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Physiological impact of waterborne crude oil exposure to reproducing polar cod (*Boreogadus saida*)

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Abstract

Climate change-driven sea ice decline is increasing access to Arctic oil reserves and shipping routes, raising the risk of oil spills. Harsh polar conditions of extreme cold, darkness, and unpredictable weather will exacerbate these risks. The polar cod (*Boreogadus saida*), a key species in the Arctic ecosystem, may be particularly affected. With its spawning window in January and February, an oil spill during the polar night could disrupt reproductive development and spawning activity.

To investigate this, adult polar cod were exposed to two concentrations (high and low) of the water-soluble fraction (WSF) of crude oil for 20 days (25.11.22–15.12.22) during late gonad development, one month before spawning. The fish were exposed using crude oil-coated gravel column, simulating the decline of polycyclic aromatic hydrocarbons (PAHs) due to weathering. Fish were sampled throughout the exposure period and spawning window.

Female polar cod exposed to high WSF levels (64.2 ± 25.35 µg/L PAHs) showed a significant advancement in reproductive development, spawning 20 days earlier than unexposed females. By the time the first unexposed female entered the regressing stage, 86% of the high-exposure group had already spawned. Low WSF exposure (19.29 ± 0.36 µg/L PAHs) resulted in a 6-day advancement. Estrogen levels did not significantly change, but elevated testosterone levels were found in the active spawning phase of the high-exposure group. In the high-exposure tank, with the greatest advancement in spawning time, a significant reduction in fertilized egg diameter was observed compared to all other experimental tanks. In males, exposure to WSF did not affect reproductive development, GSI, testosterone (T), or 11-ketotestosterone (11-KT).

These findings support the results of previous studies that exposure to crude oil in the late stages of vitellogenesis leads to an advancement in gonadal development. The present study suggests that this advancement may lead to premature spawning and reduced egg quality.

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Abbreviations

CA	Cortical alveoli
CYP19	Cytochrome P450
E2	Estradiol-17ß
Esr	Estrogen receptor
FSH	Follicle stimulating hormone
GE	Germinal epithelium
GSI	Gonadosomatic Index
GVBD	Germinal vesicle breakdown
HSI	Hepatosomatic Index
HydrO/Es	Hydrated oocytes or hyaline eggs
K	Condition factor
LH	Luteinizing hormone
MIH	Maturation-inducing hormone
PAH	Polycyclic Aromatic Hydrocarbons
PG	Primary Growth oocytes
POFs	Post-ovulatory follicles
ResEs	Residual eggs
RIA	Radioimmunoassay
Sc1	Primary spermatocyte
Sc2	Secondary spermatocytes
Sg1	Primary spermatogonia
Sg2	Secondary spermatogonia
St	Spermatids
Sz	Spermatozoa
T	Testosterone
Vtg	Vitellogenin
VTG1	Primary vitellogenic oocytes
VTG2	Secondary vitellogenic oocytes
VTG3	Tertiary vitellogenic oocytes
WSF	Water soluble fraction
Zrp	Zona radiata protein
11-KT	11-ketotestosterone

1. Introduction

1.1 Oil exploration and crude oil pollution in the arctic

Climate change is reshaping environments worldwide, significantly impacting ecosystems, resource accessibility, and potential environmental risks. In the Arctic, climate change and increasing accessibility due to rapidly decreasing sea ice thickness and coverage elevate the risk of oil pollution (Harsem et al., 2011; Zhang et al., 2024). Currently, the Arctic contributes about 10% of global oil production (Lindholt & Glomsrød, 2011) and is estimated to contain 90 billion barrels of undiscovered oil resources, or approximately 13% of the world's total (Bird et al., 2008). Most of these resources (84%) are projected to be offshore. Depending on emission scenarios, the first ice-free Arctic summer could occur between 2030 and 2050 (Kim et al., 2023), making these untapped oil reserves more accessible. Additionally, not only could oil production increase, but the potential rise in shipping activity along the Northwest Passage and the Northern Sea Route, as well as tourism, could exacerbate the risk of oil spills if the Arctic becomes ice-free (Maher et al., 2014; Mudryk et al., 2021).

The harsh Arctic conditions, particularly during winter, characterized by extreme cold temperatures, continuous darkness, and unpredictable weather patterns, along with its remote location, make oil production processes particularly difficult (AMAP, 2007). Furthermore, the loss of sea ice due to climate change may also increase the risks associated with oil operations and transportation due to more unstable ice conditions, higher waves, and an increase in extreme weather events (Dong et al., 2022; Kolstad & Bracegirdle, 2008; Zhang et al., 2024). Additionally, cleaning up an oil spill in Arctic conditions poses unique challenges. The remoteness, lack of infrastructure, and harsh conditions increase the oil spill response time because equipment, vessels, and personnel must be mobilized over vast distances. The extreme weather conditions during the polar night make the methods typically used in oil spill response operations less effective and difficult to implement (Bambulyak et al., 2014; Pavlov, 2020). The sea ice may incorporate the oil and carry it, making it inaccessible for oil spill clean-up methods and prolonging its bioavailability (Faksness & Brandvik, 2008; Wilkinson et al., 2017).

Crude oil consists of a complex mixture of hydrocarbons and non-hydrocarbons containing small amounts of sulfur, nitrogen, and other trace elements (Mutiara Sari & Kolmetz, 2016). The main components are paraffins, naphthenes, and aromatics. The composition of these

components varies significantly based on their geological origin (Faksness et al., 2008; Melbye et al., 2009; Speight, 2006).

After an oil spill, the crude oil undergoes weathering, a process in which environmental factors such as temperature, sunlight, and oxygen break down or alter the chemical composition of substances like crude oil over time (Tarr et al., 2016). This process begins with evaporation, dispersion, dissolution, and the spreading of oil droplets into the water column, followed by oxidation, sedimentation, and biodegradation of the oil particles. These weathering processes depend on environmental conditions and oil composition (Tarr et al., 2016). The low water temperature of the Arctic slows down the weathering process, making crude oil available for a longer time in the ecosystem and prolonging its potentially harmful impacts (Payne et al., 1991; Truu, 2023). The fraction of compounds that dissolves in water, is called the water-soluble fraction (WSF). This fraction consists of semi-volatile and soluble compounds such as naphthalene and aromatics, while volatile compounds in crude oil, such as BTEX (Benzene, Toluene, Ethylbenzene, and Xylenes) will readily evaporate. As a result, the composition of the WSF differs from that of the crude oil itself (Bobra, 1992; Saeed & Al-Mutairi, 2000). The WSF plays a particularly important role in the case of an oil spill, as it represents the portion of crude oil that is bioavailable to organisms and, therefore, can cause harmful effects to the aquatic ecosystem (Lari et al., 2016)

Crude oil contains numerous toxic compounds, including volatile organic compounds (BTEX), heavy metals, and polycyclic aromatic hydrocarbons (PAHs), all of which can have detrimental effects on organisms (Buskey et al., 2016; Chinedu & Chukwuemeka, 2018; Kang et al., 2014). Although PAHs make up only a small fraction of crude oil (Sammarco et al., 2013) , they are often linked to most of the known toxic effects. Fish can rapidly absorb PAHs from the water through their gills and skin, or through the gastrointestinal tract via contaminated food (Meador et al., 1995). The toxic effects of crude oil on fish, especially in early life stages, are well-documented. Crude oil exposure can lead to developmental abnormalities in young fish, resulting in deformities such as skeletal, cardiovascular, fin, and yolk sac malformations (Carls et al., 1999; Esbaugh et al., 2016; Heintz et al., 2000; Incardona et al., 2015; Incardona et al., 2004; Nahrgang, Dubourg, et al., 2016). These developmental deformities, if not lethal, may decrease growth, alter swimming and feeding behavior and reduce survival rates over time (Price & Mager, 2020; Vignet et al., 2014). For example *Coryphaena hippurus* it is shown that exposure to PAHs resulted in reduction in swimming efficiency maximal metabolic rate and cardiac output in young adults (Mager et al., 2014; Nelson et al., 2016). Aside from

developmental issues, these toxic effects are associated to long-term ecological impact on the aquatic ecosystem where an oil spill occurs, negatively affecting the growth and survival of entire fish populations (Pasparakis et al., 2019; Peterson et al., 2003). Long-term contamination by crude oil affects species at various trophic levels, disrupting complex food web dynamics and potentially compromising ecosystem stability.

1.2 Reproductive development in teleost

In most teleost species, oocytes develop from primordial germ cells into spawning-ready eggs in a yearly cycle. In iteroparous species such as polar cod, this cycle is repeated multiple times throughout their lifetime (Nahrgang et al., 2014). Polar cod belong to the group-synchronous species (Nahrgang, Storhaug, et al., 2016), which means that all oocytes that will be released in a given year develop together and are then released in a short spawning period, while primary growth oocytes in an earlier developmental stage for the following spawning events are already present in the ovaries (McMillan, 2007; Wallace & Selman, 1981).

Even though the development of oocytes into eggs is a continuous process, it is commonly split into multiple phases: the primary growth stage, the cortical alveolus stage, vitellogenesis, and oocyte maturation (Lubzens et al., 2010; McMillan, 2007; Selman et al., 1993).

During the primary growth stage, oogonia proliferate by mitosis and become primary oocytes (PG). These primary oocytes begin meiosis but are arrested in the diplotene stage of the first prophase. Throughout this stage, the oocytes can increase in volume by up to 1000 times, primarily due to an increase of the cytoplasm and the proliferation of organelles (McMillan, 2007). Additionally, pre-follicular cells proliferate and form a follicle epithelium that fully encloses the oocytes (McMillan, 2007). In the primary growth stage the development of the oocytes seem to happen independently from the pituitary (Khoo, 1979).

In the cortical alveolus stage (CAs), cortical alveoli appear and gradually fill most of the ooplasm of the oocytes. Cortical alveoli are membrane-limited vesicles containing proteins and carbohydrates and are released upon fertilization (Selman et al., 1993). Lipids start aggregate into small droplets around the nucleus and the zona radiata (zona pellucida) forms between the oocyte and the follicular cells, which will later become the eggshell (McMillan, 2007). The transition of the oocytes to the cortical alveolus stage is controlled and dependent on the pituitary (Khoo, 1979). In salmon, it has been shown that the synthesis of cortical alveoli is correlated with an increase in plasma and pituitary FSH, plasma estradiol-17β (E2), and plasma

insulin-like growth factor 1 (IGF-I) levels (Campbell et al., 2006). It has been shown that in some fish, treatment with E2 and IGF-I can induce cortical alveoli formation in oocytes (Katti et al., 2017; Khoo, 1979; Lokman et al., 2007).

During vitellogenesis, oocytes absorb lipids and vitellogenins (vtg), synthesized in the liver under E2 regulation and transported to the ovary, where they break down into yolk proteins (McMillan, 2007; Wallaert & Babin, 1992). This phase leads to significant oocyte growth and zona radiata thickening due to liver-synthesized zona radiata proteins (Modig et al., 2006). Passively, through the uptake of lipoproteins, and vitellogenin, vitamins are also taken up by the oocytes (Palace & Werner, 2006)

Vitellogenesis is regulated through the hypothalamus-pituitary-gonad-liver axis as sown in Figure 1 (Arukwe & Goksøyr, 2003; Takahashi & Ogiwara, 2023). Gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the release of gonadotropins, GtH I (FSH), from the pituitary gland, which in turn stimulates the production of estrogen in the ovaries (Young et al., 2005). Estradiol-17β (E2) is produced when androgens, such as testosterone, synthesized in the thecal layer of the follicle epithelium, are aromatized to E2 in the granulosa layer by the enzyme cytochrome P450 aromatase (CYP19) (Kagawa et al., 1982; Nagahama, 2000). E2 stimulates the production of vitellogenin and zona radiata protein in the liver, which are then transported via the bloodstream to the ovaries, aiding in yolk accumulation and zona radiata development in the oocytes (Oppen-Berntsen et al., 1992; Tata & Smith, 1979).

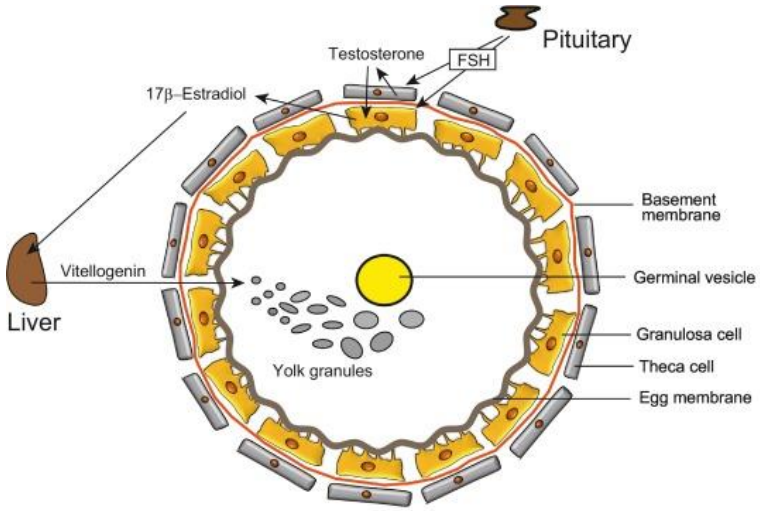

Figure 1. Schematic diagram showing the hormonal regulation of vitellogenesis in fish.(Takahashi & Ogiwara, 2023)

During oocyte maturation, meiosis resumes from the diplotene stage to metaphase II, where it halts until fertilization, and the oocyte undergoes hydration to enhance buoyancy and aid in osmoregulation (Cerdà et al., 2007). This process is regulated by a shift from GtH I to GtH II (LH), reducing estradiol-17β and increasing the maturation-inducing hormone (MIH) 17α,20β-DP, which promotes meiosis by inducing germinal vesicle breakdown (GVBD) (Nagahama & Yamashita, 2008) (Figure 2). GVBD triggers meiotic spindle formation, and within 5 hours, the oocytes reach metaphase II, with the first polar body extruded at the animal pole (McMillan, 2007). After reaching metaphase II, eggs are ready for ovulation, induced by genomic actions of 17α,20β-DP (Goetz & Theofan, 1979; Pinter & Thomas, 1999).

After ovulation and the spawning of the eggs, only the post-ovulatory follicles (POFs) of the oocytes are left. All oocytes remaining in the ovary that did not spawn will undergo atresia. After a period of regeneration, the development of the oocytes for the next spawning event will begin (McMillan, 2007).

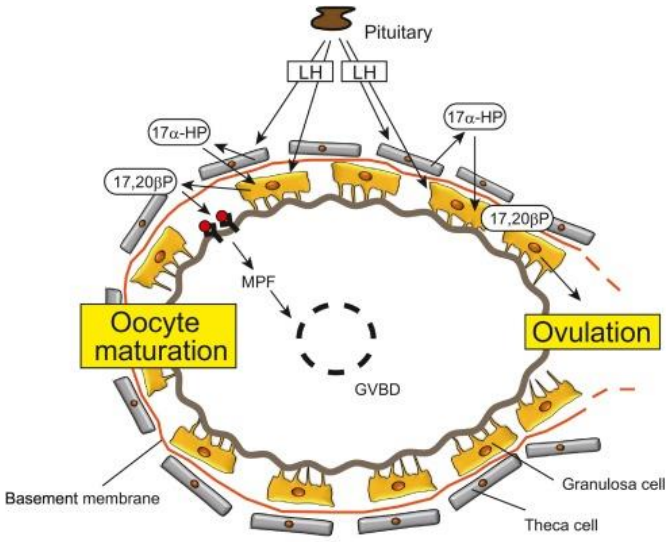

Figure 2. Schematic diagram showing the hormonal regulation of oocyte maturation and ovulation in fish. (Takahashi & Ogiwara, 2023)

Spermatogenesis is the developmental process in male fish where spermatogonial stem cells transform into mature spermatozoa through several stages of cell division and differentiation. An important role is played by Sertoli cells, which envelop the germ cells, forming the walls of the spermatogenic cysts in which spermatogenesis takes place (Lara et al., 2020).

Spermatogenesis is divided into three phases: the proliferative phase, the meiotic phase, and the spermiogenic phase (Schulz et al., 2010). In the proliferative phase, spermatogonial stem

cells undergo mitotic divisions to form differentiated spermatogonia. During the meiotic phase, the spermatogonia differentiate into spermatocytes, which then undergo meiotic divisions to produce haploid spermatids (Miura & Miura, 2011). In the spermiogenic phase, spermatids transform into mature spermatozoa. In this final differentiation, they develop a flagellum, eliminate excess cytoplasm, and undergo nuclear condensation (Schulz et al., 2010).

Spermatogenesis is controlled by the hypothalamic-pituitary-gonadal axis. GnRH released from the hypothalamus stimulates the pituitary gland to secrete FSH and LH. LH then acts on Leydig cells, triggering the production of testosterone, which is converted to 11-ketotestosterone (11-KT). Both FSH and the hormones testosterone and 11-KT interact with Sertoli cells, playing a key role in regulating spermatogonial proliferation, meiosis, and spermiogenesis (Miura & Miura, 2011; Schulz et al., 2010).

1.3 Effects on crude oil on reproduction of fish

Most studies on the effects of crude oil are conducted on early life stages, which show a high sensitivity to crude oil, while studies on the more resilient adults are less common. Nevertheless, research on the effects of crude oil on reproduction has been conducted in multiple teleost species.

Exposure to crude oil has shown mixed results in adult fish, but many studies indicate delayed reproduction or reduced gonadosomatic index (GSI). For example, in Atlantic cod, chronic exposure to crude oil has been linked to reduced GSI (Khan, 2013; Kime, 1995), comparable to the negative impacts on reproductive development observed with other pollutants affecting reproductive health (Hassanin et al., 2002; Kime, 1995; Singh & Srivastava, 2015). Furthermore, in many studies where fish were exposed to crude oil early in their gonad development, a delay in reproductive development was reported (Bender et al., 2018; Khan, 2013; Tetreault et al., 2003; Thomas & Budiantara, 1995; Truscott et al., 1983). For example, Bender et al. (2018) showed that female polar cod exposed to crude oil in June were more frequently found in earlier maturation stages seven months later, during the spawning window, compared to unexposed females.

A similar impairment in reproduction can also be found in the wild after exposure to crude oil due to an oil spill. For instance, a field study in polluted Newark Bay, New Jersey, showed that the reproductive health of killifish was compromised, with underdeveloped gonads compared to those from non-polluted areas (Bugel et al., 2010)). Similar findings were observed for the

English sole (*Parophrys vetulus)* in Puget Sound, Washington (Casillas et al., 1991), and in killifish after the Deepwater Horizon oil spill (Whitehead et al., 2012).

On the other hand, other studies have shown no noncable effect resulting from crude oil exposure. For example no effect on the GSI of Atlantic cod exposed to alkylphenols was found (Sundt & Björkblom, 2011). In polar cod, neither chronic dietary exposure (Bender et al., 2016) nor short-term exposure (Strople et al., 2023) to crude oil are shown to have a significant effect on the GSI.

Crude oil and its compounds have been linked to endocrine disruption on multiple occasions (Kim et al., 2016; Martin-Skilton et al., 2006). Reductions in steroid hormones, such as estradiol and testosterone, have been observed in female flounder (Rocha Monteiro et al., 2000), in rainbow trout (Tintos et al., 2006) and *sculpin* (Tetreault et al., 2003) after exposure to crude oil. Conversely, other studies found no effects of crude oil on steroid hormone levels (Bender et al., 2016; Pollino & Holdway, 2002; Strople et al., 2023). Crude oil has been shown to exhibit anti-estrogenic effects in females by decreasing the RNA expression of hepatic genes such as estrogen receptor α (ERα), vitellogenin and zona radiata protein (Bilbao et al., 2010; Navas & Segner, 2000; Safe & Wormke, 2003; Salaberria et al., 2014). Decreased ERα expression in response to exposure to crude oil or its compounds has been documented in multiple species, such as *Chelon labrosus* (Bilbao et al., 2010) , *Danio rerio* (Salaberria et al., 2014), *Gadus morhua* (Yadetie et al., 2018) and *Boreogadus saida* (Yadetie et al., 2021). Fish vitellogenin and zona radiata protein can serve as sensitive biomarkers for estrogen and may help identify estrogenic or anti-estrogenic xenobiotics (Arukwe et al., 2000; Arukwe & Goksøyr, 2003). Following exposure to benzo[a]pyrene, both a decrease in Vtg and Zrp levels was observed in rainbow trout (Navas & Segner, 2000; Woźny et al., 2008)

In males, exposure to crude oil also shows mixed effects, causing delays in spermatogenesis (Khan, 2013) in some cases while having no effects in other experiments (Strople et al., 2023). Similarly, research has shown that exposure to crude oil can lead to lower levels of 11-KTT in some studies (Pollino & Holdway, 2002; Tetreault et al., 2003), while other investigations have reported an increse in testosterone levels (Evanson & Van Der Kraak, 2001).

The timing of crude oil exposure is crucial in determining its effects on female fish reproductive development. Exposure during early gonad development delays maturation (Bender et al., 2018; Khan, 2013; Truscott et al., 1983)(Bender et al., 2018; Khan, 2013; Truscott et al., 1983),

while late-stage exposure, near spawning, accelerates reproductive development (Strople et al., 2023; Whipple et al., 1978). Strople et al. (2023) exposed polar cod to decreasing levels of the water-soluble fraction (WSF) of crude oil throughout the spawning season (December to April). After just 47 days of exposure (by late January), researchers found that the exposed fish had already spawned, while most unexposed fish had not. Although transcriptome mapping revealed the differential regulation of 947 hepatic genes, and the relative expression of estrogen receptor 1 (esr1) and vitellogenin (vtg) were reduced in the exposed fish, no effect on steroid hormone levels was found. These inconclusive results make it difficult to determine the mechanisms behind the advancement in reproductive development. Additionally, elevated PAH levels were found in the eggs, indicating parental transfer of oil-associated compounds from the mother to the eggs. No effect of exposure to WSF of crude oil throughout the spawning period on gonad development or steroid hormone levels in male polar cod was found.

A significant limitation of this study was the sampling time points. Only three sampling dates were utilized (December 14, 2018; January 31, 2019; and April 25, 2019), with only the January sampling occurring within the spawning window for the fish. This limitation made it difficult to analyze how and when this advancement in reproductive development occurred. Furthermore, it was challenging to adjust most measured endpoints, such as vtg α and esr1 expression, hormone levels, or GSI for maturation stage, making the interpretation of those results difficult.

1.4 Polar cod: a keystone species of the arctic ecosystem

In the Arctic marine ecosystem, polar cod (*Boreogadus saida*) is a keystone species, linking lower trophic levels, like zooplankton, to higher trophic levels such as mammals, seabirds, and other fish (Bradstreet, 1986; Hobson & Welch, 1992; Planque et al., 2014). Polar cod plays a crucial role in energy transfer within the Arctic marine ecosystem.

As a circum-Arctic species, polar cod is prevalent in both the open waters and ice-covered regions of the Arctic shelf seas (Craig et al., 1982; Marsh et al., 2020). Polar cod have evolved unique adaptations that enable them to thrive in the sub-zero environments. For instance, they produce antifreeze proteins, which allow them to withstand temperatures as low as -1.8°C (Harding et al., 2003; Hop & Gjøsæter, 2013). It is hypothesized that polar cod spawn beneath the sea ice, which offers early life stages, such as larvae and juveniles, both a refuge from predators and a reliable food source. However, the ongoing retreat of sea ice driven by climate

change may result in the loss of these essential habitats (Bouchard et al., 2017; Herbig et al., 2023). Polar cod are particularly susceptible to disruptions in ecological interactions, including the invasion of Atlantic species into the Arctic and the rising temperatures associated with climate change, which can further intensify their vulnerability (Bender et al., 2021; Dahlke et al., 2018). Climate change stresses on polar cod can have synergistic adverse effects when combined with anthropogenic pollution, such as in the case of oil spills, where rising temperatures can worsen the environmental impact (Bender et al., 2021).

Polar cod have a maximum lifespan of around seven (more common number) years. Females typically reach maturity at three years, while males reach maturity at two years of age (Hop & Gjøsæter, 2013; Nahrgang et al., 2014). The spawning period for polar cod takes place during the polar night, from January to March in the Barents Sea (Graham & Hop, 1995).

Polar cod exhibit seasonal migrations, moving from high-latitude arctic in the summer to more southern areas winter. This migration pattern helps them avoid predators and allows them to access more stable and abundant food sources, including zooplankton, during colder months (Aune et al., 2021).

1.5 Study aims

The present study was conducted to follow up on the research by Strople et al. (2023), further investigating the effects of exposure to the WSF of crude oil during the gonad development stage on reproductive development in polar cod. This study employed a more comprehensive sampling approach to address the limitations identified in the previous work.

The aim of our study was to investigate how exposure to the water-soluble fraction (WSF) of crude oil during the later stages of gonadal development affects the timing of reproductive development and spawning. We expected to observe a similar advancement as found in the precious study by Strople et al. (2023). Furthermore, we hope that through a more comprehensive sampling period, we will gain a more detailed understanding of when during development these changes occur and how much earlier spawning takes place after exposure. An additional objective was to assess whether the accelerated spawning induced by crude oil exposure caused eggs to be released prematurely. Finally, we aimed to examine how exposure to crude oil might impact plasma hormone levels, including estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT), during reproductive development. This investigation will provide

insights into potential interactions with endocrine signaling pathways and the likely anti-estrogenic effects of crude oil.

We hypothesized that:

1. As observed in Strople et al. (2023), exposure to crude oil late in gonad development accelerate reproductive development and lead to earlier spawning.
2. This advancement in reproductive development would be dose-dependent, with higher crude oil exposure resulting in a greater advancement compared to lower concentrations.
3. The advancement in reproductive development would lead to the release of smaller, premature eggs.
4. Steroid hormone levels will vary across different developmental stages, and a reduction in estradiol following crude oil exposure is expected due to the likely anti-estrogenic effects of crude oil.

Because of this, polar cod were exposed to the WSF of crude oil using a crude oil-covered gravel column, following the experimental design of Carls et al. (1999). The fish were exposed for 20 days during the late vitellogenesis phase, prior to the onset of spawning (from 25.11.2021 to 15.12.2021). Throughout the entire spawning window, samples were collected to gain a more comprehensive understanding of the effects of crude oil on reproductive development. This included examining developmental stages in relation to other measured parameters, such as gonadosomatic index (GSI), oocyte diameter, and steroid hormone levels. Additionally, two different concentrations of crude oil were used to explore the dose-dependent nature of the expected effects.

2. Methods:

All experiments were performed in accordance with the Animal Welfare Act and approved by the Norwegian animal research authority, mattilsynet (ID 28343, and ID 25790 for tagging).

2.2 Polar cod collection and husbandry

Polar cod were collected in Rijpfjorden in Svalbard and transported to the Tromsø Aquaculture Research Station in Kårvika in September 2020. Prior to the experiment, the fish were kept in a 3000 L housing tank. The light and temperature regime (1.5 - 3 °C) was set to simulate the *in situ* conditions in Svalbard (79°N), and the dissolved oxygen was kept above 80%. The fish were fed daily with Calanus sp. (Zooca). On 13.01.2021, the fish were sexed using ultrasound and tagged with external tags from Floy® Tag.

2.3 Experimental design and set-up

Polar cod were randomly transferred to 15 experimental tanks on November 19th, 2021. The tanks received filtered seawater (2 °C) with a 5 L/min inflow, oxygen saturation of over 80 %, and light matching the Rijpfjorden photoperiod (Svalbard). The tanks were randomly assigned to treatment groups in the experimental room to avoid influences due to location across treatment groups (Figure 3). Each 300 L flow-through tank contained 25 fish with a balanced sex ratio of males and females.

Polar cod were allocated to three treatment groups: one exposed to a low concentration of a water-soluble fraction of crude oil, another to a high concentration, and a control group that remained unexposed. The experimental setup consisted of five replicate tanks per treatment. The water-soluble fraction of crude oil was obtained using oiled rock columns, following the experimental design of Carls et al. (1999). These columns were prepared according to the methodology outlined by Nahrgang et al. (2010). This system simulates the gradual decrease in crude oil compound concentrations due to weathering, mimicking the aftermath of an oil spill. For the oiled rock columns, gravel from Berg Betong AS, ranging in size from 1 to 16 mm in diameter, were first washed and then coated with Goliat Kobbe crude oil from Vår Energi AS at concentrations of 18.75 g. The control gravels were kept uncoated. PVC columns were than filled with 90 kg of dried clean gravel for the control group, and 20 or 90 kg gravels of oil coated gravels for the low and high treatment groups respectively. The preparation of the gravel columns took place between October 27th and November 16th, 2021. Before the exposure, the

columns were flushed with a flow rate of 6.1 – 6.2 L/min for 24 hours, from November 18 to 19, to remove the volatile benzene, toluene, ethylene and xylene (BTEX) compounds of the crude oil and reduce the risk for baseline toxicity to occur. The exposure of polar cod to the water-soluble fraction of the crude oil took place for 20 days, from November 25 to December 15 at a flow rate of 5 L/min. Throughout the whole experiment the cod were daily fed with *Calanus* sp. Fish were starved 1 day before sampling.

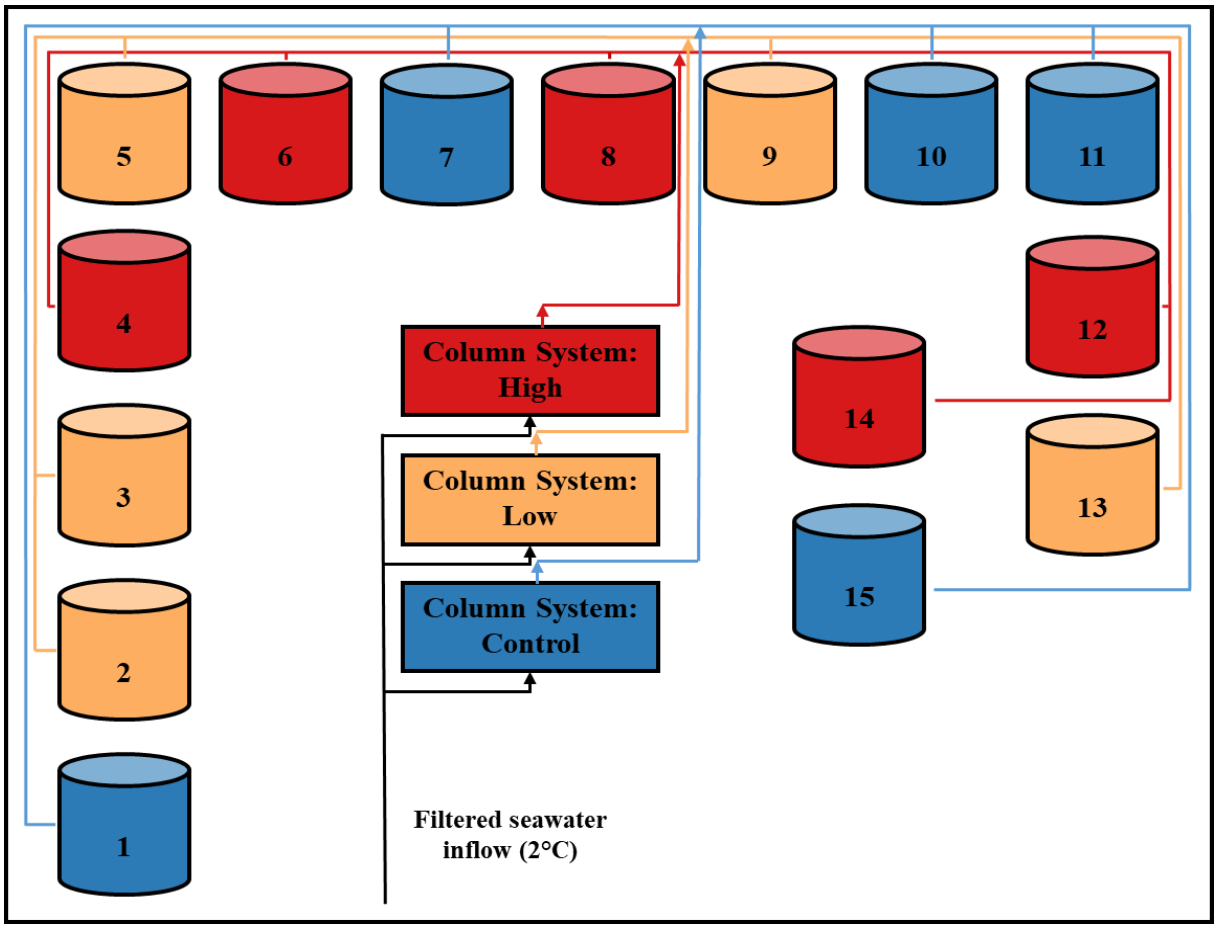

Figure 3. Experimental setup with 15 experimental tanks arranged in the experimental room. The tanks were divided into three treatment groups, each containing five tanks. The control group received water from columns containing clean gravels (blue), the low (yellow) and high (red) groups received the water-soluble fraction of crude oil from 20 kg and 90 kg oil-coated gravel respectively. The water from the column systems were flushed with filtered seawater into the experimental tanks with a flow rate of 5 L/min. The distribution of the treatment tanks inside of the experimental room was random.

2.4 Water and tissue sampling

Water samples (n = 24) for chemical analysis were collected from each of the three different exposure treatments (with two replicates per treatment) on days 0, 4, 10, and 20 of exposure to the water-soluble fraction of crude oil These samples were taken to assess the concentration

and composition of 44 PAHs in the water-soluble fraction of crude oil within the experimental tanks over the course of the exposure period.

On the day prior to transfer to the experimental setup (18.11.2021), twenty fish were sampled from the housing tank to provide a pre-experimental baseline for the biological endpoints analysed. Additional samples were taken during the exposure period on December 6 (11 days of exposure) and again at the end of the exposure period on December 15 (20 days of exposure). During the spawning period, fish were sampled multiple times, from mid-January to late February 2022, on the following dates January 19, January 26, February 2, February 8–9, February 15–16, and February 22, these dates correspond to 55, 62, 69, 75-76, 82-83 and 89 days after the start of the experiment. (Figure 4).

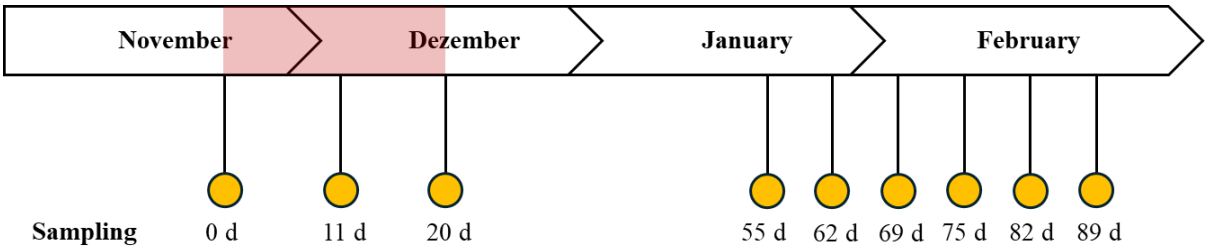

Figure 4. Experimental timeline showing the exposure period from the November 25 to Dezember 15 (red) and the sampling time points represented as day post exposure stared.

For sampling, fish were removed from the tank and euthanized with an overdose of 50 mg/L Tricaine Methanesulfonate (MS222) dissolved in seawater. Blood samples were collected from the caudal vein, centrifugation for 30 min at 4 ◦ C and 3500 rpm (Sorvall RC 5 B Plus centrifuge) and the blood plasma was stored at – 80 °C for further analysis. Sex, total weight (to the nearest 0.01 g), and the total fish length (to the nearest 0.1 cm) were documented. Fish were then dissected to collect the bile, gonads, and liver. The gonad samples were taken from the mid-section of the gonad and preserved in 4% neutral-buffered formalin for histological analysis. Liver weight and gonad weight (to the nearest 0.01 g), and somatic weight (to the nearest 0.01 g) were recorded. From these, the gonadosomatic index (GSI) was calculated using the following equation:

$$\mathrm{GSI} = \frac{\text{total gonad weight (g)} * 100}{\text{total weight(g)}}$$

Other morphological traits such as hepatosomatic index (HSI) and condition factor (K) were calculated using the following equation:

$$\mathrm{HSI} = \frac{\text{total liver weight (g)} * 100}{\text{somatic weight (g)}}$$

$$\mathrm{K} = 100 * \frac{\text{total weight (g)}}{\text{total length (cm)}^3}$$

The spawning activity of the fish in the tanks was monitored once every dy and freshly spawned buoyant eggs were collected from from egg-collectors installed in each tank. The total egg volume collected from each tank was then divided into three replicate samples and stored in a climate room at 3 °C. Microscope images of the eggs were taken a day after their collection. For this the eggs were transferred to a watch glass and immediately photographed with a Leica MC170 HD camera connected to a Leica M205C stereo microscope. From the images, the egg diameters (mm) could be measured using ImageJ software. It is important to note that eggs collected could originate from one or several females in the tank. Furthermore, the time between collection and photographs could range from minutes after spawning to up to 24 hours.

2.5 Biological and chemical analyses

2.5.1 Quantification of PAHs in water samples

The analysis of the water samples for 44 PAHs was conducted by SINTEF Ocean in Trondheim using GC-MS/MS, following the methods described by Sørensen et al. (2016a) and (2016b). Dichloromethane (DCM) was used to extract the organic phase from the water samples. The extract was then concentrated to approximately 1 mL using a TurboVap® evaporator. Prior to extraction, surrogate internal standards (SIS: 10 µg o-terphenyl, 252.3 ng naphthalene-d8, 48.0 ng phenanthrene-d10, 50.0 ng chrysene-d12, and 50.8 ng perylene-d12) were added to each sample. After extraction and before analysis, GC-MS/MS recovery internal standards (RIS: 10 µg 5α-androstane, 100 ng fluorene-d10, and 100 ng acenaphthene-d10) were also added to the samples. A laboratory blank (800 mL of Milli-Q water) was extracted using the same procedure for quality control.

For analysis, an Agilent 7890 gas chromatograph coupled with an Agilent 7010B triple quadrupole mass spectrometer equipped with an electron ionization source and collision cell was used. High-purity helium served as the carrier gas.

Target PAH analytes were identified using two distinct MRM transitions, and the most intense peak was used for quantification (Sørensen et al., 2016b). Alkylated PAH clusters were

determined using molecular ion-based MRM transitions, in line with the approach outlined in Sørensen et al. (2016a). System performance was monitored by incorporating standards throughout the sample sequence, accepting a variability of up to 25% as satisfactory.

2.5.2 Gonad histology and oocyte diameter

Following collection, the gonad samples were left in formalin for 4 days. They were then washed twice in a 70% ethanol bath and embedded in paraffin wax using the Citadel 2000. The preservation process in the Citadel 2000 involved multiple baths, in the following order: 96% ethanol, 100% ethanol, a 50/50 mixture of 100% ethanol and Histo-Clear, Histo-Clear alone, a 50/50 mixture of Histo-Clear and paraffin wax, and finally a paraffin wax bath. The samples were embedded in paraffin wax on to cassettes and stores in the fridge (4 °C).

Using a microtome (Leica RM2235), the samples were sliced into 5 µm sections. For each sample, 3 replicates from different depths (~100 µm apart), each containing 3 – 5 slices on the same microscope slide, were prepared to obtain the best possible sections, where structures could be most clearly identified despite any interference from slicing, staining, or fixation. Slides were then stained with hematoxylin/eosin and fixated with a coverslip. Images of the slices were taken using a Primo Star Zeiss microscope equipped with a camera and analyzed with ImageJ software.

Gonad histology was used to identify the developmental stage of the reproductive development of each female, following the classification system of Brown-Peterson et al. (2011) as done by Strople at al. (2023). The developmental stage classified as Developing, Spawning Capable, Actively Spawning, and Regressing. An early regressing sub-phase was added to include individuals that had just spawned and were in a stage between Actively Spawning and Regressing (Table 1).

For female fish, samples were classified into various developmental stages based on the presence or absence of different oocyte maturation stages. The presence of primary vitellogenic oocytes (VTG1) and secondary vitellogenic oocytes (VTG2) indicated that the sample was in the development phase. If tertiary vitellogenic oocytes (VTG3) were present, the sample was in the spawning-capable phase. Samples with hydrated oocytes or hyaline eggs (HydrO/Es) were in the active spawning phase. The presence of residual eggs (ResEs) and post-ovulatory follicles (POFs) indicated early regression, while samples with only post-ovulatory follicles (POFs) were in the regressing stage. Primary growth oocytes (PGs) could be found in all

developmental stages. Pictures of those developmental stages can be found in Supplement figure 1. Oocyte maturation stages were identified by consulting literature on fish gonad histology (Brown-Peterson et al., 2011; McMillan, 2007) as well as research done on polar cod specifically (Nahrgang, Storhaug, et al., 2016; Strople et al., 2023).

For each female fish in the developing, spawning capable and active spawning phase the diameter of 10 oocytes was measured using ImageJ software. Considering that oocytes are not perfectly round, the diameter was measured along two diagonals, and the mean of these measurements was calculated. Unfortunately, due to the difficulty of preparing HydrO/Es for histology, many lost their shape completely and could not be used for diameter measurement. Consequently, the diameter of 1-day-old fertilized eggs was also measured. Using ImageJ, the diameter of 10 fertilized eggs for each of the replicates was measured when possible (a few replicates did not contain 10 fertilized eggs). If possible, multiple clutches from the same tank were sampled.

For the determination of the gonad developmental stage in male fish the classification from Brown-Peterson et al. (2011) was used (Table 2). Identification of the different phases was based on the structure of the germinal epithelium (GE) and sperm development. Previously published literature on male gonad development in polar cod was used for visual comparison (Nahrgang, Storhaug, et al., 2016; Strople, 2020). Since most males remained in the spawning-capable phase throughout our experimental period, this phase was divided into three sub-phases: Early, Mid, and Late. Pictures of those developmental stages can be found in Supplement figure 2.

Table 1. Developmental stages of female gonads based on Brown-Peterson et al. (2011) and Strople at al. (2023). Stages were determined by the presence of different oocyte maturation stages (primary growth oocyte (PG), cortical alveoli oocyte (CA), primary vitellogenic oocytes (VTG1), secondary vitellogenic oocytes (VTG2), tertiary vitellogenic oocytes (VTG3), hydrated oocyte/hyaline eggs (HydgO/Es), post-ovulatory follicles (POFs) and residual eggs (ResEs))

Stages	Description	Oocyte maturation stage
Developing	Ovaries begin to develop, not spawning ready, Oocytes develop from PG over CA to VTG1/2	VTG1 or VTG 2
Spawning capable	Fish ware able to spawn, fish finish vitellogenesis	VTG 3
Subphase: Active spawning	Finished Oocytes undergo hydration and spawn	HydgO/Es
Regressing	Eggs were spent, gonads recover from spawning, only the POF were left behind	POFs
Subphase: Early regressing	Fish had just spawned, many residual eggs remained	POFs and ResEs

Table 2. Developmental stages of male gonads based on Brown-Peterson et al. (2011) and Strople at al. (2023). Stages were determent by sperm development (secondary spermatocyte (Sc2), primary spermatogonia (Sg1), secondary spermatogonia (Sg2), spermatid (St), spermatozoa (Sz)) and germinal epithelium (GE) structure.

Stages	Description	Histology
Developing	Spermatocytes enter meiosis, and active spermatogenesis occur	Sg2, Sc1, Sc2, St and Sz can be present GE continuous throughout
Spawning capable		Sz in lumen of lobules and/or sperm ducts
Subphase: Early	Spermatocysts throughout testis, active spermatogenesis, Sz are ready to be spawned	Continuous GE in all lobules
Subphase: Mid		Continuous GE near periphery and discontinuous near ducts
Subphase: Late		Discontinuous GE
Regressing	Sperm is spent, Sz present is noticeably reduced, Little to no active spermatogenesis	Residual Sz present in lumen and sperm ducts Residual GE present in the lumen

2.5.3 Steroid hormone analysis

Plasma concentrations of estradiol-17β (E2, females only), 11-ketotestosterone (11KT, males only) and testosterone (T, both sexes) were measured using radioimmunoassay (RIA), according to Schulz (1985). In this assay, an antigen (the hormones) from the sample competes with a known amount of radioactively labeled (^{3}H) antigen for the binding sites of a limited amount of antibody. Thus, the amount of antigen in the sample is inversely related to the measured radioactivity from the labeled antigen. Using a standard curve, the amount of antigen (hormone) in the sample can be determined.

For this analysis, steroids were extracted from plasma by mixing it with 4 mL of diethyl ether (DEE). The water phase was frozen using liquid nitrogen, allowing the ether phase to be decanted into a fresh glass tube. The ether was then evaporated in a 45°C water bath under a stream of N_2 gas. Finally, the extract was reconstituted in RIA buffer (57.8 mM sodium phosphate in pH 7 with 0.9% NaCl, 0.01% gelatin and 0.05% sodium azide) at a volume three times that of the original plasma sample.

For the RIA, 100 µL of extracted plasma was added to a borosilicate glass incubation tube and mixed with 50 µL of tracer solution (^{3}H-labeled steroid diluted to approximately 8200 cpm/50 µL RIA buffer) and 200 µL of antiserum solution (antisera diluted 1:9 in RIA buffer). The mixture was incubated overnight at 4°C. A standard curve, consisting of nine serial dilutions of commercially available sex steroid hormones, along with standards for total binding and non-specific binding, was included alongside each assay.

On the following day, 300 µL of cold dextran-coated charcoal solution (1 g charcoal and 0.1 g dextran T70 in 100 mL RIA buffer) was added to each sample and incubated for 5 minutes to bind and remove any steroid hormones not attached to the antibodies. The samples were centrifuged for 5 minutes at 5000 rpm at 4°C, and the supernatant was decanted into counting tubes. The supernatant was then mixed with 7 mL of Ultima Gold XR scintillation liquid, and β-radiation was measured for 5 minutes using a liquid scintillation analyzer. Each sample was then compared to the standard curve run in the same assay, allowing for calculation of hormone concentrations in the plasma sample given in µg/L.

2.6 Statistical analysis

All statistical analyses were performed using R version 4.1.2. The normality of the data was assessed using both a Q-Q plot for visual inspection and the Shapiro-Wilk test for formal statistical testing. The assumption of normality was met for oocyte diameter data, which justified the use of a two-way analysis of variance (ANOVA) to examine the effects of time, development, and treatment on the diameter of developing oocytes. A two-way ANOVA was used to analyze the effect of time, treatment, and tank on the mean diameter of the fertilized eggs.

For variables like the GSI and hormone levels, where the normality assumption was violated, we used the non-parametric Kruskal-Wallis ANOVA. This test was followed by post-hoc pairwise comparisons using the Wilcoxon rank-sum test, which helped identify which pairs of

groups were significantly different from each other. Due to the strong differences between developmental stages, the effect of treatment on GSI and hormone levels was analyzed separately for each developmental stage. Potential tank effects on the GSI, hormone levels and oocyte diameter were considered, but no effect of the tank on these variables was found. All Comparisons were considered significant at a p-values ≤ 0.15. Data are presented as mean (95% confidence interval (CI)). The water analysis data are presented as mean ± standard deviation (SD), due to them only consisting out of tow replicates.

3. Results

3.1 Total PAH concentration

The mean total PAH concentration (Σ44PAHs) in the high exposure tanks (64.2 ± 25.35 µg/L) was three times higher than in the low exposure tanks (19.29 ± 0.36 µg/L) at the onset of the exposure (Figure 5). Throughout the 20-day exposure period, the concentration of PAHs decreased in all exposed tanks, reaching 8.23 ± 0.33 µg/L in the high treatment and 1.68 ± 0.01 µg/L in the low treatment. In contrast, the PAH concentration in the control tank remained consistently between 0.03 and 0.09 µg/L. The exact PAH composition throughout the exposure period in the different treatments can be found in the Appendix (Supplement figures 3-5). In both the low and high treatments, 2-ring PAHs initially accounted for 97 ± 2.1 % and 96 ± 1.6 % of the total concentration, respectively. However, this proportion declined over time, with 2-ring PAHs representing only 87.6 ± 0.6 % for the low treatment and 89.3 ± 0.3 % for the high treatment of all measured PAHs on the last day of exposure. The remaining PAHs were primarily represented by 3-ring PAHs, ranging from 3–12% over time, with smaller contributions from 4-ring PAHs, which ranged from 0.05–0.3%. Only trace amounts of 5-ring PAHs (<0.0012%) were detected.

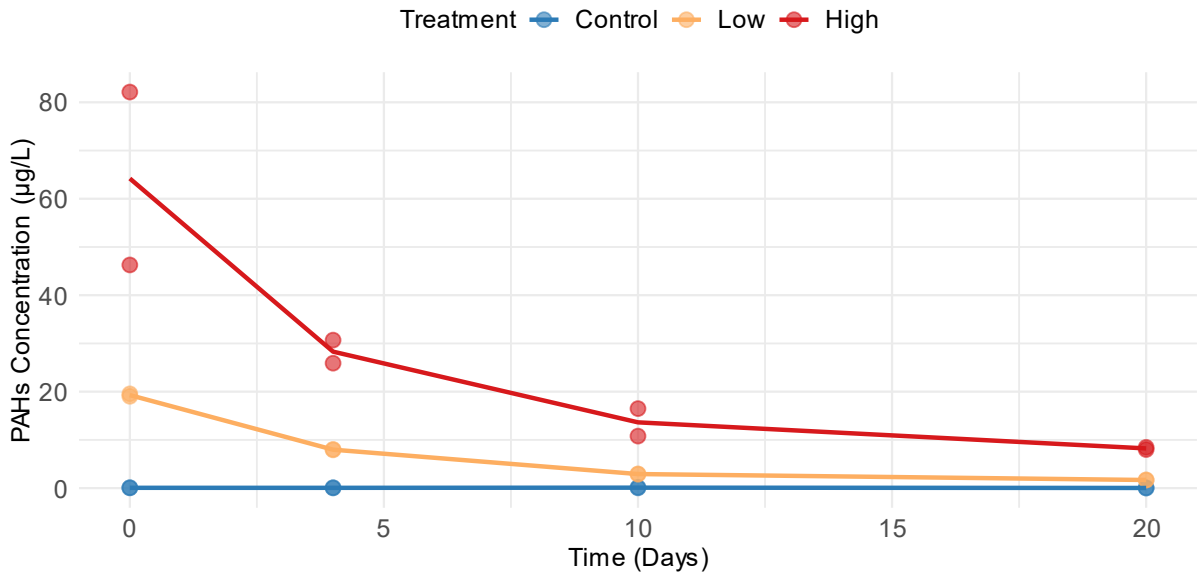

Figure 5. Total PAH concentrations (Σ44PAHs) in water samples from experimental tanks throughout the exposure period. Samples were exposed to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. Σ44PAHs is represented by individual data points for two replicates per treatment and time point (dots), with the regression line showing the mean concentration of these replicates over time.

3.2 General fish health

Throughout the experiment, three fish died, with one death occurring in each treatment group, indicating that these mortalities could not be attributed to crude oil exposure. The hepatosomatic index (HSI) and condition factor (K) showed no significant differences between the treatment groups (Table 3). Overall, no visible health defects related to exposure to the water-soluble fraction of crude oil were observed during the experiment.

Table 3. Hepatosomatic index (HSI) and condition factor (K) and sex ratio of fish exposed to control, low, and high concentrations of the WSF of crude oil. HSI and K are given as mean (95% CI)

Treatment	HSI	K	Sex ratio (M/F)
Control	18.6 (15.8, 21.4)	0.83 (0.8, 0.86)	76/63
Low	17.9 (15.8, 20)	0.8 (0.77, 0.83)	53/59
High	17.8 (16.7, 19)	0.82 (0.74, 0.9)	54/62

3.3 Histology: Gonad development

At the start of the experiment in November, 8 out of 10 females were in the developing stage (n=10), one was in the spawning-capable stage, and one was immature. Throughout the exposure period (25.11.2021–15.12.2021), the female gonads develop from VTG1 oocytes in the developing stage to VTG3 oocytes in the spawning-capable stage. By January, all the females from all treatments were in the spawning-capable stage, with the presence of vtg3 oocytes. In the control group, spawning activity was recorded from 2.2.2022. The majority of the females spawned in mid-February. The first female with gonads in the regressing stage, was sampled on February 8th. In the end of February (22nd), 73% of the sampled female in the control treatment had spawned and were in the regressing stage (Figure 6).

A similar progression in gonadal maturation was observed in both the low and high treatment groups. The low treatment group initiated spawning at the beginning of February, with the first female in the regressing phase recorded on February 2nd, a week before the first control female was found in the regressing stage. On February 16th, 85% of all sampled females in this group had reached the regressing phase.

The High treatment group started spawning 3 weeks before the control group. In the high treatment group, the first female in the regressing phase was documented as early as January 19th. By February 16th, all females exposed to high levels of the water-soluble fraction of crude oil had spawned (Figure 6).

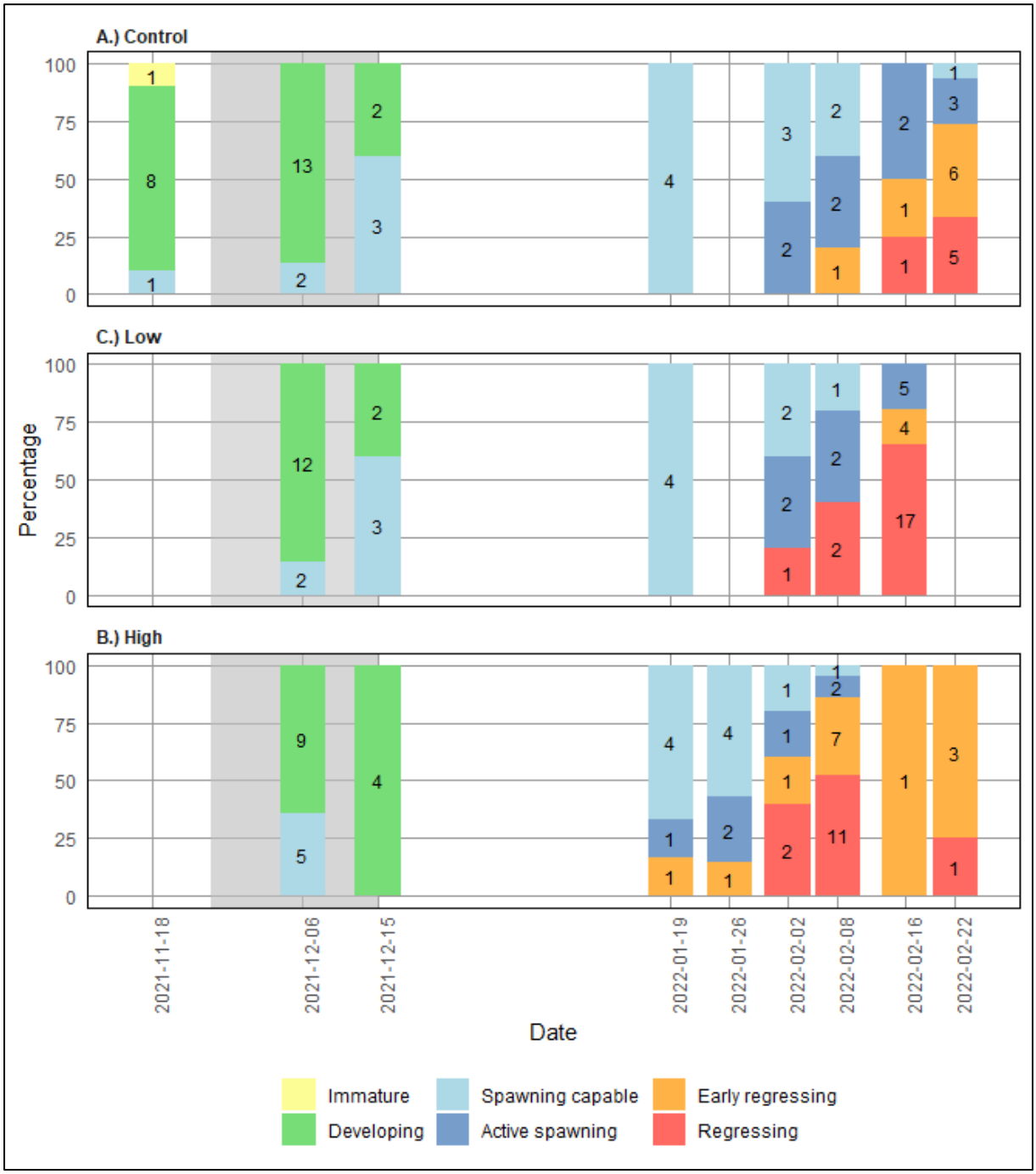

Figure 6. Fraction of the developmental stages of female polar cod throughout the spawning period. Fish were exposed to control (A), low (B), and high (C) concentrations of the water-soluble fraction of crude oil for 20 days at the beginning of the experimental period (grey area). The stages are classified based on the guidelines from Brown-Peterson et al. (2011). Sample counts are represented by the small black numbers.

In male polar cod, 9 out of 10 sampled fish were already in the spawning-capable phase at the start of the experiment, with only one still in the developmental phase. Most male fish (159 out of 183 fish) remained in this phase throughout the study with only 24 males found in the regressing stage by the end the experiment. By examining the sub-phases of the spawning-capable phase, we observed that the fish transitioned from the early to mid-spawning-capable phase in November and entered the late spawning-capable phase from mid December till February. The first male in the regressing stage was recorded on the 8th February, indicating the spawning started before this date. No differences in gonadal development were found between the different treatment groups (Figure 7.

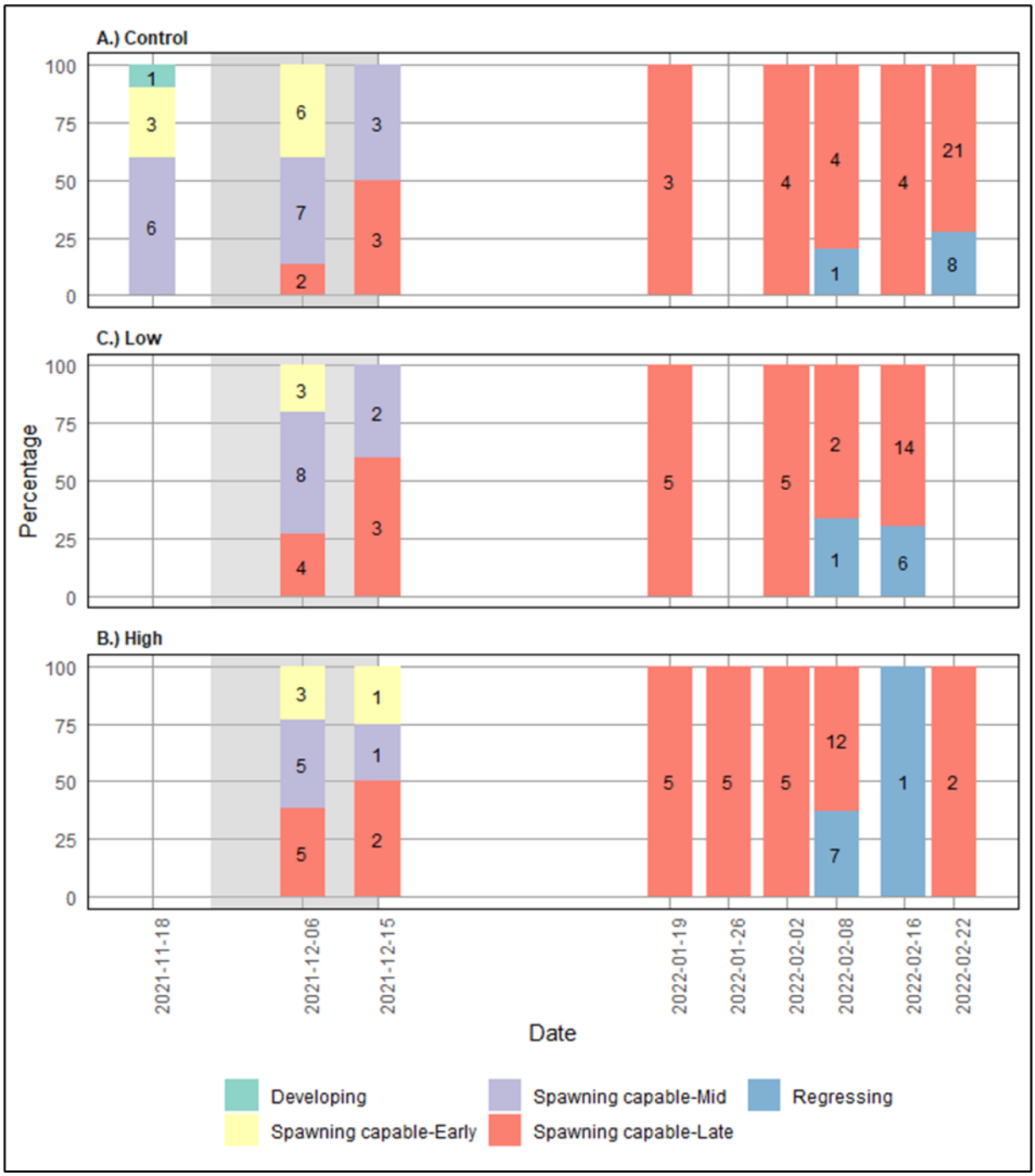

Figure 7. Fraction of the developmental stages of male polar cod throughout the spawning period. Fish were exposed to control (A), low (B), and high (C) concentrations of the water-soluble fraction of crude oil for 20 days at the beginning of the experimental period (grey area). The stages are classified based on the guidelines from Brown-Peterson et al. (2011). Sample counts are represented by the small black numbers.

3.4 Gonadosomatic index (GSI)

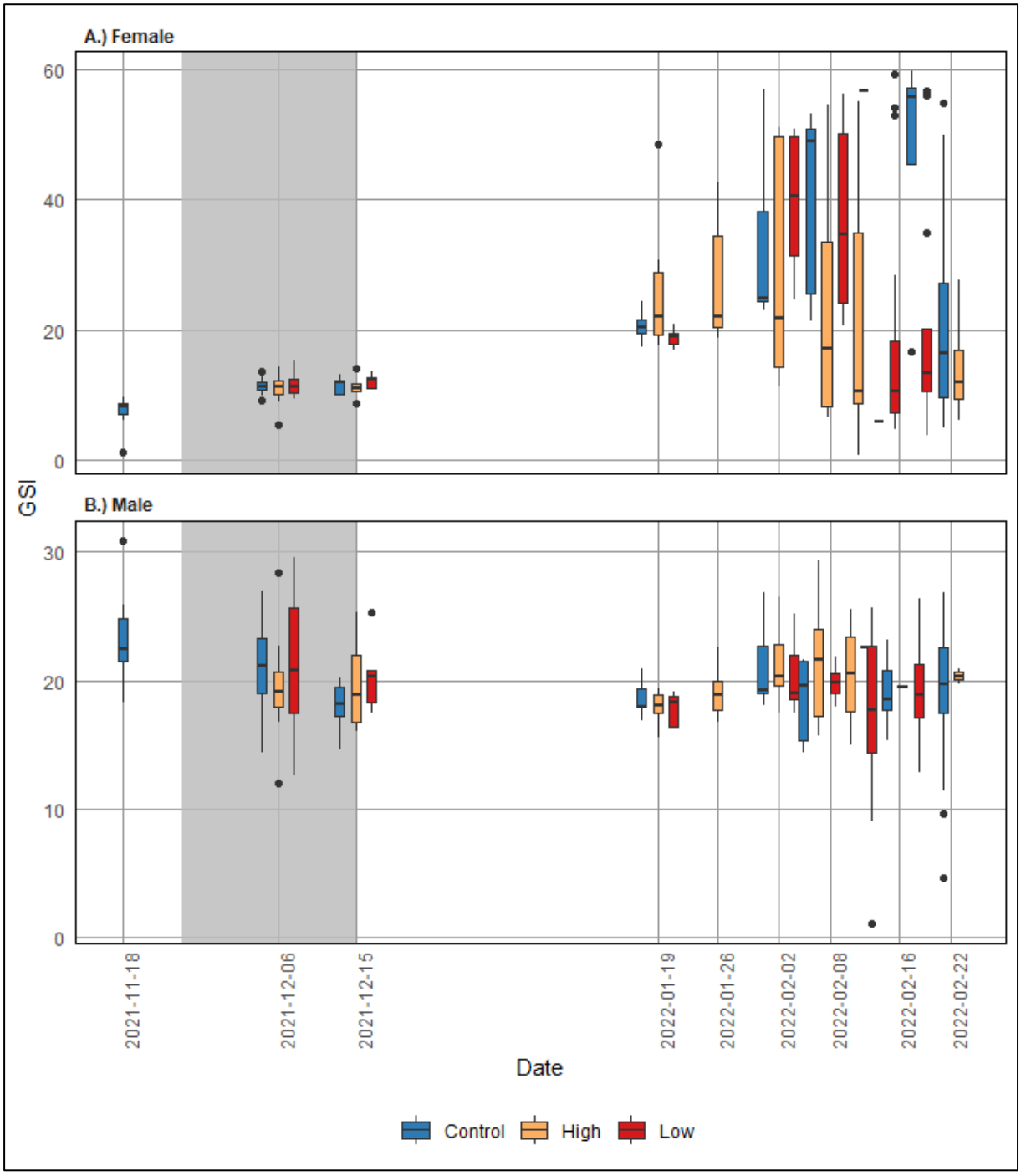

Figure 8. Gonadosomatic index (GSI, %) of female (A) and male (B) polar cod over the experimental period. The fish were exposed in the exposure period (grey area) to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots feature the median (line), the 25%-75% percentiles (box), the non-outlier range shown by whiskers, and dots representing any outliers.

The GSI of the females underwent significant changes throughout the experiment. Initially, and during the exposure period, the GSI remained low, with an average value of approximately 11.4 % (10.7, 12.1). However, the GSI increased as the oocytes developed and spawning approached in early February, before declining again at the end of February following spawning (Figure 8). The control treatment reached a maximum average of 57 % (55.1, 58.9) on February 16. In contrast, both exposure treatments peaked earlier, with lower maximum values than the control. The low treatment reached its peak on February 8, with a maximum average of 39.3 % (24.9, 53.7), while the high treatment peaked on February 2, with a maximum average of 29.6 (95% 12.6, 46.6). The changes in GSI were strongly correlated with the developmental phases ($p < 0.0001$) (Figure 9). No significant differences in GSI were observed between the treatment groups when accounting for the different reproductive phases.

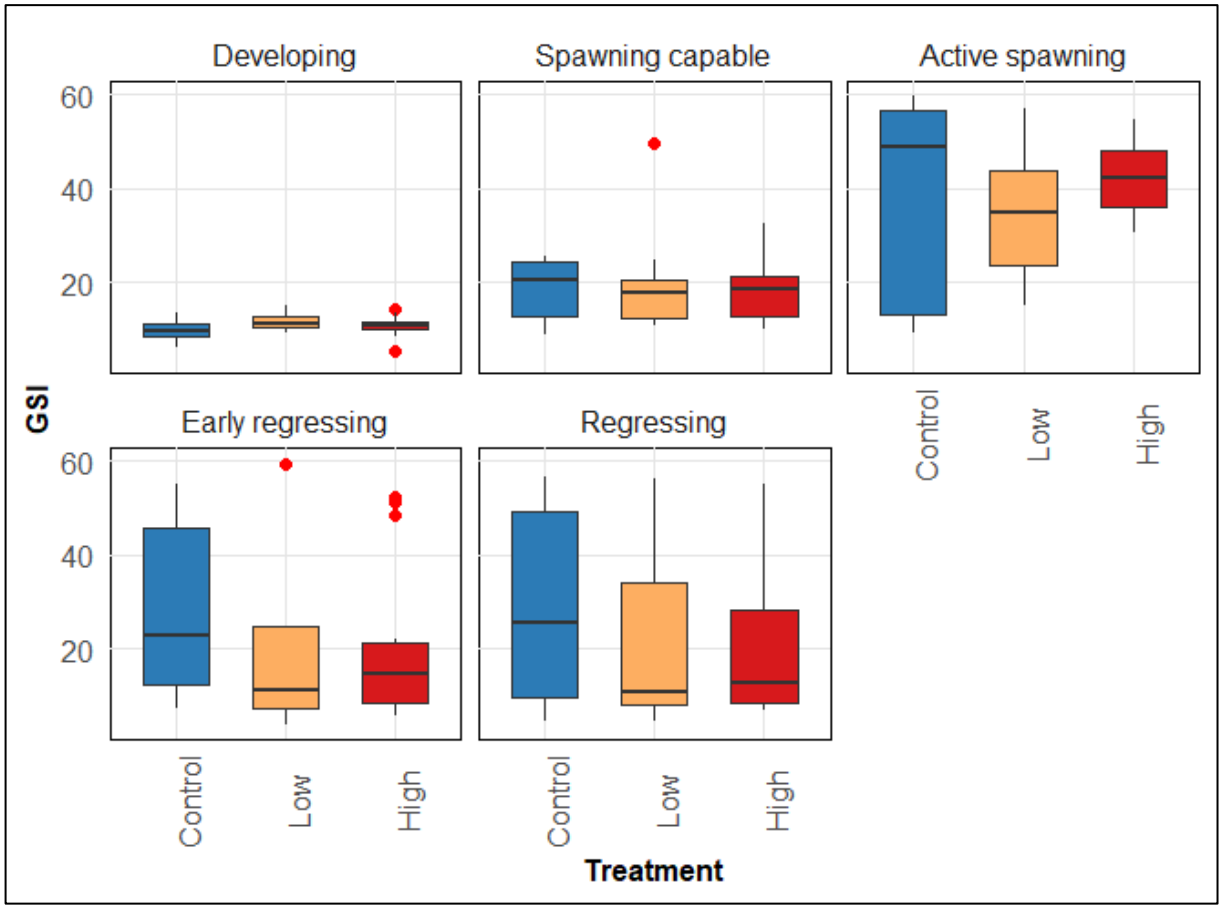

Figure 9. The Gonadosomatic Index (GSI; gonad weight/total weight) of female polar cod in different developmental phases after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots.

The GSI did not vary over the course of the experiment in any of the treatment groups for the males averaging at 20 (19.4, 20.4) ranging from 4.6 to 30.9 (Figure 8).

3.5 Oocyte and egg diameter

The measured oocyte diameter increased significantly ($p < 0.0001$) throughout the maturation of the oocytes, with the largest diameter observed in the spawning-ready, hydrated eggs during the active spawning phase (Supplement Figure 6).

The different treatments did not significantly affect the oocyte diameter ($p = 0.156$). However, the hydrated eggs in the active spawning phase tended to be smaller in the high treatment compared to the control treatment (Figure 10), with a mean diameter of 898 µm (890, 906) in the high treatment group, compared to 907 µm (894, 920) in the control group. It is important to note that only a small sample size of eggs in the active spawning phase could be analyzed, as most hydrated eggs lost their shape during preparation for histological analysis.

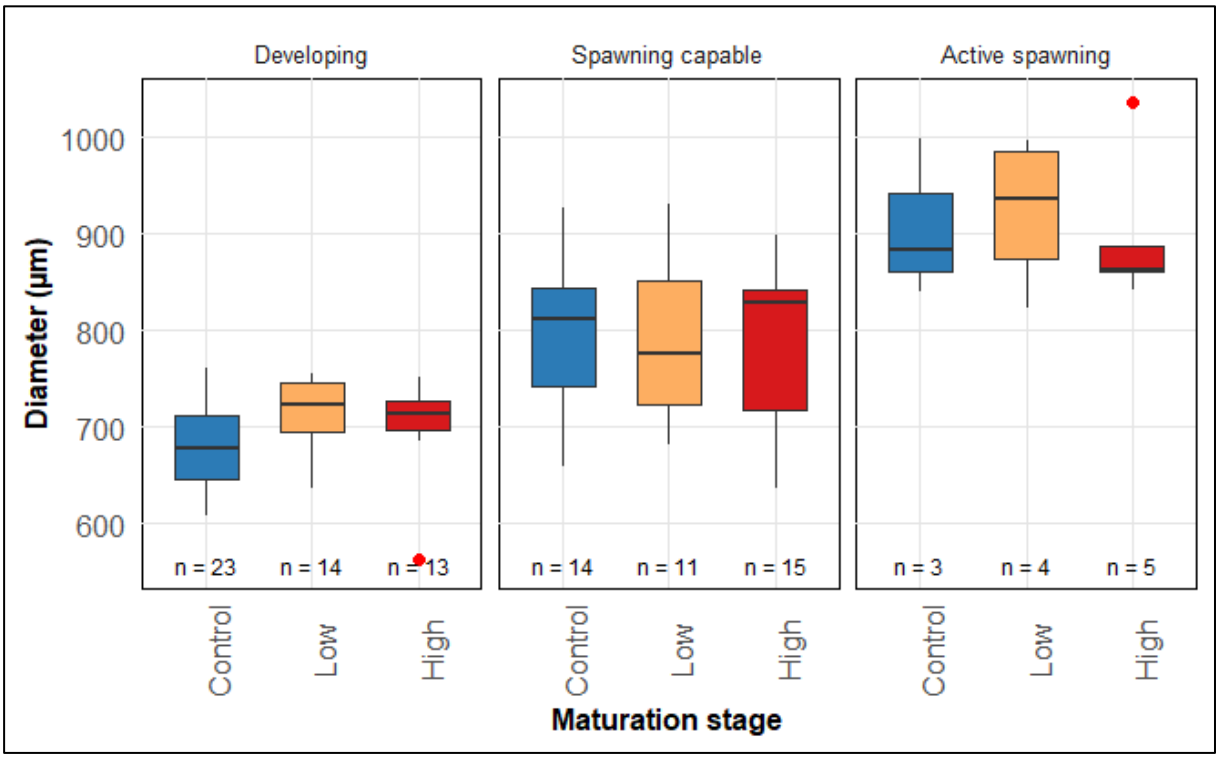

Figure 8 Oocyte diameter (µm) after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil, compared across different maturation stages of the oocytes. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots. Sample counts are indicated by the small black numbers.

The diameter of the fertilized eggs differed significantly between treatments ($p = 0.00915$), replicate tanks ($p < 0.0001$) and date of collection ($p < 0.0001$). This difference mainly resulted from the significant differences observed in one of the replicate tanks in the high treatment (Tank 12) compared to most other tanks in the experiment (Figure 11). Fish in tank 12 were the first to spawn among all tanks, starting to spawn on the 22nd of January. The eggs from tank 12 had a mean diameter of 1.60 mm (1.58, 1.62) much lower than the average diameter of 1.66 mm (1.65, 1.67) of all tanks.

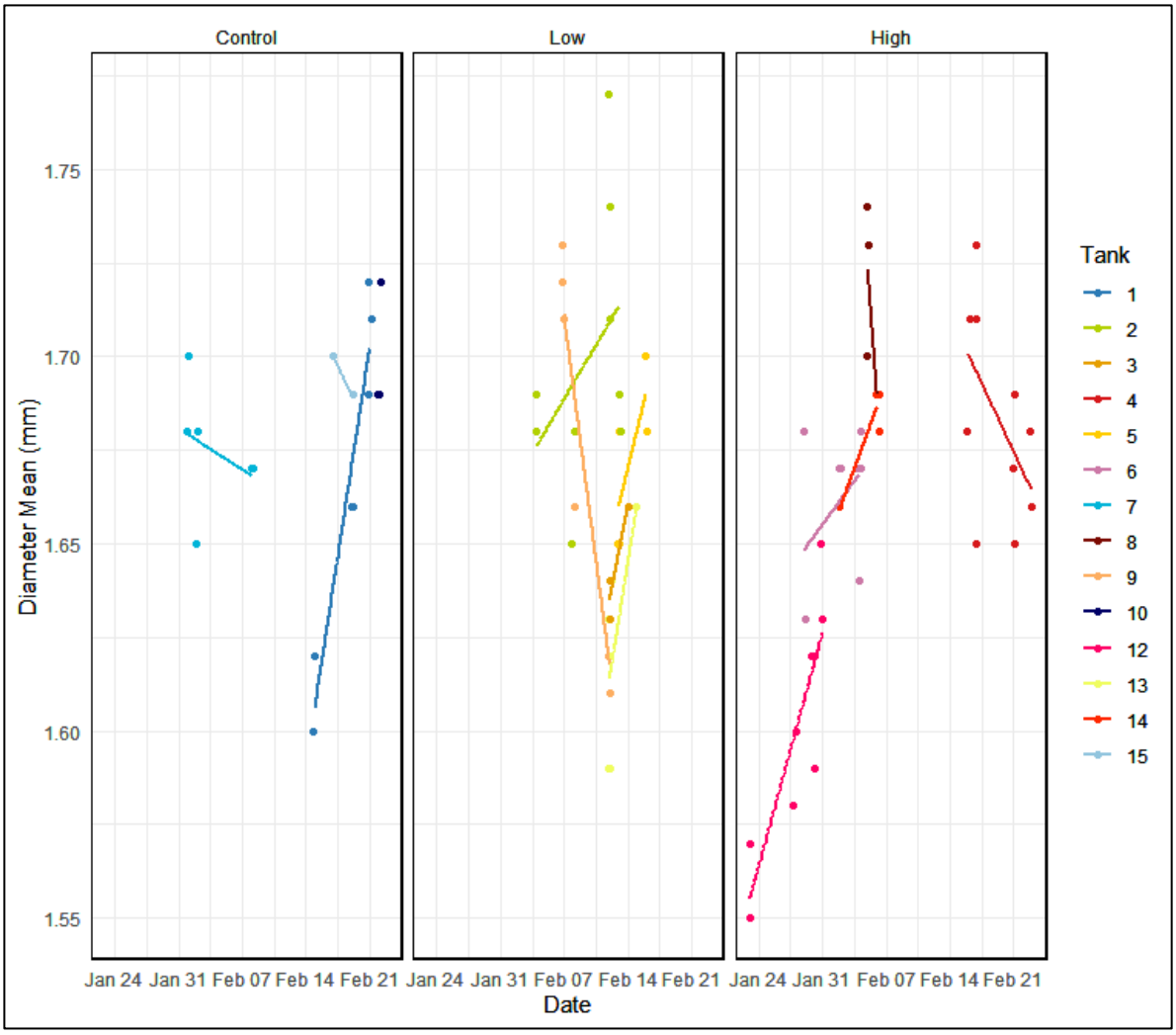

Figure 11. Mean diameter (mm) of 1-day-old fertilized egg batches collected in different replicate tanks after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil throughout the spawning window. Mean diameter of individually collected egg batches are represented as dots, and regression lines for each tank were represented as colored lines. Note that each egg batch represents eggs from one or more females that spawned simultaneously in the tank.

3.6 Steroid hormone analysis

The testosterone levels in the plasma of female polar cod increased throughout the maturation of the oocytes until they decreased again when the eggs were ripe and ready to spawn, remaining low during the regression phase. Consequently, a significant change in testosterone levels could be measured over time ($p = 0.0002$) and between the different developmental stages ($p < 0.0001$). The treatment did not significantly affect the testosterone levels of the females ($p = 0.0646$). However, when examining the treatment effects on the different developmental stages separately, it is notable that during the active spawning phase, elevated levels of testosterone ($p = 0.0111$) in the high treatment were measured compared to the control and low crude oil treatments (Figure 12). A testosterone concentration of 18.5 ng/mL (0.1, 36.9) were found in the high exposure group at the active spawning phase. In male polar cod, the

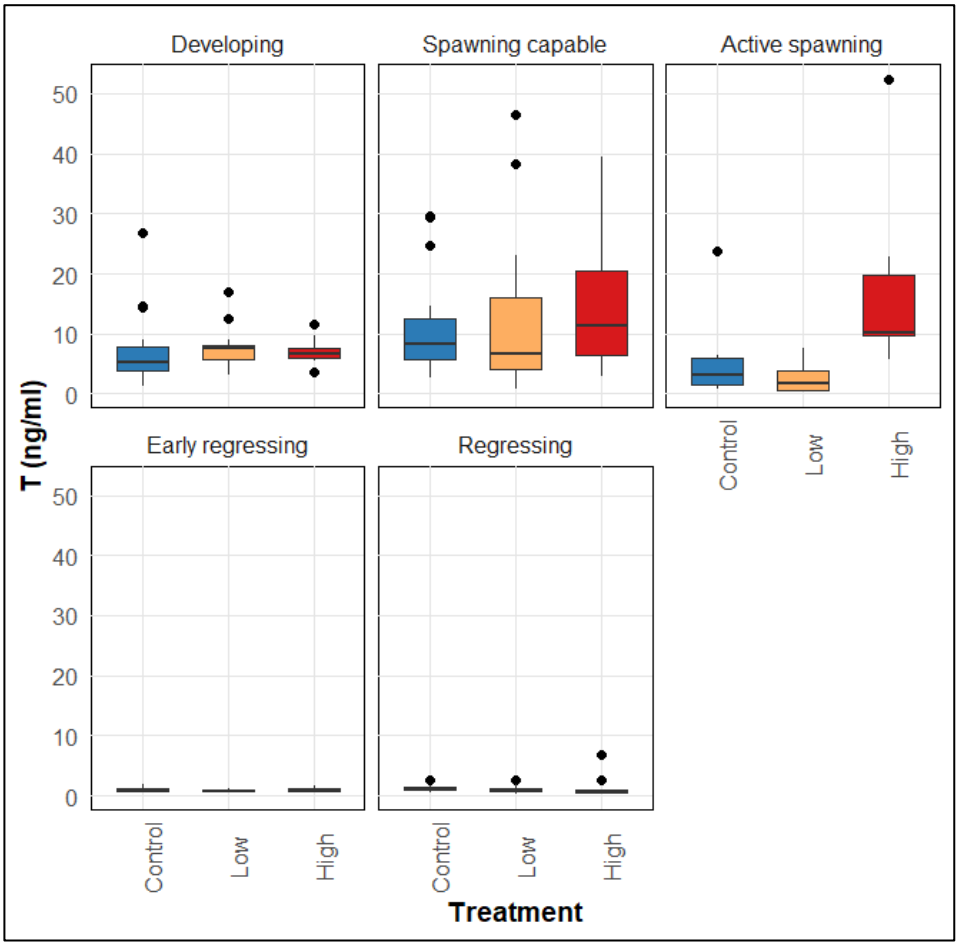

Figure 12. Levels (ng/ml) of Testosterone (T) in the plasma of female polar cod in different developmental stages after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots.

testosterone levels remained mostly constant throughout the experiment and did not differ significantly between dates, developmental stages, and treatments (Supplement Figure 7).

The measured E2 levels in female polar cod were strongly correlated with the development of the oocytes ($p < 0.0001$), following a similar pattern to T, with increased plasma levels in the developing and spawning-capable phases, and lower levels during the active spawning and regression phases. No effect of the treatment ($p = 0.971$) with crude oil on the E2 levels was detected (Figure 13). The ratio of E2/T was highest in the developing phase 1.59 (1.36, 1.81) and the lowest in the active spawning phase 0.32 (0.16, 0.48) Even though the E2/T ratio was significantly different between developmental stages ($p = 0.0015$), no significant treatment effect could be found ($p = 0.9572$) (Supplement Figure 8).

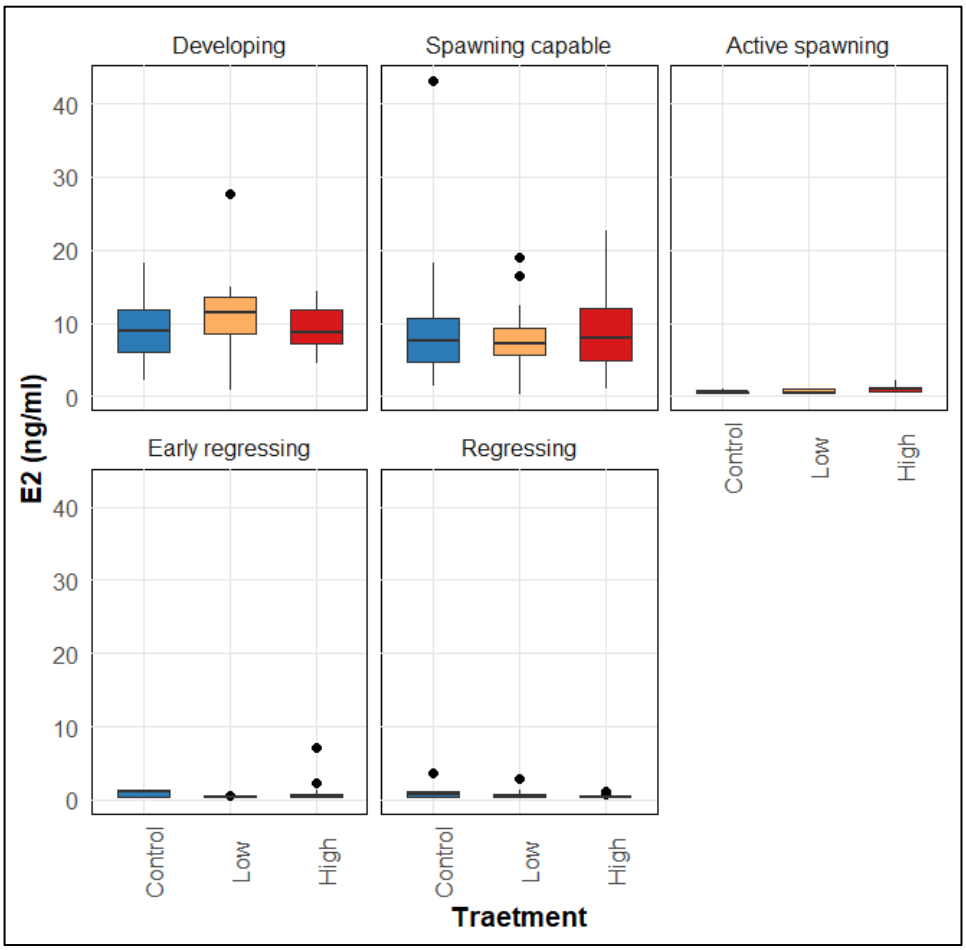

Figure 13. Levels (ng/ml) of Estradiol-17β (E2) in the plasma of female polar cod in different developmental stages after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots.

The plasma levels of 11-KT did not differ between developmental stages ($p = 0.0752$) and treatments ($p = 0.4461$). However, when analyzing the changes throughout the sub-phases of the spawning capable phase, there was a slight increase from the early to late phase ($p = 0.0057$) (Figure 14).

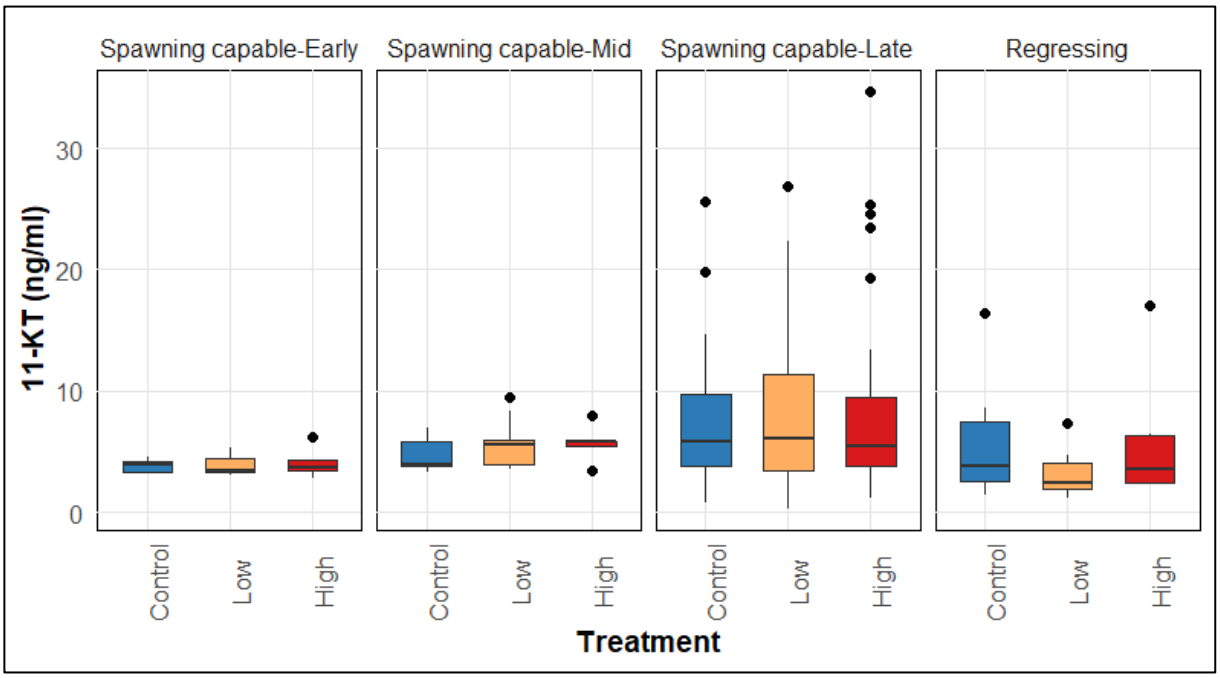

Figure 14. Levels (ng/ml) of 11-ketotestosterone (11-KT) in the plasma of male polar cod in different developmental stages after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots.

4. Discussion

4.1 Aqueous PAH concentrations

Initial aqueous concentrations of sum 44 PAHs in both the low (19.29 ± 0.36 µg/L) and high (64.2 ± 25.35 µg/L) treatment groups were within the range found in other similar exposure studies on fish (Carls et al., 2000; Nahrgang et al., 2010; Strople et al., 2023). Nahrgang et al. (2010) used sum 26 PAHs concentrations from 15 to 40 µg/L in an experiment with the same exposure method. These measured PAH concentrations also fall within the range of environmentally relevant levels reported after oil spills (González et al., 2006; Short & Harris, 1996; Wade et al., 2011). For instance, in the months following the Deepwater Horizon oil spill (April 20–July 15) in the Gulf of Mexico, water samples collected from the surrounding area revealed an average concentration of 47 µg/L of total PAHs, with levels ranging from 1231 µg/L to undetectable (<0 µg/L) between May and August, depending on sampling point and location (Sammarco et al., 2013).

As expected, the use of the crude oil-coated rock column resulted in a decrease in total PAH concentration over time, resembling the natural weathering process of crude oil in the environment as shown in previous experiments (Carls et al., 2000; Nahrgang et al., 2010; Strople et al., 2023). It has been shown that due to evaporation and dissolution, PAHs with lower molecular weights and fewer alkyl substituents are lost faster during weathering, while larger PAHs remain behind. As a result, while the 2-ring PAHs, such as naphthalene, constitute the majority of the initial PAH concentration (~95%), they dissipate rapidly over time. In contrast, the heavier 3- and 4-ring PAHs persist for a longer duration, leading to a more balanced distribution of PAHs over time (Roques et al., 1994; Short & Heintz, 1997).

The measured PAHs represent only a small fraction of the total hydrocarbons in the water-soluble fraction. Even though only the PAH levels in the surrounding water were measured, it can be anticipated that these PAHs, along with other compounds from the water-soluble fraction of crude oil, would be absorbed into the fish's body, accumulated in various tissues throughout the body and biotransformed into metabolites.

4.2 Advancement in gonad development of female polar cod

Exposure to the water-soluble fraction of crude oil led to an advancement in gonad development, with 86% of females in the high-exposure treatment having already spawned by the second week of February (08.02), when the first females in the control treatment began spawning. Our comprehensive sampling throughout the spawning period revealed that exposure to the high treatment (64.2 ± 25.35 µg/L of initial PAHs in the WSF) resulted in an approximately 20-day advancement in spawning. In comparison, the low treatment (19.29 ± 0.36 µg/L of initial PAHs in the WSF) led to a 6-day advancement. A similar advancement was observed in the previous study by Strople et al. (2023), which employed a comparable experimental setup but with fewer sampling points. The crude oil concentration used by Strople et al. (2023) had an initial PAH level similar (2.5 ± 4.3 µg/L) to our low-exposure treatment, yet the advancement they observed after 46 days of WSF exposure was significantly more pronounced, resembling the advancement seen in our high-exposure group. It is important to note that the spawning period in their experiment appeared earlier than in ours, with unexposed control female cod beginning to spawn by the end of January. Furthermore, their exposure period began in mid-December, 20 days later than the start of ours, and did not end after 20 days. Instead, it continued throughout the spawning window. The later timing of crude oil exposure to polar cod in their study, compared to ours and the prolonged exposure, may account for the differences in the extent of the observed effects. Furthermore, their sampling was limited to just 46 days after exposure (January 31st), with no other time points during the spawning window, preventing to determine how many days the exposed females spawned before the unexposed females. Another study on the effects of exposure to the WSF of crude oil during the late phase of vitellogenesis on gonad development in female *Platichthys stellatus* also showed an advancement in the Egg maturation (Whipple et al., 1978). No notable difference in the GSI was observed in the present study following exposure to crude oil, similar to the findings of Strople et al. (2023).

In contrast, most other research on the effects of crude oil or crude oil related compounds exposure on the reproductive development of adult fish reported a delay in development or a reduction in the GSI(Bender et al., 2018; Casillas et al., 1991; Khan, 2013; Meier et al., 2007; Thomas & Budiantara, 1995). In most of those studies the female fishes were exposed to crude oil before the vitellogenic stage or during early vitellogenesis. The timing of exposure to crude oil may play a crucial role in determining the impact on timing of reproductive development. If crude oil exposure occurs late in vitellogenesis, after most of the necessary yolk has already

accumulated in the oocytes, changes in factors such as estrogen levels and vitellogenin may no longer significantly affect the development of the oocytes (Navas & Segner, 2000). By this point, the gonadal development may have progressed to a stage where further arrest or reversing of the maturation is no longer possible (Rideout et al., 2005). It would be a biological disadvantage to pause or reverse oocyte development through atresia at the late stages of development. Research shows that the decision to skip spawning in years when conditions such as pollution, malnutrition, or environmental factors are unfavorable is often made early in development, sometimes at the start or even before vitellogenesis, to conserve the energy necessary for survival (Rideout et al., 2005). In Atlantic cod, it has been demonstrated that when energy reserves are too low during early vitellogenesis, oocyte development is halted as early as November, long before the onset of spawning (Skjæraasen et al., 2009).

Since reproductive development is regulated by different hormones throughout its developmental stages, exposure to crude oil may have varying effects on gonad development due to its potential interactions with these hormones. Crude oil and its compounds are linked to endocrine disruption, and some studies have shown that exposure to crude oil alters plasma concentrations of steroid hormones such as estrogen (E2), testosterone (T), and their ratio (E2/T). But the data on this are inconsistent, with some research indicating reduced steroid hormone levels after crude oil exposure (Rocha Monteiro et al., 2000; Tetreault et al., 2003; Tintos et al., 2006), while other studies found no effects of crude oil on hormone levels (Bender et al., 2016; Geraudie et al., 2014; Pollino & Holdway, 2002). It has been suggested in previous research that crude oil may have an anti-estrogenic effect. For example, multiple studies have shown that crude oils or its components result in a decrease in the expression of the estrogen receptor or vitellogenin (Bilbao et al., 2010; Salaberria et al., 2014; Strople et al., 2023; Yadetie et al., 2018). This endocrine disruption is associated with the binding and activation of the estrogen receptor by PAHs and the inhibition of androgen receptors (Thomas et al., 2009; Tollefsen et al., 2006). This could explain the delayed reproductive development in fish exposed during early vitellogenesis, where lower vitellogenin levels throughout vitellogenesis result in slower yolk formation, leading to delay in development or the skipping of the spawning in this year.

In our study, we did not observe any differences in estrogen levels between polar cod that were exposed to crude oil and those that were not exposed, at any developmental stage. However, we did find a strong correlation between estrogen levels and the developmental stages of the oocytes, with elevated estrogen levels throughout vitellogenesis. Since the females were

already in vitellogenesis at the start of the experiment, elevated estrogen levels were measured from the beginning. The plasma estrogen levels measured throughout our experiment were comparable to those reported in the spawning window by previous studies, with a value of 8.32 ± 6.43 ng/mL in January (Bender et al., 2016). Since the fish were already in vitellogenesis at the start, the vitellogenin levels in the gonads were probably already high, and most of the yolk had already accumulated in the oocytes before exposure to crude oil could have any effect. Therefore, it is possible that any potential alterations in vitellogenin levels after exposure would not slow down vitellogenesis anymore.

Exposure during the later stages of development could instead potentially influence the spawning process, leading to premature egg release and affecting egg quality. Crude oil exposure at this stage may disrupt hormones or enzymes essential for the final maturation of oocytes and spawning. Specifically, GtH II (LH) plays a crucial role in producing MIH during final maturation (Lubzens et al., 2010). Compounds in the water-soluble fraction (WSF) of crude oil may interact with LH or MIH production, leading to the advancing of the maturation process. Unfortunately, no research has yet explored these interactions. Future studies should investigate the impact of crude oil on the hormones and genes involved in final oocyte maturation.

In addition to the timing of exposure during vitelogenesis, the differing effects of crude oil exposure on gonad development may be influenced by species-specific differences. Variations in exposure methods and durations could also contribute significantly to these outcomes. For example, focusing on research involving polar cod, the exposure method can be a relevant factor. It has been shown that acute exposure to burned crude oil (48 hours of exposure with Σ26 PAH levels ranging from 101.5 ± 14.3 μg/L to 3.5 ± 1.2 μg/L, depending on the treatment) throughout early gonad development (June) results in a delay in gonad development (Bender et al., 2018). In contrast to this low-level dietary exposure (1.8 to 13.1 ng Σ26PAHs/g fish/day) from early development until spawning (June to February) did not alter the timing of gonad development (Bender et al., 2016). These two different modes of exposure will result in completely different levels, distribution patterns, and compositions of PAHs within the fish. Short, acute high waterborne exposure will initially lead to high levels of PAHs in the fish, but these levels will decrease over time due to active biotransformation and excretion after the exposure ends. In contrast, low, chronic dietary exposure will likely result in initially low levels of PAHs that accumulate within the fish over time. Furthermore, the dietary exposure route alters the systemic distribution of compounds due to first-pass metabolism. This underscores

the difficulty in comparing studies that involve different exposure concentrations, modes of exposure, and durations, as such variations in experimental designs can lead to inconsistent results and make it challenging to draw meaningful conclusions across studies

The testosterone levels in the females followed a similar pattern to that of estradiol-17β, with elevated levels during the spawning-capable phase and lower levels following spawning during the regression phase. Testosterone levels measured in this experiment during the spawning-capable phase were comparable to those reported in previous studies. For instance, Bender et al. (2016) reported plasma testosterone levels of 8.7 ± 5.5 ng/mL in polar cod during February. Interestingly, in the present study, a significant elevation in testosterone levels was observed in females when they were actively ready to spawn, and their gonads contained fully mature eggs following high exposure to the water-soluble fraction (WSF) of crude oil. Normally, plasma levels of testosterone should drop immediately after maturation before the spawning occurs to a low baseline level (Guiguen et al., 1993; Norberg et al., 2004). Hop et al. (1995) found that polar cod had a testosterone concentration of 1.21 ± 0.28 ng/mL at the time of spawning, which is significantly lower than the 18.5 ng/mL (0.1, 36.9) measured in our study. This is the first time that elevated testosterone levels have been documented following crude oil exposure. Additionally, this is the first study where fish were sampled in such detail throughout the entire spawning window, collecting detailed samples from different developmental stages throughout oocyte maturation. The reason behind the still elevated testosterone levels has not been investigated yet. It could be that, due to the advancement of the spawning time following crude oil exposure, the maturation and subsequent spawning occurred so quickly that testosterone levels had not yet dropped after the completion of vitellogenesis are change in the expression of CYP19 effecting the transformation of Testosterone into Estrogen. Whether these elevated testosterone levels have any effect on spawning and the viability of the resulting fertilized eggs is unclear.

Another indicator of endocrine disruption can be a change in the estrogen-to-testosterone (E2/T) ratio, which may signal alterations in enzymes within the steroidogenic pathway affecting the conversion of testosterone (T) to estradiol (E2) (Arukwe et al., 1997). When there are significant variations in the absolute hormone levels of individual fish, the E2/T ratio serves as a normalization metric. In the present study, the E2/T ratio varied significantly across maturation stages, with the lowest mean values 0.32 (0.16, 0.48) recorded during the active spawning phase. Although no statistically significant treatment effect on the E2/T ratio was observed, there was a trend towards lower ratios in the high treatment group during both the

active spawning phase 0.091 (0.04, 0.509) and the regressing stage. This trend could be attributed to elevated T levels in the high treatment group post-spawning. It has been shown that in female Atlantic cod, exposure to PAHs in produced water can result in a reduction of the E2/T ratio (Sundt & Björkblom, 2011).

Hormone levels can rapidly fluctuate in response to external stimuli, whereas tissue development progresses at a slower, more stable rate. Consequently, comparisons of hormone levels across identical histological developmental stages may reveal differences that are not indicative of treatment-induced alterations but rather reflect the inherent intra-specific variability in hormonal regulation.

A similar variating effect is known for the impact of stress on the maturation of oocytes in general. Stress during early ovarian development can delay ovulation, while stress during late vitellogenesis can lead to earlier spawning (Contreras-Sánchez et al., 1998; Schreck et al., 2001). Stress-induced cortisol levels are hypothesized to have modulating effects at different stages of reproductive development. During vitellogenesis, cortisol is suggested to interfere with the transcription levels of genes related to the HPG axis, leading to alterations in E2/T levels (Ye et al., 2024) . In the final maturation stage, it may have a regulatory role on the action of maturation-inducing hormones (Faught & Vijayan, 2018). The severity of the stressor plays an important role in determining the extent of the observed effects. In our experiment, the WSF of crude oil could have served as the stressor, resulting in an advancement of gonad development. In this context, it would have been interesting to measure cortisol levels as an indicator of stress. This is something that should be considered in future experiments.

4.3 Effects of crude oil on oocyte diameter and egg quality

A key question is whether final oocyte maturation occurs too early, leading to premature egg-release and potentially lower egg quality. The oocyte diameter measured in this experiment did not show any significant difference between the different crude oil treatments. During vitellogenesis, the oocyte diameter increased as expected across all treatments. This suggests that the accumulation of yolk in the oocytes was similar in both groups. Therefore, crude oil exposure did not appear to affect vitellogenesis when it occurred late during the reproductive development. The histology preparation method was unsuitable for the delicate, hydrated eggs, causing structural damage and preventing accurate diameter measurements. This resulted in a small sample size, which may have obscured potential effects, and a larger sample number

could have yielded different results. Due to this loss, the interpretation of these results should be approached with caution.

As a result, the size of freshly spawned fertilized eggs was also measured. There were no significant differences between treatments overall. However, one tank exposed to high levels of the WSF of crude oil spawned significantly earlier than the other tanks, and the egg size in this tank was also significantly smaller than in all the others. This could indicate that high levels of crude oil exposure may lead to such a strong advancement in reproductive timing that smaller eggs, potentially of lower quality, are released prematurely.

It is well known that the quality of offspring is correlated with egg size (Morrongiello et al., 2012; Trippel, 1998). Studies have shown that egg size directly correlates with the size of the offspring after hatching, giving them a better chance of survival and an advantage when competing for food (Bagenal, 1969; Magnuson, 1962; Ojanguren et al., 1996). In addition, in situations where food is scarce, fish hatched from larger eggs tend to take longer to starve, giving them a better chance of survival.(Marsh, 1986). Because of this, it could be that high crude oil exposure could lead to delayed effects in terms of survival of hatched larvae in the next generation. In addition, it has also been shown that PAHs can be maternally transferred from the mother to the eggs, potentially leading to significant long-term effects on the survival and development of the embryo (Carls et al., 2000; Strople et al., 2023).

4.4 Impact of crude oil on male gonadal development

In the present study, we did not focus extensively on male polar cod. However, we observed that most male polar cod were already in the spawning-capable phase at the beginning of the experiment and remained in this phase throughout the entire study. As a result, no effects of crude oil exposure on their reproductive development were observed. It is known that male polar cod reach the spawning-capable phase earlier than females and remain in this phase longer (Lear, 1980; Nahrgang et al., 2014), which may give them the advantage of being able to fertilize eggs over an extended period, reducing the chances of mismatches in the spawning window between males and females.

Other studies on the effects of crude oil on the reproductive development of male polar cod have also failed to find any impact on gonadal development, regardless of whether the exposure occurred early or late in spermatogenesis, or whether it was an acute or chronic exposure (Bender et al., 2018; Bender et al., 2016; Strople et al., 2023). However, it has been shown that

long-term exposure to the WSF of crude oil can disrupt spermatogenesis in male Atlantic cod (Khan, 2013; Kiceniuk & Khan, 1987). Similar to females, the effects of crude oil may vary significantly depending on species-specific responses, exposure timepoint throughout the gonad development, exposure duration, and the method of exposure, making comparisons with previous experiments unreliable.

The steroid hormone analysis of testosterone and 11-KT levels in male polar cod did not show significant differences between treatments or developmental sub-stages throughout the experiment. The mean testosterone level was 9.84 ± 6.08 ng/mL, and the mean 11-KT level was 6.63 ± 5.65 ng/mL, which are in a similar range to the testosterone concentrations (11.7 ± 7.1 ng/mL) and 11-KT concentrations (3.38 ± 1.87 ng/mL) measured in polar cod during February in previous experiments (Bender et al., 2016). Other studies on the effects of crude oil on steroid hormone levels throughout the spawning window have yielded mixed results, with some showing an increase (Evanson & Van Der Kraak, 2001), others a decrease (Pollino & Holdway, 2002), and some showing no change at all (Bender et al., 2016; Strople et al., 2023). In the study conducted by Strople et al. (2023), which had a similar experimental setup to the present study, no effects were found on steroid hormone levels in male polar cod following 47 days of crude oil exposure.

Due to time constraints, the quality of sperm, such as sperm motility or velocity, was measured but has not yet been analyzed. Previous studies have reported no effect of crude oil exposure on sperm motility in polar cod and capelin (Beirão et al., 2018; Strople et al., 2023). Contrasting, when Atlantic cod were exposed to produced water containing 0.57 μg/L of total PAHs for 12 days, a reduction in the quantity of mature sperm was observed (Sundt & Björkblom, 2011). Furthermore, there is evidence suggesting a potential paternal effect of crude oil exposure on offspring. For instance, a decrease in DNA methylation has been observed in the offspring of zebrafish following parental exposure (Bautista et al., 2020). Because of this, further research on the effects of crude oil on male polar cod are necessary

4.5 Future perspectives:

The present study, in comparison with previous studies on polar cod, highlights that varying responses to crude oil exposure can occur even within a single species (Bender et al., 2018; Bender et al., 2016; Geraudie et al., 2014; Strople et al., 2023). Determining whether the contrasting results arise from differences in exposure timing, duration, or methodology is

challenging. To address this, it would be valuable to conduct a series of experiments with a consistent exposure method but varying time points throughout gonadal development. This approach would help clarify whether the delayed spawning observed with early exposure and the advancement of spawning with late exposure are consistent, or if these divergent effects are simply a result of differences in experimental design rather than exposure timing. Furthermore, it would be interesting to follow up on the specific timepoint in gonadal development of the female polar cod where the shift from delayed to advanced spawning occurs, expecting that the more advanced vitellogenesis is the more like exposure to crude oil will lead to advance in the reproductive development instead of a delay in development.

Additionally, further investigation into the mechanisms behind the advancing effect of late crude oil exposure on gonad development of female polar cod is needed. This could involve exploring possible interactions between crude oil compounds, such as PAHs, and hormones involved in the final maturation of oocytes. Further investigation into endocrine-disruptive pathways should include a more detailed analysis of gene expression for proteins such as estrogen receptor 1 (esr1) and vitellogenin (vtg).Alternatively, if this advancement is not caused by an endocrine-disrupting interaction of crude oil itself, it may be linked to a stress response in the fish, leading to elevated cortisol levels, which have been shown to have endocrine-disrupting effects. Therefore, measuring cortisol levels should be considered in future studies.

The possibility that high exposure to crude oil in the late stages of vitellogenesis may lead to premature spawning of smaller eggs, combined with the knowledge that there is maternal transfer of PAHs to the eggs (Strople et al., 2023), highlights the need for future work focusing on the potential maternal effects of crude oil exposure on the survival of the offspring. Understanding these effects will be crucial for assessing the broader ecological consequences of crude oil pollution on fish populations.

5. Conclusion:

The present study highlights the impacts of crude oil exposure on the reproductive development of polar cod. Exposure to the water-soluble fraction of crude oil during late vitellogenesis led to an advancement in gonad development and earlier spawning in females, with no clear evidence of an endocrine-disruptive pathway. In contrast, no significant effects on gonadal development were observed in males.

Additionally, this the study also identified a crucial gap in understanding how timing and the method of exposure influence reproductive outcomes. Notably, the influence of crude oil exposure on reproductive development varies significantly based on when during the reproductive cycle the exposure occurs. These findings align with earlier research showing that exposure during early vitellogenesis typically delays reproductive development, while exposure later in development may advance spawning without significant changes in plasma levels during late exposure. This study expands on the findings of Strople et al. (2023) by placing the observed effects of crude oil on gonad development within the context of different maturation stages and highlighting the dose-dependent nature of these effects.

Furthermore, the potential impact on egg quality in the high exposure group, suggesting premature spawning, could result in smaller eggs and a potentially lower chances of survival for the offspring. This points to possible long-term repercussions that could affect the sustainability of the population and possible long consequences for the survival of the whole population.

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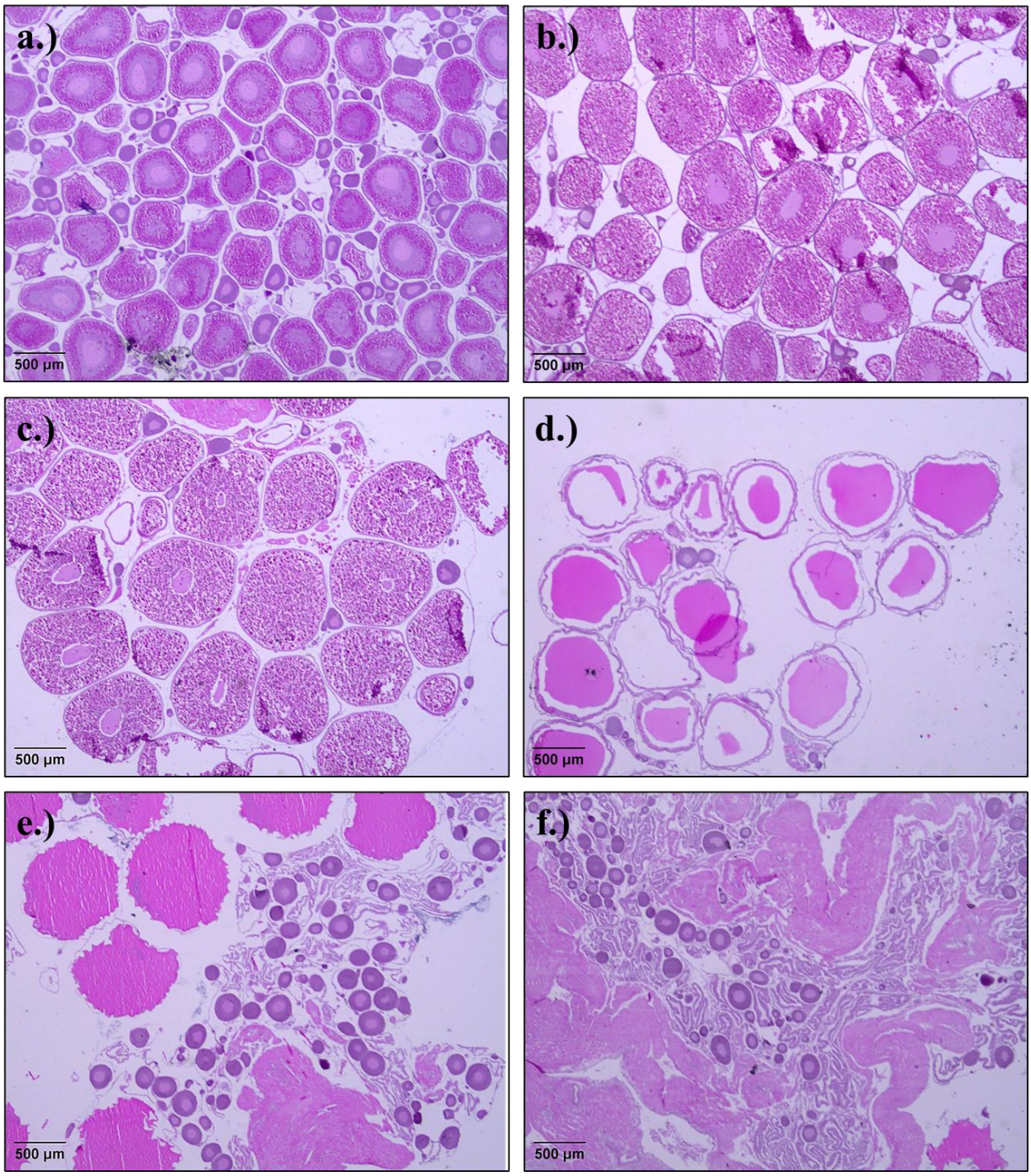
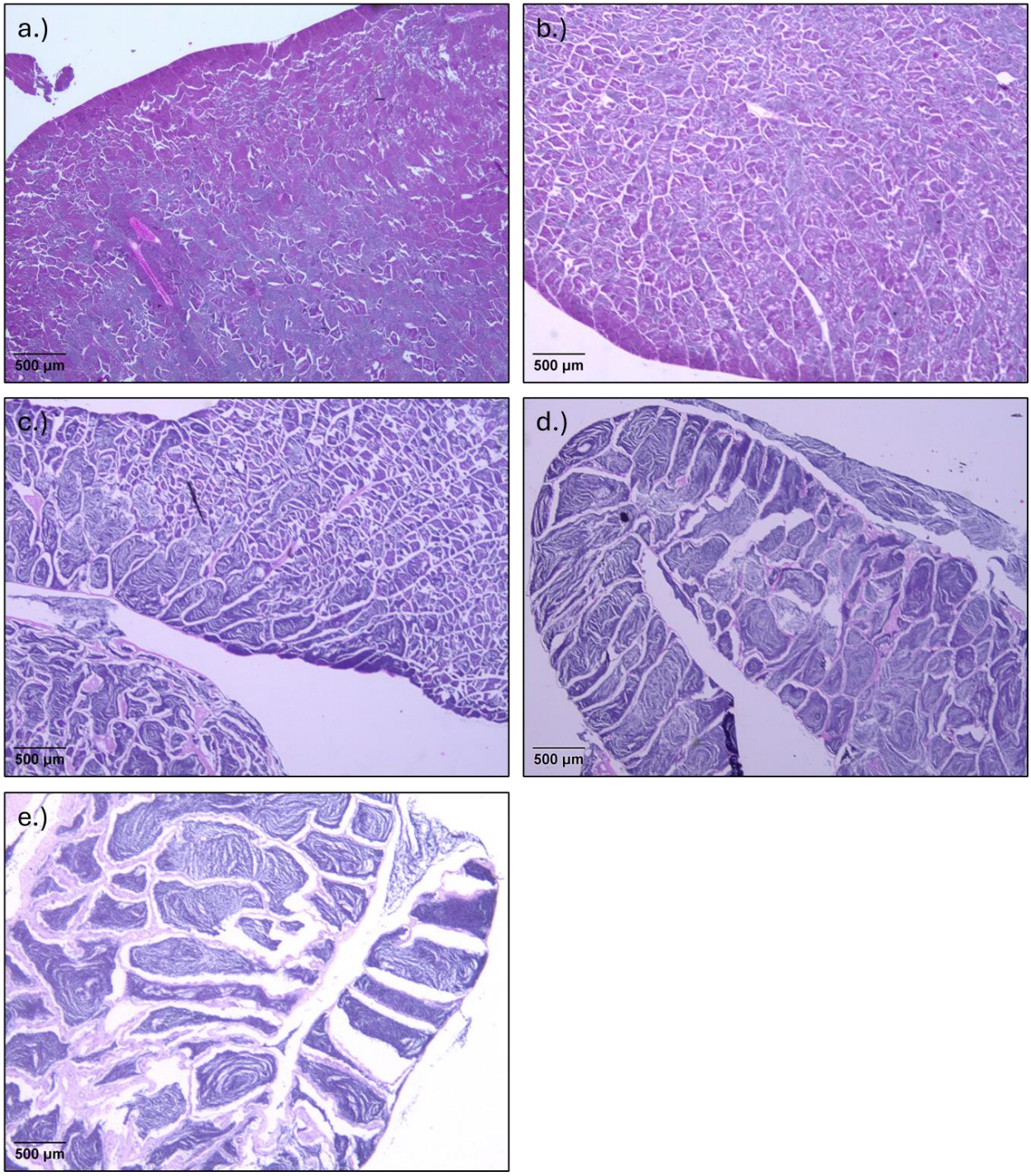
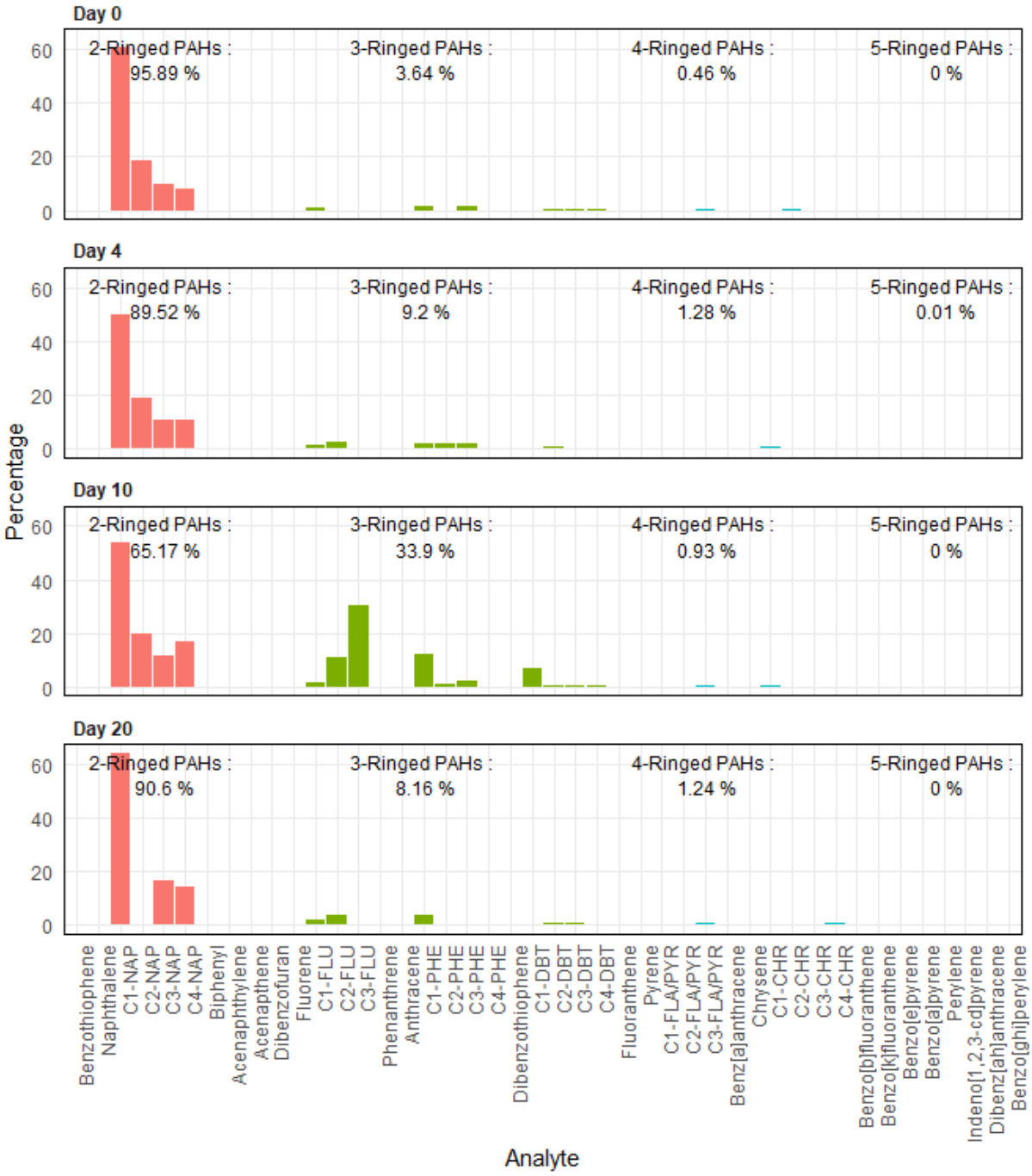
Appendix:



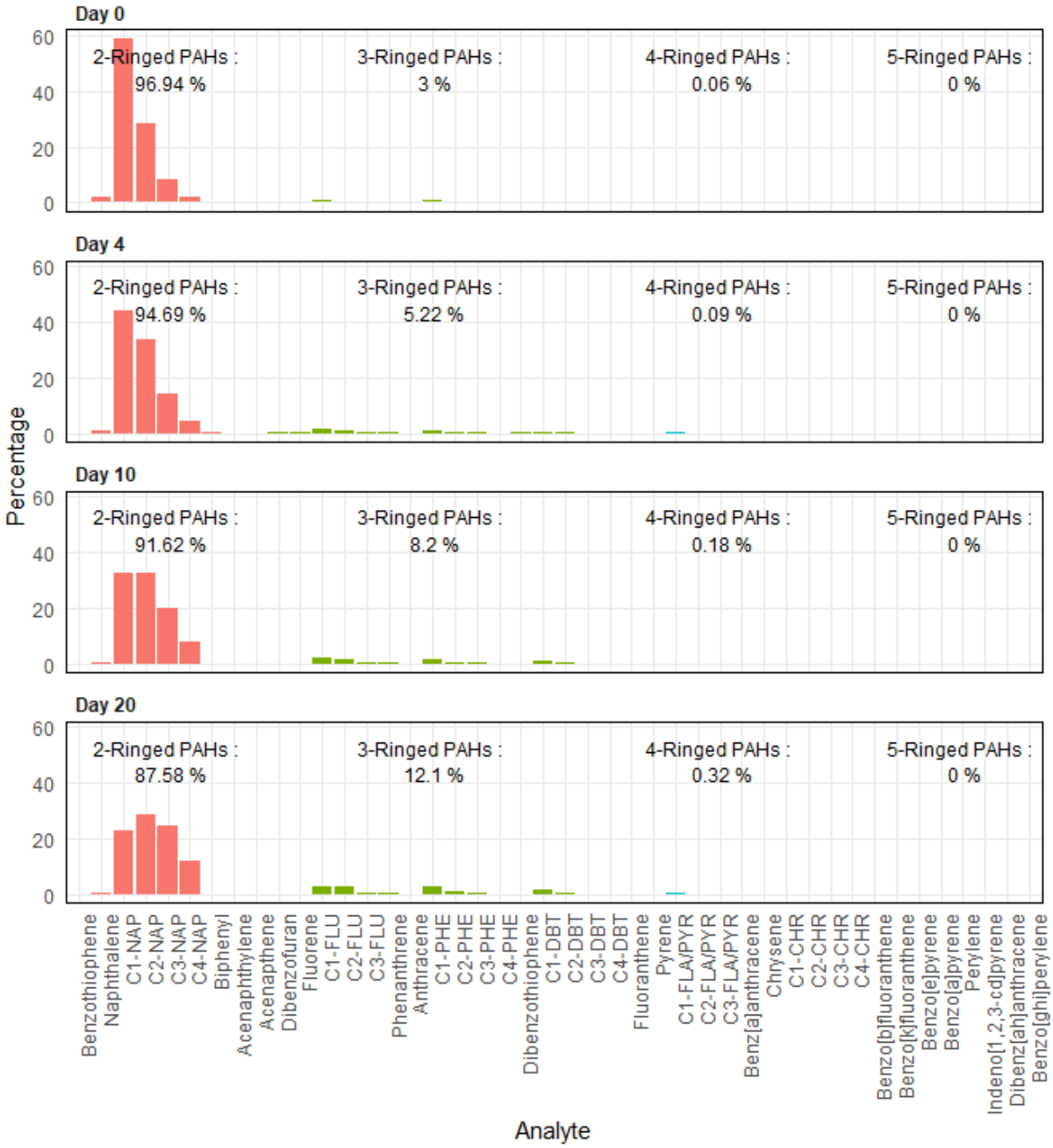
Supplement Figure 1. Gonadal developmental stages of the female polar cod: a) early developing stage with primary vitellogenic oocytes (VTG1) and primary growth oocytes; b) developing stage with secondary vitellogenic oocytes (VTG2); c) spawning-capable phase with tertiary vitellogenic oocytes (VTG3); d) active spawning phase with hyaline eggs (HydrO/Es); e) early spawning phase with residual eggs (ResEs) and post-ovulatory follicles (POFs); and f) regressing stage with POFs. (Scale bar = 500 µm)



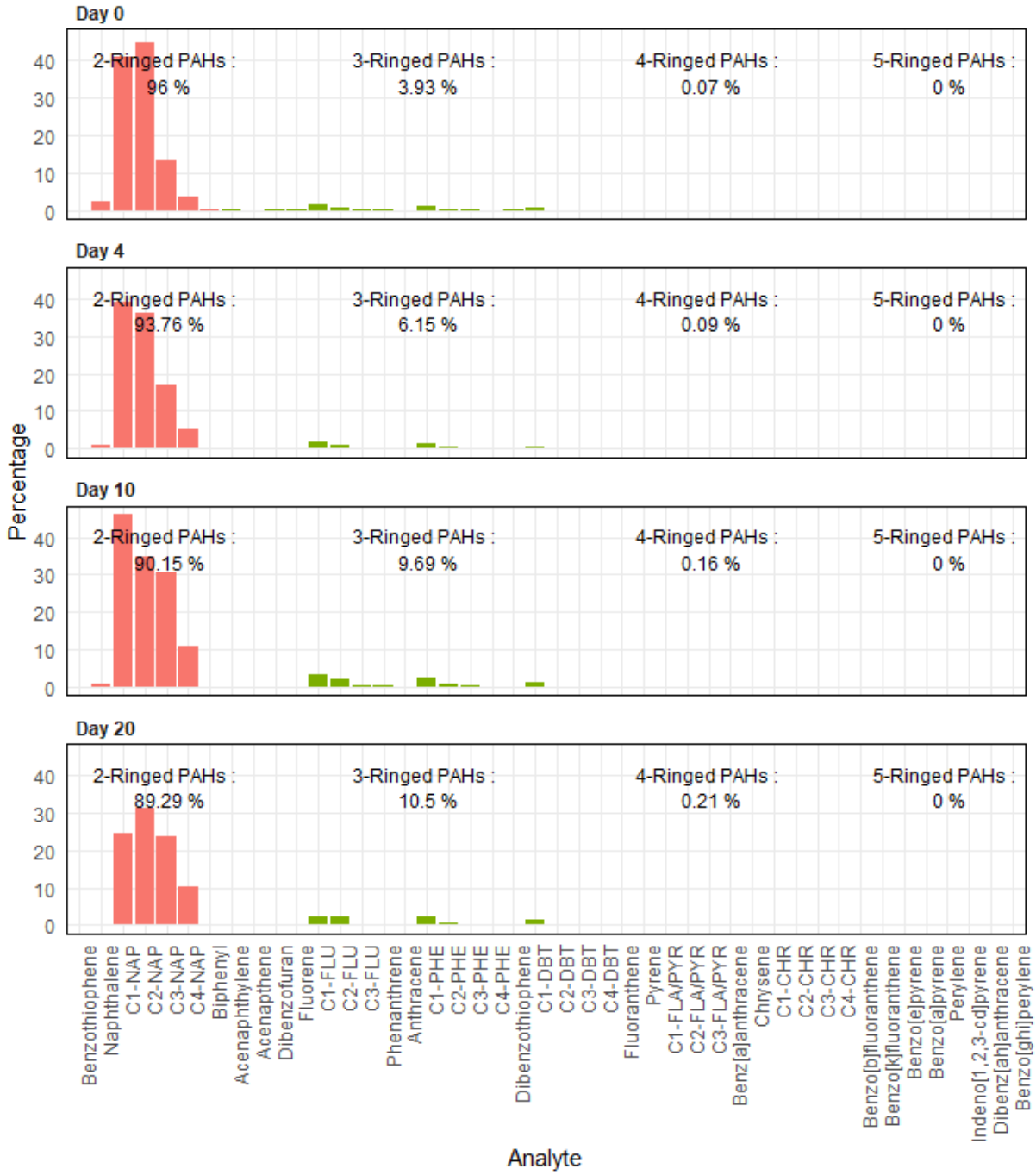
Supplement Figure 2. Gonadal developmental stages of the male polar cod: a) developing stage; b) early spawning-capable phase with continuous germinal epithelium (GE); c) mid spawning-capable phase with continuous GE near the periphery and discontinuous GE near the ducts; d) late spawning-capable phase with discontinuous GE; and e) regressing phase. (Scale bar = 500 µm)



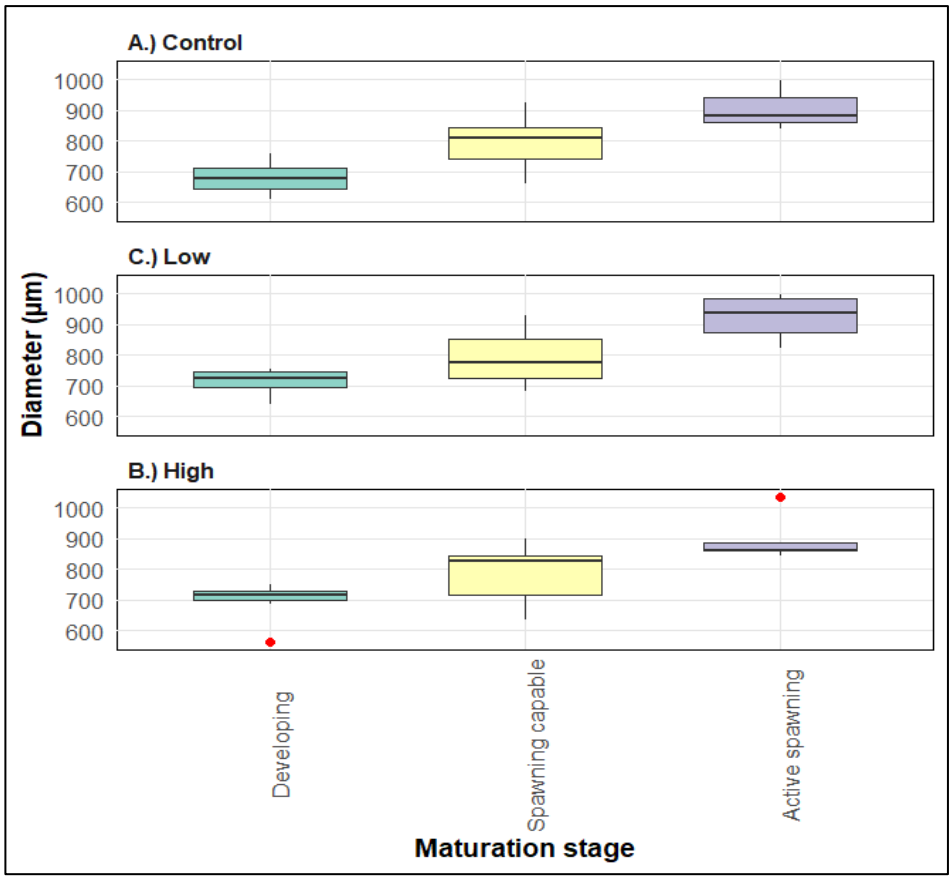
Supplement Figure 3. PAH composition expressed as a percentage of the total Σ44 PAHs in the control treatment over the exposure period. Analytes were grouped by 2-, 3-, 4-, and 5-ringed PAHs.



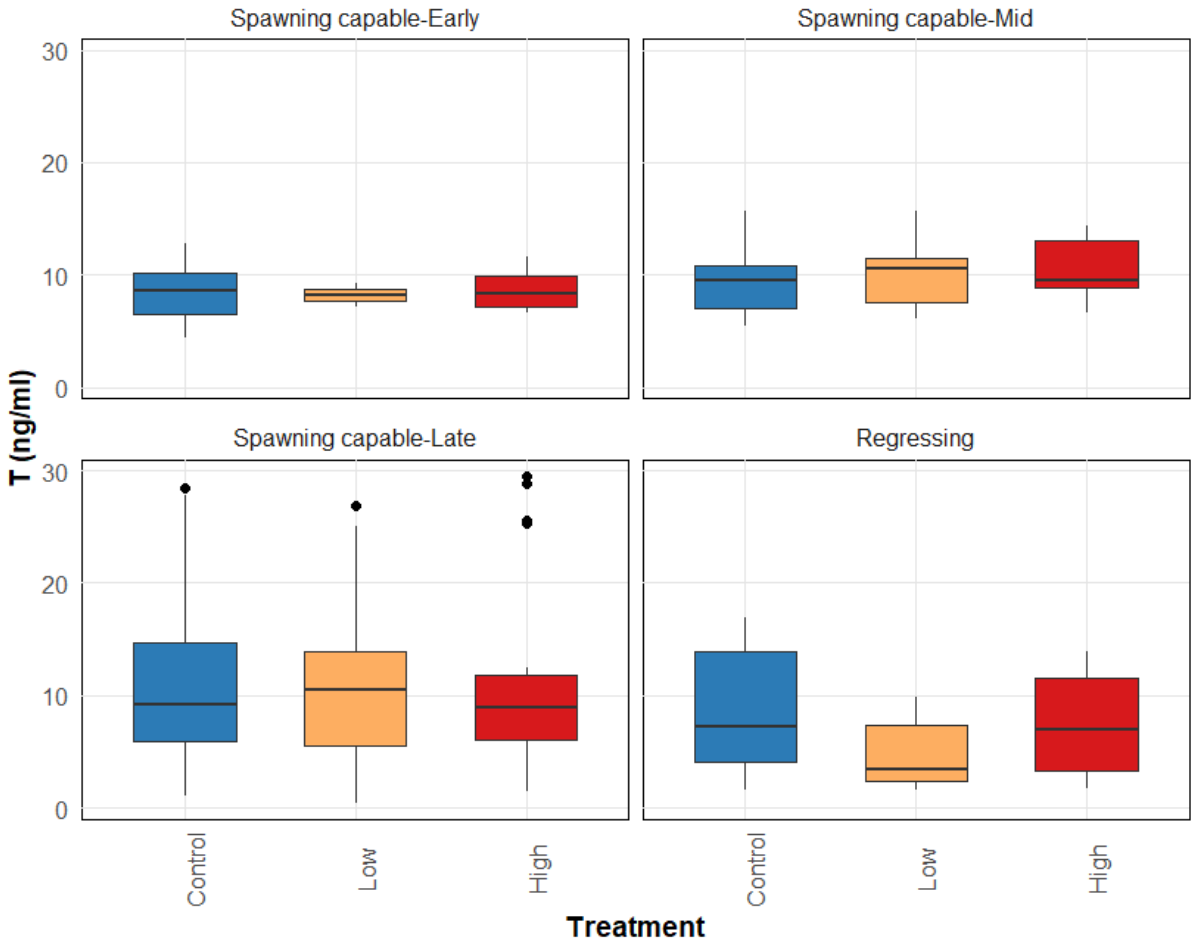
Supplement Figure 4. PAH composition expressed as a percentage of the total Σ44 PAHs in the low exposure treatment over the exposure period. Analytes were grouped by 2-, 3-, 4-, and 5-ringed PAHs.



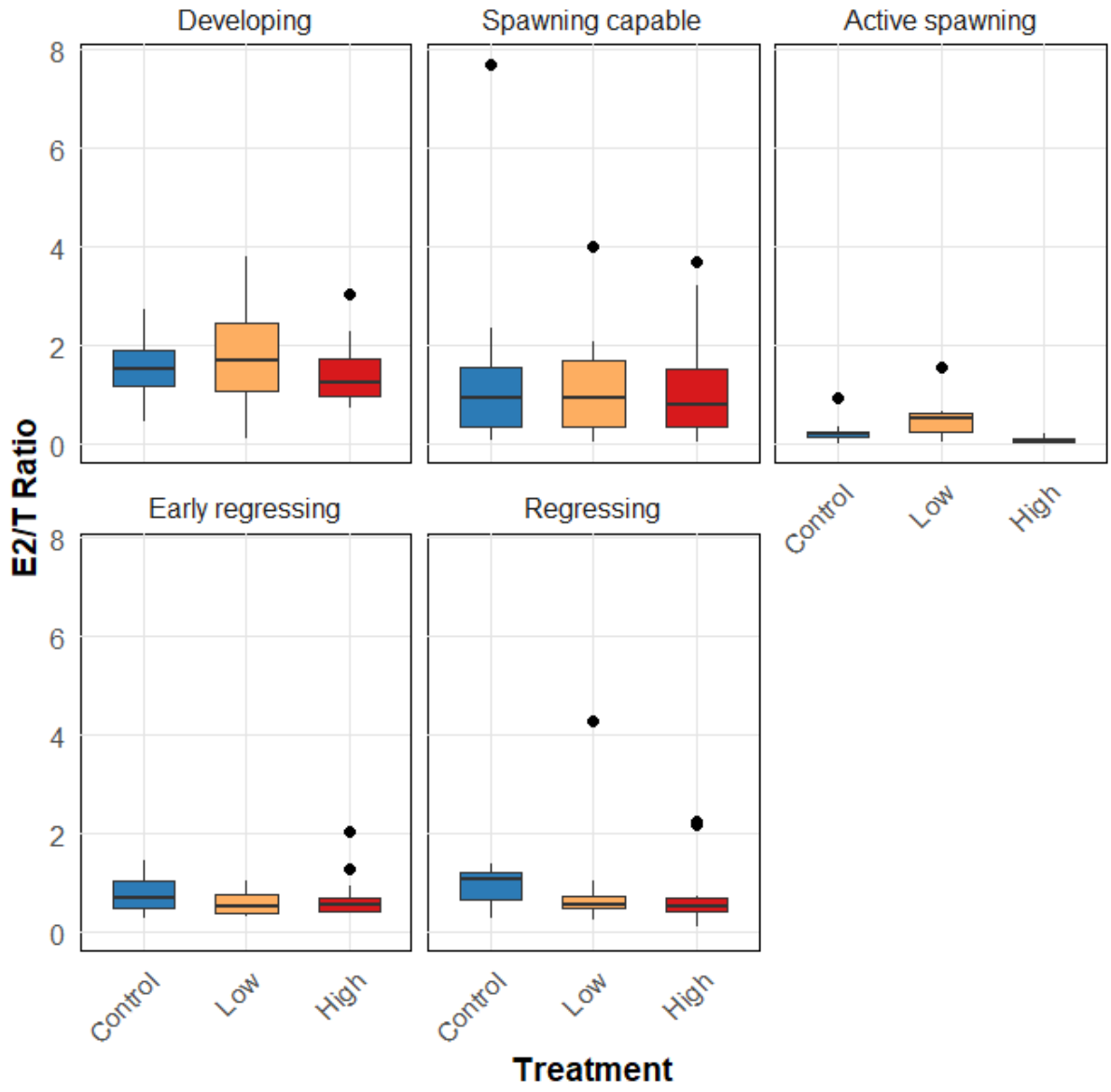
Supplement Figure 5. PAH composition expressed as a percentage of the total Σ44 PAHs in the high exposure treatment over the exposure period. Analytes were grouped by 2-, 3-, 4-, and 5-ringed PAHs.



Supplement Figure 6. Oocyte diameter (µm) throughout the different maturation stages before spawning, after exposure to control (A), low (B), and high (C) concentrations of the water-soluble fraction of crude oil. The oocytes develop from VTG1 oocytes in the developing phase (green), through VTG3 oocytes in the spawning-capable phase, to spawning-ready eggs in the active spawning phase. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots.



Supplement Figure 7. Levels (ng/ml) of Testosterone (T) in the plasma of male polar cod in different developmental stages after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots.



Supplement Figure 8. Ratio of Estradiol-17β (E2) to Testosterone (T) in the plasma of female polar cod in different developmental stages after exposure to control (blue), low (yellow), and high (red) concentrations of the water-soluble fraction of crude oil. The plots display the median (line), the 25%-75% percentiles (box), the non-outlier range (whiskers), and outliers represented by dots. For better visualization was one outlier for the high treatment group at the early regressing stage removed (value= 12.607).