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Susceptibility of polar cod (*Boreogadus saida*) to a model carcinogen / Lerebours, A.; Murzina, S.; Song, Y.; Tollefsen, K. E.; Benedetti, M.; Regoli, F.; Rotchell, J. M.; Nahrgang, J.. - In: MARINE ENVIRONMENTAL RESEARCH. - ISSN 0141-1136. - ELETTRONICO. - 170:(2021). [10.1016/j.marenvres.2021.105434]

Availability:

This version is available at: 11566/295505 since: 2024-04-08T16:13:29Z

Publisher:

Published

DOI:10.1016/j.marenvres.2021.105434

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Marine Environmental Research

Susceptibility of the key Arctic fish polar cod (*boreogadus saida*) to a model carcinogen --Manuscript Draft--

Manuscript Number:	MERE-D-21-00104R1
Article Type:	Full Length Article
Keywords:	Carcinogenesis; polar cod; Benzo(a)pyrene; cancer genes; DNA repair genes
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Abstract:	<p>Studies that aim to characterise the susceptibility of the ecologically relevant and non-model fish polar cod (<i>Boreogadus saida</i>) 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.</p> <p>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 transcript 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.</p>
Suggested Reviewers:	<p>Jim Smith jim.smith@port.ac.uk Jim Smith is an eminent scientist in the field of environmental pollution (fish).</p> <p>Lourdes Cruz lourdescruzg@gmail.com Lourdes Cruz is an excellent fish toxicologist working at MRC, UK.</p> <p>Angelique Lazartigues alazartigues@yahoo.fr Angelique Lazartigue is a great fish ecotoxicologist.</p> <p>Helene Thomas helene.thomas@univ-lr.fr</p>

	Helene Thomas has a great expertise in the area of aquatic toxicology.
Response to Reviewers:	

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

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

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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 transcript 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 $3 \cdot 10^{-4}$ $\mu\text{g BaP /g per day}$, 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 (2nd of July 2014 to 31st 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:

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

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

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-glucuronidase/arylsulphatase (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 C18 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

hexa 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 FAMTM-TAMRATM 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 BiosystemsTM ViiATM 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 ($0.03 \mu\text{g BaP/g}$ of fish/week) and high ($0.3 \mu\text{g BaP/g}$ 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 foci 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 fo BaP exposure ad biotransformation in many fish species including polar cod (Baake *et al.*, 2016; Baali *et al.*, 2016; Kammann *et al.*, 2017; 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 dietarily 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 dietary exposed 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 (Nahrgang *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 μ M 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 μ M) 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, *Platichthys 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.

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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 .

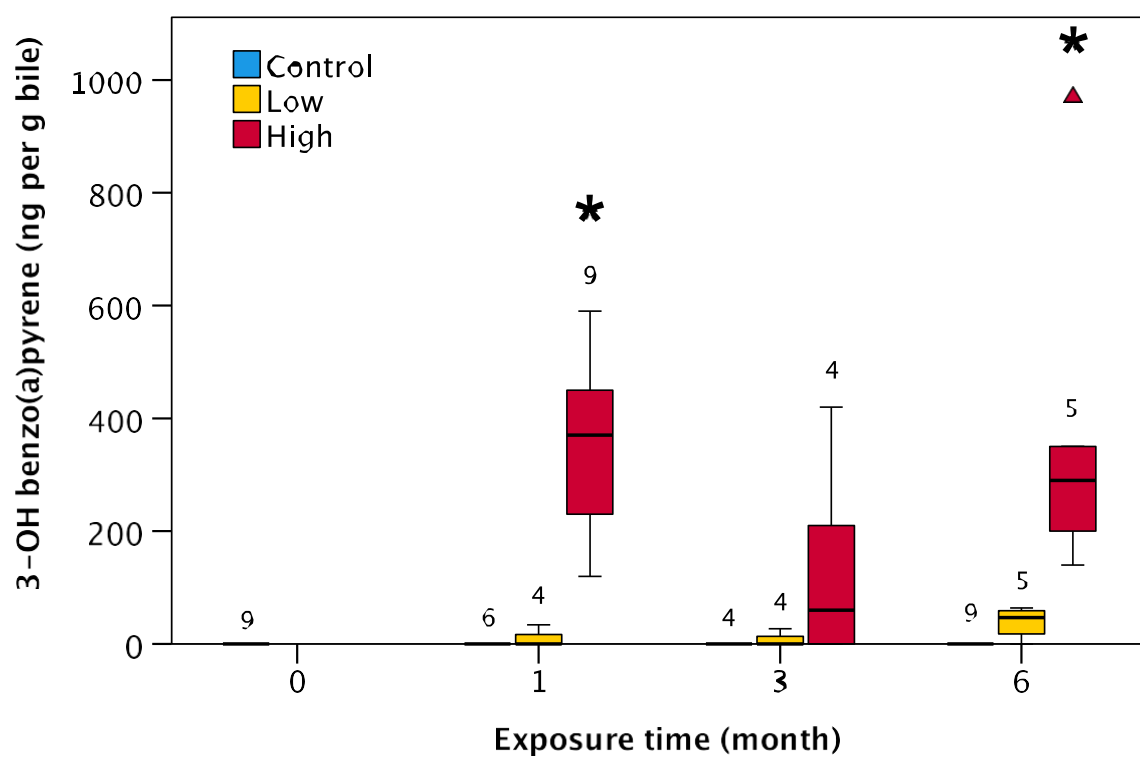
Figure 2. Relative expression of genes (mean \pm SD, arbitrary units) in liver of polar cods ($n = 10$ per treatment and time) exposed to acetone control, low and high BaP treatments after 1, 3 and 6 months. Plots represent the median (line), 25–75% percentiles (box), non-outlier range (whisker), outliers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



776 Figure 2

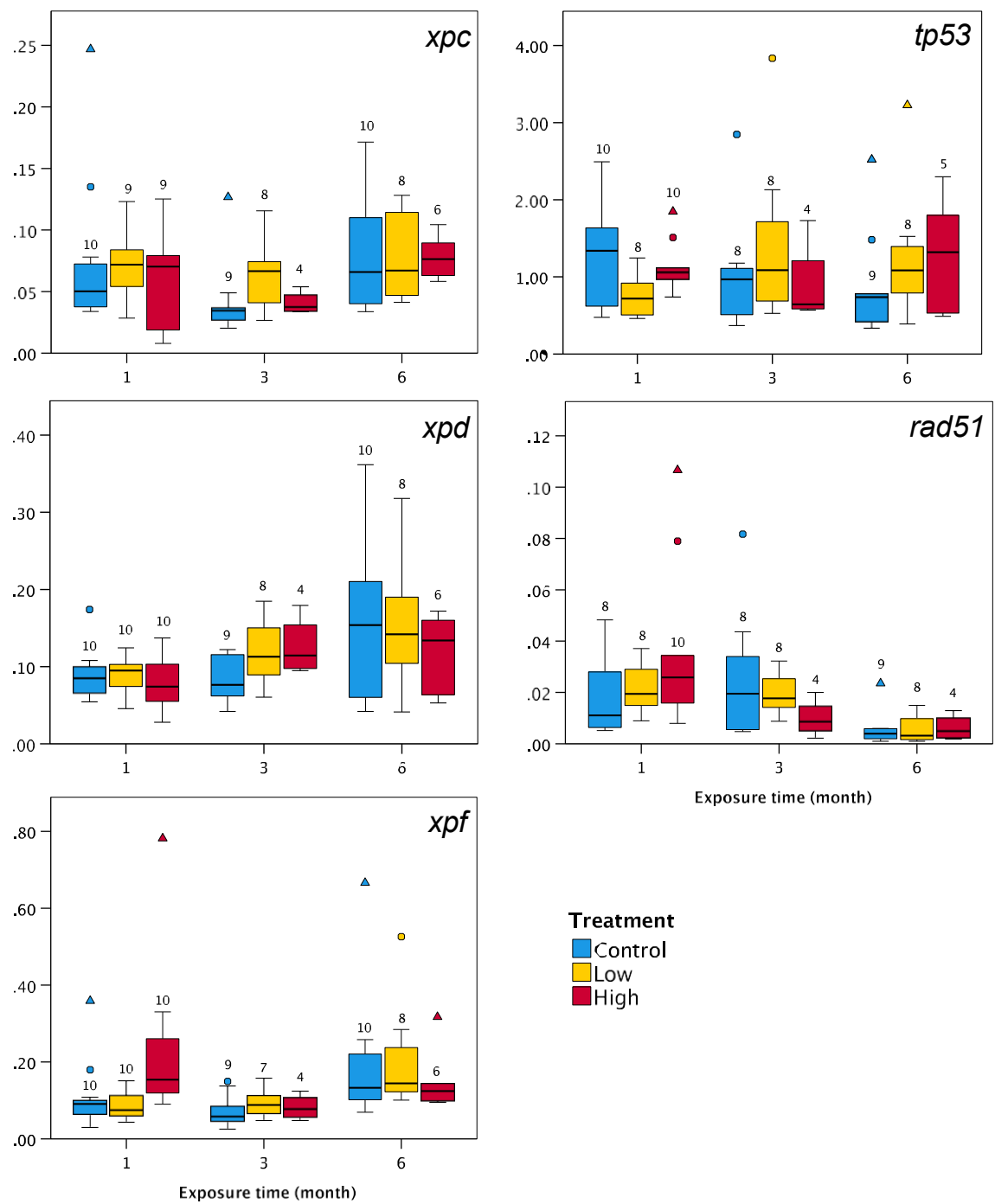


Figure 3

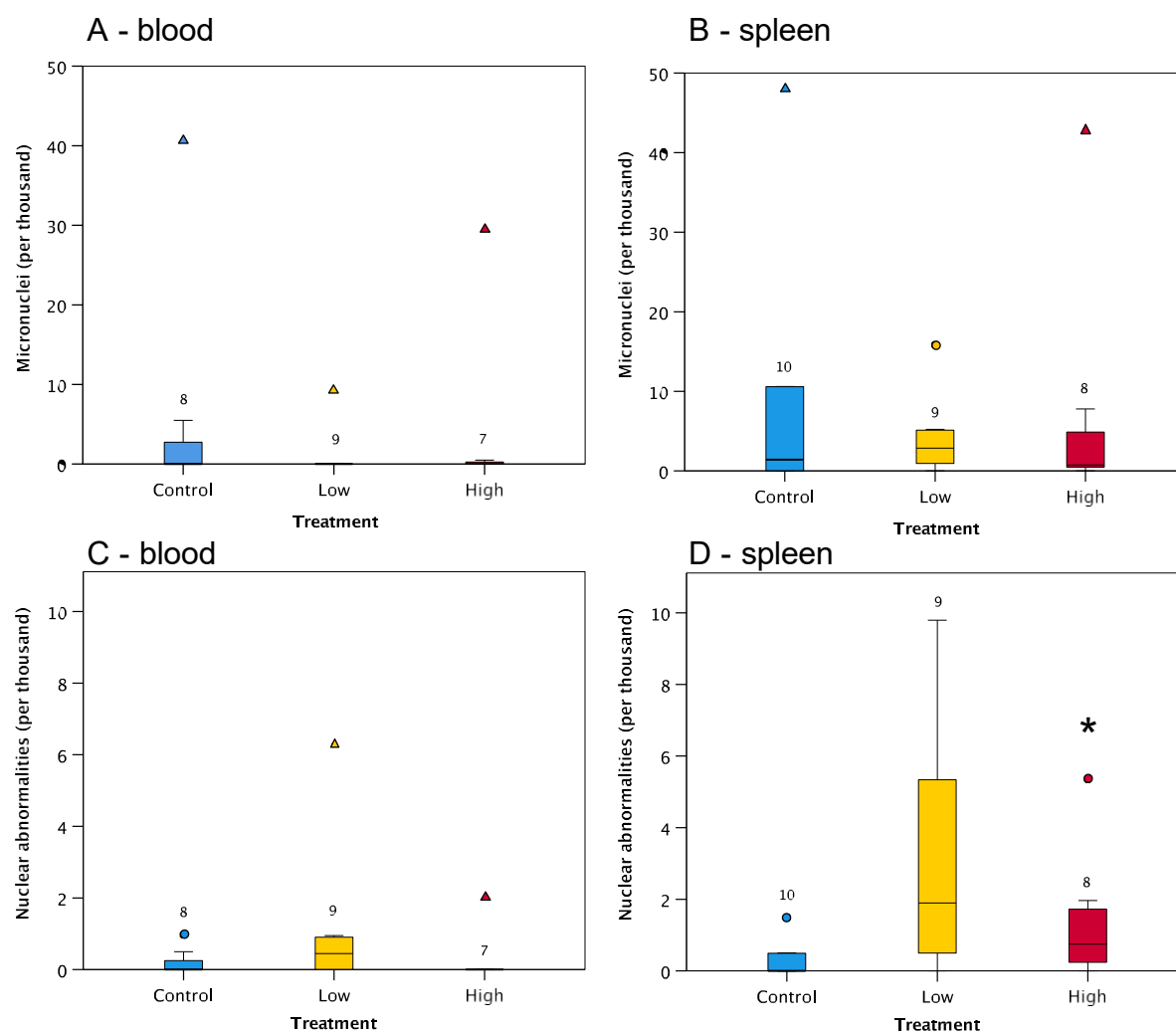


Table 1

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

800

801 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.

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> $\beta$  tubulin
TCTACTACAATGAGGCCTCAGGTAATTAAAGCAATGTCAAATGGATGACGATACTTTCTCTCGTCTGTTTCAGCTCTAACTGTT
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TGCTTTCCGGTCAGATCTTCAGGCCAGACAACTTCGTTTTTCGGCCAGAGTGGTGTGGCAACAACGGGCCAAGGGTCACTACA
CGGAAGGTGCCGAGCTGGTGGACTCTGTGCTCGACGTGGTGAGGAAAGAGGCAGAGAGCTGTGACTGCCTGCAGGGCTTCCAG
CTC

>XPC
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TACTGGTGAAGGGCTTCTGATCAGGGAGAGGCTCCAGCTACGATACGGCAA

>XPD
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GAATCCTGGAGAACATCCAGAGGAACAAGCTGATCTTCATTGAGACGCCAGATGCTGCAGAGACCAGCATGGCTCTGGAGAAA
TACCAGGAGGCATGTGAGAACGGGAGAGGAGCCATCCTTCTGTCTGTGGCCCGAGGAAAAGTGTGCGAAGGAATCGATTCTGT
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ACCTCCGGGACCAAGTTCAGATCCGGGAGAACGACTTCCCTGACGTTTCGACGCCATGCGCCATGCGGCCAGTGCCTGGCCCG
GTCATCAGGGGCAAGACGGACTACGGACTCATGATCTTCGTGACAAGCGCTACGCCCGGGCGGACAAGCGGGGGAAGCTGCC
CCGCTGGATCCAGGAGCATCAGCGACGGCAGCCTGAACCTCACGGTGGACGAGACGGTGCAGCTCTCCAAGCACTTCTCTGA
GGCAGATGGCCAGCCCTTCAGACAGGAGGACCAGCTGGGTCTGTCACTGCTGACGATAGAACAGCTGGAGTCAGAGGAGATG
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>XPF
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TCACCGCGCTCTCCAAGGAGAAGAAAGCCTTCGAACACCTCATCAGGGAGAAGGCGACCATGGTTGTGCCGGAGGAGCGAGAG
GGTCGAGAAGACACCAATCTGGACCTGGCGAGGAATCTGGAGCCCGCCAACGCTACCACCAACACCCGCAAGCAGGAGGCCA
GGACAGCCCGAGGAGCCCTCCCGGGTCATCGTGACATGCGGGAGTTCCGCAGCGAGCTGCCCTCCCTGCTGCACCGCCGCG
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GACCTGATCGGCTCGCTGCAGAGCGGCCGCTCTACACGCACTGCGTGTCCATGACGCGCTACTACAAGCGGGCCGCTGCTGCT
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GAGGAGATGAAGCGGGGCGCGGCGAGCCGCGCCGCGCGCAGGCCATCGCGGCCGAGTCGGACGCCAGGACGACGGC
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AGGCGGACAGCCTGGCCGCGCTGGCCGAGTTAGCCAGGAGAGGCTGGCGCAGGTCTGGGGCACACCGGCAACGCCAAGATG
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>rad51
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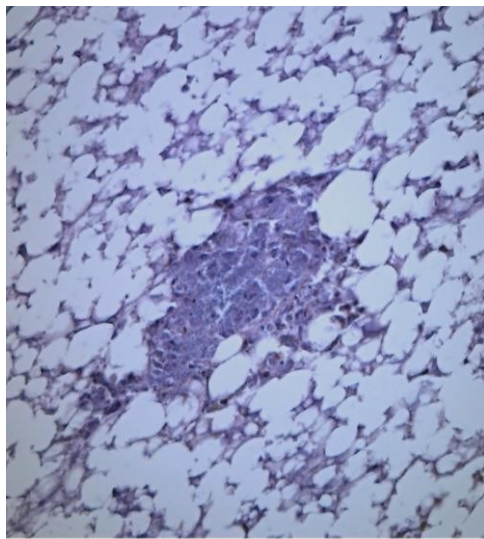


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>p53
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3 GGAGGGCAACGTTGGAGAAAAAGACGGAGGGATCCAAGCCCACCAAAAAAGAAAGAGCCCCCCCCACTCTGGCCCCACGGCT
4 CCGCCCAAGAGGGTCCTGTCCGCTCCAGCGCTGAAGAGGAGGATAAGGAGGTGTTTGTGCTACAGGTCGTTGGCCGGAAGAG
5 ATTCGAGATCCTGAGGCAGATAAACGATGCACTCGCGCTGCAGGAGAGGATGACAGTCAAGCAGGAGGTCCAAGGAGGGCCGT
6 CGCGGGGAAAGAGACGGCTGGGGGACCGGACAGACGAGGGGACCGACTGAGCGACCGTCCAACCGACCGTCCAACATAACACT
7 GCCACAGCGAACCCCGTTATTTTCTACACTTTTCTTTGTCATTCCTATTTTATTTTTTTCATAGCTTTTTTTCAGCATA
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9 AGGCCATG

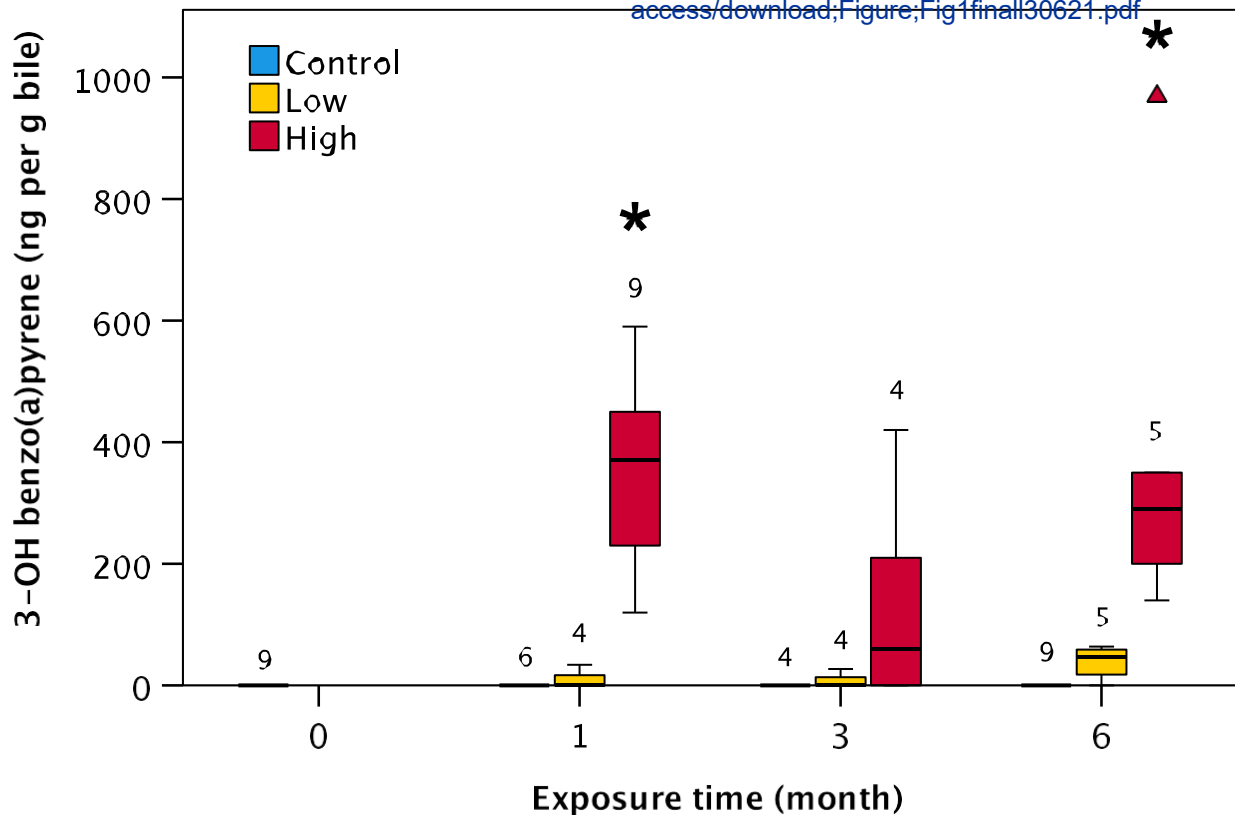
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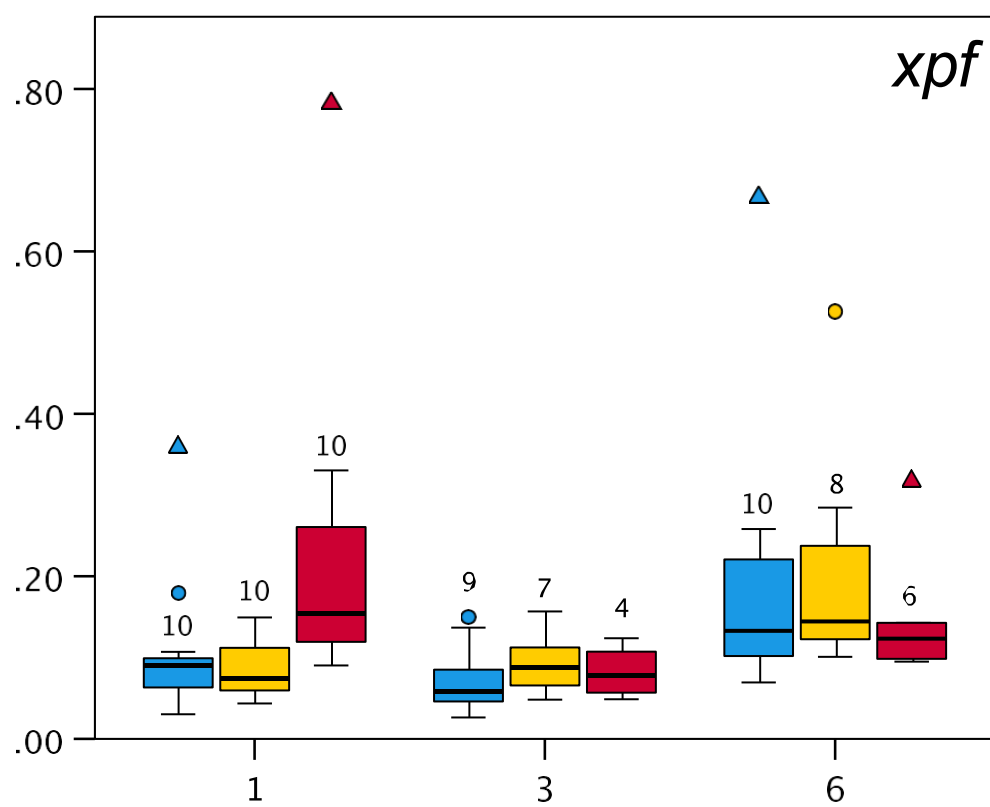
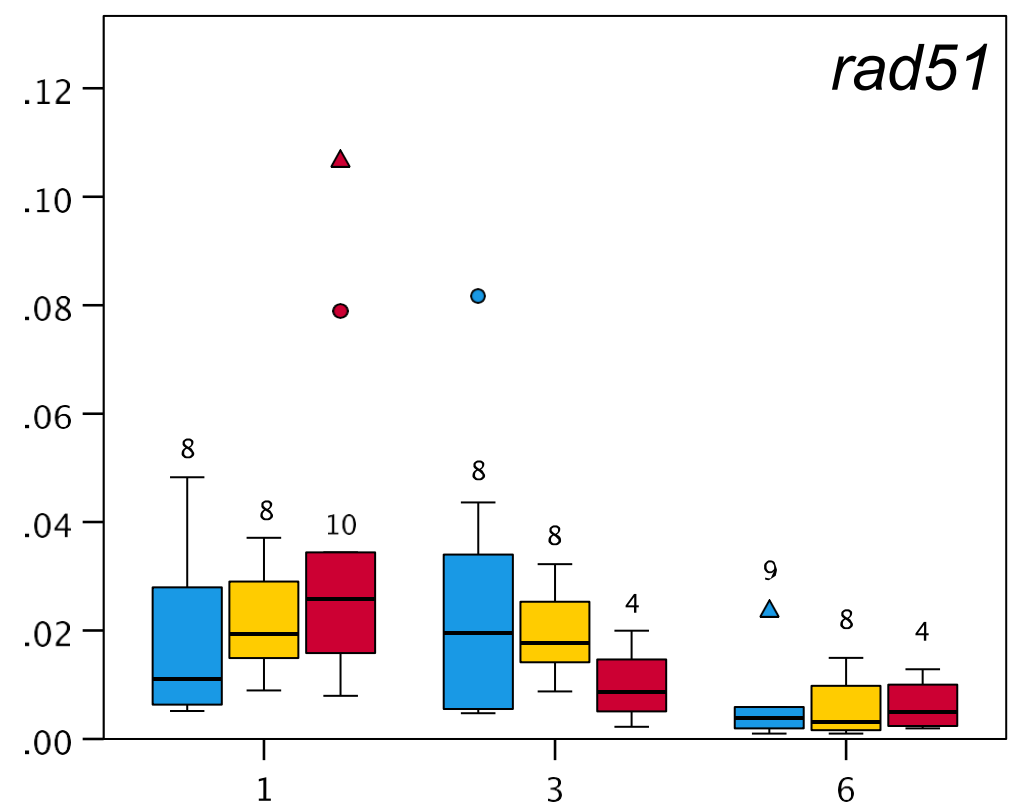
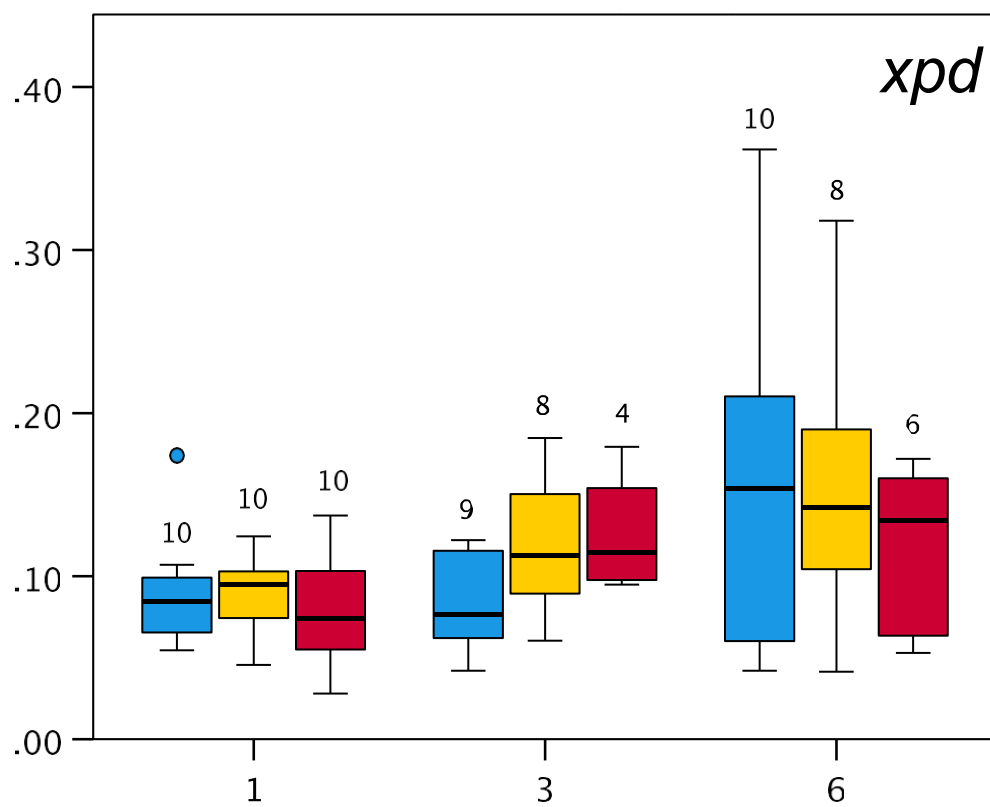
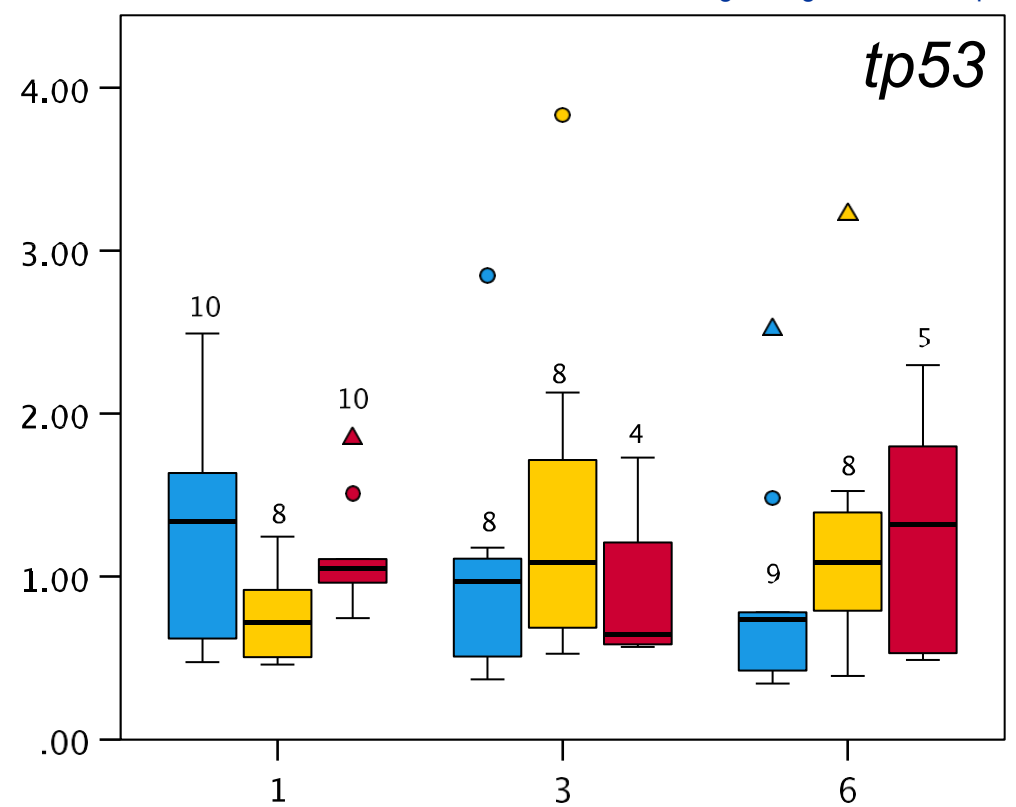
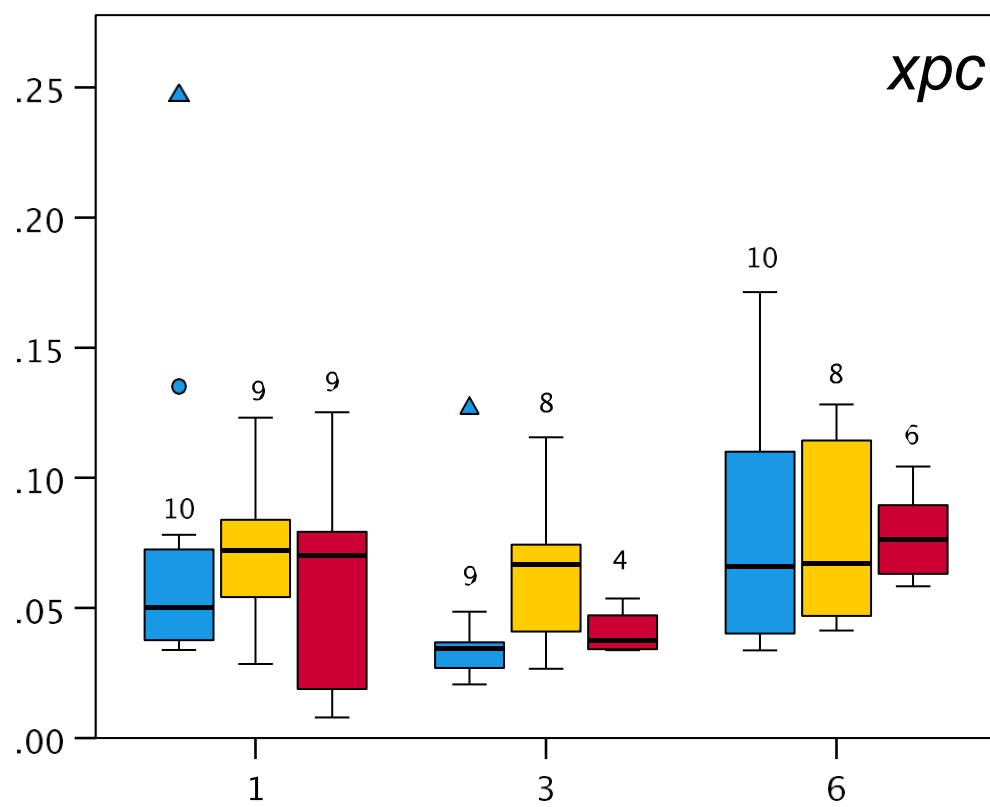
Figure S2. Basophilic foci of cellular alteration diagnosed in liver of a polar cod exposed to the low BaP concentration (magnification x40).



Figure

[Click here to access/download;Figure;Fig1final30621.pdf](#)



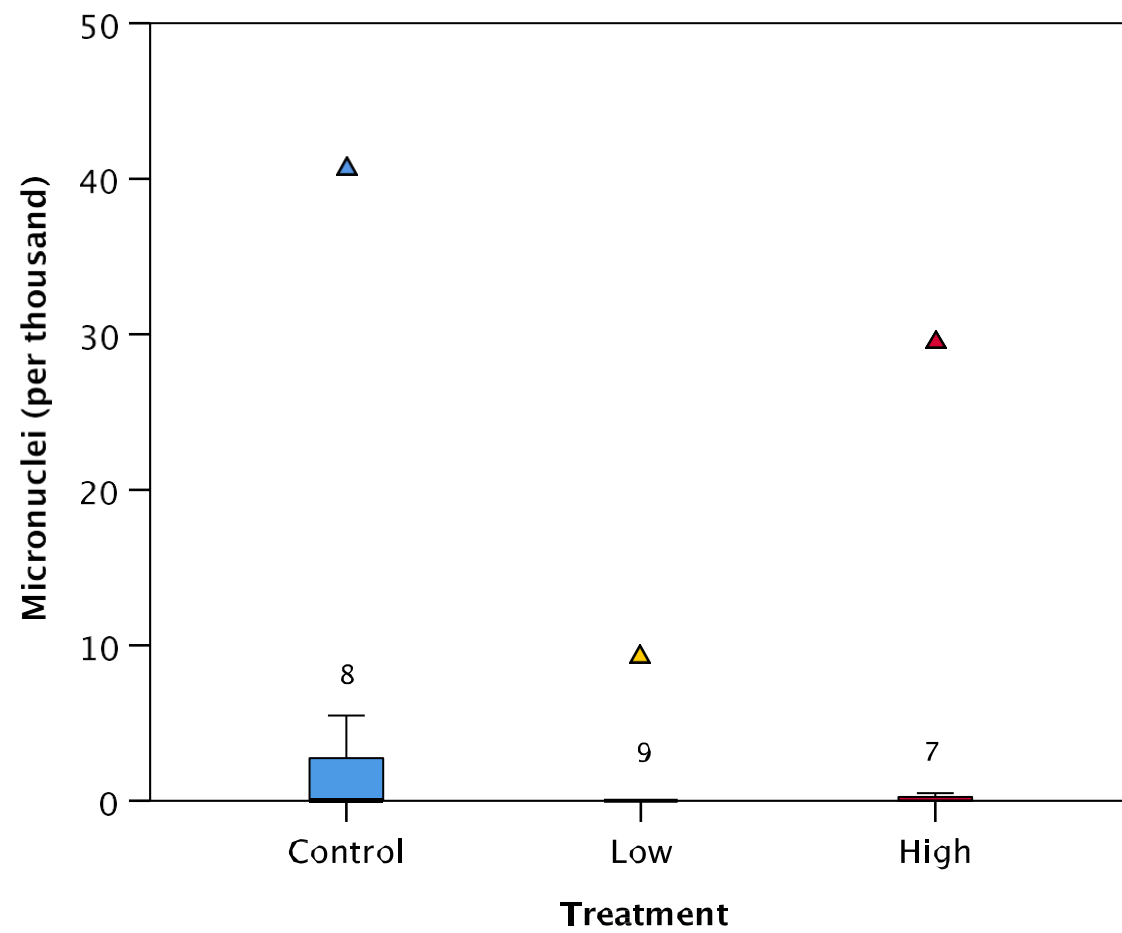
**Treatment**

Control

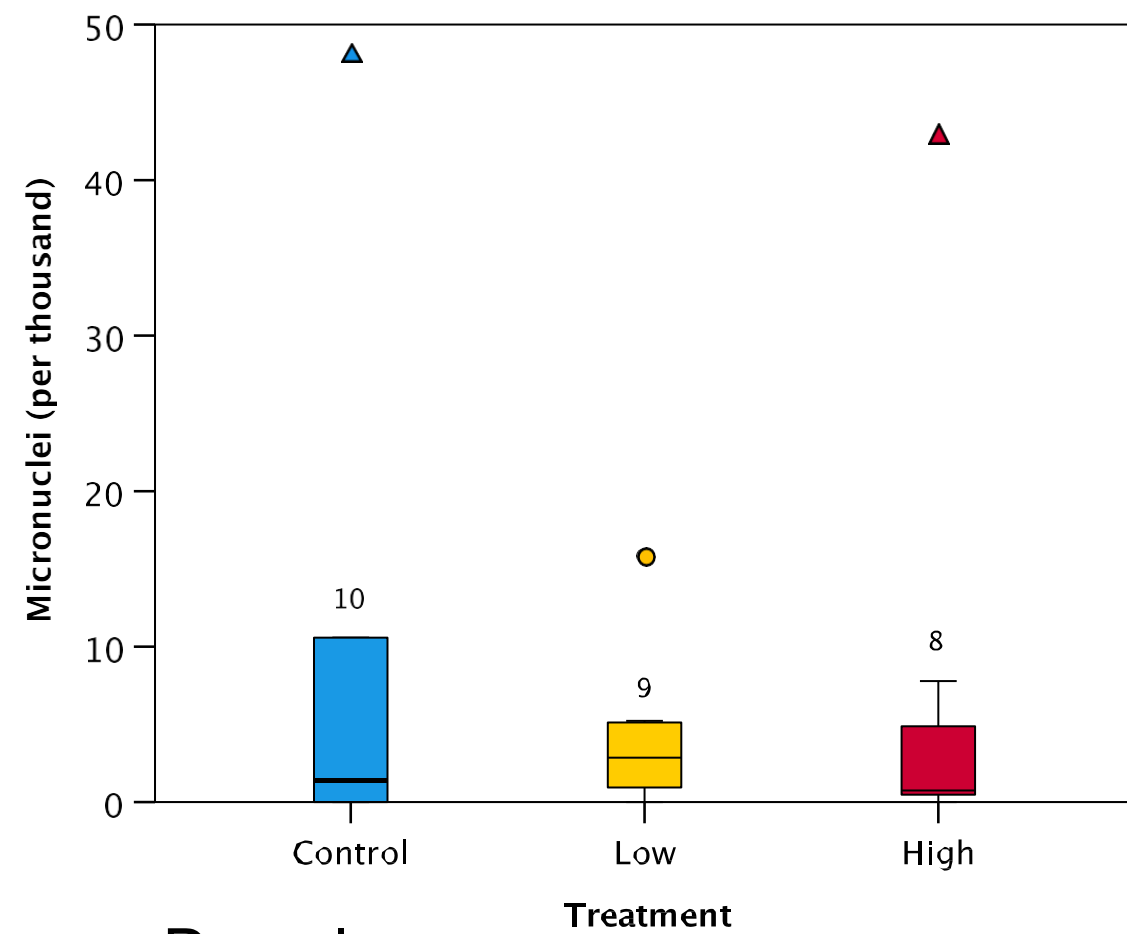
Low

High

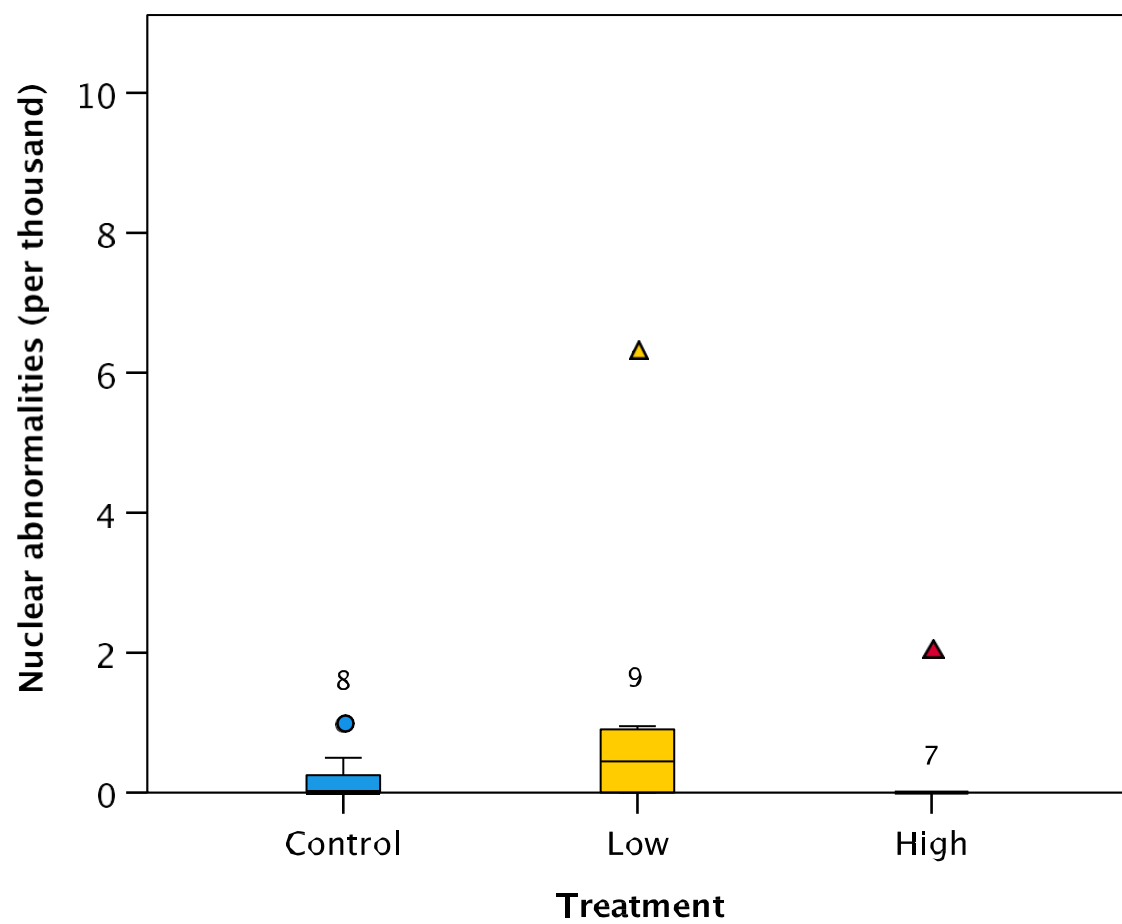
A - blood



B - spleen



C - blood



D - spleen

