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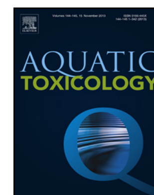

Effects of chronic dietary petroleum exposure on reproductive development in polar cod (*Boreogadus saida*)

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ABSTRACT

Increasing human activities in the Arctic raise the risk of petroleum pollution, thus posing an elevated risk for Arctic organisms to be chronically exposed to petroleum compounds. The endocrine disrupting properties of some of these compounds (i.e. polycyclic aromatic hydrocarbons [PAHs]) present in crude oil may have negative effects on the long and energy intensive reproductive development of polar cod (*Boreogadus saida*), an Arctic keystone species. In the present study, selected reproductive parameters were examined in feral polar cod exposed to crude oil via a natural diet (0.11, 0.57 and 1.14 μg crude oil/g fish/day [corresponding to low, medium and high treatments, respectively]) for 31 weeks prior to spawning. Fish maturing in the current reproductive period made up 92% of the experimental population while 5% were immature and 3% were identified as resting fish. Phase I metabolism of PAHs, indicated by ethoxyresorufin-O-deethylase (EROD) activity, showed a dose-dependent increase in high and medium crude oil treatments at week 6 and 22, respectively. Decreasing EROD activity and increasing PAH bile metabolite concentrations over the experimental period may be explained by reproductive maturity stage. Significant alterations in sperm motility were observed in crude oil exposed males compared to the controls. The investigated somatic indices (gonad and hepatic), germ cell development and plasma steroid levels (estradiol-17β [females], testosterone [males and females] and 11-ketotestosterone [males]) were not significantly altered by chronic dietary exposure to crude oil. The environmentally realistic doses polar cod were chronically exposed to in this study were likely not high enough to induce adverse effects in this ecologically important fish species. This study elucidated many baseline aspects of polar cod reproductive physiology and emphasized the influence of maturation state on biomarkers of PAH biotransformation (EROD and PAH bile metabolites).

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1. Introduction

Rapid environmental change in the Arctic (Barber et al., 2015) is enabling oil and gas exploration and exploitation in this area, shipping across the Arctic shelf seas, and tourism activities (AMAP, 2009; Eguíluz et al., 2016). The remoteness of the Arctic combined with inclement weather, unpredictable sea ice conditions, limited availability of bathymetric data, few ports, and a general lack of precedent events make operations in this area challenging (Harsem et al., 2011). These factors may increase the risk of petroleum pollution, reduce environmental monitoring possibilities, and complicate or prolong the clean up and recovery efforts in the event of an accident.

The primary toxic components in petroleum, polycyclic aromatic hydrocarbons (PAHs), are ubiquitous in the marine environment (Meador, 2006). PAHs are readily taken up by aquatic organisms. However, fish have the ability to metabolize and eliminate these compounds (Meador et al., 1995) by different biotransformation pathways including those of the cytochrome P 450 enzyme system. Even at concentrations in the lower parts per billion (Kime, 1995), PAHs have been found to be toxic to fish, inducing carcinogenic, genotoxic, and physiological impairment (Meador et al., 2006; Vignet et al., 2014). Furthermore, PAHs have been found to disrupt the endocrine system and affect reproductive function

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and growth of fish (Horng et al., 2010). Exposure to PAHs in fish has been linked to reduced investment in gonadal tissues (Booc et al., 2014) and interference with steroid metabolism (Monteiro et al., 2000). In maturing female fish, exposure to PAHs has been found to impair oocyte development, increase prevalence of atresia, and decrease steroid plasma levels of estradiol-17β (E_2) and testosterone (T) (Arukwe and Goksøyr, 2003). In males, PAH exposure has been found to suppress spermatogenesis in clams (Frouin et al., 2007) and increase testosterone (T) production in goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) by promoting testicular steroidogenesis (Evanson and Van Der Kraak, 2001).

Polar cod (*Boreogadus saida*) is a keystone species with a Pan-Arctic distribution (Bradstreet and Cross, 1982) that has been used as an indicator species in Arctic environments to understand the effects and mechanisms of oil pollution (Christiansen and George, 1995; Jonsson et al., 2010; Andersen et al., 2015; Nahrgang et al., 2010a,b). However, effects of PAH compounds on the reproductive development of this species have never been thoroughly investigated (Geraudie et al., 2014). Polar cod undertakes synchronous spawning under the sea ice in the winter months between December and March across the Arctic (Rass, 1968; Craig et al., 1982). There are apparent differences in reproductive development between males and females, such as timing of reproductive development, investment in gonadal tissue, and frequency of spawning (Nahrgang et al., 2014). Males reach maturity at a smaller size and an earlier age (Craig et al., 1982; Nahrgang et al., 2014) and gonadal investments start earlier in the season (Hop et al., 1995).

The study of reproductive physiology can provide an integrative measure of the effects of pollutants on whole organisms and important insights into the potential risks to populations. It is therefore critical to study the long-term effects of petroleum compounds at ecologically relevant doses, which may affect physiological processes in organisms, further propagating to changes at the population and ecosystem levels. The present study explores the long-term physiological effects of chronic dietary exposure to environmentally relevant levels of crude oil on reproduction in polar cod. To determine the potential effects of dietary petroleum exposure on reproductive development in polar cod, wild fish were exposed to four doses of crude oil for 31 weeks, starting in June at an early gonadal maturity phase and ending in early Feburary. Gonadosomatic index, gonad histology, plasma steroid concentrations, and sperm motility were measured. Exposure indices were measured in terms of biliary PAH metabolites and hepatic ethoxyresorufin O-deethylase (EROD) activity. We hypothesized that dietary exposure to environmentally realistic concentrations of petroleum compounds will alter physiological responses related to PAH exposure and negatively affect the reproductive development of polar cod.

2. Methods

2.1. Fish collection and husbandry

Polar cod were collected in Rijpfjorden, Billefjorden, and Kongsfjorden (Svalbard, Norway) in January 2014 during a cruise on RV Helmer Hanssen. Fish were trawled at 200 m depth using a live fish box (Holst and McDonald, 2000). Fish were kept on deck in 500 L flow-through tanks for two weeks and treated daily with Halamid® (1:500) disinfectant, while under transport to Tromsø, Norway. On the 29th of January, fish were transferred to the Tromsø Aquaculture Research Station in Kårvika. Fish were kept in a 4000 L acclimation tank at Kongsfjorden, Svalbard seawater temperatures (1.5–3 °C) to the nearest 0.5 °C as taken from mooring data (Nahrgang et al., 2014) and a light regime of 79°N. During this period, fish were fed daily until satiation on a diet of thawed *Calanus* sp. copepods from Lofoten, Norway (purchased from Calanus AS). On the 5th and 6th of June, 535 fish were selected based on length (13–17 cm fork length) and weight (11–24 g total weight) for participation in the experiment. Fish were anaesthetized until loss of equilibrium, using 5 mg/L Finquel® (Tricaine Methanesulfonate) dissolved in seawater. Fork length (±0.1 cm) and total wet weight (± 0.1 g) were recorded for each fish. Fish were then carefully tagged with a passive integrated transponder tag (Biomark®) inserted intraperitoneally, before being placed randomly in one of eight exposure tanks (n = 67 fish per tank).

2.2. Experimental design

Polar cod were exposed to dietary crude oil over the period of gonadal development starting on the 30th of June 2014 and ending on the 3rd of February 2015. Exposure tanks consisted of 300 L flow-through systems distributed randomly within the experimental room. The exposure setup consisted of four dietary crude oil treatments with two tanks per treatment. Fish received *Calanus* sp. food spiked with Kobbe crude oil (Barents Sea) at nominal concentrations of 0, 20, 100, and 200 μg crude oil/g *Calanus* sp., corresponding to control, low, medium, and high doses, respectively. Treatment food was prepared in large batches prior to the exposure period by mixing 500 g *Calanus* sp., 250 mL distilled water and 50 g gelatin, and either no (control) or one of three different nominal concentrations of crude oil. This mixture was frozen, cut into small pellets, and distributed into individual bags, corresponding to 2% of the total fish weight of each individual tank, and frozen at −20 °C until use.

Fish in each tank were fed as a group five times a week with a total ration equal to 4% body weight per feeding (Christiansen and George, 1995). *Calanus* sp. was distributed throughout the tank to reduce feeding hierarchies, although the existence of some hierarchies in the tanks was observed. On the first and fifth day of the week, fish were exposed to the dietary crude oil by receiving 2% body weight/feeding of treatment food followed by 2% body weight/feeding raw *Calanus* food. The three other feedings per week consisted of raw *Calanus* sp. amounting to 4% body weight. With this feeding regime, the effective dose was 0, 0.8, 4 and 8 μg crude oil/g fish/week (Table 1). The amount of food distributed in each tank was adjusted four times over the course of the experiment (September, October, November and January) to account for changes in total fish weight and fish removal due to sampling and mortality.

Five sampling events (n = 8 fish per tank, i.e. 16 fish per treatment) were performed 0, 6, 17, 22 and 31 weeks after dietary exposure began in June, August, October, December and January, respectively. On the 30th of June (week 0), only control fish (n = 16) were dissected, and on the 3rd of February (week 31) the remaining fish from all treatments (control[n = 12], low[n = 12], medium[n = 8] and high[n = 9]) were dissected. Each fish was anaesthetized and blood was collected from the caudal vein using a 2 mL heparinized vacuum tube (BD Vacutainer®) that was promptly set on ice until centrifugation for 30 min at 4 °C and 3500 rpm (Sorvall RC 5 B Plus centrifuge). The plasma supernatant was separated out and stored at −80 °C until steroid hormone analysis. Following blood sampling, the fish were given a sharp blow to the head before wet weight (g) and fork length (cm) were measured and liver and gonads were removed and weighed. Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated according to the following equations:

$$\mathrm{GSI} = (\mathrm{gonadweight}/\mathrm{somaticweight}) \times 100$$

$$\mathrm{HSI} = (\mathrm{liverweight}/\mathrm{somaticweight}) \times 100$$

Table 1
Summary of nominal crude oil (μg/g wwt), measured $\sum$26 polycyclic aromatic hydrocarbon (PAH) concentrations (mean ± SD, ng/g wwt) in the *Calanus sp.* diet, estimated daily doses of crude oil (μg/g fish/day), and $\sum$26 PAHs (ng/g fish/day) in fish per treatment. N is the number of fish sampled in each treatment group. Estimated doses of $\sum$26PAHs (ng/g fish/day) are based on initial mean fish total weight at the start of the experiment (week 0).

			Concentrations in diet		Doses in fish	
Treatment	N	Initial fish total wet weight (g)	Nominal conc. of crude oil (μg/g wwt)	Measured conc. of $\sum$ 26PAHs (ng/g wwt)	Estimated doses of crude oil (μg/g fish/day)	Estimated doses of $\sum$ 26PAHs (ng/g fish/day)
Control	76	16.7 ± 3.4	0	141.5 ± 103.3	0	0.8 ± 0.3
Low	60	17.8 ± 3.5	20	313.2 ± 88.0	0.11	1.8 ± 0.4
Medium	56	17.6 ± 3.7	100	1058.5 ± 237.4	0.57	7.0 ± 1.7
High	57	17.4 ± 3.4	200	2288.2 ± 214.4	1.14	13.1 ± 4.0

The middle section of the gonad was preserved in 4% neutral buffered formalin for histological analysis. The anterior section of the liver and the gallbladder were snap frozen in liquid nitrogen and stored at −80 °C until analyses of EROD activity and bile metabolites were undertaken. Remaining internal organs were removed and somatic weight of the carcass was recorded. After 31 weeks of exposure (3rd of February), male fish (n = 21) were stripped for milt for sperm motility analysis by gently massaging the abdomen and taking care to avoid contamination by urine or blood.

2.3. Determination of PAH doses in the diet

Samples from each treatment were analyzed for the 16 Environmental Protection Agency priority PAHs and 10 further alkylated naphthalenes, phenanthrenes and dibenzothiophenes compounds at Akvaplan-niva AS (Tromsø, Norway). Briefly, an internal standard containing labeled deuterated PAHs was added to the samples that were extracted by saponification with methanol/KOH followed by extraction with pentane. The extract was cleaned on gel permeation chromatography and further purified by filtration on a silica column with pentane and dichloromethane as eluents. The final extract was analyzed by gas chromatography/mass spectrometry. Triplicates were run for each treatment diet.

2.4. Analysis of PAH metabolites in bile

Bilary PAH metabolites, 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-benzo[a]pyrene, were analyzed for polar cod sampled in June, August, and February (week 0, 6 and 31, respectively). Preparation of hydrolysed bile samples was performed as described by Krahn et al. (1992). Briefly, bile (1–20 μL) was mixed with an internal standard (triphenylamine) and diluted with demineralised water (10–50 μL) and hydrolysed with b-glucuronidasearylsulphatase (20 μL, 1 h at 37 °C). Methanol (75–200 μL) was added and the sample was mixed thoroughly before centrifugation. The supernatant was then transferred to vials and analyzed. High pressure liquid chromatography (Waters 2695 Separations Module) was used to separate hydroxyl PAHs in a Waters PAH C_{18} column (4.6 × 250 mm, 5 μm particle size). The mobile phase consisted of a gradient from 40:60 acetonitrile:ammonium acetate (0.05 M, pH 4.1) to 100% acetonitrile at a flow of 1 mL/min, and the column was heated to 35 °C. A 2475 Fluorescence detector measured fluorescence at the optimum for each analyte (excitation/emissions: 1-OH-phenanthrene 256/380; 1-OH-pyrene 346/384; triphenylamine 300/360; 3-OH-benzo[*a*]pyrene 380/430). 25 μL of extract was injected for each analysis. The results were calculated by use of the internal standard method (Grung et al., 2009). The calibration standards utilized were obtained from Chiron AS, Trondheim, Norway, and were in the range 0.2–200 ng/g.

2.5. Ethoxyresorufin O-deethylase (EROD) activity

Liver samples were homogenized at 4 °C with a Precellys 24 type homogenizer in a phosphate buffer (pH 7.4) containing 150 mM KCL, 100 mM KH_2PO_4, 100 mM K_2HPO_4, 1 mM dithiothreitol and 5% glycerol. Homogenates were centrifuged (9000 × *g*, 4 °C) for 30 min. Supernatants were subsequently centrifuged (50,000 × *g*, 4 °C) for 2 h for extraction of the microsomal fraction. Pellets (microsomes) were dissolved in phosphate buffer (pH 7.4) containing 20% glycerol and stored at −80 °C until further analysis. EROD activity measurements were performed as described by Nahrgang et al. (2010b). Briefly, fluorescence was measured in a final reaction mixture containing the microsomal fraction from homogenized liver (10 μL), the substrate ethoxyresorufin (2 μM) and NADPH (0.25 mM), which started the deethylation reaction of 7-ethoxyresorufin to resorufin. Fluorescence of resorufin was measured in four replicates in the fluorimetric plate reader Synergy H1 (BioTek®, Winooski, U.S.) at the wavelength pair 540/600 nm (excitation/emission) every minute for 20 min. For each plate, a resorufin standard curve (0–0.025 μM) was included. EROD activity was normalized to the total protein content of the microsomal fraction. Total protein content was determined according to Bradford (1976), using bovine serum albumin, (0–8 μg/mL) as a standard.

2.6. Histological analysis

Gonad samples fixed in buffered formalin were rinsed, dehydrated in a series of 70% ethanol baths and embedded in paraffin wax (Aldrich, USA) overnight using Histo-clear® as a clearing agent in a Shandon Citadel 1000 (Micron AS, Moss, Norway). Tissues were then embedded into a paraffin block and sectioned at 5–7 μm thickness, using a Leitz RM 2255 microtome, and stained with haematoxylin/eosin. Each slide was examined under a Labor-Lux 11 Leitz microscope equipped with a camera (Wild Leitz AS, Oslo, Norway). Female gonadal maturity stages were based on the stage of the most advanced cohort of oocytes observed in the slices. Oocytes were categorized based on Brown-Peterson et al. (2011) into one of the following stages (including oocyte diameter measurements of each stage): primary growth (PG, 73–221 μm), cortical alveolar (CA, 268–320 μm), primary vitellogenic (Vtg I, 312–400 μm), and secondary vitellogenic a and b (Vtg IIa, 375–500 μm, VtgIIb, 500–855 μm). Oocytes in the PG stage were identified by the presence of a prominent nucleus, multiple nucleoli, and scant cytoplasm (Figure A.1A). CA stage oocytes were distinguished by the presence of cortical alveoli vesicles and visible follicular cell layer surrounding the oocyte (Figure A.1B). Vitellogenic oocytes had increasing egg shell- and follicle thickness, and were further distinguished based on the presence of yolk globules and the difference in area of cytoplasm filled with yolk. Vtg I oocytes had less than half of the cytoplasm filled by yolk globules (Figure A.1C). Vtg IIa oocytes were larger than VtgI oocytes and had between half and 2/3 of the cytoplasm filled by yolk globules,

while VtgIIb had over 2/3 of the cytoplasm filled and a centrally placed nucleus, as the nucleus migration toward the animal pole not yet started (Figure A.1D). Post-ovulatory follicle complexes (POFs) were identified by the presence of empty and collapsed follicles remaining in the ovary after spawning (Figure A.1E). Atretic oocytes were characterized by the disintegration of the nucleus and breakdown of the oocyte envelope (Figure A.1F). In October, December, and February, resting and immature females were distinguished from maturing females as those having only PG oocytes and POFs (resting females) or having only PG oocytes (immature females).

Males were separated into four progressive gonadal maturity stages based on Núñez and Duponchelle (2009): early maturing, maturing, spawning, and spent. Several males were also observed with portions of testis spent and early maturing and thus classified as such. Early maturing males were determined by the presence of early stage spermatocytes (Fig. A.2A and B of Supplementary information) while late stage spermatocytes with stronger basophilic staining were found in maturing males (Fig. A.2C and D of Supplementary information). Spawning males had spermatozoa in the lumen of lobules, and milt ran when pressure was applied to the abdomen (Fig. A.2E and F of Supplementary information). Spent males had empty lobules while spent/early maturing males were clearly spent (empty lobules), but early stage spermatocytes were also prominent (Fig. A2.G and H of Supplementary information). In October, December, and January, further division in maturity status were made between maturing and non-maturing males. Immature males were identified by testis with only early stage spermatocytes and a low GSI, and resting males had spent testis containing portions of early stage spermatocytes and a low GSI.

2.7. Steroid hormone analysis

Plasma concentrations of estradiol-17β (E_2, females only), 11-ketotestosterone (11KT, males only) and testosterone (T, both sexes) were measured using radioimmunoassay, according to Schulz (1985). A plasma pool composed of male (n = 43) and female (n = 35) wild polar cod was used as an internal reference. The cross reactivity of the E_2 and T antiserum is given by Frantzen et al. (2004) and 11KT cross reactivity is given by Johnsen et al. (2013) (for summary see Table A1 of Supplementary information). Values that fell below the level of detection (LOD) were assigned a zero value for calculations (E_2 LOD is 0.66, 11KT 0.72, and T 0.82 ng/mL plasma).

2.8. Sperm quality

Sperm motility was examined following the protocol set by Rudolfsen et al. (2005). Briefly, sperm motility analysis was conducted using an aliquot (<0.12 μL) of fresh undiluted milt placed on a 4 °C 20 μm standard count slide (Leja, Art. No. SC 20-01-C, The Netherlands) and sperm activation was induced by adding 4.5 μL chilled seawater. A video camera (Sony XC-ST50CE, Sony, Tokyo, Japan) mounted on a negative phase-contrast microscope (Olympus CH30, Olympus, Tokyo, Japan) (×10 objective) was used to record sperm activity from each male (n = 21). Sperm swimming activity was recorded for a 90 s period with two replicate trials for each male. Sperm motility was examined using computer-assisted sperm analysis, an objective tool for quantitative analysis of fish sperm quality (Kime et al., 2001). Sperm cell trajectories were analyzed using an HTM-CEROS sperm tracker (CEROS version 12; Hamilton Thorne Research, Beverly, MA, USA). The sperm analyzer was set as follows: frame rate 50 Hz; number of frames 25; minimum contrast 9; and minimum cell size 8 pixels. Five motility parameters were assessed in the present study: (1) average path velocity (VAP, μm/s), which is the velocity of the sperm head along its spatial average trajectory; (2) straight-line velocity (VSL, μm/s), which is the velocity of the sperm head along its linear track between its initial and final positions; (3) mean curvilinear velocity (VCL, μm/s), which is the velocity of the sperm head along its real curvilinear track; (4) percentage motile sperm; and (5) percentage progressive sperm (progressive sperm cells were defined as having straightness >80% and VAP >100 μm/s). To remove the potential effect of drift, cells having VAP <20 μm/s and velocity straight line <10 μm/s were considered to be static and were excluded from the motility analysis. All recordings were analyzed 30 s, 60 s, and 90 s after activation and were done blind in respect to treatment.

Spermatocrit was measured as a proxy for sperm density (Rakitin et al., 1999). Milt was collect from the stripped male fish using hematocrit tubes (n = 2/male). Tubes, blocked by clay at one end, were spun down in a centrifuge for 5 min at 4500*g* (Eppendorf centrifuge 5415C). The length of the entire milt sample and the length of the packed sperm cells were measured to create a ratio of sperm cells to the seminal fluid in the milt.

2.9. Statistical analysis

All statistical analyses were conducted with R 3.1.1 (R Core Team, 2014). After satisfying the assumptions of normal distribution and equal variance, a one-way analysis of variance (ANOVA) was used to test firstly for difference between sexes and secondly for differences between treatments on the continuous factors of EROD activity, PAH bile metabolite concentrations, GSI and HSI, and plasma sex steroid levels followed by a subsequent *post hoc* test on differences between means (Tukey's honest significant difference test). Variables that violated the assumption of normality and homogenous variance were tested using the Kruskal-Wallis test by ranks. Pearson's correlation test was used to explore the relationship between parameters. Mature, and immature/resting fish were treated separately for all analyses. Comparisons were considered significantly different than control when $p \leq 0.05$ level. Values are reported as mean ± standard deviation (SD).

Distribution of maturity stages and frequencies of atresia and POFs were analyzed using a Fishers exact test with the null hypothesis that treatment groups have the same frequency of maturity stages, atresia and POFs at a given time. A linear mixed effect model was created for each sperm motility parameter with the R package nlme (Pinheiro et al., 2016). Fixed effects were treatment and time after activation. The interaction between treatment and time after activation was also tested in the model. Random effects included fish identity nested within trial to control for variance within the same individuals tested across different times and trials. The full model was compared against a model excluding the 2-way interaction and a third model further excluding the treatment factor. All significant terms were maintained in the models and competing models were selected based on their AIC values. Each model satisfied the assumptions of parametric analysis and autocorrelation was checked. When a fixed factor indicated significant differences, pairwise comparisons using *t*-tests were assessed between treatments and the control following the linear model output in R.

3. Results

3.1. Morphometric data

At the start of the experiment on the 30th of June, the mean fork length and total weight of fish was 14.7 ± 0.9 cm and 17.3 ± 3.5 g, respectively (n = 535). No significant differences were found among tanks or treatments with regard to initial length and weight (p = 0.73 and 0.16, respectively). Fish mortality was not significantly different amongst the treatment groups with a mean frequency of 56 ± 3%. Fish that perished (n = 301) had lower condition indices

Table 2
Mean concentration (ng/g food ± SD) of 26 PAHs and Σ26PAHs for each treatment (control, low, medium, and high treatment). Four replicates were analyzed for each treatment group except for control. Values under limit of detection (LOD) are not included in Σ26PAH calculations.

PAH composition	Control	Low	Medium	High
Naphthalene	10.33 ± 3.14	13.65 ± 2.10	39.97 ± 8.06	97.05 ± 5.12
C1-Naphthalene	22.97 ± 7.00	33.61 ± 5.71	108.53 ± 38.30	258.83 ± 4.54
C2-Naphthalene	29.95 ± 11.15	62.24 ± 13.17	207.47 ± 28.65	446.74 ± 12.25
C3-Naphthalene	67.12 ± 36.22	147.81 ± 42.0	533.03 ± 156.04	1140.99 ± 63.83
Phenanthrene	<3.05	5.78 ± 1.31	13.95 ± 1.21	34.69 ± 4.70
Antracene	<0.33	<0.33	<0.33	0.43
C1-Phenanthrene/anthracene	7.29 ± 5.21	9.81	23.47 ± 3.76	53.57 ± 16.79
C2-Phenanthrene/anthracene	20.01 ± 9.25	25.33 ± 12.56	61.60 ± 14.00	154.10 ± 29.49
C3-Phenanthrene/anthracene	15.68 ± 8.93	14.04 ± 5.49	27.85 ± 5.12	64.22 ± 9.41
Dibenzothiophene	<0.51	1	1.74 ± 0.27	5.07 ± 1.07
C1-Dibenzothiophene	1.63	1.80 ± 0.09	4.89 ± 0.99	12.9 ± 4.06
C2-dibenzothiophene	6.19 ± 0.12	9.35	11.34 ± 3.03	31.86 ± 6.48
C3-dibenzothiophene	6.39 ± 0.42	6.21 ± 2.93	10.76 ± 1.99	25.39 ± 3.88
Acenaphthylene	<0.15	<0.15	<0.15	<0.15
Acenaphthene	<1.06	1.18	1.74 ± 0.50	3.08 ± 0.14
Fluorine	1.13 ± 0.30	2.49 ± 1.01	6.85 ± 1.82	14.06 ± 2.52
Fluoranthene	<1.98	2.14 ± 0.09	2.99 ± 0.82	4.51 ± 0.93
Pyrene	<3.32	<3.32	<3.32	3.52
Benzo(a)anthracene	<0.51	<0.51	<0.51	0.57
Chrysene	1.01 ± 0.22	2.35 ± 0.55	2.15 ± 0.44	3.54 ± 0.36
Benzo(b)fluoranthene	<0.71	<0.71	<0.71	0.76
Benzo(k)fluoranthene	<0.23	<0.23	0.37	0.34 ± 0.07
Benzo(a)pyrene	<0.34	<0.34	<0.34	0.35
Indeno(1,2,3-cd)pyrene	<0.73	<0.73	<0.73	0.95
Benzo(ghi)perylene	<0.61	<0.61	<0.61	0.91
Dibenzo(a,h)anthracene	<0.26	<0.26	<0.26	0.31
SUM 26 PAHs, ng/g:	141.5 ± 103.3	313.2 ± 88.0	1058.5 ± 237.4	2288.2 ± 214.4

(somatic weight, fork length, and HSI) compared to fish sampled in the experiment (Fig. A3 of Supplementary information). The sex ratio of the sampled specimens was unbalanced overall, with 68 females and 181 males (Table A2 of Supplementary information). Somatic weight and fork length of maturing polar cod did not differ significantly between crude oil exposed fish and control fish or between sexes at any time over the exposure period (Table A2 of Supplementary information). The mean somatic weight in June was 14.1 ± 2.7 g (n = 64) and, by February, the mean somatic weight had increased to 23.4 ± 5.2 g (n = 48), a mean increase of 58.8 ± 24% ($p < 0.01$). The mean length in February was 16.9 ± 1.1 cm, a significant increase of 6.8 ± 5% from the start of the study in June ($p < 0.01$). The somatic weight of immature fish (n = 13, 5% of sampled fish) was 30.5 ± 7% lower than in maturing fish. Resting fish (n = 7, 3% of sampled fish) had a lower somatic weight (27.9 ± 9% less) compared to maturing fish (not shown).

3.2. PAH doses and biomarkers of biotransformation

3.2.1. Diet doses

Fish were observed during feeding and the presence of food was confirmed in their stomachs at each sampling point, therefore the dose of crude oil mixed in the *Calanus* sp. pellets was considered the administered dose (Table 1). Assuming polar cod in each tank were feeding proportionally to their body weight, the ingested doses corresponded to 1.8, 6.0, and 13.1 ng $\sum$26PAHs/g fish/day in the low, medium, and high crude oil treatments, respectively (Table 1). The relationship between the amount of crude oil added to food and measured $\sum$26PAHs levels was positively linear ($R^2 = 0.99$). The $\sum$26PAHs accounted for ~1% wet weight (wwt) of crude oil in all treatments. The PAH composition was similar in all crude oil treatments with alkylated naphthalenes (e.g. C1–C3- naphthalene) accounting for approximately 80% of the overall PAH load (Table 2) and no statistical differences in the ratio of parent PAHs to their alkylated homologues across treatments were observed (results not shown).

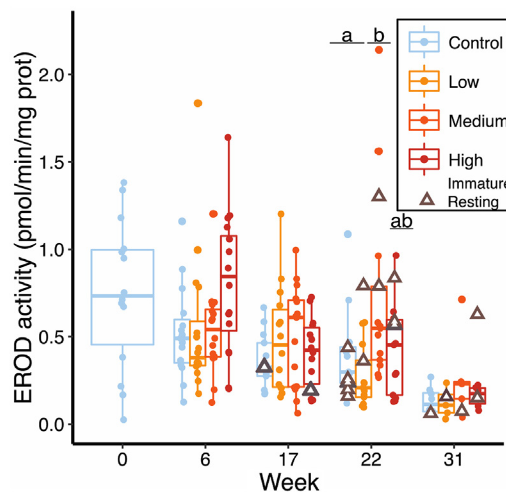

Fig. 1. Ethoxyresorufin O-deethylase (EROD) activity (pmol min/mg/proteins) in the liver of polar cod exposed to low, medium and high doses of dietary crude oil and controls. Fish (n = 16/treatment) were sampled at 0, 6, 17, 22, and 31 weeks of exposure. Boxplots represent the median (horizontal line), 1st and 3rd quartile (box), non-outlier range (whisker), outliers (points outside whiskers) of the data. Each point represents a maturing polar cod while immature and resting fish (grey triangles) are excluded from the boxplots. Treatments (only mature specimens) significantly different ($p < 0.05$) from one another are indicated by different lowercase letters.

3.2.2. Ethoxyresorufin O-deethylase (EROD) activity

Overall EROD activity in the liver was low and decreased significantly from June to February in crude oil exposed treatments and control groups (Fig. 1). EROD activity in mature fish was negatively correlated with increasing GSI ($p < 0.01$). No significant difference in EROD activity was observed in crude oil exposed groups compared to control with the exception of week 22 when the medium treatment (n = 16) had significantly higher activity compared to the control (n = 16) and low treatment (n = 15). At week 6, increased EROD activity was seen in the high treatment compared to all other groups (n = 16 in each treatment), however this difference

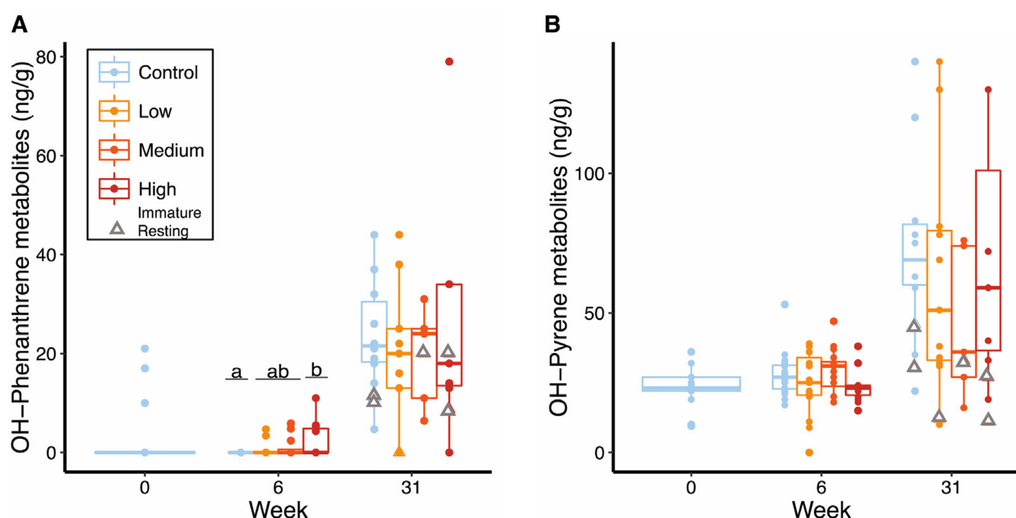

Fig. 2. PAH bile metabolites measured as (A) 1-OH-phenanthrene metabolites and (B) 1-OH-pyrene metabolites in crude oil exposed and control polar cod sampled at 0, 6, and 31 weeks of exposure. Boxplots are as in Fig. 1. Each point represents a maturing polar cod while immature and resting fish (grey triangles) appear in week 31 and are excluded from the boxplots and analysis. Treatments significantly different ($p < 0.05$) from one another are indicated by different lowercase letters.

was not statistically significant. Immature and resting fish had a dose related increase in EROD activity at week 22; however, statistical analysis was limited by the low sample size in the treatment groups (n = 5, 2, 2 and 3 in control, low, medium and high treatment groups, respectively).

3.2.3. PAH metabolites in bile

Concentrations of 1-OH-pyrene and 1-OH-phenanthrene bile metabolites increased significantly over time in all crude oil exposed and control groups (Fig. 2). Concentrations of 1-OH-phenanthrene were significantly higher in the high exposure group (n = 14) compared to control (n = 16) in August, after 6 weeks of exposure. At this time point there also was a strong correlation between diet phenanthrene concentration and 1-OH-phenanthrene bile metabolite concentration ($R^2 = 0.97$; average per treatment group). Concentrations of 3-OH-benzo[*a*]pyrene metabolites only exceeded the level of detection in a singular fish in the medium treatment in August (data not shown). Mature fish had significantly higher concentrations of 1-OH-pyrene bile metabolites (65.0 ± 38.2 ng/g, n = 46) compared to immature and resting fish at week 31 (26.5 ± 13.3 ng/g, n = 6, p = 0.02).

3.3. Effects on reproduction

3.3.1. Somatic analysis

The gonadosomatic index was significantly different between sexes and increased significantly over time (Fig. 3). Females started with a GSI of 2.2 ± 0.3% in June that remained low until February when GSI increased to 12.8 ± 6.5%. The GSI in males was 0.9 ± 0.2% in June, began to increase in October and highest GSI values were measured in February (20.1 ± 8.6%). In October (week 17), male GSI in medium treatment (n = 11) was significantly higher than GSI in the high crude oil treatment (n = 10). However, no difference was found in female GSI among treatments. In December (week 22), the GSI of both sexes began to diverge into two significantly different groups, one of which identified the maturing fish cohort with an increasing GSI towards spawning, and the other as non-maturing, (immature and resting) fish maintained a low GSI (2.0 ± 2.3%) regardless of sex and time. The most advanced gonadal development was observed in February with one male reaching a GSI of 34.7% and one female reaching a GSI of 20.9%. The hepatosomatic index did not show significant differences among treatments, with an exception of males in October (week 17) showing significantly higher HSI in control (9.5 ± 2.6%, n = 9) compared to the high (6.8 ± 1.4%, n = 10) treatment group (p = 0.04, data not shown). HSI was significantly higher in female fish compared to males ($p < 0.01$) and increased significantly over time ($p < 0.01$) with highest HSI values measured in February, reaching 13.3 ± 2.2% in females (n = 14) and 11.1 ± 3.7% in males (n = 27). GSI and HSI values for mature fish were positively correlated (R = 0.58, $p < 0.01$). Immature fish and resting fish had similar HSI values to mature fish.

3.3.2. Histological analysis of gonads

No differences were found among treatments when comparing the female gonadal maturation stage distributions (Fig. 4A). In June, ovaries were in the first stage of oogenesis, PG; the most advanced oocyte cohorts reached CA stage by August, VtgI by October, and VtgIIb by December. In February, all maturing females had VtgIIb oocytes present in ovaries regardless of treatment. Immature gonads were found in 17.6% of control females and 21.4% of females in the low exposure group while only a singular resting female was identified in the high treatment group. The experiment was terminated before any females reached the final stage of vitellogenesis, final oocyte maturation and ovulation. No difference in prevalence of atretic oocytes or POFs was observed in crude oil exposed females compared to control (data not shown). Atretic oocytes were observed in some females sampled in October (n = 7) and February (n = 2) while POFs (n = 16) were found at all sampling points and in all maturity stages.

No difference was found among treatments when comparing the male gonadal maturation stage distributions (Fig. 4B). In June and August, males were either spent with empty lobules or in the early maturing stage with early stage spermatocytes. By October and December, most males (89%) were in a maturing stage with late stage spermatocytes. In February, 75% of maturing males could be stripped for milt and spermatozoa was observed in the sperm ducts in the histological preparations of actively spawning males. Immature males (n = 8) were sampled in December with early stage spermatocytes in testis and a low GSI (2.19 ± 2.35%). Resting males (n = 6) were sampled in October, December, and February with a low GSI (1.09 ± 0.65%).

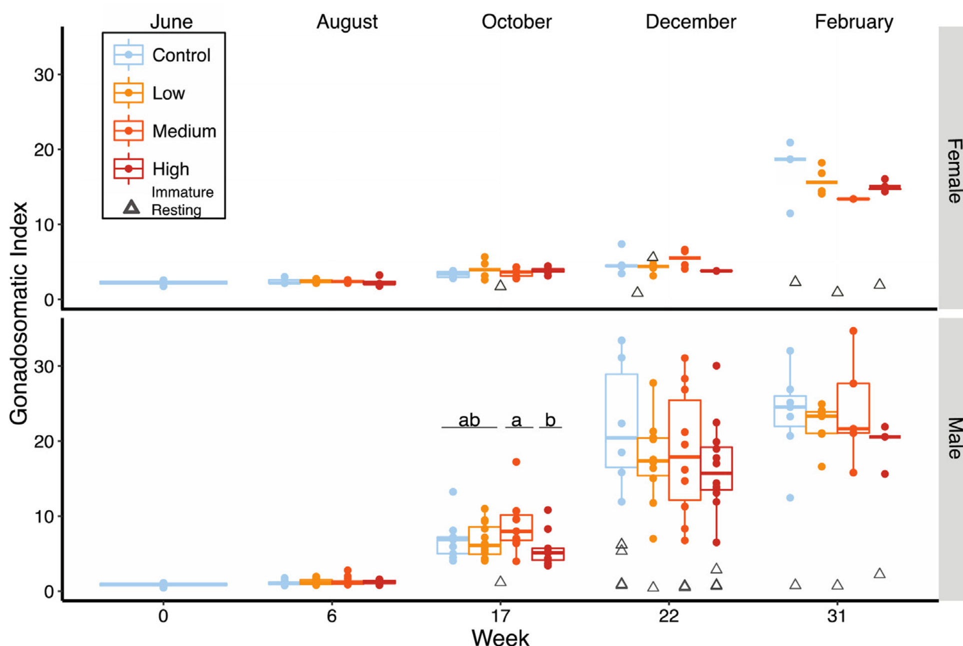

Fig. 3. Gonadosomatic Index of maturing female polar cod (top panel) and male polar cod (bottom panel) in different crude oil treatment groups sampled after 0, 6, 17, 22, and 31 weeks of exposure. Boxplots are as in Fig. 1 where different treatment groups are distinguished by color, each point representation a singular fish and significant differences between treatment groups and control are indicated by different lowercase letters ($p < 0.05$). For treatment groups where $n < 5$ only the median line is shown.

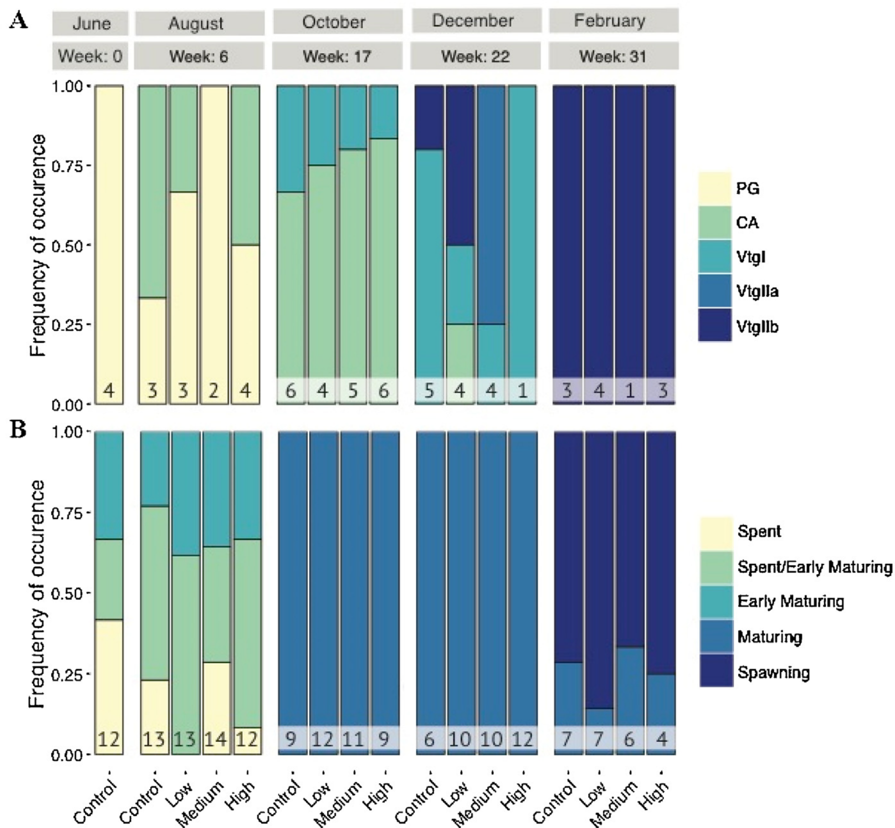

Fig. 4. Histological analysis of gonadal maturity stages in maturing (A) female and (B) male polar cod over a period of gonadal development from June to February. The frequency of occurrence of each maturity stage, represented by different colors, in each treatment group after 0, 6, 17, 22, and 31 weeks of exposure to dietary crude oil. Number of fish in each sex sampled from each treatment is noted at the base of every column. No significant difference was found between crude oil treatment groups and control with regard to gonadal maturity stage frequency of occurence. Immature and resting are not included in this figure.

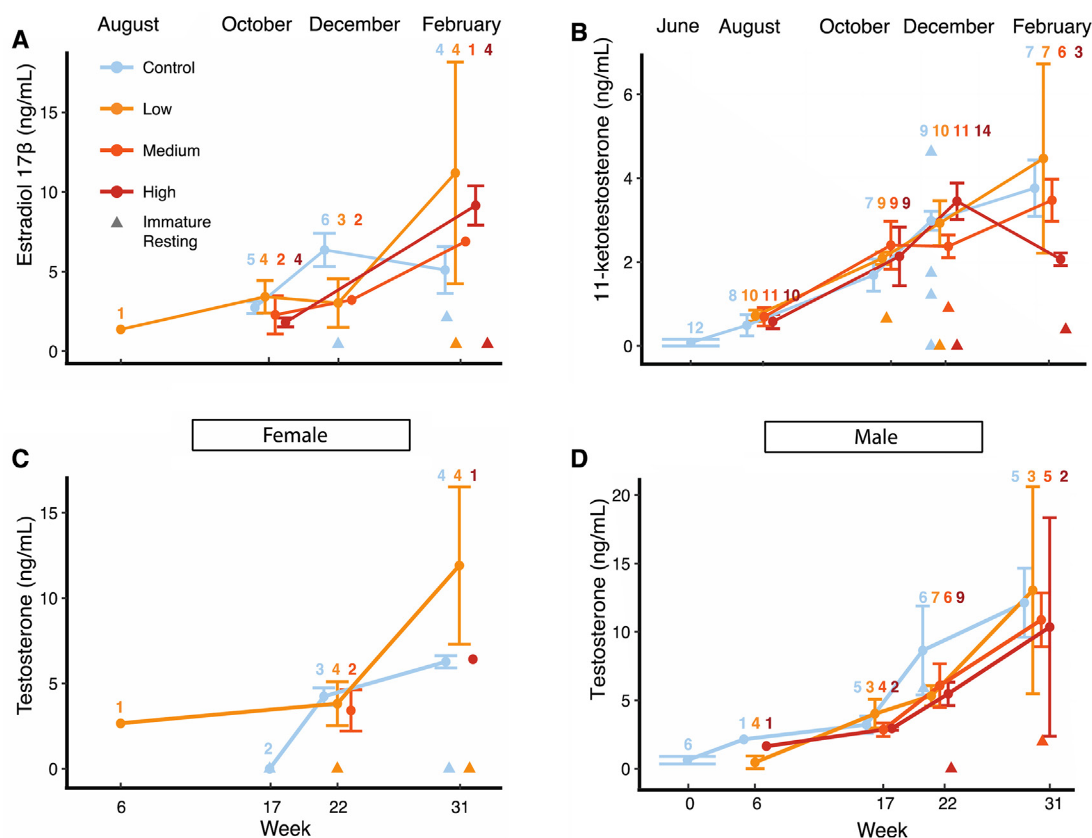

Fig. 5. Plasma concentrations displayed by mean ± standard error of (A) estradiol-17β in maturing female polar cod; (B) 11-ketotestosterone in maturing male polar cod; (C) testosterone in maturing female polar cod; (D) testosterone in maturing male polar cod after 0, 6,17, 22 and 31 weeks of exposure to different crude oil doses and controls. Different treatment groups are distinguished by color with immature fish and resting fish (triangles) excluded from trend lines. Sample size is displayed above each mean. No significant differences were found between crude oil treatment groups and control with regard to concentrations of sex steroids in blood plasma.

3.3.3. Plasma steroid concentrations

3.3.3.1. Estradiol-17β. Estradiol-17β (E_2) levels were not significantly different among treatments neither for any time point nor within a singular gonadal maturity stage although great variation was found in Vtg IIb females (Fig. 5A). In August, plasma levels of E_2 was low (1.37 ng/mL, n = 1). A significant increase occurred over time and with progressive gonadal maturity stages, and maximum E_2 levels were reached in February (8.32 ± 6.43 ng/mL, n = 39). The female with the highest E_2 level (25 ng/mL) exhibited the most advanced oocyte maturity stage (Vtg IIb) in February (week 31). The temporal changes in E_2 were significantly and positively correlated with the changes in GSI (R = 0.59), HSI (R = 0.46), and somatic weight (R = 0.31). The immature and resting females had E_2 levels at or slightly above the LOD (0.66 ng/mL, n = 4).

3.3.3.2. 11-ketotestosterone. 11-ketotestosterone (11KT) levels in males were not significantly different among treatments (Fig. 5B). Plasma levels of 11KT in males rose steadily with advancing maturity stage throughout the exposure period with low values in June (0.08 ± 0.27 ng/mL) and maximum values in February (3.38 ± 1.87 ng/mL). In February, maturing males in the high treatment (n = 3) had 11KT levels 55% lower than the control group (n = 7), although not statistically signficiant. Immature males and those in early maturing and spent stages had generally low 11KT levels (<1.0 ng/mL, n = 9), but higher levels were found in control males than in the exposed males (<5.0 ng/mL, n = 4). Increasing plasma 11KT levels were significantly correlated with increasing GSI (R = 0.59).

3.3.3.3. Testosterone. Testosterone (T) levels in females were not significantly different among treatments (Fig. 5C). Plasma levels of T in female fish were low in August (2.6 ng/mL). Testosterone levels remained low until February when an increase occurred in maturing females (mean 8.7 ± 5.5 ng/mL) corresponding to the entry into Vtg II gonadal maturity stage. The temporal changes in T were significantly correlated with the increase in GSI (R = 0.62). Immature females had T levels close to LOD (0.6 ± 0.3 ng/mL, n = 4). Plasma T levels in males were not significantly different among treatments (Fig. 5D). Maturing males had greater levels of T at all time points compared to maturing females. In maturing males, plasma T levels were low (0.62 ± 0.6 ng/mL) in June and rose to 6.2 ± 3.9 ng/mL in December and continued increasing to 11.7 ± 7.1 ng/mL in February. Testosterone levels in males were significantly correlated with GSI (R = 0.54), HSI (R = 0.31), somatic weight (R = 0.40) and maturity stage. Immature and resting male fish had low T levels (2.6 ± 3.0 ng/mL, n = 3).

3.3.4. Sperm quality

Crude oil exposure negatively affected sperm cell curvilinear path velocity (VCL) (F = 2.9, p = 0.051) (Fig. 6A). The VCL of sperm in the medium treatment was significantly reduced compared to the control treatment (p = 0.038). Percentage motile sperm was also affected by crude oil exposure (F = 2.5, p = 0.074), although not significantly at the 5% threshold. A higher percentage of motile sperm was measured in the low crude oil exposure group compared to control group (p = 0.018) (Fig. 6B). The percentage of motile sperm was on average over 80% in all groups. Although not significant at the 5% threshold, males in crude oil treatments had lower

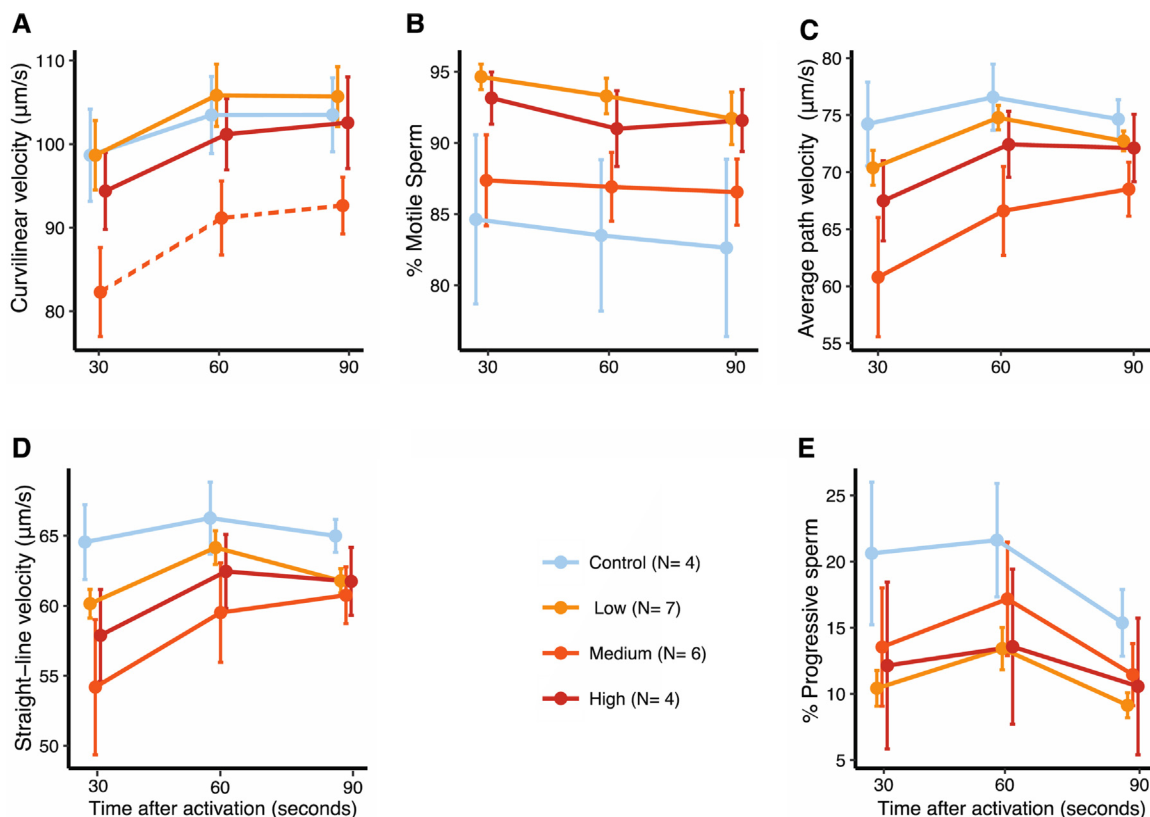

Fig. 6. Sperm motility measurements at 30, 60, and 90 s after activation from male fish stripped in week 31 (February 3rd) displayed by mean ± standard error: (A) Curvilinear velocity; (B) Percentage motile sperm; (C) Average path velocity of sperm; (D) Straight-line velocity; (E) Percentage progressive sperm. Significant differences from the control group across all times after activation are denoted by dashed line for the distinguished group ($p < 0.05$).

VAP ($F = 2.3$, $p = 0.09$) and lower VSL ($F = 1.3$, $p = 0.28$) compared to control males (Fig. 6C and D). Contrary to observations of the percentage of motile sperm, the percentage of progressive sperm fell on average by 30 ± 2.6% in all crude oil treatments compared to the control treatment (20.5 ± 3.03%) ($F = 1.25$, $p = 0.3$) (Fig. 6E). Time after activation (30, 60 and 90 s) was significant in all models ($F > 3.7$, $p < 0.05$). No interaction between the time after activation and treatment was found for any sperm motility parameter.

Spermatocrit was measured for 16 male fish in February. The mean spermatocrit was 0.97 ± 0.02 and no significant differences were found between treatments (data not shown).

4. Discussion

4.1. Effect on exposure indices

In the current study, maturing polar cod were exposed for 31 weeks to four different dietary doses of crude oil through a natural diet (measured concentration of Σ26 PAHs were at 141, 313, 1058 and 2288 ng/g in diet). These PAH doses are considered environmentally realistic and represent concentrations planktivorous fish may encounter in zooplankton communities after an oil spill (Salas et al., 2006) or in areas with chronic oil pollution (Carls et al., 2006). Zooplankton communities sampled six months after the Prestige fuel oil spill off the Northwest coast of Spain had concentrations (4.2–152 ng/g Σ14 PAHs) (Salas et al., 2006) within the range measured in the present study (24.9–152.6 ng/g Σ14 PAHs). The concentration of Σ26PAHs in the control (141 ± 103 ng/g) and low dose (313 ± 88 ng/g) of the present study resembled concentrations found in natural copepod assemblages (ΣPAHs = 120–256 ng/g) in the oil shipping port of Valdez, Alaska (Carls et al., 2006). The presence of PAHs in the control food is evidence for the ubiquity of these compounds even in relatively pristine marine environments like Lofoten, Norway, where the *Calanus* sp. for this experiment was collected (Green et al., 2013).

The dietary route may play a significant role in the exposure of marine fishes to lipophilic contaminants (i.e. PAHs) (George et al., 1995; Meador et al., 2006; Nahrgang et al., 2010b), especially for polar cod, a largely demersal fish (Geoffroy et al., 2016) with a high assimilation efficiency (Hop et al., 1997). Other studies that have explored the effects of dietary crude oil exposure on polar cod have used doses exceeding those of the present study (calculated exposure 0, 0.11, 0.57, and 1.14 μg crude oil/g fish/day equating to 0.8, 1.8, 7 and 13.1 ng $\sum$26PAHs/g fish/day in the control, low, medium and high treatments, respectively). Polar cod exposed to North Sea crude oil at doses two orders of magnitude higher (571–1285 ng Σ26PAHs/g fish/day) revealed substantial responses of exposure biomarkers (hepatic EROD activity and PAH metabolites in bile) after 4 weeks (Nahrgang et al., 2010b). Polar cod exposed to a crude oil dose three times higher than in the present study (average dose 3.8 μg crude oil/g fish/day) for 53 days during gonadal development had reduced growth (Christiansen and George, 1995) and elevated EROD activity compared to control fish (George et al., 1995). In a comparable study with regard to the duration (seven month exposure) and dose (2.8 μg crude oil in food/g fish/day), mature rainbow trout showed no effects of treatment on timing of spawning, fertilization or hatching success (Hodgins et al., 1977). A recent study by Bakke et al. (2016) found that maturing polar cod, when exposed to a single dose of radioactively labeled B[a]P (1.15 ± 0.36 μg/g fish) or phenanthrene (0.4 ± 0.12 μg/g fish), absorbed compounds into intestines, liver, and bile within two days following administration and the compounds remained in the fish tissue for over 30 days, thus exhibiting a long term bioavailability of ingested PAHs in polar cod.

EROD activity is a sensitive biomarker for the exposure to PAHs in fish and is used to assess the activity of the phase I cytochrome P450 1A1 (CYP1A1), an important enzyme in PAH biotransformation (Stegeman and Lech, 1991). The low overall EROD activity in this study may be an indication of a low effective dose of PAHs received by the polar cod liver. However, increased EROD activity was seen in crude oil-exposed fish compared to control at given time points (week 6 and 22), indicating the induction of PAH biotransformation upon crude oil exposure at these time points. Low molecular weight PAHs such as napthalenes, phenanthrenes, and fluoranthene, which made up the majority of PAHs measured in this study, have been found to cause either no effect or inhibit EROD activity in Nile tilapia *(Oreochromis niloticus)* (Pathiratne and Hemachandra, 2010) and in California halibut *(Paralichthys californicus)* (Seruto et al., 2005). The overall low amounts of PAHs in the exposure diet and the domination of lower weight PAHs with a potential inhibitory action on EROD activity may have limited the induction of responses at the doses used in the present study. Furthermore, inclusion of intestinal EROD activity to identify potential metabolism occurring prior to systemic uptake of the PAHs and alkylated PAHs would have provided additional information on the dietary exposure, especially at low levels (James et al., 1997; Van Veld et al., 1990). Such complementary analyses may allow the comparison of tissue-specific biotransformation capacity and has previously been found relevant for polar cod exposed to dietary crude oil (Nahrgang et al., 2010b).

The decreasing EROD activity over time was negatively correlated with maturity and similar declines have been observed in numerous other fish species during sexual maturation (Arukwe and Goksøyr, 1997; Whyte et al., 2000). Furthermore, crude oil-exposed immature and resting fish had higher EROD activity compared to control and maturing fish, although the number of resting and immature fish was too low to secure a robust statistical comparison. The suppression of the CYP1A1 enzyme activity in maturing fish of both sexes may be an adaptive response to maintain high steroid hormone levels necessary for endocrine regulation of reproductive development (Arukwe et al., 2008; Förlin and Hansson, 1982). For instance, E_2 has been shown to have a suppressive action on CYP1A catalytic activity through competition for binding sites as well as at pre-translational levels of CYP1A (Navas and Segner, 2001). Also, cross-talk between the signaling pathways involving the aryl hydrocarbon receptor (AhR), which regulates CYP1A expression, and the estrogen receptor (ER), which regulates vitellogenin expression, has been explored in the past decade although mechanisms are still unclear (Bemanian et al., 2004; Gräns et al., 2010; Mortensen et al., 2007; Mortensen and Arukwe 2007; Kirby et al., 2007). Inhibition of EROD catalytic activity may thus represent a possible mechanism to explain low EROD activity measured in maturing crude oil exposed fish in the present study.

A higher concentration of OH-phenanthrene bile metabolites in the high treatment group compared to control in August, and a strong correlation between phenanthrene diet concentration and 1-OH-phenanthrene bile metabolite concentration, verifies that PAHs accumulated after dietary exposure to crude oil (Aas et al., 2000; Nahrgang et al., 2010b). The lack of dose-dependent responses in bile metabolites concentrations at other time points may be due to the limitations of bile metabolites as a long-term response indicator (Collier and Varanasi, 1991). Dietary exposure to crude oil compared to waterborne exposure in polar cod resulted in less concentrated PAH bile metabolites (Nahrgang et al., 2010b) due to reduced systemic availability of PAHs (Ingebrigtsen et al., 2000). Furthermore, biotransformation and accumulation of PAH metabolites in bile may have been limited by a general low activity of CYP1A1 (EROD activity), most likely associated with the maturation processes. Additionally, a continuous (daily) feeding regime induces a regular emptying of the gallbladder, leading to a potential lack of significant PAH metabolite bioaccumulation in the gallbladder over time and thus low metabolite levels.

Pyrene and phenanthrene PAH metabolites were quantified in the bile of control fish, possibly due to the background PAHs found in the natural *Calanus* sp. diet (141 ± 103 ng Σ26PAHs/g food). Interestingly, mature fish in all treatments showed an increased concentration of OH-phenanthrene and OH-pyrene bile metabolites from August to February, while levels in immature specimens remained low. Although maturation-associated endogenous compounds with structural features resembling fluorescent PAHs (e.g. steroids) are also excreted in the bile and may potentially interfere with HPLC analysis (Honour, 2006), the specificity of the HPLC method used herein has likely limited such artifacts. It is more likely that life-stage/maturation-associated differences in the accumulation of PAHs or changes in total Phase I or Phase-II biotransformation in polar cod (Nahrgang et al., 2010b) have caused this apparent discrepancy. However, the rapid and substantial biotransformation of PAHs in fish (Meador et al., 1995) suggests that a combination of analyses of PAH-metabolites and a larger assembly of biotransformation enzymes would likely be the best strategy to decipher these maturation-specific differences.

4.2. Effects on reproductive parameters

Gonadal development was not significantly affected by exposure to dietary crude oil in polar cod in this study and appeared normal compared to histological studies from wild specimens (Nahrgang et al., 2016a) and levels of atresia were low compared to those observed by Geraudie et al. (2014). The temporal occurrence of atresia may reflect a period of oogenesis where females fine tune the energy resources used in reproduction as atresia allows for the reabsorption of energy rich oocytes (Hardardottir et al., 2001). The presence of POFs in February from the previous spawning season (12–13 months prior) is quite remarkable compared to other fish species, although cold temperatures may prolong degradation of POFs such as seen in Atlantic herring (*Clupea harengus*) and Dover sole (*Microstomus pacificus*) (Hunter et al., 1992; Brown-Peterson et al., 2011).

The present study is the first to present dynamic changes of sex steroid levels in maturing polar cod. The increasing concentrations in all sex steroid hormones measured over time matched expected profiles of maturing fish with increasing GSI and advancing oogenesis and spermatogenesis. Previous studies quantifying polar cod sex steroid hormones found levels that were an order of magnitude lower than in the present study. These low levels may be explained by the maturity stage of the fish as Hop et al. (1995) looked at fish actively or very close to spawning and Geraudie et al. (2014) most probably measured levels in previtellogenic polar cod. Hop et al. (1995) found lower E_2 levels in actively spawning females and mature females who had not reached ovulation (0.272 and 0.831 ng/mL) than what the present study reported in females at all gonadal maturity stages (0.43–25 ng/mL for PG through Vtg II maturity stages, respectively). Plasma E_2 and T levels are shown to drop back to basal levels at or immediately after spawning in Atlantic cod (Norberg et al., 2004) and Arctic char (Frantzen et al., 2004). Contrary to the plasma profiles measured in the present study, Geraudie et al. (2014) found no increase in polar cod T levels over time in males (0.023–0.149 ng/mL) or in E_2 levels in females (0.015–0.187 ng/mL). The plasma profiles of immature fish in the present study were comparable to those measured in Geraudie et al. (2014).

The absence of treatment effects on GSI, HSI and sex steroid levels in this study may be due to insufficient doses necessary to elicit an endocrine disrupting effect. Indeed, deviation in GSI is a valuable measure of long term exposure and may result from smaller, less mature oocytes and spermatocytes with a higher frequency

of atresia in the ovarian tissue and lesions within the testicular tissue (Kime, 1995). However, somatic indices may not always be very sensitive endpoints for assessing effects associated with gonadal development. For instance, no effect on GSI was observed in Atlantic cod exposed for 12 weeks to produced water (PW), which contains endocrine disrupting alkylphenols, although there were significant decreases in plasma E_2 levels and an increase in the frequency of atresia in exposed female cod (Sundt and Björkblom, 2011). In the present study, final maturation and spawning was not achieved within the experimental period. Fish would most likely have reached spawning in early March as was seen in feral polar cod not included in this experiment but taken from the same trawl and held under the same laboratory conditions (Bender ML et al. unpublished). Significant differences in sex steroid levels between crude oil treatment groups and the controls at a later maturity stage cannot be ruled out, and possible effects of crude oil may therefore have been missed. One year after crude oil exposure from the Exxon Valdez oil spill, plasma E_2 levels were still reduced in wild dolly varden (*Salvelinus malma*) and yellowfin sole (*Limanda aspera*) (Sol et al., 2000).

Certain parameters of sperm motility were negatively affected by dietary crude oil exposure. In exposed males, relatively high percentages of motile sperm (>87%) and low percentages of progressive sperm (<15%) were measured illustrating that sperm was in motion but conservatively so, which could potentially affect fertilization success. In Atlantic cod, percentage progressive sperm was shown to be the most indicative sperm motility parameter of fertilization success (Rudolfsen et al., 2008). Adverse effects of PAHs and other petroleum-related compounds on male gonads and sperm quality have been reported previously. For instance, sperm motility decreased in spottail shiners (*Notropis hudsonius*) naturally exposed to polluted waters around Montreal, Canada (Aravindakshan et al., 2004), white sucker (*Catostomus commersonii*) exposed to bleached kraft mill effluent containing PAHs and PCB compounds (McMaster et al., 1992) and in Pacific oysters (*Crassostrea gigas*) exposed to PAHs (Jeong and Cho, 2005). Exposure of polar cod to PW during reproductive development (28 days) resulted in reduced spermatogenesis and increased prevalence of histopathology (Geraudie et al., 2014). However, in the present study no alteration in spermatogenesis or obvious histopathologies were observed in the male testes in February to explain the reduced sperm motility in crude oil exposed polar cod. Polar cod used in this study were most likely to spawn a month later in March; therefore, final capacitation of spermatozoa may have been incomplete and thus not representative of motility of sperm at the true spawning time. Observed differences in sperm motility seen in crude oil exposed males could be due to a delay in capacitation not observed at the histological level or through possible endocrine-mediated effects of PAHs on spermatozoa development (Aravindakshan et al., 2004; Abdelrahim et al., 2006). Oxidative stress elicited by PAHs (Hannam et al., 2010) may also provide a possible mechanism to explain reduced sperm motility (Kao et al., 2008). Spermatozoa are susceptible to oxygen-induced damage due to large quantities of polyunsaturated fatty acids in the plasma membranes of spermatozoa (Alvarez and Storey, 1995) and low cytoplasmic concentrations of antioxidant enzymes necessary to repair damage (Saleh and Agarwal, 2002).

Reproduction in captive polar cod has been found to be an extreme energy investment with 87% of initial energy in liver used for reproductive costs under gonadal development (Hop et al., 1995). Thus, post-spawning mortality may be substantial. This is confirmed by field observations of polar cod perishing in large number after spawning (Moskalenko, 1964). In the present study, histological analyses revealed iteroparous female and male individuals, a finding supported by previous studies (Graham and Hop, 1995; Nahrgang et al., 2016a). In these individuals, ovaries contained both vitellogenic oocytes and POFs and testes contained both spent and early maturing fractions indicating previous spawning and intention to spawn in an upcoming season. Hence, the relatively high fish mortality in the present study (∼56%) could be related to the reduced condition of post-spawning fish and the cost of reproduction in a previous season. Generally, reduced somatic indices (GSI and HSI) in perished fish may indicate that a deficiency in energy reserves could explain the reduced survival of polar cod in this experiment. Analysis of the perished polar cod revealed that the likelihood of survival was not related to crude oil exposure, sex, or the initial weight of the fish (unpublished data). The presence of endoparasites in the fish body cavity (nematodes) was correlated with a higher stochastical risk of mortality, suggesting that fish with lower body indices may have had a compromised immune defense.

4.3. Conclusion and outlook

The investigated endpoints of weight and length, somatic indices, timing in gonadal development, and sex steroid hormone levels were not significantly altered by chronic dietary exposure to crude oil. However, altered sperm motility was seen in measures of sperm velocity. The ecologically realistic doses used in this chronic exposure study, exhibited by low EROD activity and PAH bile metabolite concentrations, may not have been high enough to induce adverse effects on the investigated parameters of reproduction. Furthermore, the utility of widely-used PAH exposure biomarkers (EROD activity and bile metabolites concentrations) may be reduced when polar cod are reaching the final stages of reproduction and these maturation-specific interactions need to be further investigated.

Pollutants may manifest effects on reproduction through endocrine disruption or by altering energy investment, which may impede a fish's ability to overcome other natural physiological stresses (Peterson et al., 2003). This is especially relevant for polar cod, a species already experiencing environmental changes in sea-ice cover (Stroeve et al., 2007), rising sea surface temperatures (Belkin, 2009), poleward moving competitors (Renaud et al., 2012; Hop and Gjøsæter, 2013), and increased freshwater discharge (Peterson et al., 2006), all of which may have the potential to alter the timing and success of reproduction (Bouchard and Fortier, 2011). The tolerance of polar cod reproductive development to crude oil exposure has been explored in this study but many aspects are yet to be investigated such as final maturation stages, fecundity and fertilization success, maternal and paternal effects, energy investment, and survival of early life stages (Nahrgang et al., 2016b).

Ethics statement

Permission to carry out this experiment was granted by the Norwegian Animal Welfare Authority in 2014 (ID 6571). Permission to carry out work with radioactive materials was given by the Norwegian Radioactive Regulatory body (Strålevern, project number 2014-13).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.10.005.

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