: Role of dietary phytochemicals in chemoprevention. Pharmacological Reports. 67: 996-1009.

Köhler A, Ellesat K. 2008. Nuclear changes in blood, early liver anomalies and hepatocellular cancers in flounder (*Platichthys flesus* L.) as prognostic indicator for a higher cancer risk? Marine Environmental Research, 66 : 149-150

Krahn, MM, Burrows DG, Ylitalo GM, Brown DW, Wigren CA, Collier TK, Chan SL, Varanasi U. 1992. Mass-spectrometric analysis for aromatic-compounds in bile of fish sampled after the Exxon-Valdez oil-spill. Environmental Science and Technology, 26: 116–126.

Kumar A, Yadav J, Mohan R. 2021. Spatio-temporal change and variability of Barents-Kara sea ice, in the Arctic: Ocean and atmospheric implications. Science of the Total Environment, 753: 142046.

Lee, JW, Kim, YH, Yoon, S, Lee SK. 2014. Cytochrome P450 System Expression and DNA Adduct Formation in the Liver of *Zacco platypus* Following Waterborne Benzo(a)pyrene

Exposure: Implications for Biomarker Determination. Environmental Toxicology, 29: 1032-1042.

Lerebours A, Bignell JP, Stentiford GD, Feist SW, Lyons BP, Rotchell JM. 2013. Advanced diagnostics applied to fish liver tumours: relating pathology to underlying molecular aetiology. Marine Pollution Bulletin, 72 : 94-98.

Lerebours A, Stentiford GD, Lyons BP, Bignell JP, Derocles SA, Rotchell JM. 2014. Genetic alterations and cancer formation in a European flatfish at sites of different contaminant burdens. Environmental Science and Technology, 48 :10448-10455.

Lerebours A, Chapman E, Lyons BP, Bignell JP, Stentiford GD, Rotchell JM. 2017. Hepatocellular adenoma in a European flatfish (*Limanda limanda*): genetic alterations in laser-capture micro-dissected tissue and global transcriptomic approach. Marine Pollution Bulletin, 119: 120-127.

Li Y, Li H, Spitsbergen JM, Gong Z. 2017. Males develop faster and more severe hepatocellular carcinoma than females in krasV12 transgenic zebrafish. Scientific Reports, 7: 41280.

Li H, Li Y, Lu JW, Huo X, Gong Z. 2019. Liver-specific androgen receptor knockout attenuates early liver tumor development in zebrafish. Scientific Reports, 9: 10645.

Lyons, BP, Thain, JE, Stentiford, GD, Hylland, K, Davies, IM, Vethaak, AD. 2010. Using biological effects tools to define Good Environmental Status under the European Union Marine Strategy Framework Directive. Marine Pollution Bulletin, 60 : 1647-1651.

Malins, DC, Krahn MM, Brown DW, Rhodes LD, Myers MS, Mccain BB, Chan SL. 1985. Toxic-Chemicals in Marine Sediment and Biota from Mukilteo, Washington - Relationships with Hepatic Neoplasms and Other Hepatic-Lesions in English Sole (*Parophrys Vetulus*). Journal of the National Cancer Institute, 74: 487-494.

Maria VL, Correia AC, Santos MA. 2002. *Anguilla anguilla* L. biochemical and genotoxic responses to benzo[a]pyrene. Ecotoxicology and Environmental Safety, 53: 86-92.

Meier WN, Hovelsrud GK, van Oort BEH, Key JR, Kovacs KM, Michel C, Haas C, Mats A G, Gerland S, Donald KP, Makshtas A, Reist JD. 2014. Arctic sea ice in transformation: A review of recent observed changes and impacts on biology and human activity. Reviews of Geophysics, 51: 185–217

Myers, MS, Landahl JT, Krahn MM, McCain BB. 1991. Relationships between hepatic neoplasms and related lesions and exposure to toxic chemicals in marine fish from the U.S. west coast. Environmental Health Perspectives, 90: 7-15.

Nahrgang J, Camus L, Gonzalez P, Goksøyr A, Christiansen J, Hop H, 2009. PAH biomarker responses in polar cod (*Boreogadus saida*) exposed to benzo(a)pyrene. Aquatic Toxicology. 14, 309-319.

Nahrgang J, Camus L, Broms F, Christiansen JS, Hop H. 2010a. Seasonal baseline levels of physiological and biochemical parameters in polar cod (*Boreogadus saida*): implications for environmental monitoring. Marine Pollution Bulletin, 60: 1336–1345.

Nahrgang J, Camus L, Carls MG, Gonzalez P, Jonsson M, Taban IC, Bechmann RK, Christiansen JS, Hop H. 2010b. Biomarker responses in polar cod (*Boreogadus saida*) exposed to the water soluble fraction of crude oil. Aquatic Toxicology, 97: 234–242.

Nahrgang J, Camus L, Gonzalez P, Jonsson M, Christiansen JS, Hop H. 2010c. Biomarker responses in polar cod (*Boreogadus saida*) exposed to dietary crude oil. Aquatic Toxicology, 96: 77–83.

Nahrgang J, Dubourg P, Frantzen M, Storch D, Dahlke F, Meador JP. 2016. Early life stages of an arctic keystone species (*Boreogadus saida*) show high sensitivity to a water-soluble fraction of crude oil. Environmental Pollution, 218: 605–614.

Nahrgang J, Bender ML, Meier S, Nechev J, Berge J, Frantzen M. 2019. Growth and metabolism of adult polar cod (*Boreogadus saida*) in response to dietary crude oil. Ecotoxicology and Environmental Safety, 180: 53–62.

Nogueira PR, Lourenço J, Mendo S, Rotchell LM. 2006. Mutation analysis of ras gene in the liver of European eel (*Anguilla anguilla* L.) exposed to benzo[a]pyrene. Marine Pollution Bulletin, 52: 1611-1616.

Phillips and Artlt. 2007. The ^{32}P-postlabeling assay for DNA adducts. Nature Protocols, 2: 2772–2781.

Ploch SA, King LC, Kohan MJ, Di Giulio RT.1998. Comparative *in Vitro* and *in Vivo*

Benzo[*a*]pyrene–DNA Adduct Formation and Its Relationship to CYP1A Activity in Two Species of Ictalurid Catfish. Toxicology and applied pharmacology 149: 90–98.

Poirier et al 2019. Intestinal Polycyclic Aromatic Hydrocarbon-DNA Adducts in a Population of Beluga Whales with High Levels of Gastrointestinal Cancers Environ Mol Mutagen, 60: 29–41.

Rastogi A, Richa, Kumar A, Tyagi MB, Sinha RP. 2010. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. Journal of Nucleic Acids, 2010: 592980.

Reinardy KC, Dharamshi J, Jha AN, Henry TB. 2013. Changes in expression profiles of genes associated with DNA repair following induction of DNA damage in larval zebrafish *Danio rerio*. Mutagenesis, 28: 601-608.

Rei-Salgueiro L, Costa J, Ferreira M Reil-Henriques MA. 2011. Evaluation of 3-hydroxy-benzo[*a*]pyrene levels in Nile tilapia (*Oreochromis niloticus*) after waterborne exposure to Benzo[*a*]pyrene. Ecotoxicology, 2040-2054.

Reichert WL, Myers MS, Peck-Miller K, French B, Anulacion BF, Collier TK, Stein JE, Varanasi U. 1998. Molecular epizootiology of genotoxic events in marine fish: Linking contaminant exposure, DNA damage, and tissue-level alterations. Mutation Research, 411: 215-225

Rose, WL, French, BL, Reichert, WL, Faisal, M. 2001. Persistence of benzo[a]pyrene–DNA adducts in hematopoietic tissues and blood of the mummichog, *Fundulus heteroclitus*. Aquatic Toxicology, 52: 319–328.

Rose, WL, French, BL, Reichert, WL, Faisal, M., 2000. DNA adducts in hematopoietic tissues and blood of the mummichog (*Fundulus heteroclitus*) from a creosote-contaminated site in the Elizabeth River, Virginia. Marine Environmental Research, 50: 581–589.

Rotchell, JM, Blair, JB, Shim, JK, Hawkins, WE, Ostrander, GK. 2001. Cloning of the Retinoblastoma cDNA from the Japanese medaka (*Oryzias latipes*) and preliminary evidence of mutational alterations in chemically-induced retinoblastomas. Gene, 263: 231-237.

Seriani R, Tavares Ranzani-Paiva M J., Silva- Souza A. T., Silva Roseli N. 2011. Hematology, micronuclei and nuclear abnormalities in fishes from São Francisco River, Minas Gerais state, Brazil. Acta Scientiarum Biological Sciences. 33: 107–112.

Shirmohammadi M., Mohammad N. S., Ronagh T., Movahedinia A., Hamidian G. 2018. Using cell apoptosis, micronuclei and immune alternations as biomarkers of phenanthrene exposure in yellowfin seabream (*Acanthopagrus latus*). Fish and Shellfish Immunology, 72: 37-47.

Song Y, Nahrgang Y, Tollefden KE. 2019. Transcriptomic analysis reveals dose-dependent modes of action of benzo(a)pyrene in polar cod (*Boreogadus saida*). Science of the total environment, 653: 176-189.

Stentiford, GD, Bignell, JP, Lyons, BP, Thain, JE, Feist, SW. 2010. Effect of age on liver pathology and other diseases in flatfish: Implications for assessment of marine ecological health status. Marine Ecoloy Progress Series, 411: 215-230.

Teles M, Pacheco M, Santos MA. 2003. *Anguilla anguilla* L. liver ethoxyresorufin O-deethylation, Glutathione S-transferase, erythrocytic nuclear abnormalities, and endocrine responses to naphthalene and β-naphthoflavone. Ecotoxicology and Environmental Safety, 55: 98-107

Varasini U, Nishimoto M, Reichert WL, Le Eberhart BT. 1986. Comparative Metabolism of Benzo(a)pyrene and Covalent Binding to Hepatic DNA in English Sole, Starry Flounder, and Rat. Cancer Research, 46: 3817-3824.

Varasini U, Stein JE, Nishimoto M, Reichert WL, Collier TK. 1987. Chemical Carcinogenesis in Feral Fish: Uptake, Activation, and Detoxication of Organic Xenobiotics. Environmental Health Perspectives, 71: 155-170.

Vethaak, AD, Rheinallt Tap. 1992. Fish disease as a monitor for marine pollution: the case of the North Sea. Reviews in Fish Biology and Fisheries, 2: 1-32.

Vieweg I, Bilbao E, Meador JP, Cancio I, Bender ML, Cajaraville MP, Nahrgang J. 2018. Effects of dietary crude oil exposure on molecular and physiological parameters related to lipid homeostasis in polar cod (*Boreogadus saida*). Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology. 206-207, 54–64.

Vogelbein, WK, Fournie JW, Van Veld PA, Huggett RJ. 1990. Hepatic neoplasms in the mummichog *Fundulus heteroclitus* from a creosote-contaminated site. Cancer Research 50: 5978-5986.

Wallace MI, Cottier FR, Berge J, Tarling GA, Griffits C, et al. 2010. Comparison of zooplankton vertical migration in an ice-free and a seasonally ice-covered Arctic fjord: An insight into the influence of sea ice cover on zooplankton behavior. Limnology and Oceanography 55: 831–845.

Wang L, Camusb AC, Donga W, Thorntona C, Willetta KL. 2010. Expression of CYP1C1 and CYP1A in *Fundulus heteroclitus* during PAH-induced carcinogenesis. Aquatic Toxicology, 99: 439–447.

Welch HE, Bergmann MA, Siferd TD, Martin KA, Curtis MF, Crawford RE Conover RJ, Hop H. 1992. Energy flow through the marine ecosystem of the Lancaster Sound region, Arctic Canada. Arctic 45, 343-375.

Willett, K, Steinberg M, Thomsen J, Narasimhan TR, Safe S, McDonald S, Beatty K, Kennicutt, MC. 1995. Exposure of killifish to benzo[a]pyrene: comparative metabolism, DNA adduct formation and aryl hydrocarbon (Ah) receptor agonist activities. Comparative Biochemistry and Physiology. Part B: Biochemistry and Molecular Biology, 112: 93–103.

Wills, LP, Jung D, Koehrn K, Zhu S, Willett KL, Hinton DE, di Giulio RT. 2010. Comparative chronic liver toxicity of benzo[a]pyrene in two populations of the atlantic killifish (*Fundulus heteroclitus*) with different exposure histories. Environmental Health Perspectives, 118: 1376-1381.

Zaja R, Popovic M, Loncar J, Smital T. 2016. Functional characterization of rainbow trout (*Oncorhynchus mykiss*) Abcg2a (Bcrp) transporter. Comparative Biochemistry and Physiology, Part C, 190: 15–2.

Zhu S, Li L, Thornton C, Carvalho P, Avery BA, Willett KL. 2008. Simultaneous determination of benzo[*a*]pyrene and eight of its metabolites in *Fundulus heteroclitus* bile using ultra-performance liquid chromatography with mass spectrometry. Journal of Chromatography B, 863: 141-149.

Figure and Table Legend.

Figure 1. Concentrations of biliary 3-OH-benzo[a]pyrene (ng/g bile, n = 4 to 9) in fish exposed to three treatments of BaP (acetone control, low and high) during 0, 1, 3 and 6 months. Plots represent the median (line), 25–75% percentiles (box), non-outlier range (whisker), outliers (circle) and extreme values (coloured triangle). The effect of the dose and time on the metabolite concentrations were assessed using the Kruskal-Wallis rank test. When significant, a Wilcoxon test and a Bonferroni correction were applied. Asterisks (*) show significant difference from the control treatment (p < 0.05). Numbers above boxes represent the n.

Figure 2. Relative expression of genes (mean ± SD, arbitrary units) in liver of polar cods (n = 10 per treatment and time) exposed to acetone control, low and high BaP treatments after 1, 3 and 6 months. Plots represent the median (line), 25–75% percentiles (box), non-outlier range

(whisker), outliers (circle) and extreme values (coloured triangle). The effect of the dose on the gene expression levels was assessed using the Kruskal-Wallis rank test. No significant differences ($p > 0.05$) among treatments were found. Numbers above boxes represent the *n*.

Figure 3. DNA damage in the form of micronuclei per thousand in the blood (A) and spleen (B) and nuclear abnormalities in the blood (C) and spleen (D) of polar cod sampled after 6 month of exposure. Plots represent the median (line), 25–75% percentiles (box), non-outlier range (whisker), outliers (circle) and extreme values (coloured triangle). The effect of the dose on the number of micronuclei and nuclear abnormalities was assessed using the Kruskal-Wallis rank test. When significant, a Wilcoxon test and a Bonferroni correction were applied. Asterisks (*) show significant difference from the control treatment ($p < 0.05$). Numbers above boxes represent the *n*.

Table 1. Sequences of primer pairs and FAM/TAMRA probes used in RT-qPCR reactions for each of the target genes studied. *β tubulin* was used as the reference gene.

Table 2. Fulton condition (K), hepatosomatic index (HSI), gonadosomatic index (GSI), liver, gonad and body weight (g), and fork length (cm) (mean ± SD, $n = 10$) and sex ratio determined after 0, 1, 3, and 6 months of exposure to different BaP treatments (acetone control, low and high exposures).

Figure 1

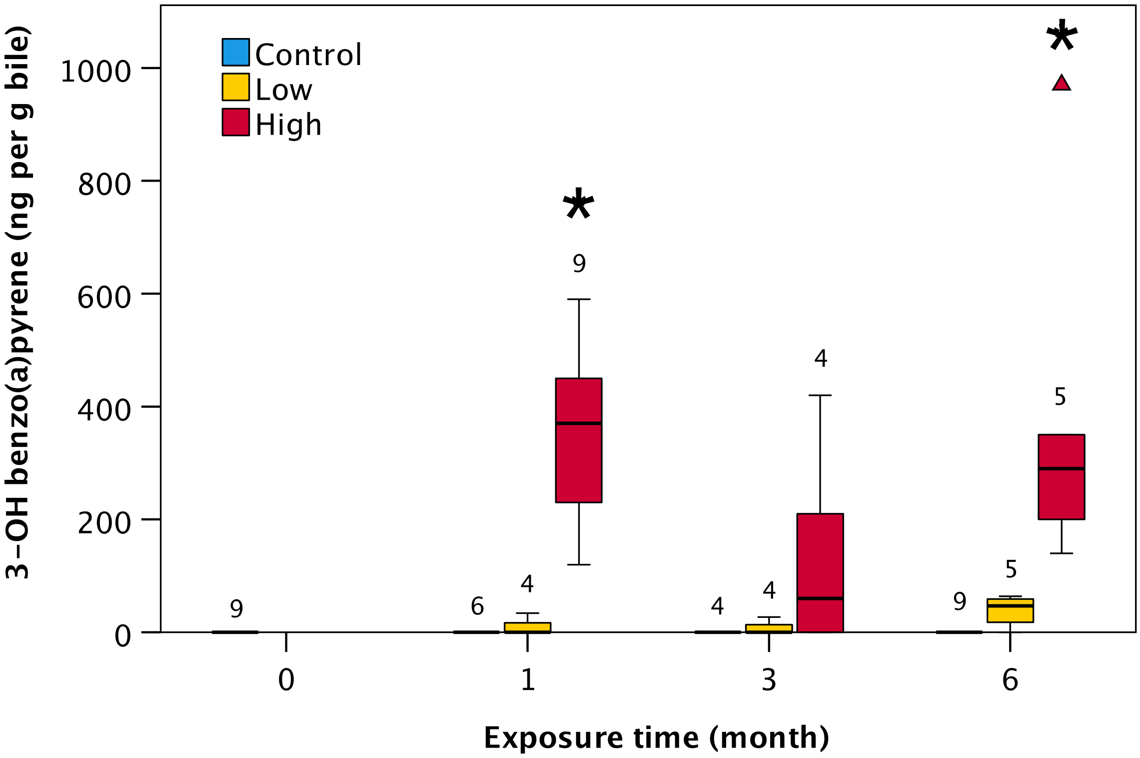
Control
Low
High
3-OH benzo(a)pyrene (ng per g bile)
1000
800
600
400
200
0
*
*
9
6
4
9
4
4
4
9
5
5
0
1
3
6
Exposure time (month)

Figure 2

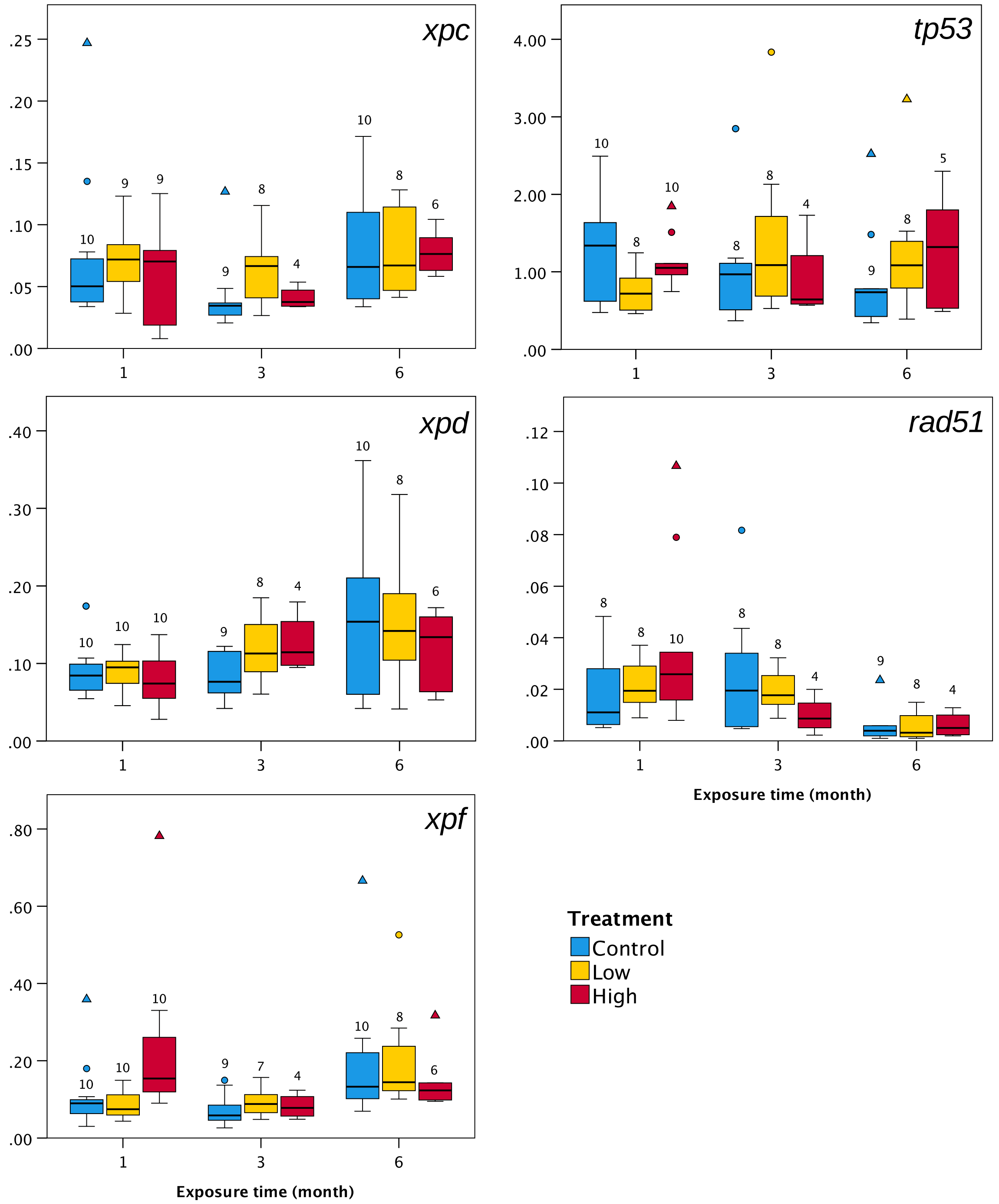
xpc
tp53
xpd
rad51
xpf
Exposure time (month)
Treatment
Control
Low
High

Figure 3

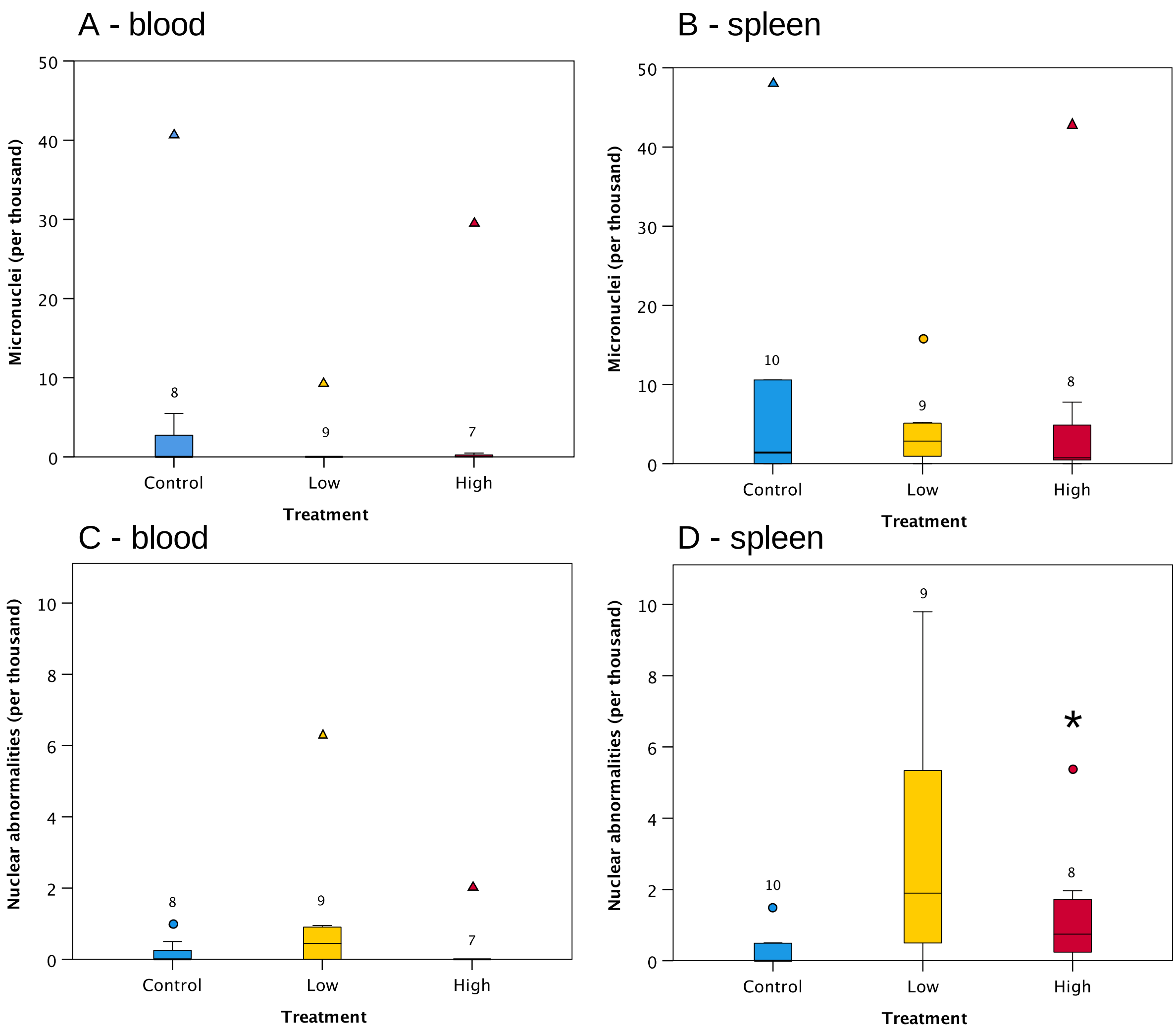
A - blood
B - spleen
C - blood
D - spleen
Micronuclei (per thousand)
Nuclear abnormalities (per thousand)
Control
Low
High
Treatment
0
10
20
30
40
50
2
4
6
8
7
9
*

Table 1

Gene name	**Primers and Probe Sequences 5'-3'**
β tubulin	F: GCCCGGCACCATGGA R: TGGCCGAAAACGAAGTTGTC P: TCCGGTGCTTTCGGTCAGATCTTCA
XPC	F: GCTTCGACTTCCATGGAGGAT R: CTTCGTGCTCCTCACACACAA P: CGCATGCTGTGACCGACGGCTAC
XPD	F: TCATGTTCGGAGTCCCTTATGTT R: GGAACTGGTCCCGGAGGTA P: ACACACAGAGCCGCATTCTGAAGGC
XPF	F: ATCTGGACCTGGCGAGGAA R: TCCTGCTTTGCGGGTGTT P: CTGGAGCCCGCCAACGCTACC
Rad51	F: AAGAAGCCGATTGGAGGAAAC R: CGCCCCTTCCTCAGGTACA P: TCATGGCCCACGCCTCCACC
tp53	F: CCTCTGAGGGGCATGTTCTC R: GGGGCTCTTTCTTTTTTTTGG P: TCCTGGGCGCGACCGCA

Table 2

	0 month	1 month			3 months			6 months		
		Control	**Low**	**High**	**Control**	**Low**	**High**	**Control**	**Low**	**High**
Fork l. (cm)	15.9 ± 1.6	16.4 ± 1	16.7 ± 1.6	16.9 ± 1.7	17.8 ± 1.6	16.8 ± 1.9	17.3 ± 1.5	16.4 ± 1.5	17.2 ± 0.7	17.3 ± 1.2
Total w. (g)	25.6 ± 7.1	29.3 ± 5.6	32.4 ± 8.8	33.2 ± 9.1	38.3 ± 11.6	32.1 ± 8.5	35.6 ± 10.4	32.7 ± 10	37.3 ± 4.5	36.7 ± 8
K	5.2 ± 0.5	5.2 ± 0.4	5.5 ± 0.5	5.4 ± 0.3	5.3 ± 0.7	5.4 ± 0.5	5.3 ± 0.4	5.2 ± 0.7	5.3 ± 0.4	5 ± 0.4
Liver w. (g)	2 ± 0.9	2.2 ± 0.7	2.5 ± 0.9	2.6 ± 0.7	2.7 ± 1.2	2.3 ± 0.6	2.8 ± 1	2.7 ± 0.9	2.6 ± 0.5	3 ± 0.9
HSI	9.6 ± 3.3	9.4 ± 2.1	9.4 ± 2.1	10.4 ± 2.8	8.6 ± 2.6	9.3 ± 2.3	9.8 ± 2.1	12 ± 3.9	9.5 ± 2	11.3 ± 2.3
Gonad w. (g)	0.3 ± 0.2	0.5 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	1.4 ± 0.5	1.1 ± 0.6	1.6 ± 0.9	4.2 ± 2.3	5.6 ± 1.3	5 ± 1.8
GSI	1.5 ± 0.7	2 ± 0.5	2.2 ± 1	1.8 ± 0.6	4.6 ± 1.2	4.1 ± 1.8	5.6 ± 2.3	16.9 ± 6.8	20.8 ± 3.9	19.3 ± 6.4
Sex ratio	40	60	30	50	50	40	30	50	22	50

Supplementary material

Figure S1. Putative coding sequences used to design the primers and probe for the 6 genes studied. The pink marks indicate the location of the introns in the DNA sequence. Primers were designed to overlap the introns whenever possible to check the specificity of the qPCR reactions.

```
>β tubulin
TCTACTACAATGAGGCCTCAGGTAATTAAAGCAATGTCAAATGGATGACGATACTTTCTCTCGTCTGTTCAGCTCTAACTGTT
TCTAATCTATTAGGTGGCAAATACGTCCCCCGCGCTGTTCTGGTCGATCTTGAGCCCGGCACCATGGACTCTGTGAGGTCCGG
TGCTTTCGGTCAGATCTTCAGGCCAGACAACTTCGTTTTCGGCCAGAGTGGTGCTGGCAACAACTGGGCCAAGGGTCACTACA
CGGAAGGTGCCGAGCTGGTGGACTCTGTGCTCGACGTGGTGAGGAAAGAGGCAGAGAGCTGTGACTGCCTGCAGGGCTTCCAG
CTC

>XPC
ACGGGCTTCGACTTCCATGGAGGATACTCGCATGCTGTGACCGACGGCTACATTGTGTGTGAGGAGCACGAAGAGATTCTCAG
AGCAGCCTGGGAGGAAGATCAAGCGCTCCAGAAACAGAAGGAGATTGAGAAGCGAGAGAAGCGGGCCACCACCAACTGGAAGC
TACTGGTGAAGGGCCTTCTGATCAGGGAGAGGCTCCAGCTACGATACGGCAA

>XPD
CGGGGTGGTCCCTGACGGCATCGTGGCGTTCTTCACCAGCTACATGTACATGGAGAACATCGTGGCGTCCTGGTATGAACAGG
GAATCCTGGAGAACATCCAGAGGAACAAGCTGATCTTCATTGAGACGCCAGATGCTGCAGAGACCAGCATGGCTCTGGAGAAA
TACCAGGAGGCATGTGAGAACGGGAGAGGAGCCATCCTTCTGTCTGTGGCCCGAGGAAAAGTGTCGGAAGGAATCGATTTTGT
GCACCACTTTGGTCGGGCAGTGATCATGTTCGGAGTCCCTTATGTTTACACACAGAGCCGCATTCTGAAGGCGCGTCTGGAGT
ACCTCCGGGACCAGTTCCAGATCCGGGAGAACGACTTCCTGACGTTCGACGCCATGCGCCATGCGGCCCAGTGCGTGGGCCGG
GTCATCAGGGGCAAGACGGACTACGGACTCATGATCTTCGCTGACAAGCGCTACGCCCGGGCGGACAAGCGGGGGAAGCTGCC
CCGCTGGATCCAGGAGCACATCAGCGACGGCAGCCTGAACCTCACGGTGGACGAGACGGTGCAGCTCTCCAAGCACTTCCTGA
GGCAGATGGCCCAGCCCTTCAGACAGGAGGACCAGCTGGGTCTGTCACTGCTGACGATAGAACAGCTGGAGTCAGAGGAGATG
CTGAAGAAGATCAGCCAAATGGCTCACCAGGCCTGACCACAT

>XPF
GATCTACAAGGCCAACCGCCCCGGGAAGACGCTGCGGGTGTATTTCCTGATCTATGGAGGATCCACAGAGGAACAGAAGTATC
TCACCGCGCTCTCCAAGGAGAAGAAAGCCTTCGAACACCTCATCAGGGAGAAGGCGACCATGGTTGTGCCGGAGGAGCGAGAG
GGTCGAGAAGACACCAATCTGGACCTGGCGAGGAATCTGGAGCCCGCCAACGCTACCACCAACACCCGCAAAGCAGGAGGCCA
GGACCAGCCCAGGGAGCCCTCCCGGGTCATCGTGGACATGCGGGAGTTCCGCAGCGAGCTGCCCTCCCTGCTGCACCGCCGCG
GGCTGGACATCGAGCCGGTCACCCTGGAGGTGGGCGACTACATCCTGACGGCGGACACCTGCGTGGAGCGCAAGAGCGTGAGC
GACCTGATCGGCTCGCTGCAGAGCGGCCGCCTCTACACGCAGTGCGTGTCCATGACGCGCTACTACAAGCGGGCCGTGCTGCT
CATCGAGTTCGACCCGGCCAAGCCCTTCTCGCTGGTGGCGCGCTCCGAGTTCCGCCACGAGCTGTCGGCCAACGACGTCACGT
CCAAGCTGACGCTGCTCACCCTGCACTTCCCGCGCCTGCGCCTCCTCTGGTGCCCCTCGCCCCACGCCACGGCCGAGCTCTTC
GAGGAGATGAAGCGGGGCCGCGGCGAGCCGGACGCCGCCGCCGCGCAGGCCATCGCGGCCGAGTCGGACGCCAGGACGACGGC
GGAGCTGTACAACCCGGCGCCGTACGACTTCCTGCTGAAGATGCCGGGGGTCAACGCCAAGAACGTGCGGGCGCTGGTGAGCA
AGGCGGACAGCCTGGCCGCGCTGGCCGAGTTCAGCCAGGAGAGGCTGGCGCAGGTCCTGGGGCACACCGGCAACGCCAAGATG
CTCTACGAGTTCCTGCACAACGTGGCCGACGTGCCCGCGCCAGCTGCCAAGGGCAGACGGACGTGAAGGGAAAGACT

>rad51
ATGGCTATGAGGAGTGAAGTGCGTTTGGAGGAGGAGGTAGAGGTGGAGGAGAACTTCGGCCCTCAGCCCATCAGTCGACTGGA
GCAAAGCGGTGTGAGCAGCAGTGACCTGAAGAAGCTGGAGGAGGCGGGCTTCCACACCATCGAGGCCGTGGCCTACACCCCCA
AGAAAGAGCTGCTCCACATCAAGGGCATCAGCGAGGCCAAGGCCGACAAGATCCTGGCGGAGGCAGCCAAGCTGGTGCCCATG
GGCTTCACCACGGCAACGGAGTTCCACCAGCGGCGGGCGGAGATCATCCAGATCTCCACCGGCTCCAAGGAGCTGGACAAATT
GTTAATGGGGGGAATGGAGACGGGTTCCATCACGGAGATGTTTGGAGAGTTCCGGACGGGGAAGACACAGCTGTGCCACACTC
TGGCTGTCACCTGCCAGCTGCCCATCGACCAGGGCGGGGGAGAGGGCAAGGCCATGTACATCGACACCGAGGGAACCTTCCGG
CCGGAGCGCCTGCTGGCCGTGGCCGAGAGGTACGGGCTGGTGGGCAGCGACGTCCTGGACAACGTGGCGTACGCCCGGGCCTT
CAACACCGACCACCAGACCCAGCTGCTGTACCAGGCCTCCGCCATGATGGCAGAGTCACGCTATGCCCTGCTCATCGTGGACA
GCGCCACCGCTCTGTACCGGACGGACTACTCCGGTCGGGGGGAGCTGTCGGCCCGGCAGGGCCACCTCGGCCGCTTCCTCCGC
ATGCTGCTGCGGCTGGCCGACGAGTTTGGCGTTGCCGTGGTGATCACCAACCAGGTGGTGGCCCAGGTGGACGGGGCGGCCAT
GTTCTCGGCCGACCCCAAGAAGCCGATTGGAGGAAACATCATGGCCCACGCCTCCACCACGCGGCTGTACCTGAGGAAGGGGC
GGGGAGAGACCCGGATCTGTAAGATCTACGACTCCCCCTGCCTCCCGGAGTCGGAGGCCATGTTCGCCATCAACGCCGACGGC
GTGGGCGACGCCAAGGACTGA
```

```
>p53
CGACGCTCCTTCTGAACTACATGTGCAACAGCTCCTGCATGGGAGGGATGAACCGGAGAGCCATCCTGACCATCCTGACCCTG
GAGTCCTCTGAGGGGCATGTTCTCGGGCGGCGTTGCTTCGAGGTGCGCGTCTGTGCCTGTCCTGGGCGCGACCGCAAGACGGA
GGAGGGCAACGTGGAGAAAAAGACGGAGGGATCCAAGCCCACCAAAAAAAAGAAAGAGCCCCCCCACTCCGGCCCCCACGGCT
CCGCCCAAGAGGGTCCTGTCCGCCTCCAGCGCTGAAGAGGAGGATAAGGAGGTGTTTGTGCTACAGGTCGTTGGCCGGAAGAG
ATTCGAGATCCTGAGGCAGATAAACGATGCACTCGCGCTGCAGGAGAGGATGACAGTCAAGCAGGAGGTCCAAGGAGGGCCGT
CGCGGGGAAAGAGACGGCTGGGGGACCGGACAGACGAGGGGACCGACTGAGCGACCGTCCAACCGACCGTCCAACATAACACT
GCCACAGCGAACCCCGTTATTTTCTACACTTTTCTTTTGTCATTCCTATTTTTATTTTTTTTCATAGCTTTTTTTTCAGCATA
TAGTTTTATATACAATGTATTTTTATTTTTCATGTATTTTTTTCTTTCTTGATACTAATTCTTTTTTATTTCGTTTTTATAAG
AGGCCATG
```

Figure S2. Basophilic foci of cellular alteration diagnosed in liver of a polar cod exposed to the low BaP concentration (magnification x40).