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

2

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25

26 **Abstract**

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

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

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## 51 **Introduction**

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

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

76 DNA adducts are used as markers for exposure and potential genotoxic effects. Exposure to  
77 BaP specifically caused DNA adducts in fish such as pale chub (*Zacco platypus*) (Lee *et al.*,  
78 2014) and killifish species (*Fundulus grandis* and *F. similis*) (Willett *et al.*, 1995; Rose *et al.*,  
79 2000, 2001). Moreover, BaP exposure was associated with neoplastic lesions in brown  
80 bullhead (*Ameiurus nebulosus*) (Ploch *et al.*, 1998), English sole (*Parophrys vetulus*)  
81 (Reichert *et al.*, 1998) and rainbow trout (Hendricks *et al.*, 1985). PAH-induced lesions have  
82 also recently been suggested in marine mammals such as harbour porpoises (*Phocoena*  
83 *phocoena*) (Acevedo-Whitehouse *et al.*, 2018) and beluga whales (Poirier *et al.*, 2019).  
84 Pollution induced cancer affects many aquatic species and represents a growing concern for  
85 aquatic wildlife (for a review see Baines *et al.*, 2021).

86 Tumourigenesis is a progressive process characterised by different stages for which  
87 the underlying molecular steps and the role of environmental exposure are not always well-  
88 known. Nonetheless, liver tumourigenesis has been well studied in flatfish (Stentiford *et al.*,  
89 2010; Lerebours *et al.*, 2013; 2014; 2017) and in the model fish Japanese medaka (*Oryzias*  
90 *latipes*) (Rotchell *et al.*, 2001) and zebrafish (*Danio rerio*) (Li *et al.*, 2017; 2019). DNA  
91 repair mechanisms have been associated with tumour formation perturbing several steps of  
92 the nucleotide excision repair (NER) pathway, which recognises and repairs DNA adducts  
93 induced by numerous environmental mutagens, including PAHs (Gillet and Schärer, 2006;  
94 Rastogi *et al.*, 2010). While such mechanisms involved in the development of tumours in  
95 certain fish species are well characterised, a substantial knowledge gap exists for non-model  
96 and ecologically important species inhabiting remote regions in particular. The Arctic is  
97 currently experiencing a rapid decline in sea ice (Kumar *et al.*, 2021) that may lead to a  
98 significant increase in marine shipping (Ho *et al.*, 2010), oil and gas exploration and  
99 operation (Elias, 2018), and tourism (Meier *et al.*, 2014) and associated release of potential  
100 carcinogenic contaminants (Elias, 2018). The polar cod (*Boreogadus saida*) is a keystone fish

101 species in the arctic marine ecosystem due to its abundance, distribution and central role in  
102 the food web (Welch *et al.*, 1992). Polar cod has been considered a model fish for arctic  
103 ecotoxicology studies (Jonsson *et al.*, 2010; Nahrgang *et al.*, 2009, 2010a,b,c). The toxicity of  
104 petroleum compounds on the physiology of polar cod has been well studied (Geraudie *et al.*,  
105 2014; Bender *et al.*, 2016; Nahrgang *et al.*, 2016; Vieweg *et al.*, 2018; Nahrgang *et al.*, 2019)  
106 but the tumourigenic potential of a potent carcinogen remains unknown in that species. A few  
107 studies however have reported a potential susceptibility to carcinogenic contaminants  
108 including BaP. The hepatic metabolism of BaP is particularly efficient in polar cod and a  
109 significant increase of covalently bound reactive intermediates of BaP in the bile of fish has  
110 been found after dietary exposure to BaP (Ingebrigtsen *et al.*, 2000; Bakke *et al.*, 2016).  
111 These reactive intermediates were found to induce the formation of DNA adducts in the liver  
112 of that species (Aas *et al.*, 2003). This genotoxic effect can in turn result in cellular  
113 abnormalities and cancer initiation. Finally, a recent study showed that expression of genes  
114 involved in DNA repair and cell cycle regulation processes was modified in liver of polar cod  
115 dietary exposed to BaP (Song *et al.*, 2019).

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

124 The sampling times were selected because carcinogenesis is a long-term process.  
125 They were comparable to the exposure durations used in several studies interested in

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

135

## 136 **Methods**

### 137 *Fish collection and exposure*

138 Adult polar cod (4 years old) were collected along the west coast of the Svalbard archipelago  
139 (Norway) onboard RV Helmer Hanssen in January 2014 using a Campelen bottom trawl (at  
140 200m depth) and a fish-lift (Holst and McDonald, 2000). At the Tromsø aquaculture research  
141 station (Havbrukstasjon i Tromsø), fish were kept in 3000 L acclimation tank under a natural  
142 light and temperature (1.5 - 3 °C) regime of 79°N (based on mooring data in Wallace *et al.*,  
143 2010). During this period, fish were fed until satiation with thawed *Calanus sp.* copepods  
144 (*Calanus AS*, Tromsø). Ninety fish were selected based on similar length ( $15 \pm 1$  cm) and  
145 weight ( $25 \pm 7$  g) for the experiment (June 2014).

146 Polar cod were dietarily exposed to 0, 0.03 and 0.3 µg BaP per gram fish per week,  
147 for 6 months (2<sup>nd</sup> of July 2014 to 31<sup>st</sup> January 2015). The experiment was conducted in  
148 compliance with the policies of the Norwegian animal welfare authorities (application ID  
149 6571). Briefly, a BaP (Sigma Aldrich, St. Louis, USA) solution in acetone was mixed with  
150 *Calanus spp* (*Calanus AS*) to yield 0.5 or 5 µg BaP per g feed or acetone alone (acetone

151 control). The acetone was volatilized by constant stirring on a magnetic stirrer for 2.5 hours  
152 at 30 °C. Small pellets were then created with the addition of 0.5 mL gelatin per g feed. Fish  
153 were fed pellets corresponding to 4% of their body wet weight (bw) 5 days a week. On the  
154 first, third and fifth day of a week, fish were exposed to dietary BaP or a solvent control by  
155 receiving the 2% bw exposed feed (or solvent control) and 2% bw of unexposed feed (no  
156 BaP, no acetone). Feeding was done by distributing the pellets to the surface of the tank.  
157 Thus, feeding hierarchies may have occurred resulting in some intra-tank individual exposure  
158 variations. On the remaining 2 days of a week, all fish were fed 4% bw of unexposed feed.  
159 The amount of food given to each tank was adjusted at each sampling point to account for  
160 both growth and sampling of specimens. With this feeding regime, the fish nominally  
161 received an average of 0, 0.03 and 0.3 µg BaP per gram of fish per week. After 1 (2<sup>nd</sup>  
162 August), 3 (3<sup>rd</sup> October) and 6 (31<sup>th</sup> of January) months, 10 fish per condition were  
163 anaesthetized and killed by a sharp blow to the head. Total body weight (g) and fork length  
164 (cm) were measured and the presence of parasites recorded. Liver and gonads were removed  
165 and weighed. Bile was snap frozen in liquid nitrogen and stored at -80°C until 3-OH BaP  
166 metabolite determination. A liver section was snap frozen in liquid nitrogen and stored at -  
167 80°C for molecular analyses. During the final sampling (6 months of exposure), a  
168 standardized liver cross-section was fixed for 24 hrs in neutral buffered formaldehyde (4%)  
169 before being transferred to 70% ethanol for subsequent histological assessment. Blood and  
170 spleen samples were preserved in Carnoy solution (3 methanol: 1 acetic acid) and stored at  
171 +4°C for subsequent identification of nuclear abnormalities and micronuclei. Finally, somatic  
172 weight (g) was determined as weight of eviscerated fish. Gonadosomatic index (GSI) and  
173 hepatosomatic index (HSI) were calculated as follows:

174 
$$\text{GSI} = (\text{gonad weight} / \text{somatic weight}) \times 100$$

175 
$$\text{HSI} = (\text{liver weight} / \text{somatic weight}) \times 100.$$



176 *3-OH-benzo[a]pyrene measurement*

177 Biliary 3-OH-benzo[a]pyrene metabolite concentration was determined after 1, 3 and 6  
178 months of exposure following the procedure detailed in Song *et al.*, (2018). Preparation of  
179 hydrolysed bile samples was performed as described in Krahn *et al.*, (1992). Briefly, bile (1-  
180 20  $\mu$  L) was mixed with an internal standard (triphenylamine) and diluted with demineralised  
181 water (10-50  $\mu$  L) and hydrolysed with  $\beta$ -glucuronidasearylsulphatase (20  $\mu$  L, 1 h at 37  
182  $^{\circ}$  C). Methanol (75-200  $\mu$  L) was added and the sample was mixed thoroughly before  
183 centrifugation. The supernatant was then transferred to vials and analysed. High pressure  
184 liquid chromatography (Waters 2695 Separations Module) was used to separate 3-OH-BaP in  
185 a Waters PAH C18 column (4.6  $\times$  250 mm, 5  $\mu$  m particle size). The mobile phase consisted  
186 of a gradient from 40:60 acetonitrile:ammonium acetate (0.05 M, pH 4.1) to 100%  
187 acetonitrile at a flow rate of 1 mL/min, and the column was heated to 35  $^{\circ}$  C. A 2475  
188 fluorescence detector measured fluorescence at the optimum for each analyte  
189 (excitation/emissions: 380/430). A total of 25  $\mu$  L extract was injected for each analysis. The  
190 results were calculated by use of the internal standard method (Grung *et al.*, 2009). The  
191 calibration standards utilized were obtained from Chiron AS, Trondheim, Norway, and were  
192 in the range 0.2-200 ng/g. Values below the limit of detection were considered as equal to 0  
193 ng/g in the analyses.

194 *Histopathological analyses*

195 Livers were processed in a vacuum infiltration processor (Shandon Citadel 1000) using  
196 standard histological protocols (Feist *et al.*, 2004). The tissues were embedded in paraffin  
197 using an STP-120 spin tissue processor (Thermo Fisher Scientific, USA). Sections of 4  $\mu$ m  
198 thickness were cut using a microtome HM 450 (Thermo Fisher Scientific, USA) and  
199 subsequently stained with haematoxylin and eosin (H&E). The liver sections were examined

200 for microscopic pre-tumour and tumour lesions according to BEQUALM and ICES criteria  
201 (Feist *et al.*, 2004). The pre-tumour lesions sought were the vacuolated, basophilic and  
202 eosinophilic foci of cellular alteration (FCA). Tumour lesions were the benign hepatocellular  
203 adenoma and the malignant hepatocellular carcinoma (HCC). Lesions associated to nuclear  
204 and cellular polymorphism, cell death, inflammation and regeneration were also examined. A  
205 total of 5, 6 and 4 fish were assessed from control, low and high exposure condition,  
206 respectively.

#### 207 *Micronucleus test and nuclear abnormalities*

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

#### 219 *Gene expression analyses*

220 Following 1, 3 and 6 months of exposure, a cross section of each liver, next to the one  
221 dedicated to histological analyses at 6 months was used for gene transcriptional response  
222 analyses. Total RNAs were extracted using the High Pure RNA Tissue kit (Roche  
223 Diagnostics Ltd, West Sussex, U.K.) according to the supplier's instructions which included  
224 a DNase treatment. RNA quality (integrity of 18S and 28S ribosomal bands) was evaluated

225 by electrophoresis on a 1% agarose-formaldehyde gel. RNA purity was assessed by  
226 measuring the ratios of absorbance:  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  using a spectrophotometer  
227 (NanoDrop, ThermoFisher). All samples were of high purity (ratios' values > 2.1).

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

249

## 250 *Statistical analyses*

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

## 258 **Results**

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

265 The dietary BaP exposure of polar cod led to a dose-dependent production of biliary  
266 3-OH BaP metabolites for the low ( $0.03 \mu\text{g BaP/g}$  of fish/week) and high ( $0.3 \mu\text{g BaP/g}$  of  
267 fish/week) BaP exposure conditions after 1 and 6 months of exposure (Figure 1). The  
268 concentrations of bile 3-OH BaP ranged from 20 to 40 ng/g of bile for the low exposure  
269 condition and were approximately 10 times higher, from 132 to 390 ng/g of bile, for the  
270 highest exposure condition.

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

274 The number of micronuclei recorded in the blood and spleen of polar cod ( $p > 0.05$ )  
275 exposed to BaP did not significantly vary as compared to control (Figure 3 A, B). Nuclear  
276 abnormalities in polar cod spleen were significantly increased in the high dose group ( $p =$   
277  $0.03$ ), while close to significant in the low dose group ( $p = 0.057$ ) (Figure 3 C, D). No  
278 significant nuclear abnormalities were observed after BaP exposure in blood cells ( $p > 0.05$ ).

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

### 283 **Discussion**

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

294 The bile concentration of 3-OH-BaP has been used as an indicator of BaP exposure  
295 and biotransformation in many fish species including polar cod (Baake *et al.*, 2016; Baali *et*  
296 *al.*, 2016; Kammann *et al.*, 2017; Song *et al.*, 2019). Indeed, previous studies led on polar cod  
297 exposed to either PAHs or crude oil have shown a very high correlation between bile  
298 metabolites of PAHs and both cyp1a mRNA expression and EROD activity (Bakke *et al.*,

299 2016, Bender *et al.*, 2016, Vieweg *et al.*, 2018, Nahrgang *et al.*, 2019, Song *et al.*, 2019). The  
300 increase in biliary 3-OH-BaP metabolite concentrations after 1 and 6 months exposure  
301 supported that of a similar, albeit shorter, exposure study (Song *et al.*, 2019). When exposed  
302 to a four times higher exposure dose (1.2 µg BaP/ g of fish/ week) than the highest dose used  
303 in the present study, a 3-OH-BaP concentration of 800 ng/ g of bile was found, which was  
304 two to six times higher than the metabolite concentration range identified in our study. The  
305 reactive BaP intermediates have been found to accumulate and covalently bind DNA in the  
306 biliary system of polar cod one month after exposure to a single dietary concentration (Baake  
307 *et al.*, 2016) equivalent to the cumulative dose received in the high BaP exposure condition  
308 during the first month of our study. Those reactive BaP metabolites covalently bind to  
309 biological molecules such nucleic acids and form DNA adducts that can lead to tumour  
310 formation. For instance, higher levels of BaP-7,8-diol metabolites and DNA binding activity  
311 were found in bile of English sole (*Parophrys vetulus*) a fish species more sensitive to  
312 carcinogenesis than the more resistant starry flounder (*Platichthys stellatus*) (Varanasi *et al.*,  
313 1986). In polar cod dietarily exposed to higher BaP concentrations (from 5 µg BaP/ g of fish  
314 in a single injection) DNA adducts were found (Aas *et al.*, 2003), revealing an increased risk  
315 of liver tumour formation later on. Indeed, 50% of rainbow trout displayed pre-tumour  
316 (basophilic FCA) and tumour (HCC) liver lesions after six months of exposure to a similar  
317 dose injected intraperitoneally (Hendricks *et al.*, 1985). In their study, 25 % of the trouts  
318 displayed similar liver lesions after twelve months of dietary exposure to a high dose of BaP  
319 (estimated to 1-2 mg BaP/ g fish/ week) (Hendricks *et al.*, 1985). In the present study, the  
320 potential genotoxic damage generated by the BaP metabolites produced did not cause  
321 significant tumour lesions. This could be the result of several factors potentially in  
322 combination, including low dose, low exposure duration and effective DNA repair  
323 mechanisms.

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

349 and 60.9 µg BaP/g of fish/ week, genes involved in the excision DNA repair process (such as  
350 *hmg2b* and *rad23a*) were differentially expressed (Song *et al.*, 2019). Shorter timepoints  
351 may have also been necessary to observe a gene expression modulation as an early response  
352 to stressors. In the liver of polar cod, transcriptional responses of genes involved in DNA  
353 damage repair were changed after two weeks of dietary exposure to BaP (Song *et al.*, 2019).  
354 Some studies using a reference genotoxic compound showed that DNA damage was rapidly  
355 repaired with increased transcription of DNA repair genes such as *rad51* in zebrafish larvae,  
356 as early as 6 hours (Reinardy *et al.* 2013). The addition of early sampling times seems  
357 relevant to include in future studies.

358 Other biological processes such as detoxification mechanisms could have prevented  
359 polar cod from the genotoxic effects of BaP exposure. Activation of detoxification events  
360 could explain the resistance of polar cod to BaP exposure and the absence of liver tumours in  
361 the present study. Variation in the expression of genes and proteins belonging to the  
362 cytochrome P450 family involved in phase I of BaP detoxification process has been well  
363 described in liver of fish (Nahgang *et al.*, 2009; Lee *et al.*, 2014; Colli-Dula *et al.*, 2018).  
364 Interestingly, *cyp1a1* and *cyp1b1* genes were upregulated in liver of polar cod following  
365 dietary exposure to 60.9 µg BaP/g of fish/ week but were not differentially expressed after  
366 exposure to a lower dose of BaP (1.2 µg BaP/g of fish/ week) (Song *et al.*, 2019). This  
367 suggests a dose threshold for activating the detoxification mechanisms during a chronic  
368 exposure. Activation of genes and proteins involved in phase II detoxification process has  
369 been also described in liver of fish exposed to BaP (Nahgang *et al.*, 2009). For instance,  
370 *gstA1* gene expression was modified in the liver of Nile tilapia exposed to 3 µg BaP/ g of  
371 fish/ week (Colli-Dula *et al.*, 2018). Interestingly, GST activity was higher in starry flounder,  
372 a tumour resistant species, than in English sole, a tumour sensitive species, after exposure to  
373 a BaP dose that induced carcinogenesis (Varanasi *et al.*, 1987). Finally, phase III



374 detoxification process based on active efflux of chemicals by ATP-binding cassette (ABC)  
375 transporters could be involved in BaP elimination. For example, a rainbow trout ABCG2  
376 transporter was found to interact with BaP (Zaja *et al.*, 2016).

377 In accordance with relatively low biliary BaP metabolite concentrations observed  
378 throughout the exposure and limited responses of genes involved in DNA damage  
379 identification and repair, no effect of dietary BaP on the micronuclei and nuclear  
380 abnormalities was recorded in the present study except in spleen where nuclear abnormalities  
381 increased in the high dose group. Micronuclei are formed during the anaphase stage of the  
382 cell division. They are considered as a reliable index of chromosomal breakage,  
383 chromosomal loss and cellular spindle malfunction (Bolognesi and Hayashi, 2011).  
384 Additionally, micronuclei constitute an irreversible form of genotoxic damage compared to  
385 DNA strand breaks and their induction are regulated by a large number of experimental  
386 carcinogens, including chlorinated hydrocarbons, benzidine, aflatoxins, methylcholanthrene,  
387 and common carcinogenic pollutants, such as PAHs, heavy metals, and pesticides (Bolognesi  
388 and Hayashi, 2011). Many research studies reported the increased in micronuclei frequency  
389 in erythrocytes of different fish species exposed to PAHs (Shirmohammadi *et al.*, 2018).  
390 Contrary to micronuclei, nuclear abnormalities origin has not been clearly explained; some  
391 suggest that nuclear abnormalities can be a primary response, prior to the micronuclei  
392 formation, highlighting their relevance in the evaluation of genotoxic damage (Bolognesi and  
393 Hayashi, 2011; Seriani *et al.*, 2011). An increase of erythrocytic nuclear abnormalities and  
394 strand breaks was observed in eels (*Anguilla anguilla* L.) and juvenile sea bass  
395 (*Dicentrarchus labrax*) exposed to a range of 0.3 to 2.7  $\mu$ M of BaP and naphthalene (Maria *et*  
396 *al.*, 2002; Teles *et al.*, 2003; Gravato and Santos, 2002), while on the contrary, lower  
397 concentrations of BaP (0.1  $\mu$ M) did not affect DNA integrity (Nogueira *et al.*, 2006). The  
398 induction of micronuclei and other nuclear abnormalities were also caused by crude oil

399 exposure in turbot (*Scophthalmus maximus*) and Atlantic cod (*Gadus morua*) (Baršienė *et al.*,  
400 2004; 2006). The exposure duration and levels are extremely important in determining  
401 micronuclei and nuclear abnormalities formation; long-term chemical exposures can cause  
402 genetic changes and consequently physiological alterations or pathologies including cancer  
403 development (Depledge and Hopkin, 1995). In a study led on the European flounder,  
404 *Platichthys flesus*, Köhler and Ellesat, (2008), first suggested that nuclear anomalies inside  
405 liver lesions of hepatocellular cancers were correlated with micronuclei frequencies in fish  
406 blood and that the histopathological grading of cancers from preneoplastic, benign to  
407 malignant types was clearly associated with micronuclei increase.

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

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751 **Figure and Table Legend.**

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

761 **Figure 2.** Relative expression of genes (mean  $\pm$  SD, arbitrary units) in liver of polar cods ( $n =$   
762 10 per treatment and time) exposed to acetone control, low and high BaP treatments after 1, 3  
763 and 6 months. Plots represent the median (line), 25–75% percentiles (box), non-outlier range



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

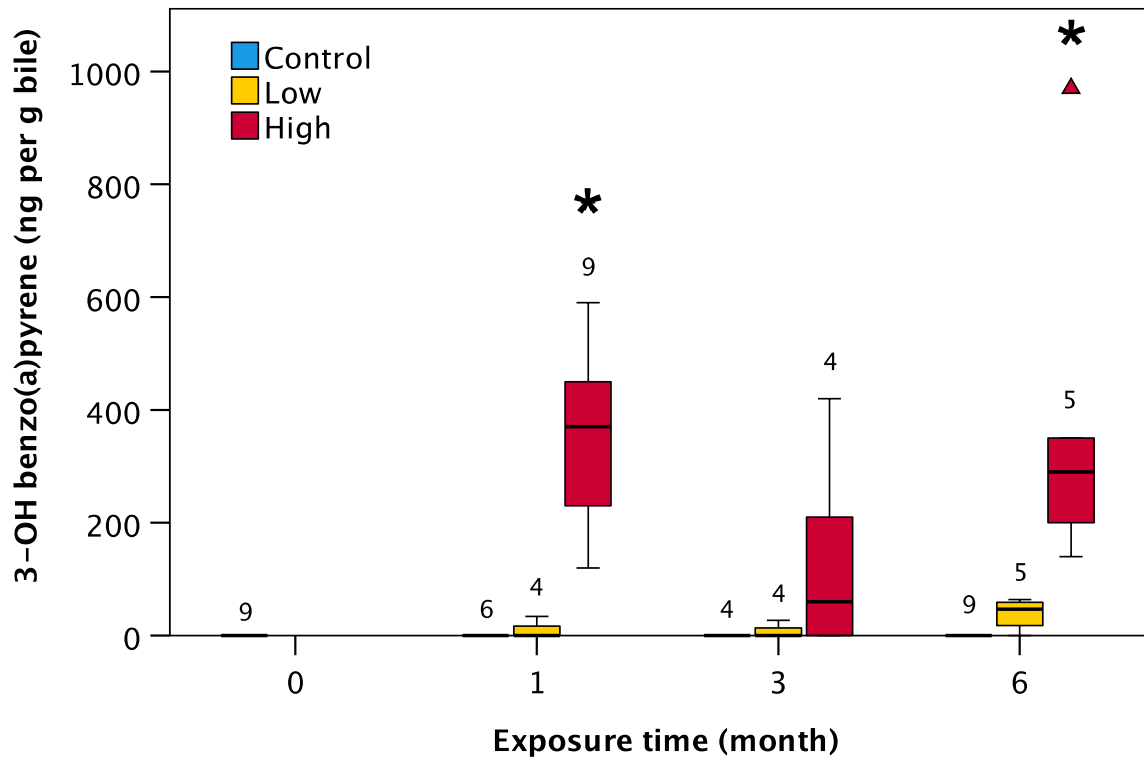
767 **Figure 3.** DNA damage in the form of micronuclei per thousand in the blood (A) and spleen  
768 (B) and nuclear abnormalities in the blood (C) and spleen (D) of polar cod sampled after 6  
769 month of exposure. Plots represent the median (line), 25–75% percentiles (box), non-outlier  
770 range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

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

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

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782 Figure 1



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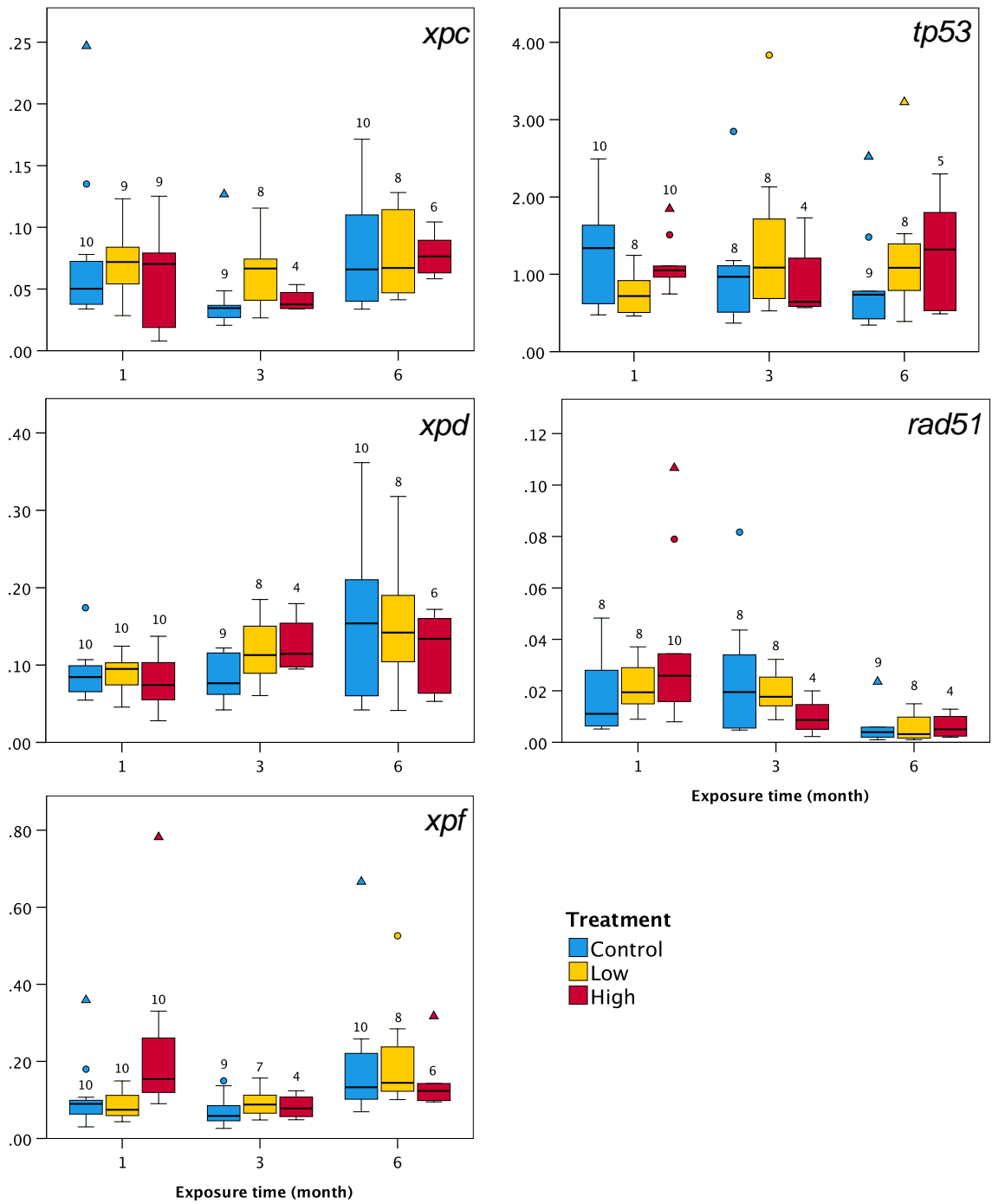
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794 Figure 2



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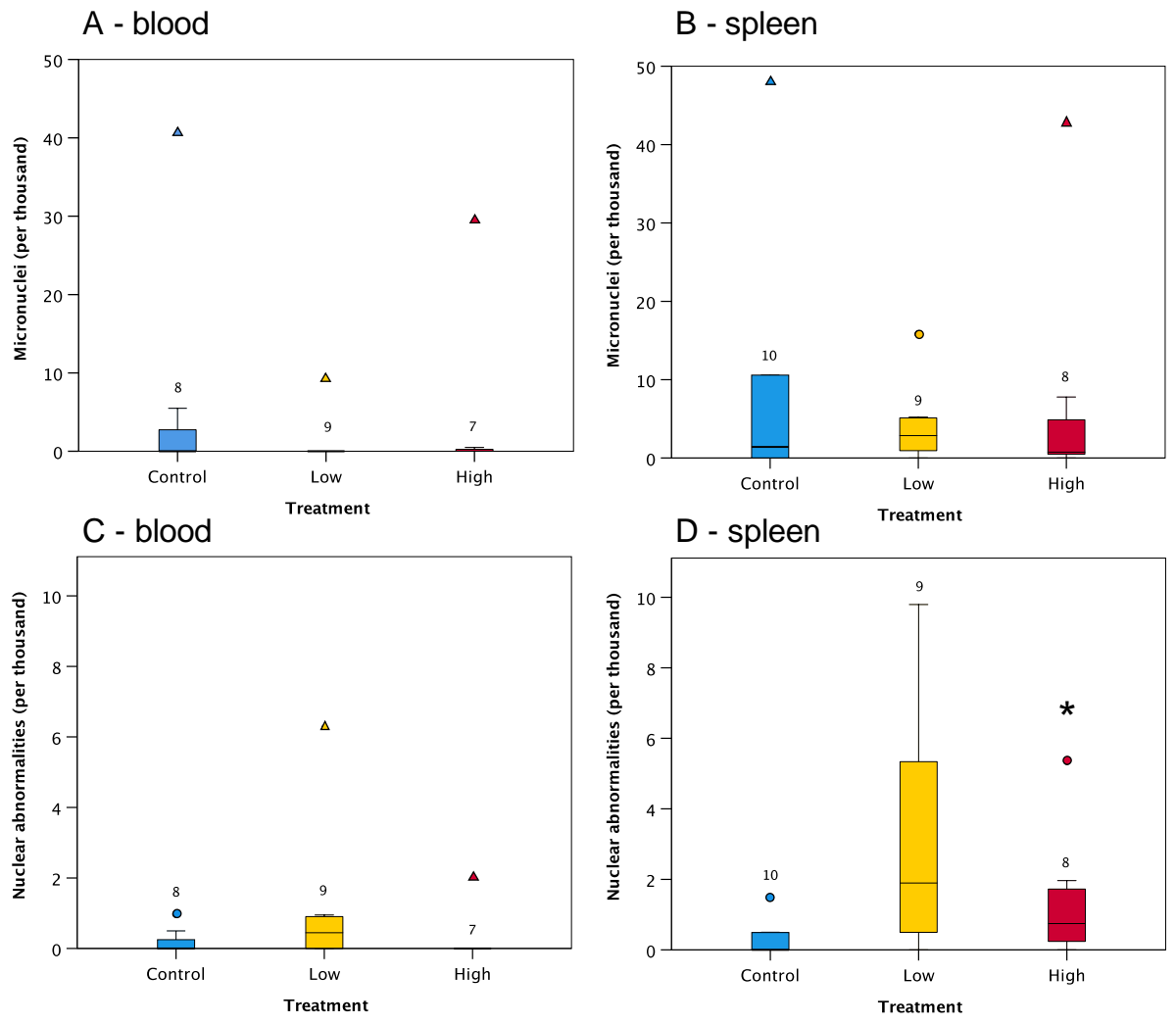
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801 Figure 3



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<b>Gene name</b>	<b>Primers and Probe Sequences 5'-3'</b>
<i>β tubulin</i>	F: GCCCGGCACCATGGA R: TGGCCGAAAACGAAGTTGTC P: TCCGGTGCTTTCGGTCAGATCTCA
<i>XPC</i>	F: GCTTCGACTTCCATGGAGGAT R: CTTCGTGCTCCTCACACACAA P: CGCATGCTGTGACCGACGGCTAC
<i>XPD</i>	F: TCATGTTCCGGAGTCCCTTATGTT R: GGAAGTGGTCCCGGAGGTA P: ACACACAGAGCCGCATTCTGAAGGC
<i>XPF</i>	F: ATCTGGACCTGGCGAGGAA R: TCCTGCTTTGCGGGTGTT P: CTGGAGCCCGCCAACGCTACC
<i>Rad51</i>	F: AAGAAGCCGATTGGAGGAAAC R: CGCCCCTTCCTCAGGTACA P: TCATGGCCCACGCCTCCACC
<i>tp53</i>	F: CCTCTGAGGGGCATGTTCTC R: GGGGCTCTTTCTTTTTTTTGG P: TCCTGGGCGCGACCGCA

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819 Table 2

	0 month	1 month			3 months			6 months		
		Control	Low	High	Control	Low	High	Control	Low	High
<b>Fork l. (cm)</b>	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
<b>Total w. (g)</b>	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
<b>K</b>	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
<b>Liver w. (g)</b>	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
<b>HSI</b>	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
<b>Gonad w. (g)</b>	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
<b>GSI</b>	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
<b>Sex ratio</b>	40	60	30	50	50	40	30	50	22	50

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## 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*

```
TCTACTACAATGAGGCCTCAGGTAATTAAGCAATGTCAAATGGATGACGATACTTCTCTCGTCTGTTTCAGCTCTAACTGTT
TCTAATCTATTAGGTGGCAAATACGTCCCCCGCGCTGTTCTGGTTCGATCTTGAGCCCCGGCACCATGGACTCTGTGAGGTCCGG
TGCTTTCGGTCAGATCTTCAGGCCAGACAACCTTCGTTTTCCGCCAGAGTGGTGCTGGCAACAACCTGGGCCAAGGGTCACTACA
CGGAAGTGCCGAGCTGGTGACTCTGTGCTCGACGTGGTGAGGAAAGAGGCAGAGAGCTGTGACTGCCTGCAGGGCTTCCAG
CTC
```

>*XPC*

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ACGGGCTTCGACTTCCATGGAGGATACTCGCATGCTGTGACCGACGGCTACATTGTGTGTGAGGAGCACGAAGAGATTCTCAG
AGCAGCTGGGAGGAAGATCAAGCGCTCCAGAAACAGAGAGAGATTGAGAAGCGAGAGAAGCGGGCCACCACCAACTGGAAGC
TACTGGTGAAGGGCTTCTGATCAGGGAGAGGCTCCAGCTACGATACGCCAA
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>*XPD*

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CGGGGTGGTCCCTGACGGCATCGTGGCGTTCCTTACCAGCTACATGTACATGGAGAACATCGTGGCGTCCGGTATGAACAAG
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TACCAGGAGGCATGTGAGAACGGGAGAGGAGCCATCCTTCTGTCTGTGGCCGAGGAAAAGTGTGGAAGGAATCGATTTTCT
GCACCACTTTCGGTTCGGCAGTGCATGTTTCGGAGTCCCTTATGTTTACACACAGAGCCGCATTTCTGAAAGCGCGTCTGGAGT
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GTGATCAGGGGCAAGACGACTACGGACTCATGATCTTTCGCTGACAAACGCTACGCCGGGCGGACAAGCGGGGAAGCTGCC
CCGCTGGATCCAGGAGCACATCAGCGACGGCAGCCTGAACCTCACGGTGGACGAGACGGTGCAGCTCTCCAAGCACTTCCTGA
GGCAGATGGCCAGCCCTTCAACAGGAGGACCAGCTGGGTCTGTCACTGCTGACGATAGAACAGCTGGAGTCAGAGGAGATG
CTGAAGAAGATCAGCCAAATGGCTCACCAGGCCTGACCACAT
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>*XPF*

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GATCTACAAGGCCAACCGCCCCGGGAAGACGCTGCGGGTGTATTTTCTGATCTATGGAGGATCCACAGAGGAACAGAAGTATC
TCACCGCGCTCTCCAAGGAGAAGAAAGCCTTCGAACACCTCATCAGGGAGAAGGCGACCATGGTTGTGCCGAGGAGCGAGAG
GGTTCGAGAAGACACCAATCTGGACCTGGCGAGGAATCTGGAGCCCGCCAACGCTACCACCAACACCCGCAAGCAGGAGGCCA
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GGCTGGATCATCGAGCCGCTCACCTGGAGGTGGGGACTACATCCTGACGGCGGACACCTGCGTGGAGCGCAAGAGCGTGAGC
GACCTGATCGGCTCGCTGCAGAGCGGCCCGCTTACACGCAGTGCCTGTCCATGACGCGCTACTACAAGCGCGCCGTGCTGCT
CATCGAGTTCGACCCGGCCAAGCCCTTCTCGTGGTGGCGCGCTCCGAGTTCGCCACGAGCTGTGCGCCAACGACGTCACGT
CCAAGCTGACGCTGCTACCCCTGCATTCGCGCGCTGGCCCTCTCTGGTGCCCTCGCCCCACGCCAGCGCCGAGCTCTTC
GAGGAGATGAAGCGGGGCGCGGCGAGCCGAGCAGCCGCGCCCGCGCAGGCCATCGCGGCCGATCGGACGCCAGGACGAGCGG
GGAGCTGTACAACCCGGCGCCGTACGACTTCTGCTGAAGATGCCGGGGTCAACGCCAAGAACGTCGGGGCGCTGGTGAGCA
AGGCGGACAGCCTGGCCGCGCTGGCCGAGTTCAGCCAGGAGAGGCTGGCGCAGGCTCCTGGGGCACACCGGCCAACGCCAAGATG
CTCTACGAGTTCCTGCACAACGTGGCCGACGTGCCCGCCAGCTGCCAAGGGCAGACGGACGTGAAGGGAAGACT
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>*rad51*

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ATGGCTATGAGGAGTGAAGTGCCTTTGGAGGAGGAGGTAGAGGTGGAGGAGAACTTCGGCCCTCAGCCCATCAGTTCGACTGGA
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AGAAAGAGCTGCTCCACATCAAGGGCATCAGCGAGGCCAAGGCCGACAAGATCCTGCGGAGGCAGCCAAGCTGGTGCCCATG
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GTTAATGGGGGAATGGAGACGGGTTCATCACGGAGATGTTTGGAGAGTTCGGACGGGGAAGACACAGCTGTGCCACACTC
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GCGCCACCGCTCTGTACCGGACGGACTACTCCGGTTCGGGGGAGCTGTCCGGCCGGCAGGGCCACTCGGCCGCTTCTCCGC
ATGCTGCTGCGGCTGGCCGACGAGTTTGGCGTTGCCGTGGTGCATCCCAACAGGTGGTGGCCAGGTGGACGGGGCGGCCAT
GTTCTCGGCCGACCCCAAGAAGCCGATTGGAGGAAACATCATGGCCACGCTCCACCACGCGCTGTACCTGAGGAAGGGG
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GTGGGCGACGCCAAGGACTGA
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>p53

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CGACGCTCCTTCTGAACTACATGTGCAACAGCTCCTGCATGGGAGGGATGAACCGGAGAGCCATCCTGACCATCCTGACCCTG
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GGAGGGCAACGTGGAGAAAAAGACGGAGGGATCCAAGCCACCAAAAAAAGAAAAGAGCCCCCACTCGGCCCCACGGCT
CCGCCAAGAGGGTCTGTCCGCTCCAGCGCTGAAGAGGAGGATAAGGAGGTGTTTGTGCTACAGGTCGTTGGCCGGAAGAG
ATTTCGAGATCCTGAGGCAGATAAACGATGCACTCGCGCTGCAGGAGAGGATGACAGTCAAGCAGGAGGTCCAAGGAGGGCCGT
CGCGGGAAAGAGACGGCTGGGGGACCGGACAGACGAGGGGACCGACTGAGCGACCGTCCAACCGACCGTCCAACATAACACT
GCCACAGCGAACCCCGTTATTTTCTACACTTTTCTTTTGTCAATTCCTATTTTATTTTTTTTCATAGCTTTTTTTTCAGCATA
TAGTTTTATATAACAATGTATTTTTATTTTTCATGTATTTTTTCTTTCTTGATACTAATTCTTTTTTATTTTCGTTTTTATAAG
AGGCCATG
```

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

