Title

Effects of dietary crude oil exposure on molecular and physiological parameters related to lipid homeostasis in polar cod (*Boreogadus saida*)

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Abstract

Polar cod is an abundant Arctic key species, inhabiting an ecosystem that is subjected to rapid climate change and increased petroleum related activities. Few studies have investigated biological effects of crude oil on lipid metabolism in this species, despite lipids being a crucial compound for Arctic species to adapt to the high seasonality in food abundance in their habitat. This study examines the effects of dietary crude oil exposure on transcription levels of genes related to lipid metabolism (*peroxisome proliferator-activated receptors [ppar-α, ppar-γ], retinoic X receptor [rxr-β], palmitoyl-CoA oxidase [aox1], cytochrome P4507A1* [*cyp7α1*]), reproduction (*vitellogenin [vtg-β], gonad aromatase* [*cyp19a1*]) and biotransformation (*cytochrome P4501A1 [cyp1a1], aryl hydrocarbon receptor [ahr2]*). Exposure effects were also examined through plasma chemistry parameters. Additional fish were exposed to a PPAR-α agonist (WY-14,643) to investigate the role of PPAR-α in their lipid metabolism. The dose-dependent up-regulation of *cyp1a1* reflected the activation of genes related to PAH biotransformation upon crude oil exposure. The crude oil exposure did not significantly alter the mRNA expression of genes involved in lipid homeostasis except for *cyp7α1* transcription levels. Plasma levels of cholesterol and alanine transaminase showed significant alterations in fish exposed to crude oil at the end of the experiment. WY exposure induced a down-regulation of *ppar*-α, an effect contrary to studies performed on other fish species. In conclusion, this study showed clear effects of dietary crude oil exposure at environmentally relevant concentrations on xenobiotic biotransformation but revealed only weak alterations in the lipid metabolism of polar cod.

Key words: biotransformation, crude oil, lipid metabolism, peroxisome proliferator-activated receptors, plasma chemistry, polar cod, reproduction.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are regarded as the primary toxic constituents in crude oil and are commonly studied with regard to biological effects of petroleum exposure in fish (e.g. Kane Driscoll et al., 2010; Vignet et al., 2014). Several effects have been related to PAH exposure in fish such as reduced growth (Meador et al., 2006; Vignet et al., 2014), diminished biological fitness (Kennedy and Farrell, 2006), immune dysfunction (Reynaud and Deschaux, 2006) and impaired reproduction (Nicolas, 1999). PAHs have also been shown to cause peroxisome proliferation in fish, a response characterized by an increased number and volume density of peroxisomes, usually accompanied by the transcriptional up-regulation of peroxisomal β-oxidation genes (Cajaraville et al., 2003). This process is suggested to be mediated through a subfamily of nuclear receptors called peroxisome proliferator activated receptors (PPARs) (Cajaraville et al., 2003), which also have been recognized as important lipid sensors and transcription factors that regulate lipid homeostasis in mammals (Feige et al., 2006). The three PPAR isotypes (α, β/δ, γ) are identified in marine fish (Andersen et al., 2000; Leaver et al., 2005; Raingeard et al., 2009) and a study on sea bass (*Dicentrarchus labrax*) suggested similar functions of marine fish PPARs as in mammals (Boukouvala et al., 2004). Although PAHs are identified as ligands for PPAR-α in human cells (Kim et al., 2005), this interaction is not known for fish. However, several studies have shown that petroleum compounds affect transcription levels of genes related to lipid metabolism (Bilbao et al., 2010; Adeogun et al., 2016; Xu et al., 2016; Cocci et al., 2017). Furthermore, PAH exposure altered lipid plasma parameters in Chinook salmon (*Oncorhynchus tshawytscha*) in a similar pattern of that found in starving fish (Meador et al., 2006). The physiological fasting response has been related to an up-regulation of PPAR-α transcription in mammals (Leone et al., 1999) and consequently, the effects of PAHs on lipid metabolism in fish could potentially be governed by the key regulator of lipid homeostasis, PPAR-α.

The Arctic is undergoing rapid climatic changes and climate models predict an ice-free Arctic Ocean during summer month by the middle of this century (IPCC, 2013). A reduction in sea ice unveils new opportunities for the petroleum industry, allowing exploration of petroleum resources on the Arctic continental shelves. The exploitation of petroleum resources in Arctic waters would, however, increase

the risk of marine oil spills in this pristine ecosystem which could have detrimental effects on fish health and ultimately on fish populations (Peterson et al., 2003). Hence, the present study aimed to investigate the biological effects of crude oil exposure on an Arctic fish species that has been studied comprehensively during the last years. Polar cod (*Boreogadus saida*) is an important key species of the Arctic marine ecosystem that is highly abundant and circumpolar distributed in Arctic waters (Hop and Gjøsæter, 2013) Itis an energy-rich and favored food item for Arctic marine predators such as sea birds and marine mammals and thereby represents an essential trophic link in the marine ecosystem of the Arctic (Hop and Gjøsæter, 2013). The high seasonality in light availability in this environment causes strong fluctuations in the availability of food for polar cod throughout the year and requires the rapid accumulation of energy in the form of lipids during summer month. This allows polar cod to survive months with sparse food available in the water column and it also enables the successful reproduction in winter. The present study examined the effects of crude oil exposure on important metabolic processes related tolipid homeostasis, reproduction and xenobiotic biotransformation in polar cod (*Boreogadus saida*). Although biotransformation of PAHs has been previously investigated (Nahrgang et al., 2010a; 2010b), only few studies have examined the effects of petroleum-related compounds on processes relevant for lipid homeostasis and reproductive development in this species (Geraudie et al., 2014; Andersen et al., 2015; Bender et al., 2016). We hypothesized that crude oil exposure would affect mRNA expression of genes relevant for key processes in lipid metabolism (*ppar-α, ppar-γ, retinoic X receptor [rxr-β], palmitoyl-coenzyme A oxidase [aox1], cytochrome P4507A1 [cyp7α1]),* reproduction (*vitellogenin [vtg-β], gonad aromatase* [*cyp19a1*]) and biotransformation metabolism (*cytochrome P4501A1 [cyp1a1], aryl hydrocarbon receptor 2 [ahr2]*). We also expected crude oil to alter physiological indicators for lipid metabolism, as was found in salmon after dietary PAH exposure (Meador et al., 2006).

To study the biological effects of crude oil exposure we performed an experiment with wild polar cod that were exposed to three different crude oil doses at environmentally relevant concentrations for 4 weeks. Samples for molecular and physiological analyses were taken at five time points during the experiment and analyzed for endpoints related to lipid metabolism, reproduction and

100 biotransformation. In addition, samples were also used for a parallel study that examined the effects of
101 crude oil exposure on the antioxidant defense system and further endpoints related to
102 biotransformation processes in polar cod, published in Vieweg et al. (2017). As PPARs have been
103 suggested to mediate the adverse effects of PAH exposure on lipid homeostasis in marine organisms
104 (Cajaraville et al. 2003, Bilbao et al 2010), the present study included a potent peroxisome
105 proliferator (WY-14,643 [WY]) as additional treatment in the exposure experiment. WY is a PPAR-α
106 agonist and *aox1* regulator in mammals (Berger and Moller, 2002) and fish (Colliar et al., 2011;
107 Urbatzka et al., 2015) and was used to investigate the potential role of PPARs in regulating lipid
108 metabolism in polar cod. Previous experimental work on polar cod suggested dietary exposure as a
109 relevant exposure route of lipophilic petroleum compounds (George et al., 1995; Nahrgang et al.,
110 2010b; Bender et al., 2016). Polar cod shows slow gastrointestinal evacuation rates (Hop and Tonn,
111 1998) and high assimilation efficiencies (Hop et al., 1997), which was suggested to cause a high
112 metabolic absorption of petroleum compounds (Nahrgang et al., 2010b). Other experimental fish
113 studies have identified food as an important pathway for crude oil compounds to enter the organism
114 and elicit adverse effects (e.g. Saborido-Rey et al., 2007; Martin-Skilton et al., 2008; Olsvik et al.,
115 2011; Bratberg et al., 2013).

116 **2. Materials and methods**

117 **2.1 Fish sampling and rearing**

118 Polar cod were caught by trawling in Billefjorden and Rijpfjorden (Svalbard, Norway, latitude 79° N)
119 during late January 2013, using the same trawling set-up as described in Nahrgang et al. (2010b). The
120 research vessel R/V Helmer Hanssen, owned by the UiT-The Arctic University of Norway, is
121 authorized by the Norwegian Fishery Directorate to perform bottom trawling to catch fish for
122 scientific purposes. Fish were kept on board the research vessel in 500 L tanks supplied by constant
123 running seawater until transferred to the research facilities of UiT-The Arctic University of Norway in
124 Kårvika (Norway, latitude 69° N). Here, polar cod were kept in 60 µm filtered seawater supplied from
125 the nearest fjord (Kvalsundet) with water flow at 7-10 L/min and temperature of 3 to 4 °C. Fish were
126 acclimated for 3 months to the laboratory conditions. During acclimation, polar cod were given frozen

C*alanus* sp. (purchased from CALANUS AS) *ad libitum* three times per week. One month prior to the start of the experiment, 250 fish were distributed into six experimental tanks (300 L) with 40 fish allocated to each of the 5 treatment tanks (3 crude oil treatments, 1 treatments for the PPAR-α model agonist WY and 1 treatment tank for the solvent control) and 50 fish allocated to the control tank. During this final acclimation step and the subsequent experiment, seawater supplied to the tanks was maintained at a mean (±SD) water temperature of 3.6 ºC (± 0.3) and a mean dissolved oxygen level of 91.7 % (± 5.2). The light regime in the tanks reflected *in situ* conditions in Svalbard (latitude 69º N) between April and May that is civil twilight, with 24 hours daylight and lower light intensities during night. The experimental work was done in accordance with the laws of the Animal Welfare Act and regulations of the Norwegian Animals Research Authority (ID 5271). The experimental work was performed by the lead author, who has the necessary training and certificate (FELASA Category C) to perform experimental work with animals.

2.2 Experimental design

The set-up of the study consisted of two parallel feeding experiments, where polar cod specimens were exposed for 32 days to either Goliat Kobbe crude oil at four different doses (control, low, medium and high) or to the PPAR-α model agonist WY-14.643 (WY) and the appurtenant solvent control (acetone). Kobbe crude oil is a light crude oil that is produced and transported in the Barents Sea (Sørheim and Moldestad, 2008), hence a crude oil type that polar cod could be exposed to in a potential oil spill in Arctic waters. The feed preparation for the crude oil treatments and a detailed PAH composition of the fish feed are described in detail in Vieweg et al. (2017). Briefly, *Calanus* sp., a relevant and important natural food of polar cod (Hop and Gjøsæter, 2013), was mixed with four different doses of crude oil (0, 0.5, 2, 4 mg crude oil/ g feed) without any solvent vehicle with a magnetic stirrer for 5 minutes. For the WY feeding experiment, the WY chemical was at first dissolved in acetone (16.8 µg/ µL acetone) and subsequently mixed to *Calanus* sp. at a final concentration of 1.7 mg WY per g feed (Table 1). The appurtenant solvent control (So-Co) was prepared by mixing 101 µL acetone per g *Calanus* sp. Following, the acetone was volatilized both from the WY and So-Co treatments by constant stirring on a magnetic stirrer for 2.5 hours at 30 ºC.

For all six feed preparations, starch (20% of the total feed weight) was added in order to increase the consistency of the feed and to allow force-feeding with minimal regurgitation from the fish. Food was supplied to the fish through force-feeding in order to control the exact dose received by each individual fish.

Every 4th day and in total 8 times, fish were force-fed 0.6 g feed with either of the 6 feed treatments, aiming to provide the fish with approx. 3% of their body weight (based on the mean total weight of all fish at the experiment start). The feed was carefully introduced into the fish`s throat with the help of a 1 mL Tuberculin syringe. The mean (± SD) dietary crude oil doses received by the fish corresponded to 0, 3.9 ± 0.9, 15.5 ± 3.0 and 26.3 ± 6.0 µg crude oil/ g fish/ day for control, low, medium, and high doses, respectively (Table 1). The WY dose corresponded to 11.8 ± 2.3 µg WY/ g fish/ day (Table 1). Directly after feeding, fish were kept under observation to control for regurgitation and a maximum of 5% feed loss was anticipated per fish and feeding, based upon results in a previous pilot experiment.

Fish were sampled every 8th day (n=10 fish/ treatment) for a total of five time points (day 0, 8, 16, 24 and 32), whereas only 10 control fish were sampled at the experiment start (day 0). At each sampling point, fish were anesthetized in a Finquel® bath (50 mg Finquel®/L water) and blood was sampled; thereafter fish were sacrificed with a sharp blow to the head. Blood (approximately 0.3 mL) was taken with a heparinized syringe from the caudal vein and transferred to a heparinized vial. The samples were kept on ice until centrifugation for 30 minutes at 4 ºC (3500 rpm). Total and somatic (excluding gut, liver and gonads) weights (± 0.1 g), total length (± 0.1 cm) and sex of each fish was recorded. Sections of liver and gonad were immersed in RNAlater® (Ambion, Thermofisher Scientific) right after being dissected out of the fish, the samples were kept on ice until they were snap frozen in liquid nitrogen and stored at -80 º C until molecular analyses (approx. 3 months of storage). For histological analysis, the middle section of the gonad was stored in 4% neutral buffered formalin (v:v). Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated according to the following equations: GSI = (gonad weight/ somatic weight) x 100 and HSI = (liver weight/ somatic weight) x 100.

2.3 Gene transcription levels

2.3.1 RNA extraction, cDNA synthesis and sequencing of target genes

In order to obtain total RNA from liver, 50 - 100 mg tissue of 8 -10 fish per treatment group from each time point (Table 2) was homogenized individually in TRIZOL® reagent (Invitrogen, Thermofisher Scientific), using a Precellys 24 tissue homogenizer (Bertin Technologies). RNA was purified with the RNeasy® MinElute® Cleanup kit (Qiagen). RNA extraction and purification from gonad samples was performed with the help of the RiboPureKit (Ambion Inc, Thermofisher Scientific), where 50 – 100 mg sample of the same fish specimens as for the liver extraction (Table 2) was homogenized in TRI Reagent® in the same homogenizer. RNA purity and concentration was verified and checked in a BioTek Epoch Multi-Volume Spectrophotometer System (Biotek) and in a Bioanalyzer (Agilent Technologies). 1 µg of total RNA was used as template for first-strand cDNA synthesis in a total volume of 40 µL reaction volume, using the Affinity Script Multiple Temperature cDNA Synthesis Kit according to the manufacture's protocol (Agilent Technologies) with a temperature of 50°C for a 1 hour duration for the cDNA synthesis. Degenerate primers were designed for *ppar-α, ppar-γ, rxr-β, aox1, cyp7α1, vtg-β, cyp19a1* and *18srRNA* for polar cod based on conserved regions of the aligned target sequences of phylogenetically related teleost fish species from GenBank. PCRs were cycled both in liver and gonad samples of polar cod and positive amplicons were sent for sequencing to the Sequencing and Genotyping Service of the University of the Basque Country. Partial sequences were confirmed with BLAST analysis (GenBank) and used to design specific primers for polar cod (Table 3) with the help of PRIMER EXPRESS 3.0 (Applied Biosystems, Thermofisher Scientific) and PRIMER 3 software. Primers for *cyp1a1, ahr2* and *β-actin* were obtained based on published polar cod sequences in GenBank (GenBank accession number **EU682947**, **EU682946** and **EU682944**, respectively).

2.3.2 Quantification of gene transcription levels

Transcription levels of *cyp1a1, ahr2, ppar-α, ppar-γ, rxr-β, aox1, cyp7α1, vtg-β, β-actin* (in liver) and *cyp19a1* and *18srRNA* (in gonad) were measured by quantitative real-time PCR (qPCR) using SYBR® Green fluorescence dye master mix (Roche). Each PCR reaction contained a total volume of 20 µl (10

207 µL SYBR® Green fluorescence dye master mix, 0.25 or 0.5 µL of specific primers depending on
208 primer concentration [Table 3], 2 µL cDNA and 7.5 or 7.75 µL RNAse free water) run in 96-well
209 plates (clear transparency in plate plastic) for gonad samples and run in 384-well plates (clear
210 transparency in plate plastic) for liver samples. The latter plates were filled with machine-aid on the
211 robotic workstation Freedom EVO® 75 (Tecan). Following, the plates were sealed with qPCR plate
212 seals (Thermofisher Scientific) and shortly spun down in the centrifuge (1000 rpm, 1 minute, 4 °C).
213 All samples were run in triplicate on a ViiA ™ Real-time PCR system (Applied Biosystems,
214 Thermofisher Scientific) and for each run the plate contained samples from all time points and
215 treatments of the experiment. The qPCR conditions were optimized for each primer (Table 3) with
216 composite samples containing aliquots of all liver or gonad samples, respectively. Each PCR included
217 a standard curve in order to calculate the amplification efficiency of each qPCR, which was close to
218 100% efficiencies for all qPCR runs except for *ppar-γ* (61 %) and *aox1* (70%). Furthermore, triplicates
219 of non-template control reactions as well as retro-transcription controls were included in each run in
220 order to check the presence of non-specific amplicons and verify the analytical sensitivity and
221 specificity of the assay. Relative gene transcription levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak
222 and Schmittgen, 2001) after correction for qPCR efficiencies and normalization to transcription levels
223 of an endogenous reference gene (Pfaffl, 2001) with a coefficient of variation below 5 %. The
224 transcription levels of the endogenous reference genes 18S ribosomal RNA (*18srRNA*) (Genbank
225 accession number: **KT985386**), elongation factor 1-a (*Eefa1*) (Genbank accession number:
226 **ABD62881.1**) and *β-actin* (*β-actin*) (Genbank accession number: **EU682944**) were tested in polar cod
227 liver and gonad samples from the different crude oil treatments and time points of the experiment in
228 order to select the adequate reference gene for the data normalization. Since the coefficient of
229 variability of *β-actin* in the liver and *18srRNA* in gonad was lower than 5%, such genes were selected
230 as reference gene for each target tissue. Mean values of transcription levels in control samples from
231 day 0 were used as calibrators.

2.4 Histological analysis of gonads

The formalin-fixed sections of polar cod gonad from the same fish specimens as those analyzed for gene transcription (Table 2) were rinsed and dehydrated in a series of 70% ethanol baths (v:v), and embedded in paraffin wax using Histo-clear® as a clearing agent in a Shandon Citadel 1000 (Micron AS) overnight. Tissues were then embedded into a paraffin block, sectioned at 5 μm thickness using a LEITZ RM 2255 microtome and stained with hematoxylin/eosin. For each fish, 8 to 12 histological slices were prepared. Each slide was examined using a LEICA Laborlux S light microscope at 40 to 250 x magnification in order to determine the gonadal maturation stage and indications of previous spawning. Gonadal maturation stages in polar cod females and males are described in more detail in Bender et al. (2016). Briefly, female ovarian sections (n=95) were categorized by oocyte stage of the most advanced cohort of oocytes observed in the slices. All female oocytes showed primary growth (PG) oocytes (Figure S1), which is indicative of fish being either immature or in a resting stage. The presence of post-ovulatory follicles (POFs) (Figure S1) indicate that the fish spawned in the previous season. Male testicular sections (n=121) were categorized into four progressive gonadal maturity stages classified as testis that were resting, maturing, ripe, or spent (Figure S2). In ripe males, spermatozoa were present in the lumen of lobules but no milt was released from the abdomen of the fish when pressure was applied. Spawning of the male fish was not observed during the experiment.

2.5 Plasma chemistry

Plasma chemistry analyses were run on replicate composite samples containing 3 or 4 individual fish for each sex and dose from the day 32 samples. Because there were few individuals for each sex and only limited quantities of blood were retrieved from each fish, only 1 or 2 composite samples for each sex were generated. Therefore the results for the sex-specific composites were analyzed together in order to increase the number of replicates per treatment. Blood plasma samples were analyzed by using an automated blood chemistry analyzer (VetTest 8008), following the method described in Meador et al. (2011). Plasma was analyzed for albumin, alanine transaminase (ALT), calcium, cholesterol, creatinine, glucose, inorganic phosphate, total protein, total globulins, triacylglycerols (TAGs), alkaline phosphatase, lipase and amylase. Quality control (Index Vetrol control solution lot

number J3910) was run prior to the analyses to verify the VetTest optic groups and the integrity of the test slides.

2.6 Statistical analyses

Treatment effects in fish exposed to crude oil (low, medium, high) and WY were tested by relating responses to control fish or So-Co fish, respectively. Significant differences in transcription levels for *cyp1a1*, *ahr2*, *ppar-α, ppar-γ, rxr-β, aox1*, and *cyp7α1* related to treatment and the exposure time were tested for significance by two-way analysis of variance (ANOVA) after log-transformation of the data in order to comply the assumption of normal distribution (tested by Shapiro-Wilk Normality test) and homogeneity of variance (Levene´s test). For these analyses, fish samples from day 0 were removed as this time point only includes the control treatment. Significant differences found were followed by a *post hoc* test on differences between means with the Tukey`s honest significant difference test. The data set showed an unbalanced ratio of male and female fish in several treatment groups (Table 2). Hence, differences in transcription levels related to fish sex were tested on a reduced data set that included only treatment groups with a balanced sex ratio (Table 2). First, differences in transcription levels were tested for all genes by an independent t-test in the control samples and mean transcription levels did not differ significantly between females and males for all genes except of *vtg-β* and *cyp19a1*. Hence, sex-related differences in transcription levels of *cyp1a1, ahr2, ppar-α, ppar-γ, rxr-β, aox1, and* cyp7α1 were tested by one-way ANOVA. For *vtg-β* and *cyp19a1*, differences in transcription levels related to treatment and time point were tested separately for female and male fish. Differences in frequency of gonadal maturation stage in fish related to treatment and time points were analyzed in male fish by Fisher`s Exact test.

Statistical analyses of plasma chemistry data were performed for each parameter analyzed at day 32 by one-way ANOVA. Control *versus* treatment differences were determined with Fisher's protected least significant difference (PLSD) *post hoc* test. Temporal statistical comparison of plasma chemistry data was done between day 0 and day 32 for the control groups. Significant results found in plasma parameters at day 32 are presented by least square linear regression. In all cases, differences in mean

values were considered statistically significant at $\alpha \leq 0.05$ level and data is presented as means and standard error of the mean (SEM), except if otherwise stated.

Statistical analysis and plotting of data for the gene transcription data were performed with the R project language (R Core Team, 2014), while SYSTAT 11 and Statview 5.0. were used for the plasma chemistry data.

3. Results

3.1 Fish mortality, morphometry and gonad maturation status

Fish mortality in the experiment was generally low (2.8 %) and no significant effect of treatment was found with regard to fish mortality. Fish were visually selected for the experiment based on similar total length and weight (mean total length [±SD]: 16.8 ± 1.2 cm; mean total weight [±SD]: 20.2 ± 4.3 g) and these measures did not significantly differ among treatments and time points. The sex ratio in the experiment was generally skewed toward more males (n=139) than females (n=101); this is especially evident in the medium crude oil treatment group at day 16 that contained only male fish (n=9) (Table 2). Polar cod males and females cannot be distinguished based on their phenotype, hence an unbalanced sex ratio is a common challenge in experiments performed with polar cod.

The GSI values were higher in males compared to females in the beginning of the experiment but decreased over time, while GSI was relatively stable in female fish throughout the experimental period (Table 3). All female fish exhibited PG oocytes indicative of females being either in a stage of regeneration or immaturity. POFs were only found in three females from the control group (day 24), low treatment (day 8) and high treatment (day 32) and indicated recent spawning. In male fish, the gonadal maturation stages in fish were significantly different between the time points when all treatments were combined (Fisher`s Exact test, $p<0.001$), changing from 60% males with maturing gonads at day 0 to 5% and then no males in the maturing stage at day 24 and day 32, respectively (Figure S3). At day 24 and day 32, male fish exhibited mostly spent gonads (Figure S3). The progress in male maturation was also reflected in GSI values, which decreased significantly from day 0 to the following time points of the experiment ($GSI = -0.3 \cdot day + 9.6$, $r^2=0.89$). The crude oil and WY

311 treatments did not affect the composition of gonad maturation stages in the treatment groups (Fisher´s
312 Exact test, p>0.05), neither did the treatments show effects on the GSI values (Table 3).

313 **3.2 Gene responses after dietary exposure to crude oil and the mammalian PPAR agonist WY**
314 Analyses of PAH levels in the fish feed showed a positive linear relationship with increasing nominal
315 concentrations of crude oil added to the feed (Table 1). Crude oil treatments induced a significant and
316 dose-dependent up-regulation of *cyp1a1* in liver for most time points (Figure 1A) and the comparison
317 of the *cyp1a1* responses between time points showed significant differences (Table S4). No treatment
318 effect was found in transcription levels of *ahr2*, however the *ahr2* responses differed significantly
319 between time points for the control and low treatments (Figure 1B, Table S4). Effects of dietary crude
320 oil on the transcription of genes related to lipid metabolism were only found in isolated cases (Figure
321 2, Table S4). The crude oil treatment did not significantly affect the transcription levels of genes
322 related to the regulation of lipid metabolism (*ppar-α*, *ppar-γ*) (Figures 2A and 2B, Table S4), β-
323 oxidation of fatty acids (*aox1*) (Figure 2D, Table S4) and reproduction (*vtg-β* and *cyp19α1*) (Figure 3,
324 Table S4). However, a significant treatment effect was found in *cyp7α1* at day 24 where fish exposed
325 to low and high crude oil doses showed a down-regulation of *cyp7α1* compared to control fish (Figure
326 2E, Table S4). Furthermore, at day 32, polar cod in the control and medium treatment group showed a
327 significant up-regulation of *rxr-β* transcription compared to earlier time points (Figure 2C, Table S4).
328 There was a general pattern of high variability in gene transcription levels among individual fish that
329 further resulted in high variability within treatment groups, as can be exemplified in the transcription
330 levels of *aox1* (Figure 2D) and of the genes related to reproduction (Figure 3).

331 The mammalian PPAR-α agonist WY induced a significant down-regulation of *ppar-α* at day 16
332 (Figure 4A), whereas no significant regulation of the PPAR target genes, *aox1* and *cyp7α1* was found
333 in exposed polar cod (Figure 4B and Figure 4C, respectively).

334 The sex and gonadal maturation stage of polar cod did not significantly affect transcription levels in
335 *cyp1a1, ahr2, ppar-α, ppar-γ, rxr-β, aox1,* and *cyp7α1* but transcription levels in genes related to
336 reproduction (*vtg-β* and gonadal *cyp19a1*) were different between female and male fish. Higher
337 transcription levels were found in females and males for *vtg-β* and *cyp19a1*, respectively (Figure 3).

3.3 Plasma chemistry

In the crude oil treatments at day 32, polar cod showed increased levels for several plasma parameters (TAGs, total protein, glucose, globulin, cholesterol, inorganic phosphate and albumin) compared to control fish (Table 4) with the latter three parameters exhibiting a dose-related response (Figure 5). The highest crude oil dose provoked significantly higher levels of cholesterol (Figure 5B) and close to significant differences for inorganic phosphate levels ($p=0.0058$) and albumin ($p=0.066$) compared to control (Figure 5A and Figure 5D, respectively). The enzyme alanine transaminase (ALT) alone exhibited declining levels that were dose-responsive, and significantly lower for the medium and high crude oil treatments compared to the control (Figure 5C). In all fish, levels of alkaline phosphatase (ALKP), lipase and amylase were below the limit of detection, except for one positive value for ALKP in the So-Co treatment.

4. Discussion

4.1 Effects of crude oil on genes related to biotransformation

The dose-dependent response of *cyp1a1* towards crude oil treatment showed an up-regulation of xenobiotic detoxification processes in polar cod. This was further supported by Vieweg et al. (2017), showing a dose-dependent increase of enzymatic 7-ethoxyresorufin-O-deethylase (EROD) activity and biliary PAH metabolites (naphthalene- and pyrene-type metabolites) in the fish of the same experiment. This indicated that PAHs were bioavailable to the fish over the course of the experiment. Transcription levels of *ahr2* were not significantly changed by the crude oil exposure and a potential explanation for the lack of *ahr2* induction can be inferred from the study of Andersen et al. (2015). The latter study showed a strong transcriptional up-regulation of an *ahr* repressor (*aryl-hydrocarbon receptor repressor b* [*ahrr*]) in crude oil exposed polar cod. Hence, crude oil exposure may induce a negative feedback mechanism via *ahrr* on *ahr2* transcription despite the up-regulation of *cyp1a1*.

The responses of the exposure biomarkers were considerably lower compared to previous polar cod studies, showing a two-fold lower up-regulation of CYP1A activity both at transcriptional and enzyme level in comparison to the findings by Nahrgang et al. (2010b) and one magnitude lower CYP1A activity

compared to the dietary exposure experiment by George et al. (1995). These differences could be related to the lower crude oil doses used in our study (3.9, 15.5, 26.3 µg crude oil/ g fish/ day) compared to previous polar cod experiments (50, 100 and 15.4, 61.8 µg crude oil/ g fish/day) (George et al., 1995; Nahrgang et al., 2010b, respectively). Dietary exposure experiments simulating conditions for benthic fish after the Prestige oil spill chose food doses even one order of magnitude higher (1.7 - 49.5 mg fuel oil/ g fish) (Saborido-Rey et al., 2007; Martin-Skilton et al., 2008) compared to the present study. The aim of our study was, however, to expose polar cod to a range of crude oil concentrations that pelagic fish could encounter after an oil spill. Adult polar cod are regarded as demersal species and feed mainly on pelagic zooplankton (Hop and Gjøsæter, 2013), which have been shown to bioaccumulate petroleum compounds from the water column (Agersted et al., 2018). Bratberg et al. (2013) provided environmentally relevant exposure levels for pelagic fish (1.65 and 82.5 µg crude oil/ g fish) that are based on measurements of PAH concentrations in Atlantic cod tissue after an oil spill in the North Sea (Grøsvik et al., 2008). Olsvik et al. (2011) suggested an even wider range of exposure doses (9, 90 and 900 µg crude oil/ g fish) that pelagic fish could encounter in their planktonic food after a hypothetical oil blowout lasting for 60 days.

4.2 Effects of crude oil on genes and plasma parameters related to lipid metabolism

The dietary exposure to crude oil caused only few significant changes at transcriptional and physiological level in exposed fish. A down-regulation of *cyp7α1* transcription was found at day 24 and prolonged crude oil exposure caused significant changes in plasma chemistry parameters in exposed polar cod at day 32 (increased cholesterol and decreased ALT levels). Cholesterol characterizes the nutritional status in fish (Sheridan and Mommsen, 1991) and ALT is a liver enzyme that is involved in the catabolism of amino acids. These parameters were also affected by PAH exposure in salmonids, inducing physiological condition in the exposed fish that were similar to those of starving fish (Meador et al., 2006). Hence, changes in cholesterol and ALT levels in crude oil exposed polar cod may indicate a shift towards enhanced energy mobilization and could potentially indicate altered lipid homeostasis in these fish. This hypothesis is further supported by the dose-related upward trend of albumin in exposed fish at day 32, as albumin is an important plasma carrier protein

that facilitates free fatty acids transport. A down-regulation of *cyp7α1* transcription upon crude oil exposure has also been described in another polar cod experiment (Andersen et al., 2015). CYP7A1 is the key enzyme in bile acid synthesis and is involved in cholesterol metabolism pathway through feedback mechanisms primarily governed by the farnesoid X receptor (FXR) and liver X receptor (LXR) (Desvergne et al., 2006). Excess hepatic cholesterol levels activate the conversion of cholesterol to bile acids governed by LXR. An excess in bile acid levels activates, in turn, the FXR, which indirectly inhibits the expression of *cyp7α1* and thereby the metabolic breakdown of cholesterol (Desvergne et al., 2006). Petroleum compounds have been suggested to affect cholesterol homeostasis at transcriptional level in fish species such as mahi-mahi (*Coryphaena hippurus*) (Xu et al., 2016), brown trout (*Salmo trutta*) (Meland et al., 2011) and Gilthead seabream (*Sparus aurata*) (Cocci et al., 2017). The treatment effects found on *cyp7α1* transcription and plasma chemistry were, however, limited to the later time points of the present experiment (days 24 and 32). This could be related to the relatively low crude oil doses used for the exposure, implying longer periods of time needed to develop effects.

The crude oil treatment did not affect transcription levels of *ppar-α* and *–γ* in exposed polar cod, which is in contrast to recent experimental studies on freshwater and marine fish. Waterborne exposure to relatively high levels of PAHs caused a significant activation of PPAR isotypes at transcription level next to induced CYP1A activity in exposed fish (Adeogun et al., 2016; Cocci et al., 2017). Furthermore, the study by Bilbao et al. (2010) showed that fuel oil exposure induced the transcriptional up-regulation of *ppar-α, rxr* and *aox1* in thicklip grey mullets (*Chelon labrosus*), as well as typical effects associated to peroxisome proliferation in marine organisms. These studies indicate that petroleum compounds could cause the disruption of the lipid homeostasis in fish, which ultimately can result in an unbalanced energy budget and have adverse consequences for the fish health. The results of the present study did not show significant effects of Kobbe crude oil on the transcription levels of *ppar-α*, *ppar-γ*, *rxr-β* and aox1, hence could not confirm the suggested implication of PPAR-α in the toxicity of crude oil in polar cod. However, as discussed before polar cod were exposed to relatively low crude oil doses that may not have been high enough to induce

significant effects in endpoints related to lipid metabolism. Furthermore, it is important to highlight that polar cod were exposed through the food, which is in contrast to the waterborne exposure in the studies on thicklip grey mullets (Bilbao et al., 2010), seabream (Cocci et al., 2017) and tilapia species (Adeogun et al., 2016). If thermodynamic equilibrium occurs between diet and water, whole-body uptake of PAHs may be similar from these two exposure routes (Meador et al., 1995), however, additional factors such as differential tissue metabolism and enterohepatic circulation may result in disparate internal PAH concentration in fish. Based on this, dietary doses may thus underestimate effect concentrations for nuclear receptors compared to waterborne uptake via the gills.

4.3 Effects of WY on the gene transcription of *ppar-α* and associated target genes

The chemical WY, which is described as a potent peroxisome proliferator and PPAR-α agonist for mammals (e.g. Berger and Moller, 2002) and fish (e.g. Leaver et al., 2005; Colliar et al., 2011; Urbatzka et al., 2015) did not cause the up-regulation of *ppar-α* and associated target genes in exposed polar cod of the present study. Thus, dietary WY exposure did not induce the expected up-regulation of *ppar-α* and *aox1* in WY-exposed polar cod but induced the opposite response and no response, respectively. The same transcriptional response was also found in zebrafish (*Danio rerio*) after WY exposure (Eide et al., 2014) and the authors suggested *ppar-γ* to be involved in this response, although no mechanistic explanation was provided for this hypothesis. An experimental study on turbot (*Scophthalmus maximus*) did, however, show a clear transcriptional induction of *ppar-α* and the PPAR-α target gene *aox1* as well as a significant decrease in plasma cholesterol upon WY exposure (Urbatzka et al., 2015). Responses of PPAR-α target genes *aox1* and *cyp7α1* were not observed upon WY exposure, neither did cholesterol levels change in WY-exposed polar cod (Table S5). Despite the fact that the WY dose (50 µg WY/ g fish) was similar in the study by Urbatzka et al. (2015) and the present study, it is important to remember that the former research group exposed turbot through intraperitoneal injection, which may increase the bioavailability of WY for the organism and thereby prevent the direct comparison of the results. Furthermore, the structure of the ligand-binding domain of PPAR-α differs between fish species (Leaver et al., 2005) and the sequence of the polar cod PPAR-α ligand-binding domain was found to be significantly different from other fish species such as Atlantic cod (*Gadus morhua*) (Bilbao et al.,

unpublished). This could have implications for the ligand affinity of polar cod PPAR-α ligand-binding domain to ligands such as WY and potentially explain the lack of up-regulation of *ppar-α* and its target genes.

4.4 Effects of crude oil on genes related to reproduction

Dietary exposure to crude oil did not induce effects on the selected molecular endpoints related to vitellogenesis (*vtg-β*) and steroidogenesis (*cyp19α1*) in polar cod, which concurs with findings of other experimental studies on the effects of petroleum compounds in fish (Bilbao et al., 2010; Holth et al., 2014). The lack of effects might also be related to the low exposure levels used in the present experiment and to the fact that male and female individuals were differentially represented in each experimental group. Higher *cyp19α1* transcription levels in males compared to females could be related to the different reproductive stages between fish sex (Figure S3) and cannot be explained by the functional role of the gonadal aromatase. CYP19A1 is the major regulator of E_2-production, predominantly expressed in fish ovaries. Female fish ovaries were in an immature/resting state and transcription levels of *cyp19α1* vary in accordance to the reproductive phases with lower transcription levels in resting ovaries compared to vitellogenic stages (Sardi et al., 2015). In male fish, the gonad maturation progressed over the course of the experiment and transcription levels of *cyp19a1* should be considered as very low and just above background levels.

Conclusion

The dietary crude oil exposure did not show significant effects on the mRNA expression of most genes studied in the present study, which are related to important processes in lipid metabolism (*ppar-α*, *ppar-γ*, *rxr-β*, *aox1*) and reproduction (*vtg-β*, *cyp19α1*). This might be related to the relatively low crude oil doses used in the present experiment, though reflecting environmentally relevant levels of petroleum compounds that polar cod could be exposed to in their habitat after an oil spill. The exposure affected, however, transcription levels of *cyp1a1,* which is indicative of a successful exposure and bioavailability of the contaminant. Crude oil exposure effects were observed for *cyp7α1* after prolonged exposure (day 24) and as increased cholesterol and decreased alanine transaminase plasma levels at day 32. These effects suggest an alteration in lipid homeostasis in exposed fish

despite relatively low but environmentally relevant exposure doses to crude oil. Further work is required in order to understand the significance of the present results and their potential implications for the fitness and survival of polar cod, especially during winter months. The dietary exposure to crude oil and the mammalian PPAR-α agonist WY did not cause effects in polar cod that are associated with peroxisome proliferation as seen in mammals and other fish species. Whether this is related to a distinctive substrate specificity of PPARs in polar cod needs to be further investigated through for instance functional and substrate-binding assays.

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Figure captions

Figure 1. Transcription levels of *cyp1a1* (A) and *ahr2* (B) in liver samples of polar cod. Fish were exposed through the diet to 0 (control), 3.9 µg (low), 15.5 µg (medium) and 26.3 µg (high) crude oil/ g fish/ day. Boxplots represent the median (horizontal line), 1. – 3.quartile (box), non-outlier range (whisker), outlier (points) of the data. Significant differences among treatments and time points are indicated by different lowercase and uppercase letters, respectively (two-way ANOVA and *post hoc* [Tukey`s honest significant difference test], $p<0.05$).

Figure 2. Transcription levels of *ppar-α* (A), *ppar-γ* (B), *rxr-β* (C), *aox1* (D), and *cyp7α1* (E) in liver samples of polar cod. Fish were exposed through the diet to 0 (control), 3.9 µg (low), 15.5 µg (medium) and 26.3 µg (high) crude oil/ g fish/ day. Plots as in Figure 1, where treatments and time point significantly different are indicated by different lowercase and uppercase letters, respectively (two-way ANOVA and *post hoc* [Tukey`s honest significant difference test], $p<0.05$).

Figure 3. Transcription level of *vtg-β* (A) in liver and *cyp19a1* (B) in gonad samples of female and male polar cod. Fish were exposed through the diet to 0 (control), 3.9 µg (low), 15.5 µg (medium) and 26.3 µg (high) crude oil/ g fish/ day. Plots as in Figure 1.

Figure 4. Transcription levels of *ppar-α* (A), *aox1* (B), and *cyp7α1* (C) in polar cod liver samples. Fish were exposed through the diet to 11.8 µg WY-14,643 (WY) / g fish/ day, acetone as solvent control (So-Co) and no treatment at the start of the experiment (Day 0). Treatments significantly different are indicated by different lowercase letters (one-way ANOVA and *post hoc* [Tukey`s honest significant difference test], $p<0.05$).

Figure 5. Linear regressions between selected plasma chemistry parameters and nominal exposure concentrations for day 32 of polar cod exposed through the diet to 0 (Control), 3.9 µg (Low), 15.5 µg (Medium) and 26.3 µg (High) crude oil/ g fish/ day. Inorganic phosphate (Phos) (A), alanine transaminase (ALT) (B), cholesterol (Chol) (C) and albumin (D) are the selected parameters shown. Treatments significantly different from control (ANOVA and Fisher's protected least significant difference (PLSD) *post hoc* test, $p<0.05$) indicated by asterisks.

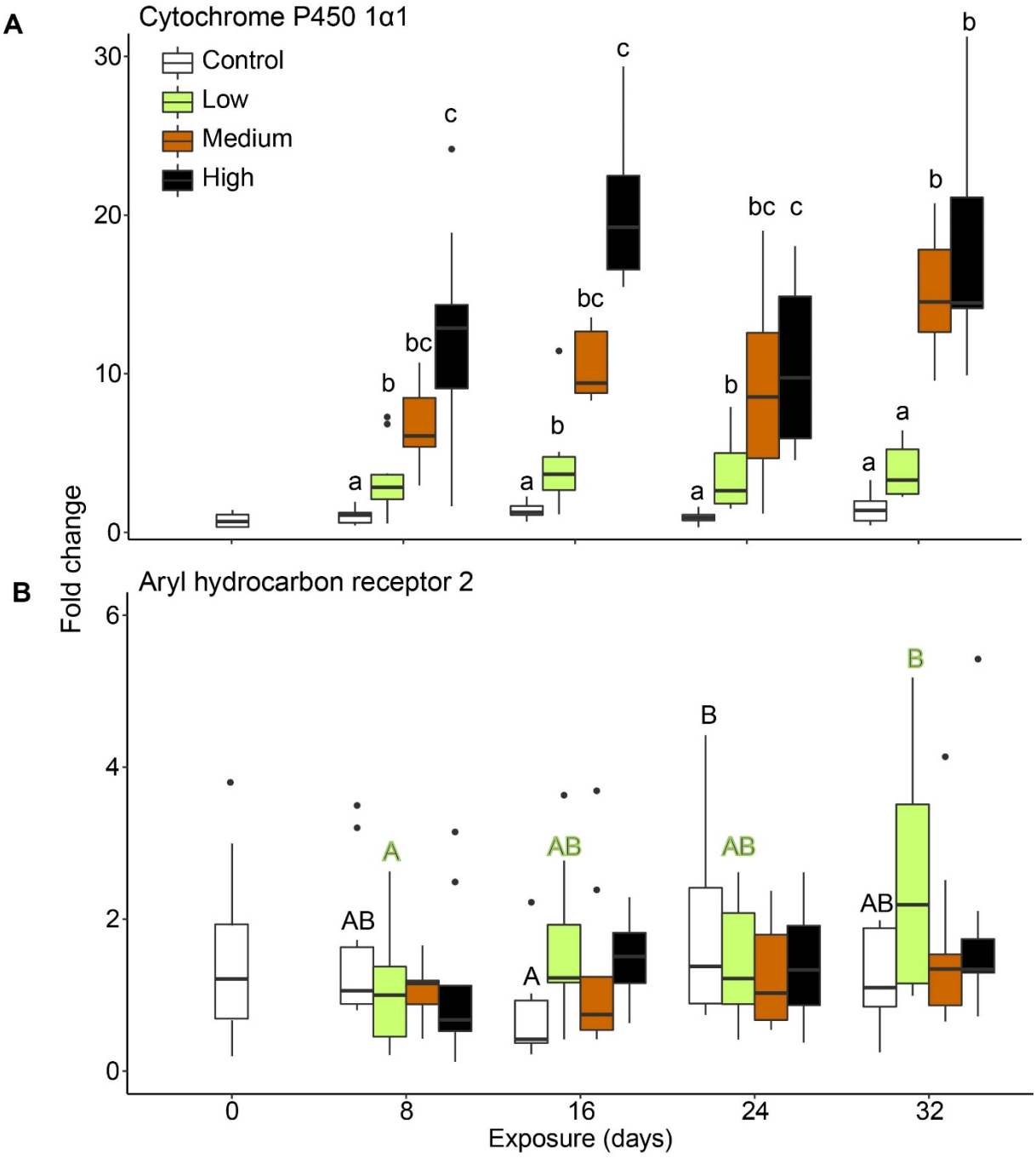
Caption supplementary material

S1 Figure. Histological sections of female polar cod gonads (A) with primary growth oocytes; (B) illustrates a post-ovulatory follicle (POF) together with primary growth oocytes.

S2 Figure. Histological sections of male polar cod gonads (A) during the maturing stage with early stage spermatocytes at 10X magnification; (B) Maturing stage at 40X magnification; (C) Ripe testis with spermatozoa at 10X magnification; (D) High magnification of ripe testis with individual spermatozoa in spawning ducts at 40x magnification; (E) Spent testis at 10X magnification; (F) Spent testis with empty lumen space at 40X magnification; (G) Resting testis with some sections still filled with spermatocytes at 10X magnification; and (H) Higher magnification (at 40X) of resting testis

S3 Figure. Percentage distribution of gonadal maturation stages in female at day 0 (n=5), day 8 (n=18), day 16 (n=10), day 24 (n=18) and day 32 (n=15) and male polar cod at day 0 (n=5), day 8 (n=20), day 16 (n=28), day 24 (n=20) and day 32 (n=24). Stages shown at each time point of the experiment (days of exposure), including fish of all the crude oil treatments (control, low, medium, high crude oil doses). Gonadosomatic index (GSI in % [± standard error]) given for each time point and sex.

Figure 1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Exposure (days)

Figure 2

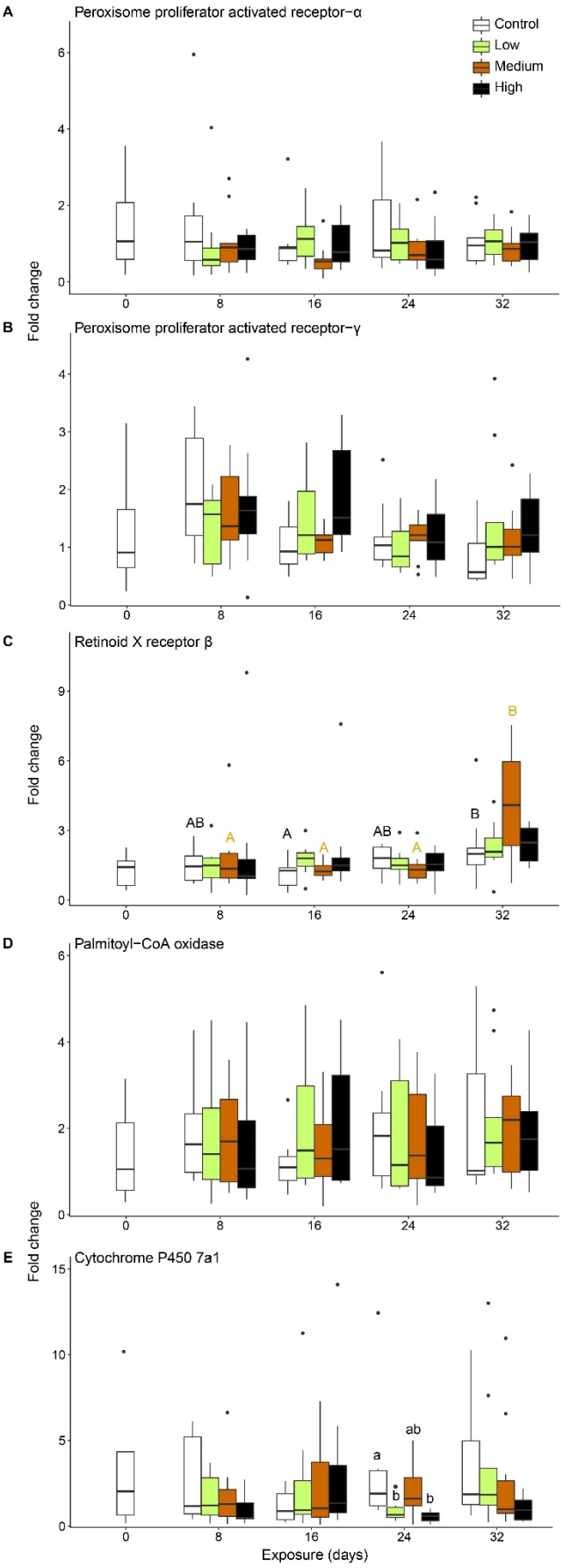

A
Peroxisome proliferator activated receptor−α
Control
Low
Medium
High
Fold change
B
Peroxisome proliferator activated receptor−γ
C
Retinoid X receptor β
AB
A
A
A
AB
A
B
B
D
Palmitoyl−CoA oxidase
E
Cytochrome P450 7a1
a
b
ab
b
Exposure (days)

Figure 3

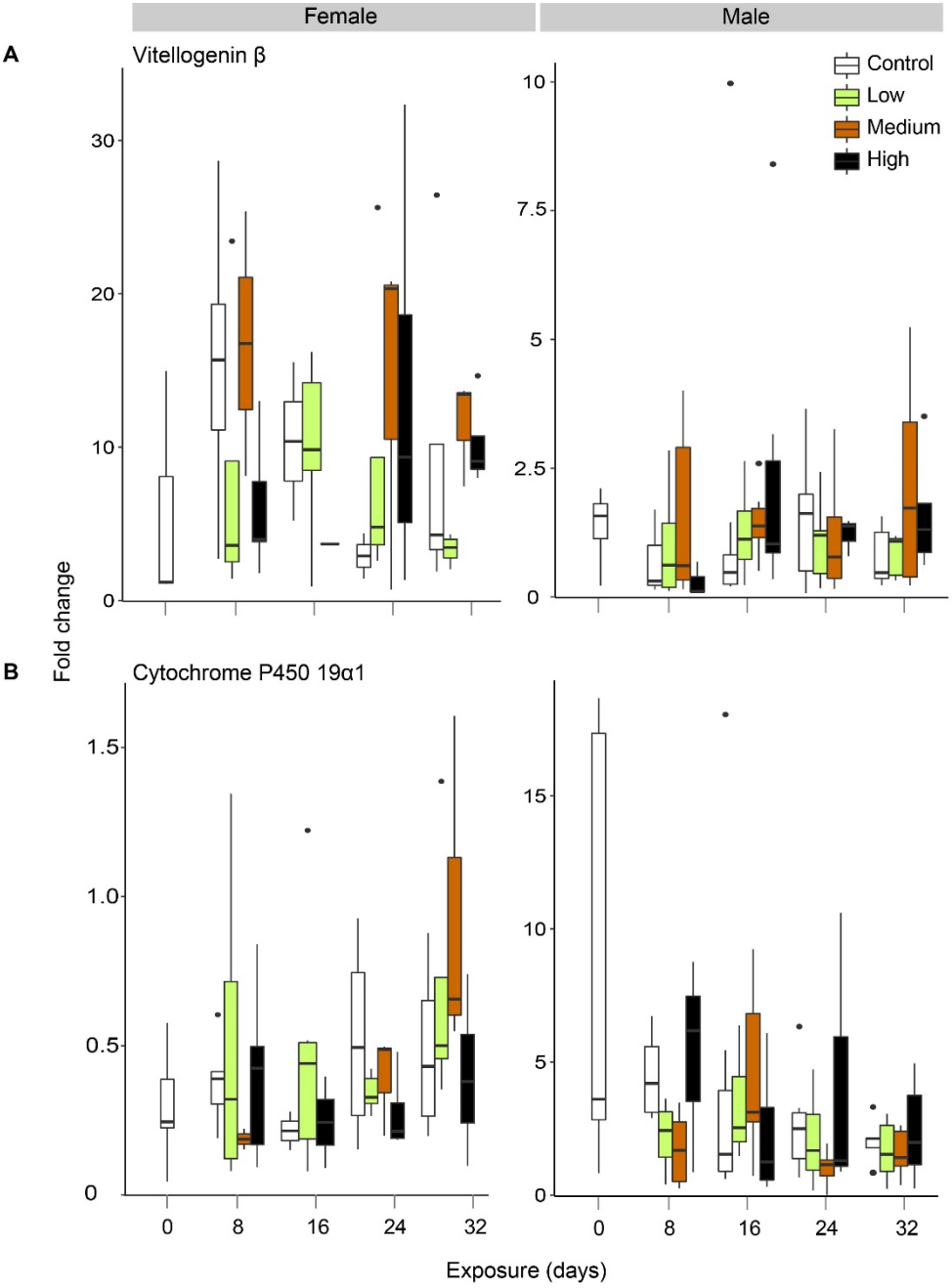

Female
Male
A
Vitellogenin β
Control
Low
Medium
High
Fold change
B
Cytochrome P450 19α1
Exposure (days)

Figure 4

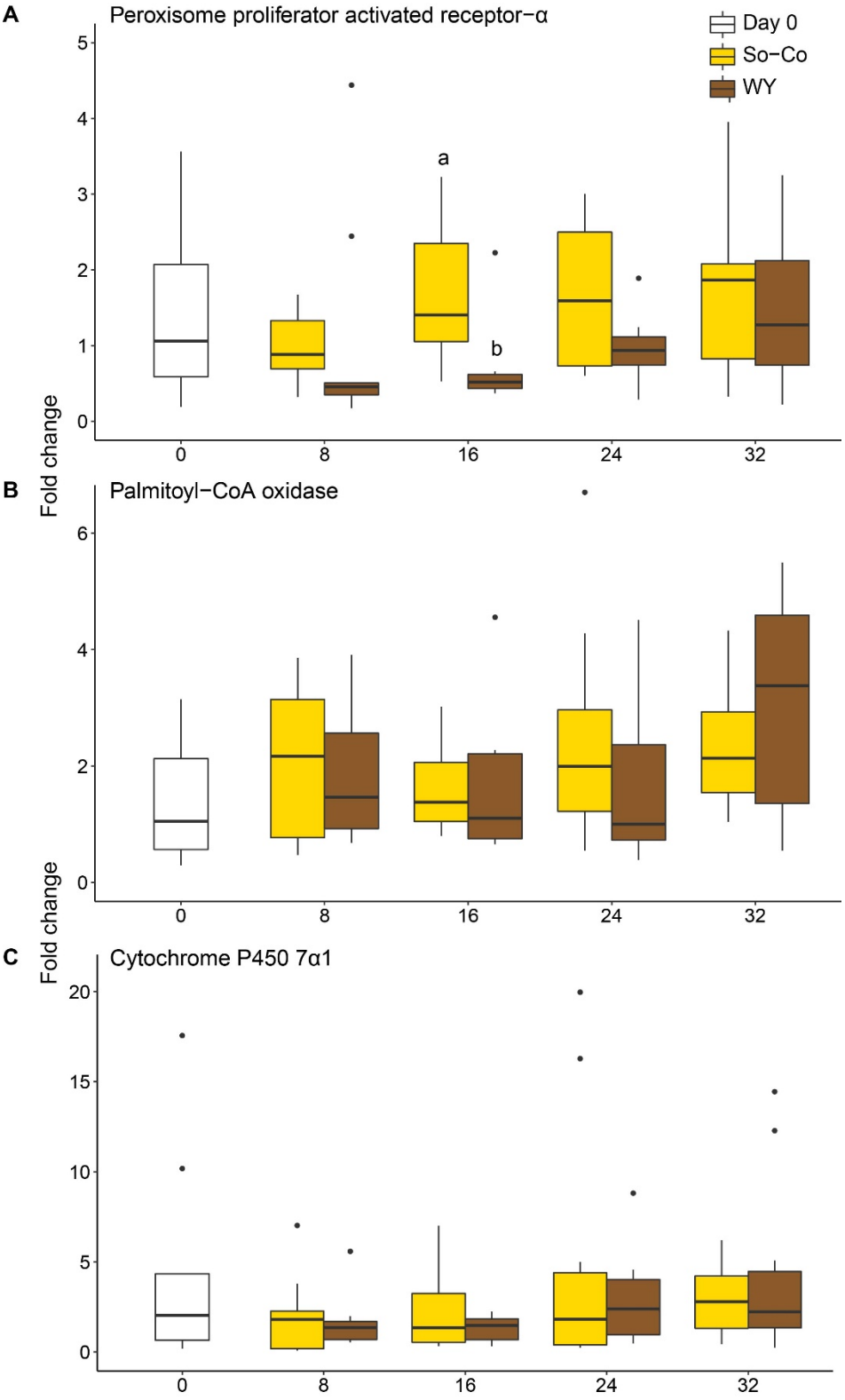

A
Peroxisome proliferator activated receptor−α
Day 0
So−Co
WY
a
b
Fold change
0
1
2
3
4
5
0
8
16
24
32
B
Palmitoyl−CoA oxidase
Fold change
0
2
4
6
0
8
16
24
32
C
Cytochrome P450 7α1
Fold change
0
5
10
15
20
0
8
16
24
32

Figure 5

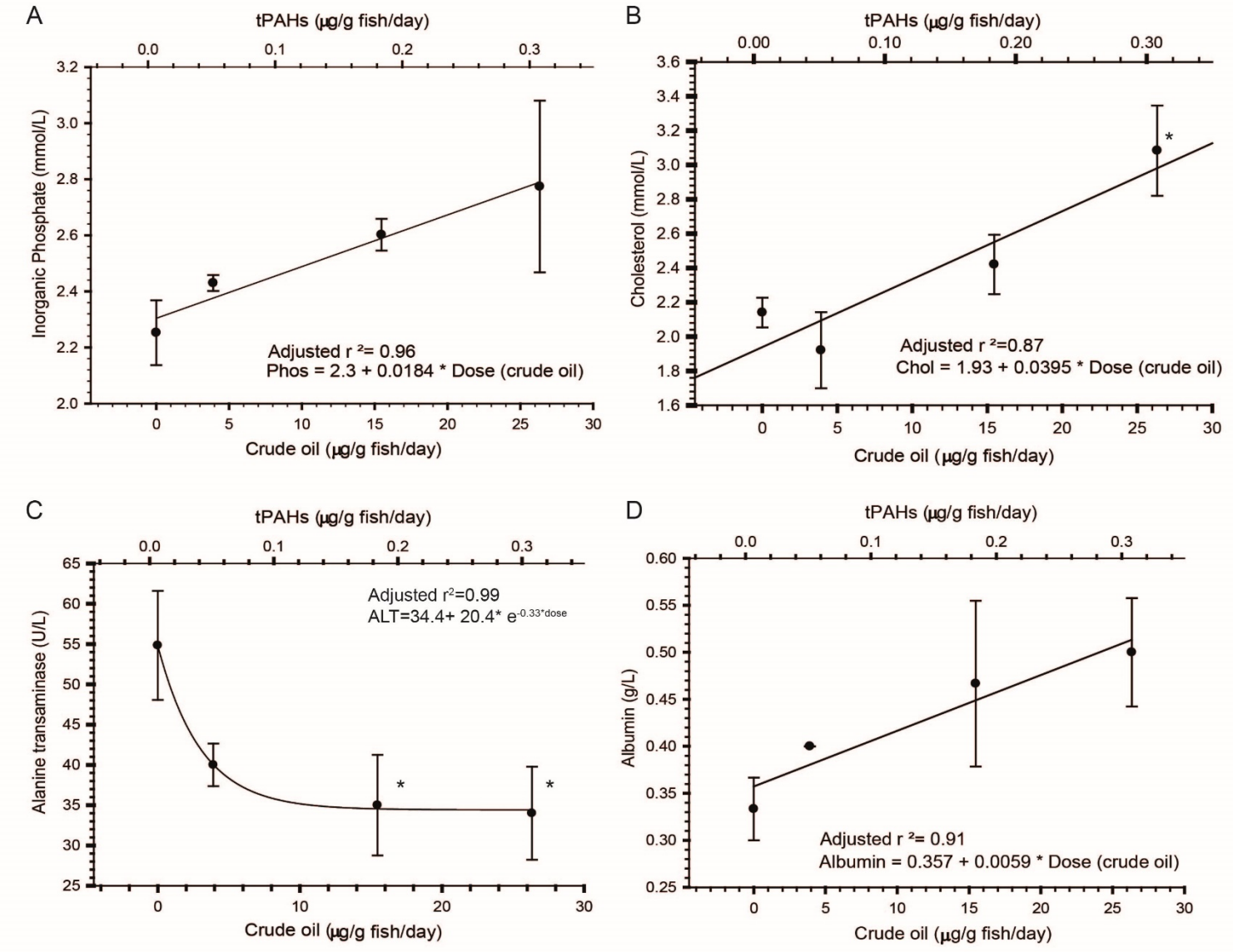

A
tPAHs (µg/g fish/day)
0.0
0.1
0.2
0.3
Inorganic Phosphate (mmol/L)
Adjusted r ²= 0.96
Phos = 2.3 + 0.0184 * Dose (crude oil)
Crude oil (µg/g fish/day)
B
tPAHs (µg/g fish/day)
0.00
0.10
0.20
0.30
Cholesterol (mmol/L)
Adjusted r ²=0.87
Chol = 1.93 + 0.0395 * Dose (crude oil)
Crude oil (µg/g fish/day)
C
tPAHs (µg/g fish/day)
Alanine transaminase (U/L)
Adjusted r²=0.99
ALT=34.4+ 20.4* e-0.33*dose
Crude oil (µg/g fish/day)
D
tPAHs (µg/g fish/day)
Albumin (g/L)
Adjusted r ²= 0.91
Albumin = 0.357 + 0.0059 * Dose (crude oil)
Crude oil (µg/g fish/day)

Table 1. Nominal concentrations and dietary doses given to polar cod during the 4 week experiment.

Treatment	mg WY/ g feed[a]		µg WY/ g fish[b] / feeding[c]	µg WY/ g fish[b]/ day	
WY-14,643 [d]					
Solvent Control	0	-	0	0	-
WY	1.7	-	47.1 ± 9.2	11.8 ± 2.3	-
	mg CR/ g feed[a]	µg PAH[e,f] / g feed	µg CR/ g fish[b] / feeding[c]	µg CR/ g fish[b]/ day	µg PAH[e]/g fish[b]/ day
Crude oil					
Control	0	0.08	0	0	$6*10^{-4}$
Low	0.5	6.5	15.6 ± 3.4	3.9 ± 0.9	0.051 ± 0.01
Medium	2	22.9	61.9 ± 12.2	15.5 ± 3.0	0.177 ± 0.03
High	4	48.2	105.3 ± 23.8	26.3 ± 6.0	0.312 ± 0.08

a Concentrations given per g *Calanus spp.* (fish feed)

b Doses given per mean total fish weight (± standard deviation), which was measured at the start of experiment

c Fish were fed every 4th day

d Mammalian peroxisome proliferator

e Sum of 26 PAHs and individual PAH levels are specified in S4 Table

f Correlation crude oil/ PAH in fish feed: PAH dose [µg PAH/ g food] = dose [mg crude oil]* 0.003 + 0.008 (r^2 = 0.99)

CR – crude oil; PAH - Polycyclic aromatic hydrocarbons; WY – WY-14,643

Table 2. Morphometrics[a] of polar cod that were sampled in the crude oil and WY-14,643 feeding experiment. Treatment groups marked with * were used for statistical testing of fish sex as important confounding factor of gene transcription.

Day	Treatment	n	Sex ratio	GSI (%)		HSI (%)	
			females : males	females	males	females	males
0	Control *	10	5:5	2.6 ± 0.2	10.9 ± 3.1	2.5 ± 0.3	2.6 ± 0.5
8	Control *	10	6:4	2.8 ± 0.2	10.0 ± 3.7	2.4 ± 0.3	4.7 ± 2.3
	Low *	10	4:6	2.6 ± 0.7	3.8 ± 1.0	3.1 ± 1.4	3.1 ± 0.6
	Medium	9	2:7	2.4 ± 0.3	5.8 ± 2.0	1.9 ± 0.4	4.0 ± 0.8
	High	9	6:3	2.6 ± 0.3	10.3 ± 2.9	1.9 ± 0.3	3.0 ± 0.7
	So-Co	9	3:6	2.0 ± 0.2	6.9 ± 2.8	5.1 ± 2.6	3.7 ± 0.8
	WY*	10	6:4	2.2 ± 0.4	6.2 ± 1.9	3.0 ± 0.5	3.8 ± 0.7
16	Control	10	2:8	2.7 ± 0.4	4.0 ± 1.3	4.1 ± 0.5	3.0 ± 0.4
	Low *	10	6:4	2.4 ± 0.1	1.8 ± 0.5	1.6 ± 0.2	3.3 ± 0.9
	Medium	9	0:9	-	3.8 ± 0.8	-	2.3 ± 0.3
	High	9	2:7	4.9 ± 2.3	3.4 ± 1.0	2.0 ± 0.3	2.7 ± 0.3
	So-Co	8	2:6	2.3 ± 0.1	5.9 ± 2.0	1.9 ± 0.1	2.0 ± 0.3
	WY	9	3:6	3.4 ± 0.9	2.6 ± 0.6	1.6 ± 0.1	2.7 ± 0.6
24	Control *	9	4:5	2.6 ± 0.2	2.1 ± 0.8	1.9 ± 0.1	2.5 ± 0.3
	Low *	10	5:5	2.1 ± 0.1	3.3 ± 2.3	1.9 ± 0.2	2.7 ± 0.6
	Medium	10	3:7	2.2 ± 0.2	4.7 ± 1.7	2.8 ± 0.6	2.6 ± 0.2
	High	9	6:3	2.4 ± 0.2	1.7 ± 0.2	3.0 ± 0.6	2.9 ± 0.5
	So-Co	10	7:3	2.4 ± 0.2	2.6 ± 0.7	2.0 ± 0.2	2.1 ± 0.2
	WY	8	GSI 1:7	2.1	3.4 ± 1.1		
		10	HSI 3:7			2.0 ± 0.1	2.1 ± 0.2
32	Control *	10	4:6	2.1 ± 0.1	1.3 ± 0.2	1.8 ± 0.2	2.0 ± 0.2
	Low *	9	4:5	2.5 ± 0.2	3.1 ± 1.2	2.2 ± 0.2	1.9 ± 0.2
	Medium	10	3:7	2.0 ± 0.2	1.5 ± 0.3	2.3 ± 0.4	2.7 ± 0.2
	High *	10	4:6	2.4 ± 0.2	1.3 ± 0.4	2.4 ± 0.2	3.2 ± 0.3
	So-Co *	10	6:4	2.1 ± 0.1	1.6 ± 0.2	2.0 ± 0.3	2.2 ± 0.3
	WY *	10	5:5	2.1 ± 0.1	1.4 ± 0.2	1.7 ± 0.2	2.9 ± 0.5

a Values are shown as mean ± standard error

n – number of fish; GSI - gonadosomatic index; HSI - hepatosomatic index

Table 3. Quantitative PCR conditions for the amplification of target genes in polar cod liver and gonad.

Gene name	GenBank[a]	Size[b]	Sequence[c]	Melt.temp.[d]	Conc.[e]	Sample dil.[f]
Liver						
cytochrome p450 1α1 (cyp1a1)	EU682947	80	FW: CGTGCTCGCCGACAGAAAC RV: AGGGCAGGAAGGAGGAGTGA	58	1.25	1: 29
aryl hydrocarbon receptor 2 (ahr2)	EU682946	84	FW: GCAGCGACCAGGTGAATTATG RV: GGAAACGGCAGACGAAGCT	56	1.25	1: 29
peroxisome proliferator activated receptor α (ppar-α)	KT985390	146	FW: GGCCCGGCAGATCTACGA RV: GTCTTCCCGGTGAGGATGGT	60	0.625	1: 9
peroxisome proliferator activated receptor γ (ppar-γ)	KT985389	80	FW: TGATGAACAATGACGGCACACT RV: GGCTTGCGTAGGCTCTTGAG	58	0.625	1: 4
retinoic X receptor β (rxr-β)	KT985387	80	FW: GGCAATATTTGACCGGGTTCT RV: GACAGCCTAGCTCCGTCTTGTC	56	1.25	1: 29
palmitoyl-Co A oxidase (aox1)	KT985388	80	FW: GGCATCGTGCTCTCCCAAT RV: TCTCCTGCGCGGATCTCT	56	1.25	1: 2
vitellogenin β (vtg β)	KT985393	80	FW: GCAACCCTGAAGGAAAGCAA RV: GGAGCGGTGTTCTTGGTCAT	57	1.25	1: 29
cholesterol 7-alpha-monooxygenase (cyp7α1)	KT985385	82	FW: GGCAGGTGGACGGCATCT RV: CCAGCTCCTTGCCAAAGAG	56	1.25	1: 19
β-actin	EU682944	80	FW: CCGCTGAGAGGGAAATCGT RV: GGTGCTCATCTCCTGCTCGAA	57	1.25	1: 29
Gonad						
cytochrome 19a1 (cyp19a1)	KT985391	80	FW: CGCTACTTCCAGCCGTTTG RV: AGGATGGACTTCATCATCACCAT	58	0.625	1: 9
18s ribosomal RNA (18srRNA)	KT985386	87	FW: CGAATGTCTGCCCTATCAACTTT RV: CCGGAATCGAACCCTGATT	57	1.25	1: 99

a GenBank accession number

b Primers size shown in base pairs

c Primer sequence in 5'- 3' orientation

d Primer melting temperatur in °C

e Primer concentration shown in pMol/µl

f Sample dilution in cDNA (sample) : RNAse-free water (v:v)

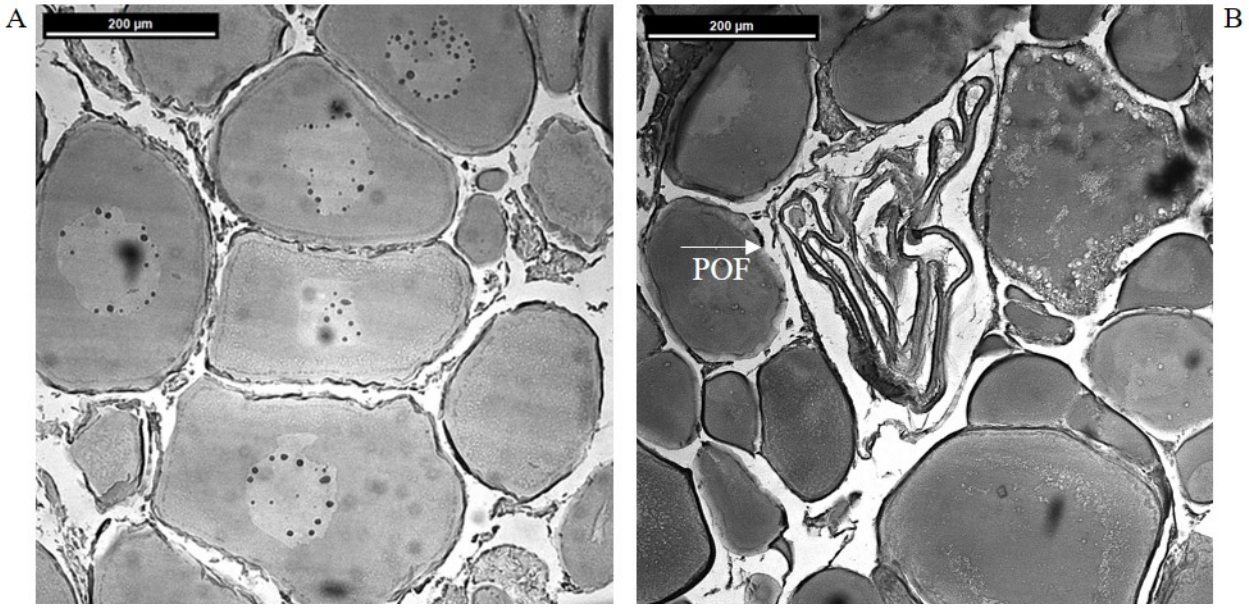
FW- forward; RV – reverse

Table 4. Plasma parameter levels[a] in polar cod exposed to different crude oil doses at the end of experiment (day 32). Numbers in bold indicate values significant different from control at day 32 (ANOVA, $p<0.05$) and values close to significance are indicated by * ($p=0.066$) and ^ ($p=0.058$).

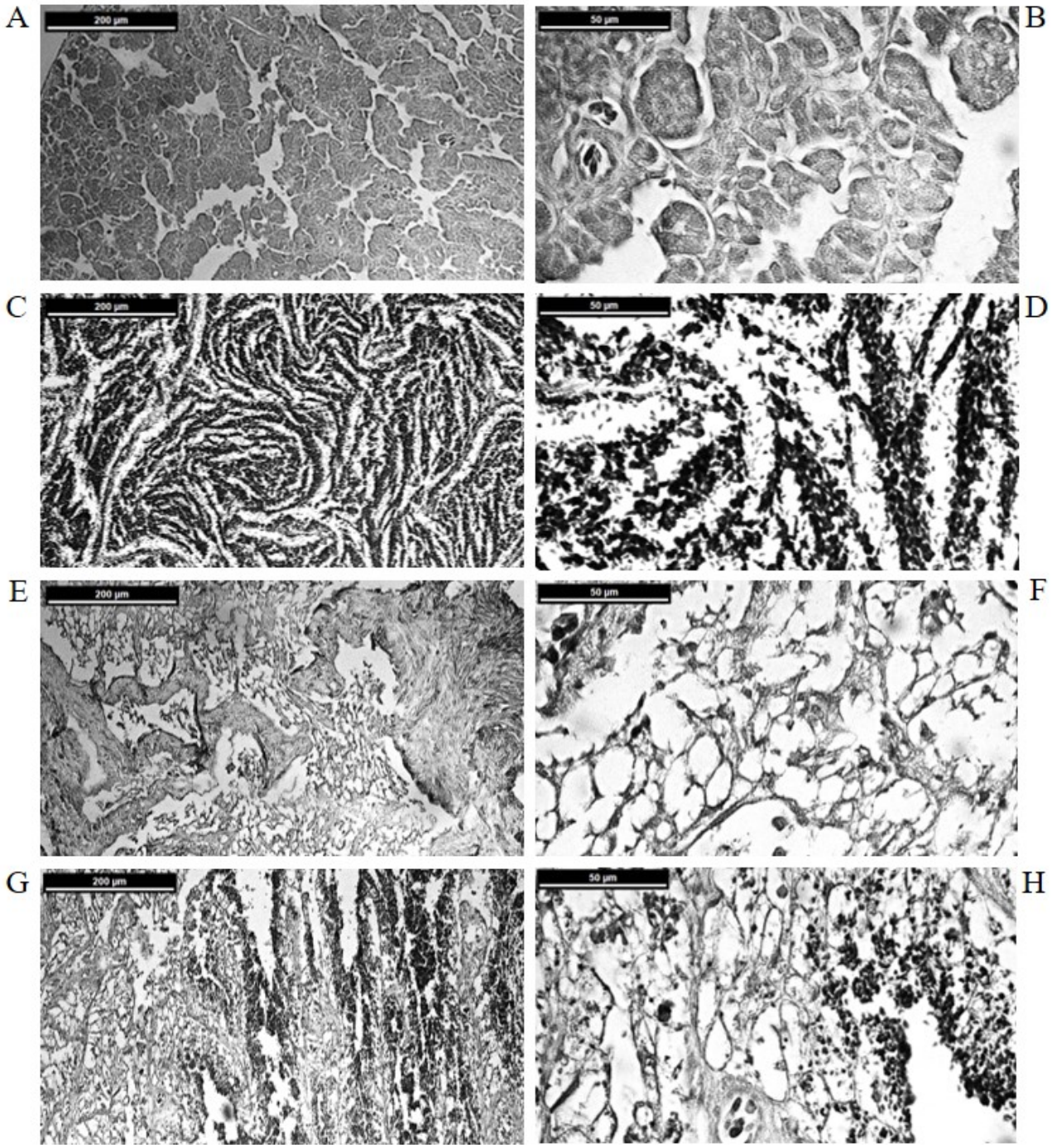
Plasma chemistry	Day 32			
	Control	Low	Medium	High
Albumin (g/ L)	0.3 ± 0.03	0.4 ± 0	0.5 ± 0.1	* 0.5 ± 0.06
Alanine transaminase (U/ L)[b]	54.8 ± 6.8	40 ± 2.7	**35.0 ± 6.2**	**34.0 ± 5.8**
Calcium (mmol/L)	2.2 ± 0.1	2.0 ± 0.08	2.1 ± 0.05	2.2 ± 0.1
Cholesterol (mmol/L)	2.1 ± 0.1	1.9 ± 0.2	2.4 ± 0.2	**3.1 ± 0.3**
Creatinine (µmol/L)	33.9 ± 3.9	29.5 ± 7.8	35.4 ± 5.1	26.5 ± 5.1
Total globulins (g/ L)	1.2 ± 0.2	1.3 ± 0.1	1.6 ± 0.2	1.4 ± 0.06
Glucose (mmol/L)	3.7 ± 0.1	3.8 ± 0.2	3.5 ± 0.2	4.5 ± 0.7
Inorganic phosphate (mmol/L)	2.3 ± 0.1	2.4 ± 0.02	2.6 ± 0.06	^ 2.8 ± 0.3
Total protein (g/ L)	1.5 ± 0.3	1.7 ± 0.1	2.0 ± 0.3	1.9 ± 0.03
Triacylglycerides (nmol/L)	0.05 ± 0.01	0.05 ± 0.02	0.08 ± 0.03	0.07 ± 0.02

a Mean concentration (± standard error) based on composite samples (n= 3-4 fish) including both fish sexes

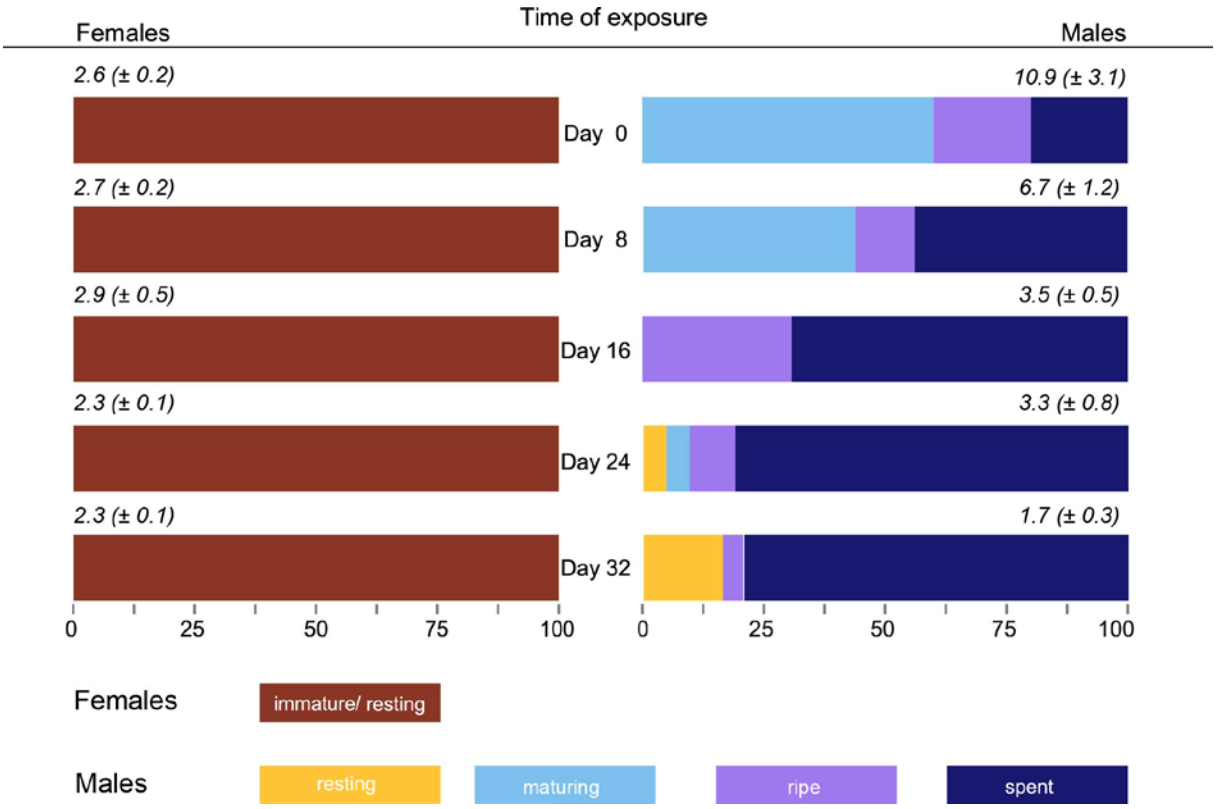
b U/L for enzyme units per liter



S1 Figure.



S2 Figure.


Time of exposure
Females
Males
2.6 (± 0.2)
10.9 (± 3.1)
Day 0
2.7 (± 0.2)
6.7 (± 1.2)
Day 8
2.9 (± 0.5)
3.5 (± 0.5)
Day 16
2.3 (± 0.1)
3.3 (± 0.8)
Day 24
2.3 (± 0.1)
1.7 (± 0.3)
Day 32
0
25
50
75
100
0
25
50
75
100
Females
immature/ resting
Males
resting
maturing
ripe
spent

S3 Figure.

S4 Table. Summary output of two-way ANOVA testing differences in gene transcription levels related to dietary crude oil treatment (control, low, medium and high crude oil doses) time point, and interaction of these factors. P-values in bold indicate significant differences.

	cyp1a1	*ahr2*	*ppar-α*	*ppar-γ*	*rxr-β*	*aox1*	*cyp7α1*	*vtg-β*	*cyp19α1*
Fish sex combined								**Female fish**	
Factor treatment									
Mean square [a]	37.6	0.2	1.0	0.2	0.1	0.1	3.5	1.4	0.4
F ratio	111.0	0.5	1.8	0.7	0.3	0.2	2.9	1.2	0.7
p value	**<0.001**	0.66	0.14	0.53	0.82	0.91	**0.038**	0.33	0.53
Factor time point									
Mean square [a]	1.9	1.7	0.4	0.7	3.1	0.5	0.9	1.0	0.9
F ratio	5.5	3.9	0.7	2.3	8.0	0.9	0.8	0.8	1.6
p value	**0.001**	**0.01**	0.56	0.008	**<0.001**	0.42	0.51	0.52	0.19
Interaction treatment time point*									
Mean square [a]	0.2	1.0	0.4	0.39	0.4	0.3	1.8	0.7	0.3
F ratio	0.5	2.2	0.7	1.31	1.5	0.5	1.4	0.6	0.5
p value	0.87	**0.02**	0.56	0.272	0.17	0.87	0.18	0.75	0.81
								Male fish	
Factor treatment									
Mean square [a]								1.2	1.3
F ratio								1.0	1.0
p value								0.402	0.387
Factor time point									
Mean square [a]								1.5	2.7
F ratio								1.2	2.1
p value								0.33	0.089
Interaction treatment time point*									
Mean square [a]								0.7	1.6
F ratio								0.6	1.2
p value								0.79	0.3

a mean squares = sum of square/ degree of freedom

S5 Table. Plasma parameter levels[a] in polar cod exposed to WY-14,643 and solvent control (acetone) at the end of experiment. Numbers in bold indicate values significantly different from control (one-way ANOVA and *post hoc*, $p<0.05$).

Plasma chemistry	Day 32	
	Solvent Control	WY-14,643
Albumin (g/ L)	0.5 ± 0.2	0.4 ± 0.03
Alanine transaminase (U/ L)[b]	39.0 ± 4.0	39.0 ± 2.9
Calcium (mmol/L)	2.3 ± 0.2	2.3 ± 0.1
Cholesterol (mmol/L)	1.7 ± 0.2	2.3 ± 0.5
Creatinine (µmol/L)	53.0 ± 13.5	29.5 ± 7.8
Total globulins (g/ L)	0.7 ± 0.2	**1.3 ± 0.06**
Glucose (mmol/L)	4.2 ± 0.5	4.4 ± 0.4
Inorganic phosphate (mmol/L)	6.5 ± 0.5	**2.7 ± 0.06**
Total protein (g/ L)	1.2 ± 0.2	**1.8 ± 0.09**
Triacylglycerides (nmol/L)	0.05 ± 0.02	0.1 ± 0.05

a Mean concentration (± standard error) based on composite samples (n= 3-4 fish) including both fish sexes

b U/L for enzyme units per liter