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

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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*
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} µg 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 Scharer, 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 ± 1 cm) and
145 weight (25 ± 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 (2nd of July 2014 to 31st 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 (2nd
162 August), 3 (3rd October) and 6 (31th 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 GSI = (gonad weight/somatic weight) x 100
175 HSI = (liver weight/ somatic weight) x 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 μL) was mixed with an internal standard (triphenylamine) and diluted with demineralised
181 water (10‐50 μL) and hydrolysed with β-glucuronidasearylsulphatase (20 μL, 1 h at 37
182 °C). Methanol (75‐200 μL) 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 × 250 mm, 5 μm 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 °C. A 2475
188 fluorescence detector measured fluorescence at the optimum for each analyte
189 (excitation/emissions: 380/430). A total of 25 μL 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 µm
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 µg 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™-
235 TAMRA™ 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 µL final volume using 1x of TaqMan®
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™ ViiA™ 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 (*β tubulin*, *hprt1* and *28S*) on all the samples
246 using the NormFinder algorithm. The expression of the *β 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 α 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 ± 0.7 to 20.8 ± 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 µg BaP/g of fish/week) and high (0.3 µg 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 μg 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 μg 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 μg 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 *hmgb2b* 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 (Nahrgang *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 µM 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 µM) 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 *Platichthy 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.

422

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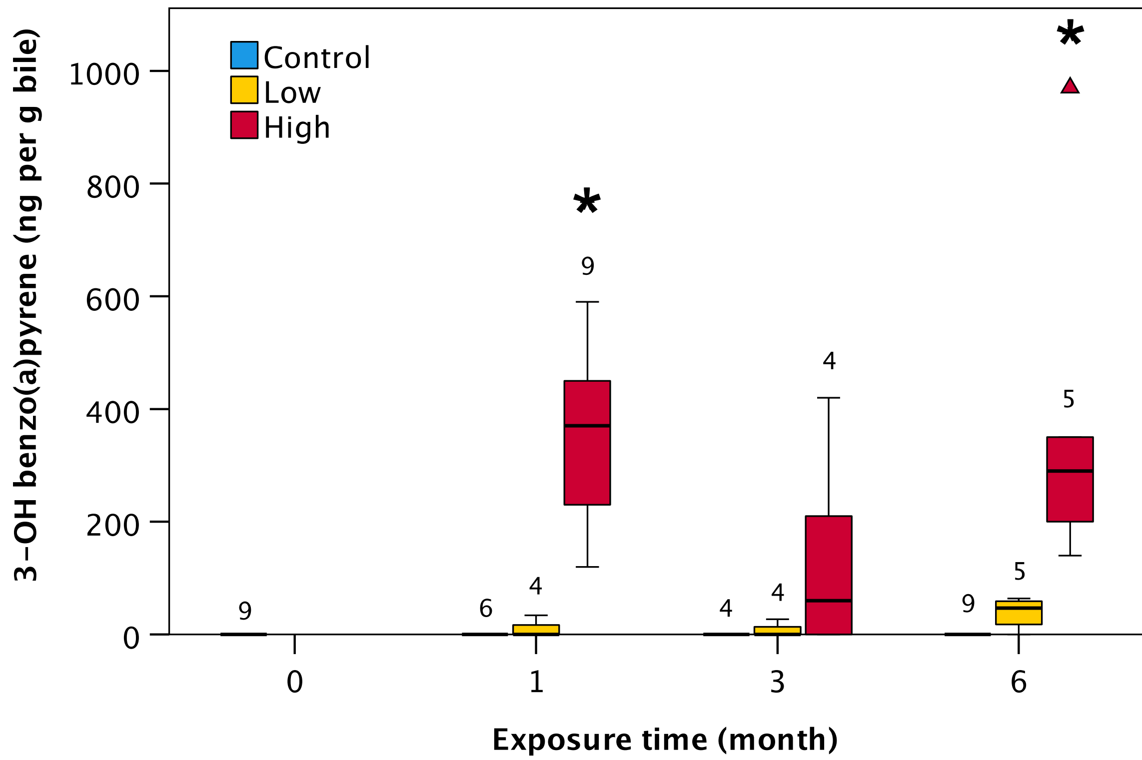
751 **Figure and Table Legend.**
752
753 **Figure 1.** Concentrations of biliary 3-OH-benzo[a]pyrene (ng/g bile, n = 4 to 9) in fish
754 exposed to three treatments of BaP (acetone control, low and high) during 0, 1, 3 and 6
755 months. Plots represent the median (line), 25–75% percentiles (box), non-outlier 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 ± 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 ± 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).
781
782 Figure 1



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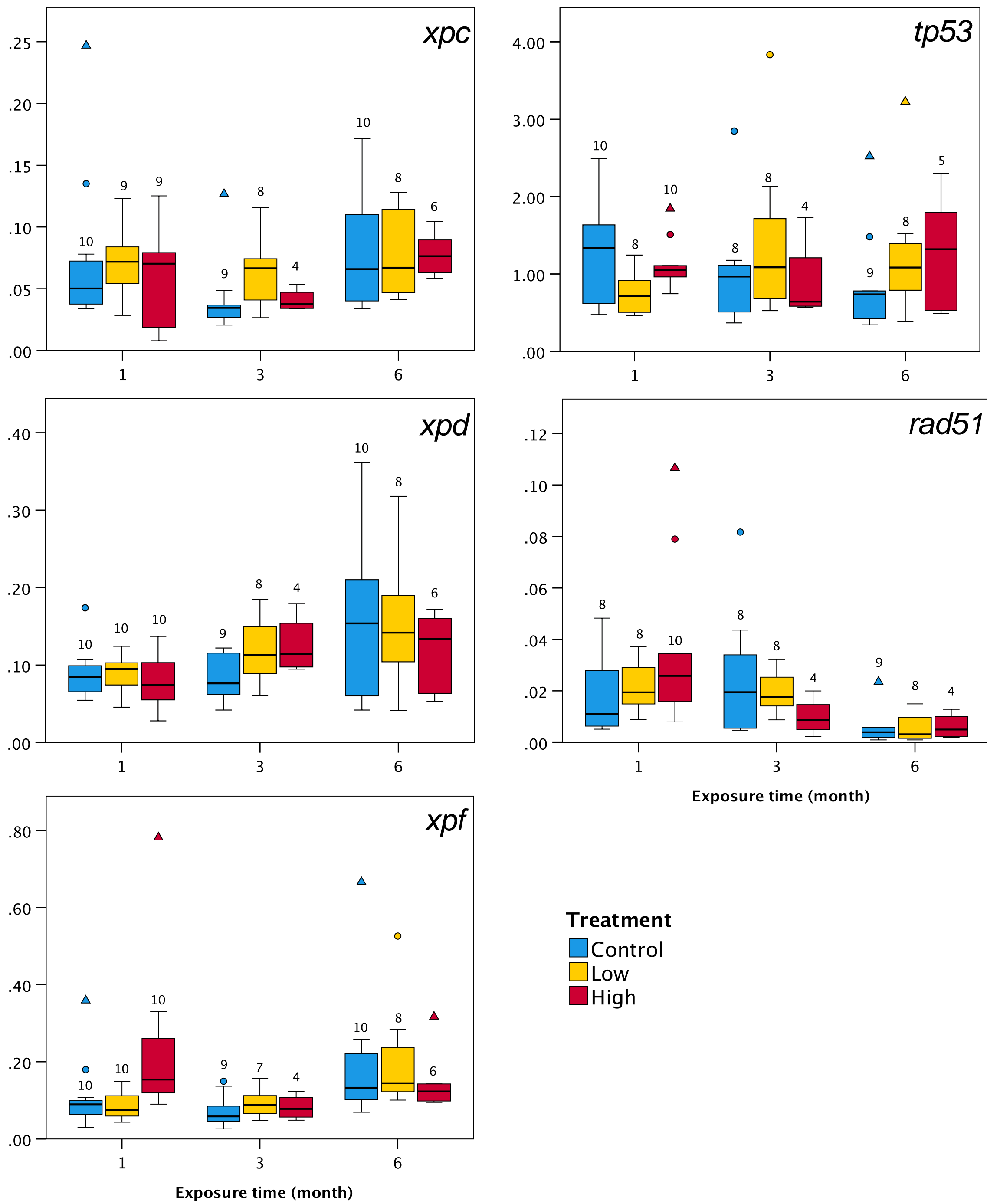
794 Figure 2



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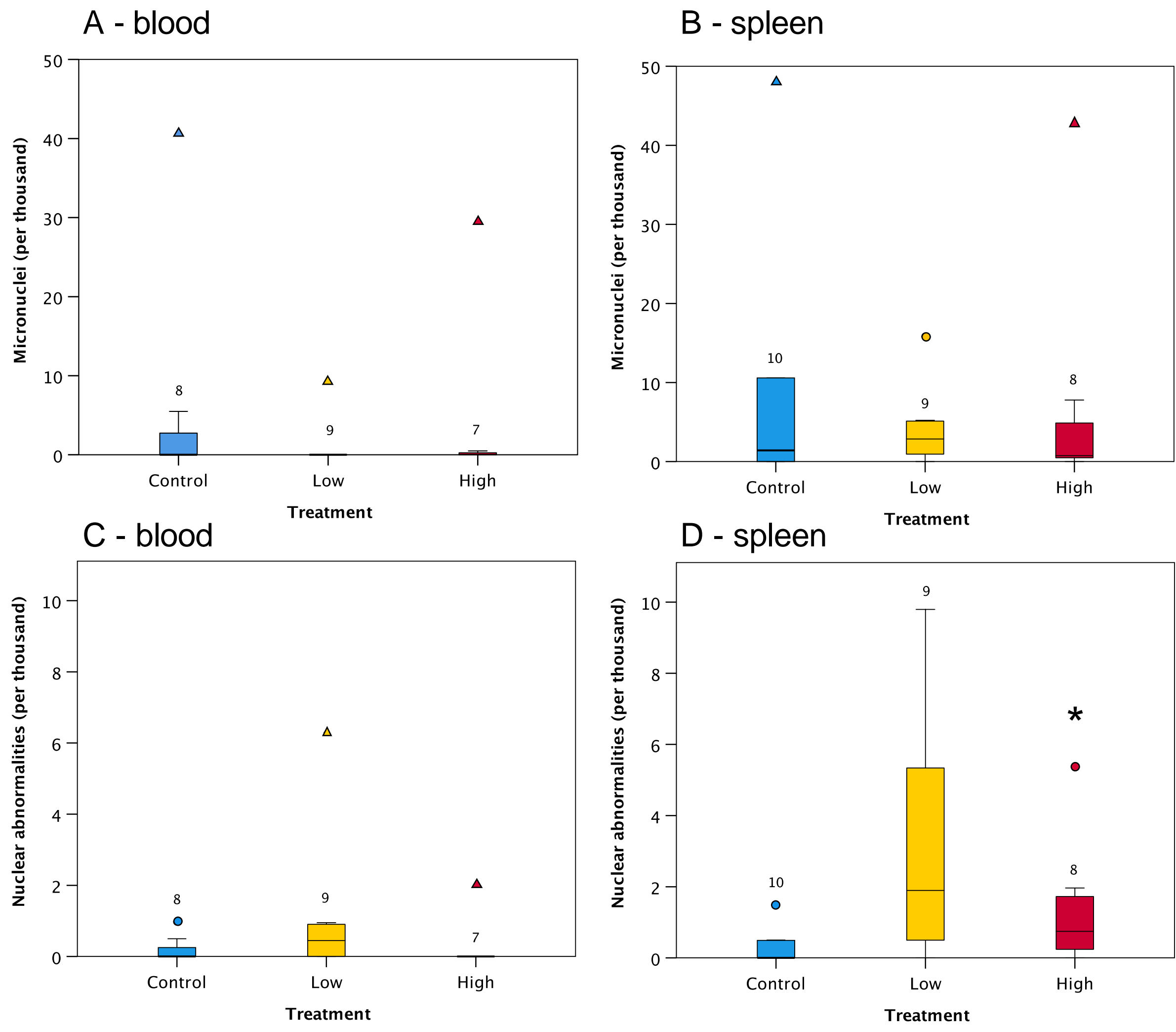
801 Figure 3



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806 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

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819 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

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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*
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).