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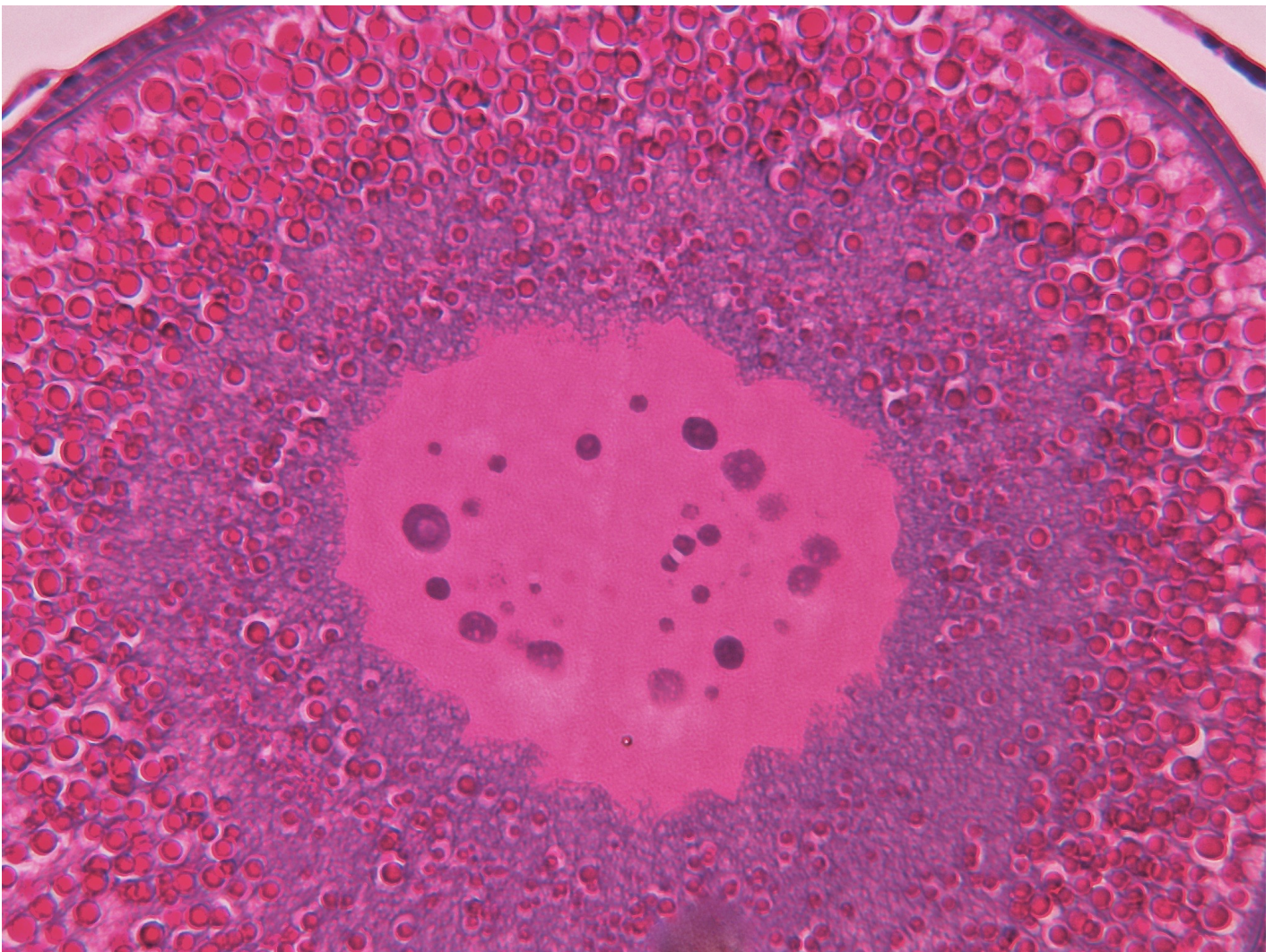Department of Arctic and Marine Biology

Long-term reproductive effects of chronic dietary petroleum exposure on polar cod (*Boreogadus saida*)

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Front Page: Polar cod (*Boreogadus saida*) oocyte in the initial stage of vitellogenesis sampled from the Long Term Experiment (this study) on October 29th 2014. Magnification 250X

Acknowledgements

When it is polar night, cold, dark, and damp, when your fingers no longer remember what they were doing, when your headlight makes your world tiny, then you really hope that there is good motivation for continuing. Running experiments on arctic fish in arctic conditions for one year can be isolating. But many people made sure that my experience was far from that. My fellow down jacket clade colleagues, which made this project possible, provided guidance, and asked questions with multiple exclamation marks at all stages of the *Long Term Experiment* are undoubtedly deserving of my utmost gratitude.

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Abstract

Abstract

Increased human activities in the Arctic pose a high risk for Arctic organisms to be chronically exposed to petroleum compounds. The endocrine disrupting properties of some of these compounds coupled with the metabolic costs of detoxification may have negative effects on the long and energy intensive reproductive development of polar cod, an Arctic keystone species. In the present study, selected reproductive parameters were examined in wild caught polar cod from Svalbard exposed to crude oil through a natural diet (0.11, 0.57 and 1.14 μg crude oil g^{-1} fish day^{-1}) over a 31-week period prior to spawning. In the experimental period from June to February, fish experienced a light and temperature regime from Svalbard (79°N). Fish maturing in the current reproductive period made up 84% of experimental population while 7% were identified as resting fish, which would most likely not spawn this season. Portions of the male and female population were confirmed to be iteroparous. Males began investing in gonadal development in October, 2-3 months earlier in the season than females and 75% of maturing males could be stripped when the experiment concluded in February. Sex steroid hormone plasma levels (estradoil-17β (females), testosterone (males and females) and 11-ketotestosterone (males)) were low in immature and fish in early maturation and then steadily increased with increasing gonadal weight in maturing fish. The investigated endpoints of growth, investment and timing in gonadal development, and sex steroid hormone levels were not significantly altered by chronic dietary exposure to crude oil. However, reduced sperm motility was seen in measures of progressive sperm and sperm velocity in low and high crude oil exposures. A trend towards a delay in onset of vitellogenesis in crude oil exposed females was observed. Tradeoffs between pollutant detoxification and reproductive investment may have influenced maturation in exposed males. This study created novel data on polar cod reproductive physiology. However, the ecologically realistic doses used in this chronic exposure study were likely not high enough to induce widespread endocrine disruption effects.

Keywords: *Boreogadus saida*, crude oil, reproduction, oogenesis, spermatogenesis, sex steroid hormones, sperm motility, chronic dietary exposure

Abbreviations

AMAP	Arctic Monitoring and Assessment Program
BaP	Benzo[a]pyrene
bw	body weight
CA	Cortical alveoli
CYP	Cytochrome P450
DDT	dichlorodiphenyltrichloroethane
E_2	Estradiol-17ß
ER	Estrogen receptor
EVOS	Exxon Valdez Oil Spill
FL	Fork length
FSH	Follicle stimulating hormone
GSI	Gonadosomatic Index
HSI	Hepatosomatic Index
LH	luteinizing hormone
LOD	level of detection
PAH	Polycyclic Aromatic Hydrocarbons
PG	Primary Growth
ppb	part per billion
PW	Produced water
RIA	Radioimmunoassay
eSc	Early spermatocytes
lSc	Late spermatocytes
SGR	Specific growth rate
St	Spermatids
Sz	Spermatozoa
T	Testosterone
TW	Total weight
VAP	Average path velocity
VCL	Curvilinear velocity
Vtg	Vitellogenin
Vtg I	Vitellogenesis I
Vtg II	Vitellogenesis II
VSL	Straight line velocity
WSF	Water soluble fraction
ww	wet weight
*Zr*p	*Zona radiata* protein
11KT	11- ketotestosterone

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Introduction

1. The Arctic keystone species, polar cod (Boreogadus saida)

Polar cod hold a keystone role in the food web of the Arctic Ocean (Bradstreet and Cross, 1982) based on their abundance and efficiency in transferring energy from lower to higher trophic levels, essentially from zooplankton to marine mammals and sea birds (Craig et al., 1982; Hobson and Welch, 1992). With a pan-Arctic distribution, polar cod is the most abundant and widespread fish species in the Arctic, and influences the distribution and movements of marine mammals and seabirds (Bradstreet, 1982; Gradinger and Bluhm, 2004). Polar cod feed mostly on pelagic zooplankton such as copepods (*Calanus sp.)* and amphipods (Renaud et al., 2012; Walkusz et al., 2013). Adults are found at depth while juveniles are found in the epipelagic layers (Geoffroy et al., 2015). However, despite its central ecological role, little information exists on the reproductive biology of polar cod (Sekerak, 1982; Hop et al., 1995).

Synchronous broadcast spawning occurs under the sea ice in the winter months between December and March in both the western and eastern Arctic, although exact spawning locations are largely unknown due to the high dispersal of positively buoyant eggs (Rass, 1968; Bradstreet, 1982; Craig et al., 1982). Polar cod are described as an r-selected species due to its small size (<30 cm fork length), short lifespan (5 years avg, 7-8 max), and early age at maturity (2-3 years) (Craig et al., 1982). However, polar cod also express attributes of a k-selected species such as a slow growth rate and relatively low fecundity (Hop et al., 1995). Like other externally fertilizing, broadcast spawning fish (Levitan, 2005), polar cod lack any obvious sexual dimorphism. Yet, there are apparent differences in reproductive strategy between males and females, such as timing of reproductive development, investment in gonadal tissue, and frequency of spawning (Nahrgang et al., 2014, Nahrgang et al., accepted). 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). Maturing polar cod in captivity have been shown to draw energy predominately from the liver and somatic tissue for gonadal development (Hop et al., 1995). In Arctic waters, females dominate the upper age classes and grow to a larger size

than males, indicating a gender difference in reproductive strategies (Nahrgang et al., 2014).

2. Fish Reproduction

The success of any living species hinges on its ability to reproduce. Reproduction is a sensitive, seasonal, and cyclic phenomenon in most teleost fish (Hoar, 1969). In the Arctic, reproduction must be synchronized with the extreme seasonality in light, temperature, and food availability (Grebmeier et al., 2006). Environmental changes such as water temperature and photoperiod provide cues to the central nervous system, triggering maturation. In female teleosts, oogenesis is the series of cytological stages in which oogonia undergo meiosis to become mature eggs capable of supporting a developing embryo (Schneider, 1996). In the ovary, oogonia undergo mitotic divisions to become primary growth oocytes (PG). In the proceeding stage, cortical alveoli oocytes (CA) increase in size, develop a steroid-producing follicle made up of granulosa (g) and theca cells, and form cortical alveoli in the periphery of the cytoplasm (Blazer, 2002).

One of the major events in oogenesis is vitellogenesis, which is responsible for the main transfer of energy into the developing oocyte (Brooks et al., 1997). During vitellogenesis, developing oocytes incorporate *zona radiata* proteins (*Zrp*) into the single cell layer *zona radiata* (Zr) and sequester hepatically derived vitellogenin proteins (Vtg) into the cytoplasm (Patiño and Sullivan, 2002). Vitellogenesis is further advanced with the formation and fusion of yolk globules in the cytoplasm, migration of the nucleus to the animal pole, and hydration of the oocyte before ovulation (Blazer, 2002). The maturation of oocytes, including the production of Vtg and *Zrp* and its uptake by oocytes, is under the control of the gonadal steroid Estradiol-17β (E_2) and is regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Hoar, 1969; Brooks et al., 1997; Nagahama, 2000) (Figure 1). Gonadotropin-releasing hormone (GnRH) is synthesized in the hypothalamus and regulates the hormone cascade involving the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which induce and regulate E_2 production (Okuzawa, 2002; Patiño and Sullivan, 2002). E_2 is the major estrogen in female teleosts

and stimulates the production of Vtg and oocyte development (Okuzawa, 2002; Patiño and Sullivan, 2002). Theca cells synthesize Testosterone (T) which is subsequently aromatized by cytochrome P450 aromatase (CYP45019a1) to E_2 by the granulosa cells (Nagahama, 2000).
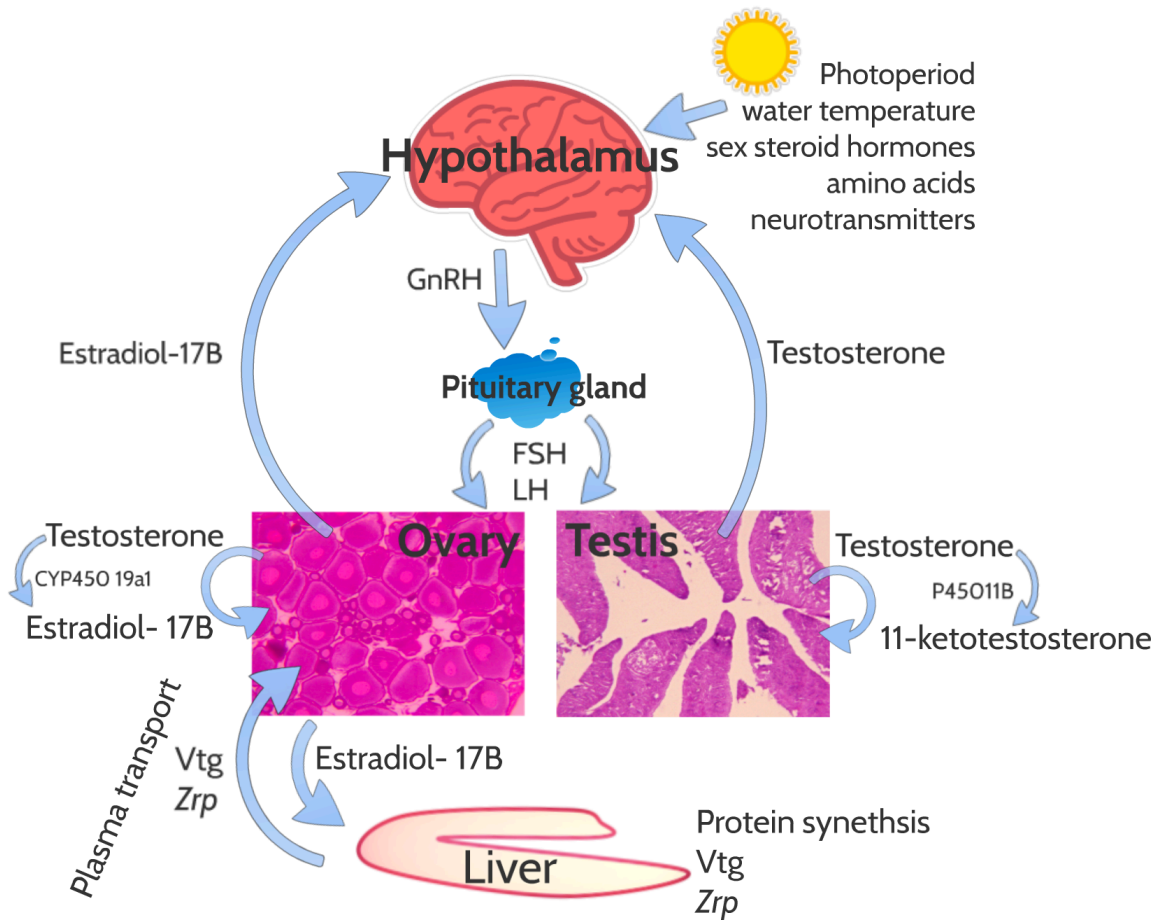

Figure 1. Schematic representation of the hypothalamic-pituitary-gonadal axis (HPG) and liver tissue during oogenic protein synthesis in female teleosts (left) and in spermatogenesis in male teleosts (right). The HPG is regulated through the negative feedback mechanism by estradiol-17β and Testosterone. Adapted from Arukwe and Goksøyr 2003. (GnRH= gonadotropin-releasing hormone, FSH=follicle stimulating hormone, LH= luteinizing hormone, Vtg= vitellogenin protein, *Zrp= Zona radiata* protein, CYP450 = cytochrome P450 aromatase, P45011B= 11β-hydroxylase).

In males, spermatogenesis describes the series of cytological stages from primordial germ cells, spermatogonia, to mature, flagellated spermatozoa (Almeida et al., 2008). In this process, spermatogonia grow and divide to form primary (early) spermatocytes (eSc) and then undergo further division to secondary (late) spermatocytes (lSc). Secondary spermatocytes divide into spermatids (St), which metamorphose into functional swimming

gametes, called spermatozoa (Sz). The testicular units in gadoids are organized into lobules (L) bound by connective tissue (Almeida et al., 2008). Similarly to females, reproductive endocrine activities in males are regulated by the hypothalamic-pituitary-gonadal (HPG) axis through a hormone cascade involving GnRH, FSH, and LH. In the testis, Sertoli cells are the main receptor of FSH and support survival and development of the germ cells (Nagahama, 2000). LH stimulates the production of androgens required for spermatogenesis (Schulz and Miura, 2002). The major androgen in most male teleost fishes is 11-ketotestosterone (11KT), which is physiologically relevant in maintaining all stages of spermatogenesis and is derived from T by the 11β-hydroxylase (P45011β) enzyme (Matsubara et al., 2003; Miura and Miura, 2003).

The biosynthesis pathway of sex steroid hormones proceeds sequentially from acetate to cholesterol, progesterone, T, and eventually E_2 (females) and 11KT (males) (Nagahama, 2000). The metabolic pathways involved in T and E_2 production are very ancient phylogenetically and are highly conserved between the animal and even plant kingdoms (Hoar, 1969). Theses hormones can be found in the gonadal tissue as well as circulating in the blood plasma (Nagahama, 2000), the latter reflecting the advancement of ovarian and testicular development of gonadal tissue (i.e. increased GSI, increased oocyte growth, spermatogonial proliferation) (Hoar, 1969). In females, early and mid-vitellogenesis are characterized by elevated E_2 plasma levels, whereas a completion of vitellogenesis and ovulation is marked by reduced E_2 levels and increased levels of T as seen in Atlantic cod (*Gadus morhua*) (Norberg et al., 2004), Arctic charr (*Salvelinus alpinus*) (Frantzen et al., 1997), and goldfish (*Carassius auratus*) (Kobayashi et al., 2002). A T peak is commonly occurring simultaneously with ovulation in female fish such as Arctic charr (Frantzen et al., 1997) and Atlantic cod (Norberg et al., 2004). T and 11KT levels in males tend to be low or undetectable in immature fish and increased towards maturity. T levels tend to be elevated several months before spawning with peak levels occurring shortly before and during spawning such as seen in Atlantic cod (Norberg et al., 2004) while 11KT levels tend to peak at the start of spawning and thereafter decrease such as seen in Arctic charr (Frantzen et al., 2004). Sex steroid hormones in polar cod (E_2, T, and 11KT) have been previously quantified at spawning with actively spawning females exhibiting lower E_2

levels than maturing females, whose eggs were not yet hydrated (Hop et al., 1995). The spawning male polar cod measured had a higher level of T than of 11KT (Hop et al., 1995). Thus far, no study has investigated temporal plasma profiles of sex steroid hormone levels in polar cod.

3. *Effects of pollution on reproduction*

Pollutants (either synthetic or natural compounds) can cause reproductive dysfunction through endocrine disruption, either by direct action on the oogenesis and spermatogenesis, or indirectly by modulation of the HPG axis (Nicolas, 1999; Goksøyr, 2006). This process has led to many instances of developmental and reproductive problems in Atlantic salmon *(Salmo salar)*, Atlantic cod, rainbow trout (*Oncorhychus mykiss*), and medaka (*Oryzias latipes*) (Arukwe and Goksøyr, 2003 and references therein; Goksøyr, 2006; Tollefsen et al., 2011). However, the molecular mechanisms of endocrine disruption by pollutants are not fully understood. For example, xeno-estrogens (e.g. bisphenol-A, DDT, etc.) can bind with high affinity to the estrogen receptor (ER), due to their homologous structure, thereby blocking the site for natural estrogens and either initiating or inhibiting natural production of Vtg and *Zr*p (Arukwe and Goksøyr, 2003). A review of pollutants and fish reproduction by Kime (1995) found that exposure to 1 parts per billion (ppb, 1 ng/g) of a pollutant is sufficient to produce harmful effects on reproduction, especially over a long exposure period. Alterations to reproductive effort can also arise via energy trade-offs between detoxification of pollutants and reproductive investment (Calow, 1989). Due to the sensitivity of these processes, reproduction is especially vulnerable to even low levels of pollution, and the effects of which may be difficult to detect, and potential consequences may take generations to emerge (Kime, 1995; Peterson et al., 2003).

Crude oil is a complex mixture containing both hydrocarbons, such as alkanes, cycloalkanes and aromatic hydrocarbons, and non-hydrocarbon compounds, the composition of which varies greatly across geographic regions (Wu et al., 2012). Polycyclic aromatic hydrocarbons (PAHs) are compounds, with two or more fused

benzene rings and often containing alkyl side groups, that make up only a small fraction of crude oil. Nevertheless, they are considered the primary toxic components in crude oil (Meador, 2006). PAHs are readily taken up by aquatic organisms, however most teleosts have the ability to metabolize and eliminate these compounds (Meador et al., 1995). Even at concentrations in the lower ppb range, PAHs have been found to be toxic to fish, inducing carcinogenic, genotoxic, endocrine disrupting effects and physiological impairment (Kime, 1995; Rice et al., 2000; Meador et al., 2006; Vignet et al., 2014). Furthermore, PAHs have been found to disrupt the endocrine system and affect reproductive function and growth of humans and wildlife (Horng et al., 2010). Exposure to PAHs in fish has been linked to reduced investment in gonadal tissues (Booc et al., 2014) and inhibition of sex steroid synthesis and degradation of steroid receptors (Seruto et al., 2005; Ohtake et al., 2007). In maturing female fish, exposure to PAHs has been found to inhibit oocyte development and maturation (especially during vitellogenesis), increase follicular atresia of both yolked and previtellogenic oocytes, cause abnormal yolk deposition within oocytes, and lead to abnormal egg maturation and production (Arukwe and Goksøyr, 2003 and reference therein). In males, PAH exposure has been found to suppress spermatogenesis in clams (Frouin et al., 2007) and increase T production in goldfish and rainbow trout by promoting testicular steroidogenesis (Evanson and Van Der Kraak, 2001).

Furthermore, phase I cytochrome P450 (CYP1A1), an important enzyme in the detoxification process of PAHs, has been found to interact with the ER and interfere with the production of Vtg and *Zrp* in females and induce the production of Vtg in males (e.g. Navas and Segner, 2001; Gräns et al., 2010). CYP1A1 is the most studied biomarker of exposure to pollution (Stegeman and Lech, 1991). Cross-talk between CYP1A1 and ER pathways can result in the disruption of Vtg synthesis (Gräns et al., 2010). PAHs and alkylphenols form only a small group of well-known hydrocarbons found in crude oil, which otherwise contains many poorly characterized compounds collectively referred to as 'unresolved complex mixtures'. These mixtures have been observed to exert additional toxic effects, may be more resistant to weathering, and have been found in high concentrations in mussels (*Mytilus edulis*) from polluted areas (Booth et al., 2007; Melbye

et al., 2009). Nevertheless, PAHs are routinely quantified in toxicology studies on crude oil and used as a proxy for toxic dose.

4. *Oil Pollution in the Arctic*

The Arctic is undergoing rapid environmental change exemplified by amplified climate change and declining sea ice extent (Barber et al., 2015). The Arctic is home to indigenous peoples, an oil and gas development area, and a tourism destination (AMAP, 2009). An ice free summer in the Arctic is estimated to occur as early as 2030 (Wang and Overland, 2012). This will further facilitate shipping already occurring along the Northwest Passage and the Northern Sea route. The United States Geologic Survey estimates that nearly one quarter of the worlds undiscovered oil reserves are found in the Arctic (Gautier et al., 2009). The increased demand of petroleum products by the world market, the high probability of finding vast petroleum resources, and the increasing accessibility due to the decreasing sea ice extent will fuel the race for arctic resources (Harsem et al., 2011). The Arctic is the most sparsely inhabited area in the Northern hemisphere (AMAP, 2009). This remoteness combined with inclement weather, unpredictable sea ice conditions, limited availability of bathymetric data, few ports, and a general lack of precedent make Arctic operations challenging (Harsem et al., 2011). The risk of petroleum pollution, whether acute or chronic, is growing proportionally with development.

Weathering of spilled oil, the combined physical and chemical processes leading to oil degradation in the environment, may be significantly prolonged in Arctic environments compared to more temperate regions, due to the extreme light climate, cold temperatures, ice cover, and slow microbial activity (Brandvik and Faksness, 2009). Oil could be encapsulated in sea ice and transported potentially long distances by wind and ocean currents (Pfirman et al., 1995) to be released during spring melt both in biological hotspots such as leads and polynyas (Kuletz et al., 2015) and at times of high biological activity (Leu et al., 2015). The most well documented example of how damaging an oil spill in areas like the Arctic comes from the 1989 Exxon Valdez oil spill (EVOS) which occurred in the pristine marine environment of Prince William Sound in south-central Alaska. The

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release of 42 million liters of Alaskan North Slope crude oil contaminated 1990 kilometers of shoreline. The recovery of at least 10 species of birds, whales, and fish has still not been achieved after 25 years (EVOSTC, 2014). The unprecedented persistence of crude oil in the ecosystem made toxic compounds more bioavailable to organisms (Hayes and Michel, 1999; Peterson, 2001; Short et al., 2003). Oil was physically protected from disturbance, oxygenation, and photolysis in the sediments making weathering and degradation processes less efficient, similar to what could be expected from oil entrapped in ice. It may be hypothesized that an oil spill in the Arctic will have even greater impacts due to the extreme light climate and ice cover which will further slow weathering processes, thus increasing the bioavailability of oil to organisms. Thus there is an urgent need for the increased understanding of the long term effects of exposure to petroleum pollution in Arctic organisms.

5. *Study Aims*

Polar cod has been used as an indicator species to understand the effects and mechanisms of oil pollution (Christiansen and George, 1995a; Stange and Klungsoyr, 1997; Jonsson et al., 2009; Nahrgang et al., 2010a; Andersen et al., 2015) due to its importance in the Arctic food web and its pan-Arctic distribution. Nevertheless, effects of these compounds on the reproductive development of this species have not been thoroughly investigated. The study of reproductive physiology can provide an integrative measure of the effects of pollutants on whole organisms and provide important insights into the potential risks to populations. It is therefore critical to study the long-term effects of these toxicants 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 crude oil on reproduction in the Arctic key species, polar cod, collected in the wild. Effects of dietary exposure to petroleum have been less explored than the waterborne exposure route in polar cod, although the dietary route may play a significant role in the exposure of marine fishes, especially those with a demersal distribution (George et al., 1995; Geoffroy et al., 2015).

Growth, gonadal development, and reproductive hormone signaling were followed over a seven-month chronic exposure period starting in June, concurrent with gonadal development. More specifically, potential endocrine disrupting effects were investigated through the study of the following aspects of reproduction: (1) reproductive investment measured as gonadal and somatic weight; (2) timing of gonadal development followed through changes in gonadal weight, (3) oogenesis and spermatogenesis by histological preparations of gonads, and (4) endocrine signaling by plasma sex steroid hormone levels; and (5) evaluation of sperm quality (i.e. motility and density). We hypothesized that these endpoints would be altered and potentially decrease the reproductive success of polar cod. This may have implications for the Arctic food web.

Material and Methods

1. Fish sampling 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 from all locations were mixed and kept on deck in 500L flow-through tanks for two weeks while under transport to Troms, Norway. Fish were treated daily with Halamid® (1:500) disinfectant and dead fish were removed. On the 29th of January, fish were moved to the UiT-The Arctic University of Norway's biological station in Kårvika and were kept in a 1000 L acclimation tank under 79 ° N photoperiod with 3° C seawater until the start of the experiment. During this period, fish were fed a daily *ad libidum* diet of thawed *Calanus finmarchicus* copepods (*Calanus sp.*) from Lofoten, Norway (Calanus AS). Permission to carry out this experiment was granted by the Norwegian Animal Welfare Authority in 2014 (ID 6571).

2. Experimental design

Polar cod were exposed to dietary crude oil over the period of gonadal development starting on the 3rd of July and ending on 3rd of February 2015. Fish were randomly assigned into control or one of three treatment groups, which received nominal concentrations of Kobbe crude oil. Concentrations were 0, 20, 100, and 200 μg crude oil g^{-1} diet of *Calanus sp.*, corresponding to control, low, medium, and high doses, respectively. Treatment groups were divided into eight tanks, with two tanks for each of the three exposures and two control tanks to increase statistical power under analysis. Treatment food was prepared in large batches prior to the exposure period by mixing the following: 500g *Calanus sp.,* 250 mL distilled water, 50 g gelatin (10%), and either no or one of three different nominal concentrations of crude oil. This mixture was allowed to setup before it was cut into small pellets, then weighted and distributed into individual bags, corresponding to 2% of the total fish weight of each individual tank, and frozen at - 20° C until use.

Material and Methods

On June 5th and 6th, 535 fish were selected based on size (13 -17 cm long) and apparent condition (11-24 g) for participation in the experiment. Fish with gross deformations or skin conditions were deemed unfit. Selected fish were anaesthetized for approximately 3 minutes until loss of equilibrium, using 5 mg L^{-1} Finquel® (Tricaine Methanesulfonate) dissolved in seawater. Fork length (± 0.1 cm) and wet weight (± 0.1 gram) was recorded for each fish. Fish were then carefully tagged with a passive integrated transponder tag (Biomark®) inserted intraperitoneally behind the left pelvic fin by a sharpened tagging gun before being placed randomly in one of eight treatment tanks. Total handling time per fish was about 45 seconds. Treatment tanks consisted of 300L flow-through systems with cooled seawater. Fish were held at Kongsfjorden, Svalbard seawater temperatures (3-2° C) to the nearest 0.5°C as taken from mooring data (J. Berge pers. comm.). The eight tanks with 67 fish per tank were randomly distributed within the experimental room with regard to treatment. Fish were allowed to acclimate with a raw *Calanus sp.* diet until the 3rd of July. Fish that died between tagging and the start of the exposure were replaced with fish collected on the same cruise.
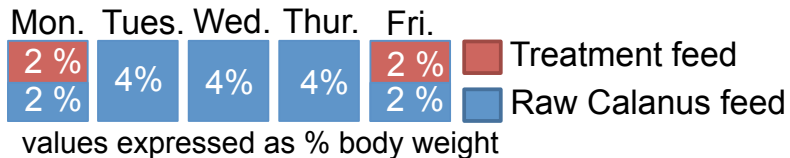

Figure 2. Feeding regime for one of the 31 identical weeks of exposure. Each box represents one day and noted inside is the percentage of body weight fed for each food type (Treatment feed in red and raw *Calanus sp.* food in blue) and treatment.

Fish in each tank were fed as a group, five times a week with a total ration equal to 4% of the total fish weight tank^{-1} feeding^{-1} (Christiansen and George, 1995) (Figure 2). On the first and last day of the week, fish were fed treatment food comprised of the prepared *Calanus sp.* gelatin pellets containing a dose of crude oil depending on treatment. This exposure was 2% of the total fish weight in the tank day^{-1} and an additional 2 % of raw *Calanus sp.* followed the treatment feeding to ensure the fish received enough food. The three other feedings per week consisted of raw *Calanus sp.* amounting to 4% of the total fish weight in the tank. With this feeding regime the effective dose was 0.0, 0.11, 0.57, and 1.14 µg crude oil g fish^{-1} day^{-1}. *Calanus sp.* was distributed throughout the tank to

reduce feeding hierarchies although observation confirmed the existence of hierarchies in the tanks. The amount of food distributed in each tank was adjusted four times over the course of the experiment following total weight check points to account for growth and removal by sampling.

Five full dissection events with eight fish from each tank (n=16 fish per treatment) were performed during the 31 weeks (215 days) of exposure (Figure 3). On the 30th of June, exposure began, and control fish (n=16) were dissected. The second sampling point occurred after six weeks of exposure on the 11th of August, the third after 17 weeks on the 29th of October, the fourth after 22 weeks on the 3rd of December, and the fifth after 31 weeks of exposure on the 3rd of February. On the 3rd of February all remaining fish (n=48) were dissected (Figure 3). Each fish was anaesthetized until loss of equilibrium. Blood was removed from the caudal vein using a 2 mL heparinized vacuum sealed collection tube (BD Vacutainer ®) and promptly set on ice until centrifugation for 30 min at 4°C and 3500 rpm for plasma separation (Sorvall RC 5B Plus centrifuge). The plasma supernatant was pipetted out and stored at -80°C until sex hormone analysis.

Following blood sampling, the fish was given a sharp blow to the head before wet weight and fork length were measured and liver and gonads were weighted and removed. The remaining internal organs were removed and somatic weight of the carcass was recorded. A section of the gonad was preserved in 4% neutral buffered formalin for histological analysis. After 31 weeks of exposure (3rd of February), male fish were stripped for milt after blood sampling by gently massaging the abdomen and taking care to avoid contamination by urine or blood (n=21). Additionally, growth was monitored four times during the experiment: after 72 (10th of September), 120 (28th of October), 152 (29th of November), and finally 193 (9th of January) days after the start of the experiment (Figure 2). Briefly, all fish were anaesthetized serially and total weight and fork length was recorded. The gonadosomatic index (GSI) was calculated using the equation GSI=100*(gonad weight/ somatic weight) and hepatosomatic index (HSI) was calculated using the equation HSI= 100*(liver weight/ somatic weight). Fulton's condition factor (K)

was calculated using the equation K= 100* (somatic weight/ (fork length ^{3})). Specific growth rate (SGR) was calculated with data from the initial fork length and total weight measurement and the four growth checks using the following formula (Cook et al., 2000):

$$\text{SGR (\% body weight gain day}^{-1})= \left[\frac{(ln \text{ Final fish wt} - ln \text{ Initial fish wt)}}{\text{days}}\right] \times 100$$

Fish that died during the experiment were recorded daily, removed from the experimental tanks and frozen for later analysis. However, this data will not be presented herein.
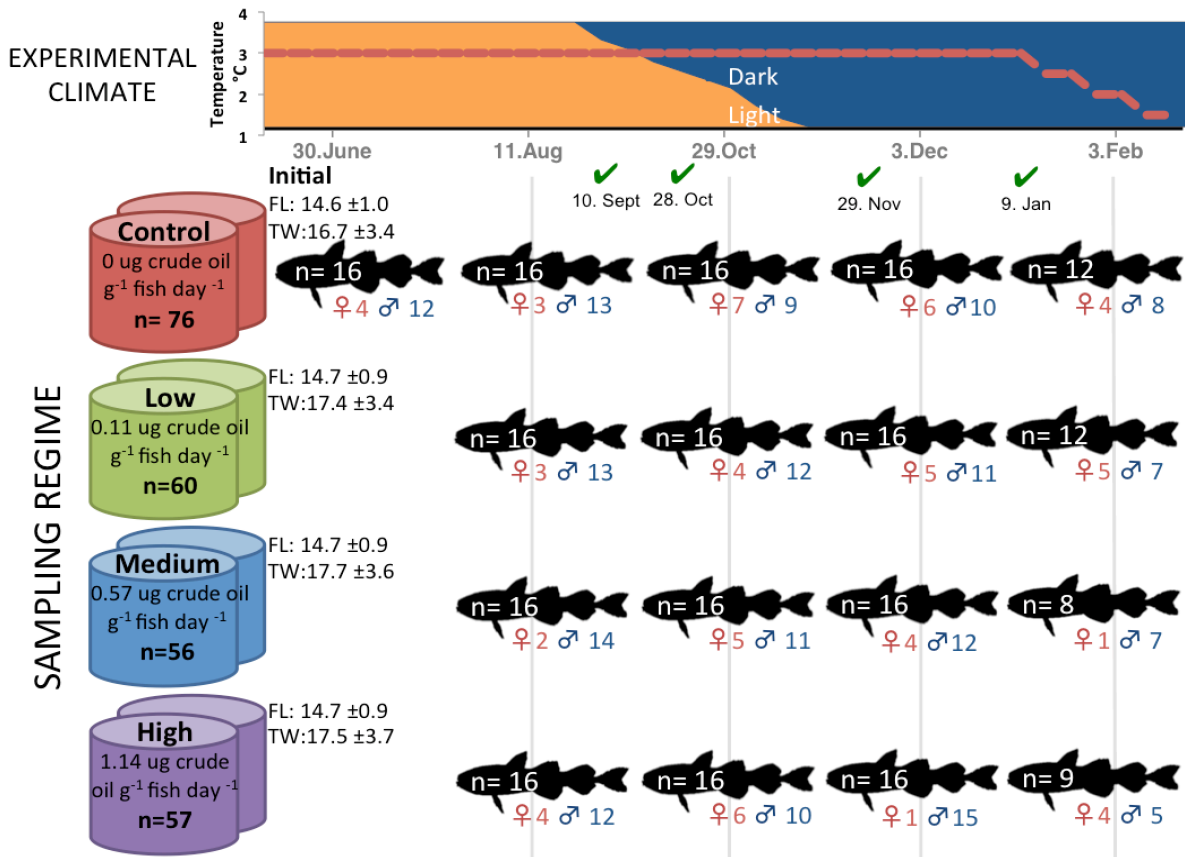

Figure 3. Experimental design with the top panel illustrating the experimental climate (seawater temperature and photoperiod) over time and timing of sampling events (grey dates). The right panel shows the sampling scheme for the different treatment ordered by increasing dose. The number of fish sampled per event in each treatment is noted inside the polar cod symbol and sex ratio is noted underneath with females in red and males in blue. Total number of fish sampled in each treatment is noted inside the treatment tanks. Initial mean fork length (FL) and total weight (TW) ± standard deviation for each treatment is to the left of each tank.

3. *Determination of PAH doses in the diet*

Calanus sp. diet samples from each treatment were analyzed for the 16 Environmental Protection Agency (EPA) priority PAHs and 10 further alkylated naphthalenes, phenanthrenes and dibenzothiophenes compounds at Unilab Analyses AS (Tromsø, Norway Spring 2015) and as described in Nahrgang et al.(2010b). 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 chromatograph/mass spectrometry. Triplicates were run for each treatment diet and mean and standard deviation were calculated. Dry weight to wet weight ratio of prepared *Calanus* sp. diet was 0.169 ± 0.005 after 24 hour drying period at 70 °C (n=8).

4. *Histological analysis*

Fixed gonad samples were rinsed of formalin and 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 7 μm thickness using a Leitz RM 2255 microtome, floated out on microscope slides, and placed overnight in an oven (60°C) before staining with haematoxylin/eosin. Haematoxylin is a basic dye that colors basophilic structures like nuclei and endoplasmic reticulum purplish-blue due to the high content of DNA and RNA in these tissues (Wheather et al., 1987). In contrast, eosin is an acidic dye adhering to basic structures like cytoplasm and connective tissue, coloring them pinkish red (Wheather et al., 1987). Two slides were prepared for each fish with 4-6 sections on each slide. Sections were taken approximately one fourth and halfway through the embedded tissue to ensure independence of replicates.

Each slide was examined under a LaborLux 11 Leitz microscope equipped with a camera (Wild Leitz AS, Oslo, Norway) at 40X and 100X magnification. A higher magnification

of 250X and 400X was used to look at certain structures (membranes, lipid droplets, spermatozoa). On female fish, only one slice per slide was analyzed for oocyte stage distribution to avoid double counting of the same oocytes (n=2 per fish). Oocytes were categorized based on Brown-Peterson et al.(2011) (Figure 3A-C). The number of oocytes was counted in a fixed area (23.75 mm^2, the area of field of view through the microscope at 40X magnification) chosen randomly within the slice into one of the following stages: primary growth (PG, 73-221 μm), cortical alveolar (CA, 268-405 μm), primary vitellogenic (Vtg I, 312-420 μm), and secondary vitellogenic (Vtg II, 714-855 μm). Oocytes in the PG stage were identified by the presence of a prominent nucleus, multiple nucleoli, and scant cytoplasm. CA stage oocytes were distinguished by the presence of cortical alveoli vesicles, *zona radiata* (Zr) eggshell, and a granulosa cell layer (g) surrounding the oocyte. Vitellogenic oocytes were distinguished based on the presence of yolk globules and the area of cytoplasm filled with yolk. Vtg I oocytes had less than 50% of the cytoplasm filled by yolk globules while Vtg II oocytes were larger and had over 50% of cytoplasm filled by yolk globules. The most advanced or leading cohort of oocytes in >10% abundance was used to characterize the gonadal maturity stage of each female and was assigned the leading cohort oocyte stage name.

Atretic oocytes were characterized by the disintegration of the nucleus and breakdown of the oocyte envelope (Figure 3D). Postovulatory follicle complexes (POFs) were identified by the presence of empty and collapsed follicles (granulosa and theca cells) remaining in the ovary post spawning (Figure 3E). Presence of atretic oocytes and POFs were noted for the entire slide. In December and February, females were divided by maturity status with resting females identified as having PG oocytes and POFs and immature females identified as having only PG oocytes.
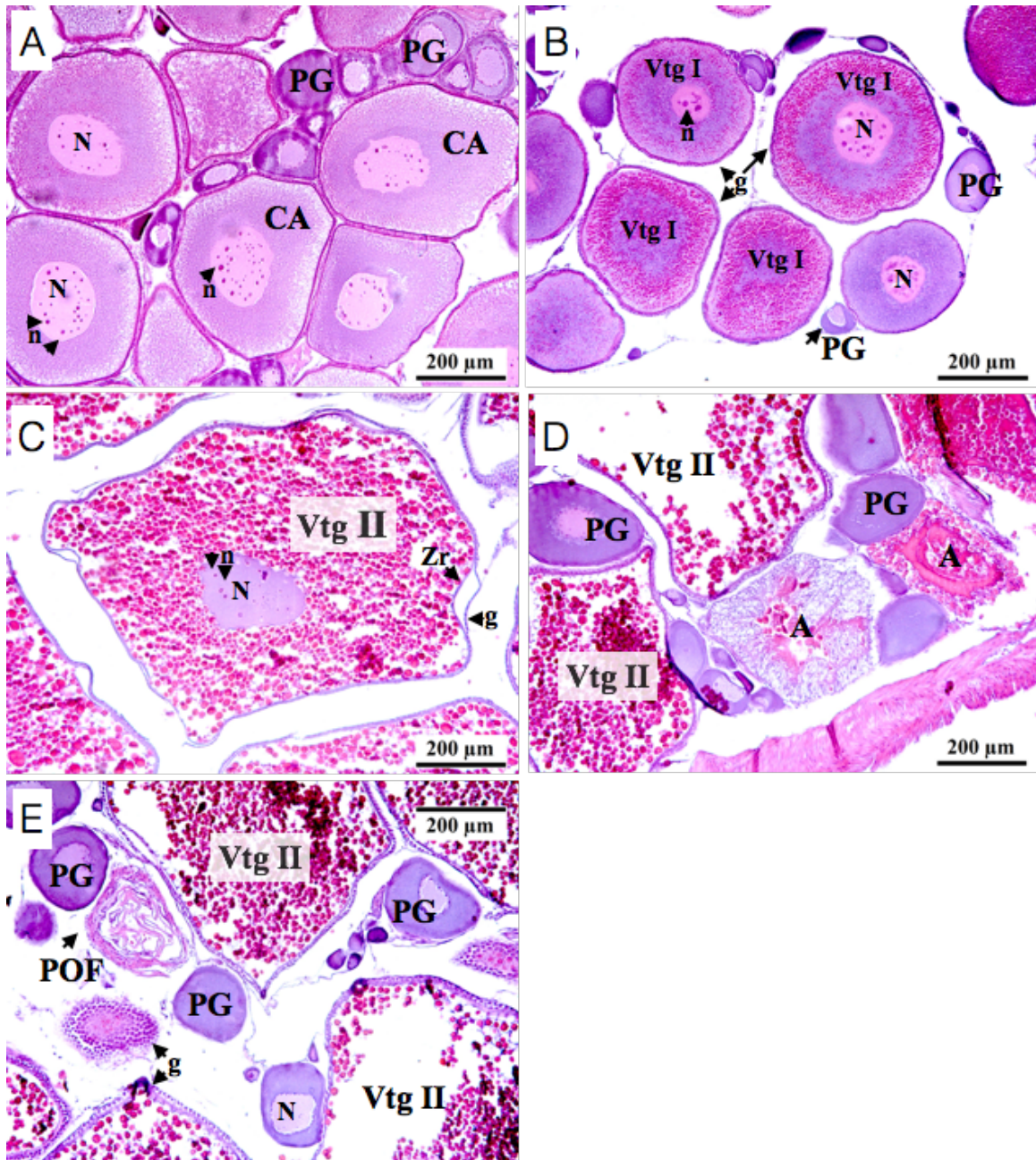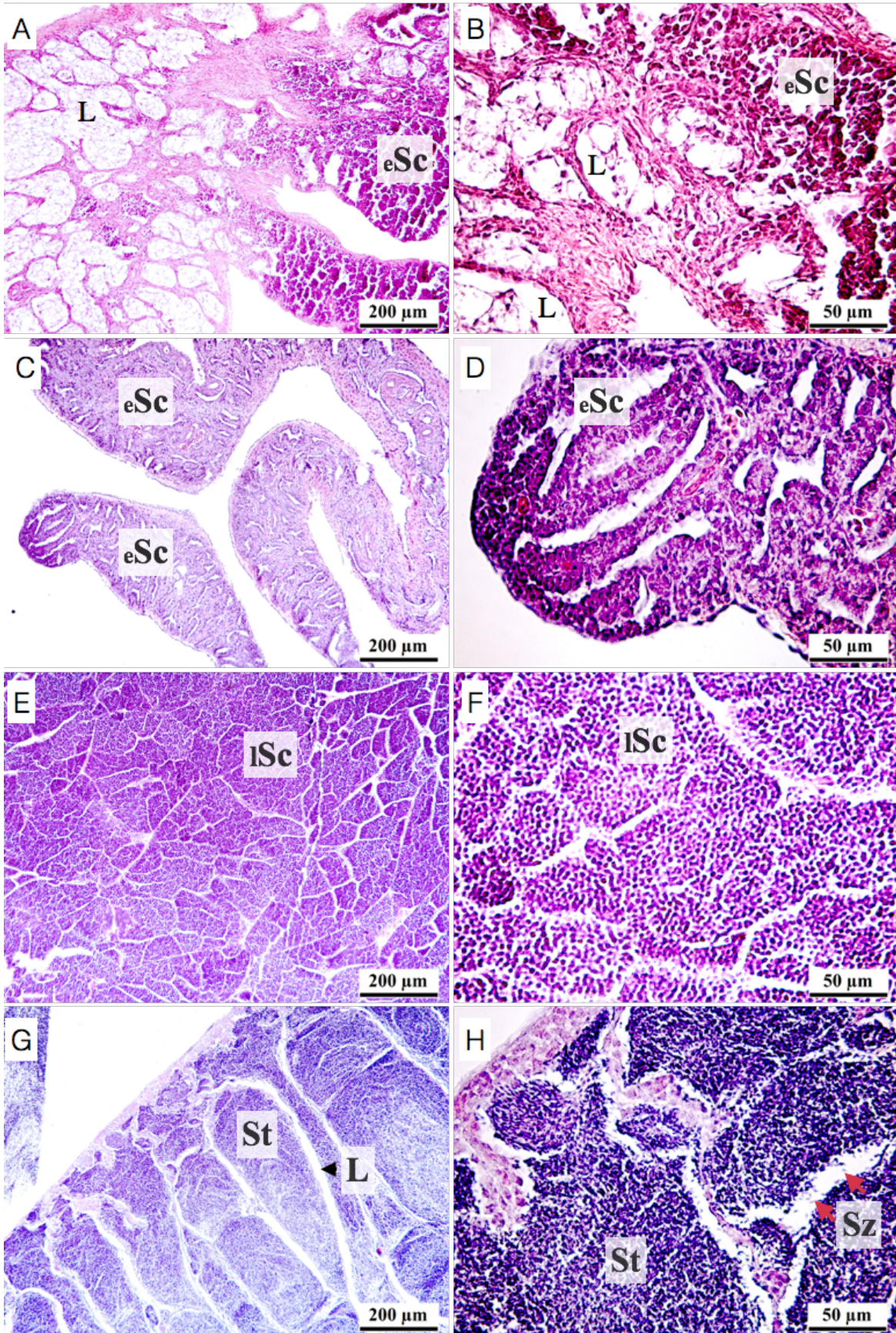
Figure 3. Photomicrographs of ovarian histology, illustrating select stages of oogenesis in polar cod: (A) Early maturation with only two stages present (PG= primary growth oocytes; CA= cortical alveolar oocyte, N= nucleus, n=nucleoli); (B) Vitellogenesis I (Vtg I) oocytes with inclusion of yolk globules in the outer periphery of the oocyte and granulosa cell layer (g); (C) Vitellogenesis II (Vtg II) oocytes with yolk globules filling cytoplasm, *zona radiata* (Zr) and granulosa cell layer (g) surrounding the oocyte; (D) Atretic oocytes (A) together with PG and Vtg II oocytes in maturing ovary; (E) Postovulatory follicle complex (POF) as evidence of previous spawning in maturing female with Vtg II and PG oocytes.

Figure 4. Photomicrographs of testicular development in polar cod: (A) Spent/early maturing testis with a portion of testis with empty lobules (L) and a portion with early stage spermatocytes (eSc); (B) Higher magnification of spent and eSc in resting male; (C) Early maturing testis full of eSc; (D) Higher magnification of early maturing testis eSc; (E) Maturing males with late spermatocytes (lSc); (F) Higher magnification of maturing males with lSc; (G) Testis ready to spawn with spermatid (St) next to lumen of lobules (L); (H) Testis ready to spawn at higher magnification with mature spermatozoa (Sz) in sperm ducts (red arrows).

Males were separated into four progressive gonadal maturity stages based on Núñez and Duponchelle (2009) from early maturing, to maturing, spawning, and spent stages (Figure 4). This was determined by presence of early stage spermatocytes in early maturing males and later stage (smaller) spermatocytes with more basophilic staining in maturing males. Spawning males had spermatid, spermatozoa in the lumen of lobules (L), and milt ran when pressure was applied to the abdomen. Spent males had empty lobules while spent/ early maturing males were clearly spent but early stage spermatocytes were also prominent. In December and January, males were divided by maturity status with immature fish, identified by a low GSI and only early stage spermatocytes, and resting males, with partially spent partially with early stage spermatocytes in testis and a low GSI.

5. *Steroid hormone analysis*

Sex steroid hormone qualification was done using radioimmunoassay (RIA) on blood plasma (Schulz, 1985). Permission to carry out work with radioactive materials was given by the Norwegian Radioactive Regulatory body (Strålevern, project number 2014-13). Ether extraction of sex steroid hormones was performed on thawed plasma samples (50-300μL) using borosilicate tubes. Plasma was thoroughly mixed with 4mL of diethyl ether before removing the inorganic phase by freezing the water phase in liquid nitrogen and evaporating the ether in a warm water bath (45°C) topped off with nitrogen gas. Hormones were re-suspended in a RIA-Buffer pH 7.0 (73 mM phosphate pH 7 with 0.9% NaCl, 0.01% gelatin and 0.05% sodium azide) with a 3X dilution of the original plasma volume. Varying volumes of blood collected from fish and the small size of the fish limited the number of hormones that could be tested. We therefore prioritized estradiol-17β (E_2, females only), 11-ketotestosterone (11KT, males only), and testosterone (T, both sexes), respectively.

The RIA allows for precise quantification of hormone levels using the competition for a limited number of antibody binding sites between hormones from the blood plasma and a known concentration of radiolabeled hormones (Schultz 1985). After overnight incubation of the sample with radioactive hormones and antiserum, hormones that are not bound to antibodies are removed and the level of radiation emitted from the sample is inversely proportional to level of hormones in the plasma. The tritium (^{3}[H]) tracer was composed of isolated radiolabeled hormones diluted in ethanol and then further diluted in RIA buffer to 10 000 counts per minute (E$_2$-GE Healthcare UK; 11KT-Amersham Biosciences, UK; T-Perkin Elmer). A standard curve of nine serial dilutions using commercially available sex steroid hormones (Sigma Aldrich, Saint Louis, US) and internal standards of total binding and nonspecific binding were run in parallel with every assay. A plasma pool composed of mixed male (n=43) and female (n=35) wild polar cod from the Barents Sea was used as an internal control. 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). All antiserums were raised in New Zealand White rabbits. Overview of assays run and coefficients of variance (CV) are given in Table 1.

In a borosilicate glass incubation tube, a dilution of extracted plasma and buffer equal to 100 μL was mixed with 50 μL^3[H] tracer solution and 200 μL antiserum solution and incubated overnight. The following day, 300 μL of chilled dextran coated coal solution (1 g coal and 0.1 dextran T70 (Amersham Biosciences)) dissolved in 100 mL cold RIA buffer) was added and allowed to incubate for five minutes to allow binding of all steroid hormones unbound to antibodies before separation by centrifugation (Hettich Rotanta 460R, UK) at 4600 rpm for 5 minutes at 4°C. The supernatant, containing steroid hormones bound to antibodies, was decanted into an antistatic scintillation tube (Perkin Elmer) and 7 mL of Ultima Gold XR ® scintillation fluid was added. Tubes were capped and shaken thoroughly for 2 minutes. Samples were counted for 5 minutes in a liquid scintillation analyzer (Perkin Elmer Tri-carb® 2900 TR) to measure beta radiation. Each assay was compared against the standard curve run in parallel and each sample was run in duplicate. The number of assays ran per steroid and the dilution factor for each steroid

tracer and antiserum varied (Table 1). Values that fell below the level of detection were assigned half the level of detection for calculations.

Table 1. The dilutions of antiserum and tritium tracer followed number of assays ran and the CV for each sex steroid hormone used in RIA. E_2 = estradiol-17β (females only); 11KT=11-ketotestosterone (males only); and T= testosterone (both sexes).

Steroid	Assays run	Samples run	Coefficient of Variance (CV)	Detection limit*
E_2	5	39	9.7%	0.66
11KT	9	152	15.5%	0.82
T	10	116	32.0%	0.72

*ng sex steroid hormone mL^{-1} plasma

6. Sperm quality

Two parameters of sperm quality, sperm motility and sperm density, were examined following the protocol set by Rudolfsen et al. (2005). Briefly, sperm motility analysis were conducted using an aliquot (<0.12 μL) of undiluted milt placed on a 4°C 20 μm standard count slide (Leja, Art. No. SC 20-01-C, The Netherlands) and sperm motility was induced in a one-step procedure 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) (X10 objective) was used to record sperm activity from each male (n=21). Sperm swimming activity was recorded for a 90 second period with two replicate trials for each male and stored on MiniDV tapes. Computer-assisted sperm analysis (CASA) is an objective tool for examining sperm motility in fish (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^{-1}), which is the velocity of the sperm head along its spatial average trajectory, (2) straight-line velocity (VSL, μm s^{-1}), 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^{-1}), which is the velocity of the sperm head along its real curvilinear track, (4) percentage motile sperm cell, and (5) percentage progressive cell

(cell having straightness >80% and VAP >100 μm s^{-1}). To remove the potential effect of drift, cells having VAP<20 μm s^{-1} and velocity straight line <10 μm s^{-1} 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.

Spermatocrit was measured as a proxy for sperm density (Rakitin et al., 1999). Milt was gently pressed from ripe male fish and, using capillary action, sucked up into small open-ended glass hematocrit tubes. One end of the tube was blocked by clay before tubes were spun down in a centrifuge for 5 minutes 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. Averages of the two replicates and standard deviations were calculated for each individual.

7. *Statistical analysis*

After satisfying the assumptions of normal distribution and equal variance, a one-way analysis of variance (ANOVA) was used to test for effects of time, sex, and treatment on the continuous factors of length and somatic weight, SGRs, GSI and HSI, plasma sex steroid levels with a subsequent *post hoc* test on differences between means (Tukey`s honestly significant differences). Variables that violated the assumption of normality were tested using a Kruskal-Wallis ANOVA. Somatic weight was used to quantify growth independent of variations in gonad, liver, and stomach weight. Pearson's correlation test was used to explore the relationship between parameters. Mean values of length and weight in each tank were used to avoid pseudoreplication bias when comparing across treatments (Hurlbert, 1984).

Maturity stage distribution and frequencies of atresia and POFs was analyzed using a chi-squared test (Zar, 1999) with the null hypothesis that treatment groups have the same frequency of different maturity stages, atresia and POFs at a single time. Coefficient of variance (CV) for RIA assays was calculated using the mean standard deviation of pool samples divided by the mean plasma levels of pool samples. A CV≤ 10% is acceptable for inter-assay variation with RIA. A linear mixed effect model was created for each sperm

motility parameter with fixed (treatment) and random effects (tank, time activation and trial). All comparisons were considered significantly different than control (unexposed) at $\alpha= 0.05$ level. However, p-values≤ 0.15 were noted as these low values indicate a substantial response, which may have biological significance (Meador et al., 2006). Values are reported as mean $\pm$ standard deviation (sd). All statistical analyses were conducted with R 3.1.1 (R core team 2014).

Results

1. Experimental set up and Mortality

At the start of the experiment in June, the mean (±SD) 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 nor variation of these variables using the standard deviation (ANOVA, p= 0.73 and 0.16, respectively across tanks) (Figure 5).
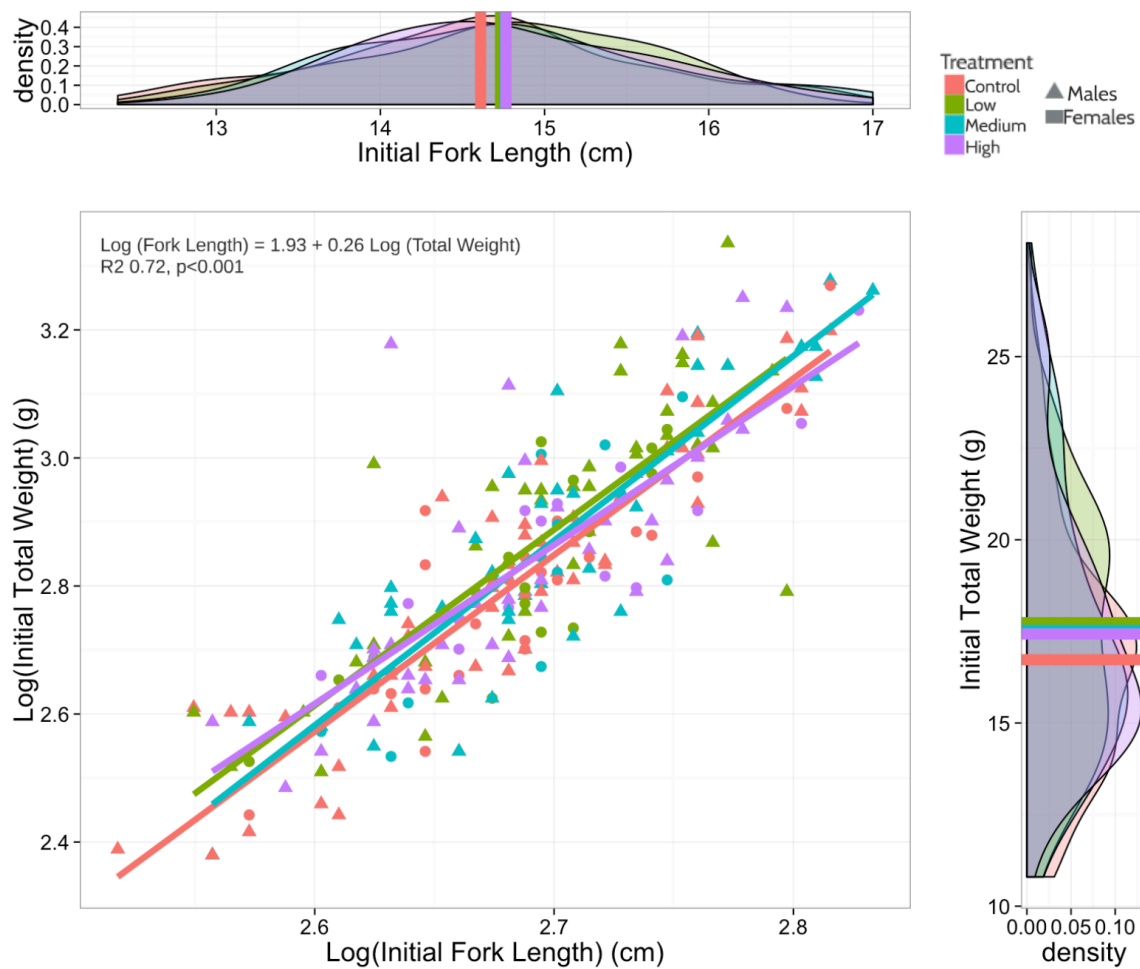

Figure 5. Initial fork length and initial total weight in log scale at the start of the experiment, in June, for females (Δ) and males ( ) divided up by treatment in different colors. Along the (Top) X-axis is a density plot displaying the relative distribution of initial fish fork length over treatment groups with mean fork length denoted by vertical colored line. Along the (Right) Y-axis is a density plot showing the relative distribution of fish initial total weights over treatment with mean weights denoted by horizontal colored lines. Equation for all treatment groups pooled is displayed.

Fish mortality was not significantly influenced by treatment (ANOVA, p=0.945) with a mean frequency of 56.5 ± 2.5% (Table2). The sex ratio was unbalanced overall, with 68 females and 181 males, and also between tanks and treatments (see Figure 3). 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 (0.0, 0.11, 0.57, and 1.14 µg crude oil g fish^{-1} day^{-1} for control, low, medium, and high treatments, respectively). Bacterial skin infections began to appear on fish in late October and became prevalent in all tanks by January (75% of fish). Endo-parasites (e.g. nematodes) were observed in 26% of fish and internal cysts in 12%, independent of treatment.

2. *Dietary PAH Concentration*

Assuming polar cod were feeding proportional to their body weight, the ingested doses corresponded to ca. 12.5, 42.3 and 91.5 ng $\sum$26PAHs g fish^{-1} week^{-1} in the low, medium, and high crude oil treatments, respectively (Table 2). There is a strong positive linear correlation between the amount of crude oil added to food and $\sum$26PAHs levels (R^2= 0.99). $\sum$26PAHs accounted for ~ 1% ww of crude oil in all treatments. The relative PAH composition was similar in all crude oil treatments with alkylated naphthalenes (e.g. C1- to C3- naphthalene) accounting for approximately 80% of the overall PAH load.

Table 2. Summary of crude oil and $\sum$ 26PAH doses in the diet and estimated doses in fish per treatment. Dose of $\sum$ 26PAHs (µg g^{-1}fish per week) was calculated using the 4% bw ration received per week, the measured concentration of PAHs in *Calanus sp.* diet and the mean total weight per treatment.

Treatment	N	Mortality	Crude oil dose µg g^{-1} diet	$\sum$ 26PAHs ng g^{-1} food	$\sum$ 26PAHs ng g^{-1} fish week^{-1}	Mean Total Weight (g) Initial	Mean Total Weight (g) Sampled
Control	76	54.9%	0	141 ± 103	5.6 ± 2.2	16.7 ± 3.3	22.4 ± 8.7
Low	60	55.2%	20	313 ± 88	12.5 ± 4.2	17.7 ± 3.5	26.4 ± 8.9
Medium	56	58.2%	100	1058 ± 237	42.3 ± 12.4	17.6 ± 3.7	25.0 ± 7.3
High	57	57.5%	200	2288 ± 214	91.5 ± 28.1	17.4 ± 3.4	24.3 ± 7.5

Table 3. Mean doses (ng/g food ± SD) of 26 PAHs and Σ26PAHs for each treatment (control, low, medium, and high treatment). Three replicate pellets were analyzed for control and medium treatment while four replicates were analyzed for low and high treatments. Values under limit of detection (LOD) are not included in calculations of Σ26PAHs.

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	313.16 ± 88.02	1058.5 ± 237.35	2288.15 ± 214.41

3. Morphometrics

Total length and somatic weight were significantly correlated for all time points and treatments ($p < 0.001$, R^2=0.79). The mean (± SD) length of fish in February was 15.7 ± 1.2 cm, a steady and significant increase of 1.0 ± 0.8 cm from June (ANOVA, p<0.001)

(Appendix A). The length of fish was not significantly different between sexes overall (ANOVA, p=0.213), therefore sexes were pooled for length analysis. Slightly longer fish were found in exposed treatment tanks than control over all time points but this was not statistically significant (ANOVA, p= 0.098) and no difference in mean standard deviation of length between treatments was found. The mean length specific growth rate (SGR) calculated from fish followed through the growth checks from June to January ranged between 0.037- 0.061 % length change day^{-1} at the highest between 29th of Nov. - 9th of Jan. and the slowest between 10th of Sept. - 28th of Oct (Table 4). Fish in the low treatment had a significantly higher length SGR in the period of 28th of Oct – 29th of Nov. compared to control (ANOVA, p=0.002).

The somatic weight of fish was not significantly different between sexes (ANOVA, p=0.196), therefore sexes were pooled for weight analysis. The mean somatic weight (± SD) in June was 14.1 ± 2.7 g, by February the mean had increased to 22.4 ± 5.5 g, a mean increase of 13.6 ± 5.9g (Kruskal-Wallis, p <0.001) (Figure 6). In December, somatic weight of immature fish (n=7) was 30.5 ± 7.4 % lower than in maturing fish; resting fish (n=6) followed a similar trend with 27.9 ± 9.3 % lower somatic weight compared to maturing fish in February (ANOVAs, p<0.001). Somatic weight did not differ significantly between treatment groups at any time point in maturing fish. Mean total weight SGR (% body weight change day^{-1}) calculated for fish followed from June to January in the growth checks was at the lowest in the period between 29th November – 9th of January (0.15 % bw change day^{-1}) and was highest in the period from 10th of September – 28th of October (0.47   % bw change day^{-1}) (Table 4). In the period from 29th of November – 9th of January fish in the medium treatment had significantly higher total weight SGR compared to control fish (ANOVA, p<0.001).

Results

Table 4. Summary statistics for fish fork length, total weight and specific growth rates for polar cod exposed to dietary crude oil followed from 5-6th of June to 9th of January during growth checks. Mean values ± standard deviation were calculated for each treatment at each checkpoint. Fork length was used to calculate % length change and total weight was used for % body weight (bw) change rates for the period indicated. The same fish were measured at each time point and the sample size for each treatment at every time point was control=15, low=20, medium=18, high=19. Significant differences from control treatment are indicated in bold (Tukey *post hoc* test, $p<0.05$).

| Date	Treatment	Fork Length (cm)	Total weight (g)		
5-6. June	Control	14.5 ± 0.69	17.1 ± 3.39		
	Low	14.5 ± 0.89	17.3 ± 3.47	Specific Growth Rate	
	Medium	14.8 ± 0.99	17.3 ± 3.45	% FL change	% bw change
	High	15.0 ± 1.14	18.2 ± 4.31	Day^{-1}	Day^{-1}
10. September				5-6th of June – 10th September	
	Control	15.0 ± 0.82	20.7 ± 5.43	0.04 ± 0.03	0.19 ± 0.11
	Low	14.4 ± 3.50	21.1 ± 5.30	0.05 ± 0.02	0.20 ± 0.10
	Medium	15.3 ± 0.94	20.5 ± 4.46	0.04 ± 0.02	0.18 ± 0.09
	High	15.6 ± 1.20	21.7 ± 5.29	0.04 ± 0.02	0.18 ± 0.09
28. October				10th September – 28th October	
	Control	15.5 ± 1.06	26.2 ± 6.98	0.06 ± 0.06	0.49 ± 0.08
	Low	15.4 ± 1.05	26.3 ± 6.76	0.03 ± 0.06	0.46 ± 0.18
	Medium	15.5 ± 0.94	26.7 ± 5.48	0.03 ± 0.02	0.55 ± 0.23
	High	15.8 ± 1.19	26.7 ± 7.31	0.03 ± 0.04	0.42 ± 0.27
29. November				28th October – 29th November	
	Control	15.6 ± 1.01	31.1 ± 9.56	0.02 ± 0.05	0.51 ± 0.16
	Low	15.9 ± 1.14	30.9 ± 7.52	**0.092 ± 0.11**	0.51 ± 0.32
	Medium	15.7 ± 0.96	30.0 ± 6.92	0.04 ± 0.03	0.35 ± 0.34
	High	15.9 ± 1.33	31.2 ± 9.03	0.01 ± 0.05	0.48 ± 0.40
9. January				29th November – 9th January	
	Control	16.0 ± 1.08	31.6 ± 9.66	0.07 ± 0.04	0.03 ± 0.21
	Low	16.1 ± 1.16	34.2 ± 7.87	0.04 ± 0.09	**0.25 ± 0.23**
	Medium	16.1 ± 1.12	32.1 ± 8.06	0.06 ± 0.08	0.15 ± 0.23
	High	16.4 ± 1.34	33.4 ± 10.08	0.08 ± 0.02	0.15 ± 0.22
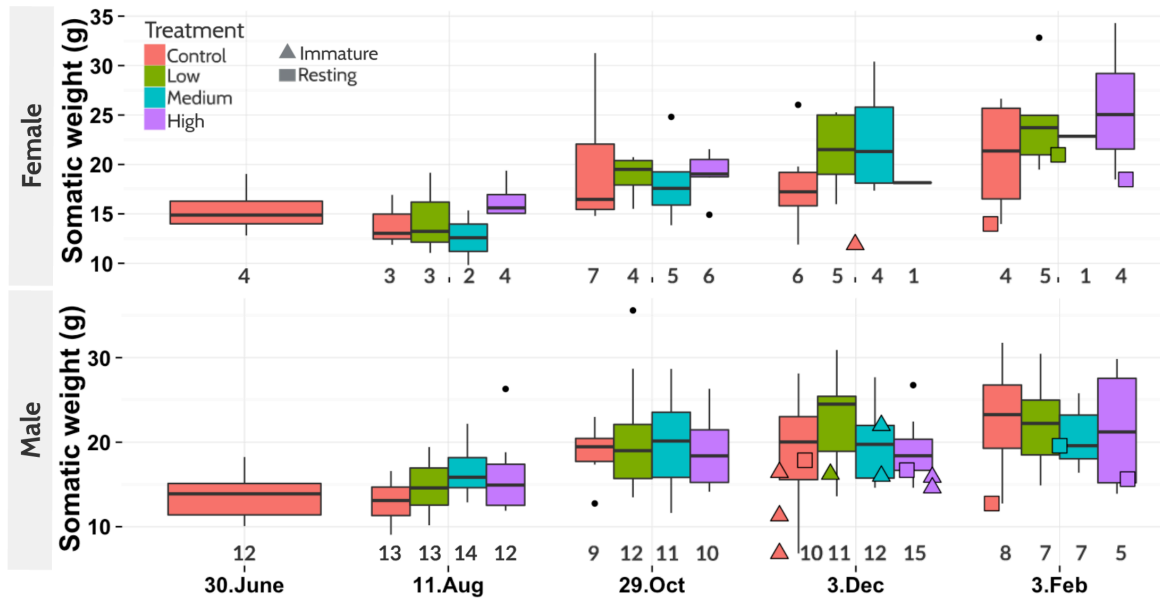
Figure 6. Somatic weight (g) records of polar cod females (top) and males (bottom) sampled over the period from the 30th of June to 3rd of February. Treatments are represented by different color boxplots and the corresponding n for each given treatment is denoted underneath. Plots represent the median (line), 25%-75% percentiles (box), non-outlier range (whisker), and outliers (dots). Immature fish (triangles) and resting fish (squares) are noted in the respective time points sampled and color fill indicates treatment.

Somatic Indices

The GSI was significantly different for sexes overall and increased significantly over time (ANOVA, p =0.004 and <0.001, respectively) (Figure 7). The GSI in males was 0.9 ± 0.2% in June, began to increase in October, and continued steadily increasing until February (20.1 ± 8.6%). Females started with a GSI of 2.2 ± 0.3% that remained low until February when GSI increased to 12.8 ± 6.5%. In December, 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 was the immature and resting fish, whose GSI remained low regardless of sex and time (2.0 ± 2.3% GSI) (ANOVA, p<0.001). In December and February, the GSI for mature, immature, and resting fish was treated separately. The maximum GSI was observed in February with a male reaching 34.7% and a female reaching 20.9%. No difference in female GSI was found between treatments. In October, male GSI in medium treatment was significantly higher than GSI in the high crude oil treatment (ANOVA, p=0.0296).
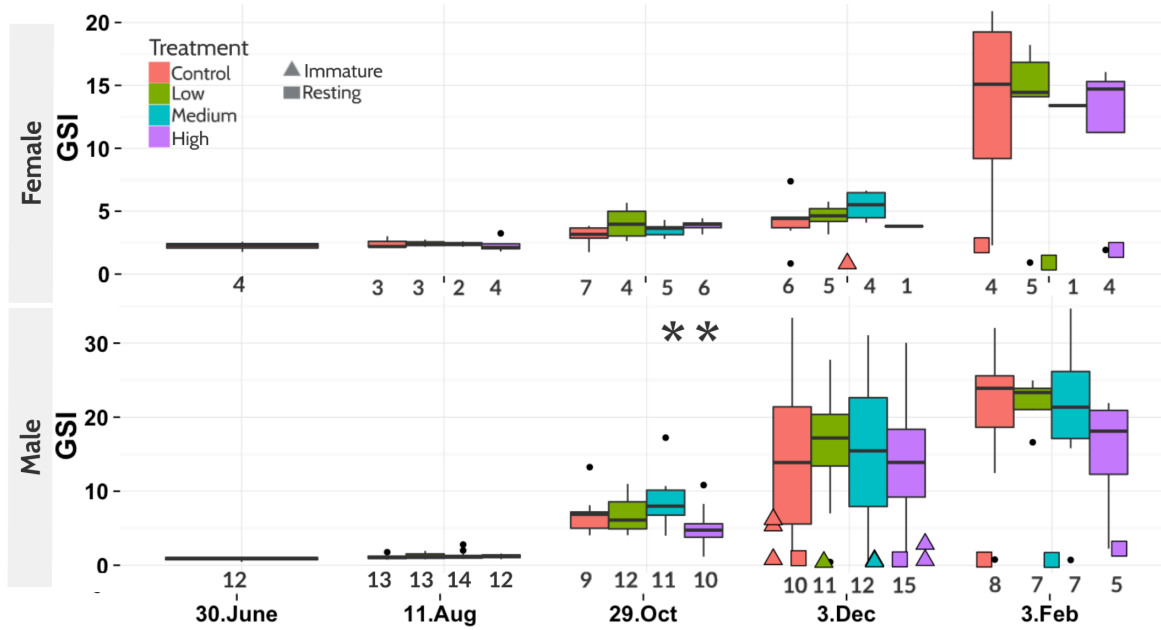
Figure 7. The gonadosomatic index (GSI) of polar cod females (top) and males (bottom) sampled over the period from the 30th of June to 3rd of February. Treatments are represented by different color boxplots and the corresponding n for each given treatment is denoted underneath. Plots represent the median (line), 25%-75% percentiles (box), non-outlier range (whisker), and outliers (dots). Immature fish (triangles) and resting fish (squares) are noted in the respective time points sampled and color fill indicates treatment. Significant differences between treatment groups are noted with an asterisk (*) above each group (α = 0.05).

The HSI was significantly different between sexes (ANOVA, p=0.015) and increased significantly over time with higher values in females compared to males (ANOVA, p<0.001) (Figure 8). Indeed, the HSI in males increased from 2.6 ± 0.7% in June to 11.2 ± 2.7% in December and remained stable until February (11.1 ± 3.7%). Similarly, females started with a HSI of 2.5 ± 0.9% that steadily increased until February with a final mean of 13.3 ± 2.2%. Immature fish and resting fish had similar HSI values to mature fish. GSI and HSI in mature fish was significantly and positively correlated (Pearsons coeff. r= 0.65, p< 0.001). HSI did not show significant differences between treatment at any time point and for neither females nor males, with an exception of males in October showing significantly higher HSI (38.2 ± 9.9%) in control compared to the high treatment group (ANOVA, p=0.04). Males and females had a similar condition factors with no effect of treatment (Appendix).
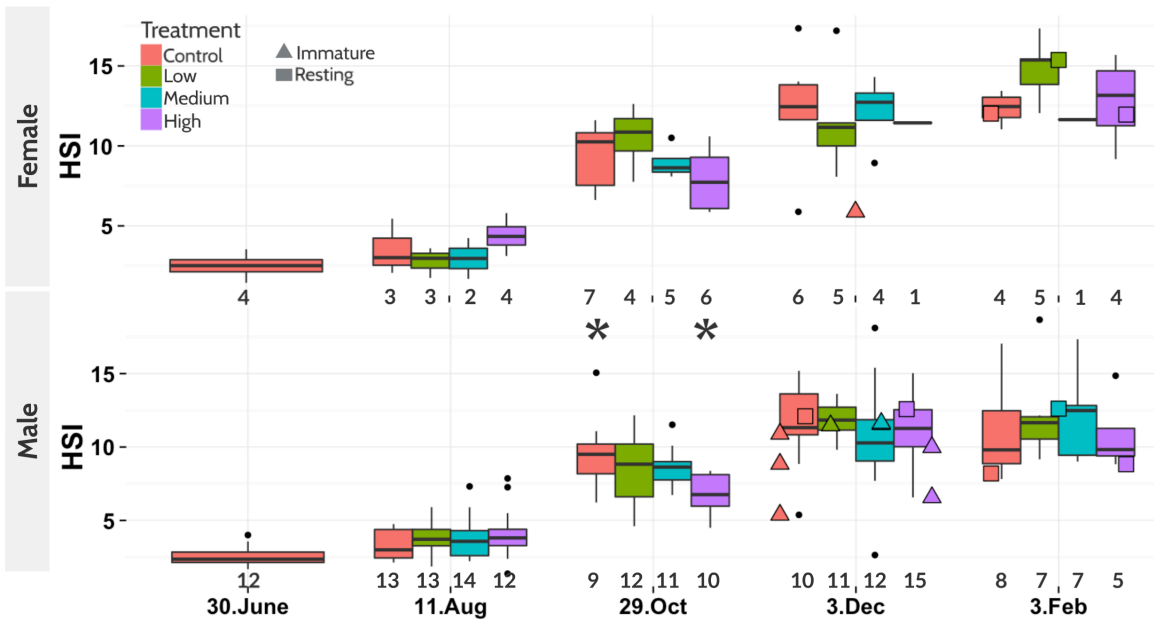
Figure 8. The hepatosomatic index (HSI) for female (top panel) and male (bottom panel) polar cod over the experimental exposure period from the 30th of June to the 3rd of February. Treatments are divided up by color boxplots and the corresponding n for each given treatment is denoted underneath. Plots represent the median (line), 25%-75% percentiles (box), non-outlier range (whisker), and outliers (dots). Immature fish (triangles) and resting fish (squares) are noted in the respective time points sampled and color fill indicates treatment. Significant differences between treatment groups are noted with an asterisk (*) above each group ($\alpha = 0.05$).

4. Histology

Oogenesis in female polar cod

From June to January, female fish underwent oogenesis from primary growth (PG) oocytes through cortical alveoli (CA) and vitellogenesis (Vtg I and II) (n= 68) (Figure 9). Progression through these stages was not uniform and females in as many as four different gonadal maturity stages were sampled at a single time point. Ovaries of individual females also had a mixed composition of oocyte stages with PG oocytes present together with all other oocyte maturity stages (see Material and Methods, Figure 3). In July, females were in the first maturing stage of oogenesis, CA, noted by the presence of empty cortical alveoli vesicles around the internal periphery of the oocyte. The majority of females remained in CA stage until December when advancement to Vtg I and Vtg II occurred; however, 22% of females entered Vtg I in October. In February, 64.2 % of females were

in Vtg II stage and 37.6 % were resting. GSI of females in Vtg II increased 2.6 fold from December to February (ANOVA, p<0.001). Females in the experiment never reached the final stage of vitellogenesis, oocyte hydration, nor ovulation. A singular immature female was sampled in December with a GSI of 0.84 % and only PG oocytes present. Atretic oocytes were observed in 20.6 % of females throughout the experiment but not in February. Females with atresia had 50.8 ± 22.9% lower GSI and tended to have lower somatic weight and HSI values (ANOVA, p=0.033, 0.073, respectively) with no regard to treatment. Post-ovulatory follicles (POFs) were found in 23.5% of females distributed over all time points and all treatments. There were no significant trends to explain the presence of POFs in females. GSI values supported the histological assessment of the gonadal maturity stages of females with increasing GSI for progressive stage of oogenesis.

By October, fewer of the females exposed to crude oil had initiated vitellogenesis (16%, 25%, 0% in low (n=4), medium (n=5), and high (n=6) treatments, respectively) where as 43% of control females (n=7) had reached Vtg I. However, this trend was not significant (chi-square test, p= 0.124) and was not observed at later time points. The number of females with atresia was highest in October (n=8). Furthermore, frequency of occurrence of atresia was higher in exposed treatments (1/8 in control, 2/4 in low, 3/5 in medium, and 2/6 in high treatments) but this was not significant (chi-square test, p= 0.69). In February, exposed females in Vtg II tended to have lower GSI than control (17.0 ± 4.9, 15.9 ± 2.0, 13.4, 15.2 ± 0.9 % GSI for control (n=4), low (n=5), medium (n=1), and high treatments (n=4), respectively) although this trend was not statistically significant.

Spermatogenesis in male polar cod

In July and August, sampled males were either spent with empty lobules or with early stage spermatocytes (Figure 10). By October, males were unanimously (with the exception of one fish) in a maturing stage with late stage spermatocytes. In December, the majority of males were in the maturing stage but 17% (n=48 total) were found in immature with testis in early maturing stage, while the remaining 4% were resting with spent testis from spawning the previous season. In February, 75% (n=27) of maturing males could be stripped for milt and spermatozoa in the sperm ducts was observed in the histological

preparations of actively spawning males. The proportion of males actively spawning did not differ between treatments.
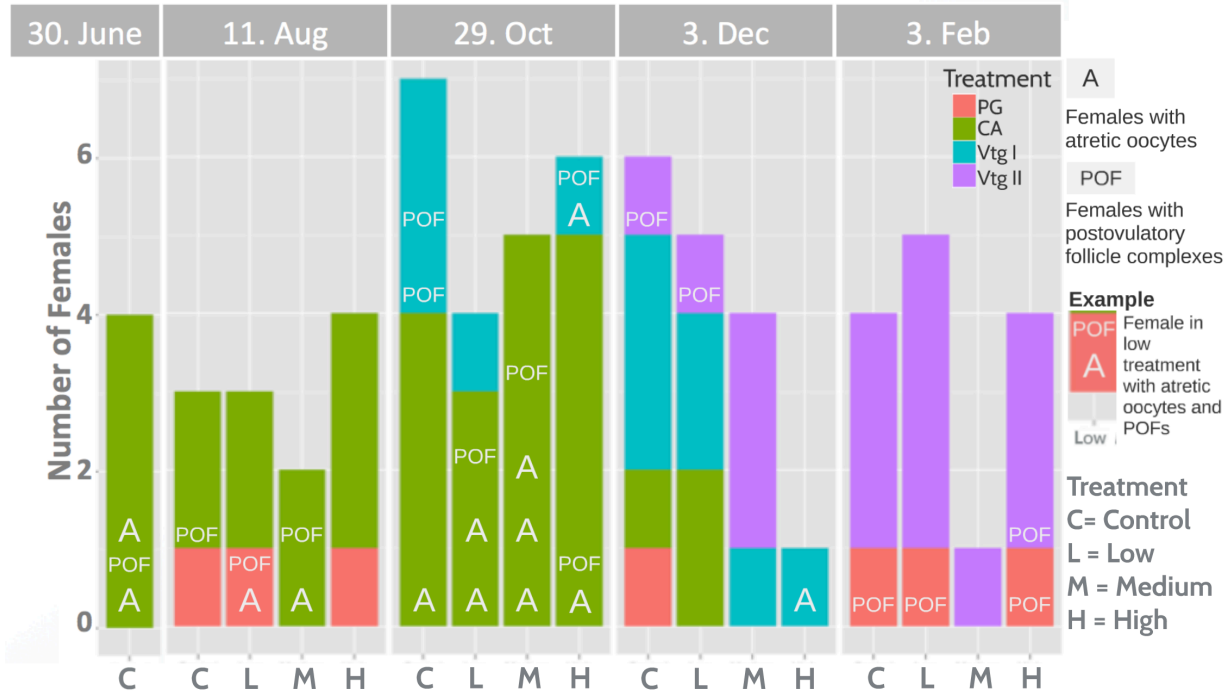

Figure 9. Number of female polar cod in each treatment group for each time point over the course of the 31-week exposure period from the 30th of June to the 3rd of February. Females in each gonadal maturity stage determined by histological analysis of ovarian slices are differentiated by color. Presence of POFs and atretic oocytes are noted inside the bar for each female. Four females had both atretic oocytes and POFs. A single immature females is represented as the pink block (PG) in December and three resting females in PG stage with POFs are found in February

GSI strongly supported the gonadal maturity stages as spent, immature and resting males had low GSI (ca. 1-2%) while maturing (12.8 %) and spawning (22.9%) males had a higher mean GSI (ANOVA, $p<0.001$). Immature males (n=9) were sampled in December with early stage spermatocytes in testis and a low GSI (2.19 ± 2.35%). Resting males (n=5) were sampled in December and February with partially spent testis and low GSI (1.09 ± 0.65%).
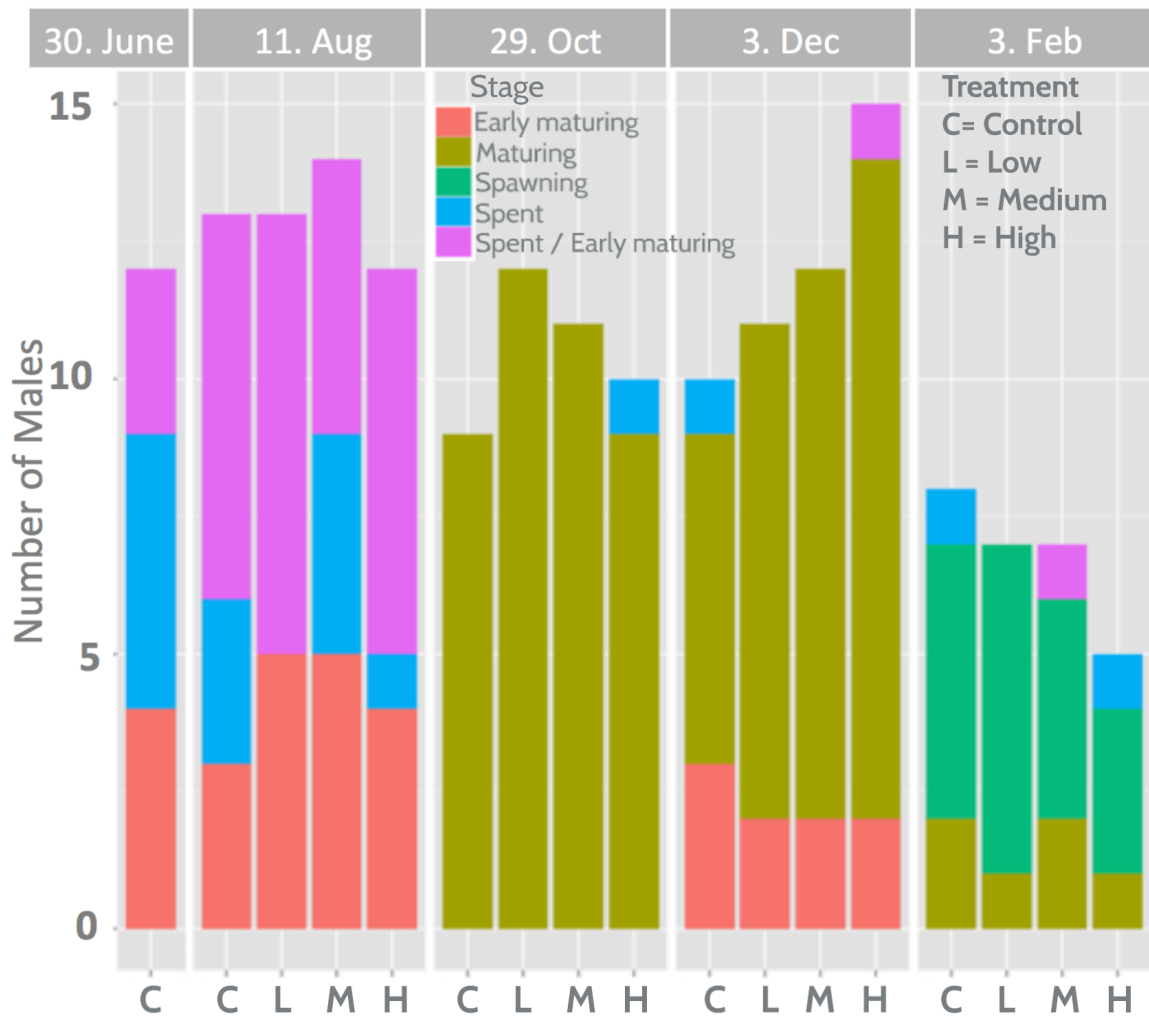
Figure 10. Number of male polar cod sampled from each treatment at every time point during the 31-week dietary crude oil exposure from the 30th of June to the 3rd of February. Gonadal maturity stage as determined by histological analysis of testicular section is differentiated by color. Immature fish are found in early maturing stage (orange) in December only and resting males (blue) are in spent or spent/ early maturing stage (purple) in December and February.

The somatic weight of immature males (i.e. early maturing) and resting males (i.e. spent and spent/early maturing) in December and February was lower than that of maturing and spawning individuals. Males in early maturing stage were sampled in June, August, and December with only males sampled in December identified as immature. Exposed immature males in low (15.1 ± 1.8g) medium (18.1 ± 2.2g) and high treatments (15.3 ± 1.2g) in December had higher somatic weight than control early maturing stage males (11.8 ± 1.6g) but sample size did not allow further statistical analysis (Figure 11A). In December, maturing males in the medium (19.37 ± 4.2) and high (19.72 ± 3.0)

treatments had significantly lower somatic weights than mature males in low treatment (24.24 ± 4.06) (Tukey *post hoc*, p=0.04 and 0.05, respectively) (Figure 11B). Spawning males in the all exposure treatments tended to weigh less (22.0 ± 5.9, 20.5 ± 3.7, 23.5 ± 8.5g in low (6), medium (4) and high (3) treatments, respectively) compared to spawning control males (5) (somatic weight 25.2 ± 5.6g), however variation in this parameter was high (Figure 11C).
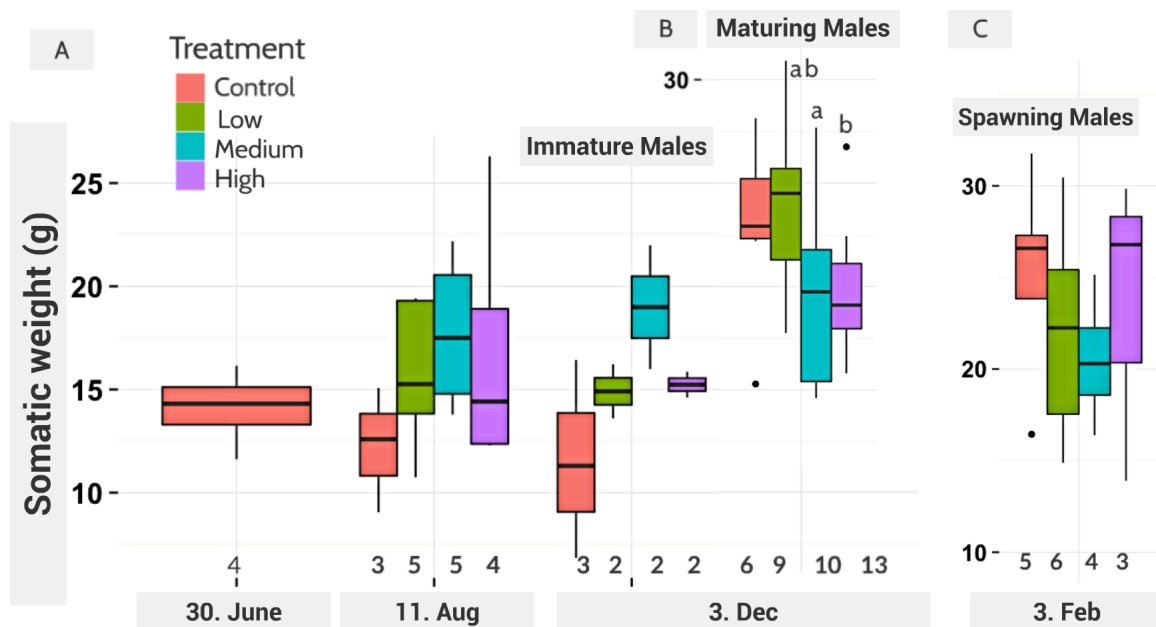

Figure 11. (A) The somatic weight (g) of male polar cod with early maturing stage testis displayed by colored boxplots for each treatment at different time points in the experiment. In December males with early maturing testis were classified as immature; (B) The somatic weight (g) of maturing male polar cod on the 3rd of December with colored boxplots for each treatment. Letters above box plots represent significant differences between treatment groups (α = 0.05); (C) The somatic weight of (g) of spawning males on the 3rd of February with colored boxplots for each treatment. Plots represent the median (line), 25%-75% percentiles (box), non-outlier range (whisker), and outliers (dots). Number of males in each category is recorded below each boxplot. Note the difference in scale between the plots A and C.

5. *Plasma Steroid Hormone Concentrations*

Estradiol-17β

In August, plasma levels of E$_2$ were low in the single female fish measured (1.37 ng mL^{-1}). A significant increase occurred over time and maximum E$_2$ levels were reached in February (8.32 ± 6.43 ng mL^{-1}, ANOVA, p=0.009)(n=39)(Figure 12A). The immature and

resting females had E_2 levels around the level of detection (LOD). Levels of E_2 significantly increased with progressive gonadal maturity stages, exhibited by a 8-fold increase between the earliest (PG) and latest stage observed (Vtg II) (ANOVA, p= 0.002). A pairwise comparison revealed that females in PG and CA stages had significantly lower E_2 levels than Vtg II fish (p=0.016 and 0.004, respectively). The female with the highest E_2 level (25 ng mL^{-1}) exhibited the most advanced oocyte maturity stage (late Vtg II). The temporal changes in E_2 were significantly and positively correlated with the changes in GSI, HSI, and somatic weight (pearson coeff. r= 0.59, 0.46, and 0.31, respectively, ANOVAs, p<0.001). Estradiol levels were not significantly different among treatments at any time point or inside a singular gonadal maturity stage although great variation was found in Vtg II females.

Testosterone

Plasma levels of T in female fish were low in June and August (2.09 ± 0.79 ng mL^{-1}) (n=26) (Figure 12B). T levels remained low until February when a sharp 7-fold increase occurred in maturing females (9.93 ± 10.05 ng mL^{-1}) corresponding to the entry into Vtg II gonadal maturity stage from proceeding maturity stages. The temporal changes in T were significantly correlated with the increase in GSI (pearson coeff. r= 0.59, ANOVA, p<0.001) but not HSI or somatic weight. Immature females had T levels close to the detection level (0.43 ng mL^{-1}). T levels in females were not significantly different over treatment with regard to time or gonadal maturity stage.

Males had greater levels of T at all time points compared to females with the rise towards final maturation occurring earlier (ANOVA, p= 0.807). In maturing males, T plasma levels were low (0.91± 0.33 ng mL^{1}) in June and rose steadily to 6.23± 4.01 ng mL^{-1} in December and further to 12.79 ± 9.89 ng mL^{-1} in February (n=90) (Figure 13A). T levels were significantly correlated with time, GSI, and maturity stage (ANOVAs, p <0.001). A 2.5-fold increase in T levels occurred between early maturing and maturing males and an addition 2.5-fold increase was seen between maturing and spawning males. Immature male fish had T levels around 2.25 ng mL^{-1}. Plasma T levels in males were not

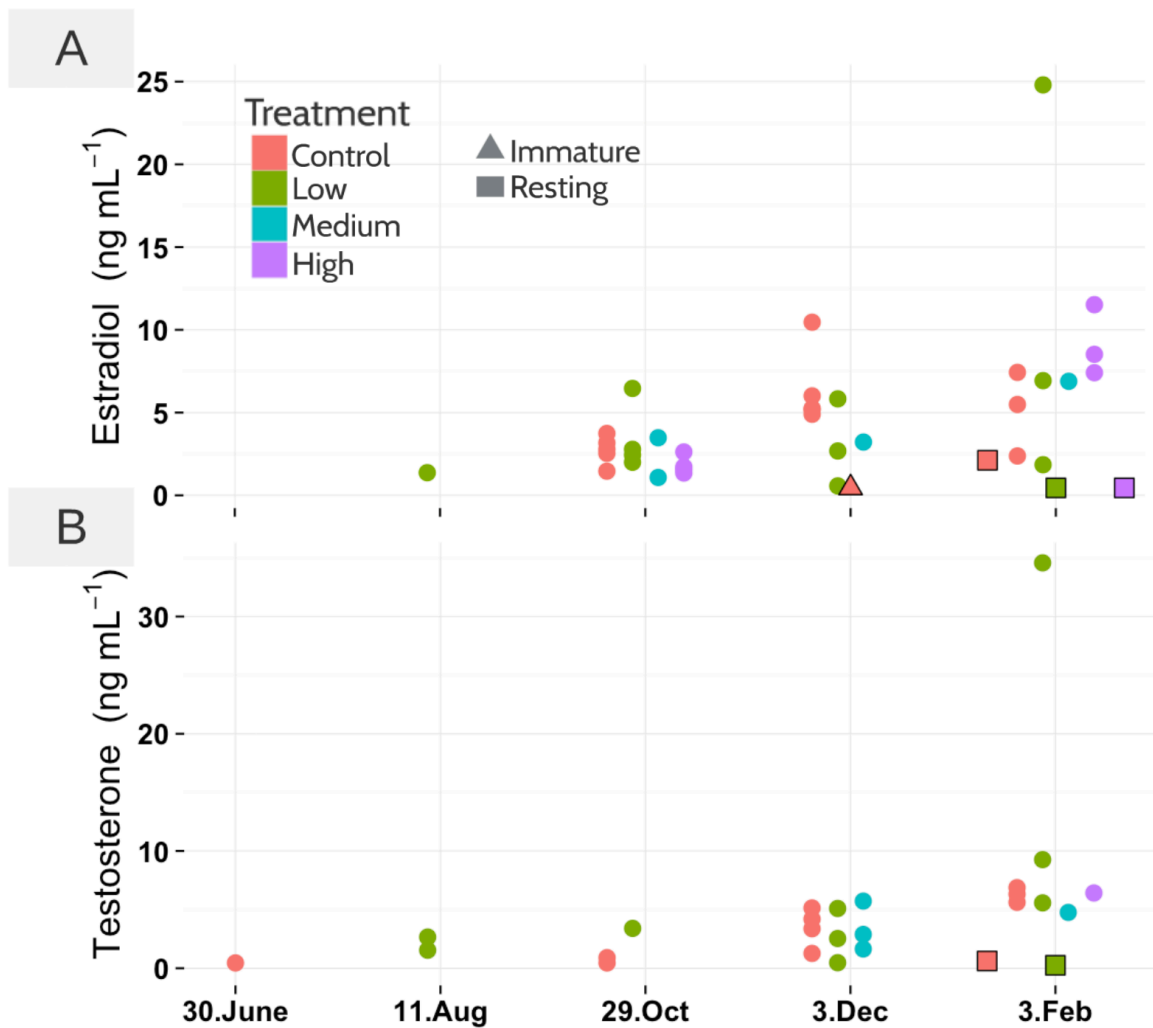significantly different over treatment at any time.

Figure 12. Plasma profiles of (A) Estradiol and (B) Testosterone in females. Treatments are displayed by color and immature fish (Δ) and resting fish ( ) are noted for each time points and filled with the appropriate treatment color. Note different scales in Y-axis for the two hormones.

11-ketotestosterone

Plasma levels of 11KT in males rose steadily throughout the exposure period with low values in July (0.49 ± 0.14 ng mL^{-1}) and maximum mean value in February (3.40 ± 1.84 ng mL^{-1}) (n=116) (Figure 13B). Immature males and those in early maturing and spent stages had low 11KT levels (< 1.0 ng mL^{-1}). Increasing plasma 11KT levels were significantly correlated with increasing GSI, time, and advancing maturity stage (Pearson coeff. r=0.59 and 0.57 for GSI and time, respectively, and ANOVA, for maturity stage all

with p<0.001). There was a 2.8-fold increase in 11KT levels between early maturing and maturing males followed by a 1.2-fold increase from maturing to spawning males. In February, maturing males in the medium treatment had 11KT levels 55% lower than the control group. T levels were on average higher than 11KT values throughout the experiment and the difference increased with time (0.40 ± 0.3 ng mL^{-1} difference in June and 9.04 ± 8.3 ng mL^{-1} in February). Plasma levels of 11KT in immature fish were above detection limits in control males only (n=3, LOD=0.82 ng mL^{-1}). No other treatment effects were seen at any time.
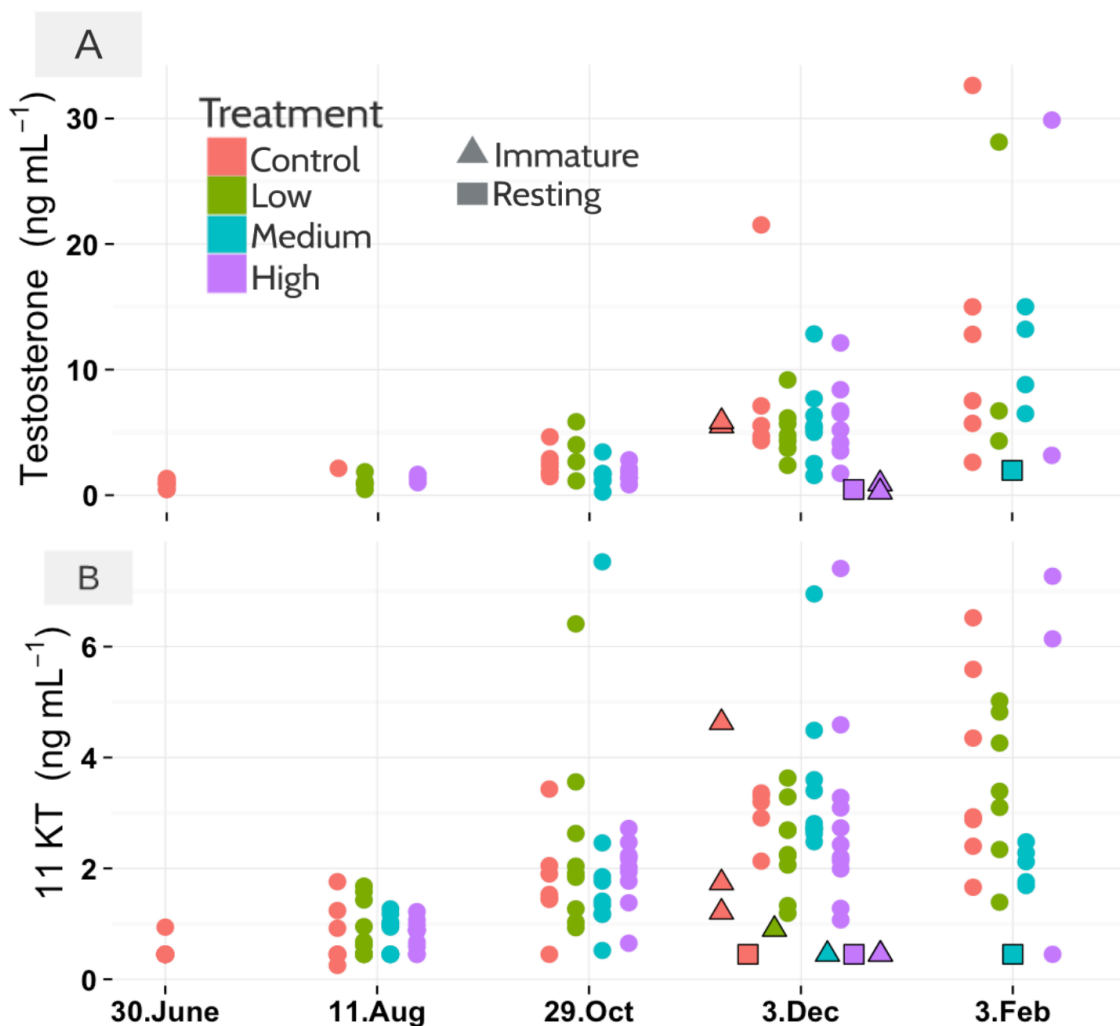

Figure 13. Plasma profiles of (A) Testosterone and (B) 11-ketotestosterone (11KT) in males. Treatments are displayed by color and immature fish (Δ) and resting fish ( ) are noted for each time points and filled with the appropriate treatment color. Note different scales in Y-axis scales.

6. Sperm Quality

Sperm velocity and motility remained relatively stable over the 90-second observation period. Significant treatment effects were seen in all analyzed parameters of velocity, percentage motile and progressive sperm (Figure 14). Exposed males in all treatments had a lower average path velocity (VAP) and straight-line velocity (VSL) than control males (significant in medium treatment $p=0.001$, and close to significant in the high treatment, $p=0.06$, respectively) (Figure 14A). The curvilinear velocity (VCL) of sperm in medium was reduced compared to control males ($p= 0.0004$) and a decreasing trend was also visible in the high treatment. The percentage of motile sperm was over 80% in all exposed males with low ($89.3 \pm 2.3\%$) and high ($87.7 \pm 2.6\%$) treatments showing significance differences from control ($79.7 \pm 4.2\%$) ($p=0.0001$ and 0.0028, respectively) (Figure 14B). Contrary to trends observed in the percentage of motile sperm, the percentage of progressive sperm fell by $30 \pm 2.6\%$ in all exposed males compared to control ($20.5 \pm 3.03\%$) and was significant or nearly so in all treatments ($p= 0.0029$, 0.0605, and 0.0433 in low ($11.8 \pm 2.7\%$), medium ($14.8 \pm 2.8\%$), and high treatments ($13.7 \pm 3.1\%$), respectively) (Figure 14C).

Spermatocrit was measured for 16 male fish in February. The mean spermatocrit measurement was 0.97 ± 0.023 and no significant difference was found between treatments (ANOVA, $p= 0.201$).
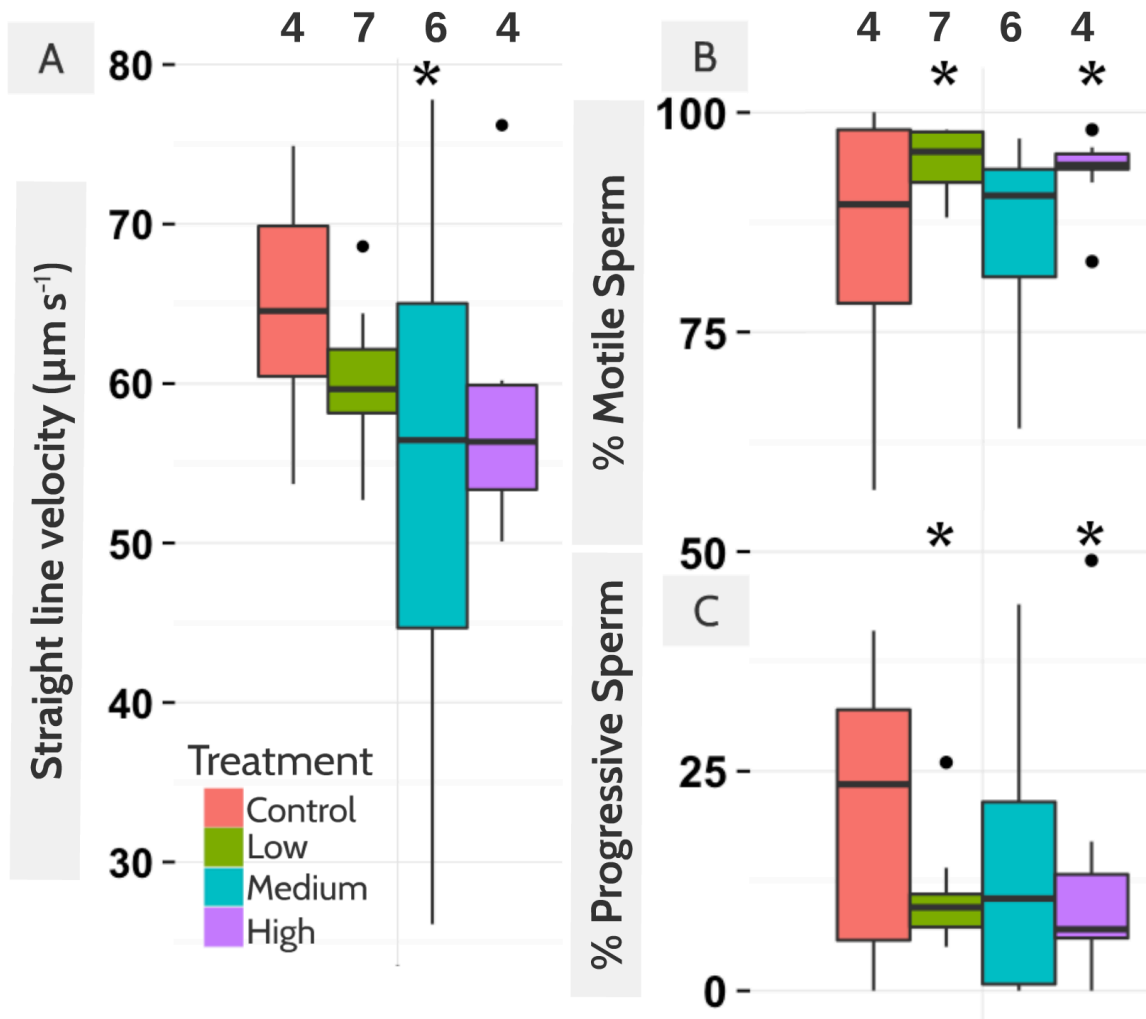
Figure 14. Sperm motility analysis from final sampling on 3rd of February: (A) Straight line velocity (VSL) of individual sperm; (B) % motile sperm; (C)% progressive sperm over different treatments. Asterisks represent a significant difference from control treatment when using a linear mixed effect model with fixed and random factors (α= 0.05).

1. Dietary crude oil doses

In the current study maturing polar cod were exposed for 34 weeks to three different dietary doses of crude oil (0.0, 0.11, 0.57, and 1.14 µg crude oil g fish^{-1} day^{-1}) with PAH loads within environmentally realistic levels. These doses can be considered environmentally realistic in light of measured concentrations 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 6 months after the Prestige fuel oil spill off the NW coast of Spain had concentrations (4.2-152 ng/g Σ14 PAHs) within the range measured in the present study (24.9-152.6 ng/g Σ14 PAHs) (Salas et al., 2006). In a simulation of the recent Deepwater Horizon oil spill in the Gulf of Mexico, copepod assemblages exposed to emulsified crude oil for 16 hours had higher body burden concentrations in the 6 PAHs measured compared to doses from the present study. Only naphthalene (97 ± 5.1 ng/g) and fluorene (14 ± 2.5 ng/g) in the high treatment dose of the present study were within the measured range of exposed copepods (33-152 and 8-42 ng/g for naphthalenes and fluorene, respectively, Almeda et al., 2013). Σ26PAHs concentrations in control (141 ± 103 ng/g) and low dose (313 ± 88 ng/g) of the present study resembled concentrations found in natural copepods assemblages (ΣPAHs = 120-256 ng/g) of the oil shipping port of Valdez, Alaska (Carls et al., 2006).

The experimental design of the present study were realistic in terms of concentrations of PAHs bioaccumulated in *C.finmarchicus* (Jensen et al., 2012). The concentration and composition of PAHs and other petroleum compounds in natural zooplankton exposed to crude oil may however diverge from that of the crude oil itself, due to compound specific biological uptake, bioaccumulation, and metabolism (Almeda et al., 2013). The food used in the present study was mixed with fresh crude oil and all compounds were thus present in their original form and concentrations. Nevertheless, copepods have been observed ingesting spilled oil and with oil droplets adhering externally to their exoskeletons after the Potomac oil spill in NW Greenland (Maurer and Kane, 1979), and these process would introduce the entire crude oil mixture to predators like polar cod.

The crude oil and subsequent PAH doses feed to polar cod in the present study were

ecologically relevant with regard to possible exposure vectors and concentrations in the natural system but below most doses used in other sub-lethal, chronic dietary exposure experiments. Few studies have explored the effects of dietary crude oil exposure on polar cod, nevertheless, the doses used in the present study (12.5-91.5 ng ΣPAHs g^{-1} fish week^{-1}) were 2 orders of magnitude lower than those used in a 4 week exposure of polar cod to North Sea crude oil (4000-9000 ng ΣPAHs g fish^{-1} week^{-1}) (Nahrgang et al., 2010b). Polar cod exposed for 53 days during gonadal development to crude oil (avg. dose 26.8 µg crude oil g^{-1} fish week^{-1}), at 3X the present study's high dose had reduced growth (Christiansen and George, 1995). In studies with juvenile salmon (*Oncorhynchus spp.*), crude oil doses (259 – 24,381 µg crude oil g fish^{-1} week^{-1}) for a 6 week exposure experiment resulted in reduced growth (Wang et al., 1993). An 8 week PAH exposure experiment (4900-154700 ng ΣPAHs g fish^{-1} week^{-1}, Meador et al., 2006) resulted in altered growth and starvation-like physiological responses. Both these studies represent doses beyond the range of doses of the present study. In a two tiered exposure study, polychaetes were placed in creosote contaminated sediments and then fed to English sole (*Parophrys vetulus*) which received an equivalent dose of 840 ng ΣPAHs g^{-1} fish week^{-1} (Rice et al., 2000), a dose nearly one order of magnitude greater than the high dose of the present study (91 ng ΣPAHs g^{-1} fish week^{-1}). Exposed English sole had impaired growth although exposure was limited to 12 days. In a comparable study with regard to the duration and dose used in the present study, rainbow trout were exposed to crude oil (20 µg crude oil in food g fish^{-1} week^{-1}) for 7 months prior to spawning and no effect of treatment was seen on the investaged reproductive parameters (Hodgins et al., 1976).

Although PAH and metabolites have not been measured in tissues and doses were low, we expected that PAHs would be absorbed into the body, distributed internally, and potentially retained over the course of the experiment. Indeed, a recent study by Bakke et al. (2015) found that maturing polar cod, when exposed to a single dose of radioactive-labeled BaP (1.15 ± 0.36 µg g^{-1} fish) or phenanthrene (0.4 ± 0.12 µg g^{-1} fish), absorbed compounds into intestines, liver, and bile within two days following administration.These compounds remained in the fish tissue for over 30 days, thus exhibiting the long term

bioavailability when ingested (Bakke et al., 2015). However, neither BaP nor phenanthrene were detected in the gonads of polar cod at any point through this experiment.

Fish ate throughout the course of the experiment, as food was present in most stomachs of sampled specimens. Reports from the Beaufort sea, Russian waters, and laboratory observations of spawning fish feeding or with full stomachs is in accordance to observations from the present study (Graham and Hop, 1995 and references therein). Therefore, individual exposure to crude oil most likely occurred over the entire experiment. The contaminated food ration given to the fish was set at 4% bw per week (20% bw per week when including uncontaminated food) for the entire experiment from June to February and while this ration may not be reflective of the limited food availability a polar cod most likely would experience in the wild, the aim was to ensure exposure to the dietary crude oil and to avoid starvation associated effects and mortality (Meador et al., 2006). However, male polar cod exposed to crude oil have exhibited preferential selection against crude oil contaminated food prepared with 200 and 400 µg crude oil g^{-1} food depending on appetite and individual preferences (i.e. aggressive males and males on reduced ration were less selective)(Christiansen and George, 1995). Food selection, at least in our higher treatment (200 µg crude oil g^{-1} food) may have resulted in different levels of exposure and may have caused some undetected variation in ingested dose between genders and individuals in the present study.

2. *Seasonal development of the gonads and hormone signaling*

The length of specimens in this study suggested that most fish in this study had reached the age of maturity (2-3 years) reported in literature (Craig et al., 1982) although 7% of fish in this study did not appear to be maturing this season. Using the mean fork length and published length-at-age equations for polar cod from Nahrgang et al. (2014), fish were approximately between 3 and 5 years old depending on their origin (Rijpfjorden, Billefjorden or Kongsfjorden). As fish were collected from these fjord systems and thereafter mixed such that the original experimental group was most certainty a blend of several populations and this may describe some of the variation observed in this study.

Discussion

After one year of captivity under 79°N photoperiod and sea temperature regime for Kongsfjorden Svalbard, reproductive development the experimental fish agreed with observations wild and other laboratory populationsaccepted. Spawning time of polar cod collected from the same cruise and held in the same laboratory conditions was in early March (pers. obs.). Comparison of oocyte maturity stages between this group of polar cod and the experimental group revealed no difference in maturity stagethus spawning of the experimental population would have mostly likely occurred one month after the final sampling (i.e. beginning of March).

The observed increasing trend in male GSI values from August to December after which values stabilized until final sampling in February (spawning) were also observed in captive and wild polar cod from the Canadian Arctic (Lear, 1979; Hop et al., 1995). However, females in the present study only began to build gonad mass from December, which was 2-3 months later than seen in wild polar cod from the Barents Sea (Nahrgang et al., submitted) and captive polar cod from the Canadian Arctic (Hop et al., 1995). Females GSI increased exponentially in December concurrent with the appearance of the first Vtg II oocytes. By early February, all maturing females had reached the middle stage of vitellogenesis (Vtg II). This difference in the onset of main gonad growth between males and females has been seen in wild polar cod populations where males tend to initiate gonadal development earlier in the season than females (Lear, 1979).

The delay in onset of Vtg I in exposed females in October was not statistically significant however, slight changes in the timing of major events in oogenesis may have eventual effects on fecundity or egg size (Kime, 1995; Arukwe and Goksøyr, 2003). Similarly, female polar cod exposed during reproductive development to environmentally realistic concentrations of produced water (PW), a petroleum waste product containing PAHs and alkylphenols, showed a delay in the onset of vitellogenesis (Geraudie et al., 2014).

Atretic oocytes were observed in 20.6 % of females between June and December, with highest occurrence in October within females in the CA stage. This temporal trend in the occurrence of atresia may reflect the period of oogenesis where females fine tune the

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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 temperature may prolong degradation of POFs and have been observed in Atlantic cod up to 1 year post spawning (Skjæraasen et al., 2012).

This is the first temporal study of sex steroid hormone levels in polar cod. The increasing trend over time in all sex steroid hormones measured matched expected profiles of maturing fish with increasing GSI and advancing oogenesis and spermatogenesis. Previous studies quantifying polar cod sex steroid hormones found levels order of magnitude lower than we have measured. 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 immature 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^{-1}) far lower than the present study reported in females at all oocyte stages (0.43- 25 ng mL^{-1} for PG through Vtg II maturity stages, respectively). Male polar cod had higher T levels than 11KT when milt was running, however, the magnitude varied with by 3-fold in the present study and 7.6 fold in Hop et al. (1995). These low levels could be explained by the spawning state of fish, as sex hormone levels drop back to basal levels at or immediately after spawning in Atlantic cod (Norberg et al., 2004) and Arctic Charr (Frantzen et al., 2004). Geraudie et al. (2014) found levels of T in males (0.023 - 0.149 ng mL^{-1}) and E_2 levels in females (0.015-0.187 ng mL^{-1}) with no increasing trend over time. These profiles are comparable with hormone profiles of immature fish in the present study.

There was no significant difference in sex steroid levels between crude oil treatment groups but as ovulation and spawning was never reached, sample size was low, and great variability existed between the few fish, possible effects of treatment may have been hidden. Plasma E_2 levels in wild Dolly varden (*Salvelinus malma*) and Yellowfin sole (*Limanda aspera*) were reduced one year after exposure from the EVOS (Sol et al., 2000). Similarly to the present study, reproductive parameters in wild fish post EVOS showed a

great deal of variability and low significance most likely due to the low exposure concentrations.

3. Investment in growth and gonadal development

Trade offs in energy allocation

A fish has a limited amount of energy for which the basic physiological processes of metabolism, growth, and reproduction compete (Holloway et al., 1990); therefore, an increase in the energetic allocation to additional processes, such as pollutant detoxification, must result in the decrease in energy allocation to other processes, such as maturation. Chronic exposure to crude oil for instance increases energy demands on the fish and energy trade-offs between detoxification and maintenance may reduce energy allocation to growth and reproduction (Rice, 1985).

In the present study, specific growth rates (SGR) in all treatments were similar to those reported for polar cod in the wild and in the laboratory. Total weight SGR reported in this study (0.15 - 0.46% bw day^{-1}) were only slightly higher than that of wild fish for their age (0.15 % bw day^{-1}, calculated from length-at-age relationship in Hop et al., 1997) but reduced compared to polar cod fed peeled deep-sea prawns (*Pandalus borealis*) from the Barents sea (0.7-0.12% bw day^{-1}, Jensen et al., 1991) and polar cod fed maximum rations of *Calanus* spp. (0.6-1% bw day^{-1}, Hop et al., 1997). The highest total weight SGRs between 10th of September and 29th of November were concurrent with an increase in gonadal investment in males. Length SGRs observed in this study (0.03-0.06% length day^{-1}) were below observed rates for polar cod from the Canadian Arctic (ca. 0.1% length day^{-1}) for similar sized fish (14.5-16.0 cm, Hop et al., 1997). While the present study found no effect of treatment on growth, impaired weight gain has been seen in female polar cod exposed to crude oil doses 20-fold higher (avg. dose 26.8 μg crude oil g^{-1} fish week^{-1}) (Christiansen and George, 1995).

Iteroparity and gender differences in polar cod

Discussion

Polar cod is considered an iteroparous species (Graham and Hop, 1995), although the reproductive strategy (iteroparity versus semelparity) has been previously debated (Nikol'skii, 1954). Reproduction in polar cod is an extreme energy investment as 87% of initial energy in liver is 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), however, laboratory studies have reported individuals repeat spawning (Graham and Hop, 1995). In the present study, histological analyses revealed iteroparous female and male individuals. In these individuals, ovaries contained vitellogenic oocytes and POFs and testis contained both spent and early maturing fractions indicating previous spawning and intention to spawn in an upcoming season. These partially spent partially regenerating testes are also found in Atlantic cod (Dahle et al., 2003). Furthermore, mortality in this study was high for mature fish (~ 56%) and was independent of treatment. This mortality could have been due to the reduced body condition of post-spawning specimens as many sampled fish had spent testes or ovaries with POFs. A preliminary analysis of the fish that perished over the course of the experiment confirmed that they had a very low condition factor and low HSI values (A. Laenger, pers.com.). These fish may have fallen below a condition threshold necessary for post-spawning survival as is seen in Atlantic salmon (Jonsson et al., 1990).

Significant gender differences have been reported in the polar cod reproductive strategy, as males are reaching maturity at a younger age, exhibiting higher GSI, and a shorter life expectancy than females (Nahrgang et al., 2014). The present study confirms these previous findings, as GSI was always higher in males compared to females. Compared to the wild source population in Svalbard fjords, GSI values in the present study in February (male 20.09 ± 8.57 %, female 12.76 ± 6.45%) were lower for mature fish of the same size class in January (male 28.2 ± 4.6%, female 20.6 ± 4.6%) (Nahrgang et al., 2014). This difference may be due to a slightly delayed spawning time in the laboratory population. In captivity, ovarian development in females has been observed to start in August (Hop et al., 1995) while we observed ovarian development occurring in October-December when females increased GSI and initiated vitellogenesis.

As the predominate source of energy used for gonadal development comes from the liver (Hop et al., 1995), the delayed start of gonadal development in females may be an explanation for the higher HSI in females throughout the experiment compared to males, who initiated gonadal development in earlier. The absence of treatment effects on GSI and HSI in this study may be due to insufficient doses to elicit an endocrine disrupting effect. Indeed, GSI is almost invariably reduced with long-term exposure to pollutants (Kime, 1995) and was also seen during a study exposing *Fundulus heteroclitus* to BaP (Booc et al., 2014). Low HSI values have also been associated with increased presence of atretic oocyte in fish such as Icelandic cod (*Gadus morhua*) (Hardardottir et al., 2001).

Increased oocyte atresia can indicate stress, low energy reserves, and has been associated with exposure to environmental contaminants (Blazer, 2002). Overall, no significant effect of exposure was seen on presence of atretic oocytes, however, at the time when atresia was most prevalent (29th of October) the control group had proportionally fewer females with atresia. Atresia may be a short-term response to exposure as in an experiment exposing polar cod to PW atresia was significantly increased after 21 days but similar between exposed and unexposed females after 28 days (Geraudie et al., 2014).

4. *Alterations in sperm quality*

Sperm motility was negatively affected by dietary crude oil exposure. Sperm VSL and percentage progressive sperm were significantly reduced in low and high crude oil treatments relative to control. In exposed males, relatively high percentages of motile sperm and low percentages of progressive sperm were measured illustrating that sperm was in motion but conservatively so, potentially reducing fertilization success for exposed males. Effects of PAHs and other petroleum related compounds on male gonads and sperm quality have been reported previously. For instance, sperm motility decreased in white sucker exposed to bleached kraft mill effluent (McMaster et al., 1992), rainbow trout exposed for one year to a phytoestrogen (Bennetau-Pelissero et al., 2001), 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). Similarly, exposure of Atlantic cod to PW for 12 weeks resulted in a reduction of mature sperm (Sundt and Björkblom, 2011). However, in the present study no alteration in spermatogenesis or obvious histopathology were observed in the male testis in February to explain the reduced sperm motility in exposed polar cod.

Unexposed polar cod exhibited a higher percentage motile sperm but lower percentage progressive sperm compared to Atlantic cod (Rudolfsen et al., 2008). In Atlantic cod, percentage progressive sperm was most indicative of fertilization success (Rudolfsen et al., 2008). The teleost sperm fertilize the egg by penetrating through a small canal called the micropyle, which is then closed after the entry of the first sperm to prevent polyspermy (Yamamoto and Kobayashi, 1992). Therefore, even minor decreases in sperm motility can have profound effects on its ability to fertilize an egg (Kime et al., 2001). The intensity of sperm competition is positively correlated with the relative investment in spermatogenesis (i.e. GSI) across fishes (Stockley et al., 1997). Thus, sperm competition may be a potentially powerful force in evolution of polar cod, a broadcast spawning species that invests heavily in gonadal development with high GSI values (maximum for males in the present study was 35% while recorded maximum in literature is <50%, Smith, 1984). Sperm motility is largely based on the endogenous ATP level (Perchec et al., 1995) and is closely associated with sperm morphology (Woods and Garside, 1996). Exposure to pollutants may result in malformed sperm which are unable to swim (Kime, 1995) or too weak to sustain swimming due to depleted ATP reserves (Jeong and Cho, 2005).

No difference was found in spermatocrit between treatment groups. Males in this study had very high spermatocrit values compared to Atlantic cod (Rakitin et al., 1999) and Arctic charr (Frantzen et al., 2004). Spermatocrit was also unaffected by exposure to bleached kraft mill effluent in white suckers (McMaster et al., 1992) but decreased in rainbow trout exposed to a phytoestrogen (Bennetau-Pelissero et al., 2001). Spermatocrit

is known to vary temporally and can be used to determine stage of spermiation in individuals starting with high levels and reducing with spawning season progression (Rakitin et al., 1999). The high spermatocrit observed in the present study may be a sign that it is still early in the spawning season, which would agree with the female's less advanced gonadal maturity stage (Vtg II).

5. *Implications and future work*

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, which are 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 freshwater discharge (Peterson et al., 2006). All of which may have the potential to alter the timing and success of the reproduction of polar cod (Bouchard and Fortier, 2011).

The design of this experiment with numerous sampling points and focus on gender-specific reproductive physiology without the actual completion of reproductive development created a challenge statistically and restricted the number of endpoints related to reproductive output and success that could be investigated. The robustness of polar cod reproduction to crude oil has been explored in this study but many aspects are yet to be investigated such as fecundity and fertilization success, maternal and paternal effects, energy and PAH concentrations in tissues, and survival of early life stages, which has recently been found to be very sensitive to crude oil exposure (Nahrgang et al., submitted). Future work will expand on the ecological components of petroleum exposure on a Pan-Arctic scale looking at *in situ* reproductive physiology of polar cod along temporal and spatial scales.

Conclusion

The investigated endpoints of growth, investment and timing in gonadal development, and sex steroid hormone levels were not significantly altered by chronic dietary exposure to crude oil. However, reduced sperm motility was seen in measures of progressive sperm and sperm velocity. Trends towards reduced reproductive investment in exposed immature males and delayed initiation of vitellogensis in exposed females were observed. Previous studies exposing polar cod to petroleum compounds have shown effects on growth and aspects of reproduction providing evidence that polar cod are sensitive to these endocrine disrupting compounds, however, the ecologically realistic doses used in this chronic exposure study were not high enough to induce such effects on reproduction. This study created novel information on aspects of basic reproductive physiology in polar cod that have not yet been elucidated especially with regard to resolution of oogenesis and spermatogenesis, sex steroid hormone profiles over a period of reproductive development, and quantification of sperm motility and spermatocrit.

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Appendix
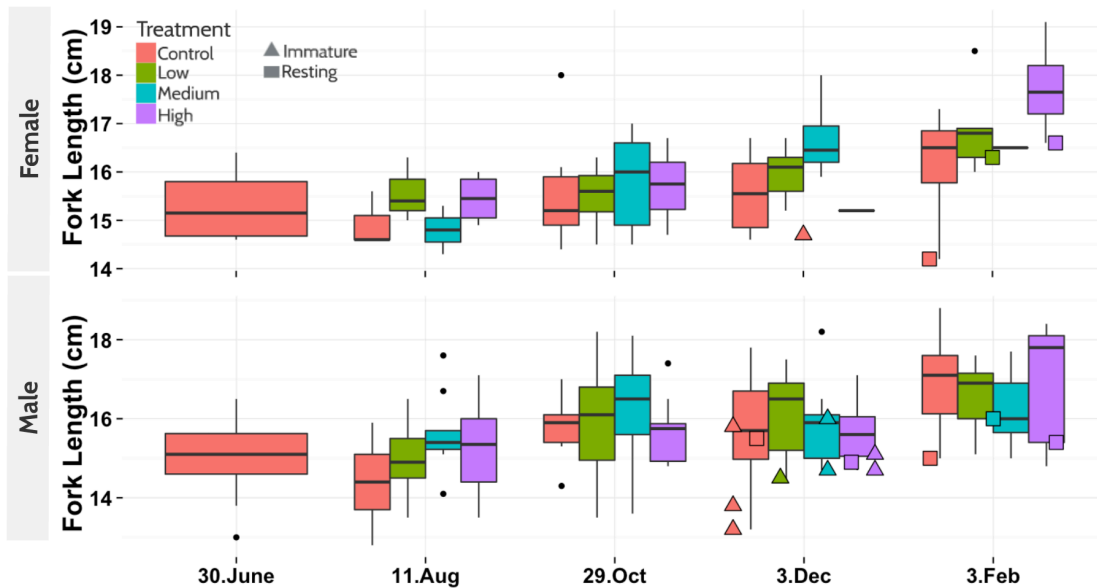
Appendix Figure 1. Fork length (cm) records of polar cod females (top) and males (bottom) sampled over the period from the 30th of June to 3rd of February. Treatments are represented by different color boxplots. Plots represent the median (line), 25%-75% percentiles (box), non-outlier range (whisker), and outliers (dots). Immature fish (triangles) and resting fish (squares) are noted in the respective time points sampled and color fill indicates treatment.
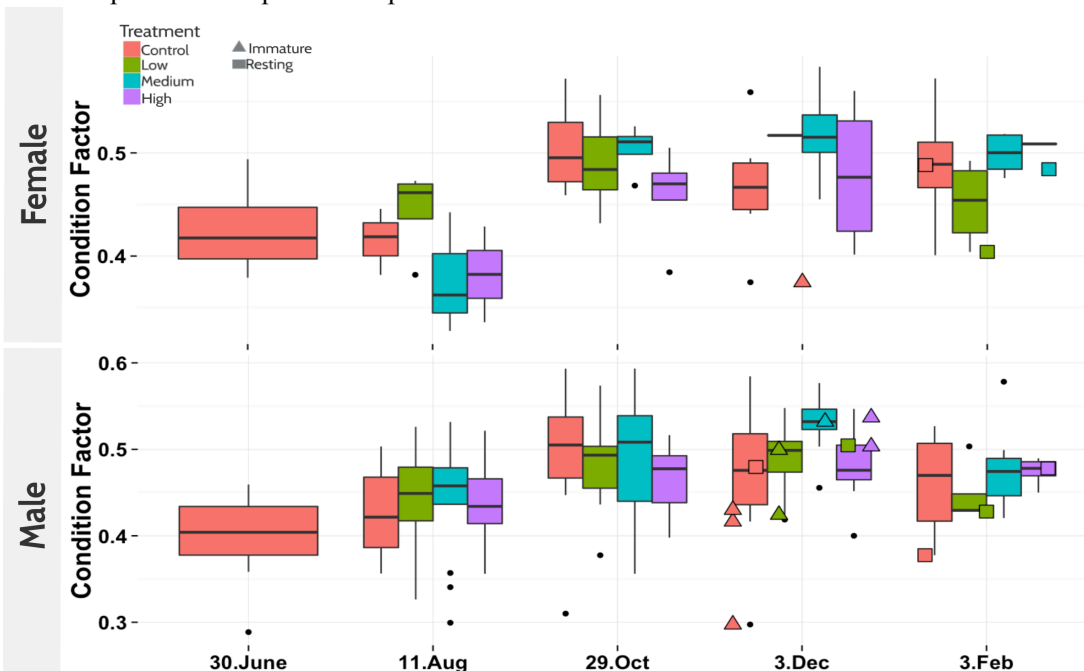
Appendix Figure 2. Fulton's condition factor (K) of polar cod females (top) and males (bottom) sampled over the period from the 30th of June to 3rd of February. Treatments are represented by different color boxplots. Plots represent the median (line), 25%-75% percentiles (box), non-outlier range (whisker), and outliers (dots). Immature fish (triangles) and resting fish (squares) are noted in the respective time points sampled and color fill indicates treatment.