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Spawning time in adult polar cod (*Boreogadus saida*) altered by crude oil exposure, independent of food availability

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ABSTRACT

Fish early life stages are well known for their sensitivity to crude oil exposure. However, the effect of crude oil exposure on adults and their gametes during their spawning period is not well studied. Polar cod, a key arctic fish, may be at risk for crude oil exposure during this potentially sensitive life stage. Additionally, this species experiences lower food availability during their spawning season, with unknown combined consequences. In the present study, wild-caught polar cod were exposed to decreasing levels of a water-soluble fraction (WSF) of crude oil or control conditions and fed either at a low or high feed ration to assess the combined effect of both stressors. Samples were taken during late gonadal development, during active spawning (spawning window), and in the post-spawning period. Histology analysis of gonads from fish sampled during the spawning window showed that oil-exposed polar cod were more likely to have spawned compared to controls. Oil-exposed females had 947 differentially regulated hepatic genes, and their eggs had a higher polycyclic aromatic hydrocarbon body burden compared to controls. Feed ration did not consistently affect polar cod's response to oil exposure for the endpoints measured, however, did alone result in decreases in some sperm motility parameters. These results suggest that polar cod's spawning period is a sensitive life event to crude oil exposure, while feed limitation may play a minor role for this supposedly capital breeder. The effects of adult exposure to crude oil on gamete quality and the next generation warrant further investigation.

KEYWORDS

crude oil; polar cod; spawning; food availability; environmental toxicology

Introduction

Early life stages (embryonic to larval period) show a high sensitivity to waterbone exposure to crude oil (Incardona et al. 2015, 2021) with potential long-term physiological consequences (Laurel et al. 2019; Mager et al. 2014; Pasparakis et al. 2016). Adult life stages are considered more robust than their early life stages (ELS) counterparts (Pasparakis et al. 2016) but these have been less well studied. As the most sensitive life stages are not necessarily those most sensitive for population viability (Kammenga et al. 1996; Ohlberger and Langangen 2015), it is of critical importance to assess the effects of oil spills on all life stages of fish in order to comprehensively understand the impact of an accidental oil spill to fish populations.

The reproductive development of fish is primarily controlled through the hypothalamus, pituitary, gonad and liver axis (HPGL). In females, vitellogenesis is the phase during which oocytes accumulate yolk and increase in size. In a simplified description, this phase is regulated by gonadotropin hormone I (GTHI) (follicle stimulating hormone (FSH)) that stimulates estrogen 17-β-estradiol (E2) production in the ovaries. Increased E2 production leads to the synthesis of vitellogenin (Vtg), a yolk precursor protein in the liver (Nagahama 1994). Once in the bloodstream, Vtg will be taken up by oocytes and converted into yolk, which is important for embryo nutrition (Nagahama 1994). After the yolk has been accumulated and the oocyte is near its final size, gonadotropin hormone II (GTHII) (luteinizing hormone (LH)) will regulate final maturation, E2 will decrease, and maturation inducing hormone (MIH) steroidogenesis will increase mediating

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ovulation and spawning (Nagahama 1994; Nagahama and Yamashita 2008). Leftover eggs (hyaline eggs) will undergo atresia which means that they will be broken down and reabsorbed. In males, gonadotrophins GTHI (FSH) and GTHII (LH) are the primary mediators of spermatogenesis (Schulz and Miura 2002) and key hormones 11-ketotestosterone (11-KT) testosterone (T), and MIH regulate the development of spermatozoa in the testis (Schulz and Miura 2002).

As reproductive development is controlled through complex changes on several levels of biological organization, crude oil exposure can impact reproductive development by affecting various mechanisms. Previous studies in several fish species show that exposure to crude oil and associated compounds can delay reproductive development and/or spawning (Bender et al. 2018; Johnson et al. 1988; Khan 2013; Kiceniuk and Khan 1987; Thomas and Budiantara 1995; Truscott et al. 1983). Levels of key steroid hormones, E2, 11-KT, and, T may be affected by petroleum compounds or polycyclic aromatic hydrocarbons (PAHs) present in the crude oil mixture (Monteiro, Reis-Henriques, and Coimbra 2000; Tetreault et al. 2003; Tintos et al. 2006). Some compounds in crude oil may show anti-estrogenic effects at high concentrations, leading to a decreased messenger RNA (mRNA) expression of key hepatic genes, such as estrogen receptor α (ERα) in females (Bilbao et al. 2010; Salaberria et al. 2014). Crude oil and field sites with high levels of PAHs have been associated with an increase in oocytes being broken down through atresia (Johnson et al. 1994; Thomas and Budiantara 1995), which may indicate decreased fecundity or skipped spawning (Rideout, Rose, and Burton 2005). Finally, crude oil exposure may also affect gamete quality. Studies have shown mixed results on sperm motility (Bautista and Burggren 2019; Beirão, Litt, and Purchase 2018; Bender et al. 2016) and PAHs may be transferred to eggs with unknown long-term consequences for the next generation (Bue, Sharr, and Seeb 1998; Carls et al. 2000).

Warming temperatures and receding ice cover provide more opportunities for shipping and oil exploration in the Arctic (Eguíluz et al. 2016; Harsem, Eide, and Heen 2011). Increased industrial activities create an inherent risk of accidental oil spills, especially during the polar night due to challenging environmental conditions such as low temperatures, 24-hour darkness, and unpredictable weather (AMAP 2007; Harsem, Eide, and Heen 2011).

Polar cod (*Boreogadus saida*), a key arctic fish species (Hop and Gjøsæter 2013; Mueter et al. 2016), spawns once a year during the polar night (January – March). Sex-specific differences in gonadal development have been reported, with males reaching a spawning capable stage several weeks earlier than females (Hop, Trudeau, and Graham 1995; Nahrgang et al. 2016). Polar cod's early life stages (embryos and larvae) have been shown to be highly sensitive to direct exposure to crude oil (Laurel et al. 2019; Nahrgang et al. 2016). However, as with other species, less is known about the potential consequences of exposure on adult fish, particularly during their spawning period. Bender et al. (2016) observed no effect on the timing of gonadal development or steroid hormones when chronic dietary exposure occurred between June and February; however, they did observe a reduction in sperm motility parameters. Female polar cod showed a delay in reproductive development following acute exposure to burned oil residues in June (steroid hormones and sperm motility not measured) (Bender et al. 2018). *Ex-vivo* exposure of polar cod liver slices to high molecular weight PAH, benzo[a]pyrene (BaP) has demonstrated an anti-estrogenic effect (Yadetie et al. 2021). Dietary exposure of BaP also led to a reduction in genes (*g6pc3*, *polr2g*, *med10*, *taf11*) involved in the estrogen pathway in polar cod (Song, Nahrgang, and Tollefsen 2019). Previous work on polar cod has also indicated a possible sex-specific response to crude oil and suggested that females may be more sensitive to crude oil exposure due to their high reproductive investment (Bender et al. 2018).

Arctic species experience a high level of seasonality in their food supply or composition due to changes in ice cover and light availability throughout the year (AMAP 2007; Berge et al. 2015; Hop and Gjøsæter 2013; Leu et al. 2011). Polar cod has been described as an opportunistic feeder, accumulating energy reserves during the summer months when feeding opportunities are higher and relying on these reserves during periods of lower food

availability in the winter (Majewski et al. 2016; Nakano et al. 2016), a period also coinciding with gonadal development (Hop, Trudeau, and Graham 1995). Polar cod has been shown to use the majority of the energy reserves accumulated in the summer for reproduction (Hop, Trudeau, and Graham 1995). Lower food availability and potentially lower energy reserves during spawning may increase the sensitivity of polar cod to oil exposure by an energy trade-off between xenobiotic metabolism and reproduction.

Here we tested the hypothesis that *in-vivo* crude oil exposure would alter the natural reproductive development of sexually mature adult polar cod and that lower food availability may further compromise the reproductive outcome. We exposed polar cod to a water-soluble fraction of crude oil for 131 days starting when polar cod were in a late stage of reproductive development until the post-spawning phase. We assessed somatic indices (gonadosomatic index (GSI) and hepatosomatic index (HSI)), gonad histology (reproductive phase and atresia), steroid hormones levels (E2, T, 11-KT), hepatic mRNA expression, (cytochrome P450 1A (*cyp1a*), vitellogenin (*vtg α*), estrogen receptor 1 (*esr1*), transcriptome response), sperm motility and PAH body burden. Based on previous studies, we predicted an up-regulation in genes involved in hepatic biotransformation, a delay in reproductive development, decreases in steroid hormone levels, a reduction in the expression of genes in the estrogen pathway, decreased sperm motility, and parental transfer of PAHs to eggs.

Materials and methods

Polar cod collection and husbandry

Polar cod were collected in November 2017 with a Campelen 1800 bottom trawl fitted with a fish-lift device (Holst and McDonald 2000) in Southeastern Hinlopen (78° 55”N, 23° 40”E) from depths between 170 and 200 m and maintained onboard the ship RV *Helmer Hanssen* in flow-through seawater tanks (2500 L). The fish were treated daily with Halamid® disinfectant (1:500; Aquatiq Chemistry). At the aquaculture research station of UiT The Arctic University of Norway in Kårvika, Norway (69°23”N, 18°10” E), polar cod were maintained in 3000 L tanks supplied with a flow-through of filtered seawater (60 µm) from the nearby fjord. Fish were fed once daily *ad libitum* with frozen *Calanus finmarchicus* (CALANUS AS, Tromsø) until the experiment started. During rearing and the experimental period, light exposure and water conditions were controlled to simulate *in-situ* conditions in Svalbard (Ny-Ålesund, 78° N). The photoperiod during the experiment was 24-hour darkness from October 29th, 2018, to February 2nd, 2019, followed by a progressively increasing amount of light during the day until 24-hour light was reached on April 22nd, 2019. The mean water temperature was maintained at an average of 1.01 ± 0.21°C. Raw data on tank conditions during the experiment is available in the data repository.

On December 5th, 2018, 472 polar cod of similar length were selected, showing an average fork length of 21.08 cm ± 1.98 (mean ± SD) and wet weight of 81.48 g ± 24.76 g. Each fish was anesthetized in a Finquel® (70 mg/L Tricaine Methanesulfonate; Sigma Aldrich) solution for morphometric measurements, sexed using ultrasound (General Electric, LOGIQ XP) based on Karlsen and Holm (1994), and marked with a unique dorsal fin tag. Polar cod were then distributed to 12–300 L experimental tanks ($n = 18$ males and 18 females per tank) and allowed to acclimatize for 10 days before the experiment began.

Experimental design

The experiment took place from December 15th, 2018, to April 25th, 2019. This period covered polar cod's spawning capable (tertiary vitellogenesis (Vtg3)) to regenerating reproductive phases (section 2.5, Figure 1).

Of the 12 experimental tanks, 6 were randomly assigned to receive the water-soluble fraction (WSF) of Barents sea Goliat crude oil (source Akvaplan-niva, Tromsø), and 6 did not receive crude oil (control). In both the oil-exposed and the control treatments, 3 tanks received a low-feed ration, and three tanks received a high-feed ration, thus resulting in 4 treatments with 3 replicate tanks for each.

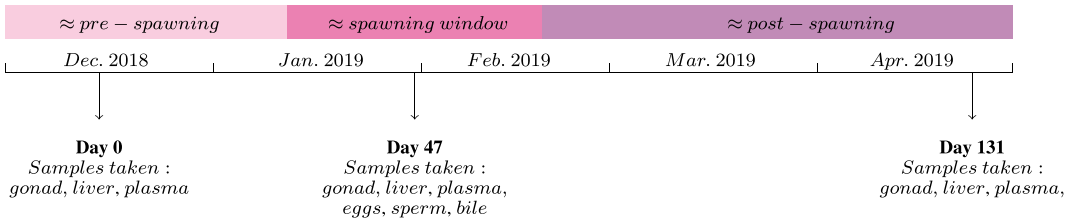

Figure 1. Experimental timeline displaying the dates of exposure, the estimated reproductive periods, and sampling time points.

The experimental set-up consisted of oiled-rock columns (Carls, Rice, and Hose 1999) to simulate an oil spill by delivering decreasing aqueous levels of oil compounds over time. Polyvinyl chloride (PVC) columns (122 × 30 cm) were filled with 45 kg of gravel coated either with crude oil (oil-exposed) or not (control). Gravel was sieved to obtain gravel pieces >4 mm, rinsed with fresh water, soaked in 5% hydrochloric acid (HCL) for 3 hours before being rinsed and soaked in 90% ethanol for 12 hours, and soaked in fresh water for 3 days followed by a final rinse. The gravel was left to dry at 60°C degrees for 48 hours. For the oil-exposed treatment, each kg of gravel was coated in 24 g of crude oil (24 g kg^{-1}) (Nahrgang et al. 2010). Seawater percolated upwards through the columns and was delivered to the experimental tanks at a rate of 900–1000 mL min^{-1}. Before the experiment started, the column effluent seawater was discarded for the first 70 hours (both oil-exposed and control) to ensure the removal of acutely toxic volatile compounds from the oil, such as monoaromatic compounds benzene, toluene, and xylene isomers (BTEX). The experiment started once the water flow through the columns was connected to the experimental tanks (day 0). In addition to water from the rock columns, all tanks received an unaltered clean seawater supply (3–4 L min^{-1}) to maintain sufficient oxygen levels and water exchange rates.

Fish were hand-fed as a group, once daily, with one of two possible feed rations of frozen *Calanus finmarchicus*. A low feed ration (1.5% somatic weight day^{-1}) represented the estimated mean feed required to cover the energetic maintenance cost of polar cod, while the high feed ration (4% somatic weight day^{-1}) was estimated to provide fish with a surplus of energy (Hop, Tonn, and Welch 1997). The amount of feed given per tank was calculated based on the average estimated somatic (gutted) weight of the fish in each tank. Somatic weight was calculated using fork-length somatic weight correlations ($y = 0.0058 \times {}^{2.9716}$, $R^2 = 0.969$) from a large data set on feral polar cod from Svalbard (Nahrgang et al. 2014).

During the experimental period, polar cod were sampled when they were in a late stage of reproductive development (Vtg3 oocytes in females and spermatozoa present in males) – day 0 (Dec. 14th 2018), during the spawning window – day 47 (Jan. 31st 2019), and during the post-spawning period – day 131 (April 25th, 2019). At each sampling event, an equal number of polar cod (day 0: $n = 6$, day 47, and 131: $n = 8$) were sampled from each tank at a balanced sex ratio to allow studying sex-specific effects of the exposure. Each polar cod was anesthetized in 100 mg/L Tricaine Methanesulfonate and blood was drawn from the caudal vein. Following extraction, blood was centrifuged for ten minutes at 3500 rpm at a temperature of 4°C (Sorvall RC 5B Plus centrifuge, Marshall Scientific) to obtain blood plasma, then stored at − 80°C. After obtaining the blood, fish were sacrificed by a sharp blow to the head, and fork length (± 0.1 cm), wet weight (± 0.1 g), gonad weight (± 0.1 g), and somatic weight (± 0.1 g) were measured. Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated based on the following equations $GSI = (Gonad\ weight/Somaticweight) \times 100$ and $HSI = (Hepatic\ weight/Somaticweight) \times 100$. Sections were cut from the middle of the gonads and transferred to 4% neutral-buffered formalin (Sigma Aldrich). A section from the anterior of the liver and gallbladder was removed, snap-frozen in liquid nitrogen, and stored at − 80°C for gene expression and PAH biliary metabolite analyses, respectively.

For the sampling during the spawning window (day 47), all fish were stripped for egg and milt samples in addition to the other samples. Thirty egg samples were snap-frozen in liquid nitrogen before being stored at − 80°C for chemical analyses. Milt samples were immediately assessed for sperm motility (section 2.4.1).

One additional tank (control treatment, high-feed ration, $n = 40$) was included for the sole purpose of assessing the onset of spawning without disturbance to the experimental fish. Starting December 28th, 2018, stripping tests were conducted on females and males, approximately every second day. This, along with histology and observational data from the experimental tanks, was used to decide the date for the spawning window sample. The final decision to begin the sampling was made when video footage (GoPro) showed frequent eggs being released in tanks 2 (oil-exposed, high-feed) and 12 (oil-exposed, low-feed).

Chemical analyses

Water & eggs

Water samples (0.8 L) were taken on exposure day 0 (tanks: 1, 2, 3, 8, 10, 12), 2 (tank 2), 4 (tank 2), 8 (tank 2), 10 (tank 2), 18 (tank 2, 5, 8, 12), 28 (tank 1, 2), and 131 (tank 1, 2). Samples were retrieved from the center of the tank roughly 30 cm under the surface using plastic tubing and transferred into duran-bottles pre-filled by sintef with 15% hydrochloric acid (HCL). Cross contamination was limited by using different tubing for oil-exposed and control tanks and flushing the tubing with clean water between samples. After collection, water samples were stored in a dark environment at 4°C until analyzed. Thirty eggs were sampled on day 47 and snap-frozen in liquid nitrogen before being stored at − 80°C. Both sample types were analyzed for PAHs and alkyl PAHs according to methods described in Sørensen et al. (2016a, 2016b). Details on the extraction and analysis of water and egg samples are provided in Supplementary Material S1.

Biliary PAH metabolites

Gall bladder samples taken at day 47 were extracted and analyzed for two PAH metabolites, 1-hydroxypyrene (pyrenol) and 9-hydroxyphenanthrene (phenanthrol), using LC-MS/MS (Zhang et al. 2015). Details on the extraction and analysis of gall bladder are provided in S1.

Biological analysis

Sperm motility

Sperm motility parameters were assessed directly after sampling with the help of the CASA system (Computer Assisted Sperm Analysis, SCA/Sperm Class Analyzer, Microptic Diagnostic Systems) based on the procedures outlined in Bender et al. (2016) and Rudolfsen et al. (2005). Sperm was activated by adding cold seawater (≈2°C) using a dilution factor of 1:200 and analyzed by pipetting 4 µL onto a chamber microscope slide (Leica standard count 2 chamber slide 20 μm) mounted to a pre-cooled specialized stage (≈2°C) provided by the CASA system (Kime et al. 2001). Motility parameters were measured with the CASA software system coupled to a video camera and a phase-contrast microscope (Nikon E-200, Tokyo – Japan) with a 10× negative contrast objective. Sperm parameters were analyzed and recorded across 4 distinct frames per duplicate sperm sample obtained from each male fish within one minute of activation. On average, there were ≈ 4000 sperm cells per frame analyzed (1600 sperm cells per sample replica). Results obtained from the sperm motility analyses were derived from the average of all 4 frames analyzed per duplicate. Recorded mean sperm motility parameters included: (1) curvilinear velocity (VCL), µm s^{-1}, (2) straight line velocity (VSL), µm s^{-1}, (3) average path velocity (VAP), µm s^{-1}, (4) Straightness (STR = VSL/VAP), and (5) Linearity (LIN = VSL/VCL).

Gonad histology

Following collection, histology samples were stepwise cleaned and preserved in bath combinations containing ethanol (70 and 96%), Histo-clear (National diagnostics), and paraffin wax according to Bender et al. (2016). Samples were sliced into 5 and 6 µM sections for males and females,

respectively. Thicker slices were taken from females due to the high lipid content in the ovaries. Slides were scanned using a digital slide scanner and NDP.view2 (Hamamatsu Photonics). Male and female reproductive phase was determined using the Brown-Peterson et al. (2011) classifications. Reproductive structures were identified based on published literature on polar cod (Bender et al. 2016; Nahrgang et al. 2016) and general teleost gonad histology (Brown-Peterson et al. 2011; McMillan and McMillan 2007). Post-ovulatory follicles (POFs) were classified into POF1, POF2, POF3, and atresia were classified into alpha and beta atresia based on the descriptions in ICES (2019). Examples of gonadal structures and reproductive phases are reported in S2 and S3, respectively.

The Brown-Peterson et al. (2011) classification worked well for most reproductive phases for males and females, but some individuals did not have a clear phase around spawning. We observed that several polar cod were in a very early stage of regression and had likely spawned close to when we took the sample on day 47. We observed samples where POFs were present in females, but many residual eggs remained (Anders Thorsen, personal communication). For males, we could see early signs of regression in the periphery of the testis with the thickening of the germinal epithelium and spermatozoa produced during the previous reproductive cycle were in contact with the Sertoli cells (Rüdiger Schulz, personal communication). To follow the standardized classification outlined by Brown-Peterson et al. (2011) we included these individuals in the regressing phase. However, a distinct phase may be better suited for similar individuals in future work, which indicates that individuals are not far into the regressing phase.

Using a Weibel grid, the ovarian volume fraction (Vv_i) was estimated for elements of the ovary in order to determine the (Vv_i) for Atresia a and b. Following the protocol described in Serrat et al. (2019), Vv_i was calculated by dividing the number of grid points hitting the ovarian element of interest by the total grid points hitting the ovarian tissue, excluding grid points overlying open space.

Each ovarian section was divided into 2000 × 2000 µM square grid. Each square represented one possible counting field on which a grid could be overlaid. A subsample of counting fields was chosen from random positions within each histology section based on the criteria outlined in S4. Weibel-grid analysis was performed in ImageJ (imagej.nih.gov/ij), using the ObjectJ plug-in (sils.fnwi.uva.nl/bcb/objectj) and Weibel-grid embedded macro (sils.fnwi.uva.nl/bcb/objectj/examples/Weibel/MD/weibel.html).

Optimization tests were performed according to Serrat et al. (2019) to find an optimal grid density and the number of counting fields (2000 × 2000 µM) necessary. Four female samples were used, each in a different phase of reproductive development (spawning capable, active spawning, regressing, regenerating). For the first test, different grid densities were compared to find an optimal grid density to avoid underestimation of Vv_i or unnecessary time consumption. The second test compared various numbers of counting fields to optimize the number of fields necessary for each subsample to capture the least frequent elements in the ovary. Following optimization, the Weibel grid was set to the optimal grid density (approximately 1190 points per 1 mm x 1 mm section), and five 2000 × 2000 µM counting fields were analyzed from each female sample from day 47 and day 131. Statistical tests were conducted to assess whether or not an individual was stripped may have affected the volume fraction of atresia.

Steroid hormone analysis

A subset of plasma samples was selected to be representative of oil exposure, feed ration, sampling time-point, and sex. Samples with the largest volume were prioritized to achieve adequate volume for the analysis until $n = 16$ per sex and sample time point were obtained. After preliminary assays, the remaining male samples from day 47 sampling were extracted and analyzed as well. Standard plasma extraction of steroid hormones was conducted according to the protocols described in the Cayman ELISA protocol and liquid solvent extraction in Metcalfe et al. (2018). Briefly, liquid solvent extraction (ethyl acetate and N-hexane, 50:50, v:v, Sigma Aldrich) was conducted on plasma samples diluted with phosphate-buffered saline (PBS) to an initial dilution of 1:10 (w:w) to increase the sample volume to an adequate

amount for analysis. Following the protocol supplied within each kit, enzyme-linked immunosorbent assay (ELISA) were conducted for: E2 (Cayman EIA kit 501,890), 11-KT (Cayman EIA kit 11-keto Testosterone 582,751), and T (Cayman EIA Testosterone kit 582,701). Two dilutions were tested for each sample, except for the E2 assays, where only undiluted samples were used due to the low levels of the hormone during the period of study.

Hepatic mRNA expression

Quantitative polymerase chain reaction

Total ribonucleic acid (tRNA) was isolated from frozen liver samples following the Tri-reagent (Sigma Aldrich) protocol. The assessment of the concentration and integrity of the RNA samples, reverse transcription, and quantitative polymerase chain reaction (qPCR) assays were performed following the procedures in Yadetie et al. (2018). Briefly, complementary DNA (cDNA) was prepared from 1 µg total RNA per sample using the iScript cDNA synthesis kit (BIO-Rad) according to the manufacturer's protocol. All cDNA samples were diluted 1:10 with nuclease-free water. Using the diluted cDNA samples, qPCR were run in replicates for 3 target genes: *vtgα*, *esr1*, *cyp1a*, and one house-keeping reference gene beta- actin (*act2b*) according to Yadetie et al. (2018). Samples were run in groups separated by sampling time and sex. Replicates were run for each reaction. Fold-change was calculated using the comparative Ct methodology (Schmittgen and Livak 2008). The average normalized result for control high-feed ration and control low-feed ration as the point from which fold change was calculated.

Primers were designed using primer 3 software (https://primer3.ut.ee/) (Table 1). The primers were previously used in work on Atlantic cod. Primers for *esr1*, *cyp1a* and *act2b* were previously used in Yadetie et al. (2018) and *vtgα* in Eide et al. (2014). These primers were compared to polar cod sequences and found to be matching.

mRNA expression with RNA sequencing

Five high-feed control and five high-feed-oil-exposed female tRNA samples from day 47 were sent for RNA sequencing (RNA-seq) by Novogene (Novogene – Genome sequencing). All female RNA samples from the high-food treatment at the day 47 sampling timepoint were analyzed using Agilent 2100 Bioanalyzer. Samples with an RNA intergrity value (RIN) value above seven were chosen for RNA-seq. If more than five samples had a sufficient RIN value, the samples with the highest values were selected first. The RNA-seq data were analyzed as described by Yadetie et al. (2018) using the recently developed RASflow pipeline (Zhang and Jonassen 2020) and the Atlantic cod reference genome (Ensembl, https://www.ensembl.org/index.html). False discovery rate (FDR) < 0.05 and fold change ≥ 2.00 (for up-regulated) or ≤ 0.5 (for downregulated) were considered differentially expressed. Mapping polar cod genes to the Atlantic cod orthologs facilitated the extraction of human (*Homo sapiens*) and zebrafish (*Danio rerio)* orthologs from the Ensembl database for pathway analysis as described before (Yadetie et al. 2018). Pathway and network analysis were conducted using the Cytoscape network visualization and analysis tools with Cluego application (Bindea et al. 2009; Shannon et al. 2003).

Statistical analysis

Statistical analysis was performed in R 4.0.5 (Team 2018). Comparisons were considered statistically significant when $p \leq 0.05$. Linear mixed effect model (lme) models were used to test all continuous response variables (gene expression, steroid hormones, GSI, VAP, VSL, and VCL) using the

Table 1. Primers used for qPCR including primer sequence, name, symbol, *Gadus morhua* ensembl id and *Boreogadus saida* gene id.

Name	*G. morhua* Ensembl gene id *B. saida* id	Primer sequence
Vitellogenin (*vtg a*)	ENSGMOG00000016966 OMLE01136441.1	5´-agactggcctggtcgtcaaa 5´-gcgaggatagaggcagggat
Estrogen receptor 1 (*esr1*)	ENSGMOG00000014898 OMLE01091583.1	5´-cgctttcggatgctccag 5´-acgagaaggccccagagttg
Cytochrome P450 1A (*cyp1a*)	ENSGMOG00000000318 OMLE01128479.1	5´-caccaggagatcaaggacaag 5´-gcaggaaggaggagtgacggaa
Beta-actin (*act2b*)	ENSGMOG00000009683 OMLE01037931.1	5´-cgacgggcaggtcatcaccatcg 5´-ccacgtcgcacttcatgatgctgt

R package "nlme." lme models were set up to include the following: two fixed predictor variables (feed group and exposure group), an interaction term (feed|exposure), and a random predictor variable (tank). Normality was assumed for continuous variables. The reproductive phase was included as a random predictor for *vtg α*, *esr1*, 11-KT, and T results. All models were arranged with feed group as the first fixed variable followed by oil exposure to employ the most rigorous test to determine oil-exposure significance. Pairwise t-test were conducted for GSI and HSI results.

A generalized linear model (glm) with a binomial distribution (ranging from 0 to 1) was used to test the ovarian volume fraction occupied by elements in the ovary and for sperm motility factors straightness and linearity (lme4). A test was conducted to assess whether the data was under or overdispersed by comparing the residual deviance/residual degrees of freedom to 1. This test returned a number under 1 indicating underdispersion, this was corrected for during analysis by using a quasibinomial, family test in the glm model.

In all models used, a backward elimination method was employed. All factors were included in the initial model. If the interaction term was not significant, the term was removed until the most parsimonious model remained. Following Vieweg et al. (2022), oil exposure was never removed from the model as this was the primary research question we aimed to address. The most parsimonious model was noted in-text (p-values).

A Chi-squared (χ^2) procedure was used to test the effects of oil exposure and feed ration on the reproductive phase. For males and females, reproductive phases were simplified into two groups: individuals that had spawned and individuals that had not spawned to satisfy the minimum cell requirements for the test. One immature female from the control high-feed group was removed from the analysis.

Results

Seven polar cod died naturally during the experiment. Four of the polar cod were from control-low-food tanks, 2 from control-high-food tanks and 1 from an oil-exposed-high food tank. None of these deaths were attributed to high levels of crude oil.

Chemical analyses

Water

In oil-exposed tanks, total PAH (tPAH) concentration ($\sum$ 44 PAHs) started at an average of 13.0 μgL^{-1} ± 4.0 SD ($n = 4$) on day 0 and decreased to a final value of 0.09 μgL^{-1} ($n = 1$) after 131 days of water being continuously flushed through the oiled-rock column. The composition of PAHs also changed throughout the experiment (Figure 2). Total PAH levels were not significantly different between feed groups during the experiment ($F(1) = 0.29$, $p = 0.596$). In control tanks the concentration of tPAH remained low throughout the experiment (0.40 μgL^{-1}± 0.30 SD).

Eggs

At day 47, eggs from oil-exposed females showed significantly higher levels ($F(1,8) = 12.02$, $p = 0.009$) of tPAH accumulation compared to eggs from control females (Figure 3). Food treatment did not affect the levels of tPAH in the eggs ($F(1,7) = 0.026$, $p = 0.87$). Naphthalenes represented the highest accumulated PAHs found in the oil-exposed eggs. On average, C3-naphthalenes were dominant in most of the oil-exposed eggs (294.7 ± 52.1 standard deviation (SD) $ng\ g^{-1}$).

Bile metabolites

At day 47, PAH metabolites, pyrenol ($F(1,19) = 0.01$, $p = 0.92$) and phenanthrol ($F(1,19) = 0.55$, $p = 0.47$) did not differ between the sexes and the sexes were pooled for further testing. Both metabolites pyrenol ($F(1,10) = 169.55$, $p < 0.0001$) and phenanthrol ($F(1,10) = 226.56$, $p < 0.0001$) were significantly higher in oil-exposed polar cod bile samples compared to control groups (Table 2).

Somatic analysis & gonad histology

Somatic analysis

Both female ($F(2,109) = 91.5$, $p < 0.0001$) and male ($F(2,125) = 56.37$, $p < 0.0001$) GSI signifi-

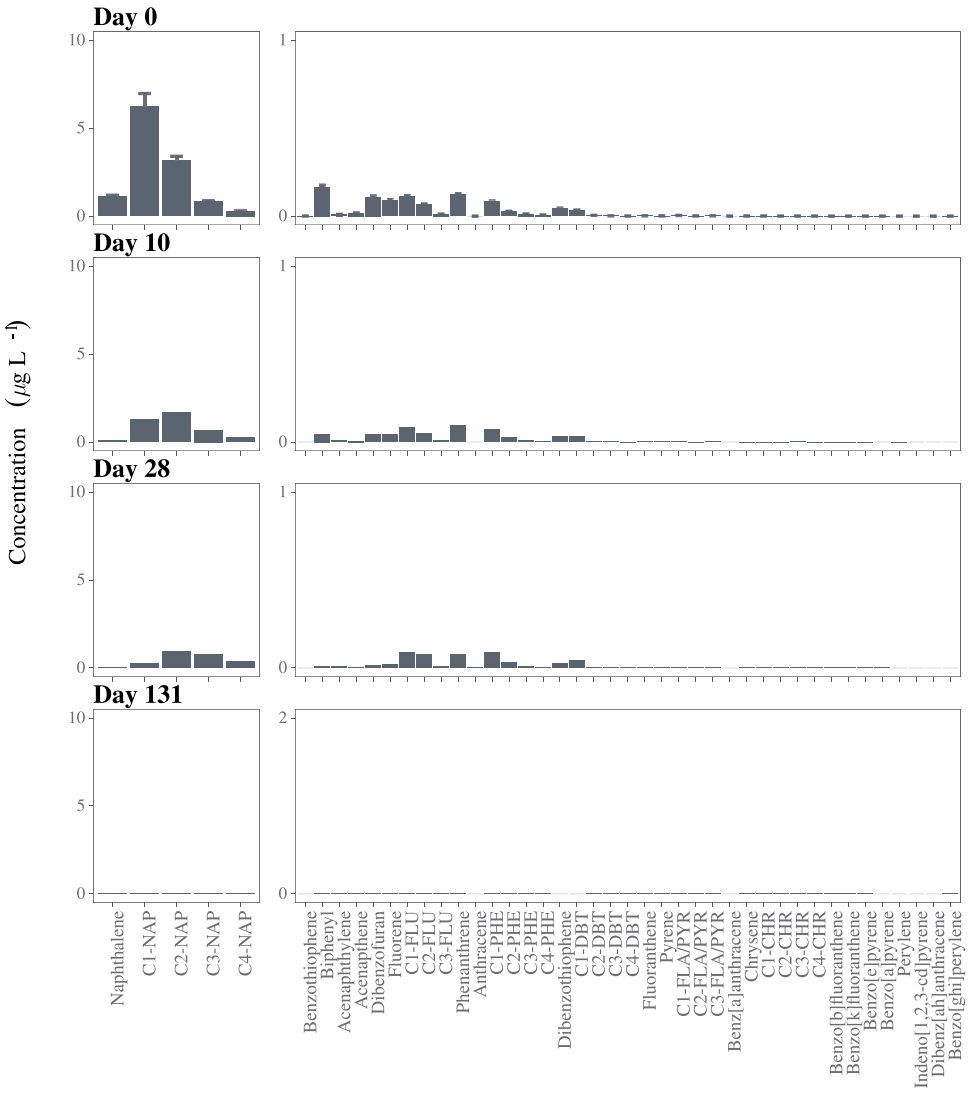

Figure 2. Average PAH composition ($\sum$ 44 PAHs) $\pm$ standard error represented as $\mu g L^{-1}$ from crude-oil-exposed water samples taken at day 0) ($n = 4$), day 10) ($n = 1$), day 28) ($n = 1$), day 131) ($n = 1$) after exposure began.

cantly changed throughout the experiment. In both sexes, GSI increased from day 0 to its highest value at day 47 and decreased to its lowest value at day 131 (Table 3). Food group did not have an effect on GSI of the control group fish for both sexes during the experiment ($F(1,4) = 0.29$, $p = 0.62$). At day 47, female GSI was 33% higher in the control-low-food compared to the exposed-low-food group and 18.4% higher in the control-high-food compared to the exposed high-food-group. However, these differences were not significant ($F(1,10) = 4.35$, $p = 0.06$). At day 131, neither oil treatment ($F(1,9) = 0.78$, $p = 0.40$) nor feed treatment ($F(1,9) = 0.25$, $p = 0.63$) resulted in a significant difference in female GSI. There were no significant differences in male GSI at day 47 or day 131.

Female ($F(2,115) = 55.12$, $p < 0.0001$) and male ($F(2,126) = 36.67$, $p < 0.0001$) HSI significantly decreased throughout the experimental period. Neither oil-exposure nor feed group affected male or female HSI at day 47. At day 131, the low-feed-group males had a significantly lower HSI ($F(1,9) = 5.32$, $p = 0.047$) compared to the high-feed males and females in the oil-exposed group had a significantly higher HSI ($F(1,9) = 11.3$, $p = 0.008$) compared to control females.

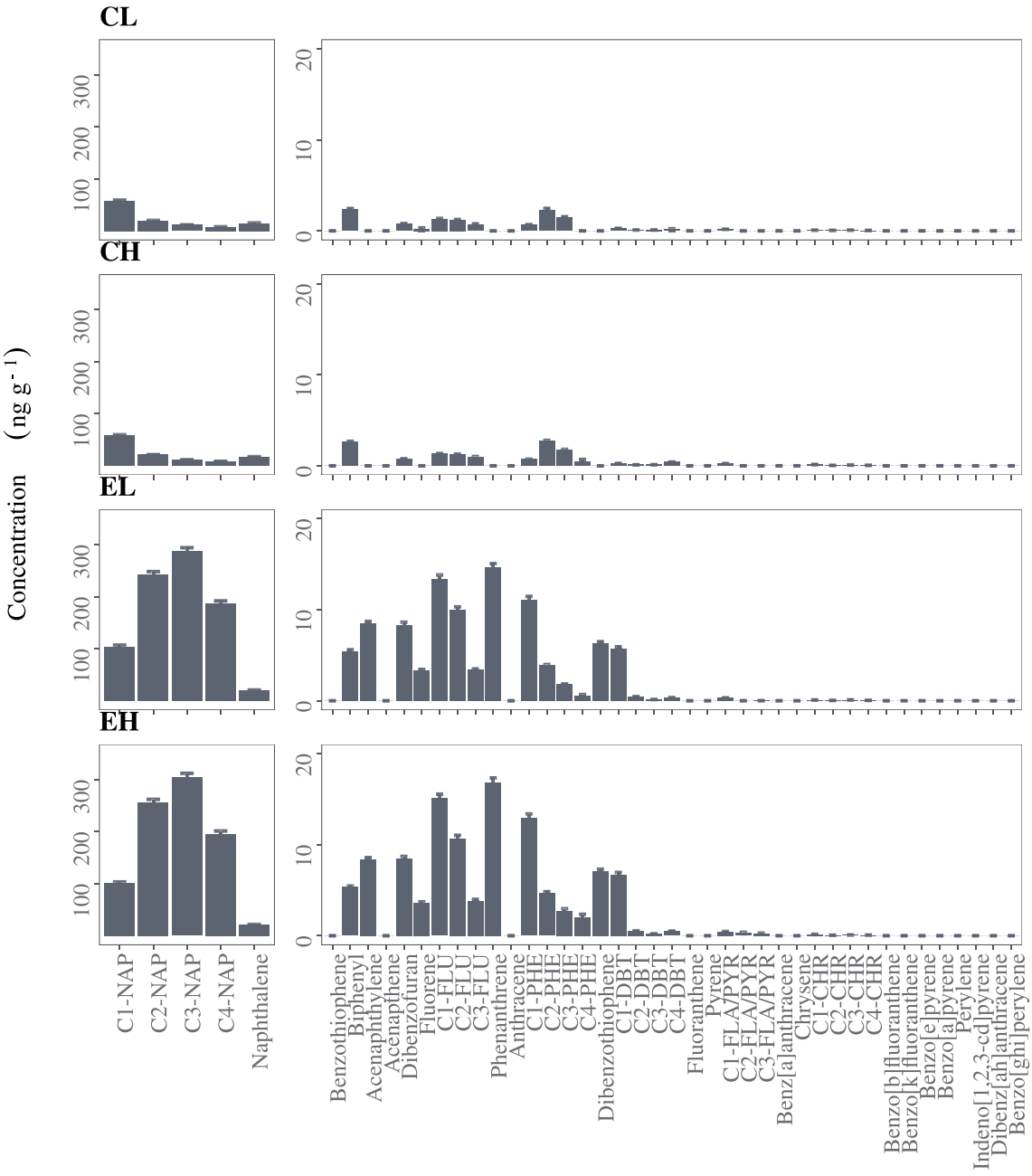

Figure 3. Average PAH composition ($\sum$ 44 PAHs)(ngg^{-1}) from eggs taken 47 days after female polar cod (*Boreogadus saida*) started to be exposed to a crude oil WSF or control conditions. CL) eggs from polar cod females in the control low-feed group ($n = 3$), CH) control high-feed group ($n = 4$), EL) oil-exposed low food ($n = 8$), and EH) oil-exposed high food ($n = 6$). Low and high food treatments received 1.5% somatic weight day^{-1} and 4% somatic weight day^{-1}, respectively.

Table 2. Bile metabolites phenanthrol and pyrenol in polar cod (*Boreogadus saida*) exposed to either a crude oil WSF or control conditions and a low-feed (1.5% somatic weight day^{-1}) or a high feed (4% somatic weight day^{-1}) ration for 47 days. CL = control low-feed, CH= control high-feed, EL= oil-exposed low-feed, EH= oil-exposed high-feed. Summary statistics reported as mean ± standard deviation (n).

	Phenanthrol	Pyrenol
CL	60.5 ± 31.5 (8)	31.0 ± 28.6 (8)
CH	51.0 ± 20.8 (8)	18.9 ± 25.1 (8)
EL	14106.8 ± 3755.4 (8)	1926.1 ± 550.0 (8)
EH	11685.3 ± 2737.8 (8)	1524.3 ± 372.2 (8)

Table 3. Gonadosomatic index (GSI) and hepatosomatic index (HSI) of polar cod (*Boreogadus saida*) exposed to either a crude oil WSF or control conditions and a low-feed (1.5% somatic weight day^{-1}) or a high feed (4% somatic weight day^{-1}) ration. CL = control low feed, CH= control high feed, EL = oil-exposed low feed, EH = oil-exposed high feed. Summary statistics reported as mean ± standard deviation (n). Significant differences among treatments for each parameter/treatment and timepoint are indicated by different letters (pairwise t-tests).

	Female GSI	Male GSI	Female HSI	Male HSI
Day 0				
CL	19.9 ± 3.75 (10)	26.2 ± 4.70 (9)^{a}	15.2 ± 2.54 (10)	10.9 ± 2.53 (9)^{a}
CH	18.4 ± 3.07 (8)	28.4 ± 6.60 (9)^{a,b}	13.3 ± 1.89 (8)	13.9 ± 4.28 (9)^{a,b}
EL	18.7 ± 3.61 (9)	31.5 ± 3.62 (10)^{b}	12.9 ± 2.88 (9)	15.0 ± 3.29 (9)^{b}
EH	17.5 ± 2.53 (7)	26.4 ± 5.50 (9)^{a}	15.2 ± 3.00 (7)	14.1 ± 2.89 (10)^{b}
Day 47				
CL	42.4 ± 14.7 (12)	30.2 ± 5.08 (11)	10.1 ± 2.11 (14)	9.57 ± 3.96 (14)
CH	35.4 ± 16.4 (10)	28.3 ± 13.5 (12)	11.6 ± 4.26 (13)	12.5 ± 9.56 (13)
EL	28.6 ± 18.9 (13)	32.1 ± 4.84 (13)	11.2 ± 4.02 (13)	8.58 ± 2.83 (13)
EH	28.9 ± 17.5 (10)	30.0 ± 6.32 (15)	10.1 ± 2.58 (11)	9.32 ± 1.82 (11)
Day 131				
CL	3.60 ± 1.69 (10)	15.7 ± 6.66 (14)	5.41 ± 2.00 (10)^{a}	4.48 ± 1.90 (14)^{a,b}
CH	4.13 ± 2.00 (12)	18.6 ± 9.84 (12)	6.47 ± 2.08 (12^{a}	6.37 ± 3.67 (12)^{a}
EL	3.57 ± 0.88 (12)	16.2 ± 3.56 (12)	9.15 ± 2.59 (12)^{b}	6.20 ± 1.40 (12)^{a,b}
EH	3.40 ± 0.73 (10)	14.5 ± 7.73 (13)	7.37 ± 1.95 (10)^{a,b}	7.44 ± 2.99 (14)^{b}

Gonad histology

At the experiment start (day 0), female and male samples were all in the spawning capable reproductive phase (Section 2.5, SI 2). Tertiary vitellogenic oocytes (Vtg3) were present in female ovaries. In Males, 90.1% were fully developed (Spawning capable – Late-GE phase) and 9.9% were almost fully developed (Spawning capable – Mid-GE phase).

At day 47 (spawning window), a significantly higher proportion of males ($\chi^2(1) = 8.17$, $p = 0.004$) and females ($\chi^2(1) = 9.20$, $p = 0.002$) had already spawned in the oil-exposed group compared to the control group (Figures 4a and 3b). At this time, 90% of oil-exposed females and 73.1% of oil-exposed males had spawned compared to 56% of the control females and 31.8% of control males. Feed group alone did not result in a significant difference in whether females ($\chi^2(1) = 0.07$, $p = 0.793$) or males ($\chi^2(1) = 3.02$, $p = 0.082$) had spawned or not.

At day 131 (post-spawning), oil-exposed females no longer showed a trend of advanced reproductive phase compared to controls (Figure 4c). Males did not show a trend of advanced reproductive regeneration in the oil-exposed group. However, the 2 individuals in the early developing phase were both from the oil-exposed group (Figure 4d). Few individual females in the regenerating phase and males in the early developing phase hindered significance testing.

Gonad histology - Weibel grid analysis

Based on Weibel-grid analysis the ovarian volume fraction occupied by alpha atresia (*Resid. Dev.* (1,36) = 1.07 $p = 0.26$) and beta atresia (*Resid. Dev.* (1,36) = 0.044 $p = 0.85$) did not significantly differ between oil-exposed and control groups at day 47. At day 131, beta atresia was significantly higher in control compared to oil-exposed females (*Resid. Dev.* (1,15) = 0.044 $p = 0.033$), but alpha atresia did not differ (*Resid. Dev.* (1,15) = 0.32, $p = 0.172$). Whether or not a female was able to be stripped did not affect the amount of atresia present in the ovary (*Resid. Dev.* (1,14) = 0.05, $p = 0.19$).

Sperm motility analyses

Sperm motility measurements taken on day 47 did not differ significantly between oil exposure and control groups. However, some measurements did differ significantly between feed groups. VSL ($F(1,9) = 6.69$, $p = 0.03$),VAP ($F(1,8) = 7.20$, $p = 0.028$), and linearity (LIN) (*Resid. Dev.* (1,35) = $p = 0.02$) were significantly lower in the low-feed males compared to the high-feed males (Table 4). VCL ($F(1,9) = 4.0$, $p = 0.08$) and Straightness (STR) (*Resid. Dev.* (1,35) = 0.21, $p = 0.07$) did not differ significantly between feed groups, but still displayed a trend of lower values in the low-feed group compared to the high-feed group.

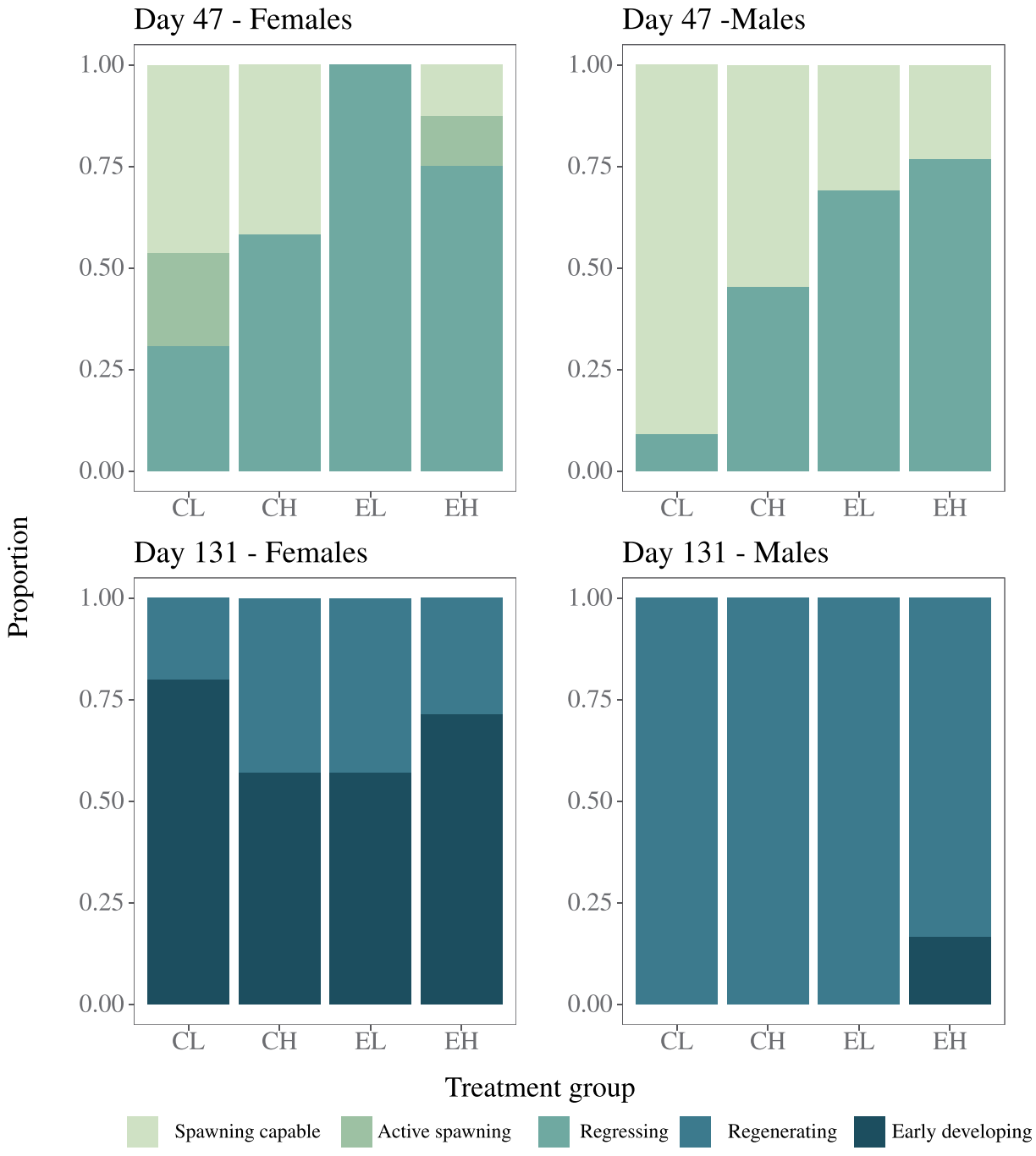

Figure 4. The proportion of polar cod (*Boreogadus saida*) in each reproductive phase between treatment groups for females at day 47, males at day 47, females at day 131, and males at day 131. Treatment was exposure to a crude oil WSF or control conditions, CL: control low-feed, CH: control high-feed, EL: oil-exposed low-feed, EH: oil-exposed high-feed. Low food groups received a food regime of 1.5% somatic weight day^{-1} and high food regimes received 4% somatic weight day^{-1}. Females day 47) $n = 12, 14, 8, 11$ males day 47) $n = 5, 7, 7, 7$ females at day 131) $n = 12, 10, 11, 14$ males at day 131) $n = 11, 5, 6, 8$.

Steroid hormones

At day 47, neither 11-KT ($F(1,17) = 2.70$, $p = 0.12$) nor T ($F(1,13) = 1.0$, $p = 0.34$) were significantly lower in oil-exposed males compared to controls (Figure 5). Feed groups and the interaction term did not significantly affect either 11-KT or T. Female steroid hormone levels (E2, T) were too low (below kit LOD) in the majority of females during the period of natural reproduction studied for any meaningful comparisons between treatment groups.

Table 4. Sperm motility measurements (VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, STR = straightness, LIN = linearity) of polar cod (*Boreogadus saida*) exposed to either a crude oil WSF or control conditions and a low-feed (1.5% somatic weight day^{-1}) or a high feed (4% somatic weight day^{-1}) ration. CL = control low feed, CH= control high feed, EL = oil-exposed low feed, EH = oil-exposed high feed. Summary statistics reported as mean ± standard deviation (n).

	VCL μm s^{-1}	VSL μm s^{-1}	VAP μm s^{-1}	STR	LIN
CL	129.2 ± 4.1 (8)	61.0 ± 2.9 (8)	79.1±2.6 (8)	0.8 ± 0.02 (8)	0.5 ± 0.03 (8)
CH	131.9 ± 6.1 (10)	64.6 ± 7.6 (10)	82.3±5.4 (10)	0.8 ± 0.1 (10)	0.5 ± 0.1 (10)
EL	126.1 ± 9.9 (10)	58.7 ± 5.9 (10)	76.4±6.7 (10)	0.5 ± 0.03 (10)	0.5 ± 0.03 (10)
EH	132.2 ± 5.8 (9)	65.2 ± 5.7 (9)	82.0±4.7 (7)	0.8 ± 0.03 (9)	0.5 ± 0.03 (9)

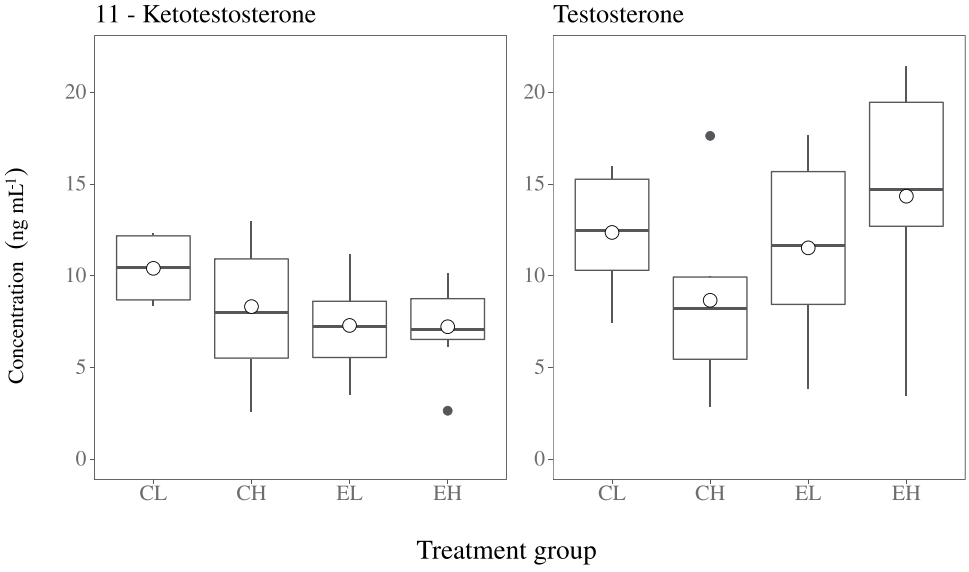

Figure 5. Levels (ng/ml) of 11 - Ketotestosterone and testosterone in plasma extracts from polar cod (*Boreogadus saida*) males exposed to a crude oil WSF or control and low or high-feed conditions for 47 days CH: control high-feed, EL: oil-exposed low-feed, EH: oil-exposed high-feed. Low and high food treatments received 1.5% somatic weight day-1 and 4% somatic weight day-1, respectively. 11 - Ketotestosterone) $n = 5, 9, 10, 7$ testosterone) $n = 7, 6, 9, 4$. White dots represent the mean. Whiskers represent the minimum and maximum values (without outliers), lower box limit – Q1, middle – median, upper limit – Q3, white circle – mean, black dots – outliers (1.5 times the interquartile range away from the upper and lower limit), numbers in parenthesis – sample size (n).

Hepatic mRNA expression

mRNA using quantitative polymerase chain reaction

At day 47, the fold-change in the relative expression of *cyp1a* was significantly higher in oil-exposed male ($F(1,8) = 32.66$, $p = 0.0004$) and female ($F(1,8) = 104.93$, $p < 0.0001$) polar cod compared to controls (Figure 6). In addition, there was a significant interaction effect between food and exposure group for both sexes (Fem: $F(1,8) = 5.35$, $p = 0.05$; Mal: $F(1,8) = 11.65$, $p = 0.009$).

At day 131, the fold-change in the relative expression of *cyp1a* remained significantly higher in oil-exposed males ($F(1,9) = 48.14$, $p = 0.0001$) and females ($F(1,10) = 42.57$ $p = 0.0001$) compared to controls. At this time, there was not a significant interaction effect between exposure and food group or significant difference between sexes for *cyp1a* expression.

Both *esr1* ($F(1,26) = 14.19$, $p = 0.0001$) (Figure 7a) and *vtg α* ($F(1,24) = 24.7$, $p < 0.0001$) (Figure 7b) differed significantly with reproductive phase in female polar cod at day 47. Due to the significant difference in the levels of both genes between reproductive phases, reproductive phase was included in the model as a random predictor. With the reproductive phase included as a random predictor, there were no longer significant differences in the expression of either *esr1* ($F(1,12) = 0.91$, $p = 0.36$) or *vtg α* ($F(1,12) = 0.05$, $p = 0.82$).

At day 131, oil exposure alone did not cause a significant difference in expression levels of *esr1* ($F = 4.86$, df = 8, $p = 0.057$). However, there was

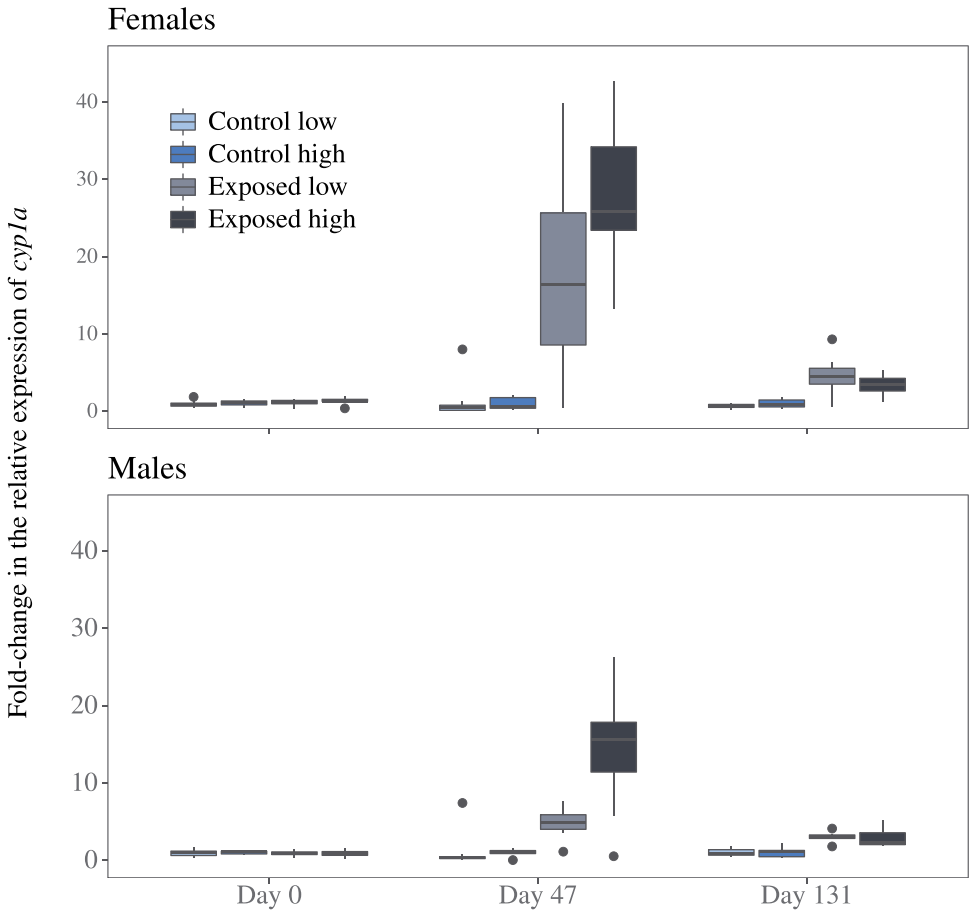

Figure 6. Fold-change in the relative gene expression of hepatic cytochrome P450 1A (cyp1a) in female and male polar cod (*Boreogadus saida*), determined by qPCR. Polar cod were exposed to a crude oil WSF over 131-days or control conditions. Low feed groups received a feed regime of 1.5% somatic weight day^{-1} and high feed regimes received 4% somatic weight. Female) *n* = day 0 = 8, 8, 8, 7 day 47 = 14, 12, 13, 9 day 131 = 9, 11, 10, 8 male) *n* = day 0 = 10, 6, 4, 8 day 47 = 11, 7, 11, 9 day 131 = 8, 8, 6, 11. Whiskers represent the minimum and maximum values (without outliers), lower box limit – Q1, middle – median, upper limit – Q3, white circle – mean, black dots – outliers (1.5 times the interquartile range away from the upper and lower limit), numbers in parenthesis – sample size (n).

a significant interaction between feed group and exposure group ($F(1,10) = 5.13$, $p = 0.047$) (Figure 7a). *vtg α* expression was significantly higher ($F(1,11) = 6.74$, $p = 0.03$) in oil-exposed females compared to control. The qPCR assays for *cyp1a*, *vtg α*, and *esr1* were validated with the RNA-seq results for the female fish (Data repository – DEGs). In both qPCR and RNAseq results *cyp1a* was upregulated in oil-exposed individuals compared to controls, *vtgα* , and *esr1* were both down-regulated in oil-exposed females compared to controls.

Transcriptome analysis using RNA-sequencing

RNA-seq results show that 947 genes were differentially expressed (false discovery rate (FDR) value < 0.05), between high-feed-oil-exposed female polar cod and high-feed-control females at day 47 (data repository – DEGs). In oil-exposed females, *cyp1a* and Aryl Hydrocarbon Receptor Repressor (*ahrr*) genes were among the top up-regulated genes. Functional enrichment analysis using human orthologs showed some of the top enriched pathways were the aryl hydrocarbon receptor (AhR) signaling pathway, oxidative stress pathway, steroid biosynthesis, and ovarian infertility pathway (Figure 8). Genes contributing to the enrichment of the ovarian infertility pathway were the zona pellucida protein (ZP2 and ZP3), prolactin receptor (PRLR), and aromatase (CYP19A1). Out of the eight genes involved in the enrichment of the steroid biosynthesis pathways, five were related to cholesterol biosynthesis, with four of these being down-regulated, NAD(P) Dependent Steroid

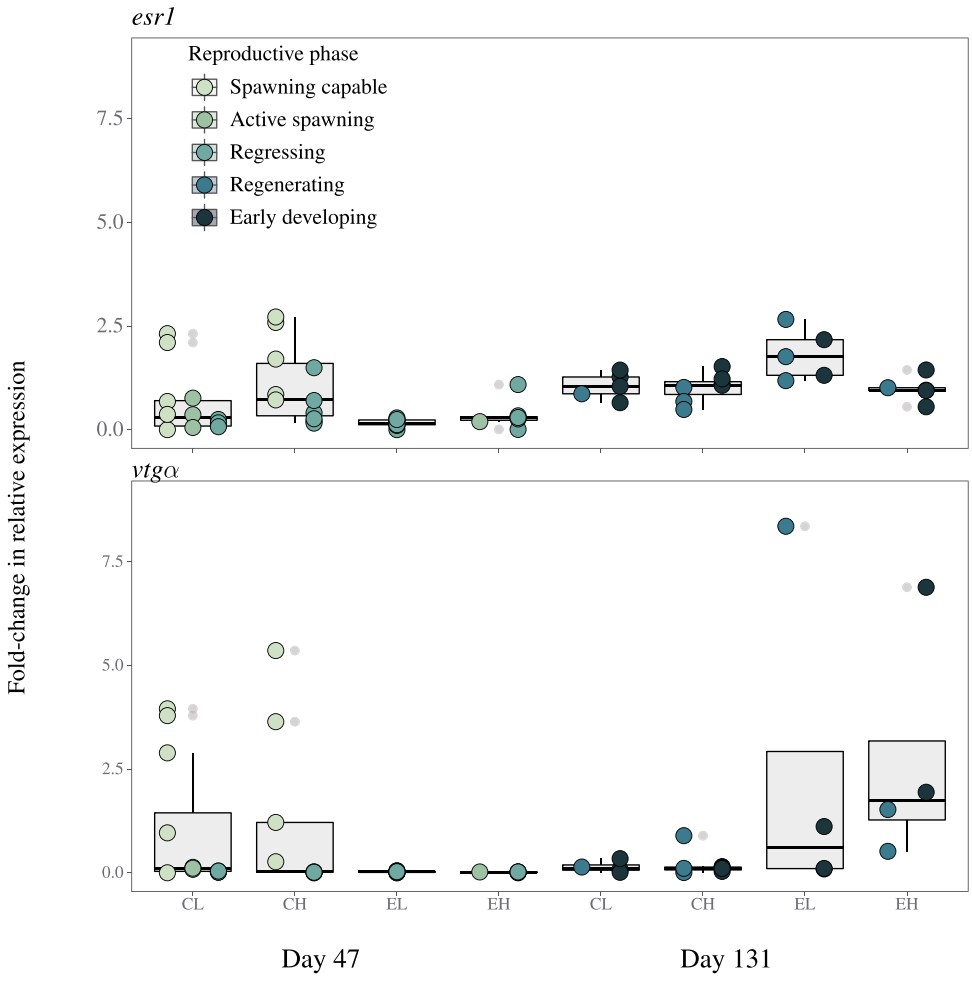

Figure 7. Fold-change in the relative expression of *esr1* and *vtg a* 47 and 131 days after exposure start in female polar cod. Exposed females were exposed to a crude oil WSF throughout the experimental period, control females were not CH: control high-feed, EL: oil-exposed low-feed, EH: oil-exposed high-feed. Low and high food treatments received 1.5% somatic weight day-1 and 4% somatic weight day-1, respectively. Reproductive phases were based on Brown-Peterson et al. (2011). esr1) day 47 = 12, 9, 10, 7 day 131 = 4, 7, 4, 8 vtg *a*) *n* = day 47 = 12, 11, 10, 7 day 131 = 5, 7, 5, 5.

Dehydrogenase-Like (NSDHL), Sterol O-acyltransferase, acyl-Coenzyme A: cholesterol acyltransferase 1 (SOAT1), 24-Dehydrocholesterol Reductase (DHCR24), and Transmembrane 7 Superfamily Member 2 (TM7SF2). Oxidative stress and related pathways such as cysteine and methionine metabolism, selenium micronutrient network, and protein folding were populated by antioxidant response genes including: microsomal glutathione transferase 1 (MGST1), glutathione S-transferase theta 2 (GSTT2), peroxiredoxin-1 (PRDX1), superoxide dismutase 1 (SOD1), and Glutamate-cysteine ligase (GCLM). FAD Dependent Oxidoreductase Domain Containing 1 (FOXRED1), and family with sequence similarity 46, member Ba (FAM46BA) were also downregulated.

Discussion

Chemical analyses

The initial levels of tPAH ($\sum$ 44 PAHs) during the experiment were within the concentration range used in similar ecotoxicological experiments studying the sensitivity of fish species to aqueous crude oil exposure (Carls et al. 2000; Nahrgang et al. 2010). These levels are also considered in the range of environmentally relevant levels documented following oil spills (Reddy and Quinn 1999; Sammarco et al. 2013). As expected, the oiled-rock column system generated a decreasing level of aqueous PAHs, with composition changing over time, as described previously by Nahrgang et al. (2010), Carls, Rice, and Hose (1999), Heintz, Short,

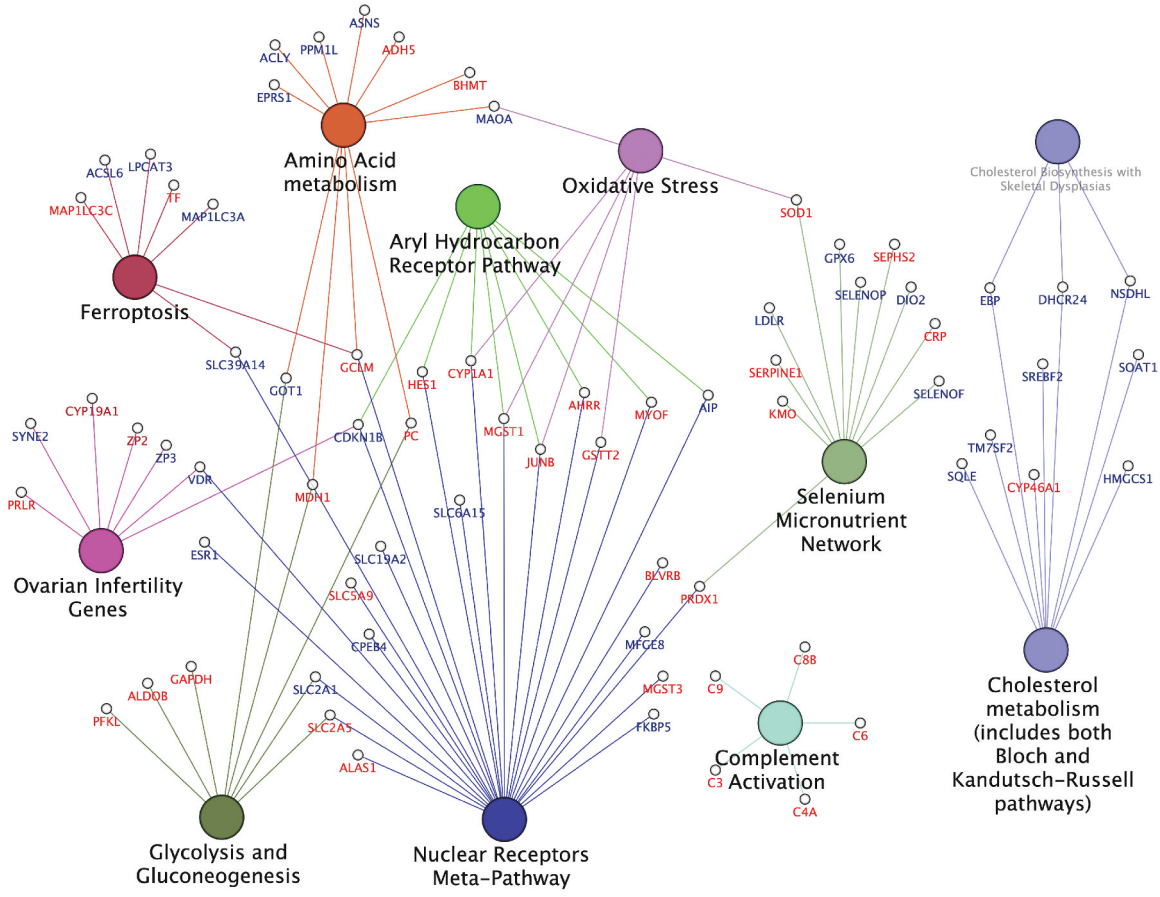

Figure 8. Networks of genes differentially expressed in female polar cod (*Boreogadus saida*) exposed to a crude oil WSF for 47 days compared or control conditions ($n = 5$ per group). Both groups were fed at a high feed ration (4% somatic weight). Significantly enriched (FDR <0.05) Wikipathways visualized in Cytoscape using the cluego plugin. Gene names in red and blue represent up-regulated and down-regulated genes, respectively. Gene names are based on human orthologs. Nodes and edges belonging to the same pathway are shown in the same color.

and Rice (1999) Incardona et al. (2014). The tPAH served as a proxy for oil exposure, although we recognize that PAHs only account for a small percentage of crude oil (Meador, Nahrgang, and Meador 2019; NRC 2003). Bile metabolites confirmed the bioavailability of 2-ring and 3-ring compounds in the oil-exposed groups.

The accumulation of PAHs in eggs indicated the parental transfer of oil-associated compounds from the water to the eggs. The PAH profile of the oil-exposed eggs was similar to the water samples taken on day 28, except that acenapthene and anthracene were not detectable in the eggs. The maternal transfer of PAHs to eggs is in line with previous work on pacific herring (*Clupea pallasii*) that showed parental transfer when adults were exposed to weathered crude oil for 8 days (Carls et al. 2000). Naphthalenes were the dominant PAH group accumulated in the eggs in both studies and the accumulation of larger PAHs was low (Carls et al. 2000). In the current study, C3-naphthalene was the dominant group compared to C2-naphthalene in Carls et al. (2000). As in the current study, acenapthene was also not detectable in pacific herring eggs, yet in contrast, anthracene was present at low levels (Carls et al. 2000). The lack of certain higher molecular weight PAHs may be due to parental partitioning that limited the amount of larger PAHs in eggs as noted in (Carls et al. 2000). Carls et al. (2000) did not observe any immediate effects on fertilization or embryos after exposure. However, bio-accumulated fractions could become available to the offspring later and affect the viability and quality of the eggs and embryos. As demonstrated in Bue, Sharr, and Seeb (1998) adult exposure of pink salmon (*Oncorhynchus gorbuscha*) to crude oil may have long-term effects on embryo survival in future

generations. Bautista and Burggren (2019) demonstrated that a 3-week parental exposure to dietary crude oil in zebrafish may be maladaptive or adaptive depending on if the embryos were raised in an oil-exposed or clean environment (Bautista and Burggren 2019). The previous studies demonstrated that other fish species might transfer PAH to their eggs, as seen in the current research, and this may have varied outcomes. In the present study, increased PAHs in eggs would also be coupled with an earlier release of the eggs with unknown consequences on egg quality and survival.

Biological response to crude oil exposure

Physiological response to crude oil

In the present study, polar cod spawned earlier in the oil-exposed group compared to the control group. This is in contrast to exposure studies that reported most often a delay in reproductive development following exposure to crude oil or petroleum compounds (Bender et al. 2018; Johnson et al. 1988; Khan 2013; Kiceniuk and Khan 1987; Thomas and Budiantara 1995; Truscott et al. 1983). To our knowledge, only one study showed an advancement in gonadal development of female starry flounder (*Platichthys stellatus*) following exposure to a water-accommodated fraction (WAF) of crude oil (Whipple et al. 1978).

In the present study, the exposure of polar cod to crude oil took place during the end of vitellogenesis. Similarly, Whipple et al. (1978), also observed an advancement in reproduction in exposed fish to the WAF during a late stage of maturation. On the contrary, when noted, most previous studies reported starting crude oil exposure primarily during earlier phases of reproduction (vitellogenesis or before). This is also the case for the previous work on polar cod that observed a delay in reproductive development after beginning exposure to burned oil residues in June (Bender et al. 2018) compared to exposure beginning in December in the current study. The timing of exposure during the reproductive cycle of the fish may be one explanation for the differences we observe between the present and previous studies as it can be a determinant of the effect of exposure (Kime 1995; Tintos et al. 2006). This has been demonstrated previously when rainbow trout exposed to NAP showed a greater decrease in E2 when exposed during early vitellogenesis compared to pre-vitellogenesis (Tintos et al. 2006).

As each reproductive phase is regulated by different hormones, crude oil exposure beginning during different phases of reproductive development may result in different outcomes. For example, previous work has shown the ability of petroleum compounds and certain PAHs present in crude oil to have an anti-estrogenic effect (Anderson, Miller, and Hinton 1996; Bilbao et al. 2010; Bugel, White, and Cooper 2010; Gao et al. 2018; Navas and Segner 2000; Salaberria et al. 2014; Woźny et al. 2008; Yadetie et al. 2018). If fish were exposed to anti-estrogenic compounds during E2-mediated vitellogenesis it may lead to decreases in E2 and the expression of genes in the estrogen pathway. This may lead to vitellogenin accumulating more slowly in the oocytes, and a subsequent delay in reproductive development. However, when exposure occurs during non-E2 mediated final maturation, or late in reproductive development, such as in the current study, an anti-estrogenic effect may not have the same physiological outcome as most oocytes are already in the final stage of vitellogenesis. At this point, most of the yolk has already been accumulated in the oocytes before exposure began. Crude oil exposure during the late stages of reproductive development may also act on different genes and hormones that are involved in final maturation. Given the limited endpoints selected in this study and the complexity of these reproductive processes, we did not pinpoint a mechanism that may be linked to the earlier spawning observed. However, investigation into hormones and genes involved in final maturation, such as MIH and LH may lead to a better understanding.

Little is known about how polar cod males and females synchronize their spawning activity. The oil-induced advancement in spawning time seen in both sexes could be due to a direct effect of the exposure. Alternatively, and given that males had already reached an advanced development stage by the time of the experiment start, it can be hypothesized that males synchronized their spawning activity to the females and that their gonadal

development was thus only indirectly affected by the exposure.

Direct comparison with the previous studies is not possible due to the variations in the exposure compound, route, set-up, and the use of different species with unique reproductive biology. Even the closely related experimental work on polar cod used a different exposure design that limits comparison (Bender et al. 2016, 2018). Future studies examining the effect of crude oil exposure beginning during different phases of reproductive development using the same exposure method may help untangle the effect of the timing of exposure from experimental design.

Although we did not observe any differences in the levels of alpha atresia we did observe a significantly higher volume fraction of beta atresia at day 131 in control compared to oil-exposed females. This result contradicts previous work and expectations (Johnson et al. 1994; Kime 1995; Rideout, Rose, and Burton 2005; Sundt and Björkblom 2011; Thomas and Budiantara 1995). However, this may be due to variations in the timing of spawning or natural variation. Oil-exposed females spawned earlier on average (before day 47) and would have had more time for residual oocytes to go through the atretic process, leading to an earlier breakdown of atretic oocytes in oil-exposed females by day 131. Atresia was notably low in all individuals, irrespective of the reproductive phase, and mainly consisted of the absorption of residual oocytes following spawning. In review, this endpoint may be more useful as an indicator of xenobiotic-related changes in the earlier phases of reproductive development when females use atresia to alter their energy investment in reproduction (Rideout, Burton, and Rose 2000).

Regarding plasma sex steroid hormone levels in males, there was no significant decrease in 11-KT or T levels in oil-exposed males compared to controls at day 47. The high natural variation in steroid hormone levels has presented difficulties in determining conclusive effects in this study and previous work (Wu et al. 2003). More directed study into hormones of interest with more individuals may help with further investigations despite the high natural variation. A continuous sampling regime over time may also allow identifying significant differences that cannot be caught based on a single sampling time point.

Finally, no sperm motility parameters tested were significantly affected by oil exposure. This finding is in line with findings from Bautista and Burggren (2019) and Beirão, Litt, and Purchase (2018), who did not see any changes in sperm motility measurements following crude oil exposure in zebrafish and capelin (*Mallotus villosus*), respectively. However, the current findings differ from those reported in previous work on polar cod (Bender et al. 2016). This is likely due to differences in the experimental design, as Bender et al. (2016) exposed polar cod to dietary crude oil from June to February. Although there were no differences in sperm motility between oil-exposed and control groups our results suggest that low feed alone may reduce sperm motility parameters in polar cod.

Molecular response to crude oil exposure

Gene expression results from target genes (males and females) using qPCR and whole transcriptome analysis using RNA-seq analysis (only females) showed the upregulation of *cyp1a*, enrichment of AhR signaling pathway, and oxidative stress pathway. The enrichment of these genes and the increased production of PAH metabolites indicate that the crude oil mixture was bioavailable to the polar cod. *Cyp1a* is a well-established biomarker for oil exposure and has been previously described in polar cod in-vivo following crude oil exposure (Nahrgang et al. 2010) and *in-vivo* and *ex-vivo* following BaP exposure (Song, Nahrgang, and Tollefsen 2019; Yadetie et al. 2021). Although cyp enzymes are involved in the metabolism of xenobiotics they can lead to increased oxidation and the generation of reactive oxygen species (ROS) that can damage cellular molecules such as DNA and proteins (Goksøyr 1995; Shankar et al. 2020). The enrichment of the genes in the AhR signaling pathways has been shown in polar cod liver tissue following exposure to crude oil (Andersen et al. 2015).

After 131 days, *cyp1a* expression was still higher in oil-exposed polar cod compared to controls despite the near control levels of tPAH present in the water samples at this time. Nahrgang et al. (2010) observed that

ethoxyresorufin-O-deethylase (EROD) enzyme activity in polar cod also remained elevated after two weeks of depuration following a four-week exposure to the WAF of crude oil (Nahrgang et al. 2010). Andersen et al. (2015) observed that biomarkers (mRNA) took 11 days to return to control levels in polar cod following 48 h exposure to mechanically dispersed crude oil. These results suggest that a recovery period exists before *cyp1a* expression returns to baseline levels in polar cod.

Female polar cod showed changes in the expression of hepatic genes involved in reproduction, including the reduction in *vtg α*, *esr1*, and other genes encoding Vtgs and Zrps. Several vitellogenesis-related genes known to be up-regulated by estrogens in the liver tissue of fish, including polar cod and Atlantic cod (Yadetie et al. 2018, 2021) were down-regulated in the crude oil exposed fish. Some of these reproduction-related genes populate the enriched ovarian infertility pathway. These estrogen-responsive genes are up-regulated by increases in E2 and therefore fluctuate during the reproductive cycle, peaking during vitellogenesis and falling around spawning (Nagler et al. 2012; Nelson and Habibi 2013; Sabo-Attwood, Kroll, and Denslow 2004). Thus, these findings had to be reviewed in light of the significant reproductive phase differences between oil-exposed and control females. The lower expression levels we observed for the genes related to vitellogenesis (*vtg α*, *esr1*, *zp2*, *zp3*) may be a secondary result of the oil-exposure leading to earlier spawning in females. This point is supported by the observation that the females with the highest levels of *vtg α* and *esr1* expression were present in the spawning capable phase and all these females were from the control group. Moreover, the effect of reproductive phase on *vtg α* and *esr1* expression was further demonstrated by the lack of statistical differences between exposed and control groups when reproductive phase was accounted for as a random predictor in the statistical model. Therefore, due to the large differences in the expression of these genes between reproductive phases, we were unable to untangle the effects of oil exposure alone from the effects of oil exposure mediated through significant changes in reproductive phase.

After 131 days of exposure, *vtg α* expression was significantly higher in oil-exposed compared to control females, and there was a significant food and oil-exposure interaction effect on *esr1* expression. Differences in the trends of *esr1* and *vtg α* between day 47 and day 131 may be due to changes in the composition and levels of PAHs in the crude oil mixture as it is weathered. Certain PAHs in the mixture may have an estrogenic or anti-estrogenic effect or varying effect with concentration. Previous studies have demonstrated a dose-dependent effect of PAH exposure on Vtg synthesis, with low PAH concentrations leading to an increase in Vtg and high PAH concentrations leading to a reduction in Vtg levels (Anderson et al. 1996; Nicolas 1999). Reversing trends in *esr1* were also shown in Bilbao et al. (2010) where *esr1* expression decreased in thicklip gray mullet *Chelon labrosus* when exposed to fuel oil but increased when exposed to weathered fuel oil. The water-soluble fraction of weathered crude oil has also been shown to have estrogenic activities in rainbow trout (*Oncorhynchus mykiss*) primary hepatocyte culture assays (Anderson, Miller, and Hinton 1996).

Pathways related to cholesterol, glucose, and amino acid metabolism were affected in oil-exposed fish. Most of the genes populating the enriched cholesterol metabolism pathway were down-regulated in crude-oil-exposed female fish. Vieweg et al. (2018) also observed the down-regulation of *cyp7a*, a gene involved in cholesterol biosynthesis, in polar cod exposed to dietary crude oil. Genes related to cholesterol, glucose, and amino acid metabolism were possibly modulated through the activated AhR pathway as previously observed in mice (Sato et al. 2008). Similarly, the AhR activation may have resulted in differential expression of immune-related complement activation pathway genes. As the AhR pathway is also known to be involved in immune responses (Rothhammer and Quintana 2019).

Our investigations into targeted reproductive genes and RNA-seq were focused on female fish as preliminary qPCR results warranted further investigation. Studies into male-specific reproductive endpoints should be followed up to investigate male sensitivity and possible sex-specific differences further.

The effect of feed treatment

Low and high-feed treatments were included to assess the combined effect of oil exposure and low-feed availability as polar cod experience a potentially lower food availability over their spawning period that could increase their sensitivity to crude oil. Contrary to our hypothesis, lower feed availability did not have an additive effect on polar cod's response to crude oil exposure. We did observe some parameters with a significant interaction effect or an effect of feed alone, such as 3 out of 5 sperm motility parameters. However, there was no consistent significant trend of lower feed causing a more pronounced response in the reproductive endpoints measured. This indicates that lower feed availability during polar cod's spawning and post-spawning period might not increase polar cod's sensitivity to oil exposure. This is in line with descriptions of polar cod as an opportunistic feeder with a natural ability to rely on energy reserves accumulated during the summer months through periods of lower feed availability (Majewski et al. 2016; Nakano et al. 2016). If this is the case, the effect of lower feed availability might have been more substantial if feed ration was reduced during the summer months when polar cod are expected to accumulate the majority of energy reserves that they would use for reproduction (Luquet and Watanabe 1986). This should be investigated in further research.

Conclusions

We observed that female and male polar cod exposed to crude oil during late vitellogeneisis spawned earlier than controls. Females had altered hepatic gene expression and produced significantly higher PAH body burden eggs. Lower feed availability in combination with oil exposure did not consistently affect female or male polar cod's sensitivity to crude oil exposure. However, lower feed availability alone did decrease some sperm motility parameters. Overall, these results suggest that polar cod's spawning period may be a sensitive life event to crude oil exposure. Still, potentially lower feed availability at this time may not increase their sensitivity. Our investigation into the hepatic gene expression and steroid hormone levels did not indicate the mechanism that could be connected to the earlier spawning observed. Further work examining how hormones (LH, MIH) necessary for final maturation may be affected by crude oil exposure may help elucidate a mechanism behind the results observed. If the earlier spawning we observed occurred without the completion of normal reproductive development it could result in the release of eggs with varied quality, and size with unknown consequences for the next generation. These points merit further study.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

Raw data from the experiment can be accessed through the following link https://doi.org/10.18710/59XOI4. RNA-seq

raw data can be accessed EMBL-EBI ArrayExpress accession number E-MTAB-12354.

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