Spawning during an oil spill

How exposure to the water-accommodated fraction of crude oil may disrupt polar cod (*Boreogadus saida*) reproduction

BIO399 Master thesis

Environmental toxicology
Biological Sciences
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Acronyms

11-KT	11-Ketotestosterone
act2b	beta-actin
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
ARNT	ah receptor nuclear translocator
BaP	benzo[a]pyrene
BP	biological pathways
BTEX	benzene, toluene, xylene isomers
CA	cortical alveoli oocyte
cDNA	complementary DNA
cyp1a	Cytochrome P450 1A
DNA	deoxyribonucleic acid
E2	17-β-estradiol
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERα	estrogen receptor α
EROD	ethoxyresorufin-O-deethylase
esr1	estrogen receptor 1
FDR	false discovery rate
FSH	follicle stimulating hormone
GE	germinal epithelium
GLM	generalized linear model
GnRH	gonadotropin-releasing hormone
GSI	gonadosomatic index
GTH	gonadotropin hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HCL	hydrochloric acid
HPGL	hypothalamus, pituitary, gonad and liver axis
HydgO	hydrating oocyte

HydgO/E	hydrated oocyte/hyaline egg
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
LME	linear mixed effect model
MIH	maturation inducing hormone
MPF	maturation promoting factor
mRNA	messenger RNA
NFW	nuclease-free water
NRT	no reverse transcriptase control
NSB	non-specific binding
NTC	no template control
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	primary growth oocytes
POF	post-ovulatory follicle
PRLR	prolactin receptor
qPCR	quantitative polymerase chain reaction
ResE	residual egg
RIN	RNA intergrity value
RNA-Seq	RNA sequencing
RNA	ribonucleic acid
RT	reverse transcriptase
SD	standard deviation
T	testosterone
TBE	tris-borate-EDTA
UCM	unresolved complex mixture
Vtg	vitellogenin
VTG1	primary vitellogenesis
VTG2	secondary vitellogenesis
VTG3	tertiary vitellogenesis
vtgα	vitellogenin

WAF	water accommodated fraction
WSF	water-soluble fraction
χ^2	chi-squared
XRE	xenobiotic response element
Zrp	zona radiata protein

Abstract

Declining ice cover in the Arctic has made it possible to extract oil reserves in areas that were once unreachable. However, all oil exploration comes with an inherent risk of spills. Conditions in the Arctic may increase this risk, especially during the polar night. Key arctic fish species, polar cod (*Boreogadus saida*), spawns during this time. In addition, they experience low energy reserves and potentially lower food availability. If an oil spill were to occur during the polar night, exposure could lead to disruption in natural reproductive development and spawning. In the present study, wild-caught polar cod were exposed to the water accommodated fraction (WAF) of crude oil and control conditions over their spawning period at either a high or low food ration. Samples taken after 47 days of exposure showed the up-regulation of Cytochrome P450 1A (*cyp1a*) relative expression in exposed individuals. At the time, results indicated that gonadal development was significantly more advanced in female polar cod from exposed groups. Of the WAF-exposed females, 90 % had already spawned compared to 56 % of the controls. Moreover, there was a significant reduction in the relative expression of estrogen receptor 1 (*esr1*) and vitellogenin ($vtg\alpha$) in exposed groups. Transcriptome mapping revealed the differential regulation of 947 hepatic genes in female polar cod. Exposure of males to the WAF did not lead to the disruption in any of the endpoints including the gonadosomatic index (GSI), testosterone (T), and 11-Ketotestosterone (11-KT). Similarly, food ration did not have a consistent effect on any of the endpoints. Contrary to the results presented, earlier studies on crude oil and petroleum compound exposure have predominately reported a delay and not an advancement in gonadal development. This difference may be explained by variations in the timing of exposure during the reproductive cycle.

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Suppliers

Materials used

Name	Supplier	Number
2-log DNA ladder	New England Biolabs	N3200
2x SYBR Green I Master	Roche	4887352001
10x loading buffer	TaKaRa	9157
Calanus finmarchicus	CALANUS AS, Lofoten	SeaFrozen
Chloroform	Sigma Aldrich	494275-50G
EIA kit Testosterone	Cayman	582251
EIA kit 11 Keto testosterone	Cayman	582251
EIA kit Estradiol	Cayman	582251
ethyl-acetate	Sigma Aldrich	141-78-6
Ethanol 96 %	VWR chemicals	64-17-5
Finquel	Sigma Aldrich	E10521
Formalin	Sigma Aldrich	HT501128
GelRed	Biotium	-
Halamid	Aquatiq Chemistry AS	H250
Instant eosin-Y alcoholic	Shandon, thermoscientific	6765040
Instant hematoxylin kit	Shandon, thermoscientific	6765015
Isopropanol	Sigma Aldrich	67-63-0
Histo-clear	National diagnostics	HS-200
iScript kit	bio-rad	180-8891
n-hexane	Sigma Aldrich	270504
Histo-wax	HistoLab	00407
Tri-Reagent	Sigma Aldrich	T9424
Vacutainer	BD	367841

Machines used

Name	Method	Vendor
Agilent 2100 Bioanalyzer	mRNA	Agilent Technologies
Digital slide scanner	Histology	Hamamatsu
Gel Doctm xr + system	Gel electrophoresis	Bio-rad
NanoDrop Microvolume Spectrophotometers and Fluorometer	RNA purity	Thermo Scientific
Rotary microtome RM2255	Histology	Leica
Shanadon Citadel 1000	Histology	Micron as
logiq xp Ultrasound	Sexing	General Electric
doppio Thermal Cycler	PCR	VWR
CFX96 Touch Real-Time pcr Detection System	qPCR	Bio-Rad
Sorvall RC 5B Plus centrifuge	Plasma separation	Marshall Scientific

Software used

Name	Supplier	URL
ImageJ	NIH	imagej.nih.gov/ij
ObjectJ plug-in	Vischer & Nastase	sils.fnwi.uva.nl/bcb/objectj
Primer 3 software	Koressaar & Remm; Untergasser et al.	bioinfo.ut.ee/primer3
NDPview	Hamamatsu	www.hamamatsu.com
Weibel-grid macro	Vischer	sils.fnwi.uva.nl/bcb/objectj/examples/Weibel/MD/weibel.html
Prism	Graphpad	www.graphpad.com/scientific-software/prism
Blast sequence comparison software	NCBI	blast.ncbi.nlm.nih.gov
Ensembl database	EMBL-EBI	www.ensembl.org
R	R Foundation	www.r-project.org

1 Introduction

1.1 Arctic oil exploration and the risk of spills

In 2008, a United States geological survey estimated that the Arctic contained approximately 90 billion barrels of undiscovered oil (USGS 2008). Of the resources assessed in the 2008 report, including oil and natural gas, 84 % were estimated to be located offshore (USGS 2008). Historically, ice cover has made it difficult to extract these resources. However, climate change and technological advancements have reduced the financial and logistical barriers to oil exploration in the Arctic (Comiso et al. 2008; Harsem et al. 2011). Although current trends in Arctic oil exploration are low compared to temperate regions, future depletion of other oil reserves, a shift in the demand for oil, and technological developments may increase the benefit of extracting these resources (Gulas et al. 2017; Harsem et al. 2011).

Harsh conditions in the Arctic may increase the risk of an oil spill, especially during the polar night (AMAP 2007; Amos 2011; Harsem et al. 2011). During this period, cold temperatures, 24-hour darkness, and unpredictable weather make working conditions difficult and add additional challenges for equipment (AMAP 2007; Harsem et al. 2011). In the event of an oil spill, ice cover can complicate clean-up by trapping and transporting oil, for which cleaning technology is still lacking (Faksness et al. 2011; Faksness & Brandvik 2008; Li et al. 2016). In addition, weather conditions and the remoteness of the Arctic can delay support teams and equipment from arriving, postponing clean-up and the drilling of potentially necessary relief wells (AMAP 2007; Amos 2011). Once crude oil enters the environment, cold temperatures can delay degradation, resulting in prolonged exposure of biota to the compounds associated with a spill (AMAP 2007; Amos 2011).

1.2 Crude oil and the water-soluble fraction

Crude oil contains thousands of different compounds, a large portion of which are unidentified and make up the unresolved complex mixture (UCM) (Marshall & Rodgers 2004). The composition and chemical properties of crude oil are highly variable depending on their geological origin (Faksness et al. 2008; Murillo et al. 2016). Crude oil is composed

of hydrocarbons (97 %) and nitrogen, oxygen, and sulfur (3 %) (NRC 1985). Although polycyclic aromatic hydrocarbons (PAHs) may account for only 0.2 – 7.0 % of crude oil (NRC 2003), they have been viewed as some of the most toxic compounds found in the mixture (J Meador & Nahrgang 2019; Pampanin & Sydnes 2013). Recent research has demonstrated that in addition to PAHs, the UCM also plays an important role in oil toxicity (Booth et al. 2007; J Meador & Nahrgang 2019; Melbye et al. 2009; Rowland et al. 2001), highlighting the importance of crude oil studies in addition to PAH specific exposure.

When crude oil is combined with water, the composition of the mixture changes as it undergoes weathering and physical movement (Faksness et al. 2008; Li et al. 2016). The components of crude oil have different fates depending on their chemical properties (Faksness et al. 2008). Some of these components become the water-soluble fraction (WSF), which has a high bioavailability for uptake by marine organisms as has been shown for PAHs (Meador et al. 1995). The term WSF is used to describe the fraction of oil under natural conditions, another term, water accommodated fraction (WAF), is used when the WSF is prepared in controlled conditions for laboratory use (Faksness et al. 2008).

The composition of the WSF differs from that of the parent oil due to the varying solubility between components of the mixture (Faksness & Brandvik 2008; Faksness et al. 2008). Volatile compounds, including benzene, toluene, xylene isomers (BTEX) make up a major part of fresh oil (Faksness et al. 2008). These volatile compounds evaporate quickly, although this can be delayed ice-covered waters (Faksness & Brandvik 2005; Faksness et al. 2008). After evaporation, the WSF is predominantly made up of semi-volatile and highly soluble compounds, naphthalene and phenols (Faksness et al. 2008).

1.3 Cytochrome P450 1A response following crude oil exposure

Cytochrome P450 1A (Cyp1a) is involved in the phase I biotransformation of xenobiotics mediated through the aryl hydrocarbon receptor (AhR). The AhR is present in the cytosol and after ligand binding undergoes conformational changes before translocating into the nucleus and binding with its dimerization partner ah receptor nuclear translocator (ARNT) (Bemanian et al. 2004). Together AhR/ARNT bind to xenobiotic response elements

(XREs) initiating the transcription of target genes including Cytochrome P450 1A (*cyp1a*) (Bemanian et al. 2004; Goksøyr 1995).

The use of Cyp1a as a biomarker for crude oil exposure has been demonstrated in key arctic fish species, polar cod (*Boreogadus saida*, Lepechin, 1774) (Nahrgang et al. 2010b; Vieweg et al. 2018) and several other teleost species (Aranguren-Abadıa et al. 2019; Goksøyr 1995; Santana et al. 2018). The level of Cyp1a can be tested through gene expression (*cyp1a*), enzyme activity with ethoxyresorufin-O-deethylase (EROD), or protein levels (Cyp1a) with immunochemical methods (Goksøyr & Larsen 1991; Sarasquete & Segner 2000).

Under natural conditions, levels of Cyp1a have been shown to vary seasonally in response to diet, temperature, and reproductive changes (Goksøyr 1995). Previous work on wild polar cod demonstrated natural changes in EROD activity throughout the year (Nahrgang et al. 2010a). Nahrgang et al. (2010a) showed that the highest levels of EROD activity occurred during the summer months and coinciding with high feeding rates. The lowest levels occurred during the spawning and post-spawning reproductive phases. Potential due to an inhibitory effect of high 17-β-estradiol (E2) on Cyp1a levels (Nahrgang et al. 2010a; Nava & Segner 2001).

1.4 Polar cod in the Arctic marine ecosystem

The Arctic has a high seasonality due to shifts in environmental conditions, ice cover and light availability throughout the year (AMAP 2007; Hop & Gjøsæter 2013; Leu et al. 2011). The polar night was once considered a period of arrest with low biological activity. However, Berge et al. (2015a) have shown that even during periods without primary production, zooplankton are still present in the water column at a potentially lower abundance and varied species composition (Berge et al. 2015a,b). These changes affect food availability for resident arctic species such as polar cod (Cusa 2016). Polar cod provide an efficient link between their marine invertebrate prey and predators, including seabirds and marine mammals (Hop & Gjøsæter 2013).

Polar cod are generalist feeders (Hop & Gjøsæter 2013) with a diet primarily made up of copepods and amphipods (Christiansen et al. 2012; Craig et al. 1982; Hop & Gjøsæter 2013; Lønne & Gulliksen 1989). In the summer months, polar cod experience high food availability and accumulate energy reserves (Hop et al. 1995). By the time the lower

food availability of winter arrives, the majority of the energy reserves accumulated in summer have already been allocated towards reproduction (Hop et al. 1995). This could leave polar cod with less energy reserves to be allocated towards other processes such as xenobiotic metabolism.

Polar cod spawn during the polar night between January and March in arctic populations (Graham & Hop 1995; Hop & Gjøsæter 2013). They have a maximum lifespan of seven years (Hop & Gjøsæter 2013). Most frequently, females mature when they are three and males when they are two years old (Hop & Gjøsæter 2013; Nahrgang et al. 2015). Females are iteroparous, capable of spawning multiple times during their lifecycle. They undergo group-synchronous ooctye development with determinate fecundity and are assumed to be pelagic broadcast spawners (Nahrgang et al. 2014). Males have been hypothesized to be semelparous, spawning once during their lifecycle (Nahrgang et al. 2014).

1.5 Gonadal development in teleosts

Gonadal development involves synchronous changes on several biological levels of organization. Disruption at any one of these levels may lead to reduced reproductive success (Casillas et al. 1991). Knowledge of the general processes that take place during gonadal development is necessary to predict and understand the outcome of teleost exposure to xenobiotics such as crude oil.

An oocyte is an ovarian cell capable of undergoing meiosis to become an egg (Arukwe et al. 2008; McMillan 2007). On a yearly cycle, in most teleost species, oocytes derived from primordial germ cells will develop into eggs (McMillan 2007). In group synchronous species such as polar cod, oocytes in the ovary develop together before being released during one spawning event (McMillan 2007).

Oogonia are derived from primordial germ cells (McMillan 2007). They multiply before going through the first stage of meiosis becoming primary growth oocytes (PG) (McMillan 2007). The oocytes are then arrested in meiosis phase I during their growth, before resumption during final oocyte maturation (Nagahama & Yamashita 2008; Senthilkumaran 2011).

Primary growth oocytes grow and develop into cortical alveoli oocytes (CAs), marked by

the appearance of white vacuoles termed cortical alevoli (McMillan 2007). During these initial phases of development, the oocyte becomes surrounded by follicle cells composed of an inner granulosa and outer theca layer (Patiño & Sullivan 2002).

After cortical alveoli development, the oocyte enters vitellogenesis and zonagenesis. During this period, yolk, necessary for embryo nutrition, is accumulated in the oocyte and zona radiata protein (Zrp) are deposited around the oocyte to form an eggshell (also known as zona radiata) (Arukwe & Goksøyr 1998, 2003; McMillan 2007).

Although vitellogenesis is a continuous process, it is usually divided into three phases, primary vitellogenesis (VTG1), secondary vitellogenesis (VTG2) and tertiary vitellogenesis (VTG3). By the end of VTG3, the oocyte is close to the size of the final egg, yolk accumulation has been completed, and no cytoplasm remains (Brown-Peterson et al. 2011). At this point, the oocyte is ready for final maturation.

During final maturation, meiosis is resumed causing the oocyte nucleus also known as the germinal vesicle (GV) to migrate towards the periphery of the cell undergoing germinal vesicle breakdown (GVBD) (Arukwe & Goksøyr 2003). At this time the oocyte is hydrated, as water content in the egg increases (McMillan 2007). When the oocyte is hydrated and the final maturation is complete, ovulation occurs. The oocyte is released from its follicle layer before travelling through the gonoducts and out into the water where it becomes a true egg (McMillan 2007; Norris & Lopez 2010).

After spawning, the ovary is primarily filled with follicles that once surrounded the oocytes, known as post-ovulatory follicles (POFs). In addition, the ovary contains leftover oocytes that will undergo atresia, the process by which oocytes are reabsorbed into the body (McMillan 2007).

In iteroparous species such as polar cod, gonadal development is a cyclical process, meaning that once spawning has been completed for one year, there is a short period of regeneration before development starts again for the next year (Brown-Peterson et al. 2011). This cycle will repeat every year that the individual is capable of reproducing (Brown-Peterson et al. 2011).

The developmental changes in the oocyte are primarily regulated through the hypothalamus, pituitary, gonad and liver axis (HPGL) (Arukwe & Goksøyr 2003; Nagahama 1994).

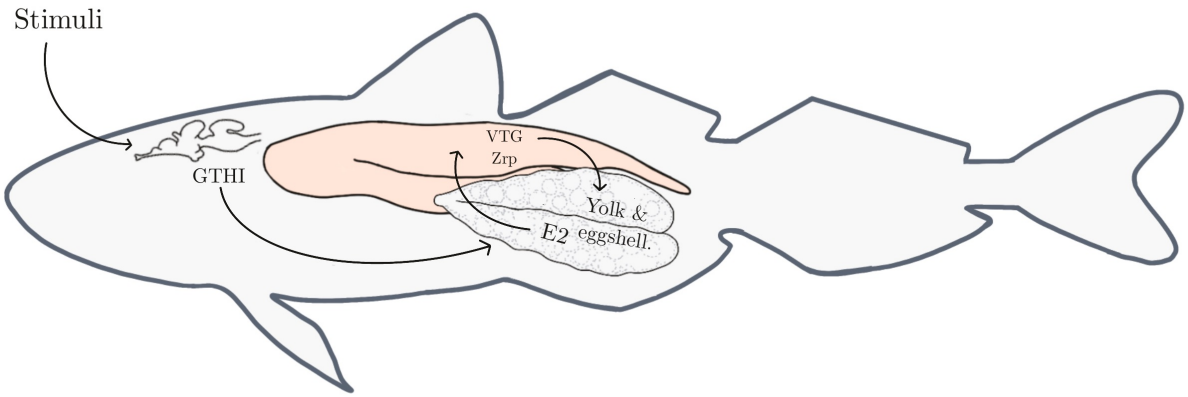


Figure 1: Diagram of the hypothalamus, pituitary, gonad and liver axis hypothalamus, pituitary, gonad and liver axis (HPGL) in teleosts and the simplified changes cause by gonadotropin hormone GTHI

These changes are illustrated in figure 1. External stimuli enter through the hypothalamus and cue the release of gonadotropin-releasing hormone (GnRH) that stimulate the pituitary gland to release gonadotropin hormones (GTHs), either GTHI, analogous to the human follicle stimulating hormone (FSH) or GTHII, analogous to the human luteinizing hormone (LH) (McMillan 2007; Nagahama 1994).

At the beginning of oocyte development, GTHI (FSH) is predominately released and involved in the growth of the oocyte and accumulation of yolk and eggshell development (Nagahama 1994). GTHI travels through the bloodstream to the gonad where it initiates steroidogenesis in the granulosa and theca layers of the oocyte, causing the conversion of cholesterol to testosterone (T) and T to E2 by Cytochrome P-450 aromatase (Cyp19) (Nagahama 2000; Nagahama 1994).

From the ovary, E2 then travels through the bloodstream to the liver where it binds to estrogen receptors (ERs), before initiating the transcription of vitellogenin (Vtg), a precursor yolk protein, and eggshell proteins such as Zrp (Arukwe & Goksøyr 2003; Nagahama 1994). The proteins then travel through the bloodstream to the ovary where they are taken up by the oocytes (McMillan 2007).

Final maturation is mediated by GTHII (LH), maturation inducing hormone (MIH), and maturation promoting factor (MPF) (Nagahama & Yamashita 2008). A surge in GTHII (LH)

initiates final maturation and the switch from E2 steroidogenesis to MIH (Senthilkumaran 2011). Finally, a drop in GTHII triggers the ovulation of oocytes, followed by spawning (Senthilkumaran 2011).

Spermatogenesis is the process by which germ cells develop into spermatozoon (Schulz & Miura 2002). Germ cells are enveloped by Sertoli cells becoming a Sertoli/germ cell unit known as a spermatocyst (Schulz & Miura 2002). Three phases are used to describe spermatogenesis (Schulz & Miura 2002). In the first, spermatogonia multiply by mitosis. In the second they go through two rounds of meiosis. In the third, they develop into spermatozoa (Schulz & Miura 2002). During this process, the spermatogonia undergo morphological changes including the reduction in cytoplasm and organelles, deoxyribonucleic acid (DNA) condensation, and the differentiation of flagellum (Schulz & Miura 2002). As in females, gonadotrophins GTHI (FSH) and GTHII (LH) are the primary mediators of spermatogenesis (Schulz & Miura 2002). These hormones regulate changes in the Sertoli and Leydig cells (involved in androgen production) (Schulz & Miura 2002).

1.6 Changes in reproductive endpoints following crude oil exposure

The effect of crude oil, PAHs, and petroleum compounds on teleost reproduction has been examined in several studies. These studies have used a variety of endpoints on different levels of biological organization, from the organism to the cellular level. Often these experiments have been conducted on model teleost species and seldom on arctic species.

Gonadosomatic index (GSI) has been used as a simple measurement that may be indicative of reproductive effects (Kime 1995). Kime (1995) reviewed the wide variety of studies that showed a reduction in GSI following xenobiotic exposure. Results from studies on PAHs and crude oil exposure have reported mixed results; some authors have noted a reduction (Bugel et al. 2010; Khan 2013; Meier et al. 2007; Truscott et al. 1983) and others, no change (Evanson & Van Der Kraak 2001; Meier et al. 2007; Pollino & Holdway 2002) in GSI.

Studies on polar cod have shown a delay in gonadal development or no effect following exposure to crude oil. Bender et al. (2018) found that polar cod females sampled

seven months after acute burned oil exposure were more frequently in earlier phases of reproduction compared to controls. In contrast, chronic exposure of polar cod to dietary crude had no effect on the timing of reproductive development (Bender et al. 2016).

In other teleost species, most studies assessing the timing of gonadal development have linked crude oil and petroleum compound exposure to delays in gonadal development, with a few exceptions. Exposure to the WSF of crude oil delayed female maturation in *Micropogonias undulatus*, and decreased the number of oocytes that underwent GVBD *in-vitro* (Thomas & Budiantara 1995). Experimental studies focusing on petroleum compounds reported a delay in oocyte development in female *Gadus morhua* after alkylphenol exposure (Meier et al. 2007), and female *Micropogonias undulatus* after naphthalene exposure (Thomas & Budiantara 1995). One study was found that reported an advancement of development following WAF of crude oil exposure in female *Platichthys stellatus* (Whipple et al. 1978).

A series of field studies compared polluted sites with high PAH levels to reference sites in Puget Sound, Washington. These studies found a delay in maturation in female *Parophrys vetulus* and a reduced ability of individuals to be induced into spawning after luteinizing hormone releasing hormone (LHRH) injections (Casillas et al. 1991; Johnson et al. 1988). However, no differences in *Pleuronectes americanus* were observed from the same sites (Johnson et al. 1994). Another field study in polluted Newark Bay, New Jersey, found a delay in gonadal maturation in female *Fundulus grandis* compared to reference sites (Bugel et al. 2010).

In males, exposure to crude oil and the WAF have been associated with an arrest or delay in spermatogensis. Water accommodated fraction exposure delayed spermatogensis in *Gadus morhua* (Khan 2013). Crude oil exposure delayed spawning in *Gadus morhua* and delayed testicular development in *Salmo salar* and *Pseudopleuronectes americanus* (Kiceniuk & Khan 1987; Truscott et al. 1983).

Low levels of atresia are natural in teleost ovaries (Rideout et al. 2000, 2005), but may increase due to low food availability (Hunter & Macewicz 1985), low condition index (Rideout et al. 2000, 2005), or exposure to xenobiotics (Kime 1995; Rideout et al. 2005). A high level of atresia can be indicative of gonadal development arrest, also known as skipped spawning (Rideout et al. 2005).

Previous experiments on teleosts following exposure to crude oil or associated compounds have found increased atresia or no changes. In the laboratory, WAF and naphthalene exposure resulted in widespread atresia in *Micropogonias undulatus* (Thomas & Budiantara 1995). Produced water exposure led to an increased level of atresia in *Gadus morhua* (Sundt & Björkblom 2011). Exposure to contaminated sites was associated with a higher instance of alpha atresia in wild *Pleuronectes americanus* (Johnson et al. 1994). However, exposure to the same contaminated sites did not cause significant differences in atresia in *Parophrys vetulus* (Johnson et al. 1988).

Although crude oil and petroleum compounds have been commonly cited as being associated with a decreases in steroid hormones, studies have shown contradicting results. In females, exposure to crude oil and associated compounds has reduced steroid production (E2, T) (Monteiro et al. 2000; Tetreault et al. 2003; Tintos et al. 2006) or had no effect (Bender et al. 2016; Pollino & Holdway 2002; Tetreault et al. 2003). In males, reduced levels of 11-Ketotestosterone (11-KT) and T have been reported in teleosts following oil-sands exposure (Tetreault et al. 2003), alkylphenol exposure (Meier et al. 2007), and WSF exposure in *Melanotaenia fluviatilis* (Pollino & Holdway 2002). An increase in T has been demonstrated after *in-vitro* PAH exposure in *Carassius auratus* (Evanson & Van Der Kraak 2001).

Researchers have identified crude oil as having predominately anti-estrogenic effects, leading to decrease of estrogen receptor α (ERα), Vtg, and zona radiata protein (Zrp). This effect has been hypothesized to be due to the cross-talk between AhR and ERα (Bemanian et al. 2004; Navas & Segner 2000; Safe & Wormke 2003; Yadetie et al. 2018).

Vtg and Zrp have been effectively used as sensitive biomarkers for estrogen pathway disruption to identify estrogenic or anti-estrogenic xenobiotics (Arukwe & Goksøyr 2003; Arukwe et al. 1997). Zona radiata protein has been previously identified as a potentially more sensitive and biologically more important biomarker than Vtg (Arukwe & Goksøyr 2003; Arukwe et al. 1997). However, Zrp has been to a lesser extent used in previous literature, as its expression seems to vary between species (Arukwe & Goksøyr 2003)

Exposure to the WAF of crude oil decreased ERα expression in *Chelon labrosus* (Bilbao et al. 2010) and *Danio rerio* (Salaberria et al. 2014). Benzo[a]pyrene (BaP), a high molecular weight PAH, commonly used in exposure studies, decreased ERα *in-vitro* in *Gadus morhua*

and Oncorhynchus mykiss (Gao et al. 2018; Yadetie et al. 2018). A negative correlation between Cyp1a and Vtg levels was found following *in-vitro* exposure of *Oncorhynchus mykiss* to AhR agonists (Anderson et al. 1996a). This trend was also observed in *Fundulus grandis* taken from polluted sites with varying levels of PAH contamination (Bugel et al. 2010). In addition, BaP *in-vivo* and *in-vitro* lead to a decrease in Vtg in *Oncorhynchus mykiss* (Anderson et al. 1996b; Navas & Segner 2000). Furthermore, Woźny et al. (2008) showed that Zrp levels decreased following BaP exposure.

In addition to assessing the expression of single genes involved in reproduction, transcriptome mapping has been useful in determining disrupted gene pathways. The technique RNA sequencing (RNA-Seq) was recently used by Yadetie et al. (2018) to look at enriched gene pathways following *ex-vivo* exposure of *Gadus morhua* liver to BaP. Results showed the enrichment of oxidative stress, AhR signalling, xenobiotic metabolism, and the down-regulation of genes involved in the estrogen pathway. The hepatic transcriptome has also been recently examined in polar cod following BaP exposure (Song et al. 2019). Song et al. (2019) showed the enrichment of oxidative stress and AhR signalling pathways, similar to Yadetie et al. (2018).

1.7 Aims of the study

As demonstrated in the studies introduced, exposure to crude oil and associated compounds can lead to changes in reproductive endpoints. Disruption in the processes involved in reproduction are especially relevant as they have the potential to affect spawning and fertilization (Casillas et al. 1991). As the Arctic becomes more available for oil exploration, it is important to understand the associated risk to arctic species, such as polar cod. In addition, which life events may be the most sensitive crude oil exposure.

There have been few reports of polar cod reproductive development during spawning or how exposure during this period may alter it. Moreover, low energy reserves during the polar night coupled with potentially lower food availability may affect their sensitivity to crude oil. The current experiment attempts to address this gap in the literature by exposing wild-caught polar cod to the WAF of Goliat crude oil, at an environmentally relevant level. Adults were exposed starting before spawning until their post-spawning period at a low and high food ration.

The research question is divided into three parts:

1. To identify any changes in natural gonadal development using measurements of GSI, reproductive phase timing, and atresia.
2. To investigate how WAF-exposure may alter levels of plasma hormones (E2, T, 11-KT) involved in reproduction.
3. To assess changes in hepatic gene expression by examining the response of crude oil biomarker (*cyp1a*), genes involved in reproduction (vitellogenin (*vtg*α) and estrogen receptor 1 (*esr1*)), and transcriptome mapping.

The current thesis investigates how WAF exposure during spawning may alter reproductive endpoints on several levels of biological organization. To limit the scope of the thesis, I focus on the alterations in reproductive endpoints in light of previous findings. For this reason, I do not discuss in detail the underlying mechanistic causes of these effects, including which components of crude oil are associated with the changes observed.

2 Materials and Methods

2.1 Polar cod collection and rearing

In November 2017, polar cod were collected by bottom trawl in Southeastern Hinlopen (78° 55'N, 23° 40'E) from depths between 170 and 200 m (Figure 2). A Campelen 1800 bottom trawl was fitted with a FISH-LIFT device to avoid injury to polar cod from the trawling procedure (Holst & McDonald 2000).

Once on-board, the fish were transferred into flow-through seawater tanks (2 500 L) that were treated daily with Halamid disinfection solution (1:500). Following the cruise, the fish were transported to the research facility of UiT the Arctic University of Norway in Kårvika (69°23'N, 18°10'E), where they were kept in a 4 000 L tank supplied with a flow-through of filtered seawater (60 μm) from the nearby fjord.

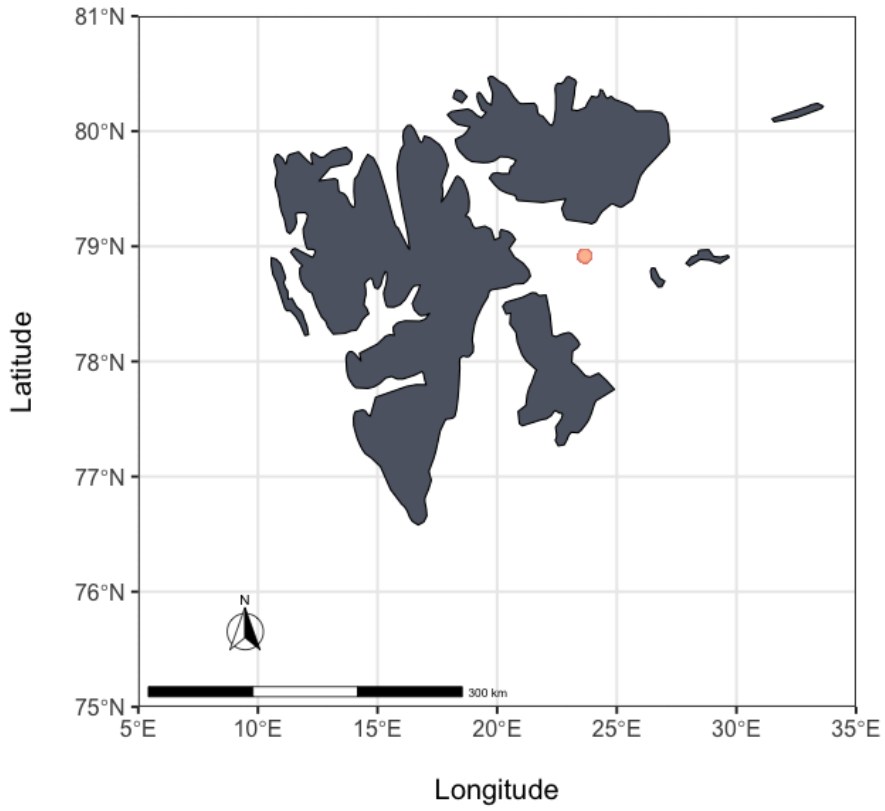


Figure 2: Capture site of wild polar cod (78° 55'N, 23° 40'E) in Southeastern Hinlopen, Svalbard, Norway.

Fish were fed daily *ad libitum* with *Calanus finmarchicus* until experiment start. The supplier information for the materials, machines, and software mentioned in the following section is available at the beginning of this thesis, following the acknowledgements section.

During rearing and throughout the experimental period, light exposure and water conditions were controlled to simulate *in-situ* conditions in Svalbard (Ny-Ålesund, 78 ° N). The photoperiod during the experiment was: 24-hour darkness from October 29th, 2018 to February 2nd, 2019, followed by an increasing amount of light until April 22nd, 2019, when 24-hour light began. During 24-hour darkness, the only light to which fish were exposed was light from a headlamp during feeding and sampling. Mean water temperature was maintained at an average of 1.01 °C ± 0.21 standard deviation (SD) and dissolved oxygen at an average of 88.23 % ± 3.62 SD.

Ten days before the start of the experiment, 472 fish were selected from the holding tank based on a similar size (average length: 21.08 ± 1.98 SD and weight: 81.48 ± 24.76 SD), corresponding to an approximate age of 4 years (Nahrgang et al. 2014). Each fish was anaesthetized using a 70 mgL^{-1} Finquel (Tricaine Methanesulfonate) dissolved in seawater. They were sexed, marked with a unique external tag (colour-coded by sex), and transferred to one of the experimental tanks (300 L). Researchers from NOFIMA AS (Øyvind Hansen and Velmurugu Puvanendran) determined sex using ultrasound based on the procedure developed for Atlantic cod (Karlsen & Holm 1994).

2.2 Experimental design

All experimental work was performed in accordance with the Animal Welfare Act and the regulations of the Norwegian Animal Research Authority (ID 17724). Ireen Vieweg, the lead researcher of this experiment, had the training and certification necessary (FELASA Category C) to perform experimental work with animals.

The polar cod were randomly distributed into 12 experimental tanks figure 3. Each tank contained 36 fish with a balanced sex ratio of male (n = 18) and female fish (n = 18) and assigned an exposure and food treatment. Tanks were arranged randomly around the room in order to distribute any differences due to location across treatment groups, such as proximity to the door affecting light exposure and proximity to the gravel columns affecting water flow (Figure 3).

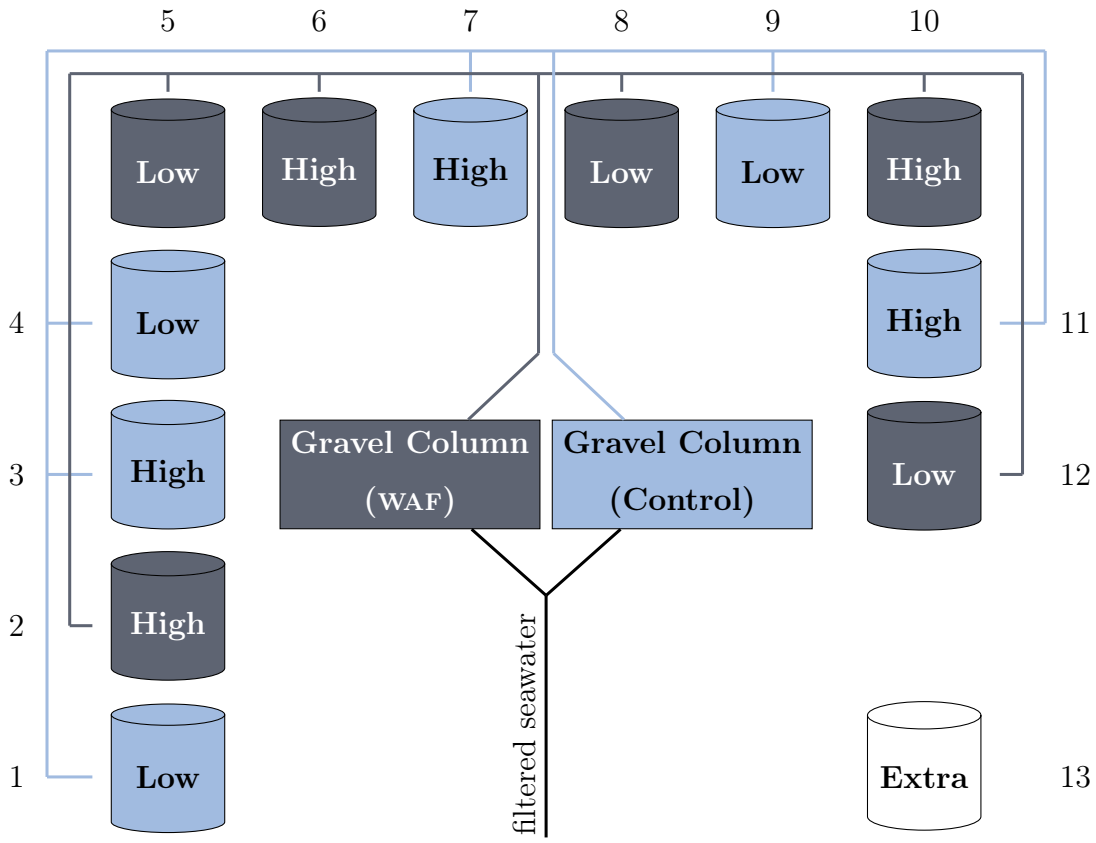


Figure 3: Experiment set-up. Thirteen tanks were arranged around the experimental room. Twelve of these tanks were used for the experiment. The 13th was used to assess the onset of spawning. Half of the experimental tanks were exposed to the WAF of crude oil (gray) and half were control (blue). Each tank also received a low (1.5 % somatic weight day^{-1}) or food high (4.0 % somatic weight day $^{-1}$) food ration (labeled on each tank).

Half the tanks were maintained under control conditions and the other half were exposed to the WAF of Goliat (Kobbe) crude oil from the Hammerfest Basin in the southwestern Norwegian Barents sea (Murillo et al. 2016). Oil exposure was produced by an oiled-rock-column system to simulate natural weathering and degradation of oil components over time (Carls et al. 2000). Before entry into the tanks, seawater was pumped through hard plastic columns (122 x 30 cm) filled with 45 kg of gravel, which was coated either with oil (exposed) or not (control).

These columns were prepared according to the methodology outlined by Nahrgang et al. (2010). After cleaning, gravel for the WAF-exposure column was soaked in 24 g of Goliat crude oil per kg of gravel. The experimental water supply from the rock columns was

maintained at a rate of 900 – 1 000 ml min^{-1}. Each tank also received a clean seawater supply (3 – 4 $Lmin^{-1}$) in order to maintain sufficient oxygen levels for the fish. Right before the experiment started, seawater was flushed through the column for 70 hours to ensure volatilization of BTEX.

In addition to the exposure treatments, each tank was assigned one of two possible food rations. Each day polar cod were fed *Calanus finmarchicus* as a group either at a high (4 % somatic weight day^{-1}) or low food (1.5 % somatic weight day^{-1}) ration. The low food ration represents the mean food required to cover the energetic maintenance cost of polar cod based on a previous experiment (Hop et al. 1997). The high food ration provided fish with a potential surplus of energy for growth, reproduction and health (Hop et al. 1997).

The amount of food given per tank was calculated based on the estimated somatic weight (gutted fish weight) of the fish in each tank. The wet weight of polar cod is known to change seasonally and shows a strong increase due to gonad growth towards spawning (Nahrgang et al. 2015). Hence, somatic weight was chosen in lieu of total weight as the reference for food intake. Somatic weight was calculated with the help of fork-length somatic weight correlations ($y = 0.0058x^{2.9716}$, $R^2 = 0.969$) from a large data set on feral polar cod from Svalbard (Appendix A; Nahrgang et al. 2014). Feed amounts were updated based on monthly growth checks (measurements of length and weight).

An additional 13^{th} tank was included in the experiment to assess the natural gonadal development of the polar cod (n = 40) under control conditions and high food ration. This made it possible to monitor the onset of spawning along with the help of histology (Section 2.3) without disturbance to the experimental fish. At the beginning of January, stripping tests were conducted on females and males from tank 13 in order to determine when spawning would likely occur. Assessing the onset of spawning was necessary as we aimed to conduct the second sampling time-point near spawning.

Fish were tested every week during the beginning of the spawning period and every second day when visual observations indicated that they were close to spawning. In addition, the tanks were monitored daily for eggs. During each test, three females and three males were anaesthetized in Finquel before light pressure was applied to their abdomen in order to stimulate the release of milt or eggs.

Fish (6 to 8) were sampled from each tank at a balanced sex ratio at experiment start (Day 0, Dec. 14th, 2018), during the spawning window (Day 47 and 48, Jan. 31st and Feb. 1st, 2019) and during the estimated post-spawning period (Day 131, April 25th, 2019). Sampling time-points will be referred to hereafter as Day 0 or before exposure start, Day 47, and Day 131, respectively.

During each sampling time-point, Figure 4 fish was anesthetized before blood was drawn from the caudal vein with a 2 ml heparinized Vacutainer. Following extraction, blood was centrifuged for ten minutes at 900 g-force at a temperature of 4 °C to obtain blood plasma that was stored at – 80 °C until further analyses. After blood samples were taken, fish were sacrificed by a sharp blow to the head and fork length (± 0.1 cm), wet weight (± 0.1 g), gonad weight (± 0.1 g), and somatic weight (gutted body weight, ± 0.1 g) were measured. Gonadosomatic index (GSI) was measured using the following equation:

$$\text{GSI} = \frac{Gonad\ weight}{Somatic\ weight} \cdot\ 100$$

Samples of the gonad, liver, and gall bladder were removed. For histology, sections were cut from the middle of the gonads and transferred to 4 % neutral-buffered formalin. Samples for gene expression analyses were cut from the anterior section of the liver, and subsequently, snap-frozen in liquid nitrogen and stored at – 80 °C. In addition to liver samples, gall bladders were removed and snap-frozen for PAH metabolite analysis. Following the Day 47 sampling time-point, freezer malfunction led to the partial thawing of some of the liver samples taken during that sampling time-point. Further assessment of these samples is discussed in section 2.4.1. At the Day 47 sampling time-point, all polar cod sampled were stripped before sampling.

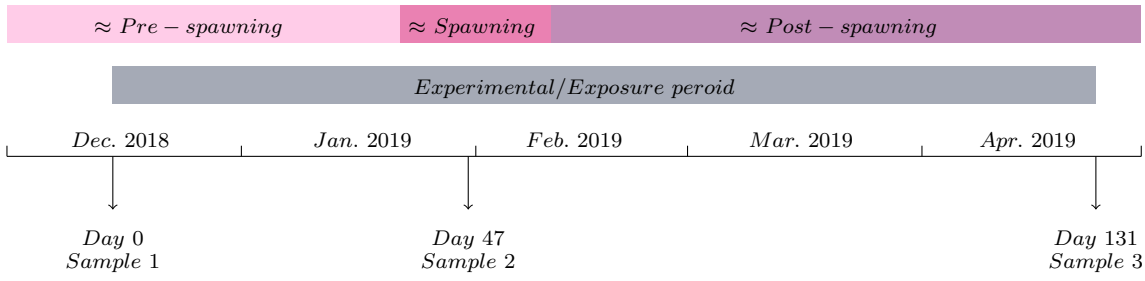


Figure 4: Experimental timeline displaying the dates of exposure, the estimated reproductive periods, and sampling time points.

Water samples for chemical analysis of total PAHs ($\sum$ 44 PAHs) were taken at days 0, 2, 4, 8, 10, 18, 28 and 131 during the experiment to monitor the decrease in oil compounds in the water of the experimental tanks. Samples were retrieved from the center of all tanks using rubber tubing and were transferred to DURAN-bottles pre-filled by SINTEF with 15 % hydrochloric acid (HCL). After collection, water samples were stored in a dark cool environment (4 °C) until being analyzed by SINTEF. Extraction and analysis of PAHs and alkyl PAHs was conducted at SINTEF using gas chromatography/tandem mass spectrometry following the procedures described by Sørensen et al.; Sørensen et al. (2016; 2016). PAH metabolites, pyrenol and phenanthrol, in gallbladder samples were analysed by SINTEF.

2.3 Gonad histology

2.3.1 Histology methods and slide preparation

Gonad histology samples were preserved and prepared for use in two different methods, reproductive phase identification and Weibel-grid analysis. Although both methods are based on the same reproductive structure identification, they were completed and analysed separately. Reproductive phase was based on the presence or absences of structures whereas Weibel-grid was used to determine an estimated ovarian volume fraction occupied by each structure.

Following collection, histology samples were left in 4 % neutral buffered formalin for at least 4 days. Samples were then cleaned in two consecutive 70 % ethanol baths and run through a ten-step process in the Shandon Citadel 1000, following the procedure outlined by Bender et al. (2016). This process consisted of ten bath combinations containing ethanol (70 and 96 %), Histo-clear, and paraffin wax to step-wise clean and preserve the samples.

The samples were then individually embedded in paraffin wax (Histo-wax), sliced into 5 µm and 6 µm sections, for males and females, respectively, using a microtome. Two replicate microscope slides were created from each gonad sample from regions approximately 50 – 60 µm apart. Slicing two replicate slides per sample from different regions in the gonad ensured that there was a back-up if a slide was broken or not of sufficient quality. Moreover,

this procedure allowed for the best slide to be chosen where the structures could be most clearly identified without interference due to fixation, slicing or staining. Three to eight slices from each section of the gonad were arranged on the same microscope slide. Slides were then stained with haematoxylin/eosin before being scanned using a digital slide scanner and analysed using NDPview and ImageJ software.

2.3.2 Female reproductive phase identification

The reproductive phase for each female was determined based on gonad histology. During analysis, the exposure and food group for each sample were unknown. The classification scheme outlined by Brown-Peterson et al. (2011) was used with the following deviations: an early regressing subphase was added to account for individuals that were in-between spawning and regressing (Table 1).

Using the NDPview mapping function, one replicate slide (with several slices) was traversed at a starting point of 5x magnification. The presence or absence (1 or 0) of each reproductive structure necessary for staging was recorded: primary growth oocytes (PG), cortical alveoli oocytes (CAs), primary vitellogenic oocytes (VTG1), secondary vitellogenic oocytes (VTG2), and tertiary vitellogenic oocytes (VTG3), hydrating oocytes (HydgOs),hydrated oocyte/hyaline eggs (HydgO/Es), atresia any stage and post-ovulatory follicles (POFs), was recorded (Table 3).

Gonad structures were identified by comparison to published literature on the general gonad histology of fish (Brown-Peterson et al. 2011; McMillan 2007), as well as specific documentation on polar cod (Bender et al. 2016; Nahrgang et al. 2015). ICES (2019) was used to classify the different stages of POFs and atresia. Due to the difficulties distinguishing between VTG2 and VTG3 based on previous literature on polar cod, a size range was added for these oocytes based on Iwamatsu (1981). Iwamatsu (1981) examined medaka oocytes with a similar final oocyte size. The size range was used as a guide and adjusted based on the size of other oocyte stages in polar cod. While this is a somewhat arbitrary cut-off because vitellogenesis is a continuous process, the addition of this size restriction aided in making a more strict division between oocytes.

Size was measured by taking the average diameter (measured perpendicular to each other) for five randomly selected oocytes, each with a GV. If the average diameter of these five

Table 1: Simplified reproductive phase classification for females based on Brown-Peterson et al. (2011). Stages were determined by the presences or absence of reproductive structures (primary growth oocytes (PG), cortical alveoli oocyte (CA), primary vitellogenic oocytes (VTG1), secondary vitellogenic oocytes (VTG2), tertiary vitellogenic oocytes (VTG3), hydrated oocyte/hyaline eggs (HydgO/Es), hydrating oocytes (HydgOs) and residual eggs (ResEs).

Phase	Presence	Absence
Immature	PG	POF3 or atresia
Developing	VTG1 or VTG2	VTG3 or hydrating oocyte (HydgO) or hydrated oocyte/hyaline egg (HydgO/E)
Subphase: Early developing	CA	VTG1,VTG2,VTG3
Spawning capable	VTG3	HydgO or HydgO/E
Subphase: Active spawning	HydgO or HydgO/E	POFs
Regressing	POFs, few ResE	Many ResE, late stage POFs
Subphase: Early regressing	POFs and many ResE	Few ResE
Regenerating	PGs and POF3 and/or beta atresia and verification that the individual is not immature	CA

oocytes was greater that 600 μM it was in stage VTG3 and if less, VTG2. The following experts from Institute of Marine Research (IMR) and Norwegian Institute for Water Research (NIVA) were consulted to verify the findings: Anders Thorsen (IMR), Marianne Frantzen (Akvaplan NIVA), Grethe Thorsheim (IMR) and Merete Fonn (IMR). A synopsis of this information can be found in Table 3.

The presence or absence of structures was translated into a final reproductive phase for each female using formulas in Excel based on the classifications outlined in Brown-Peterson et al. (2011) and summarized in Table 1.

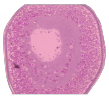
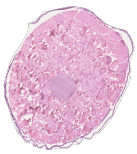
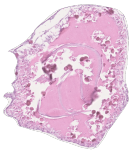
2.3.3 Male reproductive phase identification

Male reproductive phase was determined based on visual comparison with previous work on polar cod (Bender et al. 2016; Nahrgang et al. 2015) and classified based on Brown-Peterson et al. (2011). Different spawning capable sub-phases were identified using the germinal epithelium patterns outlined in Brown-Peterson et al. (2011). Different phases of sperm development were not recorded as all male testis already contained spermatozoa by the time exposure began. Therefore, these individuals were in the spawning capable phase and the further distinction between prior reproductive phases of the sperm was not necessary.

Table 2: Simplified reproductive phase classification for male polar cod based on Brown-Peterson et al. (2011) (GE = germinal epithelium).

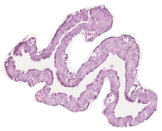
Phase	Description
Spawning capable	Spermatozoa present
Subphase: Early - GE	Continuous GE in all lobules
Subphase: Mid - GE	Continuous GE near periphery and discontinuous near ducts
Subphase: Late - GE	Discontinuous GE throughout testis
Regressing	Residual GE present in the lumen
Regenerating	Threadlike testis, lumen non-existent and GE continuous throughout

Table 3: Reproductive structures identified for reproductive phase identification and Weibel-Grid analysis, including name, picture and description. An asterisk denotes that the picture taken from polar cod samples earlier in the year and not during the experiment.

Structure	Picture	Description
Primary growth oocyte (PG)		Prominent nucleus and nucleoli with little cytoplasm.
Cortical alveoli oocyte (CA)		Presence of cortical alveoli (white vacuoles).
Primary vitellogenic oocyte (VTG1)	 *	Accumulation of yolk globules in the periphery of the cytoplasm.
Secondary vitellogentic oocyte (VTG2)	 *	Accumulation of yolk throughout the cytoplasm and an average size less than 600 μM.
Tertiary vitellogenic oocyte (VTG3)		Yolk globules that filled the cytoplasm with an oocyte size greater than 600 μM.
Hydrating oocyte (HydgO)		Yolk globules begin to fuse together, and the oocyte starts to take up water.
Hydrated oocyte / egg (HydgO/E)		Completely hydrated oocyte with no visible cytoplasm or yolk left. Hydrated oocyte and hyaline egg (egg within the ovary) combined.
Alpha atresia (Atresia A)		Both early and late alpha atresia . Starting small and progressing to larger breaks in the chorion, diverse appearance depending on the stage of the oocyte.

Continued on next page

Table 3 – continued from previous page

Structure	Picture	Description
Beta atresia (Atresia B)		Late atresia where the remaining structure is greatly reduced in size and there are no yolk contents visible, difficult to distinguish from POF3.
Post ovulatory follicle 1 (POF 1)		Large POF with a clearly defined cord-like shape and the lumen is still present. This definition is based on stages 1-3 ICES (2019).
Post ovulatory follicle 2 (POF 2)	N/A	Still large but more compact, folds are almost or not at all visible, based on stages 4-5 in ICES (2019).
Post ovulatory follicle 3 (POF 3)		Looks like a mass of disordered cells, difficult to distinguish from atresia B, based on stages 6-7 in ICES (2019).

2.3.4 Weibel-grid analysis

Weibel-grid analysis was performed for females from the Day 47 and Day 131 sampling time-points in order to determine the estimated ovarian volume fraction occupied by atresia. The Weibel-grid consists of a series of repeating line segments that can be adjusted based on the level of detail needed for the analysis, determined through grid-optimization (Weibel et al. 1966). The end of each line is categorized with the reproductive structure it overlays (Figure 5).

Using the NDPview grid function, each histology scan was divided into 2000 x 2000 μM squares and a subsample of these squares was chosen for Weibel grid analysis. I created a series of guidelines for this thesis to determine which squares would be chosen for the subsample to ensure consistency across individuals and that the subsample was representative of the whole gonad slice (Appendix B). Once the subsample of 2000 x 2000 μM squares was chosen, PNG files for each square, at 5x magnification, were exported for further

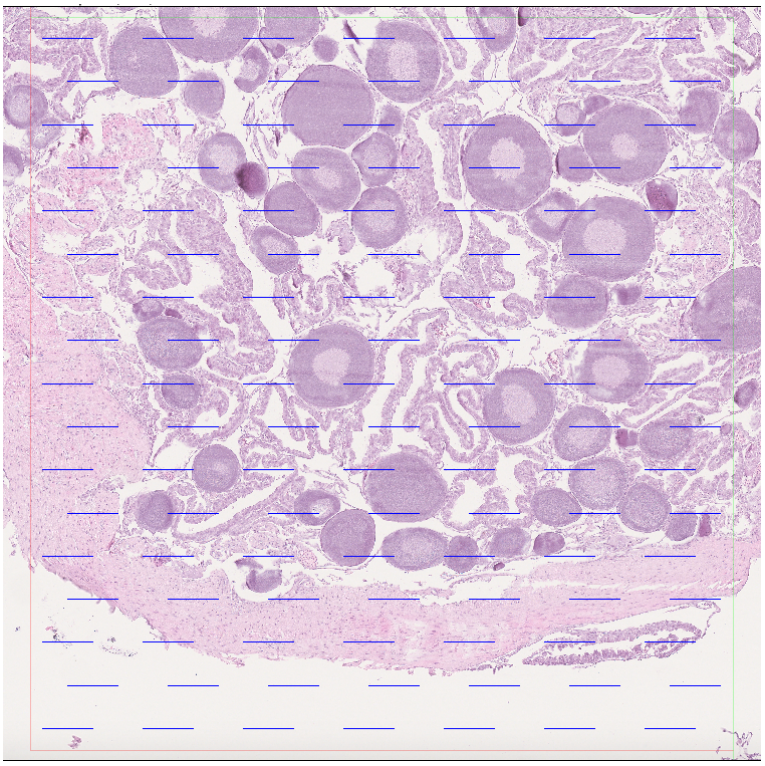


Figure 5: An example of a Weibel-grid overlaying a regressing female polar cod. This square represents one 2000 x 2000 μM PNG file, five of which were conducted for each individual.

analysis. Weibel-grid analysis was performed in Image J, using the Object J plug-in and Weibel-grid embedded macro.

Before grid analysis was performed, the Weibel-grid was optimized in order to determine the necessary grid density and area (number of 2000 x 2000 μM squares) needed for a representative sample from each gonad. To optimize the grid, four females in different reproductive phases (spawning capable, active spawning, regressing, and early developing) were chosen as test cases. Using the subset of squares from each female, grid analysis was performed several times at different grid densities on each test female. The grid density was chosen where the grid was fine enough to provide consistent results for the structures of interest.

A similar method was applied for area optimization. Results from the grid analysis (at the optimal grid density) were compared between a different number (one to nine) of 2000 x 2000 μM squares. This was conducted to determine, on average, how many squares were necessary in order to consistently capture the least frequent structures in each gonad.

Following optimization, the Weibel-grid was set to the optimal grid size (approximately

1190 points per 1 mm x 1 mm section). Then, five 2000 x 2000 μM squares were analysed from each female from the Day 47 and Day 131 sampling time-point.

The end of each grid line was then assigned a category based on the structure it overlaid. After all endpoints were assigned, the counts for each reproductive structure were exported and percentage of occurrence was calculated. The resulting data provided an estimated ovarian volume fraction for each reproductive structure.

2.4 Gene expression

2.4.1 Quantitative polymerase chain reaction

Total ribonucleic acid (RNA) was extracted from liver sections based on the TRI-reagent protocol. Liver were removed from – 80 °C storage and placed in liquid nitrogen during the procedure. Working on dry ice, a 30 – 40 mg piece of liver was obtained from each sample and transferred to an Eppendorf tube in liquid nitrogen. To avoid cross-contamination, instruments were cleaned between samples. Each sample was then homogenized in 0.7 ml TRI-reagent before being placed on ice. Once all samples in a batch were homogenized, the samples were removed from the ice and kept at room temperature for five minutes before 0.2 ml of chloroform was added and the tubes were shaken. Samples were then allowed to sit at room temperature for ten minutes before being centrifuged at 12 000 g-force for 15 minutes.

The samples were divided into three distinct layers after centrifugation. From the aqueous layer, containing total RNA, 0.4 ml (80 %) was removed and transferred to a new tube along with 0.4 ml isopropanol. The samples were then incubated at room temperature for ten minutes before being centrifuged at 12 000 g-force for ten minutes. The isopropanol was withdrawn, leaving only the RNA pellet before 1.4 ml of 75 % ethanol was added. The tubes were vortexed and subsequently centrifuged at 7 500 g-force for 5 minutes. Following this, the ethanol was removed, and the pellet left to dry for 30 minutes, before adding 50 µl of nuclease-free water (NFW). Once NFW was added, the tubes were transferred to a 60 °C oven for 30 minutes to dissolve the RNA pellet. To ensure proper dissolution, samples were vortexed halfway before being returned to the oven.

Total RNA quantity and purity were measured using NanoDrop Microvolume Spectropho-

tometers and Fluorometer. Two measurements were used to assess the purity of samples. The first measurement, 260/280, represents the ratio of absorbance at 260 nm and 280 nm. Similarly, the second measurement, 260/230, represents the ratio of absorbance at 260 nm and 230 nm. Accepted 260/280 and 260/230 values were $\sim$ 2.0, and $\sim$ between 2.0 and 2.2, respectively. The quantity of total RNA in each sample was measured using the same machine.

Agarose gel electrophoresis was conducted to assess the integrity of the RNA samples. A 1 % agarose mixture was made with 0.5 X tris-borate-EDTA (TBE). Agarose gels were prepared by adding GelRed nucleic acid gel stain to 1 % agarose (1:100). The agarose gel and GelRed mixture was added to the gel electrophoresis chamber and allowed to solidify for at least 30 minutes. Ribonucleic acid (RNA) samples were thawed and 1 μl of each sample of interest was mixed with 1 μl of 10X loading buffer and 8 μl formamide.

This mixture was then transferred to an oven (60 °C) for ten minutes for denaturation before being immediately placed on ice. The gel was submerged in 0.5 X TBE buffer, samples were loaded along with a 2-log DNA ladder, and electrophoresis was run at 80 V for 60 minutes. Using Gel DocTM XR + system, gels were visualized under UV light and photographed (Appendix C). As mentioned earlier, a freezer malfunctioned after the Day 47 sampling. Some of these liver samples showed signs of being slightly degraded. This was kept in mind during quantitative polymerase chain reaction (qPCR) analysis with the help of researcher Fekadu Yadetie.

Total RNA was diluted, and complementary DNA (cDNA) was synthesized with an iScript kit. Using the quantity of total RNA in each sample obtained from the NanoDrop, the quantity of NFW to total RNA was calculated in order to achieve a dilution of total RNA of 100 ng μl^{-1}. To synthesize cDNA, 10 μl of diluted total RNA was added to a 10 μl solution containing 4 μl 5 X iScript reaction mix, 1 μl iScript reverse transcriptase (RT), and 5 μl of NFW. The iScript kit reaction mix also contains oligo (dT) and random hexamer primers, RNase inhibitor, dNTPs and RT buffer.

All samples were run in groups based on sampling time-point and sex (eg. Day 47 males, Day 47 females) in a PCR machine on the following program: 25 °C for 5 min, 46 °C for 30 min, 95 °C for 1 min, and then stored at 4 °C. After the protocol was complete, cDNA was diluted 1:10 using NFW before being transferred to – 40°C for storage.

Using the diluted cDNA samples, qPCR were run for 4 genes: *vtgα*, *esr1*, *cyp1a*, and beta-actin (*act2b*). Samples were run in groups separated by sampling time-point and sex. In each reaction, 5 μl diluted cDNA from each sample or standard was added to the wells along with 5 μl of 2 μM primer mix and 10 μl of 2 x SYBR Green I Master. In addition, a no reverse transcriptase control (NRT) and no template control (NTC) were included as negative controls. The plate was then sealed and centrifuged at 500 g-force at 4 °C for 2 minutes.

Next, the samples were run in the Touch Real-Time PCR Detection System with an initial temperature of 95 °C for five minutes (to active the hot start enzyme) followed by 40 cycles of 95 °C for ten seconds, 60 °C for one minute. Readings were taken at the end of each cycle. Replicates were run for each reaction.

Primers were designed using primer 3 software and *Gadus morhua* gene sequences, reported in the Table 4. The primers *esr1*, *cyp1a* and *act2b* were previously used for *Gadus morhua* in Yadetie et al. (2018) and *vtgα* in Eide et al. (2014). These primers were compared by Fekadu Yadetie with polar cod sequences using blast sequence comparison software and found to match.

Table 4: Primers used for qPCR including primer sequence, name, symbol, *Gadus morhua* Ensembl ID and *Boreogadus saida* gene ID.

Name	Ensembl gene ID and B. saida ID	Primer sequence
Vitellogenin (*vtgα*)	ENSGMOG00000016966	5´-AGACTGGCCTGGTCGTCAAA
	OMLE01136441.1	5´-GCGAGGATAGAGGCAGGGAT
Estrogen receptor 1 (*esr1*)	ENSGMOG00000014898	5´-CGCTTTCGGATGCTCCAG
	OMLE01091583.1	5´-ACGAGAAGGCCCCAGAGTTG
Cytochrome P450 1A (*cyp1a*)	ENSGMOG00000000318	5´-CACCAGGAGATCAAGGACAAG
	OMLE01128479.1	5´-GCAGGAAGGAGGAGTGACGGAA
Beta-actin (*act2b*)	ENSGMOG00000009683	5´-CGACGGGCAGGTCATCACCATCG
	OMLE01037931.1	5´-CCACGTCGCACTTCATGATGCTGT

Fold change was calculated using the comparative Ct methodology outlined by Livak & Schmittgen (2001). Following this method, 2^{-Cq} was calculated with Cq values for the target gene (vtgα, *esr1* or *cyp1a*) and reference gene, *act2b*. Results were normalized by dividing the target gene results by the reference gene results. To calculate fold change, the average normalized result for control high-food ration and control low-food ration were calculated. Each result from a high food tank (exposed and control) was individually divided by the average result for high-food control. The same was completed for the low-food tanks.

The relative quantities (2^{-Cq}) of the reference gene, *act2b*, were compared between exposed and control groups using a linear mixed effect model (LME). This was completed in order to ensure that treatment did not significantly affect the reference gene used for normalization. Results showed that *act2b* did not differ significantly between exposed and control groups for Day 47 females (linear mixed effect model (LME), $p = 0.137$) Day 47 males (LME, $p = 0.853$), Day 131 females (LME, $p = 0.062$) or Day 131 males (LME, $p = 0.295$).

Each primer was tested using a standard curve created from liver samples from polar cod exposed to a compound that induces the gene of interest. For *cyp1a*, a pooled solution of individuals exposed to the WAF of crude oil from the current study was used. For *vtgα* and *esr1* samples were pooled from polar cod liver slices that were exposed to ethynylestradiol based on Yadetie et al. (2018). For each standard solution, eight RT reactions samples were pooled and serially diluted (1:2) with NFW. Using the standard curve, efficiency of the reaction was verified to be within 90 – 110 % and that the standard curve covered the range of levels observed in the samples being tested (Appendix D).

2.4.2 Transcriptome mapping with RNA sequencing

Five high-food control and five high-food exposed female samples from the Day 47 sampling were sent for RNA-Seq by Novogene (Novogene – Genome sequencing). All female RNA samples from the high-food treatment at the Day 47 sampling time-point were analysed using Agilent 2100 Bioanalyzer. Samples with an RNA intergrity value (RIN) value above seven (cut-off provided by Novogene) were chosen for RNA-Seq. If more than 5 samples had a sufficient RIN value, the samples with the highest values were selected first.

The RNA-Seq data were analyzed by Fekadu Yadetie using the recently developed workflow,

RASflow (Zhang & Jonassen 2020) and the well-annotated Atlantic cod *Gadus morhua* reference genome (Ensembl). False discovery rate (FDR) < 0.05 and fold change ≥ 2.00 (for up-regulated) or ≤ 0.67 (for down regulated) were considered differentially expressed. Mapping polar cod genes to the Atlantic cod *Gadus morhua* orthologs facilitated extraction of human *Homo sapiens* orthologs and zebrafish *Danio rerio* orthologs from the Ensembl database and pathway enrichment analysis as described in Yadetie et al. (2018).

2.5 Plasma hormone analyses

2.5.1 Plasma Extraction

A subset of plasma samples was selected to be representative of the exposure group, time point, food ration, and sex in the experiment. Steroid hormones extracted from plasma before being used in the enzyme-linked immunosorbent assay (ELISA). Standard plasma extraction was conducted following the procedure outlined in the ELISA protocol and Metcalfe et al. (2018). Plasma samples were thawed on ice and diluted with phosphate-buffered saline (PBS) to an initial dilution of 1:10 (w:w) to increase the sample volume to an adequate amount. Plasma was extracted by adding four times ethyl-acetate n-hexane solvent mixture (50:50) to each sample.

The samples were then vortexed twice for ten seconds and 80 % of the supernatant transferred to a glass vial. The same procedure was conducted three more times, and each time 90 % of the supernatant was removed. The resulting supernatant for each sample was allowed to evaporate in a heating module at 30 °C under a constant nitrogen stream for approximately 45 minutes. The resulting steroid hormones were reconstituted in 500 μl of ELISA buffer and stored at – 40 °C.

2.5.2 Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays were conducted for three hormones. The analytical procedure of the respective protocol was followed. Plasma extracts were diluted to various concentrations, so samples fell within the confined concentration range for the assay.

Each ELISA was conducted and analysed following the protocol supplied within each kit with the following specifications: all buffers and reagents were diluted or reconstituted in

MiliQ water; two dilutions were tested for each sample (Appendix E), with the exception of the E2 assays, where only undiluted samples were used due to the low levels of the hormone in the plasma extracts. Each 96-well plate contained the necessary controls, two blank wells (blk) representing the background absorbance caused by the Ellman's reagent. Two non-specific bindings (NSBs) representing the non-immunological binding of tracer to the well. Two maximum binding wells (B_0) representing the maximum amount of tracer that can be bound to the antibodies without any free hormone sample being added.

The plates were rinsed six times instead of five with ELISA wash buffer. During plate development the plate was covered and allowed to develop in the dark on a shaker for 60 – 120 minutes, until B_0 – blk was ≥ 0.3 absorbance.

The concentration of hormone in each sample was calculated from the absorbance readings. The average absorbance of the blk wells was calculated and subsequently subtracted from all wells. The average NSB and average B_0 was calculated and used to determine the corrected B_0 (B_0 – NSB). The $\%B/B_0$ was determined for all wells using the following equation:

$$\frac{\%B}{B_0} = adjusted\ absorbance - \frac{average\ \text{NSB}}{corrected\ B_0} \cdot\ 100$$

The $\%B/B_0$ for the standard replicates along with their corresponding concentration was input into Prism and used to create a standard curve. Concentrations were $\log_2$ transformed and the relationship between concentration and $\%B/B_0$ was fitted to a sigmoidal 4PL curve. Hormone concentration for each sample was then interpolated from this standard curve using $\%B/B_0$ value for each well and subsequently back transformed (2^X). The average sample concentration was calculated between duplicates and multiplied by the dilution factor. Samples were excluded from treatment comparison if $\%B/B_0$ was outside of the accurate detection range of the assay $20 > \%B/B_0 > 80$ or if samples dilutions had a disparity greater than 20 %.

2.6 Statistical Analysis

2.6.1 Linear mixed effect model

A linear mixed effect model (LME) is especially useful when the data is clustered, such as in tanks (Demidenko 2013; Laird & Ware 1982). This means that each measurement

taken (eg. fish weight) is not independent, but clustered into tank groups, making each tank, not each fish the independent variable (Demidenko 2013). The inclusion of the clustering variable, in this case tank, makes it possible to account for differences between the individual tanks and avoid pseudoreplication (Demidenko 2013).

Linear mixed effect model models include fixed predictor variables and random predictor variables. The fixed predictor variables are the ones that are of interested to the hypothesis and may impact the mean of the response variable. The random variables are not of interest for the hypothesis being tested but may influence the variance in the response variable. In the current study, an LME was chosen to test continuous response variables: gene expression, hormone level and GSI. All models employed null hypothesis testing with a cut-off p-value of 0.05. The order of predictors always was arranged in the model so that exposure was tested second to employ the most rigorous statistical test for the data.

To analyse the data using LME, a backward elimination method was employed. All factors were included in the initial model and non-significant predictors were subsequently removed one at a time until the most parsimonious model remained. An LME was set up to include the following: a response variable, two fixed predictor variable, food group and exposure group, an interaction term, and tank as the random predictor variable. In addition, reproductive phase was included as a random predictor for *vtgα* and *esr1* relative expression results. Excluding non-significant factors leads to the remaining groups having larger numbers of cases (n).

All figures are plotted using the results of the most parsimonious model (Section 3). Therefore, some plots include both food and exposure group, while others include only exposure or only food. This plotting style allows us to focus on meaningful effects.

2.6.2 Chi-squared test

Chi-squared (χ^2) can be used when the response variables under consideration are categorical. In the current study, this procedure was used to assess the effects of food group and exposure group on the reproductive phase of males and females. For females, reproductive phases were combined into two groups: females that had spawned and females that had not spawned. One immature female from the control high-food group was removed from the analysis because an immature female cannot differ in their reproductive phase.

For males, expected cell counts were not large enough to test either differences between reproductive phase or between individuals that had or had not spawned.

2.6.3 Generalized linear model

A generalized linear model (GLM) is useful in instances where the response variable is a proportion and normality cannot be assumed. For these reasons, a GLM with a binomial distribution (ranging from 0 to 1) was used to test the ovarian volume fraction occupied by each reproductive structure determined through Weibel-grid analysis. A test was conducted to assess whether the data was under or over dispersed and corrected for during analysis. A GLM model that incorporates a clustering factor, Generalized linear mixed model (GLMM) as with the LME model was not used due to the already complex model for the sample size.

3 Results

3.1 Mortality and general health

Seven polar cod died naturally during the experiment. None of these deaths were attributed to high levels of the WAF of crude oil. Several exposed polar cod displayed a lighter skin pigmentation within the first one to two months of exposure, however, no other external changes were observed.

3.2 Total PAH exposure over the experiment

Chemical analyses of water samples showed that the total PAH concentration ($\sum$ 44 PAHs) in exposed tanks started at an average of 12.5 $\mu g L^{-1}$ ($\pm$ 4.31 SD) and decreased over the experiment. In contrast, concentrations in control tanks remained low throughout (Figure 6). After 131 days of exposure, the control and exposed water samples had a similar total PAH concentration of 0.090 and 0.103 $\mu g L^{-1}$, respectively. Total PAH values and PAH composition over the experiment are presented in Appendix F and G, respectively.

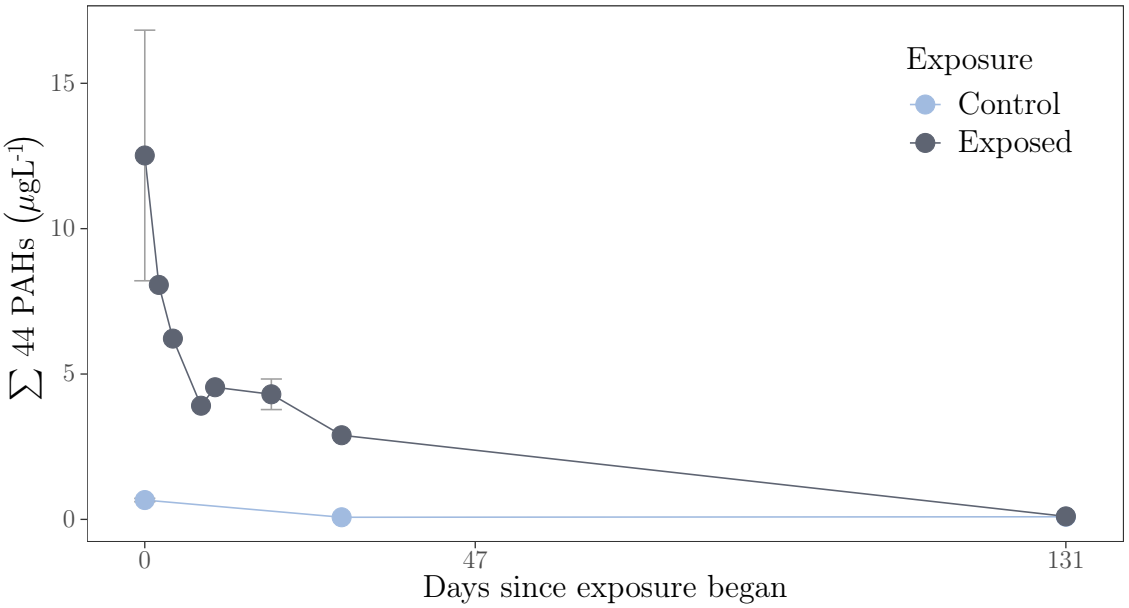


Figure 6: Mean and standard deviation of total PAH ($\sum$ 44 PAH) concentration (μgL^{-1}) in water samples taken from control (blue) and exposed tanks (gray) tanks over the experimental period. Exposed: n = 4, 1, 1, 1, 1, 4, 1, 1; Control: n = 2, 1, 1. Exposed tanks contained the water-accommodated fraction of crude oil.

3.3 Response of Cytochrome P450 1A to exposure

The fold-change in the relative expression of hepatic *cyp1a* was compared between exposure and food groups. Before exposure (Day 0), qPCR results verified that there were no significant difference in relative *cyp1a* expression between exposure groups (LME, $p = 0.281$ and $p = 0.490$) or food groups (LME, $p = 0.812$ and $p = 0.946$) for females or males, respectively.

After 47 days of exposure, relative expression of *cyp1a* was significantly higher in exposed females (LME, $p < 0.0001$, Figure 7A) and males (LME, $p < 0.0001$ Figure 7B). There was a significant interaction between exposure and food groups for both female (lme, $p = 0.05$) and male polar cod (lme, $p = 0.0001$) in comparison with controls. High-food exposed groups had the highest fold-change in *cyp1a*. Exposed female polar cod also had a higher fold-change in the relative expression of *cyp1a* compared to males. In addition, PAH metabolites were higher in exposed compared to control polar cod (Appendix H).

After 131 days of exposure, differences between exposed and control groups remained

significant for females (LME, $p = 0.0001$; Figure 7C) and males (LME, $p = 0.0002$, Figure 7D). However, this was at a lower fold-change compared to samples taken at Day 47. At this time, there was no longer a significant interaction with food group and exposure associated with relative *cyp1a* expression.

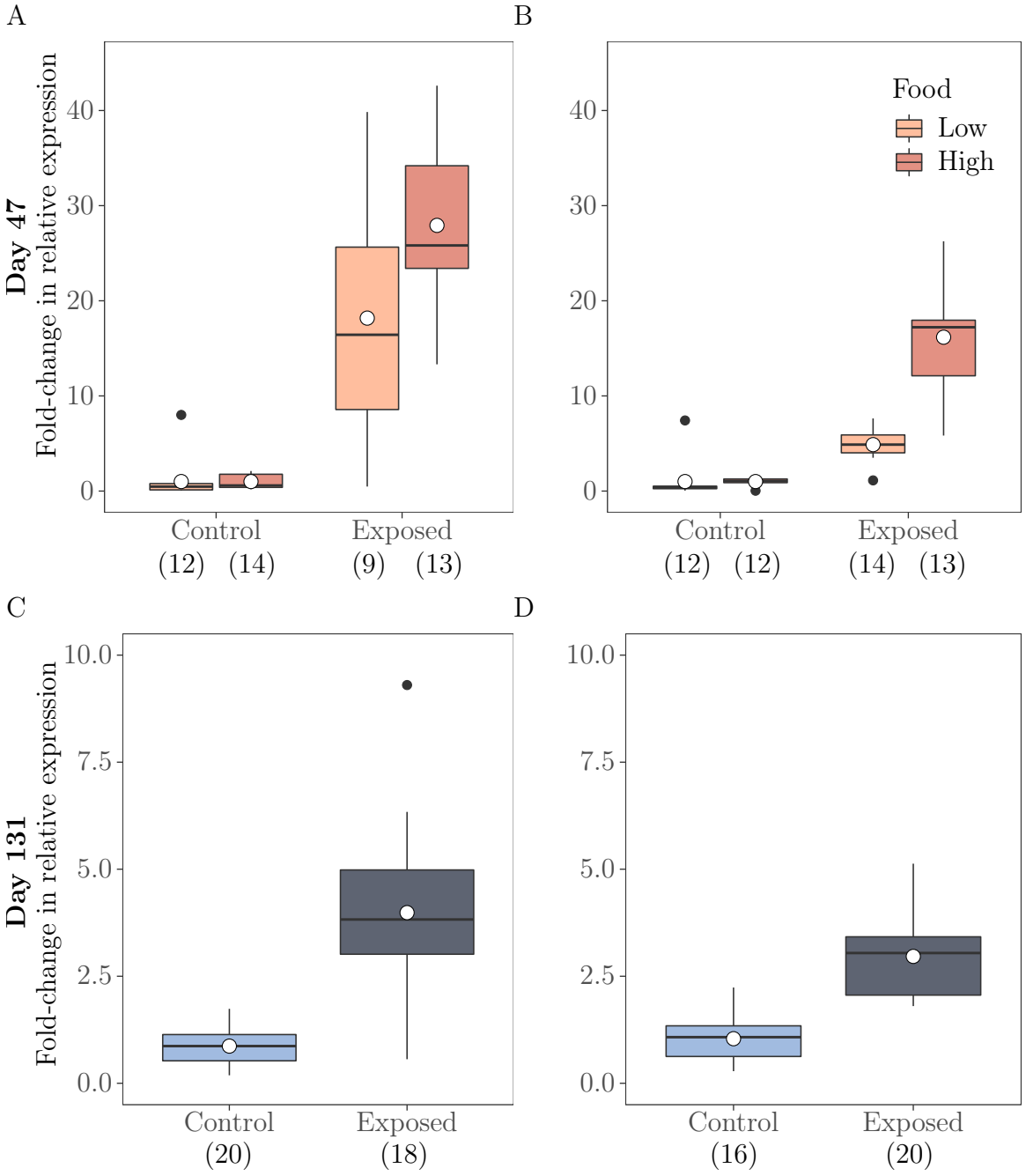


Figure 7: Fold-change in the relative expression of hepatic Cytochrome P450 1A (*cyp1a*) after 47 days of exposure in female (A) and male (B) and 131 days of exposure in (C) female and (D) male polar cod. Most parsimonious models are plotted. Exposed samples were exposed to the water-accommodated fraction (WAF) of crude oil, control samples were not. Low and high food treatments received 1.5 % somatic weight day^{-1} and 4 % somatic weight day^{-1}, respectively. Whiskers represent the minimum and maximum values (without outliers), lower box limit - Q1, middle - median, upper limit - Q3, white circle - mean, black dots - outliers (1.5 times the interquartile range away from the upper and lower limit), numbers in parenthesis - sample size (n).

3.4 Changes in gonadosomatic index during the experiment

Both female (LME, $p < 0.0001$; Figure 8A) and male (LME, $p < 0.0001$; Figure 8B) GSI significantly changed over the experiment. In both sexes, GSI was low at the beginning of the experiment (Day 0) and increased near spawning (Day 47). It decreased to its lowest value in the post-spawning phase (Day 131). Exposure was not significantly associated with either, female (LME, $p = 0.112$) or male GSI (LME, $p = 0.918$) after reproductive phase was taken into account. Further testing revealed that male GSI did not differ significantly between the Day 47 and Day 131 sampling time-point (t-test, $p = 0.176$).

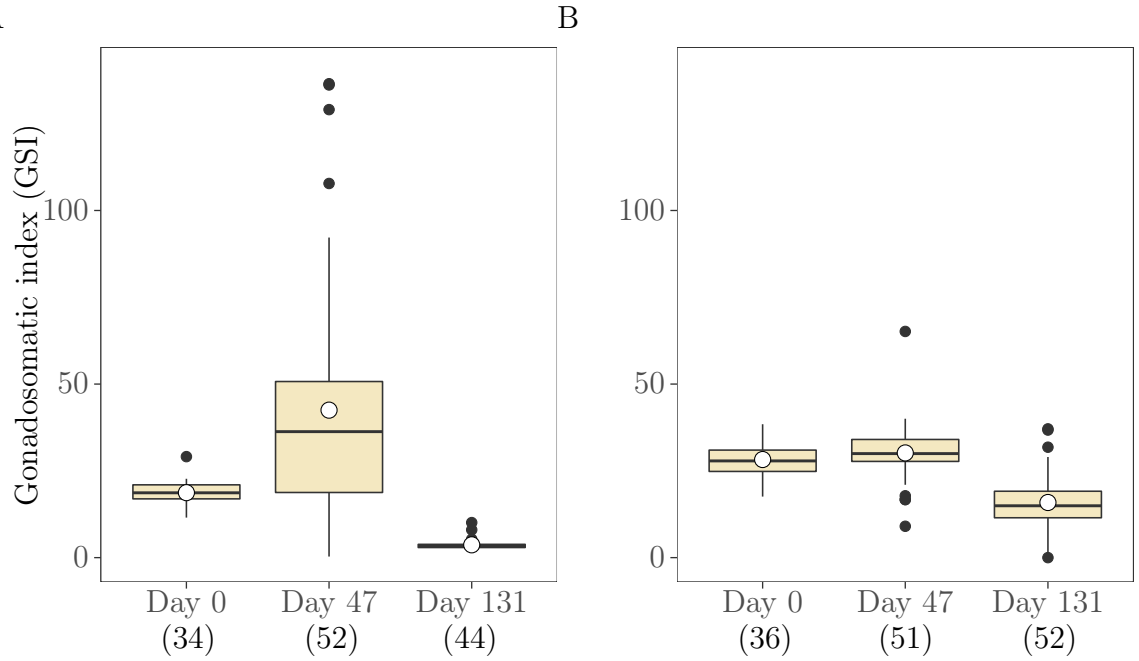


Figure 8: Gonadosomatic index GSI (gonad weigh/somatic weight) for female (A) and male (B) polar cod over the sampling time-points (Day 0, 47, and 131). Most parsimonious model plotted. Whiskers represent the minimum and maximum values (without outliers), lower box limit - Q1, middle - median, upper limit - Q3, white circle - mean, black dots - outliers (1.5 times the interquartile range away from the upper and lower limit), numbers in parenthesis - sample size (n).

3.5 Gonad histology

3.5.1 Alterations in the timing of reproductive development

Before exposure began (Day 0), females were all in the same phase of reproduction (spawning capable phase), with VTG3 oocytes (Figure 9A). After 47 days of exposure, the females sampled were no longer all in the same reproductive phase. Females were present in four different phases, with 53 % having already spawned (regressing). After 131 days of exposure, females sampled were in two different reproductive phases, either the regenerating or the early developing phase . Ovarian histology pictures for each stage are presented in Appendix I.

Before exposure (Day 0), all males were also in the spawning capable phase (figure 9B). However, different sub-phases of gonadal development. After 47 days of exposure, males were predominately still in the spawning capable phase (mid or late germinal epithelium (GE)), with only 9 % having spawned (regressing phase). This is notable in light of the finding that more than half of the females had already spawned at this sampling time-point. Testis histology pictures for each stage are presented in Appendix I

After 131 days of exposure, 19 % of males still had not spawned (late-GE). However, by this time, the majority of males had spawned and were present in the regressing or regenerating phases. At this time males were present in earlier reproductive phases compared to the females with only 6 % of males present in the regenerating phase and 35 % of females. Moreover, most females had already passed the regenerating phase.

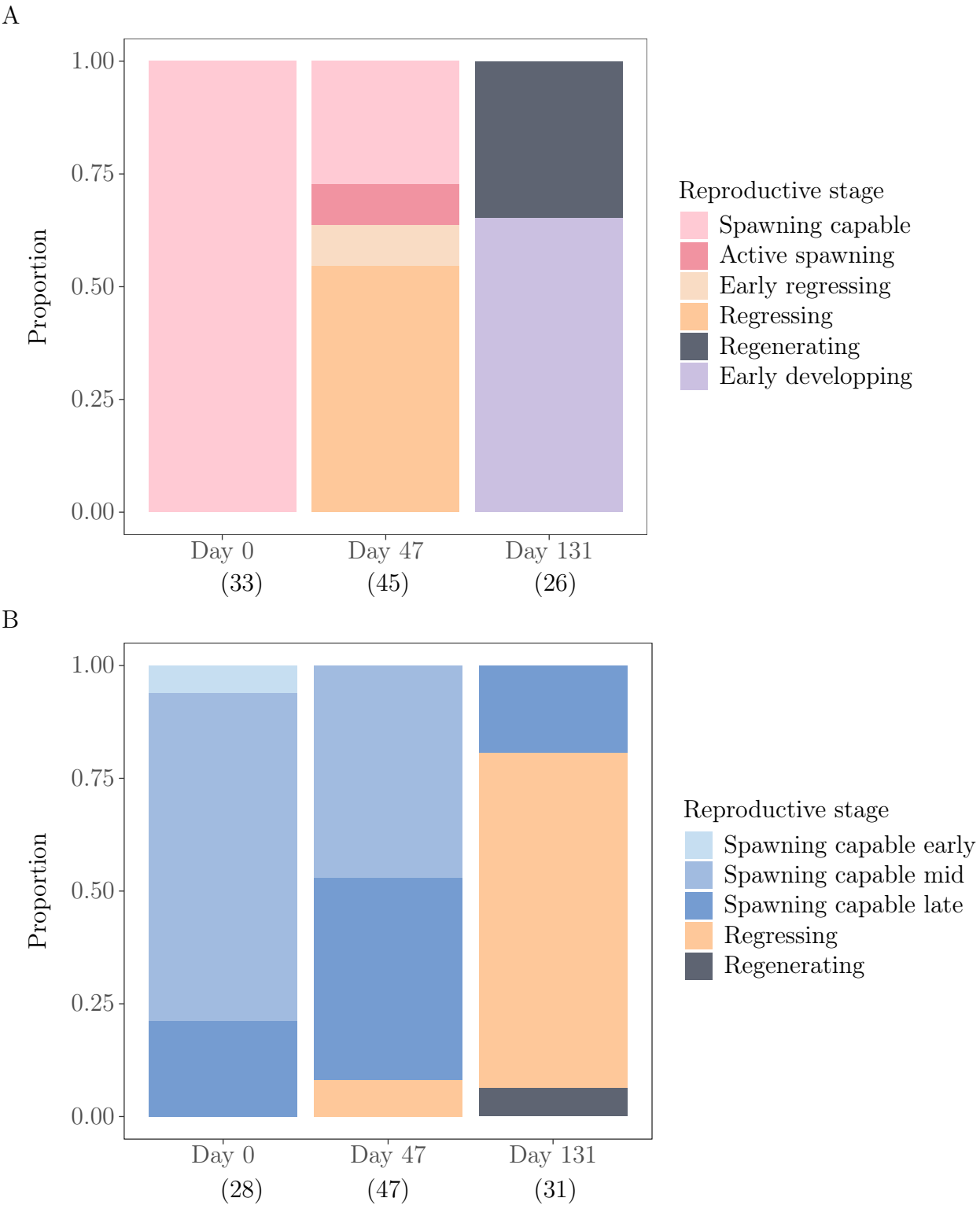


Figure 9: Reproductive stage of A) female and B) male polar cod from exposure and food groups sampled at Day 0, 47 and 131 of the experiment. Exposed samples were exposed to the water-accommodated fraction (WAF) of crude oil, control samples were not. Low and high food treatments received 1.5 % somatic weight day^{-1} and 4 % somatic weight day^{-1}, respectively. Stages based on Brown-Peterson et al. (2011). Counts represented in parenthesis (n).

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As mentioned above, after 47 days of exposure, females were distributed across four different reproductive phases. These differences were assessed in terms of exposure and food groups. Results indicated that reproductive phase differed significantly between exposure groups (χ^2, $p = 0.002$; Figure 10A). A greater proportion of female polar cod in the exposed group had already spawned compared to control females.

Due to the low sample size, food and exposure groups could not be tested in the same statistical model. However, food alone did not have a significant effect on the phase of reproduction (χ^2, $p = 0.057$). Moreover, an interaction effect could not be tested because there were no cases in select categories (spawning capable and active spawning in the exposed low-food group).

Comparison after 131 days of exposure indicated exposed females no longer showed a trend of more advanced reproductive phases compared to controls (Figure 10B). Due to the few females in the regenerating phase at this sampling time-point, the significance of this trend could not be tested.

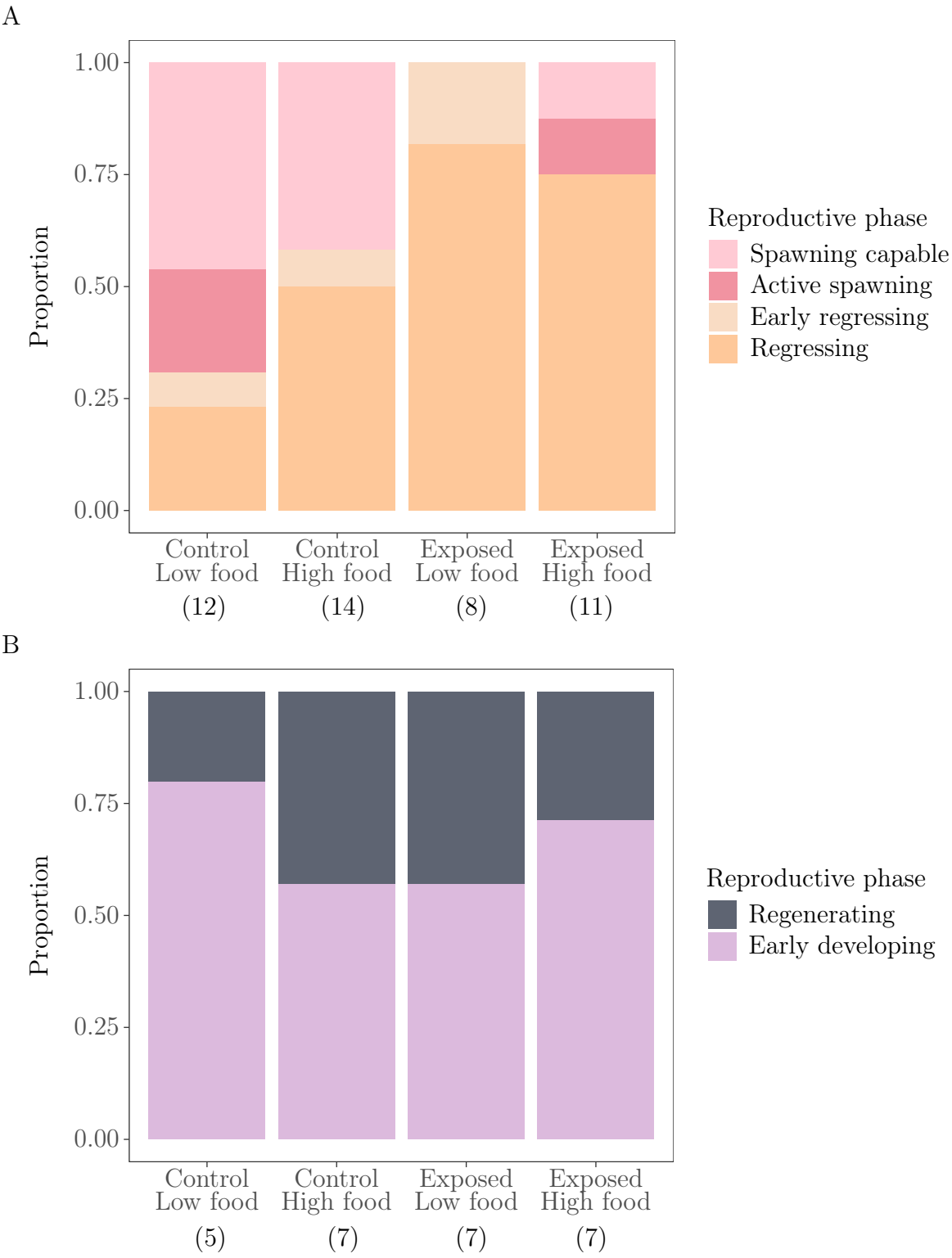


Figure 10: The proportion of female polar cod in each reproductive phase between treatment groups at A) 47 and B) 131 days after exposure. Exposed groups were exposed to the WAF of crude oil, control were not. Low food groups received a food regime of 1.5 % somatic weight day^{-1} and high food regimes received 4.5 % somatic weight day^{-1}. Numbers in brackets represent counts (n).

Differences in the reproductive phase of males could not be assessed due to low sample sizes in particular groups. Notably, at the Day 47 sampling time-point, the only males that had spawned (regressing) were in exposed tanks (Figure 11A) and at 131 days all males in the most advanced phase of development (regenerating) were also from exposed tanks (Figure 11B).

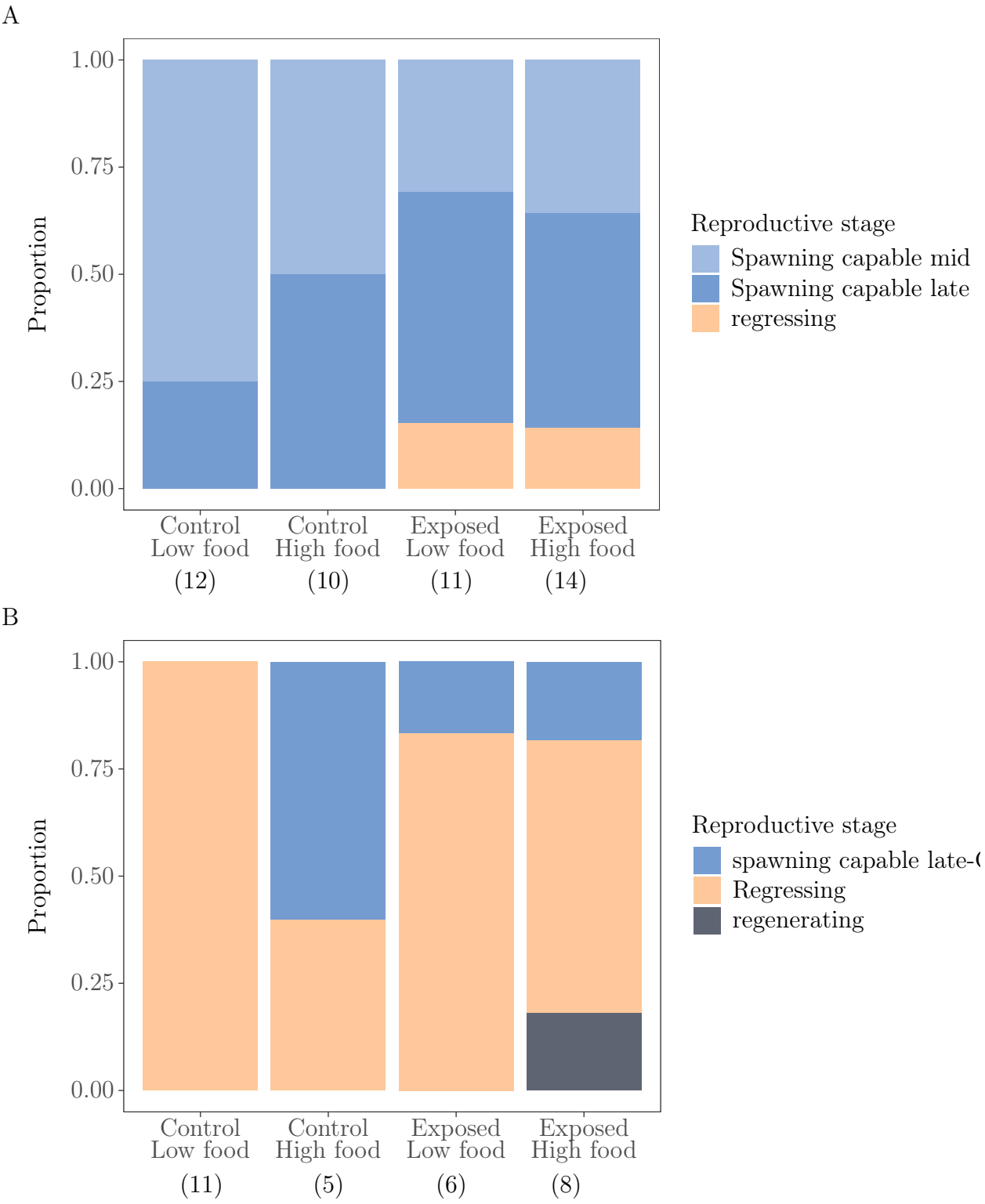


Figure 11: The proportion of male polar cod in each reproductive phase between exposure and food groups groups A) 47 and at B) 131 days after exposure. Exposed groups were exposed to the WAF of crude oil, control were not. Low food groups received a food regime of 1.5 % somatic weight day^{-1} and high food regimes received 4.5 % somatic weight day^{-1}.

3.5.2 The ovarian volume fraction occupied by atresia

Weibel-grid analysis was conducted to compare the ovarian volume fraction occupied by alpha and beta atresia. Reproductive phase had a significant impact on beta atresia (GLM, $p = 0.004$; Figure 12), but not on alpha atresia (GLM, $p = 0.180$). Similarly, exposure had a significant impact on the ovarian volume fraction occupied by beta atresia (GLM, $p = 0.004$), but not alpha atresia (GLM, $p = 0.705$). A full list of the results from Weibel-grid analysis, including the volume occupied by other reproductive structures is shown in Appendix J.

The effect of stripping on the levels of atresia was also assessed. Conducting this test was relevant due to the differences in spawning readiness observed between exposed and control groups that could potentially have led to differences in the efficacy of stripping (Figure 10). Stripping was not significantly associated with either alpha (GLM, $p = 0.721$) or beta atresia (GLM, $p = 0.234$) between individuals that were or were not able to be stripped.

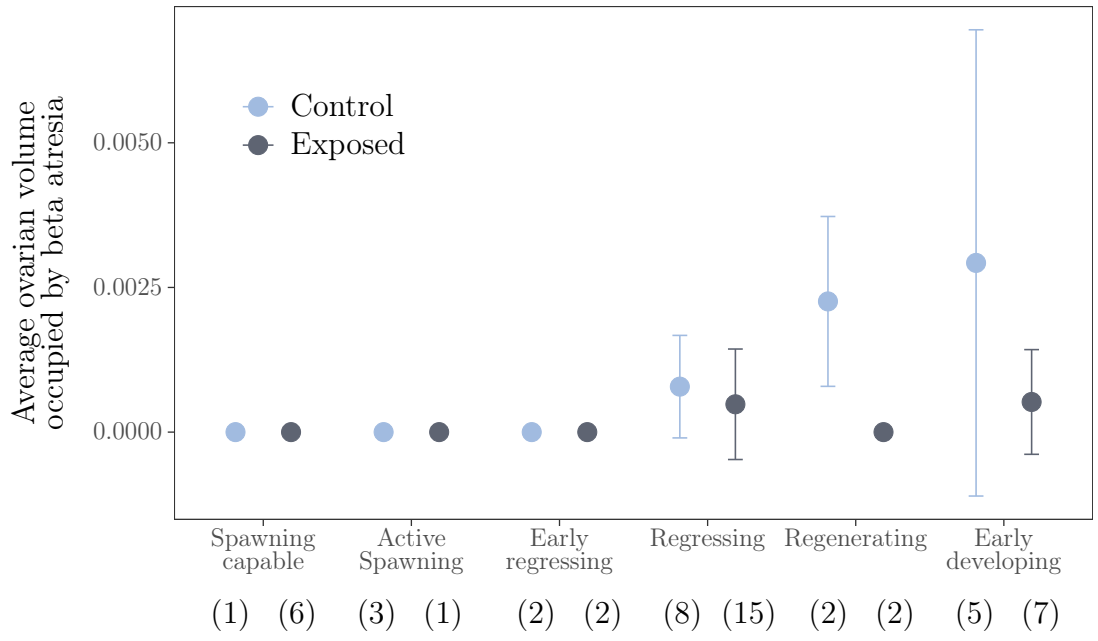


Figure 12: Mean and standard deviation of ovarian volume fraction occupied by beta atresia. Counts are reported in parentheses (n). Reproductive phases were based on Brown-Peterson et al. (2011).

3.6 Plasma hormone analyses

After 47 days, there was no significant difference between levels of 11-KT and T in males (Figure 13). The concentration of 11-KT showed a lower mean in exposed males compared to control, however, this difference was not significant (Figure 13A; LME, $p = 0.112$). Mean levels between exposure groups were similar for T and there were no significant differences between exposure groups (Figure 13B; LME, $p = 0.714$).

Unlike males, differences in plasma hormones between exposure and food groups could not be assessed in females. At the Day 47 sampling time-point, few female plasma samples had sufficient levels of E2 to fall within the range of the assay. Moreover, female plasma samples for 11-KT and T did not pass the quality control specifications outlined in the Cayman ELISA protocol (disparity < 20 % between dilutions). The levels of these hormones between reproductive phases are, however, reported in Appendix K.

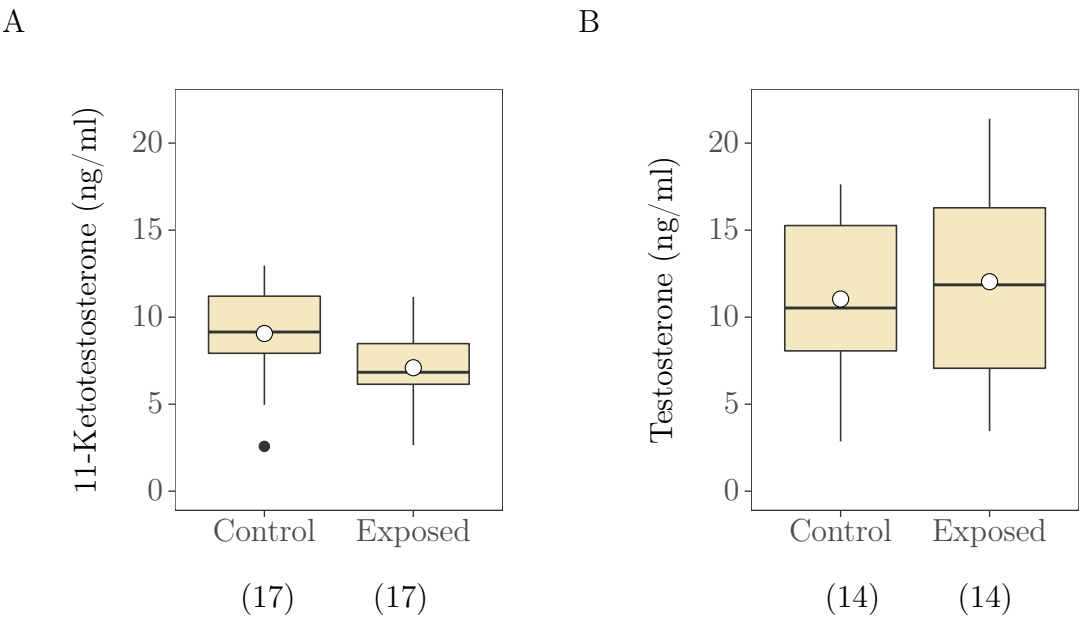


Figure 13: Level (ng/ml) of A) 11-Ketotestosterone and B) Testosterone in plasma extracts from male polar cod at Day 47 of the experiment. Most parsimonious model plotted. Exposed polar cod were exposed to the water-accommodated fractionWAF of crude oil for 47 days before sampling and control samples were not exposed to the WAF. Whiskers represent the minimum and maximum values (without outliers), lower box limit - Q1, middle - median, upper limit - Q3, white circle - mean, black dots - outliers (1.5 times the interquartile range away from the upper and lower limit), numbers in parenthesis - sample size (n).

3.7 Gene expression

3.7.1 Changes in vitellogenin alpha and estrogen receptor 1 expression

Fold-change in the hepatic relative expression of *vtgα* and *esr1* differed between exposure groups at the 47 and 131 day sampling time-points for females but not males (Figure 14). After 47 days, exposure group was significantly associated with the fold-change in relative expression of *esr1* (LME, $p = 0.046$; Figure 14A) and *vtgα* (LME, $p = 0.047$; Figure 14B). Both genes had a lower fold-change in the exposed group.

However, *esr1* and *vtgα* expression after 47 days of exposure also varied with reproductive phase. Females with the highest levels of *esr1* and *vtgα* were in the earliest reproductive phase (spawning capable) and all were in the control group. When stage was taken into account in the statistical model (as a random predictor variable), exposure was no longer significant for either *vtgα* (LME, $p = 0.760$) or *esr1* (LME, $p = 0.292$).

After 131 days of exposure, even after stage was accounted for, an interaction between food groups and exposure groups was significantly associated with the fold-change in *esr1* relative expression (LME, $p = 0.021$, Figure 14D). For *vtgα*, exposure group was significantly associated with the fold-change in relative expression and this was irrespective of stage (LME, $p = 0.025$; Figure 14D).

In males, after 47 days of exposure, neither exposure group nor food group was significantly associated with the relative expression of *vtgα* (LME, $p = 0.148$) or *esr1* (LME, $p = 0.189$). Similarly, there were no significant differences in *vtgα* (LME, $p = 0.592$) or *esr1* (LME, $p = 0.411$) after 131 days.

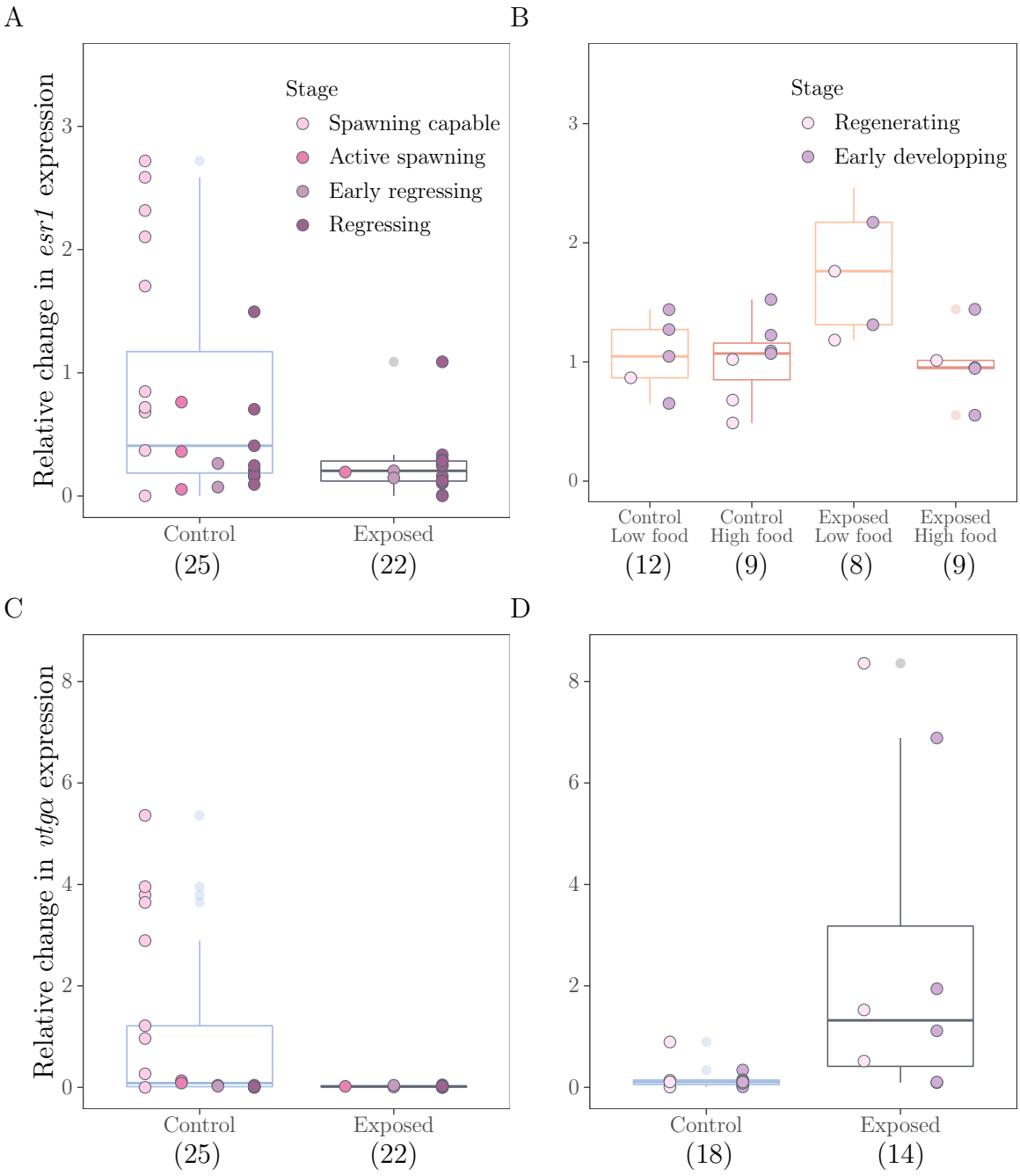


Figure 14: Fold-change in the relative expression of of *vtgα* after A) 47 days and B) 131 days of exposure and *esr1* after C) 47 and D) 131 days of exposure in female polar cod. Most parsimonious model represented. Exposed females were exposed to the water-accommodated fraction WAF of crude oil, control females were not. Low and high food treatments received 1.5 % somatic weight day^{-1} and 4 % somatic weight day^{-1}, respectively. Reproductive phases were based on Brown-Peterson et al. (2011). Whiskers represent the minimum and maximum values (without outliers), lower box limit - Q1, middle - median, upper limit - Q3, black dots - outliers (1.5 times the interquartile range away from the upper and lower limit), counts are in parenthesis (n).

3.7.2 Differentially regulated genes in female polar cod liver

Read-counts generated from RNA-Seq were mapped to the *Gadus morhua* genome. Results showed that 947 genes were differentially regulated (false discovery rate (FDR) value < 0.05), 416 genes were down-regulated and 531 genes were up-regulated between female polar cod exposed to the WAF for 47 days and controls.

Hierarchical clustering of *Danio rerio* orthologs was conducted in order to visualize the top differentially expressed genes (Figure 15). Some up-regulated genes of note include *cyp1a* and *cyp27b1*. For down-regulated genes, Zona pellucida glycoprotein 2, tandem duplicate 3 (zp 2.3) and si:dkey-4c23.3 (vtg1-1) are of note within the current scope of this thesis.

As with the qPCR gene results, reproductive phase may have also an impact on the differentially expressed genes. The fifth control individual appears as an outlier, more closely resembling the exposed females. This female was in the regressing reproductive phase, whereas the rest of the controls were in spawning capable or an unknown phase. All exposed females used in RNA-Seq were either in the regressing or unknown stages.

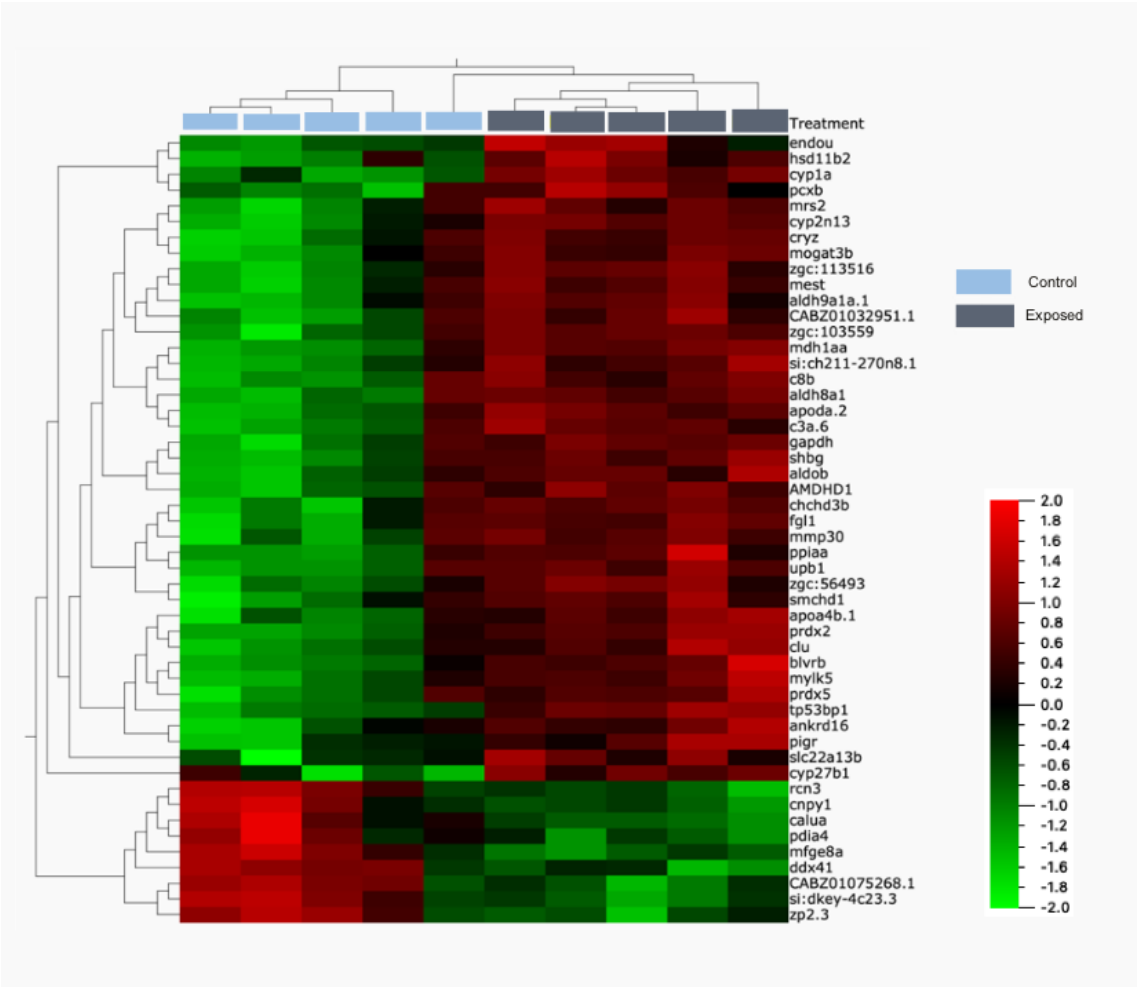


Figure 15: Hierarchical clustering of the top differentially expressed (DE) *Danio rerio* ortholog genes (FDR < 0.05, minimum fold-change of 2) between 5 WAF-exposed (gray) and 5 control (blue) polar cod females 47 days after exposure began. Log2-transformed normalized counts of RNA-Seq data was used in two-way clustering (with average linkage) using Qlucore OMICs Explorer. Relative expression levels are shown by the color scale bar (bottom right), where red and green ends represents highest and lowest relative expression, respectively. Rows represent genes and columns represent samples.

Homo sapiens orthologs of the top differentially regulated genes (FDR value < 0.05) were used to identify significantly enriched pathways by mapping the genes to WikiPathways (Table 5) and KEGG (Table 6) databases. Some of the top enriched biological process (BP) pathways were ovarian infertility and steroid biosynthesis genes. Furthermore, among the enriched pathways and of interest for the framework of this thesis were the oxidative stress pathway (above significance) and AhR signaling pathway. Genes contributing to the enrichment of the ovarian infertility pathway the up-regulation of zona pellucida

protein (ZP2 and 3), prolactin recepor (PRLR), and aromatase (CYP19A). Out of the eight genes involved in the enrichment of the steroid biosynthesis pathways, five were related to cholesterol with the four genes involved in cholesterol biosynthesis being down regulated NSDHL, SOAT1, DHCR24, TM7SF2.

Table 5: Significantly enriched biological pathways (BP) after mapping human orthologs to the Wiki database. Results are from RNA-Seq analysis of 10 high food (4 % somatic weight day^{-1}) females after 47 days of exposure. Five females were exposed to the water-accommodated fraction of crude oil and five were control.

Pathway	Adjusted *p*-value	Genes
Nuclear Receptors Meta-Pathway	4.82e-03	CDKN1B, ALAS1, MGST3, MGST1, SLC2A1, GSTT2, SLC2A3, SLC2A5, SLC5A5, PRDX1, HES1, JUNB, SLC19A2, SLC2A11, VDR, SLC6A15, MYOF, AIP, SLC39A14, ESR1, SLC5A9, CYP1A1, AHRR, BLVRB, MFGE8, GCLM, CPEB4, FKBP5
Glycolysis and gluconeogenesis	4.95e-03	PFKL, PC, GOT1, MDH1, SLC2A1, SLC2A3, ALDOB, SLC2A5, GAPDH
Selenium micronutrient network	6.94e-03	CRP, GPX6, SERPINE1, DIO2, DIO3, SELENOF, KMO, SOD1, PRDX1, SELENOP, APOB, SEPHS2, LDLR
Ovarian infertility genes	1.56e-02	CDKN1B, VDR, ZP3, CYP19A1, PRLR, ZP2, SYNE2
Aryl hydrocarbon receptor pathway	2.29e-02	CDKN1B, MYOF, MGST1, AIP, CYP1A1, AHRR, HES1, JUNB
Ferroptosis	3.84e-02	TF, MAP1LC3A, MAP1LC3C, LPCAT3, ACSL6, GCLM, SLC39A14
Amino acid metabolism	3.93e-02	ACLY, BHMT, PC, PPM1L, GOT1, MDH1, MAOA, ASNS, EPRS, GCLM, ADH5
Oxidative stress	6.00e-02*	MAOA, MGST1, CYP1A1, GSTT2, JUNB, SOD1

Table 6: Significantly enriched biological pathways (BP) after mapping human orthologs to the KEGG database. Results are from RNA-Seq analysis of 10 high-food (4 % somatic weight day-1) females after 47 days of exposure. Five females were exposed to the water-accommodated fraction of crude oil and five were control

Pathway	Adjusted p-value	Genes
Steroid biosynthesis	5.20e-05	SQLE, CYP27B1, EBP, NSDHL, SOAT1, DHCR24, CEL, TM7SF2
Vitamin digestion and absorption	2.71e-03	LMBRD1, FOLH1, RBP2, SLC23A1, APOA4, APOB, SLC19A2
Glycine, serine and threonine metabolism	8.91e-03	GAMT, GATM, BHMT, ALAS1, MAOA, PIPOX, PHGDH, AGXT
Protein processing in endoplasmic reticulum	9.74e-03	ERO1A, BCAP31, ERO1B, HSPA8, XBP1, LMAN1L, EDEM1, EIF2AK3, DERL1, SYVN1, RRBP1, PDIA4, HERPUD1, CAPN2, SEC61B, MAP3K5, SEC31A
Glycerolipid metabolism	2.25e-02	PNLIPRP2, DGAT2, TKFC, MOGAT3, AKR1A1, PNPLA3, CEL, DGKH, ALDH9A1
Ferroptosis	2.92e-02	TF, MAP1LC3A, MAP1LC3C, LPCAT3, ACSL6, GCLM, SLC39A14
Fat digestion and absorption	2.92e-02	PNLIPRP2, FABP2, DGAT2, MOGAT3, APOA4, CEL, APOB
Tryptophan metabolism	2.97e-02	TPH2, MAOA, CYP1A1, KYAT3, KMO, ALDH8A1, ALDH9A1
Pancreatic secretion	3.21e-02	PNLIPRP2, CPA1, CELA3B, CELA2A, CPB1, CCKAR, CTRL, AMY2B, RAB3D, CTRB1, CEL
Cysteine and methionine metabolism	4.73e-02	BHMT, GOT1, MDH1, DNMT3B, KYAT3, GCLM, CDO1
Protein digestion and absorption	4.78e-02	SLC8A3, CELA2A, CPA1, CELA3B, CPB1, CTRL, XPNPEP2, CTRB1, COL4A3, SLC16A10

4 Discussion

4.1 Summary of the study

This study aimed to assess how an oil spill occurring during polar cod´s spawning period may affect reproductive endpoints on several levels of biological organization. Wild-caught polar cod were exposed to the WAF of Goliat crude oil for 131 days over their spawning period. Endpoints were assessed before exposure began, Day 0, during the spawning window, Day 47, and during the post-spawning period, Day 131. Results indicate that exposure to the WAF of crude oil for 47 days led to an advancement in female gonadal development and changes in the expression of genes involved in reproduction compared to controls. In males, there was no effect of WAF on the reproductive endpoints measured.

4.2 Total PAH and Cytochrome P450 1A induction

Chemical analysis revealed that the total PAH concentration in water from exposed tanks started at approximately 12.5 $\mu g L^{-1}$ ($\sum$ 44 PAHs) and decreased over the experiment. Decreasing PAHs over time demonstrate that polar cod were exposed to weathering crude oil as described previously (Carls et al. 2000; Heintz et al. 1999; Nahrgang et al. 2010b). We recognize that PAHs only account for, on average, less than 1 % of crude oil (J Meador & Nahrgang 2019). However, total PAHs are used here as a proxy for WSF exposure.

There was a significant increase of *cyp1a* expression and higher levels of PAH metabolites after 47 days of exposure. This indicates the up-regulation of hepatic biotransformation processes and the bioavailability of PAHs and other compounds found in the crude oil to polar cod. High-food exposed polar cod had the highest relative expression of *cyp1a*. The difference may be explained by increased food ingestion leading to associated ingestion of WAF.

The results from RNA-Seq analysis of females after 47 days concur with the qPCR results with significant up-regulation of *cyp1a*. In addition, these results indicate the enrichment of gene pathways associated with xenobiotic exposure, including oxidative stress and the AhR signalling pathways. Both have been shown *in-vivo* in polar cod liver by Song et al. (2019) and in Atlantic cod liver following *ex-vivo* BaP exposure (Yadetie et al. 2018).

After 131 days of exposure, the level of PAHs in the exposed tanks reached the lowest value during the experiment (0.103 $\mu g L^{-1}$, $\sum$ 44 PAHs). However, relative *cyp1a* expression remained significantly higher in exposed polar cod compared to control, although at a lower fold-change compared to Day 47. This indicates that even when exposure to oil-related compounds has been reduced to control levels, *cyp1a* expression remains elevated for a some time. Similar results were indicated by Nahrgang et al. (2010) where hepatic EROD activity in polar cod remained elevated after 2 weeks of depuration following a 4 week exposure to the WSF of crude oil (Nahrgang et al. 2010b).

4.3 An advancement of reproductive development

Gonadal development was more advanced in most female polar cod exposed to the WAF of crude oil compared to control females. After 47 days, 90 % of exposed females had already spawned compared to 56 % of the control females.

A previous study reported advancement of gonadal development following WAF of crude oil exposure in female starry flounder (Whipple et al. 1978). However, most studies have reported a pause, delay, or arrest of ovarian maturation following exposure to crude oil, PAHs, petroleum compounds, or polluted sites in polar cod and other teleosts (Bender et al. 2018; Bugel et al. 2010; Casillas et al. 1991; Johnson et al. 1988; Meier et al. 2007; Thomas & Budiantara 1995). Other studies have reported no effect on gonadal development following exposure to crude oil or petroleum compounds (Bender et al. 2016; Johnson et al. 1994).

Differences between gonadal development results between the current and previous studies may be attributable to one or a combination of the following factors: 1) differences in the timing of exposure during the reproductive cycle, 2) differences in the species biology and life-history traits, or, 3) differences in experimental design.

The phase of reproductive development when exposure occurs is the most likely explanation for the variations we see between our findings and the previous studies. The timing of exposure during the reproductive cycle has been reported as an important determinant of the changes in reproductive endpoints following exposure (Kime 1995; Tintos et al. 2006).

Unfortunately, several studies fail to report the reproductive phase of individuals at the

start of exposure. Moreover, the large variation in terminology used historically, for different species and the lack of complete definitions makes comparison across studies difficult.

That being said, from the studies with clear reporting, most fish seem to have been exposed before or during vitellogenesis (Bender et al. 2018, 2016; Johnson et al. 1988, 1994; Thomas & Budiantara 1995). All of the females in the current study were already in late vitellogenesis when the exposure began.

Similarly,Whipple et al. (1978) exposed *Platichthys stellatus* during a late stage of maturation and saw an advancement in gonadal development. In these instances, exposure-caused changes in, for example, Vtg levels may not have the same impact as would be expected during earlier phases of vitellogenesis because most of the necessary yolk has already been accumulated (Navas & Segner 2000).

Furthermore, females may have passed the point where arresting or delaying the current reproductive cycle by massive atresia or pause was biologically likely or advantageous. As previous work has suggested that a critical period may exist before early development for oocyte development to be arrested (Rideout et al. 2000).

Another possible explanation may be species-related differences in biological sensitivity, xenobiotic metabolism or life history. The studies conducted in Puget Sound found that *Parophrys vetulus* from exposed sites were less likely to go through gonadal maturation (Casillas et al. 1991; Johnson et al. 1988), however there was no significant difference in the maturation of *Pseudopleuronectes americanus* from the same sites (Johnson et al. 1994). The authors of these studies noted that the differences in findings may be due to species-specific differences.

A study on polar cod gonadal development found a delay in gonadal development following acute burned oil exposure (Bender et al. 2018). The finding indicates that the advancement of gonadal development observed in the current study may not be due to species specific differences in polar cod.

That being said, polar cod's short lifespan may play a role in the current findings. A delay or arrest in reproductive development may only be beneficial if there is a future possibility of reproduction (Javoiš 2013). Polar cod in the current study were roughly

4 years old. In the wild, polar cod rarely age beyond 5 years old (Mueter et al. 2016). Therefore, the possibility of future reproductive success may be lower than in longer-lived species (Javoiš 2013).

Lastly, variations in study findings may be partially related to the differences seen in exposure compounds and experimental design. The experimental work conducted on polar cod were different in their exposure design (Bender et al. 2018, 2016). Bender et al. (2016) exposed polar cod to chronic Goliat crude oil from early development to post-spawning and found no effect on the timing of reproductive development. However, the authors used a dietary exposure which makes it impossible to directly compare the exposure doses due to different toxicokinetics in the fish upon exposure.

Furthermore, Bender et al. (2018) found a delay in reproductive development in polar cod. However, the researchers used acute burned oil exposure followed by seven months of depuration. These studies (Bender et al. 2018, 2016) demonstrate that even in the closely related experiments there are still enough variations in the design to impede direct comparison.

Differences in the reporting methods for the dose of exposure makes comparison across studies more difficult (J Meador & Nahrgang 2019). In addition, The use of PAHs and other petroleum components versus whole crude oil as well as different crude oil sources with varying composition (Faksness et al. 2008; Murillo et al. 2016).

After 47 days, 90 % of the females were regressing but only 9 % of the males were visibly regressing. All the males that had spawned were in the exposed group. From the results, it is unclear whether there was a difference in the coincidence of male and female spawning between exposed versus control groups. Even though males were present in different reproductive phases, all were able to be stripped. Another notable finding from the males was the presence of males still in late gonadal development during the last sampling, approximately 84 days after the majority of females had spawned.

The results from Weibel-grid analysis showed the ovarian volume fraction occupied by alpha and beta atresia. Beta atresia was significantly higher in exposed compared to control group. This is contrary to previous studies linking crude oil, PAH, petroleum compounds, and other xenobiotic exposure to an increase and or no change in atresia

(Johnson et al. 1994; Kime 1995; Rideout et al. 2005; Sundt & Björkblom 2011; Thomas & Budiantara 1995).

The difference in the volume fraction of beta atresia between exposed and control females may be linked to the variations in the timing of spawning. In exposed individuals, females on average spawned earlier, before the 47 day sampling time-point, meaning that any left-over oocytes that became atretic would have had a longer time to be reabsorbed compared to control fish. Atresia was notably very low in all individuals irrespective of the reproductive phase and mostly consisted of residual eggs being reabsorbed.

4.4 Alterations in gene expression

In female polar cod, both *vtgα* and *esr1* genes were found to be down-regulated after 47 days in exposed groups. This finding is in line with previous work demonstrating that crude oil, PAHs, and petroleum compounds may lead to inhibition of estrogen-induced gene expression. Several laboratory and field studies have reported a decrease in Vtg (protein or messenger RNA (mRNA)) (Anderson et al. 1996a; Bugel et al. 2010; Meier et al. 2007; Navas & Segner 2000; Yadetie et al. 2018) and ERα (protein and mRNA) (Bilbao et al. 2010; Salaberria et al. 2014; Yadetie et al. 2018).

The anti-estrogenic effects of AhR agonist exposure has been attributed to the cross-talk between AhR and ERα, for which several mechanistic hypotheses have been previously presented (Anderson et al. 1996a; Navas & Segner 2000; Nicolas 1999; Safe & Wormke 2003; Yadetie et al. 2018).

Although our results of a decrease in *vtgα* and *esr1* after 47 days of exposure are in keeping with previous knowledge on the anti-estrogenic effects of AhR agonists, the decreases we observe may be due to indirect effect of significant reproductive phase differences between exposed and control groups.

E2-mediated changes in *esr1* and *vtgα* expression have been shown to follow seasonal cycles corresponding to reproductive changes, with *vtgα* and *esr1* expression peaking during vitellogenesis and falling before spawning, mediated by high levels of E2 (Nagler et al. 2012; Nelson & Habibi 2013; Sabo-Attwood et al. 2004). For this reason, reproductive phase before and after spawning may have a large impact on the expression levels of

reproductive genes *esr1* and *vtgα*.

This point is further supported by our results that show higher levels of *vtgα* and *esr1* in females that are in the spawning capable phase, in a late phase of vitellogenesis. Due to the significant differences in the reproductive phase of females, there is an unequal number of females in the spawning capable phase between the exposed and control groups (ten spawning capable females in control and zero in exposed). These 10 females inflate the mean in the control group. Moreover, this point is supported by the fact that when reproductive phase was included in the statistical model, exposure is no longer significant. Furthermore, it should be noted that this is not to say that there may not be anti-estrogenic effects of crude oil exposure but due to the differences between reproductive phase we cannot make any definitive conclusions to this end.

After 131 days of exposure, *vtgα* expression was significantly higher in exposed compared to control groups, and *esr1* did not differ significantly between any treatment groups. At this time, variations between reproductive phases no longer had a significant impact on *vtgα* and *esr1* expression. The significant induction of *vtgα* is congruent with previous studies that have demonstrated a dose dependent effect of PAHs on Vtg synthesis, with low concentrations leading to an increase in Vtg and high concentrations leading to a reduction in Vtg levels (Anderson et al. 1996b; Nicolas 1999).

This finding may be attributed to the changes in PAH composition caused by the weathering process. Changes in the expression of *esr1* from 47 to 131 days of exposure likely has a similar explanation as *vtgα* whereby there may be a dose dependent effect or changes in PAH composition. Reversing trends in *esr1* were shown in Bilbao et al. (2010) where *esr1* expression decreased in *Chelon labrosus* when exposed to fuel oil but increased when exposed to weathered fuel oil.

At this time, I do not have any hypotheses for why food groups are a significant factor affecting *esr1* after 131 days of exposure but not the other sampling time points or in *vtgα*, this relationship requires further investigation.

No significant differences were observed in either *vtgα* or *esr1* in males. This may be due to the generally low levels of E2 and expression of these genes in males, which could make anti-estrogenic effects difficult to identify.

The RNA-Seq results from female polar cod after 47 days of exposure indicated that in addition to *vtgα* and *esr1*, 947 hepatic genes were differentially regulated between exposed and control females. There was a significant enrichment of ovarian infertility and steroid biosynthesis gene pathways (Human orthologs). The enrichment of ovarian infertility pathway was not observed previously in polar cod exposed to BaP (Song et al. 2019) or in *Gadus morhua* liver slices (Yadetie et al. 2018).

As with the relative expression of *vtgα* and *esr1*, some of the variation observed between the exposed and control group RNA-Seq results may be attributed to differences in the reproductive phase. For example, the decreases in VTG1-1 and ZP 2.3 could be explained by reproductive phase-related differences, as the expression of these genes were down-regulated in the control female that had already spawned.

The significance of the reproductive phase differences was unknown before RNA-Seq analysis was completed. Within the scope of this thesis, reproductive phase related differences were unable to be adjusted for. It was also difficult to establish which genes associated with pathway enrichment were due to reproductive phase and which were due to exposure. Future work on this project will look more in-depth at the differences in the RNA-Seq data and discuss the reproductive phase related effects in more detail.

4.5 Plasma hormones in males

The quantity of 11-KT and T was assessed in males after 47 days of exposure to the WAF. There was a lower mean 11-KT level in exposed males compared to control, but neither 11-KT nor T were significantly different between treatment groups. In males, researchers reported mixed results, several of which found no change in plasma hormones (Bender et al. 2016; Evanson & Van Der Kraak 2001; Meier et al. 2007; Pollino & Holdway 2002; Tetreault et al. 2003).

Truscott et al. (1983) suggested that 11-KT was more sensitive to exposure compared to T. The difference could explain why there are lower levels of 11-KT in exposed males, but no similar trend in testosterone. The high natural variation in levels of steroid hormones has presented a difficulty for determining conclusive effects in this study as well as in previous work (Wu et al. 2003).

4.6 Potential consequences of the changes observed

As noted in Casillas et al. (1991), reproductive development in teleosts involves a series of complex processes on many biological levels of organization, and disruption of any of these may have detrimental effects on reproductive success. This study demonstrated that exposure to the WAF advanced female reproductive development.

Females releasing their eggs earlier into the environment may release eggs that are not fully developed or of a lower quality which may in turn affect fertilization and survival of the eggs/embryos. Moreover, fertilization may be affected if this results in a mis-match with male spawning.

Spawning represents a high energetic cost whereby resources that could be used for growth, somatic maintenance or xenobiotic metabolism are allocated towards offspring (Arukwe & Goksøyr 2003; Hop et al. 1997). A disruption of this process leading to reduced offspring survival represents a loss of resources and fitness for an individual. Moreover, the findings demonstrate the disruption of normal hepatic gene expression, including genes involved in reproduction and other processes with unknown consequences on for parental and offspring survival.

The underlying mechanistic causes for an advancement of reproduction that is not assessed within the scope of this thesis. However, an advancement in gonadal development could be related to the anti-estrogenic effects observed following crude oil exposure and PAHs (Anderson et al. 1996a; Navas & Segner 2000; Nicolas 1999; Yadetie et al. 2018). Nevertheless, an anti-estrogenic effect may better explain why studies have predominately reported a delay in gonadal development, as E2, ERα, and Vtg are essential for yolk development and growth of oocytes during vitellogenesis.

In work by King Heiden et al. (2006) anti-estrogenic effects along with a delay in reproduction were found following TCDD exposure. In contrast to E2 mediated vitellogenesis, final oocyte maturation leading to spawning is predominately regulated by GTHII and MIH such as 17α20β-DP (Nagahama & Yamashita 2008). Moreover, work has shown that high concentrations of E2 along with T are necessary to induce GTHII and initiate final oocyte maturation, leading to spawning (Nagahama et al. 1995). These mechanistic effects cannot be discussed within the results of the current thesis and requires further

experimentation along with thorough review of the literature on the subject.

4.7 Evaluation of sampling and analyses

A total of 272 fish were sampled. However, within a few methods there were some challenges with low sample size due to the natural variation in steroid hormones and exposure caused variation in reproductive phase. When working with live animals, this is an issue that is often encountered due to well-founded restrictions limiting the unnecessary use of animals (*Animal Welfare Act* 2009). This may be mitigated in future endeavors by having more directed studies looking into select parameters and using a simpler experimental design targeted at one or a few endpoints.

Few studies have been made on this particular period of development in polar cod and use of radioimmunoassay in Bender et al. (2016). For this reason, we were unaware that the levels of E2 would be too low to compare between groups at spawning and during the post-spawning period.

This provides valuable information for future studies on polar cod using ELISA. Upcoming studies should keep in mind the low levels of certain plasma hormones during the spawning and post-spawning period and adjust their methods accordingly. Alternatively, the use of a lower dilution before extraction may allow levels of steroids to be detected by the ELISA assay. It should be kept in mind for future studies that spawning may be advanced or delayed, and this is something that should be taken into account in the experimental design. For example, another aspect studied during this experiment was viability of eggs. The reproductive phase related differences impeded any conclusive results because any control eggs stripped were not ready to be spawned and died shortly after.

Moreover, for the current study we used control fish fed at the high-food regime to assess the onset of spawning (Tank 13). However, this approach would later turn out to be problematic. As we were waiting for the fish in these tanks to spawn, many of the exposed tanks were already spawning. Future studies should take this into consideration. Method alterations such as the use of exposed and control fish to assess the onset of spawning can be used. In addition, tanks could be sampled at different times if needed for the experimental design.

After consideration, atresia may not have been a relevant endpoint for assessment after spawning as most of the atresia taking place was only the natural re-absorption of left over oocytes. I suggest that future studies assess the volume fraction of atretic oocytes before spawning or in earlier reproductive phases.

Finally, there has been a focus on female polar cod throughout this thesis due to the fact that we did not observe any differences in the reproductive endpoints measured for males and some we were unable to assess. Future studies should take into account the timing of development between these two sexes and perhaps assess the effect of exposure on males earlier in the reproductive season before they are close to being ready for spawning. Furthermore, I suggest future studies focus more on male specific end-points before any conclusions can be made between the difference in susceptibility between the sexes.

4.8 Conclusions

The present study demonstrates that exposure of female polar cod to the WAF was associated with earlier spawning compared to controls. Furthermore, exposure resulted in the differential regulation of hepatic gene expression. Food ration did not have a significant or consistent effect alone or in combination with exposure on reproductive endpoints. Indicating that potentially lower food availability during the polar night may not be a significant factor affecting the outcome of exposure for the reproductive endpoints assessed. Exposed males did not show any significant variations in the reproductive endpoints assessed, possibly due to their advanced stage before the start of the experiment.

Earlier spawning in females could result in the release of low quality eggs. In addition, this could lead to a potential mis-match with male spawning. Either of these possibilities could lead to reductions in recruitment, which on a long-term perspective could have larger consequences at higher ecological levels. For this reason, the results presented suggest that spawning may be a sensitive life event for crude oil exposure in polar cod.

This work adds to our knowledge of how an oil spill in the Arctic could have different effects on polar cod reproduction at varying times during their reproductive cycle. Moreover, that there may be gaps in our current understanding of why crude oil and associated compounds may result in varying outcomes in polar cod.

4.9 Future perspectives

In line with current research, this finding demonstrates the necessity of assessing different combinations of exposure timing in order to accurately predict the outcome. Whipple et al. (1978) found an advancement of gonadal maturation. However, the study has been mostly overlooked.

I hope that the results from this study will increase the interest in examining the effects of exposure to crude oil and petroleum compounds over different periods of the reproductive cycle. There is a need for future experiments on polar cod where adults are exposed to the same experimental set-up during different phases of reproductive development. More research is required into the underlying mechanistic effects of exposure on the timing of gonadal development in teleost species, as this has not been examined in the current thesis.

Historically, female teleost reproduction has received a greater focus in the scientific literature compared to males. Increased knowledge about male polar cod and teleost reproductive cycle would be beneficial in determining how exposure may affect their reproduction, and in turn a better understanding of the effects an oil spill could have on polar cod reproduction.

References

AMAP. 2007. Arctic oil and gas 2007. Arctic Monitoring and Assessment Programme Oslo, Norway.

Amos W. 2011. Development of Canadian Arctic offshore oil and gas drilling: lessons from the Gulf of Mexico. ELJ European Law. 20(1): 39–46.

Anderson MJ, Miller MR & Hinton DE. 1996a. In vitro modulation of 17-β-estradiol-induced vitellogenin synthesis: Effects of cytochrome P4501A1 inducing compounds on rainbow trout (*Oncorhynchus mykiss*) liver cells. Aquat Toxicol. 34(4): 327–350.

Anderson MJ, Olsen H, Matsumura F & Hinton DE. 1996b. In VivoModulation of 17β-Estradiol-Induced Vitellogenin Synthesis and Estrogen Receptor in Rainbow Trout (*Oncorhynchus mykiss*) Liver Cells by β-Naphthoflavone. Toxicol Appl Pharmacol. 137(2): 210–218.

Animal Welfare Act. 2009. www.regjeringen.no/en/dokumenter/animal-welfare-act/id571188/. Accessed: 2020-05-10.

Aranguren-Abadıa L, Lille-Langøy R, Madsen AK, Karchner SI, Franks DG, Yadetie F, Hahn ME, Goksøyr A & Karlsen OA. 2019. Molecular and functional properties of the Atlantic cod (*Gadus morhua*) aryl hydrocarbon receptors AHR1A and AHR2A. Environmental Science & Technology.

Arukwe A & Goksøyr A. 1998. Xenobiotics, xenoestrogens and reproduction disturbances in fish. Sarsia. 83(3): 225–241.

– . 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol. 2(1): 4.

Arukwe A, Knudsen FR & Goksøyr A. 1997. Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Health Perspect. 105(4): 418–422.

Arukwe A, Nordtug T, Kortner TM, Mortensen AS & Brakstad OG. 2008. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (*Danio rerio*) exposed to water-soluble fraction of crude oil. Environ Res. 107(3): 362–370.

Bemanian V, Male R & Goksøyr A. 2004. The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR-and ERα-signalling pathways. Comp hepatol. 3(1); 2.

Bender ML, Frantzen M, Camus L, Le Floch S, Palerud J & Nahrgang J. 2018. Effects of acute exposure to dispersed oil and burned oil residue on long-term survival, growth, and reproductive development in polar cod (*Boreogadus saida*). Mar Environ Res. 140: 468–477.

Bender ML, Frantzen M, Vieweg I, Falk-Petersen IB, Johnsen HK, Rudolfsen G, Tollefsen KE, Dubourg P & Nahrgang J. 2016. Effects of chronic dietary petroleum exposure on reproductive development in polar cod (*Boreogadus saida*). Aquat Toxicol. 180(196-208).

Berge J, Daase M, Renaud PE, Ambrose Jr WG, Darnis G, Last KS, Leu E, Cohen JH, Johnsen G, Moline MA, et al. 2015a. Unexpected levels of biological activity during the polar night offer new perspectives on a warming Arctic. Current Biol. 25(19): 2555–2561.

Berge J, Renaud PE, Darnis G, Cottier F, Last K, Gabrielsen TM, Johnsen G, Seuthe L, Weslawski JM, Leu E, et al. 2015b. In the dark: a review of ecosystem processes during the Arctic polar night. Prog Oceanog. 139: 258–271.

Bilbao E, Raingeard D, De Cerio OD, Ortiz-Zarragoitia M, Ruiz P, Izagirre U, Orbea A, Marigómez I, Cajaraville M & Cancio I. 2010. Effects of exposure to Prestige-like heavy fuel oil and to perfluorooctane sulfonate on conventional biomarkers and target gene transcription in the thicklip grey mullet C*helon labrosus*. Aquat Toxicol. 98(3): 282–296.

Booth AM, Sutton PA, Lewis CA, Lewis AC, Scarlett A, Chau W, Widdows J & Rowland SJ. 2007. Unresolved complex mixtures of aromatic hydrocarbons: thousands of overlooked persistent, bioaccumulative, and toxic contaminants in mussels. Environ Sci Technol. 41(2): 457–464.

Brown-Peterson NJ, Wyanski DM, Saborido-Rey F, Macewicz BJ & Lowerre-Barbieri SK. 2011. A standardized terminology for describing reproductive development in fishes. Mar Coast Fish. 3(1): 52–70.

Bugel SM, White LA & Cooper KR. 2010. Impaired reproductive health of killifish (*Fundulus heteroclitus*) inhabiting Newark Bay, NJ, a chronically contaminated estuary. Aquat Toxicol. 96(3): 182–193.

Carls MG, Hose JE, Thomas RE & Rice SD. 2000. Exposure of Pacific herring to weathered crude oil: Assessing effects on ova. Environ Toxicol Chem. 19(6): 1649–1659.

Casillas E, Misitano D, Johnson LL, Rhodes LD, Collier TK, Stein JE, McCain BB & Varanasi U. 1991. Inducibility of spawning and reproductive success of female English

sole *(Parophrys vetulus)* from urban and nonurban areas of Puget Sound, Washington. Mar Environ Res. 31(2): 99–122.

Christiansen JS, Hop H, Nilssen EM & Joensen J. 2012. Trophic ecology of sympatric Arctic gadoids, Arctogadus glacialis (Peters, 1872) and *Boreogadus saida* (Lepechin, 1774), in NE Greenland. Polar biol. 35(8): 1247–1257.

Comiso JC, Parkinson CL, Gersten R & Stock L. 2008. Accelerated decline in the Arctic sea ice cover. Geophys Res Lett. 35(1):

Craig P, Griffiths W, Haldorson L & McElderry H. 1982. Ecological studies of Arctic cod (*Boreogadus saida*) in Beaufort Sea coastal waters, Alaska. Can J Fish Aquat Sci. 39(3): 395–406.

Cusa MLJ. 2016. The effect of seasonality on polar cod (*Boreogadus saida*) dietary habits and temporal feeding strategies in Svalbard waters. MA thesis. UiT Norges arktiske universitet.

Demidenko E. 2013. Mixed models: theory and applications with R. John Wiley & Sons.

Eide M, Karlsen O, Kryvi H, Olsvik P & Goksøyr A. 2014. Precision-cut liver slices of Atlantic cod (*Gadus morhua*): an in vitro system for studying the effects of environmental contaminants. Aquat Toxicol. 153: 110–115.

Evanson M & Van Der Kraak GJ. 2001. Stimulatory effects of selected PAHs on testosterone production in goldfish and rainbow trout and possible mechanisms of action. Comp Biochem Phys C. 130(2): 249–258.

Faksness L & Brandvik PJ. 2005. Dissolution of water soluble components from oil spills encapsulated in ice. *Proceedings to the 2005 Arctic and Marine Oilspill Program Technical Seminar, Calgary, Canada*, 59–73.

Faksness L, Brandvik PJ, Daae RL, Leirvik F & Børseth JF. 2011. Large-scale oil-in-ice experiment in the Barents Sea: Monitoring of oil in water and MetOcean interactions. Mar pollut Bull. 62(5): 976–984.

Faksness L & Brandvik PJ. 2008. Distribution of water soluble components from Arctic marine oil spills—a combined laboratory and field study. Cold Reg Sci Technol. 54(2): 97–105.

Faksness L, Brandvik Per Johan S & K L. 2008. Composition of the water accommodated fractions as a function of exposure times and temperatures. Mar Pollut Bull. 56(10): 1746–1754.

Gao D, Lin J, Ou K, Chen Y, Li H, Dai Q, Yu Z, Zuo Z & Wang C. 2018. Embryonic exposure to benzo (a) pyrene inhibits reproductive capability in adult female zebrafish and correlation with DNA methylation. Environ Pollut. 240: 403–411.

Goksøyr A. 1995. Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Toxicology in Transition*. Springer, 80–95.

Goksøyr A & Larsen HE. 1991. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): I. Basal properties and induction of P450 1A1 in liver of immature and mature fish. Fish Physiol Biochem. 9(4): 339–349.

Graham M & Hop H. 1995. Aspects of reproduction and larval biology of Arctic cod (*Boreogadus saida*). Arctic. 48(2): 130–135.

Gulas S, Downton M, D'Souza K, Hayden K & Walker TR. 2017. Declining Arctic Ocean oil and gas developments: Opportunities to improve governance and environmental pollution control. Mar Policy. 75: 53–61.

Harsem Ø, Eide A & Heen K. 2011. Factors influencing future oil and gas prospects in the Arctic. Energy policy. 39(12): 8037–8045.

Heintz RA, Short JW & Rice SD. 1999. Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered Exxon Valdez crude oil. Environ Toxicol Chem. 18(3): 494–503.

Holst JC & McDonald A. 2000. FISH-LIFT: a device for sampling live fish with trawls. Fish Res. 48(1): 87–91.

Hop H & Gjøsæter H. 2013. Polar cod (*Boreogadus saida*) and capelin (Mallotus villosus) as key species in marine food webs of the Arctic and the Barents Sea. Mar Biol Res. 9(9): 878–894.

Hop H, Tonn WM & Welch HE. 1997. Bioenergetics of Arctic cod (*Boreogadus saida*) at low temperatures. Can J Fish Aquat Sci. 54(8): 1772–1784.

Hop H, Trudeau VL & Graham M. 1995. Spawning energetics of Arctic cod (*Boreogadus saida*) in relation to seasonal development of the ovary and plasma sex steroid levels. Can J Fish Aquat Sci. 52(3): 541–550.

Hunter JR & Macewicz BJ. 1985. Rates of atresia in the ovary of captive and wild northern anchovy, *Engraulis mordax*. Fish Bull. 83(2): 119–136.

ICES. 2019. Manual for the AEPM and DEPM estimation of fecundity in mackerel. Series of ICES Survey Protocols SISP 5.

Iwamatsu T. 1981. On a relationship between oocyte and follicle cells around the time of ovulation in the medaka *Oryzias latipes*. Annot Zool Jpn. 54: 17–29.

Javoiš J. 2013. A two-resource model of terminal investment. Theor Biosci. 132(2): 123–132.

Johnson LL, Casillas E, Collier TK, McCain BB & Varanasi U. 1988. Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington. Can J Fish Aquat. 45(12): 2133–2146.

Johnson LL, Stein JE, Collier TK, Casillas E & Varanasi U. 1994. Indicators of reproductive development in prespawning female winter flounder *(Pleuronectes americanus)* from urban and non-urban estuaries in the northeast United States. Sci Total Environ. 141(1-3): 241–260.

Karlsen Ø & Holm JC. 1994. Ultrasonography, a non–invasive method for sex determination in cod (*Gadus morhua*). J Fish Biol. 44(6): 965–971.

Khan R. 2013. Effects of polycyclic aromatic hydrocarbons on sexual maturity of Atlantic cod, *Gadus morhua*, following chronic exposure. Environ Pollut. 2(1): 1.

Kiceniuk J & Khan R. 1987. Effect of petroleum hydrocarbons on Atlantic cod, *Gadus morhua*, following chronic exposure. Can J Zoo. 65(3): 490–494.

Kime DE. 1995. The effects of pollution on reproduction in fish. Rev Fish Biol Fisher. 5(1): 52–95.

King Heiden T, Carvan III MJ & Hutz RJ. 2006. Inhibition of follicular development, vitellogenesis, and serum 17β-estradiol concentrations in zebrafish following chronic, sublethal dietary exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin. Toxicol Sci. 90(2): 490–499.

Koressaar T & Remm M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics. 23(10): 1289–1291.

Laird NM & Ware JH. 1982. Random-effects models for longitudinal data. Biometrics: 963–974.

Leu E, Søreide J, Hessen D, Falk-Petersen S & Berge J. 2011. Consequences of changing sea-ice cover for primary and secondary producers in the European Arctic shelf seas: timing, quantity, and quality. Prog Oceanogr. 90(1-4): 18–32.

Li P, Cai Q, Lin W, Chen B & Zhang B. 2016. Offshore oil spill response practices and emerging challenges. Mar Pollut Bull. 110(1): 6–27.

Livak KJ & Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- Δ ΔCT method. Methods. 25(4): 402–408.

Lønne O & Gulliksen B. 1989. Size, age and diet of polar cod, *Boreogadus saida* (Lepechin 1773), in ice covered waters. Polar biol. 9(3): 187–191.

Marshall AG & Rodgers RP. 2004. Petroleomics: the next grand challenge for chemical analysis. Acc Chem Res. 37(1): 53–59.

McMillan DB. 2007. Fish histology: female reproductive systems. Springer Science & Business Media.

Meador J & Nahrgang J. 2019. Characterizing Crude Oil Toxicity to Early-Life Stage Fish Based On a Complex Mixture: Are We Making Unsupported Assumptions? Environ Sci Technol. 53(19): 11080–11092.

Meador, Stein J, Reichert W & Varanasi U. 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. *Rev environ contam T.* Springer, 79–165.

Meier S, Andersen TE, Norberg B, Thorsen A, Taranger GL, Kjesbu OS, Dale R, Morton HC, Klungsøyr J & Svardal A. 2007. Effects of alkylphenols on the reproductive system of Atlantic cod (*Gadus morhua*). Aquat Toxicol. 81(2): 207–218.

Melbye AG, Brakstad OG, Hokstad JN, Gregersen IK, Hansen BH, Booth AM, Rowland SJ & Tollefsen KE. 2009. Chemical and toxicological characterization of an unresolved complex mixture-rich biodegraded crude oil. Environ Toxicol Chem. 28(9): 1815–1824.

Metcalfe SS, Kroon FJ, Beale DJ & Miller G. 2018. Development of a validation protocol of enzyme immunoassay kits used for the analysis of steroid hormones in fish plasma. J Exp Mar Biol. 499:

Monteiro PRR, Reis-Henriques MA & Coimbra J. 2000. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus L.*) Aquat Toxicol. 48(4): 549–559.

Mueter FJ, Nahrgang J, Nelson RJ & Berge J. 2016. The ecology of gadid fishes in the circumpolar Arctic with a special emphasis on the polar cod (*Boreogadus saida*).

Murillo WA, Vieth-Hillebrand A, Horsfield B & Wilkes H. 2016. Petroleum source, maturity, alteration and mixing in the southwestern Barents Sea: New insights from geochemical and isotope data. Mar Petrol Geol. 70: 119–143.

Nagahama Y. 2000. Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish. *International Symposium on the Reproductive Physiology of Fish.*

Nagahama Y. 1994. Endocrine regulation of gametogenesis in fish. Int J Dev Biol. 38(2): 217–229.

Nagahama Y & Yamashita M. 2008. Regulation of oocyte maturation in fish. Dev Growth Differentiation. 50: S195–S219.

Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T & Katsu Y. 1995. Regulation of oocyte growth and maturation in fish. *Curr Top Dev Biol. 30:* Elsevier, 103–145.

Nagler JJ, Cavileer TD, Verducci JS, Schultz IR, Hook SE & Hayton WL. 2012. Estrogen receptor mRNA expression patterns in the liver and ovary of female rainbow trout over a complete reproductive cycle. Gen Comp Endocrinol. 178(3): 556–561.

Nahrgang J, Camus L, Broms F, Christiansen JS & Hop H. 2010a. Seasonal baseline levels of physiological and biochemical parameters in polar cod (*Boreogadus saida*): Implications for environmental monitoring. Marine pollution bulletin 60(8), 1336–1345.

Nahrgang J, Camus L, Carls MG, Gonzalez P, Jönsson M, Taban IC, Bechmann RK, Christiansen JS & Hop H. 2010b. Biomarker responses in polar cod (*Boreogadus saida*) exposed to the water soluble fraction of crude oil. Aquat Toxicol. 97(3): 234–242.

Nahrgang J, Storhaug E, Murzina SA, Delmas O, Nemova NN & Berge J. 2015. Aspects of reproductive biology of wild-caught polar cod (*Boreogadus saida*) from Svalbard waters. Polar Biol. 39(6): 1155–1164.

Nahrgang J, Varpe Ø, Korshunova E, Murzina S, Hallanger IG, Vieweg I & Berge J. 2014. Gender specific reproductive strategies of an Arctic key species (*Boreogadus saida*) and implications of climate change. PLoS One. 9(5):

Nava J & Segner H. 2001. Estrogen-mediated suppression of cytochrome P4501A expression in rainbow trout hepatocytes: role of estrogen receptor. Chem Biol Interact. 138: 285–298.

Navas JM & Segner H. 2000. Antiestrogenicity of β-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. Aquat Toxicol. 51(1): 79–92.

Nelson ER & Habibi HR. 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen Comp Endocrinol. 192: 15–24.

Nicolas JM. 1999. Vitellogenesis in fish and the effects of polycyclic aromatic hydrocarbon contaminants. Aquat toxicol. 45(2-3): 77–90.

Norris DO & Lopez KH. 2010. Hormones and reproduction of vertebrates. Vol. 1. Academic Press.

NRC. 1985. Oil in the sea: inputs, fates, and effects. Vol. 1. National Academies.

– . 2003. Oil in the sea III: inputs, fates, and effects. National Academies Press (US).

Pampanin DM & Sydnes MO. 2013. Polycyclic aromatic hydrocarbons a constituent of petroleum: presence and influence in the aquatic environment. *Hydrocarbon*. Vol. 5. InTech Rijeka, Croatia, 83–118.

Patiño R & Sullivan CV. 2002. Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol Biochem. 26(1): 57–70.

Pollino CA & Holdway DA. 2002. Reproductive potential of crimson-spotted rainbowfish (*Melanotaenia fluviatilis*) following short-term exposure to bass strait crude oil and dispersed crude oil. Environ Toxicol. 17(2): 138–145.

Rideout R, Burton M & Rose G. 2000. Observations on mass atresia and skipped spawning in northern Atlantic cod, from Smith Sound, Newfoundland. J Fish Biol. 57(6): 1429–1440.

Rideout R, Rose GA & Burton MP. 2005. Skipped spawning in female iteroparous fishes. Fish Fish. 6(1): 50–72.

Rowland S, Donkin P, Smith E & Wraige E. 2001. Aromatic hydrocarbon "humps" in the marine environment: unrecognized toxins? Environ Sci. 35(13): 2640–2644.

Sabo-Attwood T, Kroll KJ & Denslow ND. 2004. Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol Cell Endocrinol. 218(1-2): 107–118.

Safe S & Wormke M. 2003. Inhibitory Aryl Hydrocarbon Receptor- Estrogen Receptor α Cross-Talk and Mechanisms of Action. Chem Res Toxicol 16. 7. 807–816.

Salaberria I, Brakstad OG, Olsen AJ, Nordtug T & Hansen BH. 2014. Endocrine and AhR-CYP1A pathway responses to the water-soluble fraction of oil in zebrafish (*Danio rerio* Hamilton). J Toxicol Env Heal A. 77(9-11): 506–515.

Santana MS, Sandrini-Neto L, Neto FF, Ribeiro CAO, Di Domenico M & Prodocimo MM. 2018. Biomarker responses in fish exposed to polycyclic aromatic hydrocarbons (PAHs): Systematic review and meta-analysis. Environ Pollut. 242: 449–461.

Sarasquete C & Segner H. 2000. Cytochrome P4501A (CYP1A) in teleostean fishes. A review of immunohistochemical studies. Sci Total Environ. 247(2-3): 313–332.

Schulz RW & Miura T. 2002. Spermatogenesis and its endocrine regulation. Fish Physiol Biochem. 26(1): 43–56.

Senthilkumaran B. 2011. Recent advances in meiotic maturation and ovulation: comparing mammals and pisces. Front Biosci. 16: 1898–1914.

Song Y, Nahrgang J & Tollefsen KE. 2019. Transcriptomic analysis reveals dose-dependent modes of action of benzo (a) pyrene in polar cod (*Boreogadus saida*). Sci Total Environ. 653: 176–189.

Sørensen L, Meier S & Mjøs SA. 2016a. Application of gas chromatography/tandem mass spectrometry to determine a wide range of petrogenic alkylated polycyclic aromatic hydrocarbons in biotic samples. Rapid Commun Mass SP. 30(18): 2052–2058.

Sørensen L, Silva MS, Booth AM & Meier S. 2016b. Optimization and comparison of miniaturized extraction techniques for PAHs from crude oil exposed Atlantic cod and haddock eggs. Anal Bioanal Chem. 408(4): 1023–1032.

Sundt RC & Björkblom C. 2011. Effects of produced water on reproductive parameters in prespawning Atlantic cod (*Gadus morhua*). J. Toxicol. Environ. Health Part A. 74(7-9): 543–554.

Tetreault GR, Mcmaster ME, Dixon DG & Parrott JL. 2003. Using reproductive endpoints in small forage fish species to evaluate the effects of Athabasca oil sands activities. Environ Toxicol Chem. 22(11): 2775–2782.

Thomas P & Budiantara L. 1995. Reproductive life history stages sensitive to oil and naphthalene in Atlantic croaker. Mar Environ Res. 39(1-4): 147–150.

Tintos A, Gesto M, Alvarez R, Míguez JM & Soengas JL. 2006. Interactive effects of naphthalene treatment and the onset of vitellogenesis on energy metabolism in liver and gonad, and plasma steroid hormones of rainbow trout *Oncorhynchus mykiss*. Comp Biochem Physiol C Toxicol Pharmacol. 144(2): 155–165.

Truscott B, Walsh J, Burton M, Payne J & Idler D. 1983. Effect of acute exposure to crude petroleum on some reproductive hormones in salmon and flounder. Comp Biochem Physiol C Comp Pharmacol. 75(1): 121–130.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M & Rozen SG. 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40(15): e115–e115.

USGS. 2008. Circum-Arctic resource appraisal: Estimates of undiscovered oil and gas north of the Arctic Circle. Tech. rep. US Geological Survey.

Vieweg I, Bilbao E, Meador J, Cancio I, Bender ML, Cajaraville MP & Nahrgang J. 2018. Effects of dietary crude oil exposure on molecular and physiological parameters related to lipid homeostasis in polar cod (*Boreogadus saida*). Comp Biochem Phys C. 206: 54–64.

Weibel ER, Kistler GS & Scherle WF. 1966. Practical stereological methods for morphometric cytology. J Cell Biol. 30(1): 23–38.

Whipple J, Yocom T, Smart D & Cohen M. 1978. Effects of chronic concentrations of petroleum hydrocarbons on gonadal maturation in starry flounder *Platichthys stellatus*. Nation Mar Fish Serv.

Woźny M, Brzuzan P, Łuczyński MK, Góra M, Bidzińska J & Jurkiewicz P. 2008. Effects of cyclopenta [c] phenanthrene and its derivatives on zona radiata protein, ERα, and CYP1A mRNA expression in liver of rainbow trout (*Oncorhynchus mykiss Walbaum*). Chemico-biological interactions. 174(1): 60–68.

Wu RS, Pollino CA, Au DW, Zheng GJ, Yuen BB & Lam PK. 2003. Evaluation of biomarkers of exposure and effect in juvenile areolated grouper *(Epinephelus areolatus)* on foodborne exposure to benzo [a] pyrene. Environ Toxicol Chem. 22(7): 1568–1573.

Yadetie F, Zhang X, Hanna EM, Aranguren-Abadía L, Eide M, Blaser N, Brun M, Jonassen I, Goksøyr A & Karlsen OA. 2018. RNA-Seq analysis of transcriptome responses in Atlantic cod (*Gadus morhua*) precision-cut liver slices exposed to benzo [a] pyrene and 17α-ethynylestradiol. Aquat Toxicol. 201: 174–186.

Zhang X & Jonassen I. 2020. RASflow: an RNA-Seq analysis workflow with Snakemake. BMC Biochem. 21(1): 1–9.

Appendices

Appendix A

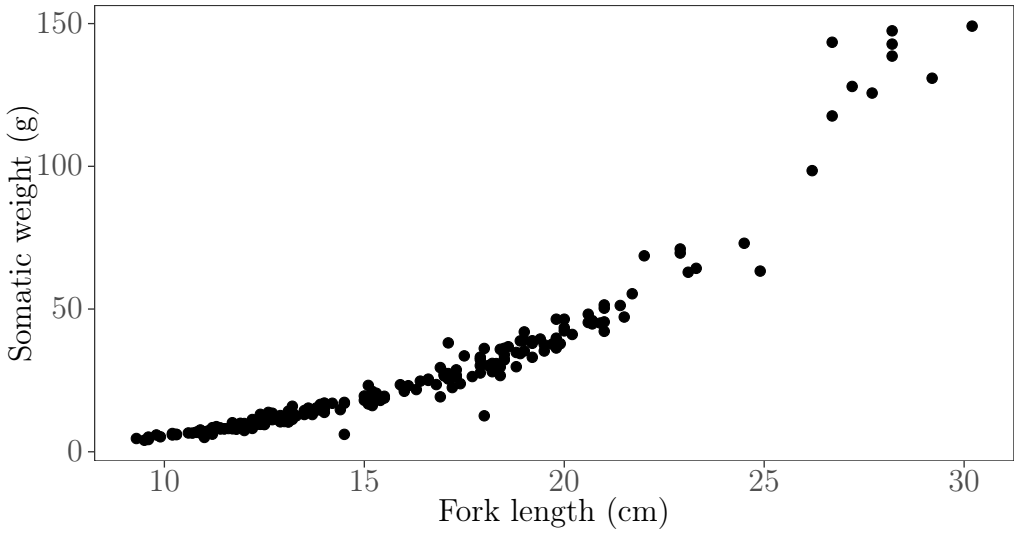


Figure 16: Fork length to somatic weight ratio from feral polar cod (males and females) used to calculate feed regimes. The Data set was collected during the Nahrgang et al. (2014) (unpublished data).

Appendix B

Guidelines for consistent sub-sample selection for Weibel-grid analysis

1. Moving left to right on each slide, the first adequate (eg. no processing problems or missing sections) slice was used.
2. The order of square selection was chosen based on the size and structure of the slice

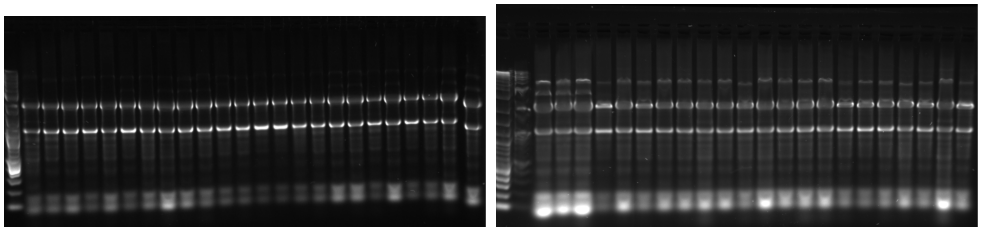
   (a) If the slice covered 5-10 squares with well-defined negative grid (space that is clearly outside the ovary wall) every suitable square was taken
   (b) For 10-20 squares with well-defined grid space every second square was taken
   (c) For 20-30 squares with well-defined grid space every third square was taken
   (d) For 5-15 squares with undefined grid space (space that is not clearly outside the gonad) every square was taken
   (e) For 15-30 with undefined every suitable second was taken
   (f) For 30-45 with undefined every third square was taken

3. A suitable square was defined by the following:

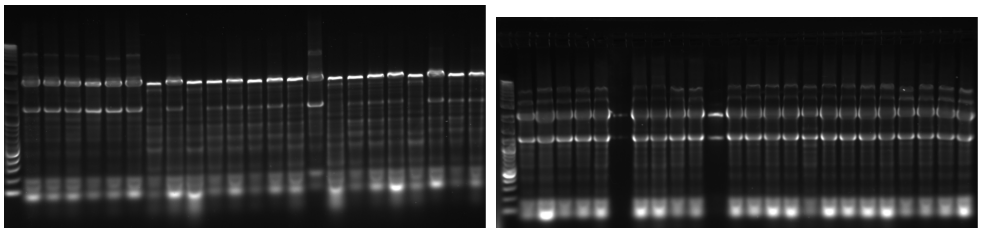
   (a) Squares that had clear negative grid or were well within the gonad, leaving no ambiguous edges
   (b) Squares with at least 25 % of the area covered by reproductive structures (not including ovarian wall)
   (c) Squares that did not contain sections that were damaged due to processing

4. When there were not enough suitable squares, squares that lacked well-defined grid space but contained the most structures were taken first, then those that had less than 25 % structures were taken, and lastly squares that contained sections damaged due to processing were taken.

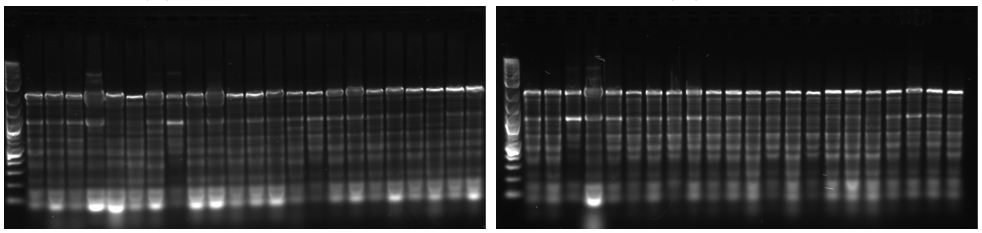
Appendix C



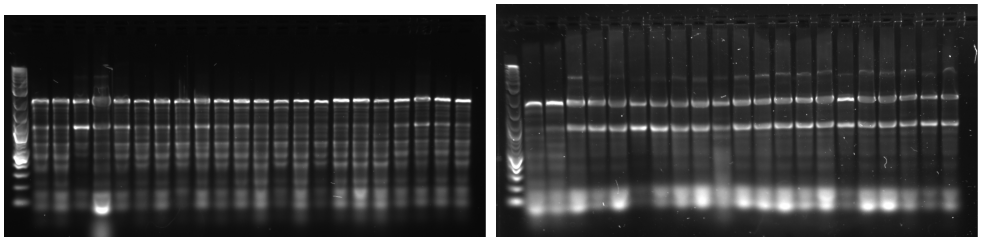
(a) Day 0 **(b)** Day 0



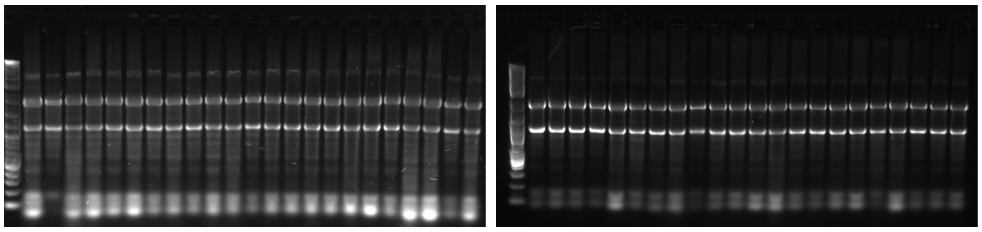
(c) Day 0 & 131 **(d)** Day 47



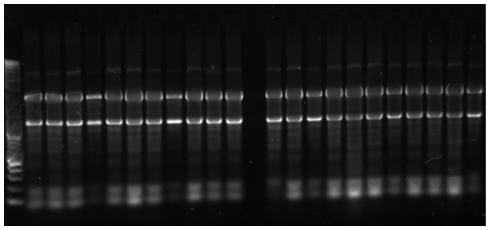
(e) Day 47 **(f)** Day 47



(g) Day 47 **(h)** Day 131



(i) Day 131 **(j)** Day 131



(k) Day 131

Appendix D

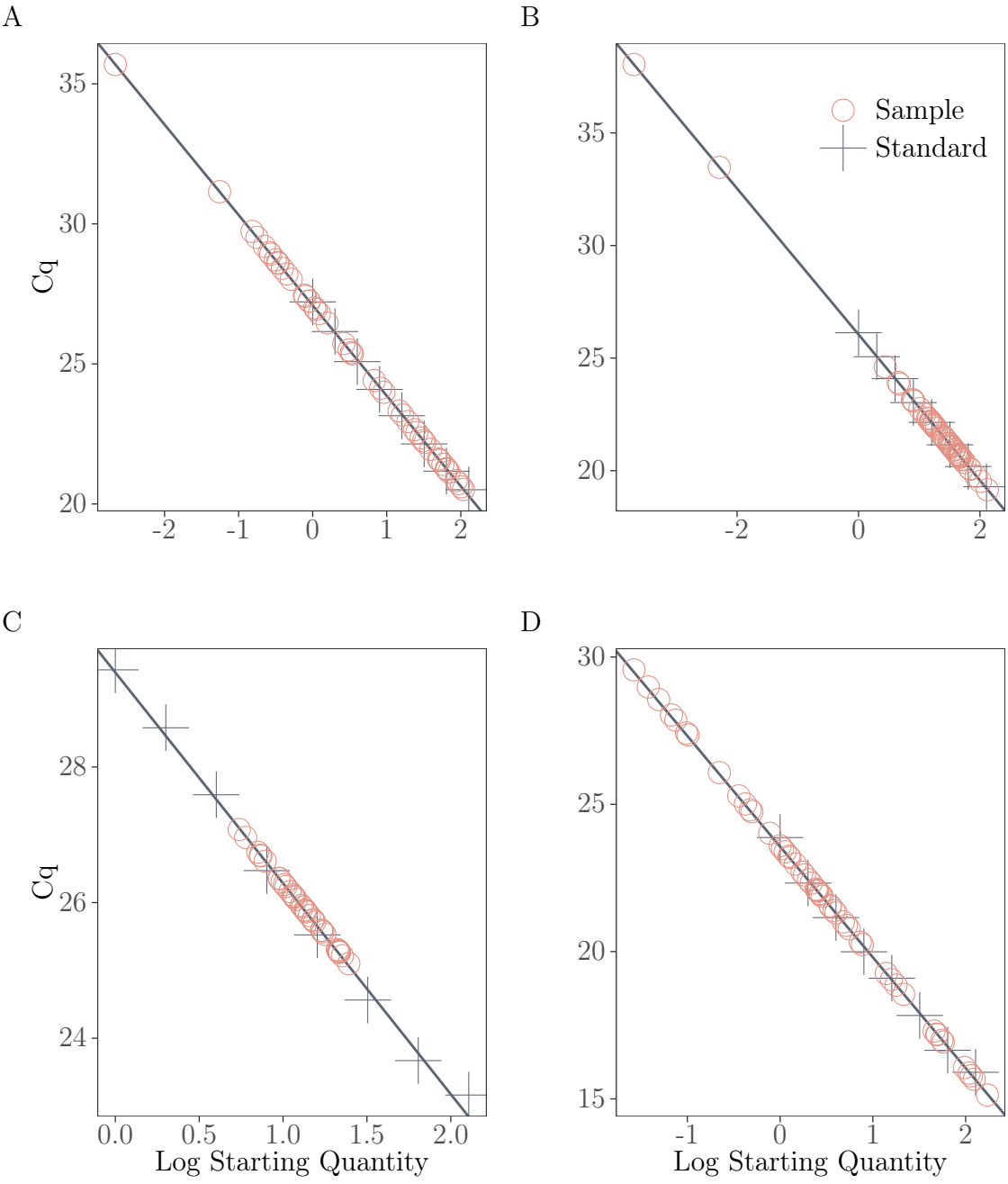


Figure 18: Standard curves for A) *cyp1a*, B) *act2b*, C) *esr1*, and D) *vtgα*. Standard samples are marked as an x and samples tested to ensure adequate range are represented as circles. Slope and percent efficiency were as follows. A) –3.23 and 102.04 B) –3.24 and 101.74 C) –3.11 and 105.01 D) –3.76 and 92.27.

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Appendix E

Table 7: Dilutions used for enzyme-linked immunosorbent assay (ELISA)

Hormone	Sampling	Female	Males
E2	Day 0	1:200, 1:100	N/A
E2	Day 47	1:10	N/A
E2	Day 131	1:10	N/A
T	Day 0	1:10, 1:100	1:300, 1:600
T	Day 47	1:15, 1:30	1:300, 1:600
T	Day 131	1:15, 1:30	1:300, 1:600
11-KT	Day 0	1:300, 1:600	NA
11-KT	Day 47	1:300, 1:600	NA
11-KT	Day 131	1:300, 1:600	NA

Appendix F

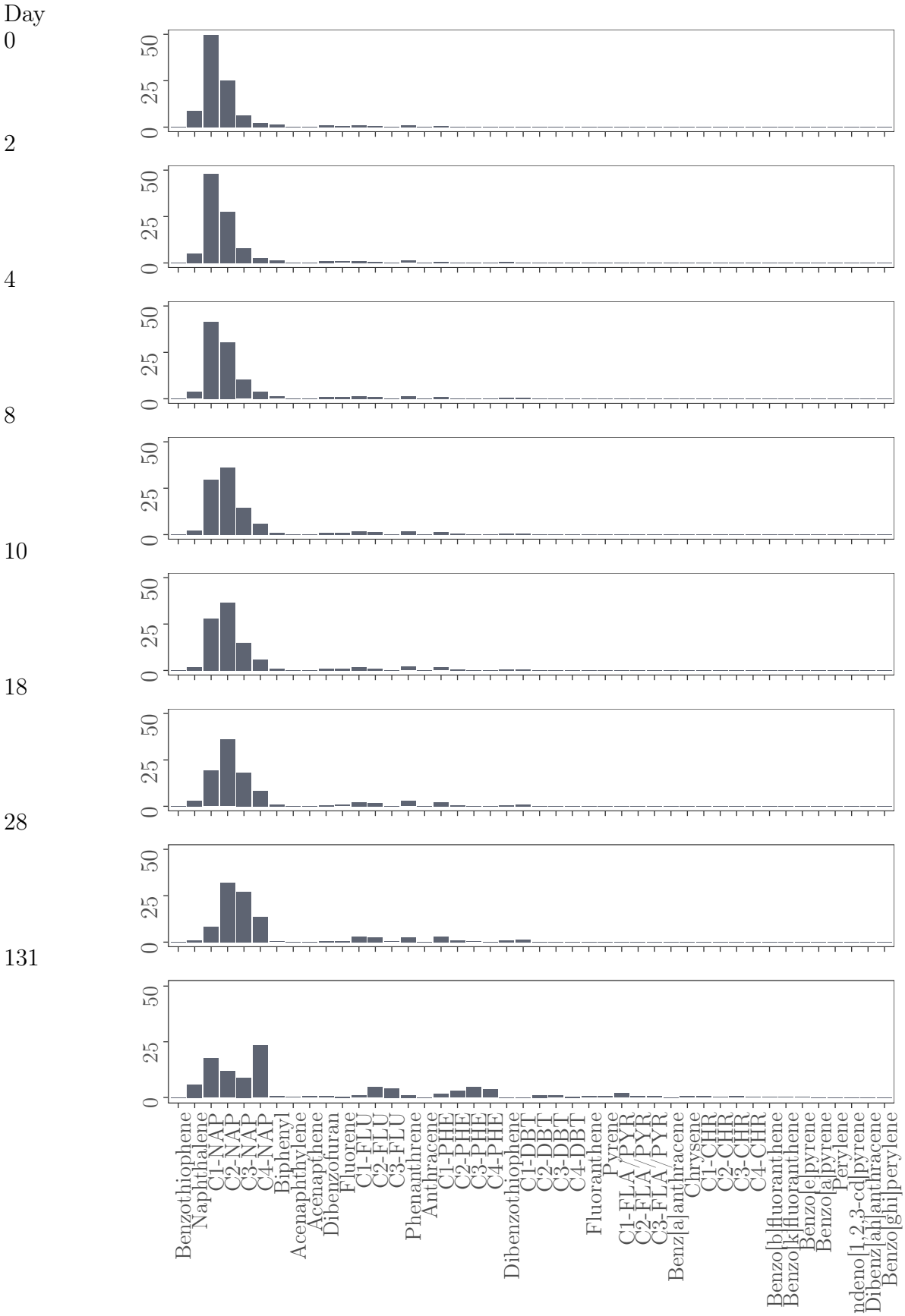


Figure 19: PAH composition represented as percent of total PAH from exposed water samples taken at A) 0, B) 2, C) 4, D) 8, E) 10, F) 18, G) 28, H) 131 days after exposure began.

Appendix G

Table 8: Total PAH ($\sum$ 44) values from water samples taken over the experimental period.

Day	Tank	Exposure	Sum PAH (μgL^{-1})
0	1	Control	0.62
0	3	Control	0.71
0	2	Exposed	7.73
0	8	Exposed	16.03
0	10	Exposed	10.02
0	12	Exposed	16.29
2	2	Exposed	8.07
4	2	Exposed	6.22
8	2	Exposed	3.91
10	2	Exposed	4.55
18	2	Exposed	4.87
18	5	Exposed	4.57
18	8	Exposed	3.68
18	12	Exposed	4.09
28	1	Control	0.07
28	2	Exposed	2.89
131	1	Control	0.09
131	2	Exposed	0.10

Appendix H

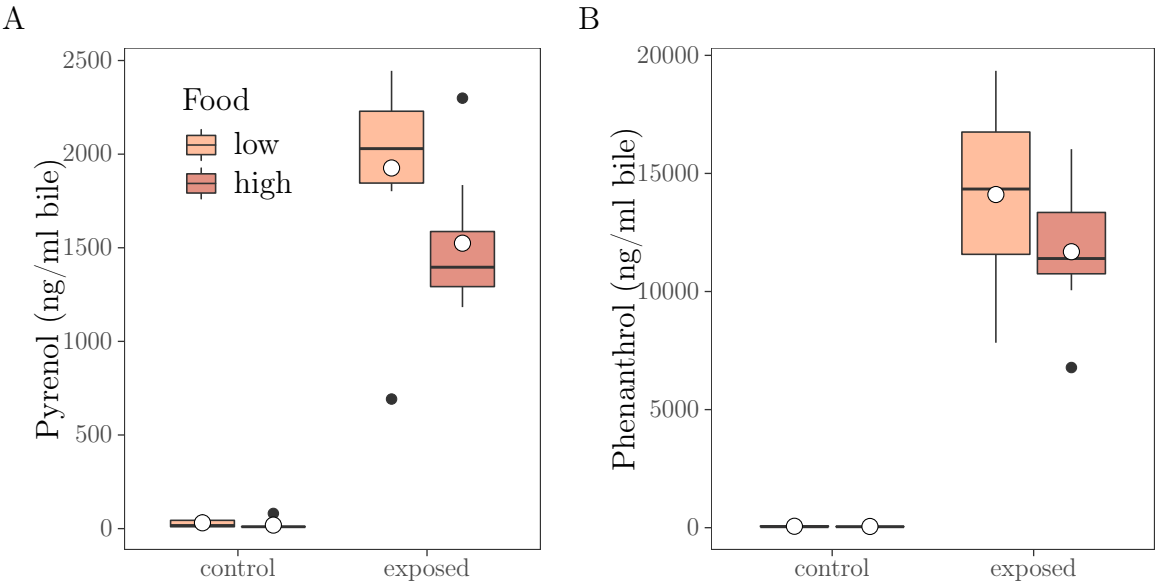


Figure 20: PAH metabolites in male and female polar cod.

Appendix I

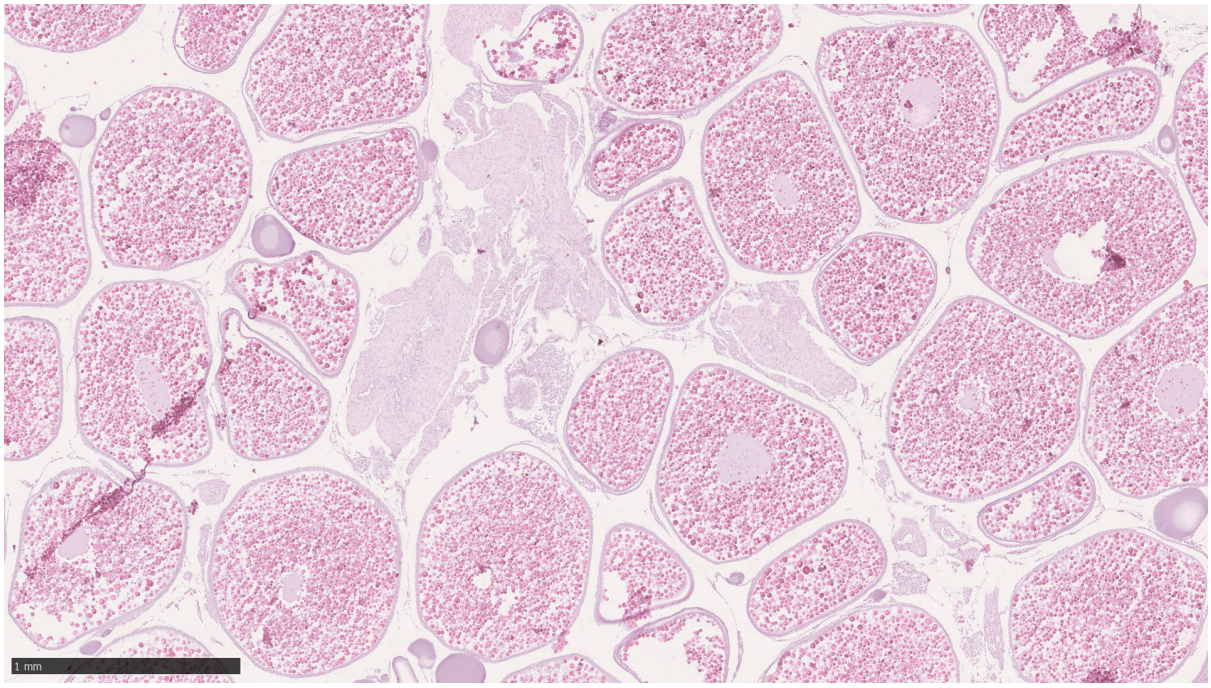


Figure 21: Spawning capable female polar cod histology (standard bar = 1 mm).

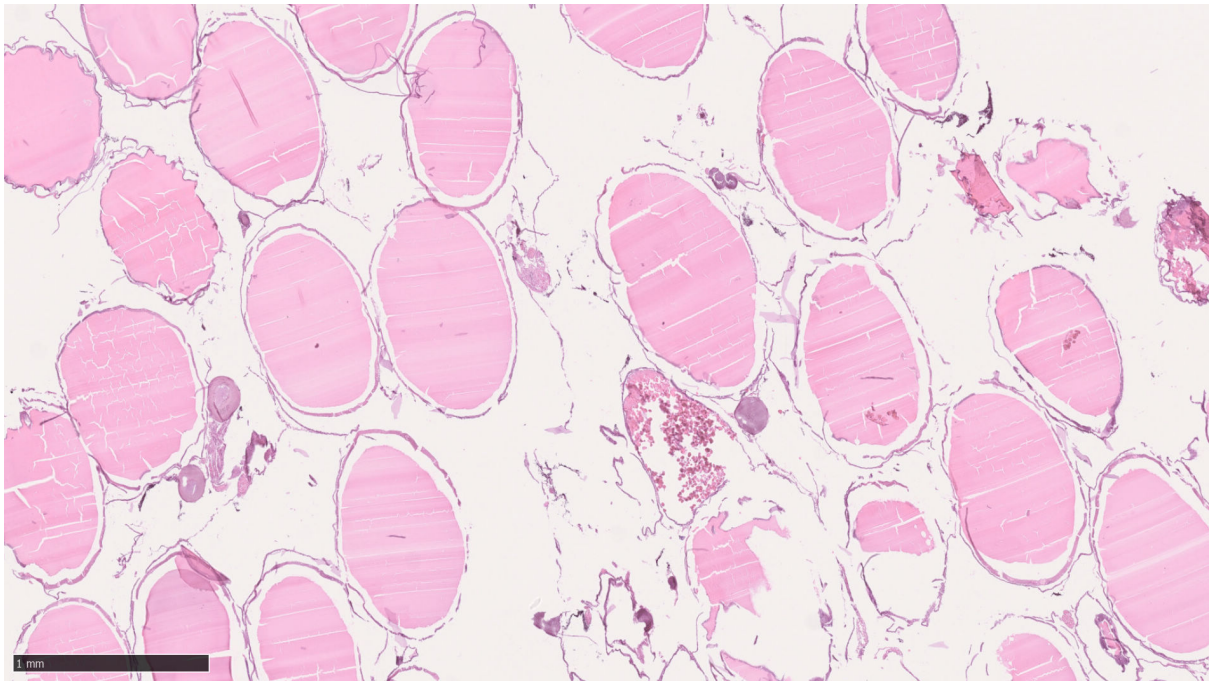


Figure 22: Active spawning (spawning capable subphase) female polar cod histology (standard bar = 1 mm).

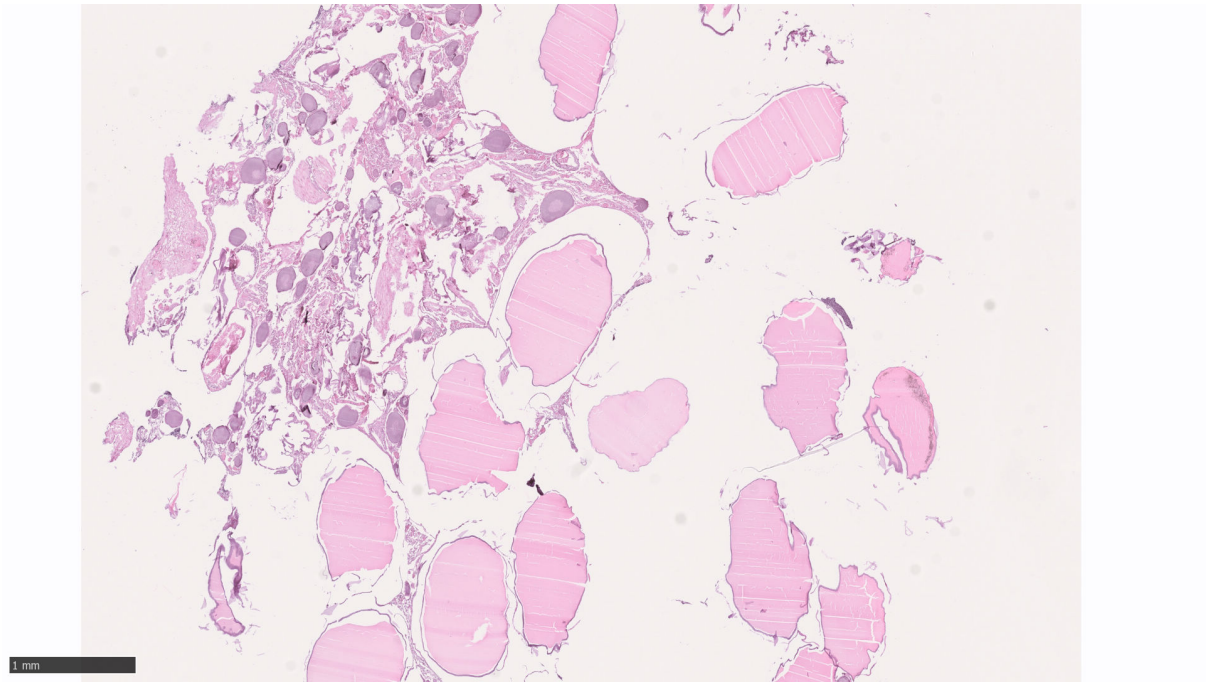


Figure 23: Early regressing (regressing subphase) female polar cod histology (standard bar = 1 mm).

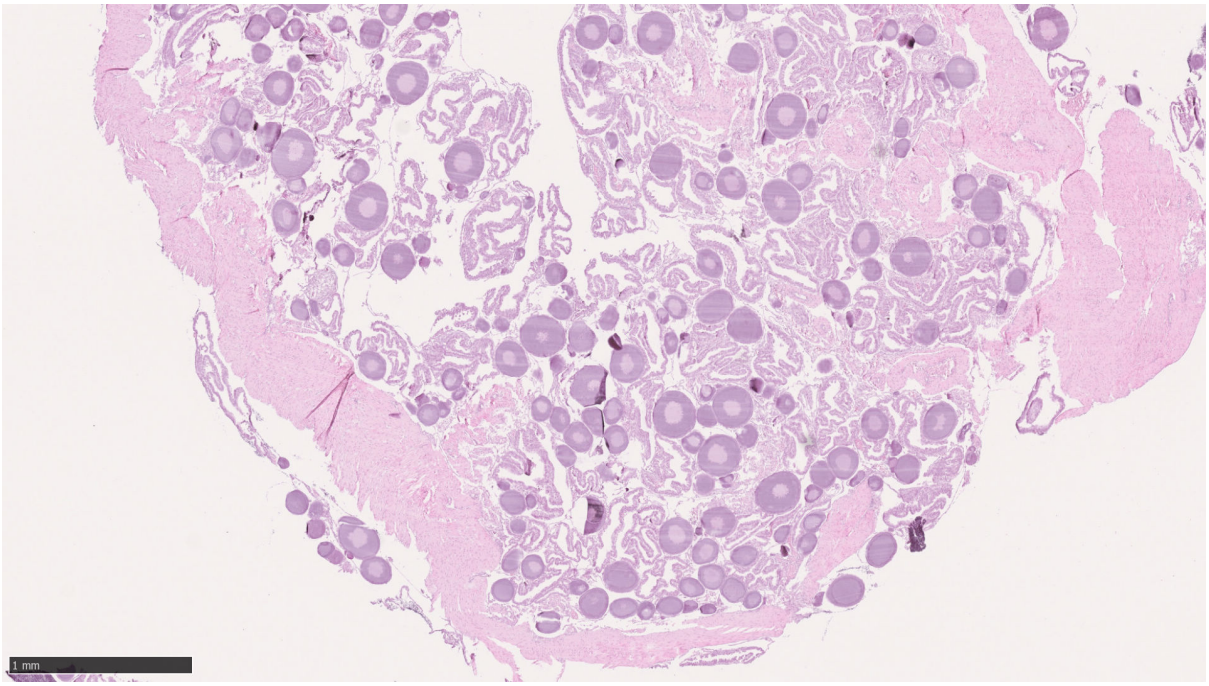


Figure 24: Regressing female polar cod histology (standard bar = 1 mm).

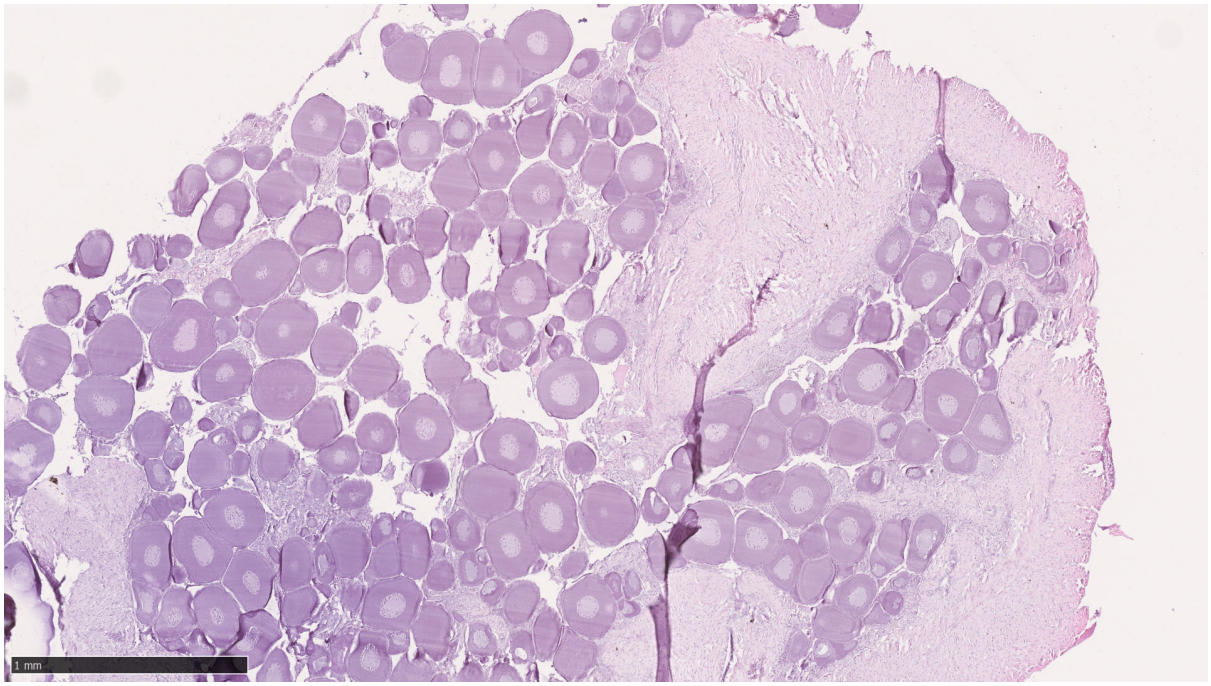


Figure 25: Regenerating female polar cod histology (standard bar = 1 mm).

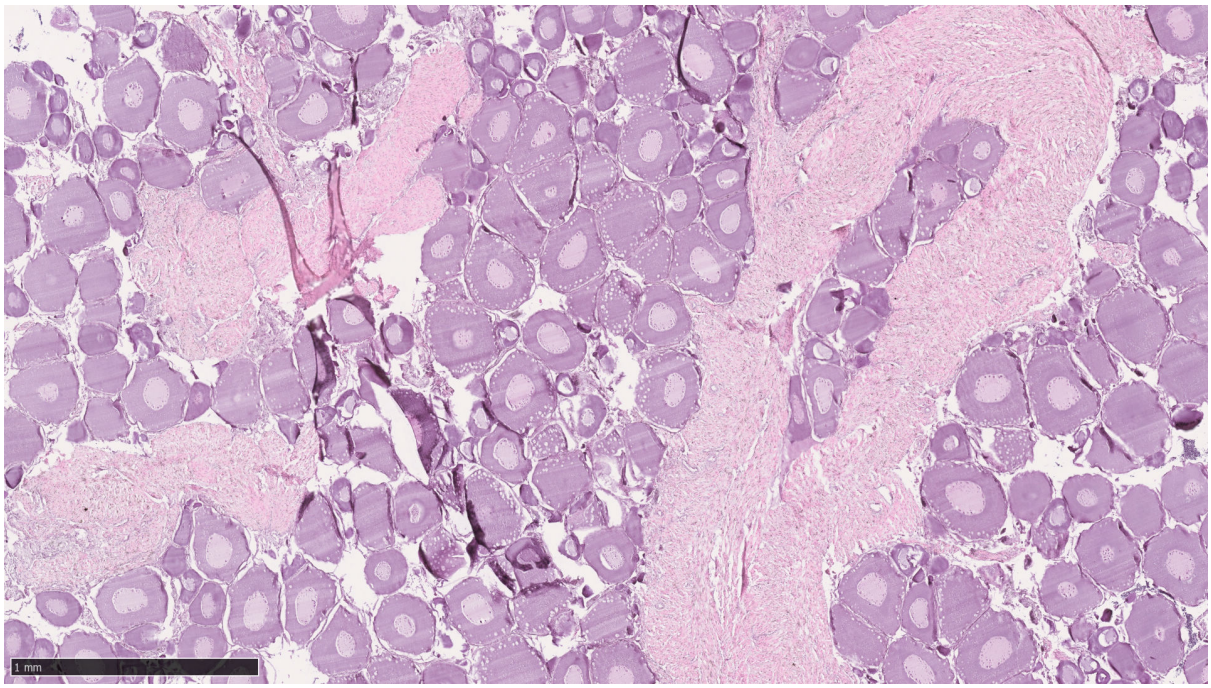


Figure 26: Early developing female polar cod histology (standard bar = 1 mm).

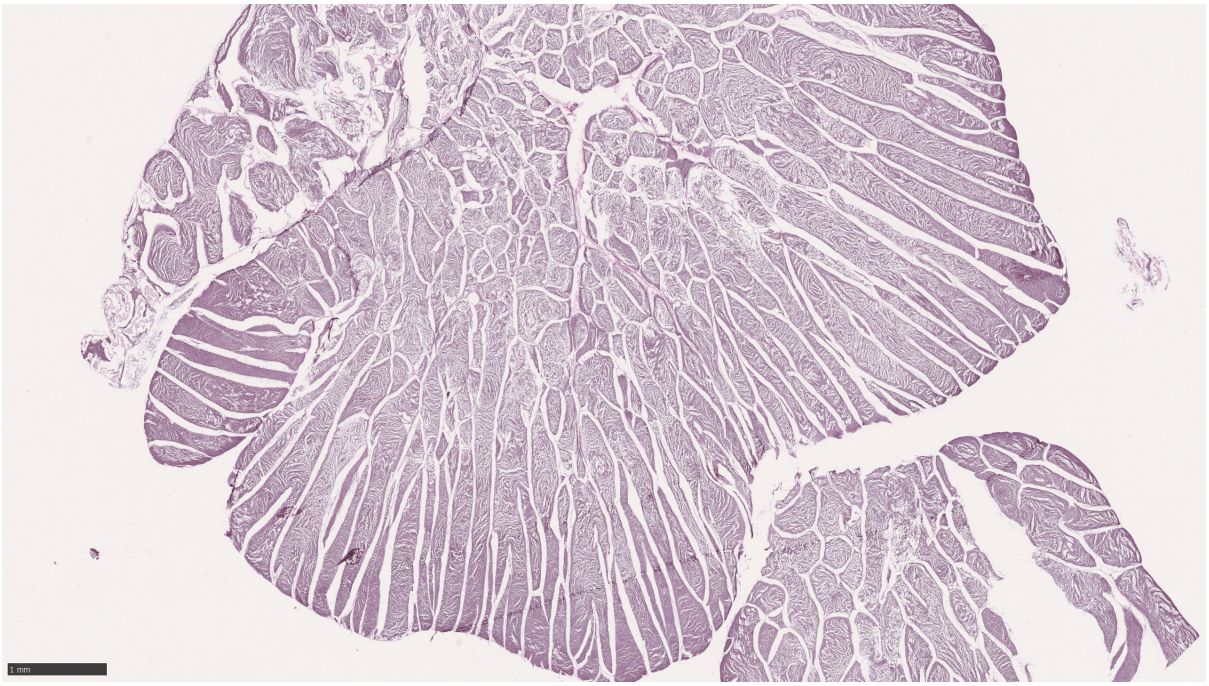


Figure 27: Mid germinal epithelium (GE) male histology (spawning capable subphase).

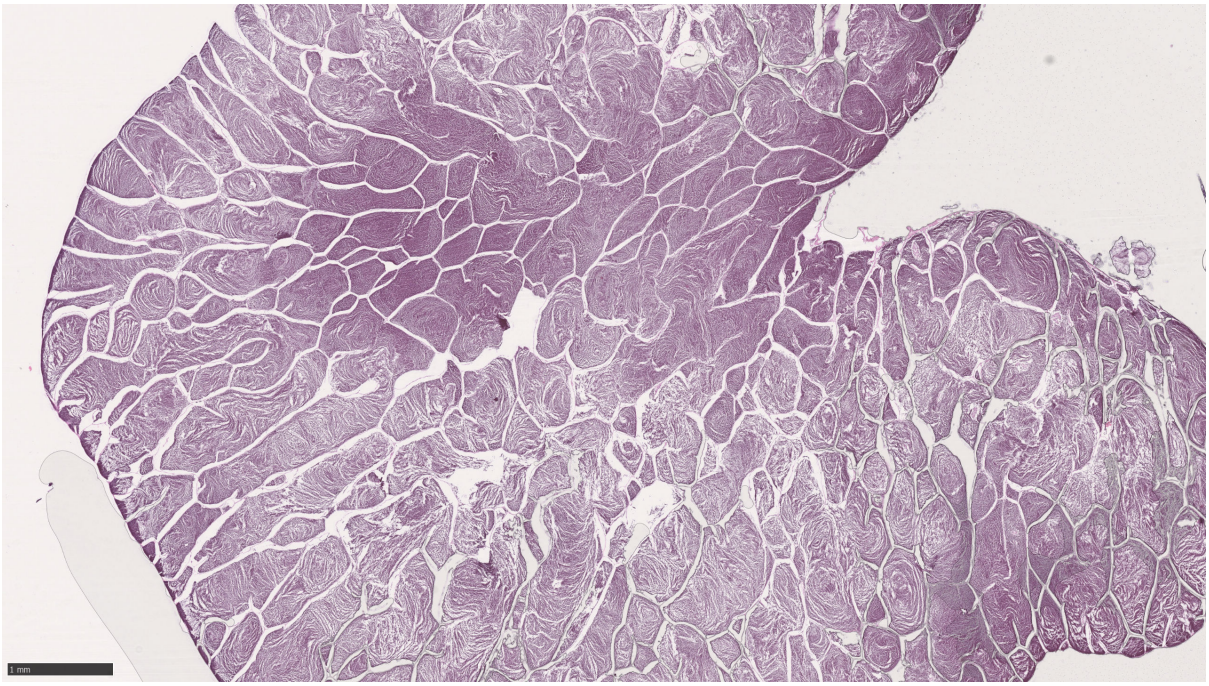


Figure 28: Late germinal epithelium (GE) male histology (spawning capable subphase).

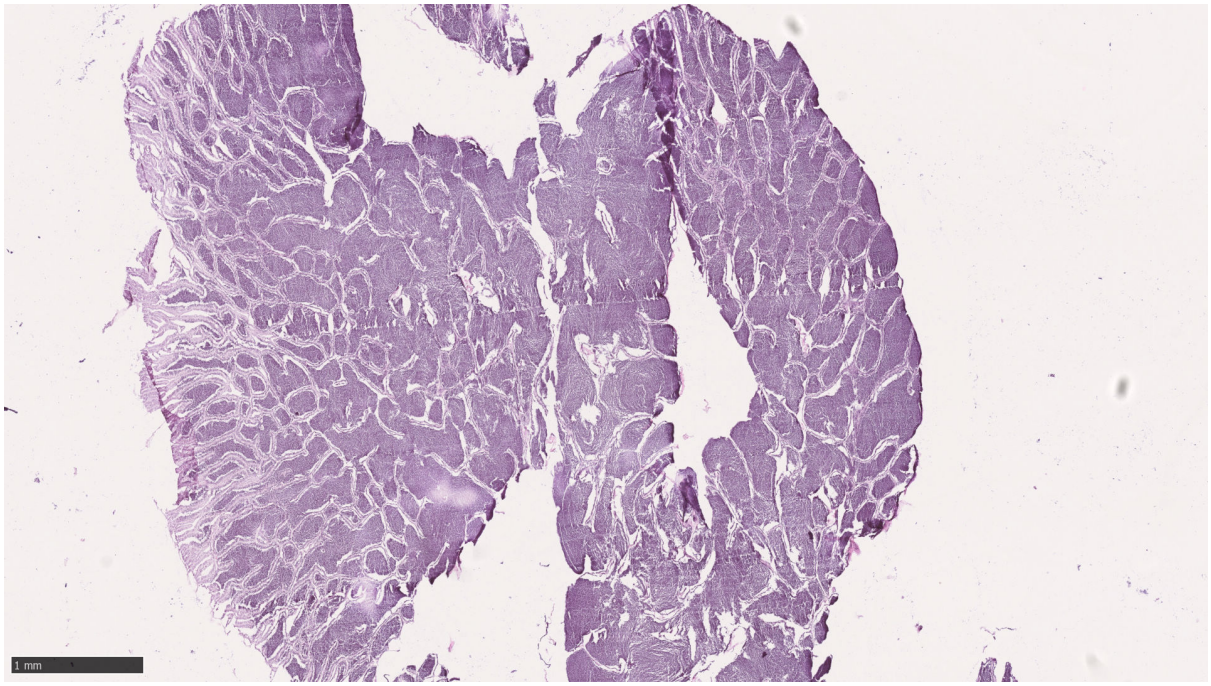


Figure 29: Regressing male histology (spawning capable subphase).

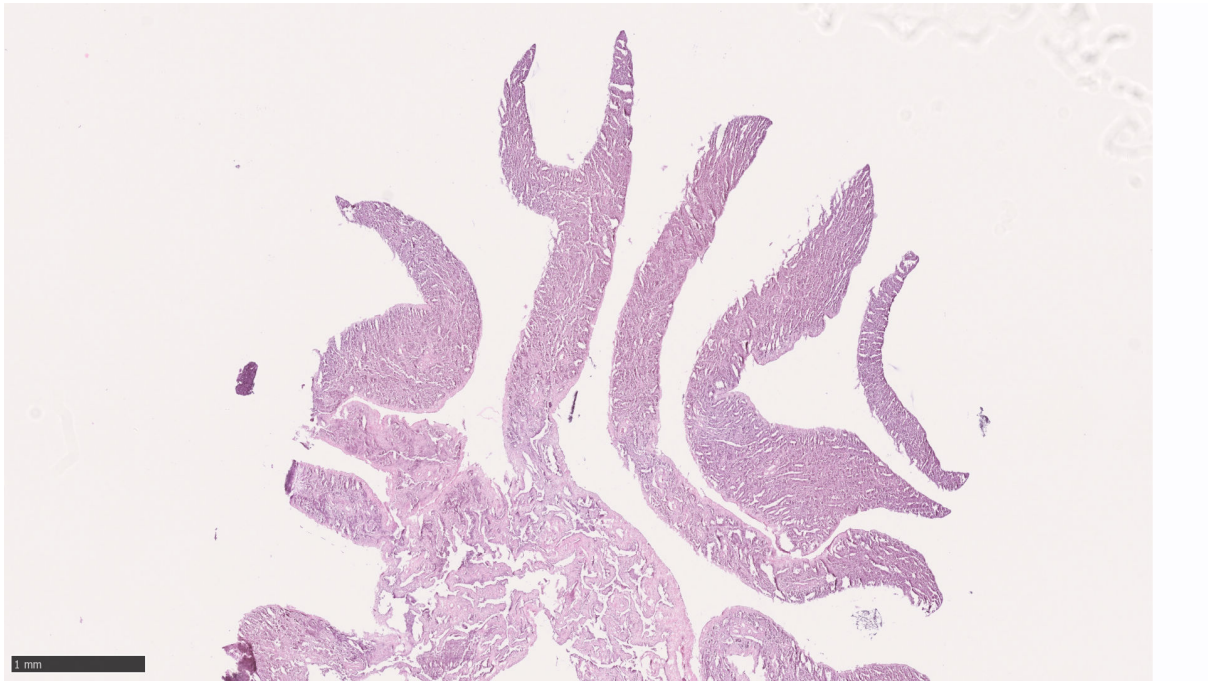
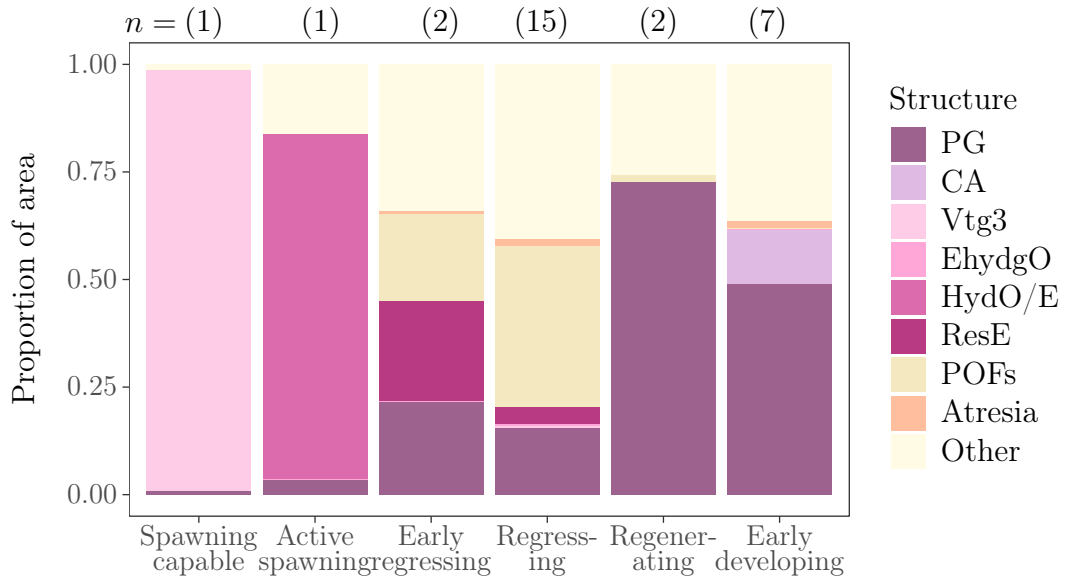


Figure 30: Regenerating male histology (spawning capable subphase).

Appendix J

A



B

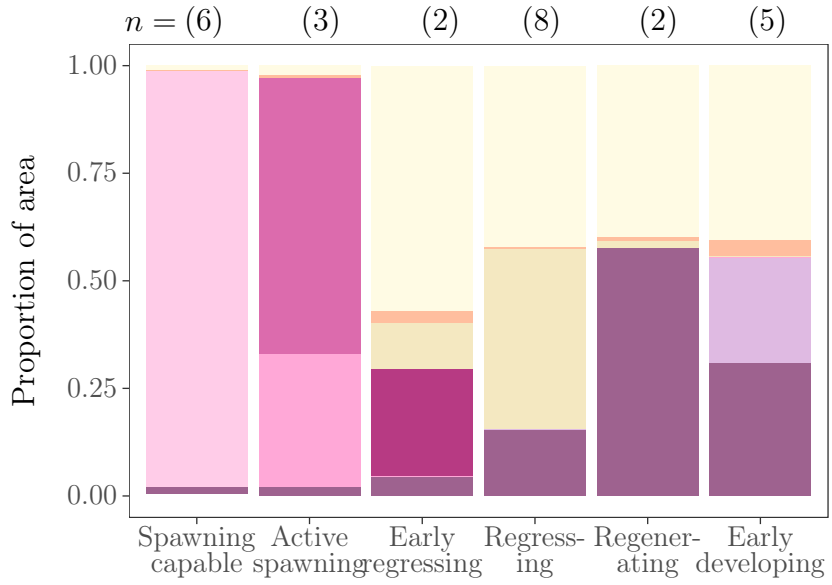


Figure 31: Average estimate of ovarian volume fraction occupied by each reproductive structure in female polar cod gonad samples in each reproductive stage in (A) Exposed and (B) Control fish. Exposed groups were exposed to the WAF of crude oil. Estimated area was determined by Weibel-grid using Image J.

Appendix K

Table 9: Summary statistics for female polar cod plasma hormone analyses, including average, standard deviation, and individual count for E2, T, and 11-KT. These values are only to display the change in hormone levels between stages and the concentration values cannot be used for further comparison. Due to the low levels of E2 in the samples, samples outside the optimal detection range were used, but only for comparison. Stages were classified using histology samples from the middle of the ovary with stage classifications based on Brown-Peterson et al. (2011).

Stage	E2	E2 SD	E2 (n)	T	T SD	T (n)	11-Kt	11-Kt SD	11-Kt (n)
Spawning capable (early)	5.24	1.36	3	-	-	-	0.83	0.33	3
Spawning capable (late)	0.42	0.24	3	14.08	3.79	1	17.41	-	1
Active spawning	0.28	0.16	2	-	-	-	9.49	3.76	2
Regressing	0.15	0.02	9	-	-	-	1.01	0.79	4
Regenerating	0.17	0.02	2	18.25	2.13	2	-	-	0
Early developing	0.11	0.02	4	20.78	0.48	2	-	-	0