UiT The Arctic University of Norway

Institute of Arctic and Marine Biology

Polar Cod in a Changing Arctic

Toxicity of crude oil on sensitive life history stages of a key Arctic species

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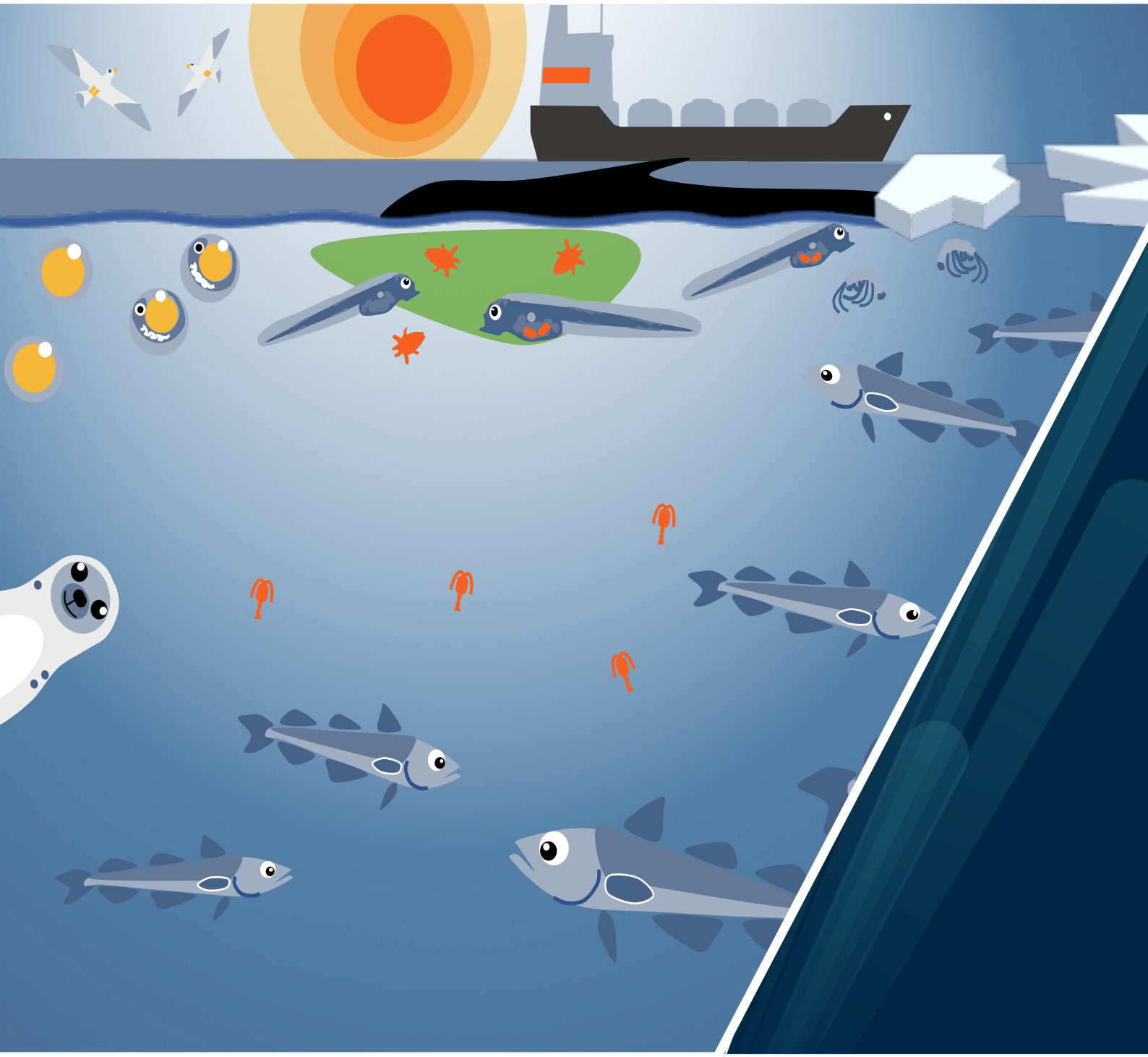

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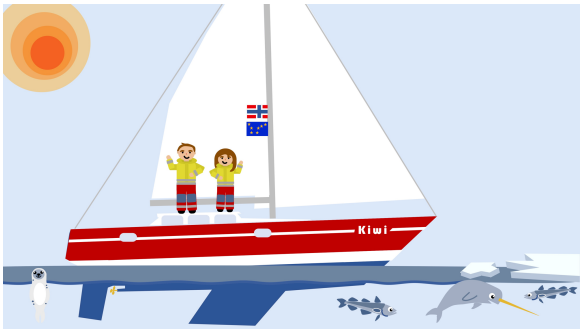
Foreword

The journey of the PhD is wavy. My motivation to work with important Arctic marine issues was instilled in me early through my upbringing in coastal Alaska, with many hours spent in the front of a sea kayak, and many summers with my eyes glued on tide pools. Channeling that motivation to continue to progress in the lab, in rough seas, and behind a desk to tell a story that was worth sharing with the world was only possible with the guidance of my supervisors, namely Jasmine Nahrgang and Marianne Frantzen. From first bringing me to Tromsø, introducing me to polar cod, and entertaining the many plans I have had for my academic progress, I thank you both dearly. And to my PhD committee of Jim Meador and Maxime Geoffroy, who provided a needed outside perspective and critical review to my work, thank you.

I would like to apologize and express my gratitude to my family in Alaska for the motivation and support even while being 8000 miles away for seven years. Your words and actions drive me to do my best every day. My husband Danny is the truest gem for encouraging, questioning, and loving me from the very beginning of this journey and for moving to Norway so that we could live out a sailing adventure in the Arctic. Now we will embark on a new journey together - Our unborn child deserves recognition for kicking me from the inside to finish this PhD!



Thank you - to my community of colleagues - Ireen, Raphaelle, Liza, Christine, Erin, and Mathilde- for your encouragement and adventures! -To my new friends at the Alaska Fisheries Science Center in Newport, Oregon who provided a perfect place to write my dissertation. While unorthodox, I would like to recognize the sailing yacht Kiwi for teaching me to be strong when I am scared and the invaluable skills of problem solving and when to ask for help.

Every trip to collect polar cod aboard the *RV Helmer Hansen* was successful, thanks to the captain and crew aboard. We thank Eni Norge, Akvaplan-niva, and SINTEF for providing the fresh Kobbe crude oil used in the present work. Keeping polar cod in the lab is no small technical or biological feat. Only due to the expertise of the staff at the UiT Biological station in Kårvika, at the FISK Akvaplan-niva marine laboratory in Tromsø, and the rotifer and artemia culturists at Nofima was this possible. Such experiments and analysis would not be feasible without additional help from researchers, students, and technicians. With that, I thank Ingeborg Hallanger, Apolline Laenger, Emma Kube, Katharina Bjarnar Løken, Merete Grung, Connie Jones, Paul Dubourg, Randi Olsen, and Ditte Secher Paludan. Lastly, I thank reviewers and statistical experts Jørgen Schou Christiansen, André Frainer, and Michael Greenacre for their assistance.

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Lastly, I thank the fish for giving their swimming souls to science.

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Summary

Environmental change in the Arctic is occurring at an unprecedented rate with a loss of sea ice, warmer sea and air temperatures, altered primary production, and northward species range expansions. While these changes may have negative impacts on many ice-associated and endemic Arctic species, positive outcomes are predicted for some human activities. Prospects of fishing, resource extraction, tourism, scientific activities, and trans-arctic shipping are increasing in the North. However, the remoteness of the Arctic combined with inclement weather, unpredictable sea ice conditions, limited mapping, few ports, and a general lack of precedent may challenge Arctic operations. The risk of petroleum pollution is growing proportionally with these activities. Using empirically-driven research as a foundation, it should be possible to limit the environmental footprint of human activity in the Arctic. The investigation of the environmental impacts of large-scale disasters, like oil spills and human activity on key species of the Arctic region, is one piece of this puzzle. Through the presented research, I aim to understand the potential future of a key Arctic fish, polar cod (*Boreogadus saida*), as a sentinel species in the knowledge-based management of the Arctic.

With a pan-Arctic distribution, polar cod is the most abundant and widespread pelagic fish species in the Arctic and plays an essential ecological role as a forage fish, influencing the distribution and movements of marine mammals and seabirds, and as subsistence food for humans. Polar cod is a small Gadidae fish endemic to the Arctic and younger age classes are found in association with sea ice. Early life stages are found in the surface waters where oil spills and warmer water temperatures are likely to be encountered, if present. Older age classes may be exposed through their zooplankton diet or transiently to an oil spill scenario. Biological impacts of oil spills and laboratory experiments provide strong evidence for the toxicity of crude oil, resulting in lethal and sublethal effects on marine organisms even at low concentrations (µg crude oil /L seawater). One approach to study the effects and sensitivity of a species is to look at responses that have importance for individual fitness – physiological and whole organism endpoints. Following changes in growth, metabolism, reproduction, and survival provide an integrated response from the lower levels of molecular, biochemical, and cellular responses to determine the risk to individual fitness, delivering more robust predictions for effects at the population and ecological level. Here, I hypothesize that exposure to simulated oil spills will result in adverse effects on individual fitness during critical life-history stages of polar cod.

This work presents four different oil spill scenarii on three physiologically different life stages of polar cod, maturing adults, post-spawning adults, and early life stages. The first two papers focus on how mature adult polar cod are physiologically affected by low levels of dietary crude oil exposure during the period of reproductive development (Paper I) and the period of post-spawning (Paper II). In line with the oil spill scenario proposed in the first two works, Paper III investigates the potential physiological effect of acute exposure to different oil spill response actions (*i.e.,* mechanically dispersed, chemically dispersed, and *in-situ* burned crude oil) on the subsequent period of reproductive development. Paper IV focuses on the sensitivity of early

life stages to low levels of crude oil dissolved in the water at two temperatures. This work follows the embryos from fertilization through first feeding. Each experiment was designed to simulate an oil spill scenario in the Arctic with cold temperatures and appropriate light cycles, fresh crude oil, and oil spill response measures that are under evaluation for Arctic use.

Together this body of research empirically supports the physiological robustness of mature stages of polar cod chronically exposed to low levels (post-spill concentrations; Papers I and II) or acutely to high (present spill) crude oil concentrations (Paper III) and the elevated sensitivity of the eggs and larvae to low levels of crude oil further amplified by a 2.3°C increase in water temperature (Paper IV). While no effect of crude oil exposure was observed on adult survival, reduced energy reserves and condition in the post-spawning stage suggests the increased physiological sensitivity of this life-history stage. The physiological effects of crude oil exposure on respiration, growth, and reproductive development were not consistent. Effects observed on sperm motility under dietary exposure and gonadal development in females exposed to burned oil residues require follow-up examination, especially in light of the importance of gamete quality to individual fitness. Furthermore, understanding the physiological state and sex of polar cod exposed is vital for result interpretation, and studies should be designed with this in mind. A small increase in temperature combined with low levels of crude oil exposure resulted in changes in egg buoyancy, smaller size at hatch, reduced growth and feeding activity, severe deformities, and increased mortality in embryos and larvae. These interactive effects of environmental stressors like climate change and petroleum pollution further support the vulnerability of polar cod early life stages.

Findings from the present work can suggest including post-spawning mortality parameters, life history stage-specific sensitivities, including delayed effects, and the development of a more accurate dose metric for crude oil exposure for future experimental, modeling, and risk assessment efforts. Ideally, a crude oil dose metric would span beyond the sum polycyclic aromatic hydrocarbon measure to include other possible toxic compounds in crude oil.

Global environmental change is not limited to increased water temperature and pollution events as this dissertation presents. Reductions in sea ice extent, ocean acidification, increasing freshwater input, southern species moving northward to alter community structures, and other pollution issues, like microplastics and emerging contaminants, all stand to affect polar cod with possible cascading effects on the entire Arctic marine ecosystem. While it can be tempting to broadly apply the one-fish-fits-all approach to potential anthropogenic impact questions in the Arctic, the understanding of life-history stage-specific sensitivities and traits, the changing ecological role, population dynamics, and general resilience to perturbations are not yet in place for polar cod. Determining how the sentinel species, polar cod, will respond to these environmental and ecological stressors and what influence this will have on the resilience of the Arctic marine ecosystem is the future aim of this research.

List of Papers

I. **Bender, M.L.,** Frantzen, M., Vieweg, I., Falk-Petersen, Johnsen, H.K., Rudolfsen, G., Tollefsen, K.E., Dubourg, P., Nahrgang, J., 2016. Effects of chronic dietary petroleum exposure on reproductive development in polar cod (*Boreogadus saida*). Aquatic Toxicology. http://dx.doi.org/10.1016/j.aquatox. 2016.10.005.

II. Nahrgang, J., **Bender, M.L.**, Meier, S., Nechev, J., Berge, J., Frantzen, M. Growth and metabolism of adult polar cod (*Boreogadus saida*) in response to dietary crude oil. Ecotoxicology and Environmental Safety. 180 (2019) 53–62. https://doi.org/10.1016/j.ecoenv.2019.04.082

III. **Bender, M.L,** Frantzen, M., Camus, L., LeFloch, S., Palerud, J., Nahrgang, J. 2018. Effects of acute exposure to dispersed oil and burned oil residue on long-term survival, growth and reproductive development in polar cod (*Boreogadus saida*). Journal of Marine Environmental Research. 140: 468-477. doi: 10.1016/j.marenvres.2018.09.005.

IV. **Bender, M.L.,** Giebichenstein, J., Teisrud, R.N., Laurent, J., Frantzen, M., Meador, J., Sørensen, L., Hansen, B.H., Reinardy, H.C., Laurel, B., Nahrgang, J. Interactive effects of crude oil pollution and warming on polar cod early life stages. Manuscript In Prep

List of Abbreviations

AhR	Aryl hydrocarbon receptor
BTEX	Compounds benzene, toluene, ethylbenzene, and xylene
BO	Burned oil residue
CYP1A	Cytochrome P 4501A
CDO	Chemically dispersed oil
DWH	Deepwater Horizon (oil spill)
ELSs	Early life stages
EPA	Environmental Protection Agency (U.S.)
ER	Estrogen Receptor
EROD	7-ethoxyresorufine-O-deethylase
EVOS	Exxon Valdez Oil Spill
GC-MS	Gas chromatography mass spectrometry
GSI	Gonadosomatic Index
HSI	Hepatosomatic Index
IPCC	Intergovernmental Panel on Climate Change
LC_{50}	Concentration of substance that induces 50% mortality
mRNA	messenger ribonucleic acid
MDO	Mechanically dispersed oil
OSR	Oil spill response
PAHs	Polycyclic Aromatic Hydrocarbons
RCP	Representative Concentration Pathways
SGR	Specific growth rate
TPH	Total petroleum hydrocarbon
UCM	Unresolved Complex Mixture
UV	Ultraviolet radiation
WSF	Water soluble fraction (of crude oil)

1 The Changing Arctic Marine Ecosystem

1.1 The Arctic as a Home

A marine biologist defines the Arctic as those waters that are cold, perhaps covered by sea ice. At the same time, a social scientist may look for Arctic people, a geographer considers everything north of the Arctic Circle, and a terrestrial biologist may use tree line as an indication of where the Arctic stops and starts (AMAP, 2010). However, each one of these definitions uses the distribution of biotic and abiotic factors that are dynamic - moving North or South or disappearing before our eyes. The Arctic is changing. As a scientist, we have to adapt.

Our duty is to understand how our Arctic home could be altered by our actions, to listen to those that have been here many generations before us, and to predict how our actions today and tomorrow will affect the Arctic for future generations. The Arctic is home to four million people (IPCC, 2019) and bordered by five nations; Norway, Russia, the U.S. (Alaska), Canada, and Denmark (Greenland). Collaborative efforts across countries on environmental policy, research, and resource exploitation may be a viable way forward; however, particular economic interest and historical precedents cloud progress on this front.

While conservation of the Arctic is on many minds and agendas, it is not the aim of this body of work. Instead, I aim to understand the potential future of a key Arctic species, polar cod (*Boreogadus saida*), as a sentinel species in the knowledge-based management of the Arctic. While polar cod are undoubtedly not on the minds of all Arctic stakeholders - this single species has provided a wealth of knowledge on the implications of possible future scenarios in the Arctic waters it inhabits.

1.2 The Current Status of the Arctic Marine Ecosystem

The Arctic has strong seasonality in light availability, which profoundly affects the biological activity and fundamental physiological processes in arctic marine ecosystems (Berge *et al.,* 2015; Figure 1). The presence of sea ice and snow cover further influence the light available for primary production (Leu *et al.,* 2011). Sea ice is a central element of the Arctic system as it acts as a barrier between the ocean and the atmosphere and reflects solar radiation. Sea ice also provides habitat for Arctic organisms, a platform for the travel and hunting of indigenous peoples, and an obstacle to access resources for other human activities. In the early spring, sea ice algae utilize the first light under the ice and are followed by a bloom of pelagic phytoplankton at the marginal ice zones (Søreide *et al.,* 2010). This spring bloom becomes the basis of the food web for planktonic grazers (*i.e.,* zooplankton; Falk-Petersen *et al.,* 2000), secondary consumers (*e.g.,* fish; Hop and Gjøsæter, 2013) and top predators (*e.g.,* seals; Ryg *et al.,* 1990) in the Arctic. The ability to accumulate resources quickly during the spring and summer months and to store those resources, often in the form of lipids (Falk-Petersen *et al.,*

2000), for the rest of the year to fuel growth and reproduction is an adaptive mechanism of many Arctic species.

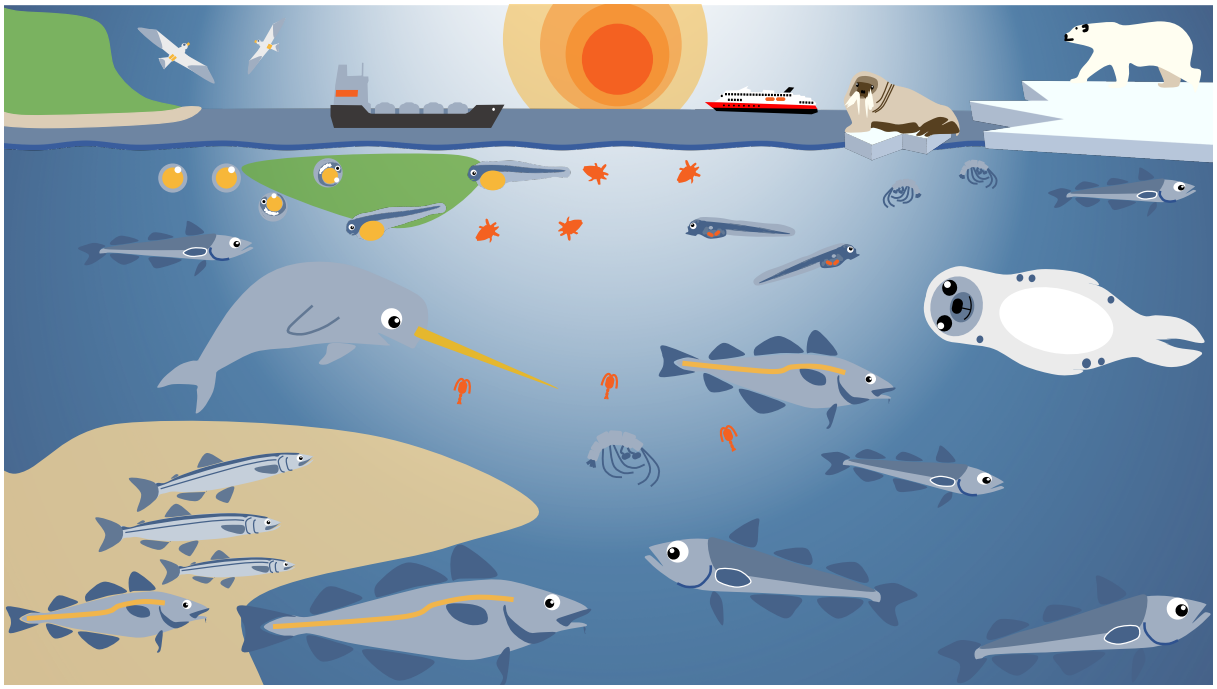

Figure 1. Schematic of the Arctic marine ecosystem with possible anthropogenic stressors.

The annual air temperatures in the Arctic is rising twice as fast as the global mean temperature increase, a phenomenon known as Arctic amplification (Richter-Menge *et al.,* 2019). The Intergovernmental Panel on Climate Change (IPCC) commonly presents future change in the context of a group of well-tuned scientific models based on levels of greenhouse gas emissions called representative concentration pathways (RCP). Two models have become highly publicized, RCP 8.5 the "business-as-usual" with little to no mitigation efforts taken scenario and the RCP 2.6 scenario which represents lowered emission, high mitigation future with a 66% chance that global warming will be limited to 2°C by 2100. In the RCP 8.5 scenario, the IPCC projected an increase in global mean surface temperature by 3.7°C until 2100 (IPCC, 2014). Global warming projections for both models predict substantial warming for the Arctic (Figure 2a; IPCC, 2019). Across the Arctic shelf seas, sea surface temperatures in August 2019 were ~1-7°C warmer than the 1982-2010 August mean (Richter-Menge *et al.,* 2019). In the newest IPCC report published based on research findings up until 2018, it is stated with high confidence that the oceans have received 90% of the excess heat of the globe and has been warming continually since the 1970s (Figure 2b,c; IPCC, 2019).

The loss in Arctic sea ice extent, unprecedented in the last 1000 years, is occurring at rate of 12.8 ± 2.3% decline every decade since 1979 (IPCC, 2019, and references within). The loss in sea ice area, thickness, and older sea ice cause positive feedback effects (i.e., sea ice albedo feedbacks) and leads to further warming of the Arctic waters (Figure 2d; IPCC, 2019). Loss in sea ice has contributed to the shifts in primary productivity seen in the Arctic Ocean with changes in light availability and stratification. In all regions of the Arctic, increasing

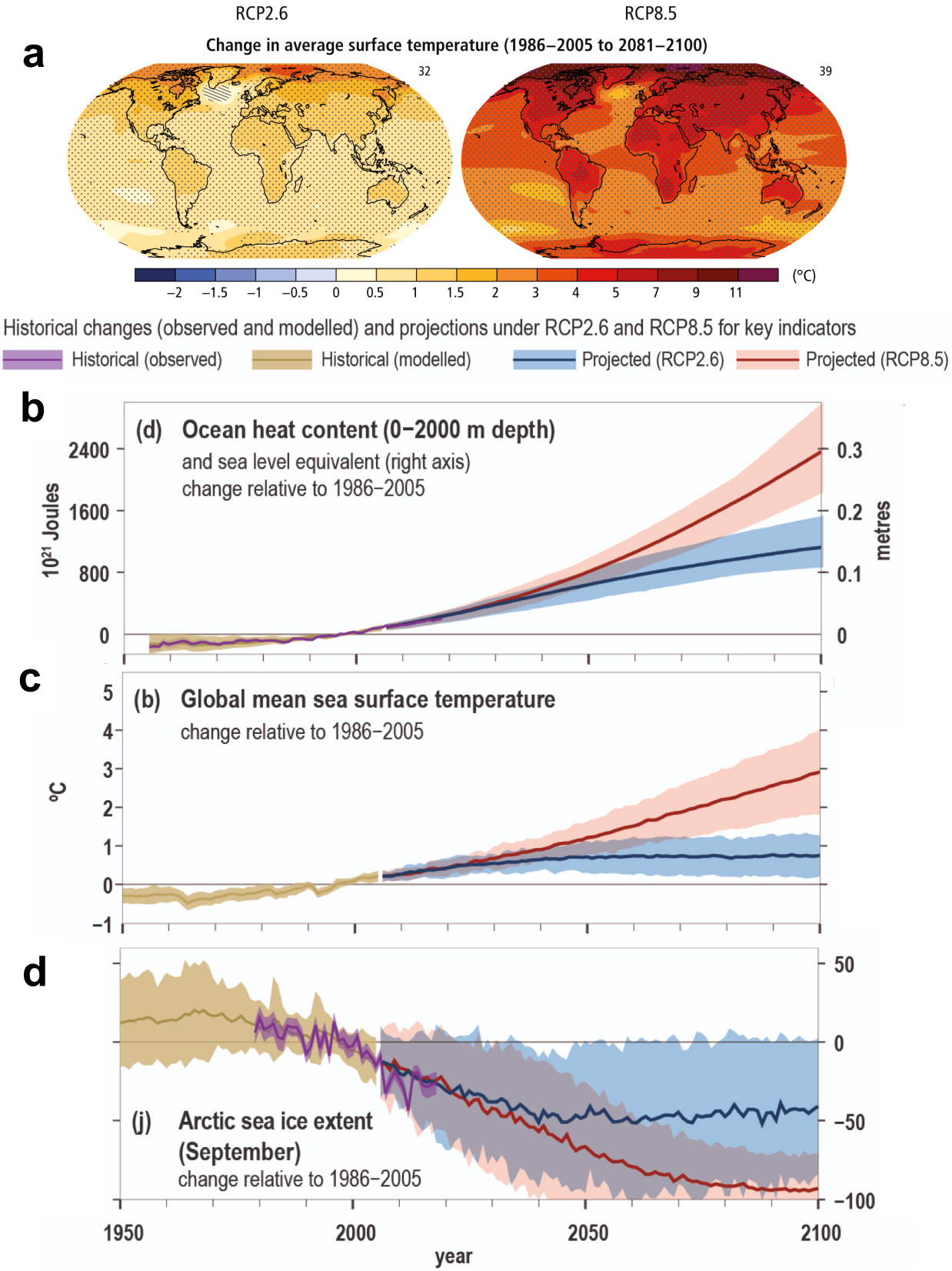

Figure 2. (a) Model Intercomparison for the 2081–2100 period under the RCP2.6 (left) and RCP8.5 (right) scenarios for the change in annual mean surface temperature. From the IPCC 2014 Fifth Assessment Report; Observed and modelled historical changes in the ocean and cryosphere since 1950, and projected future changes under low (RCP2.6) and high (RCP8.5) greenhouse gas emissions scenarios. Changes are shown for: (b) Global ocean heat content change (0–2000 m depth); (c) Global mean sea surface temperature change with likely range, (d) Arctic sea ice extent change for September. From the IPCC 2019 Summary for Policy Makers on the Ocean and the Cryosphere.

primary productivity over the 2003-2019 period has been observed with the most pronounced increases seen in the Eurasian Arctic, Barents Sea, and Greenland Sea (Richter-Menge *et al.,* 2019). Changes in Arctic primary production with blooms occurring earlier in the spring have likely driven changes in primary consumers (*i.e.,* zooplankton), which have cascading effects on important fisheries and higher trophic levels. This ocean warming and changes in primary production have very likely driven range expansions of lower latitude species into the high latitudes (Fossheim *et al.,* 2015) and resulted in the restructuring of ecosystems of the North Atlantic, Northeast Pacific, and the Arctic.

1.3 Arctic petroleum pollution risks

While global climate change has negative impacts on sea ice extent and many ice-associate species, positive consequences are predicted for some human activities (IPCC, 2019). Prospects of fishing, resource extraction, tourism, scientific activities, and trans-arctic shipping are increasing in the North. The remoteness of the Arctic combined with inclement weather, unpredictable sea ice conditions, limited availability of bathymetric data, few ports, and a general lack of precedent makes Arctic operations challenging (Harsem *et al.,* 2011). The risk of petroleum pollution, whether acute or chronic, is growing proportionally with development (Congressional Research Service, 2018; National Research Council of the National Academies of Science, 2014; Arctic Council, 2009).

A disproportionate amount of the world's oil and gas resources are located in the Arctic, both onshore and offshore (Allison and Mandler, 2018). The United States Geologic Survey estimates that 13% of the world's undiscovered oil reserves and 30% the world's undiscovered gas reserves are found in the Arctic (Gautier *et al.,* 2009) with most of these resources lying offshore (Gautier, 2011). As of 2018, three nations produce oil and gas north of the Arctic Circle: the U.S. (Alaska), Russia, and Norway, and many other countries are in exploration phases of development (Allison and Mandler, 2018). The high reliance on fossil fuels in the world economy, the high probability of finding vast petroleum resources, and the increasing accessibility due to the decreasing sea ice extent fuel the race for Arctic resources (Harsem *et al.,* 2011). However, world economics has influenced the levels of Arctic oil industry activities to date (Harsem *et al.,* 2015), environmental concerns and high risk and high project costs relative to potential returns have slowed development in many areas (Schaps, 2015). Despite this current atmosphere in the Arctic, petroleum pollution from offshore drilling operations, well blowouts, tanker spills, or vessel accidents are real risks to which prevention and mitigation plans should be in place.

Tracking of Arctic-wide vessel activities for three years, from 2015-2017, using Automatic Identification System data revealed that tens of thousands of voyages were undertaken in Arctic waters each year (Silber and Adams, 2019). Fishing vessels, primarily in the Barents, Bering, and Norwegian Seas, surpassed the operations of all other vessel types and comprised 52% of all Arctic voyages (Silber and Adams, 2019). Vessel activity related to oil and gas exploration and extraction was highest in the Norwegian, Barents, and Kara Seas. Trips to the central Arctic Ocean were limited by sea ice and dominated by research survey vessels, and ice-breakers

during the summer and fall (Silber and Adams, 2019). The two most popular shipping routes in the Arctic are the Northern Sea Route from the North Atlantic to the Pacific North of Russia and the Northwest Passage through the Canadian Arctic Archipelago. They are possible alternative shipping routes to the Suez and Panama Canal routes (Melia *et al.,* 2016). The Northern Sea Route, for example, is forecasted to cover 8% of the world's shipping needs by 2030 (Nilsen, 2020). Already the Northern Sea route is used by large ships and tankers on trans-Arctic voyages delivering supplies and materials and transporting oil and gas products (Dalaklis and Baxevani, 2017; Humpert, 2014). Transport of gas from facilities across the Barents Sea to Murmansk, Russia, via ice-breaking tankers occurs year-round (Offshore Technology Focus, 2018), evidence of the ongoing oil/gas industry on the North coast of Russia.

Looking forward, maritime transport operations in the Arctic will likely experience the most considerable growth in areas undergoing the most rapid loss in sea ice, such as along the Northern Sea Route and the Northwest Passage (Silber and Adams, 2019). Indeed, the possibility of an uncontained, large-scale oil spill from oil and gas operations, vessel operations or fishing is the single greatest threat to Arctic ecosystems (Congressional Research Service, 2018). The plausibility of a substantial discharge appears high, given the levels of activity devoted to oil/gas industries, according to Silber and Adams (2019).

1.4 The role of science

With scientific research as a foundation, it should be possible to limit the environmental footprint of human activity in the Arctic. This would require an understanding of the ecological vulnerability through an understanding of the current state of the environment, factors that drive processes and change, and then shape the future of environmentally responsible development around these findings. The investigation of the environmental impacts of the petroleum industry, large scale disasters, like oils spills, and human activity on key species of the Arctic region is merely one piece of this puzzle.

Communication of scientific results in an understandable manner will allow decision-makers and managers to anticipate and better prepare for accidents, consider how to modify responses accordingly, and to evaluate mitigation measures if an accident were to occur. A considerable international effort to draft and implement policies like the International Marine Organization legally binding rulebook, The Polar Code, a document entered into force in 2017, that aims to protect the environment and ensure safety of large ships sailing the high North. The Polar Code attempts to mitigate the concern that increased ship traffic in remote polar regions will inevitably pose an environmental risk where little risk was present previously. However, the Polar Code does not regulate heavy fuel oil, a fuel regularly used by bulk carriers or vessels smaller than 500 gross tons in the Arctic but banned in Antarctica (Schopmans, 2019). The success of the Polar Code, Arctic specific oil spill contingency plans, and simulation efforts for oil spill effects rely on the best scientific understanding for knowledge-based decision making (Carroll *et al.,* 2018).

Lastly, science focusing on understanding the future Arctic should aim to capture the intricate and confounding nature in which environment change is occurring. The Arctic is undergoing profound environmental change with declining sea ice, higher sea surface temperatures, changes in community structure, and increased human activity (IPCC Report, 2019; Carmack *et al.,* 2015); still, rarely do these changes occur in isolation. Anthropogenic stressors like climate change and environmental pollution can act synergistically to challenge an organism or population beyond the degree of any single stressor (Crain *et al.,* 2008). Climate change can amplify the adverse effects of exposure to low levels of pollutants in Arctic wildlife, which may have the potential for population-level effects (Borgå, 2019). Identifying and quantifying the cumulative impacts of anthropogenic stressors on organisms, populations, and ecosystems enables more informed societal, political, and economic decision making (Coté *et al.,* 2016; Chapin *et al.,* 2000).

2 Oil Pollution in the Arctic

2.1 Crude Oil

To understand how a potential oil spill will behave in the Arctic marine environment and the possible effects on the ecosystem, we need to review some essential properties on liquid petroleum products with a focus on crude oil. Crude oil is a naturally occurring substance generated by geological and geochemical processes inside the Earth over 10s-100s millions of years and is a critical part of our current fossil fuel dependence. A variety of petroleum products are derived from crude oil through refinement. Crude oil compositions vary from the very start of formation; thus, each type of crude oil has unique properties. These properties influence how petroleum will behave when it is released and determine its effects on biota and habitats (Transportation Research Board and National Research Council (NRC), 2003).

Crude oil and its derived petroleum products are made up principally of hydrocarbons (compounds containing only hydrogen and carbon), making up about 97% of most oils with nitrogen, sulfur, and oxygen filling the remaining 3% (NRC, 1985). Their structure characterizes the hydrocarbon compounds into the saturates, olefins, aromatics, and polar groups. The saturate group consists primarily of alkanes, which are composed of hydrogen and carbon with the maximum number of hydrogen atoms around each carbon and thus completely "saturated" (Speight, 1991). Olefins are those that contain fewer hydrogen atoms than the maximum possible (NRC, 1985). Polar compounds are those that have a significant molecular charge as a result of bonding with elements of the 3%, such as sulfur, nitrogen, or oxygen. Lastly, aromatic compounds include at least one benzene ring and account for anywhere from 1-20% of the hydrocarbons in crude oil. These benzene rings are very stable and, therefore, persistent in the environment. The more volatile monoaromatic (single-ring) compounds found in crude oil are often referred to as BTEX, or benzene, toluene, ethylbenzene, and xylene (Speight, 1991; NRC, 1985). Polycyclic aromatic (multiple-ring) hydrocarbons (PAHs) consist of at least two benzene rings, and a conventional crude oil may contain 0.2 - > 7% total PAHs. Within an unrefined oil, the abundance of aromatic hydrocarbons usually decreases with increasing molecular weight (*i.e.,* number of benzene rings). The PAHs in crude oil often

contains one or more methyl, ethyl, or occasionally higher alkyl substituents on one or more aromatic carbons. Most often, these alkylated PAHs are more abundant than the parent compounds in petroleum (Sporsol *et al.,* 1983). PAHs number in the 10000s, each with unique properties although generally nonpolar and lipophilic, and only about 100 of which have been identified and studied (Logan, 2007) (see appendix for list of commonly quantified compounds). Those compounds that have not been identified fall into the unresolved complex mixture (UCM) (Farrington and Quinn, 2015). This UCM fraction can be two orders of magnitude higher than the concentrations quantified in the PAH dose metric (Sammarco *et al.,* 2013). The UCM fraction may contain up to 250 000 different compounds and can represent 70 - 98% of the total hydrocarbons of the water soluble fraction of crude oil (Melbye *et al.,* 2009; Faksness *et al.,* 2008).

Other sources of petroleum pollution are present in the marine system, including produced water, drilling mud/cuttings, bilge and ballast water, and all types of fuel. However, these are outside the scope of the current study.

2.2 Fate of spilled oil in the marine environment

The properties of crude oil that are most important in understanding the fate of spills are viscosity, density, and solubility. Viscosity is how runny the oil is and determines how easily it can spread. Dispersion of oil occurs in both the horizontal and the vertical directions and generally in concert with mass circulation including the influence of currents and tides (Spaulding, 1995) and waves. Density determines whether a particular oil will sink or float in the marine environment. Evaporation can remove about one-third of the volume of a medium crude oil slick within the first day (Fingas, 2011), although evaporation is reduced with low temperatures as those prevailing in the Arctic. Solubility is a measure of how much oil will dissolve into the water. While generally low, at less than 100 parts per million, solubility is an essential process because the water-soluble fractions of the oil are sometimes toxic to aquatic life. Solubility decreases rapidly with increasing size of a compound and increasing number of substitutions. Although solubilization represents a minor loss process compared to evaporation or biodegradation, the concentration of compounds dissolved in water may be sufficient to have impacts on marine organisms.

Weathering is the change in physical and chemical properties of oil occurring after the oil is released. Solubilizing in water is a form of weathering as the more volatile lighter compounds (ex. BTEX compounds) evaporate (Carls *et al.,* 1999), while the remaining hydrophobic compounds remain in the slick or are found as dispersed droplets. Understanding the distribution between the dissolved phase and the oil droplets is important for determining the fate of hydrocarbons in the sea and the bioavailability of these chemicals to marine biota. Dissolved hydrocarbons in the water soluble fraction (WSF) can persist for weeks to years and can readily diffuse across gill and cell membrane surfaces, and those associated with particles can be ingested during feeding. If oil droplets, tarballs, or oil residues are present in the water column, these can adhere to gills, exoskeletons, skin or egg shells and alter the duration and exposure concentration to marine organisms (NRC, 2003).

If an oil spill were to occur in Arctic waters, the fate of the spilled oil might be very different than in warmer areas. Oil could be encapsulated in sea ice, where it is protected from evaporation, mechanical dispersion by waves, and UV degradation and thus could be transported potentially long distances by ice drift, wind, and ocean currents (Pfirman *et al.*, 1995). Oil would then be released during the spring melt in biological hotspots such as leads and polynyas (Kuletz *et al.,* 2015) and at times of high biological activity (Leu *et al.,* 2015). Environmental parameters, such as cold temperatures, sea ice, UV radiation, and biochemical constraints, like nutrient availability and primary production, control the weathering and biodegradation potential of spilled oil in Arctic seawater (Figure 3; Vergeynst *et al.*, 2018; Brandvik and Faksness, 2009). Low temperatures reduce evaporation and solubilization of oil by altering the physiochemical properties (Vergeynst *et al.,* 2018). If a spill were to overlap spatially or temporally with a phytoplankton bloom or areas of heavy sediment load, like a glacial front, the oil could form a "dirty blizzard." Oil is entrained in sinking matter, as was seen following the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (Vergeynst *et al.,* 2018). Low nutrient conditions may limit bacterial hydrocarbon degradation (Vergeynst *et al.,* 2018). These factors influence the residency time of spilled oil and prolong the time organisms can come in contact with oil compounds (National Research Council of the National Academies of Science, 2014).

Prolonged residence time is one factor determining the bioavailability of oil compounds to organisms. While a large oil spill has yet to occur in Arctic waters, an oil spill in the Arctic may have even more significant impacts due to the extreme light climate and ice cover, which will further slow weathering processes (Brandvik and Faksness, 2009) and may increase the

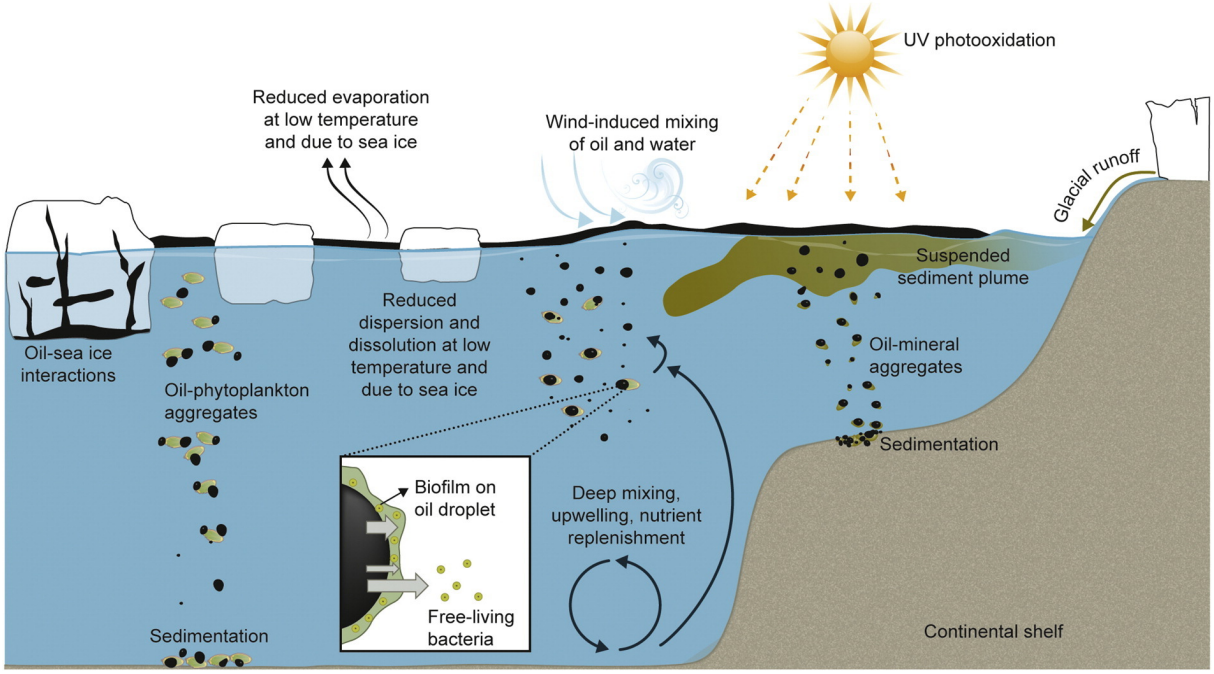

Figure 3.Schematic diagram of Arctic-specific conditions that affect oil biodegradation. From Vergeynst et al., 2018

bioavailability of oil to organisms (Fingas, 2011). Bioavailability is the amount of petroleum made available to an organism through physical, chemical, and biological processes. For example, oil entrainment in sinking matter can reduce the bioavailability for pelagic organisms while increasing the bioavailability to organisms living in the sediment. The degree of weathering, size and type of spill, oil spill response methods used, exposure routes, organisms exposed, and life stage of exposure influence the complex measure of bioavailability.

2.3 Lessons from historic oil spills

The most well-documented example of how damaging an oil spill in Arctic-like areas can be comes from the 1989 Exxon Valdez oil spill (EVOS) which occurred in the pristine marine environment of Prince William Sound in south-central Alaska, a sub-Arctic environment (62°N). The release of 42 million liters of Alaskan North Slope crude oil-contaminated 1990 kilometers of shoreline. The recovery of at least ten species of birds, whales, and commercially important fish had still not occurred after 25 years (EVOS Trustee Council, 2014). Oil was physically protected from disturbance, oxygenation, and UV degradation in the sediments making weathering and degradation processes less efficient. The unprecedented persistence of crude oil in the ecosystem made petroleum compounds more bioavailable to organisms (Short *et al.,* 2003; Hayes and Michel, 1999). Impacts of the oil spill on sea otters, orca whales, sea birds, and commercially important fish species (*i.e.,* Pacific herring) have persisted through sublethal effects at low concentrations affecting health, growth, and reproduction of these populations, as well as indirect trophic interactions (Peterson *et al.,* 2003). Oil spill response (OSR) countermeasures were largely not in place in Prince William Sound before EVOS, and this has resulted in the development and testing of many of today's oil spill contingency plans for the Arctic and around the world.

Despite caution exhibited by the industry following disasters like the EVOS, the most massive oil spill occurred in 2010 in the Gulf of Mexico. The blowout of the *Deepwater Horizon* (DWH) well released 800 million liters of light crude oil at a depth of 1600 m, a volume almost 20x greater than EVOS. In the wake of DWH, salt marshes and seabird populations were negatively impacted, but little contamination of seafood was found (Beyer *et al.,* 2016). The long-term impacts on large and commercially important fish species like bluefin tuna (*Thunnus thynnus)* and mahi-mahi (*Coryphaena hippurus*), deep-sea coral, birds, sea turtles, and crustaceans are of concern (Beyer *et al.,* 2016). While OSR measures were more readily assessable in the Gulf of Mexico, only 15- 25% of the oil was removed mechanically, similar to percentage recovered after the EVOS (Atlas and Hazen, 2011).

Fish species, such as Pacific herring (*Clupea pallasii*) and pink salmon (*Oncorhynchus gorbuscha*), were likely affected after the EVOS through physiological defects that led to reduced growth rates and higher larval and juvenile mortality with exposure to crude oil (Incardona *et al.,* 2015). While experimental studies have found support for toxic effects of oil on individuals at sumPAH concentrations ≤ 45 µg/L (Incardona *et al.,* 2015), a larger challenge is identifying persistent effects at the population level, where duration and magnitude of oil exposure is unknown (Ward *et al.,* 2017). In pink salmon embryos experimentally exposed to

5.4 µg/L sumPAHs and then released into the wild, delayed effects on growth and survival indicate the potential for population-level effects resulting from embryonic exposure to oil (Heintz *et al.,* 2000). These two species have been the focus of a number of studies because of their large value in the commercial fishery and because of the population declines observed in the wake of the EVOS. Herring collapsed four years following the oil spill and has yet to recover (Thorne and Thomas, 2008). Similarly, pink salmon returns were reduced in the years after the oil spill (Brannon *et al.,* 2012), now recovered. While linking fishery collapses following EVOS has not revealed strong correlations with crude oil exposure, a number of other factors have been proposed to explain the observed changes in fish population dynamics, including disease, changes in the ocean environment, altered spawning habitat, changes in interactions between species, and increases in predation (Thorne and Thomas, 2008; Hulson *et al.,* 2008). Linking population declines in these fish species to the EVOS remains hotly debated in the scientific community, and among stakeholders (Incardona *et al.,* 2015; Thorne and Thomas, 2008).

2.4 Toxicity of crude oil and oil spill response measures

To define the toxicity of a substance, we need to understand how it is toxic. A substance that can be harmful, poisonous, or cause an adverse effect is a toxicant. Toxicity is defined by the degree a substance is toxic. The study of toxicology aims to elucidate the dose-response relationship and the mechanisms in eliciting a response for both nature and humanmade substances to understand human health, ecological, agricultural, or other applications of such a substance. Toxicants are absorbed across membranes (*e.g.,* intestines, gills) either through passive diffusion or active transport such as in the case of lipophilic compounds adhering to dietary lipids (Vetter *et al.,* 1985). When toxicants pass this initial membrane, they enter the bloodstream where they are transported to tissues and where they act upon receptors and are metabolized, excreted, or accumulate. A given concentration/dose of a toxicant administered through a specific exposure route does not immediately equate to a predictable biological response. Factors like the toxicokinetics, life stage, duration of exposure, organism exposed, and condition of an organism need to be considered, and these factors will be reviewed in detail in the following sections. Toxicokinetics is the study of how and at what rate a toxicant enters an organism and the process the substance undergoes inside the body, including metabolism or excretion. Firstly, the organism in question is exposed to the toxicant. Organisms can be exposed to crude oil in the environment after an oil spill through ingestion or by direct contact with skin/exoskeleton, fur, feathers, or gills. While birds and mammals receive considerable media attention following an oil spill event and are highly vulnerable to oiling, whereby thermoregulation and buoyancy are affected (*e.g.,* Renner and Kuletz, 2015), this synthesis will solely focus on fish.

2.4.1 Possible modes of action and biological effects of crude oil toxicity

PAHs make up 0.2 - > 7% of the total hydrocarbons in most crude oils (Sammarco *et al.,* 2013). Nevertheless, they are the most studied toxic components in crude oil, have high lipophilicity, and can persist in the environment long after release (Pasparakis *et al.,* 2019; Carls *et al.,* 1999). PAHs are readily taken up by aquatic organisms; however, most teleosts can metabolize and eliminate these compounds (Meador *et al.,* 1995). Detoxification metabolism is induced with

the activation of the aryl hydrocarbon receptor (AhR) in vertebrates. The PAH-bound AhR joins with the AhR nuclear translocator, which moves into the nucleus and binds to the dioxin-responsive element on the DNA. Hundreds to thousands of genes are regulated, either directly or indirectly, by the AhR (Doering *et al.,* 2018). The induction of biotransformation target genes (Sherratt and Hayes, 2001) will alter lipophilic aromatic compounds to more water-soluble compounds through two main phases (Di Giulio and Hinton*,* 2008). These water soluble compounds are easier for the organism to excrete. In phase one, the CYP1A enzyme adds a polar functional group to the compound to form an intermediate metabolite. In phase two, metabolites are joined with an additional endogenous compound (e.g., glutathione, sugar derivate) to further increase the hydrophilic nature of the metabolite and to aide in excretion (*e.g.,* PAH metabolite) from the liver to the gallbladder and finally the feces (Kohle and Bock, 2007). The CYP1A enzyme and gene pathways are readily induced by crude oil exposure in fish and the catalytic activity of CYP1A, measured as 7-ethoxyresorufine-O-deethylase (EROD) activity, is commonly used as an indicator of biotransformation activity in fish upon exposure (Goksøyr and Förlin, 1992; Stegeman and Lech, 1991). Additionally, PAH metabolite levels in bile provide an indirect indication of the PAH exposure and biotransformation in the fish and are commonly analyzed in environmental assessments (Beyer *et al.,* 2010). While biotransformation is generally regarded as a detoxification mechanism, this process yields PAH metabolites that are more reactive and toxic than their parent compounds (Gerba, 2019).

Activation of the AhR can result in a range of adverse biological effects in many species, including hepatotoxicity, tissue lesions, suppression of immune system, impaired reproductive and endocrine processes, teratogenesis, altered cardiac development and function, carcinogenesis, and mortality (Doering *et al.,* 2018). In maturing adults, crude oil exposure has been shown to reduce investment in gonadal tissues (Sol *et al.,* 2000); disrupt and delay gametogenesis (Bugel *et al.,* 2010); affect growth (*e.g.,* Gravato and Guilhermino, 2009; Kerambrun *et al.,* 2012; Claireaux *et al.,* 2013; Sandrini-Neto *et al.,* 2016) and metabolism (Davoodi and Claireaux, 2007; Christiansen *et al.,* 2010; Klinger *et al.,* 2015) in fish. The mechanisms behind these effects can be multiple, including increased energy costs from detoxification metabolism and toxicity (Klinger *et al.,* 2015), behavioral changes leading to reduced nutrient assimilation (Moles and Rice, 1983; Christiansen and George, 1995), and toxicant-induced alterations in nutrient assimilation (Saborido-Rey *et al.,* 2007).

Crude oil toxicity in early life stages of fishes and its mechanistic understanding has been a rapidly expanding field of science in the last decades. Exposure to crude oil leads to lower heart rate, fluid retention, and morphological deformities (Meador and Nahrgang, 2019 and reference within). Even at low concentrations (sumPAHs < 1 parts per billion), the WSF of crude oil is toxic to fish ELSs, inducing carcinogenic, genotoxic, endocrine-disrupting effects, physiological impairment, behavioral effects, and mortality (Pasparakis *et al.,* 2019; Vignet *et al.,* 2014; Meador *et al.,* 2006; Rice *et al.,* 2001; Kime, 1995). Tradeoffs in growth, development, and behavior with detoxification, regardless of the mode of toxicity, likely also play into observed ELS effects (Pasparakis *et al.,* 2019). Many modes of toxicity are related directly to morphological effects of PAHs, for example, skeletal deformities and fluid retention (*i.e.,* edema) were associated with the inhibition of Na+/K+-ATPase and Ca^{2}+-ATPase activity

in larval fish exposed to PAHs alone (Li *et al.,* 2011). Cardiotoxicity has been linked to the disruption of ion homeostasis in cardiomyocyte cells (Brette *et al.,* 2014). With complex PAH mixtures, both AhR-independent and -dependent mechanisms may likely be active in the same cells under some conditions (Incardona, 2017). Meador and Nahrgang (2019) suggest a non-specific baseline toxicity MOA for the suite of effects observed in larval fish when exposed to the WSF of crude oil. Nonspecific baseline toxic effects are characterized by a common syndrome of effects elicited by a large variety of organic compounds at relatively high exposure concentrations, a condition possibly met under exposure to the WSF of crude oil, and not necessary PAH specific; however, mechanisms are unknown. In a recent review of work done on fish in the wake of DWH, oil toxicity was described as "a multi-target, multi-organ syndrome with substantial species-specific sensitivity differences" (Pasparakis *et al.,* 2019).

Although PAHs are well known toxicants, crude oil is composed of many thousands other organic compounds with a high bioaccumulation potential (Booth *et al.,* 2008, 2007). Research is only starting to understand toxic potential of the UCM fraction, with effects observed on fish hepatocytes (Petersen *et al.,* 2017; Melbye *et al.,* 2009) and mussels (*Mytilus edulis;* Booth *et al.,* 2007). As an example of a mode of action, unidentified, non-PAH compounds from the polar fraction of the crude oil WSF induce the AhR, but also the estrogen receptor (ER) and alter metabolic activity (Melbye *et al*., 2009). The characterization of the exposure doses for crude oil derived experiments and field studies is thus a major issue currently, as only few standardized, quantitative methods (*e.g.,* Total petroleum hydrocarbon (TPH) and PAHs) are available to characterize the composition of exposure water. The list of 16, 40, or 50 PAHs routinely analyzed are representing only a small fraction of the compounds that are bioavailable and potentially toxic to organisms and may not be adequate as proxy for whole mixture characterization (Meador and Nahrgang, 2019; Andersson and Achten, 2015).

2.4.2 Effects of oil spill response measures

The goal of an OSR is to minimize the risk and impact of a spill on the environment. Mechanical recovery of spilled oil is often the first choice in an OSR and risk mitigation as it removes the risk of further contact or damage to the ecosystem. However, chemical dispersants and *in situ* burning may be better suited in the icy waters of the Arctic (Fritt-Rasmussen *et al.,* 2015) as they require less machinery and less human resources. The use of chemical dispersants as an OSR action is intended to increase the biodegradation potential of petroleum by forming oil-surfactant micelles (Lessard and Demarco, 2000). The use of chemical dispersants thereby enhances the oil concentration in the water column for a time while reducing the surface slick and the risk for encapsulating the oil slick into the sea ice (Brandvik *et al.,* 2006). Increased concentrations of small oil droplets in the water column result in increased bioavailability of PAHs (Milinkovitch *et al.,* 2012; Ramachandran *et al.,* 2004), which has been linked to adverse effects on behavior, growth, reproduction, and survival in several fish species (Nwaizuzu *et al.,* 2016; Yu *et al.,* 2015; Wu *et al.,* 2012; Milinkovitch *et al.,* 2011; Yamada *et al.,* 2003; Gulec *et al.,* 1997). Newer dispersants (i.e. Corexit® 9500A), which was applied heavily during the DWH spill, have low acute aquatic toxicity to fish (Wise and Wise, 2011).

In situ burning is a countermeasure technology that was first implemented in 1958 along the ice-covered Mackenzie River in Northwest Canada. Since then has been used operationally during the 1989 Exxon Valdez oil spill in Alaska and extensively in the 2010 DWH oil spill in the Gulf of Mexico (Beyer *et al.,* 2016; Hunt, 2009). Burning is a quickly implemented OSR action that requires little infrastructure and, most significantly, can be effective depending on spill characteristics, leaving approximately 10% of the initial hydrocarbon load (Fingas, 2016; Buist *et al.,* 2013; Guenette and Sveum, 1995). Burning creates a burned oil residue (BO) that may sink depending on oil characteristics, in addition to toxic air pollutants like black carbon (Fritt-Rasmussen *et al.,* 2015). A limited number of studies have investigated the potential effects of BO from *in situ* burning on biota, and agreement on the low acute toxicity of burned oil applications has been reached (Fingas, 2016; Buist *et al.,* 2013). Compared to untreated and chemically dispersed oil, BO was found to be less acutely toxic in rainbow trout (*Onchoryncus mykiss*), three-spined stickleback (*Gasterosteus aculeatus*) (Blenkinsopp *et al.,* 1996), Australian bass (*Macquaria novemaculeata*) (Cohen and Nugegoda, 2006, 2000), amphipods and snails (Gulec and Holdway, 1999), and in copepods (Faksness *et al.,* 2012). However, the long-term effects of acute exposure to burned oil residues have yet to be examined.

3 Polar cod, a key Arctic forage fish species

Figure 4. Distribution of polar cod based on participation in research sampling, examination of museum voucher collections and the literature (Mecklenburg et al., 2011, 2014, 2016; Mecklenburg and Steinke 2015). Map shows the maximum distribution including rare and common sightings. From Hedges et al., 2017.

3.1.1 Polar cod ecology

In the high-Arctic and the Arctic shelf seas, polar cod (Lepechin 1774) is a foundation species (Bradstreet and Cross, 1982) due to its high abundance and efficiency in transferring energy from lower trophic levels (*i.e.,* plankton) to higher trophic levels (*i.e.,* marine mammals and sea birds) (Hobson and Welch, 1992; Craig *et al.,* 1982). With a pan-Arctic distribution, the endemic polar cod is the most abundant and widespread pelagic fish species in the Arctic (Figure 4), influences the distribution and movements of marine mammals and seabirds (Matley *et al.,* 2012; Loseto *et al.,* 2009), and is harvested as subsistence food for humans (Magdanz *et al.,* 2010). Polar cod, especially younger age classes, are found associated with sea ice (Gradinger and Bluhm 2004) even in the Arctic basins (David *et al.,* 2016) while older year classes are semi pelagic. This species is adapted to low light conditions (Jonsson *et al.,* 2014) and sub-zero temperatures through the production of antifreeze glycoproteins (Osuga and Feeney, 1978). Polar cod is a small, short-lived Gadidae closely related to the larger

commercially harvested species, Atlantic cod (*Gadus morhua*) and Pacific cod (*Gadus macrocephalus*) (Bradstreet *et al.,* 1986).

Although the commercial potential of polar cod has been recognized, historical and current fisheries for polar cod are limited to small catches, mostly in the Barents Sea by Russian and Norwegian vessels (Gjøsæter, 1995). Mature polar cod are caught in bottom trawls using a shrimp sized mesh and generally associated with waters ~ 0°C and North of the Polar front in the Barents Sea. Despite a circumpolar distribution, polar cod populations are routinely monitored only in the Barents sea as part of the annual Joint Russian-Norwegian ecosystem survey. Populations have been variable over the last 30 years, and the latest stock size is reported from 2017 at 360 x 10^6 kg with a maximum stock reported in 2006 at 1941 x10^6 kg (Gjøsæter *et al.,* 2018).

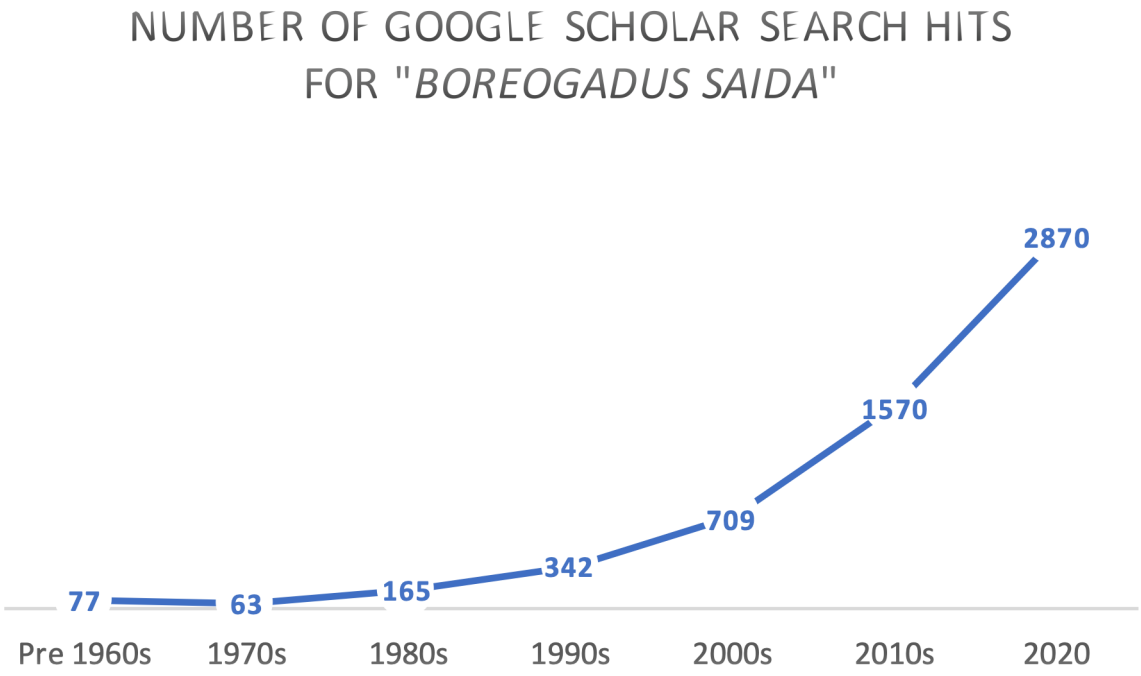

Figure 5. Number of Google Scholar search hits for "Boreogadus saida" over the past seven decades. Search performed Feb 7th 2020.

Research on polar cod has a long history, particularly in the Russian Arctic (Boitsov *et al.,* 2013; Ponomarenko, 2000; Rass, 1968; Andriyashev, 1954), but a recent surge in research interest and effort is founded in the rapid environmental change occurring in the Arctic (Wang and Overland, 2009). Polar cod research has benefitted from this surge leading to a steady increase in polar cod publications from the international research community (Figure 5). Intensive investigations on ecology (review by Mueter *et al.,* 2016; Renaud *et al.,* 2012), life history and distribution (Marsh *et al.,* 2019; David *et al.,* 2016; Geoffroy *et al.,* 2016; Bouchard *et al.,* 2016), and physiology, including impacts environmental change (Laurel *et al.,* 2018,

2016; Dahlke *et al.,* 2018; Kunz *et al.,* 2016; Nahrgang *et al.,* 2014) has solidified the role of polar cod as a sentinel Arctic species for science.

3.1.2 Polar cod life history

Polar cod utilize different habitats during its life cycle (Figure 6). Spawning occurs between December to April through the synchronous broadcast release of gametes. Although little is known of spawning behavior, there are two known spawning locations in the Barents Sea (Rass, 1968; Figure 7), and spawning likely occurs in shallow nearshore areas of the northern Bering, Beaufort, and Chukchi seas (Vestfals *et al.,* 2019). Polar cod are described as an r-selected species due to its small size (<30 cm fork length), short lifespan (5 years avg, 7-8 max), and early age at maturity (2-3 years) (Craig *et al.,* 1982). However, polar cod also express attributes of a k-selected species such as a slow growth rate and relatively low fecundity with a female carrying 10-20 thousand eggs (Nahrgang *et al.,* 2014; Hop *et al.,* 1995). Like other externally fertilizing, broadcast spawning fish (Levitan, 2005), polar cod lack any apparent sexual dimorphism. There are visible differences in reproductive strategies between males and females, such as the timing of reproductive development, investment in gonadal tissue, and frequency of spawning (Nahrgang *et al.,* 2014). Males reach maturity at a smaller size and an earlier age (Craig *et al.,* 1982), gonadal investments start earlier in the season (Hop *et al.,* 1995), and males disappear from the population at an earlier size and age than females (Nahrgang *et al.,* 2014). Maturing polar cod in captivity have been shown to draw energy predominantly from the liver and somatic tissue for gonadal development (Hop *et al.,* 1995). Adult life stages can be epipelagic (Mueter *et al.,* 2016) or occur in deeper waters (Geoffroy *et al.,* 2016; Benoit *et al.,* 2010). Polar cod are planktivorous and feed mostly on pelagic zooplankton such as copepods (*Calanus sp.)* and amphipods, like *Themisto libellula* (Cusa *et al.,* 2019; Walkusz *et al.,* 2013; Renaud *et al.,* 2012). Polar cod feed year-round, even during the Arctic winter despite low light and primary production levels (Cusa *et al.,* 2019).

The reproductive success of polar cod is related to sea-ice extent and duration, i.e., timing of ice break-up and freezing, and the formation of early polynyas (Bouchard and Fortier 2008, 2011; Fortier *et al.,* 2006). Large (1.6–1.8 mm in diameter), positively buoyant, transparent eggs are found in the surface layers and under sea ice, where they are protected from turbulence and UV damage (Graham and Hop, 1995; Hop *et al.,* 1995; Altukhov, 1981; Andriyashev, 1954). Embryos develop in 30-90 days depending on temperature with the highest embryo survival observed in temperature between -1 – 3°C (Dahlke *et al.,* 2018; Laurel *et al.,* 2018; Sakurai *et al.,* 1998; Rass, 1968). Hatching of larvae (4-8 mm in length; Bouchard and Fortier, 2011), with varying quantities of yolk reserves remaining, occurs in the spring and summer and first diet items are likely calanoid nauplii and eggs (Fortier *et al.,* 1995) present in the upper water column during the spring bloom along the sea ice edge. The larval stage duration is, on average, 149 ± 31 (SD) days (27-35 mm length) ending in the metamorphosis to pelagic juveniles (Marsh *et al.,* 2019; Bouchard and Fortier, 2011). In the surface layer, polar cod ELSs are preyed upon by seabirds (Karnovsky and Hunt, 2002) and by their adult congeners (Baranenkova *et al.,* 1966). At the onset of winter, older polar cod follow the downward

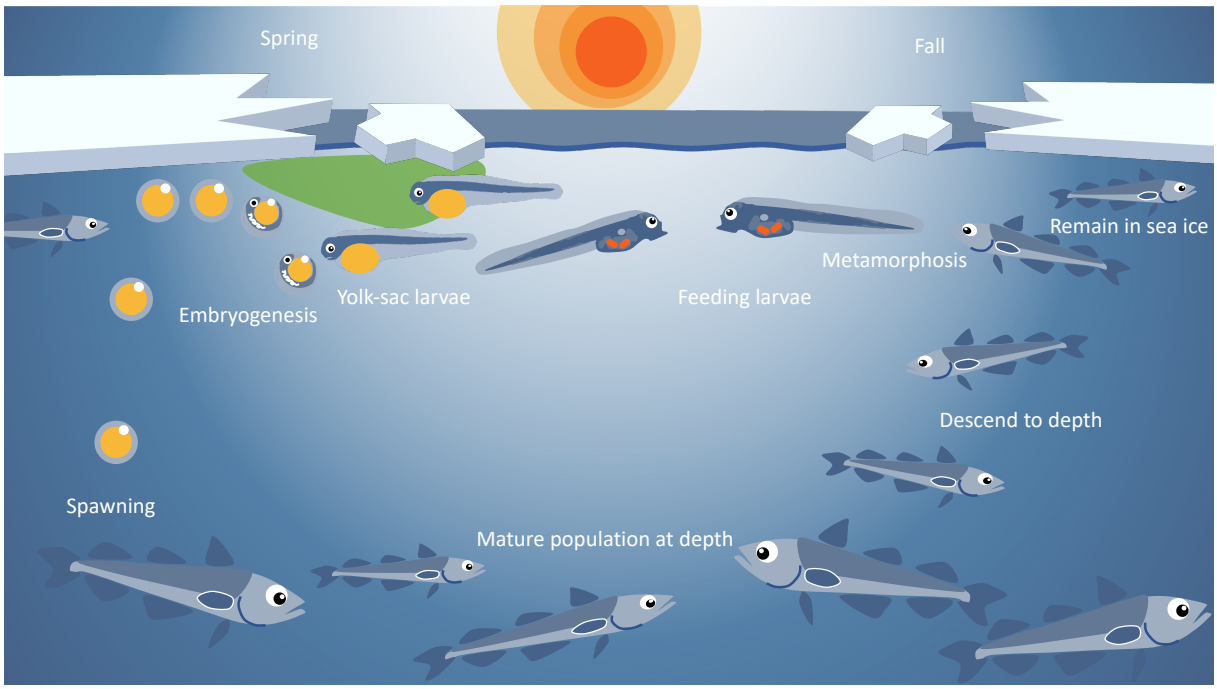

Figure 6. Schematic of the life cycle of polar cod

migration of zooplankton (Geoffroy *et al.,* 2011) and join mature aggregations at depth (Geoffroy *et al.,* 2016). Larvae and younger, smaller juveniles continue to be associated with the drifting sea-ice and are likely transported away from the shelves, as young polar cod are frequently observed and sampled in drifting sea-ice (David *et al.,* 2016; Lønne and Gulliksen, 1989).

3.1.3 State of the knowledge on polar cod responses to crude oil

Polar cod habitat overlaps with shipping lanes, fishing grounds, and areas of current and planned oil and gas operations of the Arctic. The use of polar cod as an ecotoxicological Arctic indicator organism has gained traction, especially in the field of petroleum research (Laurel *et al.,* 2019; Vieweg *et al.,* 2018; Nahrgang *et al.,* 2016; Andersen *et al.,* 2015; Jonsson *et al.,* 2010; Christiansen and George, 1995). An indicator species functions as an early warning sign for environmental health and may have predictive power for understanding larger ecological changes and cascades (e.g. changes in population size or condition of higher trophic levels).
Biomarker response relevant for petroleum biological monitoring in polar cod were characterized and showed dose-dependent responses through a variety of exposure designs and doses (Nahrgang *et al.,* 2010 a, b; Christiansen and George, 1995). These experimental studies, as well as field baseline studies verified the robust nature of certain biomarker responses in polar cod and their utility as a species suited for oil pollution monitoring in the Arctic (Nahrgang *et al.,* 2010a,b; Jonsson *et al.,* 2010). Furthermore, exposure of polar cod to OSR methods has been undertaken by Dussauze and colleagues (2014) using chemical dispersants with no additional effects observed and by Gardiner and colleagues (2013) to set lethal concentration values. Finally, only two polar cod early life stages studies have been published

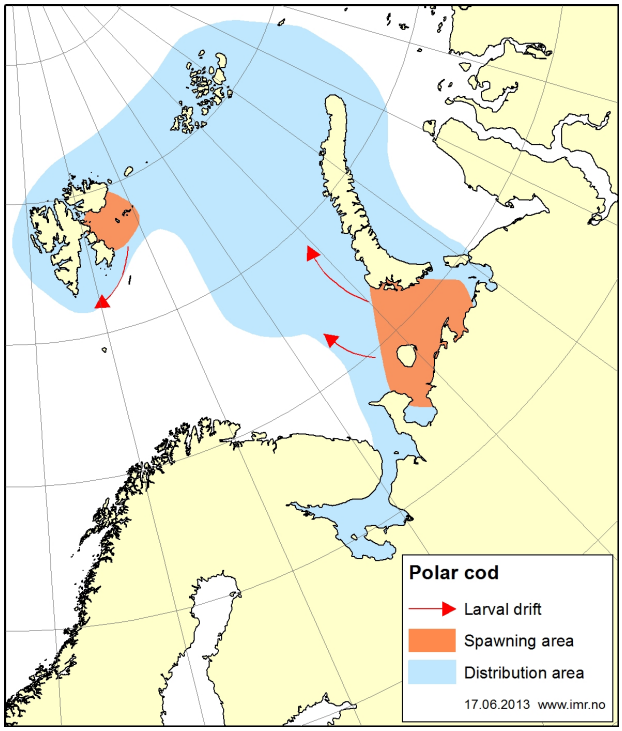

Figure 7. Distribution of polar cod stocks monitored in the Joint Russian-Norwegian ecosystem survey in the Barents Sea. Spawning areas are highlighted in orange and possible larval drift paths are depicted with red arrows. From www.hi.no/en/hi/temasider/species/polar-cod accessed on Feb 7th 2020

to date that show reduced survival and severe deformities (Nahrgang *et al.,* 2016) and long term effects on lipid allocation after transient exposures to a crude oil WSF (Laurel *et al.,* 2019).

While it can be very tempting to broadly apply the one-fish-fits-all approach to potential anthropogenic impact questions in the Arctic, the understanding of polar cod sensitivities, changing ecological role, life-history traits, population size, and general resilience to perturbations are not yet in place. Polar cod, an Arctic endemic species, is by definition only found in Arctic waters where few research stations exist and research cruise time is limited by sea ice, often with long transits and high cost of running vessels suited for sampling in the Arctic waters. Success has been found in capturing wild individuals and rearing polar cod in the lab (Kent *et al.,* 2016); however, access to live species and the high cost of cooling and maintaining seawater at temperatures ~ 0°C limits the feasibility of these projects for many facilities.

4 Scope of the work, objectives, and approaches

4.1 Scope of the work

Fishes have received much of the research attention following both EVOS and DWH, as they are commercially and ecologically important species, wide-ranging, hold various trophic levels, are relatively long-lived, are convenient to collect and hold in captivity for experimental purposes, and have high public awareness value (Pasparakis *et al.,* 2019; Whitfield and Elliott, 2002). These characteristics make fish excellent indicator species for understanding the impacts of oil spills and will be the focus of this work. The circumpolar distribution and key ecological role of polar cod make this fish species especially suited to Arctic related investigations on the biological effects of oil spills. Polar cod petroleum research has been largely focusing on adult life stages, biomarker responses, and few exposure scenarios with little ability to indicate population levels effects.

The scope of possible effects of crude oil exposure can be scaled by time, severity, level of biological organization, and whether effects are direct or indirect. The result is a murid of

potential investigative directions for an ecotoxicologist. Oil spill events provide a wealth of possible effects to find and study. However, a robust theoretical understanding is invaluable to link the cause and impact, especially in the face of litigation and substantial economic interests. Before the EVOS into today, risk assessments are based on toxicology testing using short (less than 48 hours) exposures to quantify the lethal thresholds organisms (mostly laboratory fish and invertebrates) could withstand, known as LC_{50} values (lethal concentration at which 50% of the experimental population die) (Peterson *et al.,* 2003). While the importance of this work is not in question, it fails to address possible sublethal or long-term responses. It is also irrelevant when considering the time window that oil persists in the environment.

The level of biological organization at which an effect is found, from the molecular and cellular level up to the population or ecosystem levels, provides a tool for ecotoxicologist to assess the degree of injury and the sensitivity in a particular system (Figure 8). Identifying effects lower on the scale can act as early warning signs before severe effects like mortality or a population reduction occur. One sensible approach to study the effects and sensitivity of the species is to look at responses that have importance for individual fitness – physiological and whole organism endpoints. These endpoints are above the typical biomarker levels that are appropriate for early warning signs but lack ecological relevance. It can be relatively simple to associate a toxicant with its molecular effect, but much more difficult to show that a specific molecular response (*e.g.,* biomarker response at a gene level) would be behind an ecosystem level response to a pollutant (Walker *et al.,* 2006). Measuring responses in the middle of the biological organization scale, such as changes in growth, metabolism, reproduction, and survival, provides an integrated response from the lower levels of molecular, biochemical, and cellular responses which have undergone a series of regulations with toxicant exposure (Nikinmaa and Waser, 2007). Physiology acts as an integrative response to determine the risk to individual fitness, delivering stronger predictive nature for effects at the population and ecological level (Calow and Forbes, 1997).

In the life history of an organism, certain stages can be hypothesized to be more sensitive to perturbation and, as such, result in more severe effects than if the same treatment were to be inflicted on a more robust life-history stage. By choosing presumptively sensitive and critical life history stages for exposure experiments, it may be possible to improve our predictive abilities for potential population level effects in the event of an oil spill, for example. However, scaling from individual effects at sensitive life stage to

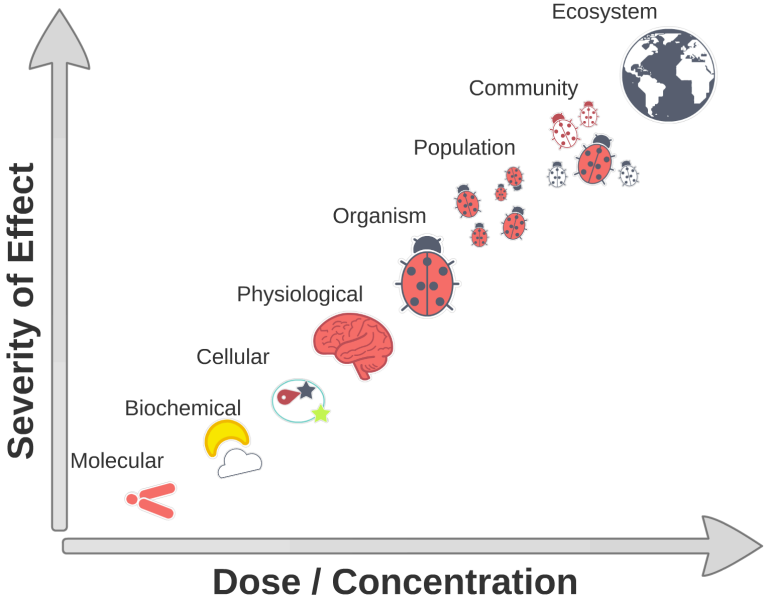

Figure 8. Schematic of the levels of biological organization and the estimated severity and dose / concentration required to elicit an effect at the given level.

population-level effects should be done conservatively as population dynamics, life history traits, and environmental factors, like changes in ocean climate, fishing pressure, or altered ecosystem structure add considerable complexity. A good example of this challenge is the herring population crash following the EVOS (Pearson *et al.,* 2012; Thorne and Thomas, 2008; see section 2.3). In the present work, we have focused on three likely sensitive life stages in polar cod; reproductive development, post-spawning, and the early life stages.

4.1.1 Period of reproductive development

Investigations into reproductive physiology can provide an integrative measure of the effects of pollutants on whole organisms and essential insights into the potential risks to populations. Sublethal crude oil exposure can have population-level impacts manifested through effects on fish reproduction a year or more after an oil spill (Whitehead *et al.,* 2012; Peterson *et al.,* 2003). Reproduction is a seasonal and cyclic phenomenon in most teleost fish (Hoar, 1969) and a key function to species survival. In the Arctic, reproduction must be synchronized with the extreme seasonality in light, temperature, and food availability to be successful (Grebmeier *et al.,* 2006). Gamete maturation in females (*i.e.,* oogenesis) and males (*i.e.,* spermatogenesis) is under tight endocrine control and modulated through sex steroid hormone cascades, involving Estradiol-17β, testosterone, and 11-ketotestosterone (Almeida *et al.,* 2008; Miura and Miura, 2003; Okuzawa, 2002; Nagahama, 2000). Following gamete development on the tissue level provides a snapshot of the maturity stage at any one time together with a more time-integrated view of recent spawning events. Individual fecundity is ultimately determined by the quantity and quality of mature gametes, for female polar cod this can be defined generally as lipid rich oocytes and for males, flagellated, swimming spermatozoa. Any alteration in the timing of maturation or quality and quantity of gamete, such as reduced sperm motility can reduce the reproductive output of an individual.

Exposure to crude oil has been shown to disrupt circulating steroid hormones levels (Sol *et al.,* 2000; Truscott *et al.,* 1983); reduce gonadal investment (Sol *et al.,* 2000); alter gamete production; and delay reproductive development (Bugel *et al*., 2010). Effects on reproduction may be modulated through AhR-dependent actions or through endocrine disruption by compounds found in crude oil (Arukwe *et al.,* 2008). Energy tradeoffs between exposure tolerance, including biotransformation, and survival, growth and reproductive development may also be a likely mechanism for crude oil toxicity. Polar cod show a high-energy investment in reproduction, compared to other gadids, with a total body weight loss of 30–50% through gonadal development and spawning (Hop *et al.,* 1995). With such a tremendous energy investment into reproduction and the importance to maintaining populations, the potential individual and physiological effects of crude oil exposure warrant further study **(Papers I and III)**.

Studies and simulated oil spills in sea ice conditions have contributed to our current understanding of oil behavior and clean up potential (Fingas, 2016; Brandvik *et al.,* 2006). Possible response efforts in the Arctic may be hampered due to the region's remoteness, insufficient mapping, and inadequate support by spill response infrastructure or contingency

plans (Nevalainen *et al.,* 2017; National Research Council of the National Academies of Science, 2014). Mechanical recovery in ice has not been effective in sea ice concentrations greater than 30% (Brandvik and Faksness, 2009). Cognizant of the deficiencies in Arctic spill recovery plans and the possible adverse effects, several Arctic nations collaborate to reduce their response time and increase their remediation readiness. Double-hulled tankers are routinely in use by oil companies, in addition to local availability of response and recovery equipment and trained personnel (Alaska Department of Environmental Conservation, 2019). International bodies, such as the Arctic Council (Arctic Council, 2009) and the International Maritime Organization, have attempted to address these shortcomings through regulations like the Polar Code. Ultimately, the fate of Arctic oil spills will depend upon a unique set of circumstances that will govern risk and impacts, including the volume of oil spilled, the chemical nature of the oil, the bioavailability of petroleum compounds, and the ecosystems impacted.

Very little work has been done on OSR responses and polar cod and no study has been done on *in situ* burned oil residues, a method well-suited for Arctic waters. Recent OSR actions have stressed the need for OSR decision making that is site- and situation-specific to adequately consider physical factors and environmental and societal resources at risk (Beyer *et al.,* 2016; Fingas, 2016). If a spill were to occur, oil would likely persist in the environment with limited removal, this chronic exposure aspect of understanding the effects of oil spills is especially relevant for Arctic species, like polar cod, and has not yet been fully investigated. However, acute exposures may also be appropriate in an oil spill response context where countermeasures are employed over a shorter time window, and polar cod could swim away from a spill. Even with acute exposure, elucidating the long-term effects of exposure on population relevant responses like reproductive development, survival and growth, will be vital in predicting and mitigating possible effects of an oil spill (Claireaux *et al.,* 2013; Forbes *et al.,* 2006) **(Paper III)**.

4.1.2 Post-spawning stage

After the release for gametes has occurred, fish are in a post-spawning stage with reduced energy reserves. The high cost of reproduction combined with seasonal pluses in food availability, in the form of spring blooms, months after spawning provides a plausible explanation for a potential of increased sensitivity in this stage. As additional support for this hypothesis, field observations report a disparity of males in the upper age classes, evidence of facultative semelparity, while females are more likely to be iteroparous and spawn multiple times in their life (Nahrgang *et al.,* 2015, 2014). Likewise, post-spawning survival is linked to remaining energy reserves and the capability of polar cod to resume feeding and build new energy stores (Hop *et al.,* 1995). Building new energy reserves is highly important feature, especially in females, for which fecundity is limited by body size, and are dependent on growing larger and reproducing over several winters to maximize fecundity (Nahrgang *et al.,* 2014).

Thus, the post-spawning stage may be a more critical time during the year, where initial low energy conditions of these specimens could have significant consequences on their capacity to cope with additional stress factors such as pollutants. Fish may experience a tradeoff between

stress tolerance, including biotransformation, and somatic growth, maintenance, and rebuilding lipid energy stores. The physiological responses of survival, growth, metabolic rate, and energy reserves (*i.e.,* lipid content) can provide integrated measures of the individual fitness **(Paper II)**.

4.1.3 Early Life Stages

To ensure survival and a strong year class, pelagic eggs and larvae must maintain their position in the water column through buoyancy control and swim bladder inflation, be able to forage when yolk reserves are exhausted and be able to grow quickly and build up reserves for overwintering while avoiding predation (Miller and Kendall, 2009). Positively buoyant embryos and larvae of polar cod have limited mobility and both a restricted horizontal distribution and a shallow vertical distribution (Bouchard *et al.,* 2017). These factors put these ELSs at a greater risk for direct exposure to environmental change and pollution events in surface waters (Geoffroy *et al.,* 2016) and during the spring melt.

4.1.3.1 Crude oil effects on ELSs

The embryonic and larval period of polar cod is long and the surface area to volume ratio is high compared to adults (Miller and Kendall, 2009), increasing the time and area available to come in contact with a potential oil spill. Generally, fish ELSs are more sensitive to toxicants (Pollino and Holdway, 2002; Petersen and Kristensen 1998), attributed to the relatively underdeveloped organs, lower metabolism during early ontogeny, and altered toxicokinetics compared to adults (Jung *et al.,* 2015; Ingvarsdottir *et al.,* 2012; Petersen and Kristensen ,1998; Paine *et al.,* 1992). Increased bioaccumulation of lipophilic compounds during the prolonged embryo stage, in concert with developmental stage-specific biotransformation rates and high lipid concentrations may elevate the body burden, the internal concentration of the compound(s), of exposed ELSs (Meador *et al.,* 2008).

Exposure to petroleum pollution in developing fish is hypothesized to act through multiple pathways causing developmental toxicity and sublethal effects that persist to later life stages (Laurel *et al.,* 2019; Mager *et al.,* 2014, Hicken *et al.,* 2011; Carls and Meador, 2009; Carls *et al.,* 1999). Exposure to crude oil triggered gene pathways involved in biotransformation, stress response, lipid metabolism, and ion regulation in Atlantic haddock (*Melanogrammus aeglefinus*) ELSs (Sørhus *et al.,* 2017). On the organismal level, crude oil exposure has resulted in changes in egg buoyancy, reductions in larval size, defects of the heart, increased incidence of edema and deformities, reduced growth, and reduced survival in many fish species (Pasparakis *et al.,* 2016; Incardona *et al.,* 2015; Carls *et al.,* 1999), including polar cod (Laurel *et al.,* 2019; Nahrgang *et al.,* 2016).

Several studies have revealed that cold water species are more sensitive to oil-induced toxicity during their ELSs (Edmunds *et al.,* 2015; Incardona *et al.,* 2015, 2014; Sørensen *et al.,* 2017; Sørhus *et al.,* 2016). Furthermore, model ecotoxicological laboratory organisms, like zebrafish (*Danio rerio*), are relatively robust to oil exposure, and comparisons of dose-effect relationship in these species are as much as two orders of magnitude higher than most marine species

(Incardona, 2017). As such many mechanistic studies on these model species use unrealistic environmental concentrations and do not accurately reflect how wild species would be affected.

4.1.3.2 Warming effects on ELSs

Polar cod stocks in the Barents Sea, Iceland-East Greenland waters, parts of the Bering Sea, Canadian Arctic Archipelago, and in Disko Bay (Greenland) are in decline, or modelled to be in decline, and retreating northward with increased water temperatures (Steiner *et al.,* 2019 and references within; Eriksen *et al.,* 2015; Astthorsson, 2015; Divoky *et al.,* 2015; Hansen *et al.,* 2012). Climate-driven changes in life-history traits have already been observed in mature polar cod (Nahrgang *et al.,* 2014). Remarkably, spawning areas in the Barents Sea are in a "hotspot of global warming" (Lind *et al.,* 2018), where loss of sea ice and warming are occurring more rapidly (Screen and Simmonds, 2010). Warmer Atlantic water masses (>2.5°C) are predicted to replace the cooler Arctic waters (<1°C) before the end of the century in the Barents Sea (Lind *et al.,* 2018).

Additionally, polar cod ELSs have a more narrow thermal window than juveniles or adults (Laurel *et al.,* 2018). Exposure of fish eggs and larvae to increased temperature can increase enzyme activity and accelerate development while decreasing the efficiency of metabolism and reducing the energy available for development (Polotis *et al.,* 2017). Furthermore, elevated temperature decreases the duration of embryogenesis, reduces size and survival of hatchings (Laurel *et al.,* 2018; Koenkner *et al.,* 2018; Hansen and Falk-Petersen 2001), increases larval growth rate (O'Dea *et al.,* 2019), and increases the frequency of deformities in polar cod (Graham and Hop, 1995) and many other fish species (Réalis-Doyelle *et al.,* 2018, 2016; Wargelius *et al.,* 2005).

4.1.3.3 Interactive effects of crude oil and warming on ELSs

Few studies have investigated the cumulative effects of temperature and crude oil exposure on ELS of fish. Those that have are focused on warm water species, such as mahi- mahi, especially in the wake of the DWH oil spill in 2010. Greater metabolic demands and higher frequency of deformities, altered cardiac function, and changes in embryo buoyancy were reported as synergistic effects of these two stressors (Perrichon *et al.,* 2018; Pasparakis *et al.,* 2016). Increased temperature may increase the metabolic demand leaving limited energetic reserves for detoxification processes in fish ELSs (Pasparakis *et al.,* 2016) and increasing the potential toxic effects of petroleum exposure. Additionally, temperature affects xenobiotic biotransformation and elimination processes in an organism (Andersen *et al.,* 2015). Ocean acidification and temperature effects investigated in polar cod ELSs under a multistressor scenario revealed a narrowing of the embryonic thermal window in polar cod (Dahlke *et al.,* 2018). Multistressor interactions with crude oil exposure and temperature have not been investigated in polar cod embryos or larvae, likely due to difficulty with first feeding stages, ethical regulations involved with first feeding larvae, and the difficulty in carrying out multistressor experiments. In light of the environmental change and pollution risk present in the Arctic (Borgå, 2019) and the vulnerability of fish embryos and larvae to environmental

stressors, a holistic investigation on the effects these factors on the health of sensitive species in a changing Arctic is warranted **(Paper IV)**.

These critical knowledge gaps form the basis of the present work and look beyond short-term toxicity to include delayed and chronic effects using physiological, biochemical, and molecular endpoints to embrace a more realistic approach to the impacts of oil spills in the Arctic.

4.2 Study Objectives

Overall Objective

Experimentally determine physiological effects of oil spills on critical life history stages of a key Arctic forage fish, polar cod, for knowledge-based management of the Arctic.

Objective I Investigate the effects of chronic dietary crude oil exposure on physiological endpoints and individual fitness including survival, reproductive development, energy reserves, metabolic rate, and growth in adult polar cod (*Paper I and Paper II*)

> **Hypothesis Ia** Dietary exposure to crude oil would adversely affect the reproductive development of polar cod
> **Hypothesis Ib** Dietary exposure to crude oil will adversely affect the individual fitness in post-spawning polar cod

Objective II Assess and rank adverse effects of acute exposure associated with oil spill remediation methods on reproductive development, survival, and growth in adult polar cod (*Paper III*)

> **Hypothesis II** Exposure to the oil spill remediation method that reduces the bioavailability of crude oil will result in the least adverse effects.

Objective III Explore the molecular and physiological effects of petroleum exposure and warmer water temperatures on polar cod early life stages (*Paper IV*)

> **Hypothesis III** Sublethal and lethal effects on polar cod embryos and feeding larvae would result from petroleum exposure and be further exacerbated under an elevated temperature.

Background objectives of doctoral work

- Practice good experimental research techniques to reduce the number of organisms involved in each study without comprising statistical power and minimize suffering in exposed organisms
- Use appropriate and applicable methods of measure that would be useful to other fields of science and for decision-makers
- Conduct work with high scientific integrity- reporting results and statistical treatment with transparency and avoiding over speculation
- Communicate findings through non-traditional and traditional scientific venues to widen the impact of the conducted research and inform stakeholders, youth, and the public

4.3 Approach & timeline

Without waiting for the next big oil spill to occur, laboratory studies can aim to answer the remaining questions in oil spill research by carefully designing and implementing experiments based on the existing scientific knowledge, untested hypotheses, and relevant new situations that may occur soon. The present body of work aims to mimic some of the possible exposure conditions organisms would likely face in the event of an Arctic oil spill. Exposure concentrations and doses were low to reduce the potential for acute mortality, and experimental durations were long and covered critical physiological bottlenecks essential for population success such as reproduction, post-spawning, and early life stages.

With a diversity of possible exposure routes, studies often chose those that are most relevant for the system and scenario in question. For example, a semi-pelagic fish would likely come in contact with water-borne crude oil (Paper III and IV) and not crude oil in sediment or an oil slick. Alternatively, dietary exposure (Paper I and II) could be relevant, especially if diet items were themselves likely to come in contact with oil compounds and to accumulate such compounds. Lipid-rich plankton, like the Arctic *Calanus* species, one of the most important prey items of polar cod, accumulate lipophilic petroleum compounds in higher concentrations than the surrounding waters (Hansen *et al.,* 2020; Agersted *et al.,* 2018) through a process called bioaccumulation**,** and can expose potential predators feeding on them as a cascading impact. The dietary route may play a significant role in the exposure of marine fishes to lipophilic contaminants (*i.e.,* PAHs; Bakke *et al.,* 2016; Nahrgang *et al.,* 2010b; Meador *et al.,* 2006; George *et al.,* 1995), especially considering the high assimilation efficiency of polar cod (Hop *et al.,* 1997). Dietary exposure is scantly studied for petroleum products but may constitute an important pathway for long-term toxicity (Agersted *et al.,* 2018). The duration of exposure is also an important factor to consider both in the field and in the laboratory. Acute exposures (Paper III), often less than 48 hours, are common for laboratory studies but may not reflect the persistence of oil. Long term persistence following an acute event was observed following the EVOS while the 87-day blowout of the DWH oil spill provided a chronic source. Thus, chronic exposure (Paper I, II, and IV) may be a more relevant exposure duration. As polar cod are a slow-growing species, reproduce once a year, and have long embryogenesis and larval periods, longer experiments were necessary to address our physiologically-based research questions.

Studies were planned and executed in an order different from that presented here. This research represents an expansion of groundwork laid down by Christiansen and George (1995) with the first studies of polar cod exposure to crude oil and the doctoral work of J. Nahrgang exploring biomarker adaptability and responses to dietary and WSF of crude oil in the adult stage (Nahrgang *et al.,* 2010 a, b, c, d; Christiansen *et al.,* 2010). The investigation performed in Paper II on the adverse outcomes of dietary exposure to crude oil in a post-spawning phase formed the bases for endpoint selection in Paper I and Paper III. Exposure levels in Paper I and II were chosen based on previous experiments (Christiansen and George, 1995) to span above and below the dose as which effects on growth were observed. Paper I levels were determined based on growth effects in Paper II that showed effects in high treatment and considerations of

the prolonged exposure. Endpoints and methods used in Paper I guided the direction of the subsequent works with reproductive development as the focus of the acute exposure, long recovery experiment (Paper III). In these studies, the physiological endpoints of growth and reproductive development provided an integrative measure of the effects of pollutants on whole organisms.

The final paper on the effect of crude oil exposure and warming on polar cod early life stages is a combination of hypotheses formed from a field study investigating climate-driven changes in life-history traits of adult polar cod (Nahrgang *et al.,* 2014) and the first study exposing polar cod embryos to crude oil (Nahrgang *et al.,* 2016). Results from the latter study revealed the need for detailed analytical chemistry with low levels of detection. In this early life stage, it is more difficult to follow specific individuals over time, multiple organisms have to be pooled to reach minimum sample mass for many analyses, and culturing and rearing through different ELSs is challenging. Conversely, working with early life stages has the benefit of a large sample number, known age and origin of organisms, reduced effect of sex, and the transparent nature of polar cod embryos and larvae provides a unique window into the internal functioning. The multi-stressor and early life stage work required the inclusion of a wider variety of endpoints and a more complex interpretation. ELSs are more amenable to extensive experimentation, which increases the likelihood of revealing sensitive endpoints (Pasparakis *et al.,* 2019). Notably, early life stages are photogenic!

5 Methods

Methods for this work are explained in brief, see attached manuscripts for in-depth, reproducible methodology. While all four experiments are unique, each addressing a slightly different approach to the effects of petroleum pollution on polar cod, they do share some key characteristics such as the wild-caught nature of the organisms; environmentally relevant levels of crude oil pollution administered over environmentally relevant durations; and the focus on physiological biomarkers of effect (Table 1). Each study was required to have permission to collect wild fish in the Svalbard Fisheries Zone by the Norwegian Fisheries Department and approval of methods, endpoints, and the number of fish used in each experiment by Norwegian Food Safety Authority. Appropriate training for students and principal investigators in the ethical treatment of aquatic animals was obtained (FELASA C level).

5.1 Collection and acclimation of mature polar cod

As a broodstock of polar cod does not yet exist in Norway, only in the US and Canada, the collection of mature polar cod from the wild was undertaken for each experiment. Wild specimens ensure a diverse population that has not experienced artificial selection in the lab and most closely resembles the phenology of the wild Barents Sea Polar cod population. However, sampling polar cod from the wild is resource-intensive as it requires a large trawling vessel, sampling permits, technical expertise for collection and maintenance of fish on board, the risk of not finding or injuring the fish in trawling process, and a significant environmental impact of bottom trawling on the seafloor.

The mature polar cod used in this work were collected from various fjord locations around Svalbard, Norway, in 2012, 2014, and 2017 on the UiT Research Vessel *Helmer Hanssen* during the fall and winter months. Fish were captured using a Campelen shrimp bottom trawl attached to fish lift (Holst and McDonald, 2000), which is shown to increases survival rates of some fish caught in bottom trawls. Fish were kept on board in flow-through tanks supplied with fresh unfiltered seawater until they reached port. Underway fish were treated daily with a disinfection agent (Halamid ® at 1:500) and were not fed.

For Paper I and Paper IV, polar cod were reared at the UiT Biological Station in Tromsø, Norway (69°N) in a 4000L acclimation tank under a natural daylight cycle of 69°N and temperatures of 2.3-2.5°C until exposures/stripping of mature gametes commenced, roughly eight and two months, respectively.

Fish were fed daily until satiation on a diet of thawed *Calanus* sp. copepods from Lofoten, Norway (purchased from Calanus AS) (Paper I and IV). For Paper II, fish were held at the experimental facilities at the University Centre in Svalbard, Norway in two 700L tanks at 6.9 ± 1.0°C in constant darkness (light climate of 8°N) and fed every three days until satiation with aquaculture feed AgloNorse TROFI AS, Tromsø, Norway during the 1.5-month acclimation period. For Paper III, fish were held at the Akvaplan-niva marine laboratory in Tromsø, Norway, in a 5000L acclimation tank for eight months supplied with 6.2 ± 0.1°C seawater and

under a Svalbard light climate. Fish were fed twice a week on a commercial marine fish feed (ration equal to 4% body weight per feeding; Skretting, 3–4 mm dry pellets).

Table 1. Overview of experimental design for the four papers presented in this dissertation

PAPER	LIFE STAGE	EXPOSURE	EXPOSURE ROUTE	EXPERIMENT LENGTH	EXPOSURE TYPE	EXPOSURE LENGTH
I	Maturing	Kobbe crude oil	Dietary	31 weeks	chronic	31 weeks
II	Post Spawning	Kobbe crude oil	Dietary	10 weeks	chronic	8 weeks
III	Maturing	Kobbe crude oil + dispersant or burned	Dispersed in water	27 weeks	acute	48 hours
IV	Early life stages	Kobbe crude oil	Water-soluble fraction	25 weeks	Decreasing chronic	Background levels in 28 – 56 days depending on temperature

5.2 Oil Exposure preparations

While the oil preparation differed for each experiment based on the intended exposure route (see Table 1), fresh Kobbe crude oil from the Barents Sea was used in all four experiments. Briefly, dietary exposure used a mix of foodstuffs, either natural *Calanus* copepods (Paper I) or aquaculture pellets (Paper II), mixed with a given dose of crude oil and gelatin for stability. The amount of food administered was based on fish weight. Food was either force-feed or distributed by hand in the tank. Dietary exposure experiments were intended to explore the potential effects of bioaccumulated crude oil in polar cod food items, a possible exposure vector for mobile fish species. The dispersed solution of Kobbe crude oil and possible oil spill response methods were used in an acute exposure scenario for Paper III. Oil was mixed into the exposure tanks with the addition of dispersant or as a burned oil residue to create the three different response measures of mechanical dispersants, chemical dispersants, or burning the oil. Lastly, paper IV employed a well-studied system of oil rock columns, developed in the wake of the Exxon Valdez Oil Spill to simulate the oil leaking out from a soiled beach over several tidal cycles to provide a decreasing yet chronic sources of the water-soluble fraction of crude oil (Carls *et al.,* 1999). Oiled rock columns are prepared using fresh crude oil and cleaned gravel mixed at the desired concentration. The gravel is then dried, placed in a Polyvinyl chloride (PVC) column, and seawater is flushed up through the column before entering the exposure tank. The fresh Kobbe crude oil was allowed to weather to reduce fish exposure to the most volatile oil compounds such as the acutely toxic BTEX (benzene, toluene, ethylbenzene, and xylene) compounds. The use of oiled rock columns in the present work aimed to mimic oil leaking from sea ice.

There are several methods for preparing a WSF or dispersed oil solution in the lab that will have a substantial impact on the composition of the aqueous fraction. Quantifying crude oil

exposure both in lab studies and in the field has traditionally been done using a few select compounds from the complex crude oil. These compounds are a) believed to be of biological significance (*i.e.,* toxic), and b) are quantifiable through standard laboratory methods. Typically, this task has fallen on a subset of PAHs usually 16, 26, 42, or 44 in number, which functions as a dose metric and allows across study and oil comparisons.

5.3 Experimental Design

Experiments were designed to control for aspects of fish welfare, size, and the environment to ensure that the varying crude oil dose/ concentration would be the likely cause of any observed effect. In the final paper of the present work, early life stages were exposed to an increased temperature treatment to simulate ocean warming in a multi-stressor experiment with crude oil exposure. Variation from individual to individual inherent in biology necessitated the use of replicate exposure tanks in all experiments. However, a common tank was used for the long-term recovery period following acute exposure to oil spill response measures in Paper III to reduce the potential tank effect on individually tagged fish. Tanks sizes were fitted to the life stages and fish number used in each experiment, water was cooled to mimic Arctic temperatures, water flow, and aeration was adjusted to maintain high oxygen levels in each tank. Outflow was filtered through charcoal to remove the remaining oil components before the water left the facilities. Sampling events during the experiments were spread to cover more subtle changes and otherwise focused on developmental milestones like vitellogenesis in mature fish or heart development and first feeding in early life stages. Balancing the number and size of sampling events with long-duration experiments requires thorough planning and hypothesis forming to define endpoints of interest with little ability to repeat experiments due to seasonal timing of events.

For each experiment, it was critical to assess the exposure dose or concentration to understand dose/ effect relationships and to compare our findings to other studies. This was done in several different ways depending on the exposure route (dietary vs. water borne) and life stage. In the dietary studies, the concentration of crude oil mixed with the food was precisely controlled, and doses were calculated based on fish weight with the reported treatment being a mass of crude oil per gram fish measure. Within the treated food itself, 16 Environmental Protection Agency priority PAHs and ten further alkylated naphthalenes, phenanthrenes, and dibenzothiophenes compounds were quantified and compiled into a sum26PAHs concentration using analytical methods detailed below. In Paper I, treated food was disturbed in each tank for fish to feed on as their appetites and social hierarchy allowed. In Paper II, fish were force feed using syringes and therefore received precisely the dose of crude oil initially intended in that treatment group. Water samples were taken at 24-hour intervals during the acute 48 hour exposure (Paper III) for total hydrocarbon content and 26 PAH quantification. Water samples from paper IV were collected from the oil flow of the oil rock columns at regular intervals from exposure start to day 56 in Paper IV for quantification of 44 PAHs. Paper IV also included PAH quantification in embryos sampled at two intervals and two-dimensional gas chromatography analysis for specific water samples taken early in the exposure when levels were highest.

In Paper I, III, and IV, additional unexposed fish were kept under the same experimental conditions in parallel with the full exposure, referred to as an "outer control" group. These fish functioned as biological timekeepers for staging sampling events around important reproductive events (Paper I) or early life developmental milestones (Paper IV) and as a control for the experimental handling stress (Paper III). When working with a species like polar cod where relatively little is known about the natural phenology, the outer control tanks provided the opportunity to establish a baseline knowledge at the same time as running an exposure experiment investigating deviations from this baseline.

In vitro fertilization was performed for paper IV using gametes strip spawned from wild caught parents. Briefly, Gametes were collected and pooled for multiple females and males, allowed to mix for 10 minutes before distribution into the respective rearing incubators.

5.4 Chemical analysis of water and biota

PAH and total hydrocarbon quantification and identification
Briefly, specific PAH quantification was performed by first adding an internal standard containing labeled deuterated PAHs to samples before extraction by saponification with methanol/KOH followed by extraction with pentane. The extract was cleaned on gel permeation chromatography and further purified by filtration on a silica column with pentane and dichloromethane as eluents. The final extract was analyzed by gas chromatography/mass spectrometry. Triplicates were run for each sample. Determination of total hydrocarbon content (n-C10 – n-C35) was performed on isooctane extracts by Gas Chromatography (GC)-Flame Ionization Detector. Each sample extract was analyzed on the GC simultaneously with control solutions (reference oil EDC95/11; Norwegian Environment Agency M-408/2015) of three known total hydrocarbon content, covering the concentration range of the sample extracts (Frantzen *et al.,* 2016).

Analysis of 26 PAHs (16 Environmental Protection Agency [EPA] priority parent PAHs and C1-C3-alkylated naphthalenes, phenanthrenes and dibenzothiophenes) concentrations in the diet and the water was performed by GC–Mass Spectrometry (GC–MS) operated in selected ion monitoring mode. Single PAH concentrations were calculated by quantification of altered deuterated standards added prior to extraction, and development of a pre-determined calibration curve of five PAH-standards at different concentrations (Frantzen *et al.,* 2016). The measured total hydrocarbon content and PAH concentrations represent dissolved components as well as oil droplets. In the determination of sum26 PAH concentrations, single components with values below the limit of detection were assigned a value of zero. Samples analyzed for the fourth Paper followed procedures detailed in Sørensen *et al.,* 2016a, and included alkyl PAH clusters determined by multiple reaction monitoring using transitions from the molecular ion, as described by Sørensen and colleagues (2016b) for 44 PAHs. The bioconcentration factor (BCF) was calculated using PAH levels in the water and the mean embryo PAH levels of the highest oil treatment using the following equation:

$$\mathrm{BCF} = \frac{PAH\ concentration_{embryos}}{PAH\ concentration_{water}}$$

Two-dimensional gas chromatography

In Paper IV, two-dimensional gas chromatography (GC-GC) was performed on water samples with the intent to explore compound diversity beyond traditional target PAH analysis. The GC-GC analysis provides high-resolution separation of potentially hundreds of compounds in a complex mixture like the WSF of crude oil (Damavandi *et al.,* 2016). Samples were analyzed by an Agilent 7890B gas chromatograph coupled with an Agilent 7250 quadrupole time of flight mass spectrometer fitted with an EI source and collision cell (Agilent Technologies, Santa Clara, CA, USA). First dimension separation was achieved using an Agilent J&W DB-1MS column. A Zoex ZX2 cryogenic modulator was used to trap and transfer continuous fractions from the first to the second-dimension columns. Second dimension separation was achieved using an Agilent J&W DB-17MS column. The source was operated at 70 eV, scan speed was 50 Hz, and scan range set at 50-450 m/z. Data were collected in Agilent Masshunter and processed using GCImage® resulting in a full chromatogram of compounds in the 2D space (Figure 9).

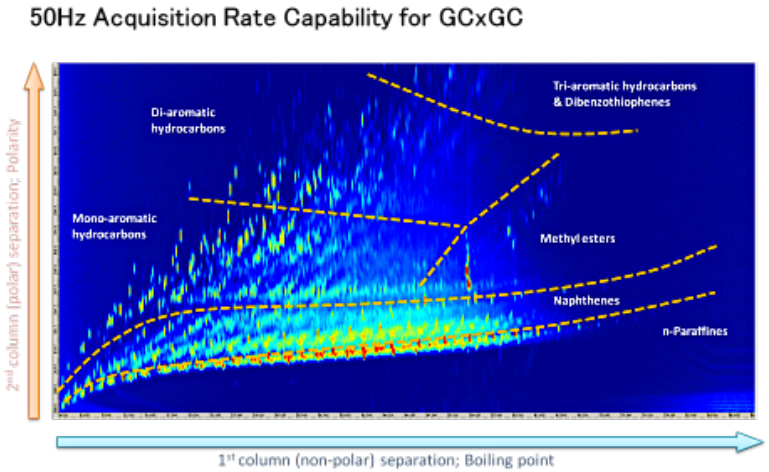

Figure 9. The two dimensional view of the full chromatogram of a petroleum example, with compound group general locations outlined (JOELUSA, 2015)

5.5 Indicators of exposure

In this section, I will briefly detail the exposure indicators used in all experiments. These indicators are metabolites, changes in mRNA expression, or protein levels that can be directly related to the exposure of an organism to a chemical, like crude oil, and identify if exposure has occurred and to what degree. Relating indicators of exposure to intended and realized exposure doses/concentrations provides critical information on the bioavailability of compounds and the toxicokinetics of the uptake, metabolism, and excretion of compounds. Without indicators of exposure confirming administered exposure, observed effects may be misattributed.

Cyp mRNA expression (Paper IV)

Indicators of exposure were analyzed for fish after a full tissue dissection followed by snap freezing of tissues and embryos. One of the earliest and most reliable indicators of PAH exposure is to investigate changes in the biotransformation pathway triggered by AhR activation (Goksøyr and Förlin, 1992; Stegeman and Lech, 1991). Quantifying the change in

regulation of the Phase I process, whereby *cyp1a* is upregulated, can be done by measuring the concentration of mRNA in the cell and was performed in Paper IV. Briefly, quantitative reverse-transcription PCR was performed for *cyp1a* and *cyp1c1,* a related cyp gene likely also involved in biotransformation but role is less understood (Wang *et al.,* 2006), on RNA extracted from embryos. RNA was extracted and cleaned using an RNeasey mini kit, with additional DNAase treatment. Control genes were validated by non-significant treatment effect on cycle threshold values. Relative fold change was calculated using the efficiency-adjusted ΔΔCt method (Pfaffl, 2001) from three control genes (*b actin, elf1a,* and *rpl4*) at each temperature.

EROD Activity (Paper I and II)

EROD activity is a sensitive biomarker for the exposure to PAHs in fish and is used to assess the activity of CYP1A, an important enzyme in PAH biotransformation Phase I (Stegeman and Lech, 1991) and was analyzed for Paper I and II. Briefly, liver samples were homogenized in a phosphate buffer. Homogenates were centrifuged, and subsequently, supernatants were centrifuged for extraction of the microsomal fraction. Pellets (microsomes) were dissolved in phosphate buffer. EROD activity measurements were performed as described by Nahrgang *et al.,* (2010b); herein, fluorescence was measured in a final reaction mixture containing the microsomal fraction from homogenized liver, the substrate ethoxyresorufin and NADPH, which started the deethylation reaction of 7-ethoxyresorufin to resorufin. Fluorescence of resorufin was measured in four replicates in the fluorimetric plate reader Synergy H1 (BioTek®, Winooski, U.S.) at the wavelength pair 540/600nm (excitation/emission) every minute for 20 min. EROD activity was normalized to the total protein content of the microsomal fraction. Total protein content was determined according to Bradford (1976).

PAH Bile Metabolites (Paper I and II)

Lastly, the final product of the biotransformation pathway, the excretable metabolite form of PAHs can was collected from the gall bladder of fish. The biliary PAH metabolites 1-OH-phenanthrene, 1-OH-pyrene, and 3-OH-benzo[a]pyrene were analyzed for Paper I and only 1-OH-phenanthrene was analyzed for Paper II. Preparation of hydrolyzed bile samples was performed as described by Krahn *et al.,* (1992). Briefly, bile was mixed with an internal standard (triphenylamine) and diluted with demineralized water and hydrolyzed with b-glucuronidasearylsulphatase. High pressure liquid chromatography (Waters 2695 Separations Module) was used to separate hydroxyl PAHs in a Waters PAH C18 column. A 2475 Fluorescence detector measured fluorescence at the optimum for each analyte (excitation/emissions: 1-OH-phenanthrene 256/380; 1-OH-pyrene 346/384; triphenylamine 300/360; 3-OH- benzo[a]pyrene 380/430). The results were calculated by the internal standard method (Grung *et al.,* 2009).

5.6 Indicators of effect

Like indicators of exposure, indicators of effect are changes in the organism, which can be quantified and linked to exposure. These range across the levels of biological organization, though concentrated at the bottom with changes in mRNA expression, concentrations of specific molecules, tissue structure, size, and function to survival. Relating indicators of effect

to indicators of exposure confirms with greater certainty the dose-effect relationship, given that both sets of indicators are specific to the treatment type. These analyses on adult polar cod form the basis of Papers I-III; to achieve this, fish were sacrificed at given intervals throughout each study (only at end of Paper III), dissected, and tissues were snap-frozen until analysis. Indicators of effect implemented in this study in the order of biological organization from molecular to organismal effects will be briefly reviewed in the following sections.

Hormone analysis (Paper I)

At intervals throughout reproductive development, fish were anesthetized, and blood was collected from the caudal vein, then centrifuged. The plasma supernatant was separated and stored at −80°C until steroid hormone analysis. Plasma concentrations of estradiol-17ß (females only), 11- ketotestosterone (males only), and testosterone (both sexes) were measured using radioimmunoassay, according to Schulz (1985). A plasma pool composed of male and female wild polar cod was used as an internal reference. The cross-reactivity of the hormone antiserum is given by Frantzen *et al.,* (2004) and Johnsen *et al.,* (2013). Values that fell below the level of detection were assigned a zero value for calculations.

Lipid Classes (Paper II)

The liver samples for lipid analysis were snap-frozen in liquid nitrogen and stored at −80°C. Briefly, the lipids of the liver samples were extracted, and the different lipid classes were separated by solid phase extraction prior to the analysis of fatty acids by gas chromatography. The total lipids of liver samples were extracted by a modified Folch method with chloroform/methanol (Folch *et al.,* 1957). The lipid extract was separated into major lipid classes by a solid phase extraction procedure adapted from the Kaluzny *et al.* (1985), using aminopropyl bonded phase columns to separate lipid mixtures into individual classes. Briefly, 0.5 ml of lipid extract was loaded in an aminopropyl modified silica mini-column. Lipids were eluded using chloroform/isopropanol acetic acid in diethyl ether, and methanol. The eluates were collected, and nonadecanoic acid was added as an internal standard. All the eluates were dried by nitrogen gas, and the fatty acids were analyzed by gas chromatography with a flame ionization detector. Before analysis by GC, all solid phase extraction fractions from the liver samples were methylated with 2.5 M dry HCl in methanol (HPLC-grade, Merck in Oslo, Norway) to obtain fatty acid methyl esters that were analyzed on gas chromatograph according to Meier *et al.* (2006). Neutral lipids, free fatty acids, and phospholipids were analyzed.

Histology (Paper I – III)

The middle section of the gonad was preserved in 4% neutral buffered formalin for histological analysis following the full fish dissection. Briefly, gonad tissues were rinsed of buffered formalin, dehydrated in a series ethanol baths, and embedded in paraffin wax (Aldrich, USA) overnight using Histo-clear® as a clearing agent in a Shandon Citadel 1000 (Micron AS, Moss, Norway). Tissues were then embedded in paraffin and sliced, using a Leica RM 2255 microtome before being stained with hematoxylin and eosin. Gonad maturity stages in females were classified using the development stage of oocytes based on Brown-Peterson *et al.* (2011) and varied in detail between studies generally covering immature, resting, and varying degrees of maturity (Figure 10). Immature and resting females had only primary growth oocytes while

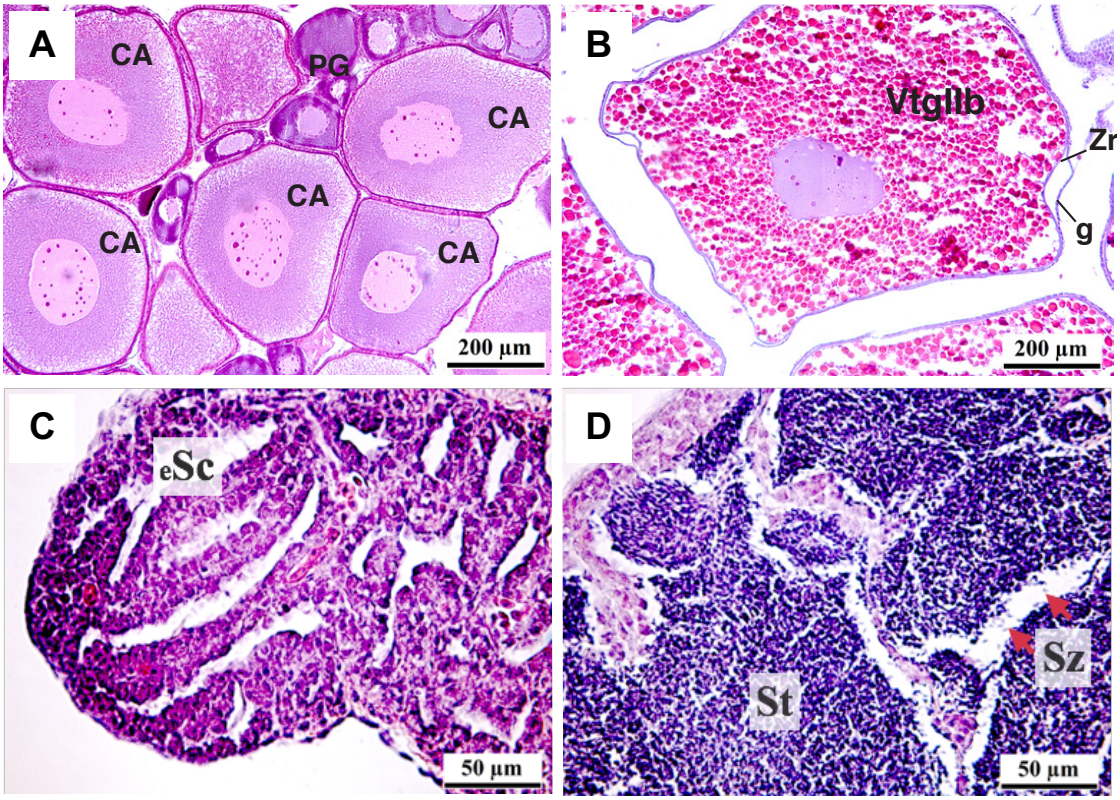

*Figure 10. Photomicrographs of ovarian histology, illustrating select stages of oogenesis in polar cod: (**A**) Developing ovary with cortical alveolar (CA) oocytes and primary growth oocytes (PG);(**B**) Advanced vitellogenesis stage (Vtg IIb) oocyte with yolk globules filling cytoplasm and evident egg shell, zona radiata (Zr) and follicle cell layer (g) around oocyte periphery; Photomicrographs of testicular development in polar cod: (**C**) Early maturing testis full of early spermatocytes (eSc); (**D**) Spawning testis at higher magnification with spermatid (St) and mature spermatozoa (Sz) in sperm ducts. From Bender et al., 2016, Supplementary Information*

maturing females had vitellogenic oocytes present. Resting females were identified by the presence of post-ovulatory follicle complexes and residual oocytes from previous spawning events with otherwise only primary growth oocytes. Maturing females exhibited different phases of oocyte development with varying extents of vitellogenin derived oil droplets in the oocyte cytoplasm. Abnormal oocyte development was noted with regard to the location of cortical alveolar vesicles and oil droplet within the oocyte. Oocyte diameter was counted for oocytes in the most advanced cohort using the image processing software (Leica DFC 295 camera attached to a Leica DM 2000 LED microscope and Leica analysis software) and then averaged for each female. Oocyte stage frequency disruption was determined by classifying all oocytes with a nucleus in an area of 20 mm^2 placed randomly on the tissue slice. Frequency counts were averaged over both replicate slides. The presence of residue oocytes was noted, and the relative frequencies of atretic oocytes were semi-quantified using a 0–3 scale for each female. Male testes were classified into the four different maturity stages of immature, resting, and maturing with either late spermatocytes stage I or with late spermatocytes stage II dominating (staging based on Núnez and Duponchelle (2009)). Immature males were identified as those with testis containing only early-stage spermatocytes, while resting males had spent testis containing portions of early stage spermatocytes with otherwise empty lumen space.

HSI, GSI, and Condition (Paper I – III)

Following blood sampling, the fish were given a sharp blow to the head before wet weight (g), and fork length (cm) were measured, and liver and gonads were removed and weighed. All remaining internal organs were removed, and the somatic weight of the carcass was recorded. Gonadosomatic index (GSI), hepatosomatic index (HSI), and condition factor were calculated according to the following equations:

$$GSI = \frac{\text{Gonad weight (g)}}{\text{Somatic weight (g)}} \text{x } 100$$

$$HSI = \frac{\text{Liver weight (g)}}{\text{Somatic weight (g)}} \text{x } 100$$

$$Condition\ factor = \frac{\text{Total weight (g)}}{\text{Total length (cm)}^3} \text{x } 100$$

Growth (Paper II and III)

Growth was recorded regular intervals by first anesthetizing, identifying through reading unique fish tags, then measuring the total weight and total length before returning fish to their tanks to recover. Specific growth rate (SGR) for individual fish for the entire experimental period was determined according to the equation:

$$SGR = \frac{\ln(W_2) - \ln(W_2)}{\text{time interval}} \text{x } 100$$

where SGR is % increase in body weight or length (Paper IV) per day. W_1 and W_2 are the total weights of the fish recorded at times 1 and 2, respectively, and time interval is the number of days between weighting events.

Respiration (Paper II)

Whole-body respiration was measured intermittently during the experiment on the same individuals using an automated intermittent flow-through respirometer equipped with eight chambers and polymer optical fiber dipping probes. Chambers were cooled and placed in individual tanks containing filtered seawater. Fish were weighed and placed individually into chambers. The automated respiration consisted in 7-min cycles of closed respirometry and flushing. Respiration values were taken from the period of 2.5 - 3 hours after the start of the cycles.

Sperm Quality (Paper I)

At the end of the experiment, males were stripped for milt by gently massaging the abdomen and taking care to avoid contamination by urine or blood. Sperm motility was examined following the protocol set by Rudolfsen *et al.* (2005). Briefly, sperm motility analysis was conducted using a small aliquot of fresh undiluted milt placed on a cooled count slide, and sperm activation was induced by adding chilled seawater. A video camera mounted on a negative phase-contrast microscope was used to record sperm activity from each male. Sperm swimming activity was recorded, and sperm motility was examined using computer-assisted

sperm analysis, an objective tool for quantitative analysis of fish sperm quality (Kime *et al.,* 2001). Sperm cell trajectories were analyzed using an HTM-CEROS sperm tracker. Five motility parameters were assessed in the present study: (1) average path velocity, which is the velocity of the sperm head along its spatial average trajectory; (2) straight-line velocity, which is the velocity of the sperm head along its linear track between its initial and final positions; (3) mean curvilinear velocity, which is the velocity of the sperm head along its real curvilinear track; (4) percentage motile sperm; and (5) percentage progressive sperm.

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

Survival (Paper I – IV)
Mortality was recorded daily over the entire experiment, and cumulative mortality was calculated for each treatment in adult exposures. Mortality in early life stage exposures was calculated using instantaneous mortality rates for the developmental stages using the equation (Houde, 1989):

$$\text{instantaneous mortality rate} = \frac{\ln N_t - \ln N_o}{-t}$$

where N_o is the abundance at the start of the developmental period, N_t is the abundance at the end of the developmental period, and t is the number of days spent in the developmental period.

5.6.1 Early life stage-specific indicators of effect

Just as specific life stages have specifics sensitivities, the indicators of effect need to be precise. This difference is based on the mostly morphological effects seen in ELSs compared to the physiological impacts observed in adult fish (Pasparakis *et al.,* 2019). Yet, not all indicators are that different, and where overlap occurs (*e.g.,* growth, mortality, mRNA expression), ELS specific methods are described in the proceeding section.

Heat shock protein mRNA expression
Heat shock protein (*hsp 70* and *hsp 8*) mRNA was quantified using the same methods as *cyp1a* and *cyp1c1* (see section on indicators of exposure).

Egg Sinking Speed and density
Embryos were sampled out of each incubator before peak hatch and transferred with a minimum amount of water to a 100 mL glass graduated cylinder filled with diluted seawater (28 ‰). The time it took for the egg to sink a given distance was recorded. The time and distance were converted into a sinking speed (cm/s) for each egg. Further conversion of sinking speed to egg density (g/mL) was performed using Stoke's Law, an average egg diameter of 1.63 mm measured from a sub-samples of eggs, and the kinetic viscosity of seawater at 28 ‰ (Knutsen *et al.,* 2001).

Size-at-hatch and yolk sac area

Early in the hatching period, 20-30 newly hatched yolk-sac larvae were sampled out of each incubator, anesthetized, and photographed at 1.6x magnification. Total length, yolk-sac area, and presence of yolk-sac edema were measured from pictures for each larva using Image J (Schneider et al. 2012).

Cardiac Activity

Newly hatched yolk-sac larvae were sampled from each incubator and placed laterally on the left side on a stage thermally controlled by a circulating cooling bath. Each larva was recorded for one minute at 4x magnification under a stereomicroscope. Heart rate was measured by counting heartbeats within a 30 second window using a manual counter. Arrhythmia was calculated using the number of frames between beats for a 20 second period and the standard deviation between the first seven beats recorded. The same observer analyzed all films.

Deformities, swim bladder inflation, and feeding success

Following confirmation of yolk-sac absorption and initiation of first feeding, larvae were sampled from each incubator, anesthetized, and photographed at 1.6x magnification. Information on larval length (measured using ImageJ), swim bladder inflation (absence/ presence), stomach fullness, and incidence of deformities were extracted from the images for each larva. Prevalence of malformations of the eyes, jaws, spin, and pericardial edema was quantified using absence/presence for each larva. A deformity index was calculated for each incubator using the sum of the scores for each deformity category divided by the total score possible for each incubator (*i.e.,* if all fish sampled were deformed (Wassenberg and Digulio, 2004).

5.7 Statistical methods

All statistical analyses were conducted with R 3.1.1 (R Core Team, 2014). After satisfying the assumptions of normal distribution and equal variance, a one-way analysis of variance (ANOVA) was used to test firstly for the difference between sexes and secondly for differences between treatments on the continuous factors followed by a subsequent post hoc test on differences between means (Tukey's honest significant difference test). Variables that violated the assumption of normality and homogenous variance were tested using the Kruskal-Wallis test by ranks. When significant results were encountered, a post hoc pairwise Dunn's test using rank sums was performed between the control and crude oil treatments. These tests were done in conjunction with a Benjamini-Hochberg adjustment on p-values to account for potential errors arising from multiple comparisons (Benjamini and Hochberg, 1995). Linear models and correlation tests (either the Pearson or Spearman method) were used to explore relationships within parameters. Distribution of maturity stages was analyzed using a Fisher's exact test with the null hypothesis that treatment groups have the same frequency of maturity stages at a given time. With a significant Fisher's exact test result, a chi-squared test was run comparing all treatment groups and control against one another. A linear mixed effect model was created for each sperm motility parameter with the R package nlme (Pinheiro *et al.,* 2016). Fixed effects were treatment, and random effects included fish identity nested within a trial to control for

variance within the same individuals tested across different times and trials. When a fixed factor indicated significant differences, pairwise comparisons using t-tests were assessed between treatments and the control following the linear model output in R. Comparisons were considered significantly different than control when $p \leq 0.05$ level. Values are reported as mean ± standard deviation (SD).

In Paper IV, statistical analysis to assess the effect of increased temperature, crude oil exposure, and the interaction of these two stressors was performed using the `nlme' package (Pinheiro *et al.,* 2016). Water chemistry results were modeled using a simple linear model and a three way interact term including time and treatments. A linear mixed effect model was run for responses with single measures in each incubator. A generalized least-squares linear model was used in instances when measurements were made on an individual fish within an incubator. Temperature and crude oil treatments were always treated as fixed factors in models to reveal nonlinear trends in concentration and response, while incubator was treated as a random factor to avoid pseudoreplication. To test the statistical significance of possible interactions between the stressors, we used log-likelihood ratio tests to compare a model with fixed effect interaction against a model omitting the interaction (Zuur *et al.,* 2009). An ANOVA was run on the best fit model. Model coefficients were extracted from models with significant interactions to assess whether interactions acted antagonistic or synergistic for each treatment combination (Folt *et al.,* 1999). An interaction coefficient <0 was categorized as congruent with stressor antagonism, a coefficient >0 was congruent with stressor synergism. The normality of model residuals was checked, and non-normally distributed data (*e.g.,* length and yolk-sac area) were log-transformed. Values are displayed as mean ± 1 standard error of the mean, and an arbitrary significance level was set at p-values <0.05.

6 Key Findings

6.1 Paper I – Chronic dietary exposure effects on reproductive development

We hypothesized that dietary exposure to low levels of crude oil in the diet would adversely affect the long and energy intensive reproductive development and individual fitness of polar cod. Feral polar cod were exposed to 0, 0.11, 0.57, or 1.14 µg crude oil / g fish/day (corresponding to control, low, medium, or high treatments, respectively) for 31 weeks before spawning. The biomarkers of exposure, PAH metabolites in bile (Fig 11A) and EROD activity, confirmed the bioavailability and metabolism of PAH compounds in polar cod after six weeks. HSI, GSI, reproductive tissue development, and plasma steroid hormone levels (Figure 11B) were not significantly altered by chronic dietary exposure to crude oil but revealed seasonal trends in gonadal development. Sperm motility (i.e., curvilinear velocity, Figure 11C) was negatively affected by dietary crude oil exposure. This study elucidated many baseline aspects of polar cod reproductive physiology and emphasized the influence of maturation state on biomarkers of PAH biotransformation (EROD and PAH bile metabolites).

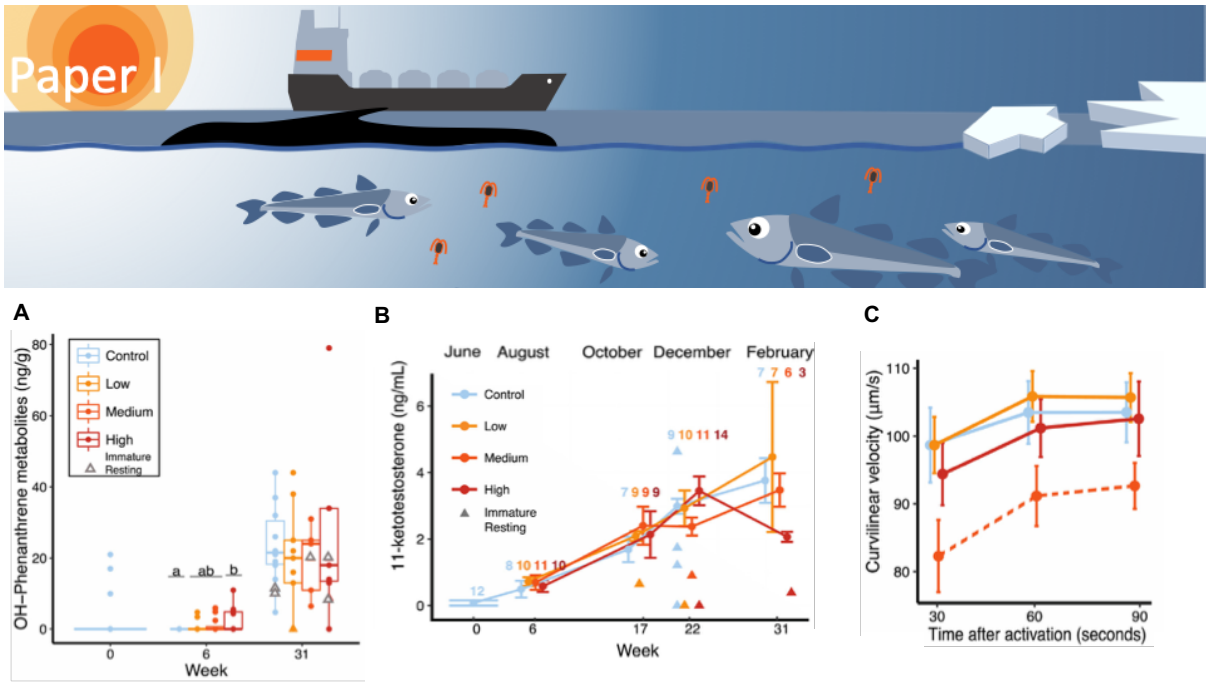

Figure 11. (A) 1-OH phenanthrene metabolites (ng/g bile dry weight) in polar cod bile with treatment distinguished by different colored boxplots and immature/resting fish represented as grey triangles. Treatment significantly different ($p < 0.05$) from one another are distinguished by different lowercase letters; (B) Plasma concentrations of 11-ketotestosterone in male polar cod after 0, 6, 17, 22, and 31 weeks of exposure to different crude oil doses and control. The number of samples in each group is displayed above the point; (C) Curvilinear velocity of sperm (µm/s) 30, 60, and 90 seconds after activation. Treatments with significant differences from control are plotted as dashed lines. Panels B and C display mean ± standard deviation for each treatment (distinguished by color).

6.2 Paper II- Dietary exposure effects on physiological parameters in post-spawning stage

We hypothesized that individuals would be in a vulnerable physiological state with poor energy stores after spawning and may be more susceptible to crude oil exposure. Feral polar cod were exposed to crude oil via force-feeding (0, 0.57, 5.71, and 28.57 µg crude oil / g fish/day [corresponding to control, low, medium, and high treatments, respectively]) for eight weeks followed by two weeks of recovery in the early spring. Significant dose-responses in exposure biomarkers (hepatic EROD activity and 1-OH phenanthrene metabolites in bile) indicated that PAHs were bioavailable already after one week of exposure (Figure 12A). Generally, all fish had low condition. The majority of females were immature and had low hepatic lipid content. Males were mostly all in a post-spawning stage and had larger hepatic energy stores than females. Most specimens showed a loss in weight with a negative SGR (Figure 12B), and this was significantly related to treatment during the first week of exposure. In individuals with reduced condition (i.e., low HSI), the effects of crude oil exposure on SGR were exaggerated (Figure 12C). Females exposed to crude oil showed a significant elevation of oxygen consumption compared to controls, although not dose-dependent. This study highlights the importance of the energy status of individuals, which is especially low during the post-spawning stage, for physiological responses to a crude oil exposure.

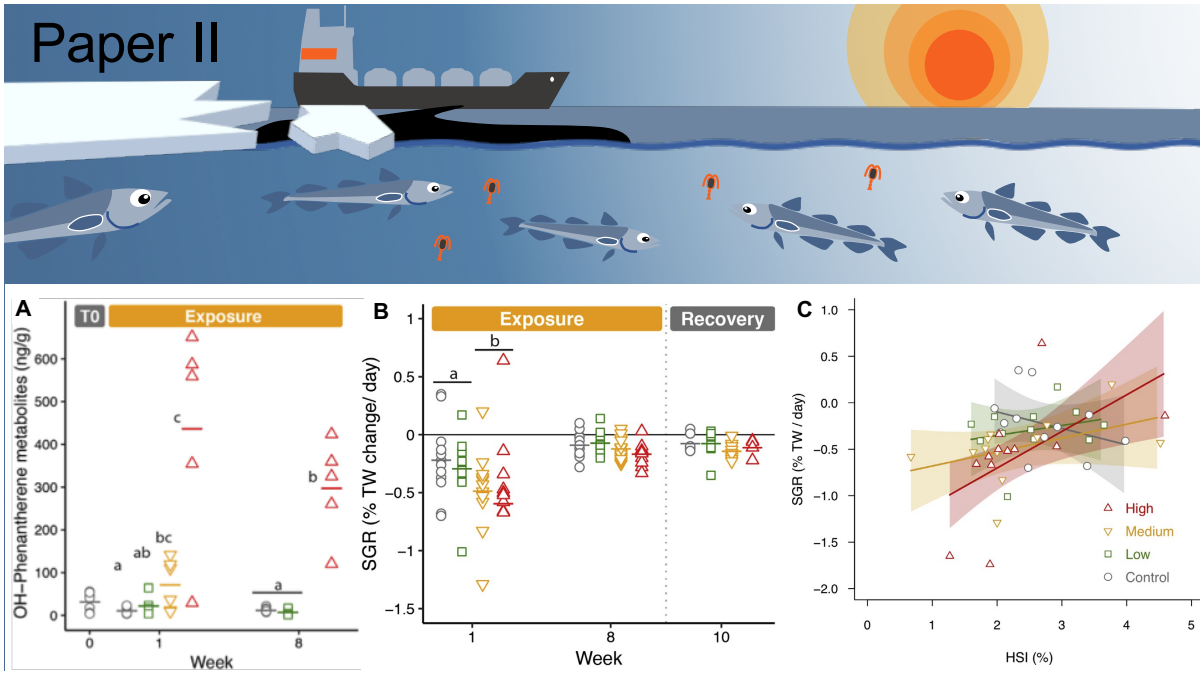

Figure 12. (A) 1-OH phenanthrene metabolites (ng/g bile dry weight) in polar cod bile; (B) Specific growth rate (SGR), % total weight (TW) of males and females between tagging to one, eight, and ten weeks; (C) The interaction of HSI (%) and treatment on the SGR of males and females in the first week of exposure. Results from linear models and 95% confidence intervals are plotted for each treatment group. Plots show individual data points distinguished by shape and color for each treatment group. For panel A and B treatment group means are represented with a dash (-), and different letters (a,b,c) indicate significant differences (Kruskal Wallis test, p<0.01) among treatments for each timepoint.

6.3 Paper III- Acute exposure to oil spill response measures during reproductive development

We hypothesized that exposure to the oil spill remediation method that reduces the bioavailability of crude oil will result in the least adverse physiological effects. Wild polar cod were acutely exposed (48 hours) to environmentally realistic concentrations of either mechanically dispersed oil (MDO), chemically dispersed oil (CDO), or burned oil residues (BO) followed by a seven-month monitoring period in clean water coinciding with the period of reproductive development. Long-term effects on survival, growth, and reproductive development were assessed. Significantly higher concentrations of PAHs were measured in the CDO and MDO treated water compared to the BO treatment (Figure 13A), confirming the increased bioavailability of chemically dispersed oil. Mechanically and chemically dispersed oil induced a transient short-term reduction in growth in the first week post-exposure. Compensatory growth was observed in the following months, and no significant long-term effects of exposure were seen in growth or mortality. Females exposed to BO residues were more frequently found in an earlier phase of gonadal maturation compared to unexposed females at the end of the experiment (Figure 13B and C). No effects of different oil spill response actions were seen in the reproductive development of males, growth, or mortality. Ultimately, this study provides information for the assessment of population consequences of different OSR actions as part of a net environmental benefit analysis.

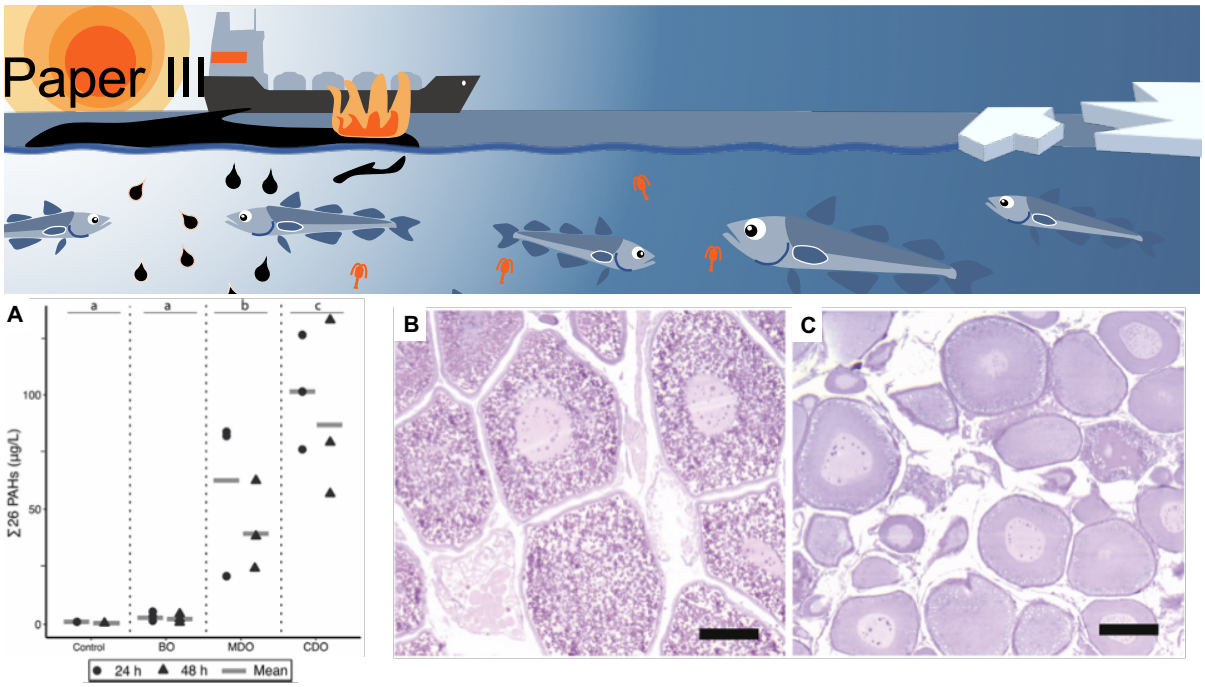

Figure 13. (A) sum26PAH concentrations after 24 hours (circles) and 48 hours (triangles) for all treatment groups with mean concentrations illustrated by black bars. Treatment mean concentrations that do not share a letter (see top of figure) are significantly different from one another ($p<0.01$); (B)Histological preparations of an advanced maturing females with vitellogenic oocytes and (B) an early maturing female with cortical alveoli vesicles. Scale bare is 200 µm in both pictures.

6.4 Paper IV- Interactive effects of crude oil pollution and warming on polar cod early life stages

We hypothesized that sublethal and lethal effects would result from petroleum exposure and be further exacerbated under an elevated water temperature. Early life stages from wild parents were fertilized *in vitro* and exposed to the water-soluble fraction of crude oil and a 2.3°C increase in water temperature from fertilization to first feeding. Embryogenic exposure to low levels of crude oil (equating to maximum 240 ng/L total PAHs in the water at the start of exposure) accumulated in embryos in high concentrations (Figure 14A). The highest crude oil treatment at the warmer temperature exhibited the presence of oil droplets when analyzed using two-dimensional gas chromatography (see section 7.2.2). Rearing at warmer temperature resulted in a shortened embryogenic period, hatching in a premature stage, higher larval growth rates, and higher mortality rates in late embryonic and larval periods. Exposure to crude oil resulted in reduced egg buoyancy (Figure 14B), reduced cardiac activity, lower incidence of feeding and swim bladder inflation, slower growth, higher mortality in larval stages, high frequencies of deformities of the eyes, jaws, and spin (Figure 14C). Synergistic effects of increased temperature and crude oil exposure resulted in greater upregulation of biotransformation-related genes, higher rates of malformation, and lower survival of larvae. In the future, a warmer, more active Arctic may threaten the sensitive early life stages of this key circumpolar forage fish.

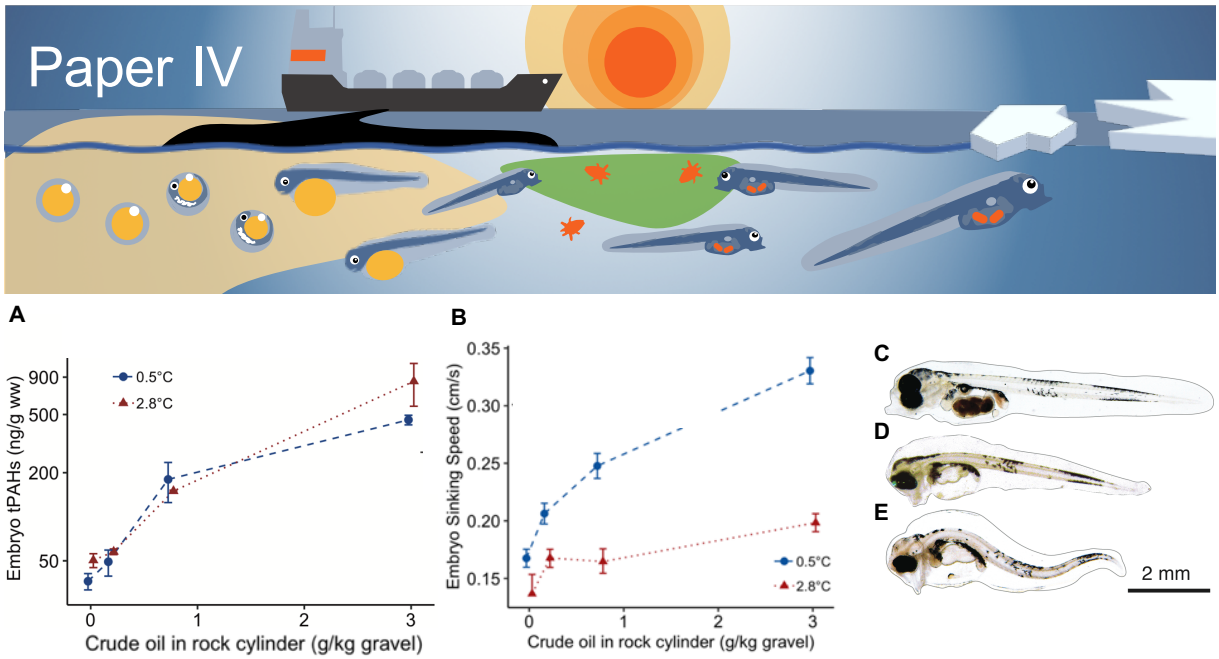

Figure 14. The effect of temperature and exposure to the WSF of crude oil on (A) total 44 PAH concentrations (ng/g wet weight) in embryos after four days on exposure/ post-fertilization displayed as treatment means ± standard error of the mean. Each treatment is comprised of two independent pools of two incubators plotted on a nonlinear scale. Colors, symbols, and line types distinguish the temperature groups; (B) normalized embryo sinking speeds displayed as treatment means (± standard error of the mean, each point includes mean values from four incubators, each incubator comprised of 20 embryo measurements); Typical phenotypes of exogenously feeding larvae exposed to (C) colder, control group; (D) colder, high oil treatment; (E) warmer, high oil treatment.

7 Discussion

7.1 Are certain polar cod life history stages more sensitive to crude exposure?

In this work, I hypothesized that specific life history stages of polar cod, a key Arctic forage fish species, would be more sensitive to crude oil exposure. Identifying vulnerable times and sensitive physiological responses to low levels of crude oil exposure would improve our predictive abilities for potential population-level effects in the event of an oil spill in the Arctic. The varied exposure routes, durations, life stages, physiological states, and chemical reporting challenges the direct comparison across the experiments included in this PhD research and in the field of ecotoxicology. However, we do see trends in individual and physiological responses that indicate increased sensitivities for specific life history stages of reproductive development, post-spawning, and early life stages.

Lethal effects of crude oil exposure were only observed in early life stages under exposure to the WSF of crude oil together with a suite of morphological and physiological effects. Sublethal effects of crude oil exposure during/ on reproductive development were observed on gamete quality (*i.e.,* sperm motility and oocyte development) but no long-term effects on growth or other physiological parameters were observed. Therefore, the period and process of reproductive development can be seen as rather robust while early life stages are more sensitive. In the post-spawning stage, effects of crude oil exposure were observed in respiration (females only). Individuals in the post-spawning stage were in the lowest condition of the adults from all the three adult exposure studies (Papers I-III) and exhibited a negative growth rate. These features support the post-spawning stage as a particularly sensitive stage in the polar cod life history.

7.2 How suitable were the exposure levels, dose metrics, and experimental designs used?

7.2.1 Crude oil exposure route and levels

Exposure levels in all experiments aimed to be environmentally and ecologically realistic. Paper I presents work from the lowest dose concentration and longest exposure duration of all dietary crude oil exposure studies in polar cod (Figure 15). Paper II has doses and durations that are more closely comparable to other studies focusing on biomarker responses and short-term effects. The crude oil doses used in the dietary exposure studies are set to represent ranges of PAH concentrations planktivorous fish may encounter in zooplankton communities after an oil spill (Salas *et al.,* 2006) or areas with chronic oil pollution (Carls *et al.,* 2006; Figure 15). The environmentally realistic doses polar cod were exposed to in Paper I were likely not high enough to induce adverse effects.

In the experimental setting, using more controlled exposure routes like force-feeding contaminated food allows for greater control over the dose and ideally a more straightforward

comparison between treatment and response. However, techniques like force-feeding risk additional stress to the organism and reduced the ecological relevance. A more realistic exposure would require a separate experiment exposing zooplankton to crude oil, either in water or emulsified with phytoplankton or sediments (See section 4.3) before being given as food items. This additional experiment would inevitably result in a change in the exposure composition as the toxicokinetics of the zooplankton would determine the eventual exposure.

In the oil spill response work, total hydrocarbon and PAH water concentrations in both mechanically and chemically dispersed oil treatments reflected environmentally realistic concentrations reported from experimental field trials and dispersant operations during actual oil spills (Sammarco *et al.,* 2013; Lessard and Demarco, 2000). The wave and tidal energy of the natural system were replicated in the experimental system using pumps to allow mechanical dispersion. While measures of hydrocarbon concentrations after *in situ* burning operations are scare, levels in the present study were below seawater concentrations measured after experimentally spilled and burned oil (Brandvik *et al.,* 2010; Daykin *et al.,* 1994). Compositional changes with burning likely occurred with the enrichment of high molecular weight PAHs, pyrogenic PAHs, and metals (Shigenaka *et al.,* 2015), altering the exposure composition.

Effects of *in situ* oil pollution have not been observed in polar cod in nature, likely due to the absence of a major oil spill event and possibly limited sampling effort around areas receiving chronic petroleum pollution (*i.e.,* estuarine areas or close to oil and gas operations in the Beaufort Sea). However, concentrations of PAH metabolites measured in wild-caught fish caught in the eastern Beaufort, a pristine area, were associated with adverse health effects (*i.e.,* lower condition; Tomy *et al.,* 2014). Likewise, our findings confirm that PAH metabolites are

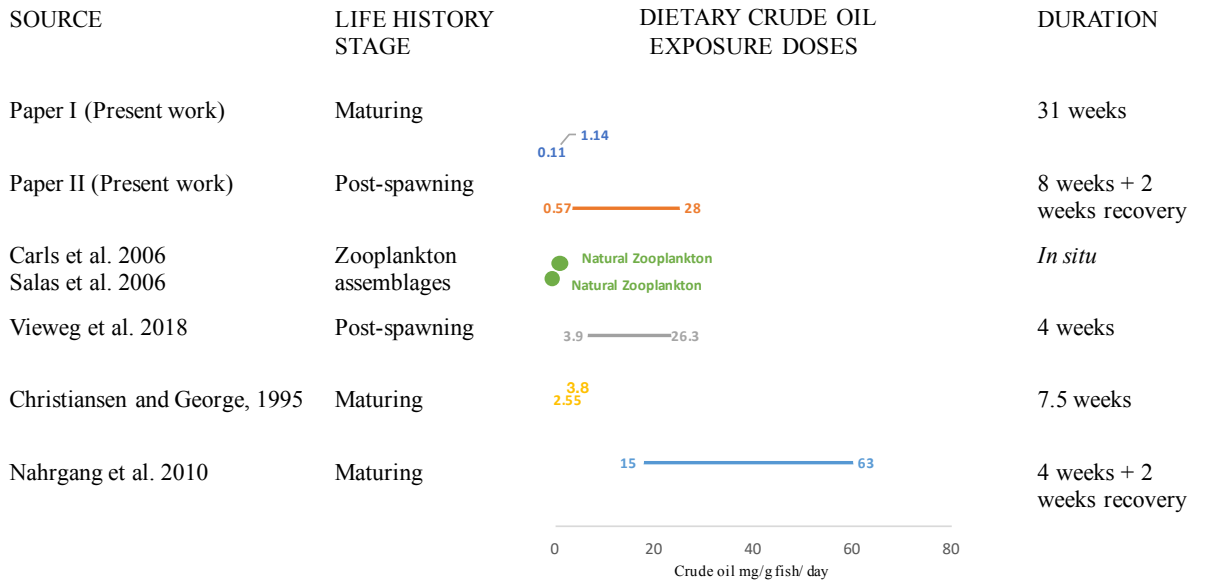

Figure 15. Comparison of dietary exposure crude oil doses for polar cod studies discussed in present work. Natural zooplankton assemblage values are presented from after the Prestige Oil spill (Salas et al., 2006) and in the crude oil export port of Valdez, Alaska (Carls et al., 2006), values are converted from sumPAH measurements used correlations from Paper I to give a rough estimate of dose relevance.

sensitive indicators of exposure to crude oil. OH-phenanthrene proved to be a more sensitive indicator than OH-pyrene. However, even under chronic exposure to dietary crude oil, metabolites were more responsive earlier in the studies at 1-8 weeks. Metabolite concentrations scaled within the two dietary studies by nearly an order of magnitude higher in the force-fed fish contra the hand-fed fish, although even control force-fed fish had measurable PAH bile metabolites. PAH background levels in control food were lower in the aquaculture feed fed to the force-fed group (80 ng/g Sum26PAHs) compared to the wild *Calanus* harvested from Lofoten, Norway provided to the hand-fed group (141 ng/g Sum26PAHs). This suggests that PAHs were otherwise present in the system. However, different analytical laboratories were used to obtain bile metabolite results, and, as mentioned, different methods and diet types were used for feeding and different foodstuffs were used, thus comparisons are not definite.

EROD activity levels in the liver were a less sensitive indicator of dietary crude oil exposure compared to bile metabolites and were only higher in exposed fish compared to unexposed fish at the higher crude oil doses (Paper II). With chronic dietary exposure during reproductive development (Paper I), EROD activity was overall low, perhaps an indication of a low effective dose of PAHs received by the liver. Maturity status was found to affect both PAH metabolites levels and EROD activity, evidence of further biological regulation of these responses by the organism. The suppression of the CYP1A1 enzyme activity in maturing fish of both sexes may be an adaptive response to maintain high steroid hormone levels necessary for endocrine regulation of reproductive development (Arukwe *et al.,* 2008; Förlin and Hansson, 1982). For instance, estradiol has been shown to have a suppressive action on pre-translational levels of CYP1A and CYP1A catalytic activity through competition for binding sites (Navas and Segner, 2001). Also, cross-talk between the signaling pathways involving the AhR, which regulates CYP1A expression, and the estrogen receptor, which regulates vitellogenin expression, has been explored in the past decade although mechanisms are still unclear (Gräns *et al.,* 2010; Mortensen *et al.,* 2007; Mortensen and Arukwe, 2007; Kirby *et al.,* 2007; Bemanian *et al.,* 2004).

The overall low amounts of PAHs in the exposure diet and the domination of lower molecular weight PAHs with potential inhibitory action on EROD activity (Whyte *et al.,* 2008) may have limited the induction of CYP1A at the doses used in the present study. Additionally, the inclusion of intestinal EROD activity to identify potential metabolism occurring before systemic uptake of the PAHs and alkylated PAHs would have provided additional information on the dietary exposure, especially at low levels (Van Veld *et al.,* 1990). Such complementary analyses may allow the comparison of tissue-specific biotransformation capacity and have previously been found relevant for polar cod exposed to dietary crude oil (Nahrgang *et al.,* 2010b).

Both temperature and crude oil concentrations used in Paper IV are likely to be encountered by eggs and larvae in the natural environment. Biophysical modeling of Barents Sea polar cod ELSs have already observed the northward retreat of spawning locations and further forecast a

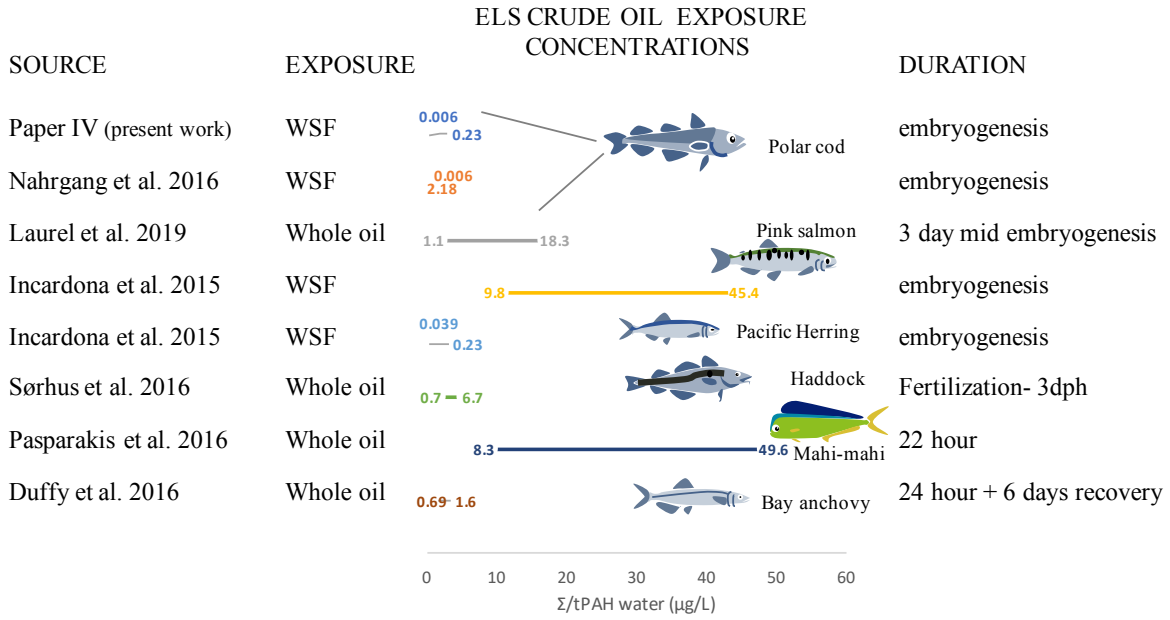

Figure 16. Comparison table of relevant ELS crude oil exposure studies from different species displayed using the sumPAH dose metric (µg/L).

recruitment collapse in polar cod ELS with sea ice reduction and increased temperature (Huserbråten *et al.,* 2019). The total PAH water concentrations measured on day 0 in the highest crude oil exposure treatments at both temperature are above what is considered hazardous for fish ELSs following an oil spill (>100 ng/L; Hodson, 2017); notably, water PAH levels fell below that threshold within the first four days of exposure. Exposure concentrations (5-237 ng/L) were within the lower range of total PAHs measured in waters in the years following EVOS (129- 126 635 ng/L; Boehm *et al.,* 2007) and within the low range of concentration measured after DWH oil spill (Echols *et al.,* 2015). The warm, high oil treatment likely altered the exposure from the water-soluble fraction to one containing bulk oil microdroplets detected through two-dimensional gas chromatography. Zebrafish embryos exposed to both the dispersed oil and the WSF at the same PAH concentrations suggest that droplet contact is not more toxic than the WSF (Carls *et al.,* 2008). However, polar cod embryos directly exposed to the dispersed oil (< 900 µg crude oil/L) in surface slick had higher bioaccumulation of PAHs and greater adverse effects compared to submerged embryos in the same treatment (Laurel *et al.,* 2019; Figure 16).

7.2.2 Dose Metrics

Results from the two-dimensional gas chromatography revealing a more comprehensive suite of compounds in the WSF and oil droplets (Figure 17) illustrates the challenges with crude oil chemistry and toxicology work today. PAHs and total hydrocarbon content have been a proxy for quantifying crude oil in most recent studies despite exposure to the WSF of crude oil or whole oil dispersed in the water (Figure 16). However, low effect concentrations (<100 ng/L sumPAHs) are reported in ELSs exposed to the WSF fraction of crude oil, yet single compound PAH exposure studies cannot reproduce these effects without scaling the PAH concentrations orders of magnitude higher (Meador and Nahrgang, 2019). As PAHs are just a small fraction of the organic compounds found in the WSF of crude oil, the potentially large toxic contribution

by other compounds is not considered (Meador and Nahrgang, 2019). Additionally, the UCM fraction is resistant to weathering (Melbye *et al.,* 2009); contains a high fraction of polar compounds, alkylphenols, alkylbenzenes (C3−C6), and alkylated aromatic heterocycles; and these compounds are in high abundance relative to the PAHs used in the dose metric (Barron *et al.,* 1999). This issue with PAHs as a dose metric makes cross study comparisons, species sensitivity comparisons, and concentration-based oil spill modelling scenarios challenging.

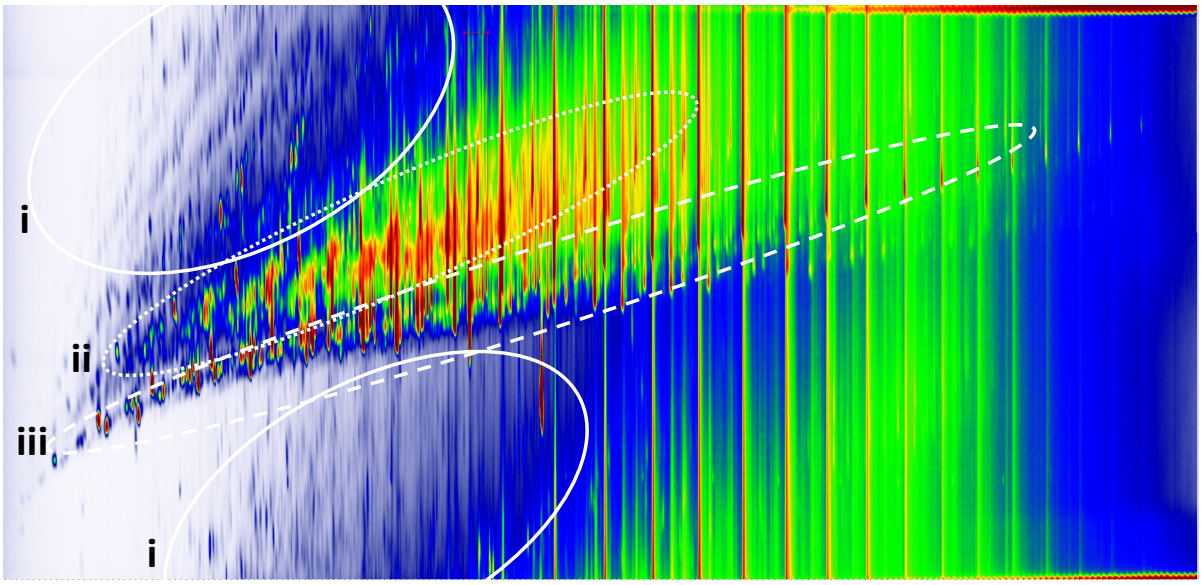

Figure 17. GCxGC total ion chromatogram of water sample extract "T0 3°C High" treatment of Paper IV. Circles mark elution ranges of i) PAHs, ii) monoaromatic compounds and iii) aliphatic compounds. Warmer colors represent higher relative concentrations. Areas outside indicated areas a result of compounds "wrapping around" the second dimension separation.

In the water of the warm, high oil treatment (Paper IV) bulk oil was present, likely due to the relatively low viscosity, low pour point, and low asphaltenic content, and thus lower retention of the fresh Goliat Kobbe crude oil in the gravel cylinders (Sorheim and Moldestad, 2008; Sørensen *et al.,* 2014). This same phenomenon was not observed in the cold, high oil treatment and so we proceed cautiously with our analysis of potential synergistic effects. As evidenced by the two-dimensional gas chromatography analysis, we see that using standard PAH quantification techniques in a whole crude oil exposure scenario risks underestimating the contribution and the mechanistic understanding of the effects of semi-polar, polar, and monoaromatic compounds, which have been demonstrated to be of toxicological significance and worthy of additional investigation (Meador and Nahrgang, 2019; Sørensen *et al.,* 2019). However, the presence of oil droplets in the system alone may not be linked to increased toxicity as many ELS studies have shown embryos to be mainly affected by the WSF of crude oil (Sørensen *et al.,* 2017; Carls *et al.,* 2008). Crude oil droplets, when present, adhere to the chorion of polar cod embryos (Laurel *et al.,* 2019) and, thus, may increase the internal concentrations of the toxic fraction, such as larger and alkylated PAH homologues (Sørensen *et al.,* 2017). On the contrary, we observed the opposite trend with higher BCFs of larger and more alkylated PAHs in the cold group compared to the warm incubated embryos, evidence

that oil droplets were likely not altering bioavailability and that elimination of PAHs was occurring. The K_{ow} predicted BCFs were lower than calculated BCFs for most PAHs, and this may be explained by our dynamic exposure design which did not allow for a steady state to establish and the high lipid content of polar cod eggs (11-17% total lipids; Laurel *et al.,* 2018) which are not accurately predicted by the K_{ow} metric. Determining internal concentrations in ELSs studies and not only exposure concentrations would provide a better understanding of the compounds that are bioavailable and should be expanded beyond PAH screening to include other possible toxic compounds in crude oil.

7.2.3 Experiments viewed *ex post facto*

Each experimental design had strengths and challenges. Early experiments (Papers II and I) struggled with sex and treatment balance, especially in the face of mortality. Physiological state (post-spawning vs. immature vs. ripe) and varied conditions at the start of the experiment, while environmentally relevant, confounded certain analysis and reduced the statistical power of analyses. Since the execution of these experiments, ultrasound techniques have been developed to determine sex non-invasively in polar cod (Vieweg *et al.,* In Prep). This technique will help dramatically to improve study design and reduce the number of fish needed in a future experiment. Using wild-caught fish leads to more variability in life history and condition than a lab-reared population, and such considerable variation should be planned for in sample size calculations.

Recovery periods in long-term exposure studies should be a goal in themselves and be run over multiple weeks when possible. A two week recovery may be enough to look at biochemical responses returning to baseline, but it is not sufficient for identifying long term physiological and sublethal irreversible/reversible effects. Recovery time after exposure was employed in Paper II and III and allowed for delayed effects to become evident. Recovery time is an advantage when using acute exposures, but with a longer exposure as was used in Paper II, no changes were seen in the recovery period. In the case of the early life stages under a decreasing WSF exposure with no exact exposure length, the delayed effects of embryogenic exposure seen in yolk-sac and feeding larvae were severe and widespread, further cementing the need for long term observations / recovery periods, even at exposure low concentrations. Using longer recovery time does necessitate ongoing sampling effort, a larger number of organisms used in an experiment, and, thus larger resource investment to gain higher biological relevance.

7.3 Did crude oil exposure affect reproductive development in polar cod?

Investment into gonadal development, measured through the GSI, was not affected by crude oil exposure, while liver tissue investment, measured through HSI, seemed to be a valuable covariate in understanding crude oil exposure on growth (Paper II). Treatment effects on GSI were infrequent and transient; however, GSI helps distinguish maturity stages between immature and mature individuals when in-depth histological analysis are not available.
Gonadal development assessed through histological preparations provided a snapshot of the reproductive development stage at any one time together with a more time-integrated view of

recent spawning events. Through gonadal histology, it was possible to verify that polar cod of both sexes are iteroparous and can spawn multiple times in their lives, at least when held in captivity. Including maturity status as a covariate in the analysis reduced the variability in other response measures (*e.g.,* PAH bile metabolites, EROD activity, and sex steroid hormone levels). While no substantial effects of crude oil exposure emerged from gonadal tissue analysis, less mature oocytes were present in females acutely exposed to the burned oil residue compared to the other OSR measures. Possibly, females in the BO treatment were not able to invest as heavily into oocyte development. This snapshot does not reveal the mechanism of delay or whether it would result in reduced fecundity of exposed individuals. No differences in maturity stage distributions or histopathologies were observed in males exposed to crude oil. Likely, gonadal development is prioritized even under stressful conditions, and therefore this endpoint is robust to low levels of crude oil pollution.

Sex steroid hormone levels were extracted from the blood plasma and quantified using radioimmunoassay. Unfortunately, this assay requires volumes > 300 µL of plasma, and that volume becomes a rather challenging job to collect with fish weighing less than 20 grams. Therefore, steroid levels were not available for every fish sampled. Great variability was observed over time in all three hormones measured with no effect of crude oil exposure. Despite the endocrine-disrupting properties of PAHs and crude oil mixtures with the potential to impair vitellogenesis in fish (Melbye *et al.,* 2009; Hylland, 2006; Arukwe and Goksøyr, 2003), the exposure levels used in Paper I were likely not high enough to induce this response. This was the first study to follow the development of sex steroid hormones over time and revealed a build-up of sex steroid hormones over reproductive development, a common feature in other fish with a determined spawning season (*i.e.,* Atlantic cod; Norberg *et al.,* 2004).

Sperm motility, an indicator for gamete quality and maturation for males only, to the author´s knowledge, has never been investigated in polar cod previously. This measure proved to be easy to collect, analyze and yielded sensitive results with implications for fertilization success. Reduced sperm motility was observed in the absence of histopathologies, delayed gamete development, or alterations in sex steroid hormone levels. While mechanisms remain unclear, it is possible that exposed males were delayed in sperm maturation although not visible in histological examinations or that oxidative stress linked to biotransformation may explain reduced sperm motility (Koa *et al.,* 2008). Sperm motility data was collected in males that were ripe approximately one month before females, and as such, it is unknown how much sperm motility would change before spawning. In crude oil exposed males, relatively high percentages of motile sperm (>87%) and low percentages of progressive sperm (<15%) were measured, illustrating that sperm were still in motion, but not at high velocities. While studies on effects of crude oil exposure on sperm motility are few, sperm fertilization ability of capelin (*Mallotus villosus*), an Arctic and sub-Arctic forage fish, was reduced with *in vitro* sperm exposure to crude oil and dispersants, but this was not related to a reduction in sperm motility (Beirao *et al.,* 2018).

Lastly, while only isolated effects were seen on reproduction parameters in Papers I and III, none of these studies reached a stage of spawning where the actual fitness, fertilization success

or quality of eggs could be evaluated. This shortcoming is due to the number of remaining fish after long experiments with mortality, as well as, time and financial constraints. However, the reduced gamete quality observed (*i.e.,* slower sperm or delayed gamete maturation) may diminish the reproductive output for the individual (Schreck *et al.,* 2001) and therefore directly impact the individual fitness with implications for population fitness.

7.4 Did crude oil exposure affect energy consumption or growth in polar cod?

Dietary crude oil exposure led to an increased standard metabolic rate, especially in females, and may indicate an energy tradeoff between routine metabolism and detoxification during the post-spawning phase. An increase in standard metabolic rate may contribute to higher energy consumption and reduced energy stores in these fish. Conversely, Christiansen *et al.,* (2010) found reduced standard metabolic rate with exposure to the WSF of crude oil both acutely and for the following four weeks. No effect of WSF crude exposure was seen in adult mahi-mahi standard metabolic rate, but a reduction was measured in maximum metabolic rate (Stieglitz *et al.,* 2016). This reduction is attributed to impaired oxygen uptake and/or oxygen transport capabilities and thus limiting the overall metabolic capability of the fish while swimming with the potential to reduce the overall fitness if effects are long-term (Stieglitz *et al.,* 2016).

Total liver lipid content and lipid class composition was analyzed during the post-spawning phase and revealed stark sex differences correlated with HSI, maturity stage, and reproductive history and not with crude oil treatment. However, the sample size was too small to overcome high variability during statistical analysis. A second paper exploring growth, survival, and lipid levels of the fish from the long-term dietary exposure study (Paper I) is in preparation. Preliminary analyses reveal no effect of crude oil exposure on growth or survival, but alterations were observed in lipid classes between treatment groups, though not dose-dependent. Changes in lipid levels in polar cod larvae have been associated with embryogenic exposure to dispersed crude oil, and this may have strong implications for overwintering success likely driven by latent effects of crude oil exposure on lipid allocation and metabolism (Laurel *et al.,* 2019). While lipid content and lipid class composition are powerful tools to quantify energy reserves with population-level implications for overwintering success and reproductive investment, the complexity of interpreting lipid class fluctuations over time and between tissues reduces the utility of this tool with a smaller sample size.

Growth was followed in three out of the four studies as a robust, integrative, physiological response. Growth information can be taken without removing the fish from the experiment and, as such, can provide individual-specific long-term effects with good temporal resolution. Crude oil exposure effects on growth were only short-term in adult fish occurring after initial exposure to both dietary crude oil and chemically and mechanically dispersed oil treatments. These transient reductions were recoverable with compensatory growth observed following reductions and are not thought to be a long-term effect of exposure at the given doses and concentrations. While reductions in growth have been seen with dietary exposure to crude oil in polar cod (Christiansen and George, 1995), higher doses may be the explanation (see Figure

15). Growth in ELSs was positively affected by temperature and negatively affected by crude oil exposure, likely with consequences for larval survival and overwintering success. Higher larval growth rates and higher mortality was observed with increased temperature potentially placing the surviving, more tolerant individuals on better survival trajectories after hatch.

One hypothesis of this work is that a tradeoff between somatic growth and maintenance and stress tolerance (including biotransformation) will occur with exposure to crude oil. This tradeoff and its complexity is illustrated by the relationship between growth and HSI, a good proxy for energy reserves, under exposure to dietary crude oil in the post-spawning stage (Paper II). With increasing crude oil dose, energy low fish (*i.e.,* low HSI) were more likely to exhibit accelerated weight loss compared to fish with higher energy reserves (Figure 12c). Handling stress may have also contributed to stress in the experimental group. In this post-spawning stage the lowest liver reserves ever published for polar cod were observed (Vieweg *et al.,* 2018; Nahrgang et al 2010 a, b). The energy status of the fish is related to spawning history, age, and season and these factors, in turn, influence the ability of the fish to cope with the additional energy demands that exposure requires without making tradeoffs at the physiological level. Energy tradeoffs are also suggested as mechanisms for delayed oocyte development in the burned oil residue exposed females and the transient growth reduction following exposure to dispersed oil OSR measures.

7.5 Did crude oil exposure affect survival in polar cod?

Mortality, an integrated response of stress, was observed in all studies but never related to crude oil exposure except in the late embryogenic and larval stages of Paper IV. Mortality in adults may be related to previous spawning events, as seen in Paper III in larger unexposed fish, and further supports the vulnerability of the post-spawning individuals. As mortality reached 56% in some experiments (Paper I) and can therefore drastically alter a study design, a further investigation into the factors leading to reduced survival (*e.g.,* handling stress, infections, parasitism, low condition) is warranted. Mortality, while easy to measure, does not seem to be a sensitive response for crude oil exposure in adult polar cod at the levels used in the present studies but more an indication of sensitivities of life history stages. Regardless, mortality should still be considered in polar cod studies as "survivor bias" may occur, leading to an overestimate of the robustness of polar cod to a given crude oil exposure when weaker individuals in the experimental population have been removed by non dose-dependent mortality.

In a review summarizing work done after DWH on ELSs, mortality was the most reported endpoint in studies, with the greatest effect range, and the most sensitive, an interesting result considering its middle position in the biological organization scheme (Pasparakis *et al.,* 2019). In this case, earlier warning signs at lower levels of biological organization would presumably also be present at even lower concentrations. However, larval mortality observed at low nominal oil concentrations (equating to 690-1600 ng/L sum50PAHs) in the DWH experimental work was attributed to delayed effects of acute exposure (Duffy *et al.,* 2016; Figure 16). This PAH concentration was higher than what polar cod embryos were exposed to in the present work (max 250 ng/L Sum44PAHs) but also shorter in duration compared to the dynamic

exposure and the levels delivered through the oiled rock column system. In the present work, elevated dose-dependent mortality was only observed 1 - 2 months after exposure began and can thus also be discussed as delayed mortality and possibly the indirect results of starvation as severe deformities hampered feeding success.

7.6 How were early life stages affected by warming and crude oil exposure?

7.6.1 Effects of warming

Rearing at a warmer temperature lower embryonic survival and resulted in premature hatching of the warm reared larvae, determined by the smaller size and lack of facial structures like jaws or advanced eye and digestive system development, compared to the cold reared larvae. This finding corroborates the high thermal sensitivity of polar cod embryos (Laurel *et al.,* 2018). Larvae reared at 2.8°C hatched one month earlier than those reared at 0.5°C and had elevated heat shock (*hsp 70*) mRNA expression indicating cellular/biochemical adjustments with temperature. In the cold-reared larvae, the larger size and more advanced developmental stage at hatch resulted in exogenous feeding shortly after hatch. Hatching at smaller sizes may leave warm-reared larvae more vulnerable to predation and less fit to forage in the wild (Porter and Bailey, 2007). On the contrary, higher larval growth rates were measured in the warm-reared larvae, which potentially place surviving, more tolerant individuals on better survival trajectories after hatch. Growth and survival rates are both stage-specific and temperature-dependent, even within the larval stage of polar cod (Koekner *et al.,* 2018). Accelerated larval growth is likely associated with higher energetic demands and may increase the vulnerability of growing larvae to alterations in food quality and abundance (Laurel *et al.,* 2018), a predicted change in the Arctic ecosystem with ongoing warming (Weydmann *et al.,* 2014).

In fact, spawning areas in the Barents sea are in a 'hotspot of global warming' (Lind *et al.,* 2018), where the loss of sea ice and warming are occurring more rapidly (Screen and Simmonds, 2010). Warmer Atlantic water masses (>2.5°C) are predicted to replace the cooler Arctic waters (<1°C), where polar cod spawn, before the end of the century (Lind *et al.,* 2018). Similarly, climate models using the IPCC business-as-usual RCP 8.5 climate change scenario predict a 1°C increase in sea surface temperature in the next 50 years around Svalbard (Hanssen-Bauer *et al.,* 2018). Likewise, biophysical models of polar cod ELS predict an "imminent recruitment collapse" if the observed sea ice loss and ocean warming continue (Huserbråten *et al.,* 2019; Dahlke *et al.,* 2018). Direct climate-related changes have also been observed to impaired growth and reproduction in adult polar cod from warmer, Atlantic influenced areas compared to more Arctic areas (Nahrgang *et al.,* 2014).

7.6.2 Effects of crude oil exposure

Effects of crude oil on early life stages were widespread, including sublethal and lethal responses from the late embryonic stage until first feeding. The adverse effect of crude oil exposure on embryo buoyancy, measured as embryo sinking speed in less saline water and converted to egg density, may have implications on early life survival, vertical and horizontal

distribution and dispersal of ELSs (Myksvoll *et al.,* 2014), and, ultimately, year-class abundance (Sundby and Kristiansen, 2015). Similar reductions in buoyancy were observed in mahi-mahi embryos exposed to crude oil and higher temperatures (Pasparakis *et al.,* 2017; 2016). Egg buoyancy changes over a more extended period of embryogenesis in exposed mahi-mahi embryos suggests that embryos are actively avoiding unfavorable conditions by decreasing buoyancy and then later returning to pre-exposure buoyancy (Pasparakis *et al.,* 2019b). While a mechanistic understanding of changes in egg buoyancy was outside the scope of this study, exposure to crude oil has likely disrupted osmoregulation of the normally hyperosmotic, buoyant eggs. In haddock, embryonic exposure to crude oil caused differential expression of pathway related to osmoregulation (Sørhus *et al.,* 2017). While depletion of yolk reserves or altered lipid metabolism would likely alter buoyancy in embryos, yolk-sac area measurements in larvae shortly after hatch did not statistically differ with crude oil exposure. Differences between the temperature groups may be explained by changes in body structure, *i.e.,* greater length at hatch in the cold reared group, as proteins are the heaviest component of embryos (Sundby and Kristiansen, 2015).

The reduction in heart rate and increased heart arrhythmia are a well-studied response to petroleum exposure in fish ELSs (*i.e.,* pink salmon, pacific herring, and haddock; Figure 16) (Sørhus *et al.,* 2017; Incardona *et al.,* 2015). Deformities likely hamper the swimming, foraging ability, and mechanisms to feed in larvae resulting in the high correlation between feeding success and presence of deformities. The high incidence of craniofacial deformities for polar cod exposed to low concentrations of crude oil has been observed in other studies (Nahrgang *et al.,* 2016; Laurel *et al.,* 2019). The timing of the loss of severely deformed larvae matches temperature corrected starvation windows determined experimentally for polar cod larvae (35-50 days post hatch; Laurel *et al.,* 2018) and, thus, mortality may be an indirect delayed effect of crude oil exposure.

7.6.3 Interactive effects

Interactive effects of temperature and crude oil exposure were observed in 64% (14 of 22) of the responses measured and spread across all developmental stages from embryos to feeding larvae and at multiple levels of biological organization from mRNA expression to rates of deformities and mortality. Within the interaction effects, 43% (6 of 14) of these response interactions found to be strong ($p<0.05$) synergistic effects, most notably in the survival of yolk-sac and feeding larval stages. Multiple stressors, in the case of synergism, have a larger impact on the organism than the sum of the individual stressors (*i.e.,* an additive response) and contribute to a realistic understanding of the biological responses of organisms in a more complex environment. For example, higher mortality rates in the warm treatment under crude oil exposure could be driving selection for more robust, albeit deformed larvae in these treatments. As exogenous feeding larvae, it is possible that the warm, high oil group were tolerant to the multiple stressor scenario with the more robust individuals remaining after the periods of elevated mortality in the yolk-sac and feeding larval periods (Gunderson et al, 2016). However, even with higher rates of deformities (*i.e.,* jaw, eye, spinal) at lower oil exposures, warm treated fish were as able to feed and reached similar developmental milestones, such as swim bladder inflation, as cold reared larvae exposed to oil.

Under a multi-stressor scenario, polar cod will likely be at an ecological disadvantage in the future Arctic. These interactive effects will future challenge our predictive ability. Interactive effects have been observed for polar cod ELSs with exposure to ocean acidification and warming (Dalhke *et al.,* 2018). Still, this field remains rather unexplored despite the environmental relevance of multi-stressor work.

8 Conclusions and future perspectives

This work presents four different oil spill scenarios on three physiologically different life stages of the Arctic key forage fish, polar cod. Generally, polar cod adults are relatively robust to low levels of crude oil exposure, and oil spill response countermeasures, as no effect of crude oil exposure was observed on survival in any adult exposure. Reduced energy reserves and condition in the post-spawning stage suggests the increased physiological sensitivity of this life-history stage and warrants further investigation. The physiological effects of crude oil exposure on respiration, growth, and reproductive development were not consistent across time and sex, were not dose-dependent, or were not observed. Effects seen on sperm motility under dietary exposure and gonadal development in females exposed to burned oil residues require follow-up examination, especially in light of the importance of gamete quality to individual fitness. Furthermore, understanding the physiological state and sex of polar cod exposed is important for result interpretation, and studies should be designed with this in mind. Early life stages, as predicted, were the most sensitive life stage. The addition of a second stressor, temperature, worsened the effect of crude oil exposure at the higher levels of biological organization (*i.e.,* mortality). Despite the expectation that small effect sizes would result from small increases in temperature and low levels of crude oil exposure (O'Dea *et al.,* 2019; Liess *et al.,* 2016), the magnitude of the effects observed in this study was high, further supporting the vulnerability of polar cod early life stages to environmental stressors like climate change and petroleum pollution.

Polar cod have proven amiable as an indicator species with a few notable limitations and prominent advantages. The ecological relevance as a circumpolar forage fish, the ability to undergo its entire lifecycle in laboratory conditions, and relatively simple rearing provided cold water is in supply will likely pave the way for continued research on polar cod. Data on basic biological and anthropogenic effects are necessary for ongoing risk assessments and model building.

The field of Arctic ecotoxicology is relatively new, but the future is bright as the Arctic will continue to be a home for millions of people, undergoing rapid environmental change, and hold many valuable natural resources. Improving our understanding of the Arctic ecosystem and how anthropogenic activities impact the structure and function of the ecosystem can be improved through an expansion of methodologies such as including -omics technology to elucidate affected pathways, even in non-model organisms. Additionally, transparent data publishing would ensure that even studies employing a variety of methods will have enough information reported to allow for conservative comparisons. This would be further improved with a concerted effort to report dose/concentration measurements and internal concentrations when appropriate. Improvements in experimental design, including using the best available methods for sex determination and appropriate numbers of organisms to ensure robust statistical analysis, should be a priority moving forward. Designing experiments that span generations (*e.g.,* multi- and transgenerational) would provide a look into an unknown, little studied process, which can be a substantial exposure route for polar cod eggs when females are exposed

to crude oil (J. Nahrgang unpublished data). In the present work, continued analysis is underway for both Paper I and Paper IV, and follow-up studies will explore physiological endpoints of importance (*i.e.,* growth, survival, and lipid content) and growth, oxygen consumption, and behavior in the respective studies. Inclusion of this biological understanding of sensitive life stages and delayed effects together with the knowledge of other key species in the environment (*e.g.,* forage fish, zooplankton, primary producers) into oil spill scenario models would provide a much-needed insight into the impacts an oil spill would have on the population and the ecosystem of the Arctic.

In fact, an estimation of the hypothetical impact of a large-scale oil spill, equating to more than double the size of the EVOS, was performed for polar cod in the Beaufort Sea using acute toxicity data for juvenile polar cod (LC_{50} at 30000 ng/L sum26 PAHs; Gallaway *et al.,* 2017). Results from this study attribute a large-scale oil spill to the loss in the reproductive output of 7300 polar cod females. In a population whose size is in the billions in the U.S. Arctic alone, "the loss of 7300 females' reproductive output would be insignificant" (Gallaway *et al.,* 2017). Findings from the present work can suggest including post-spawning mortality parameters, life history stage-specific sensitivities, delayed/ latent effects of exposure, and the development of a more accurate dose metric for crude oil exposure for future experimental, modeling, and risk assessment efforts. Ideally, a crude oil dose metric would span beyond the sumPAH measure to include other possible toxic compounds in crude oil.

Global environmental change is not limited to increased water temperature and the acute and chronic petroleum pollution events as this dissertation presents. Reductions in sea ice extent, ocean acidification, increasing freshwater input, southern species moving northward to alter community structures and other pollution issues, like microplastics and emerging contaminants, all stand to affect polar cod (Dalhke *et al.,* 2018; McNicholl *et al.,* 2018; Kühn *et al.,* 2018; Christiansen, 2017; Bouchard and Fortier, 2011) with possible cascading effects to the entire Arctic marine ecosystem. For example, in the Barents Sea from the period of 2004 to 2012, the extent of Arctic water masses decreased in association with a northward shift of the boundary separating the Arctic and boreal (southern) fish communities (Fossheim *et al.,* 2015a,b). During these years, the boreal fish community expanded northward while the Arctic fish community retracted (Fossheim *et al.,* 2015a,b). Polar cod, in particular, respond quickly to changes in temperature and ice extent (Marsh and Mueter, 2019; Alabia *et al.,* 2018) and are sensitive to habitat change (Nahrgang *et al.,* 2014). Increased primary and secondary productivity has likely favored the boreal fish species competitively over the northern species. The larger predatory species (*i.e.,* Atlantic cod) might also exert feeding pressure on the smaller Arctic species (*i.e.,* polar cod) (Fossheim *et al.,* 2015). Determining how the sentinel species, polar cod, will respond to these environmental and ecological stressors and what influence this will have on the resilience of the Arctic marine ecosystem is the future aim of this research.

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Methods

2.1. Fish collection and husbandry

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

2.2. Experimental design

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

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

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

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

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

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

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

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

2.3. Determination of PAH doses in the diet

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

2.4. Analysis of PAH metabolites in bile

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

2.5. Ethoxyresorufin O-deethylase (EROD) activity

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

2.6. Histological analysis

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

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

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

2.7. Steroid hormone analysis

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

2.8. Sperm quality

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

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

2.9. Statistical analysis

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

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

3. Results

3.1. Morphometric data

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

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

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

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

3.2. PAH doses and biomarkers of biotransformation

3.2.1. Diet doses

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

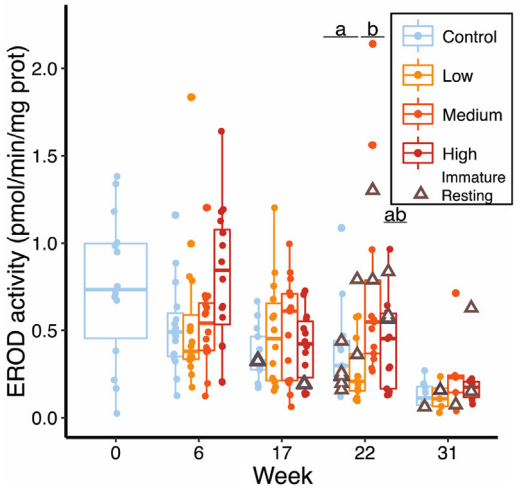

Fig. 1. Ethoxyresorufin O-deethylase (EROD) activity (pmol min/mg/proteins) in the liver of polar cod exposed to low, medium and high doses of dietary crude oil and controls. Fish (n = 16/treatment) were sampled at 0, 6, 17, 22, and 31 weeks of exposure. Boxplots represent the median (horizontal line),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

3.2.2. Ethoxyresorufin O-deethylase (EROD) activity

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

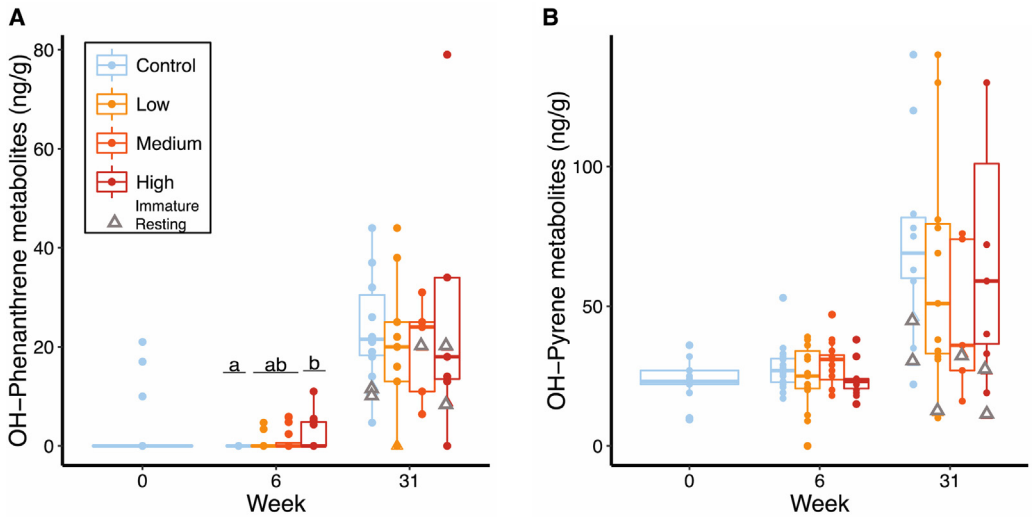

Fig. 2. PAH bile metabolites measured as (A) 1-OH-phenanthrene metabolites and (B) 1-OH-pyrene metabolites in crude oil exposed and control polar cod sampled at 0, 6, and 31 weeks of exposure. Boxplots are as in Fig. 1. Each point represents a maturing polar cod while immature and resting fish (grey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

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

3.2.3. PAH metabolites in bile

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

3.3. Effects on reproduction

3.3.1. Somatic analysis

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

3.3.2. Histological analysis of gonads

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

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

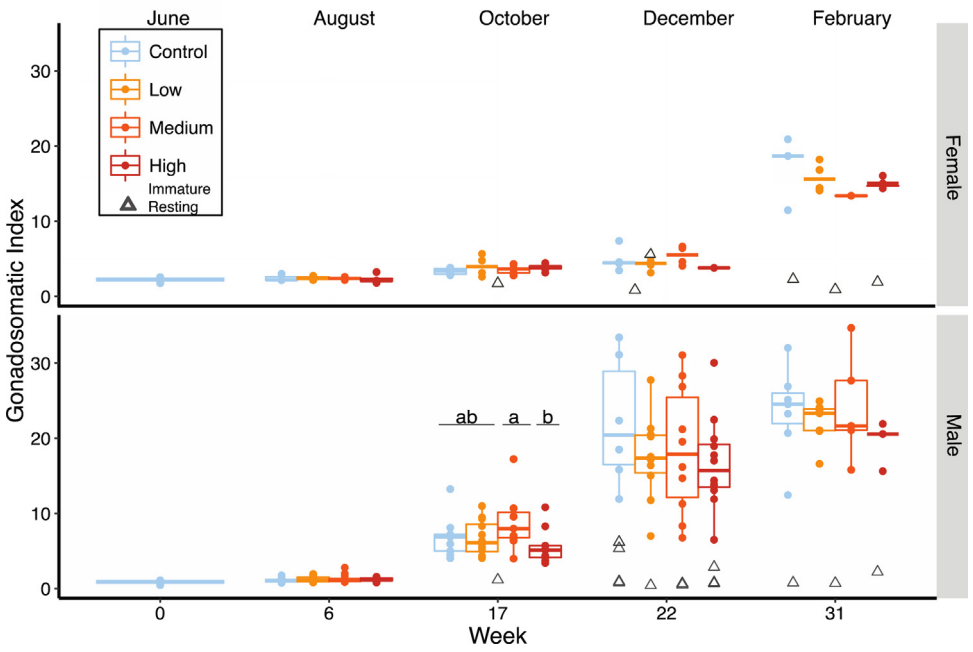

Fig. 3. Gonadosomatic Index of maturing female polar cod (top panel) and male polar cod (bottom panel) in different crude oil treatment groups sampled after 0, 6, 17, 22, and 31 weeks of exposure. Boxplots are as in Fig. 1 where different treatment groups are distinguished by color, each point representation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

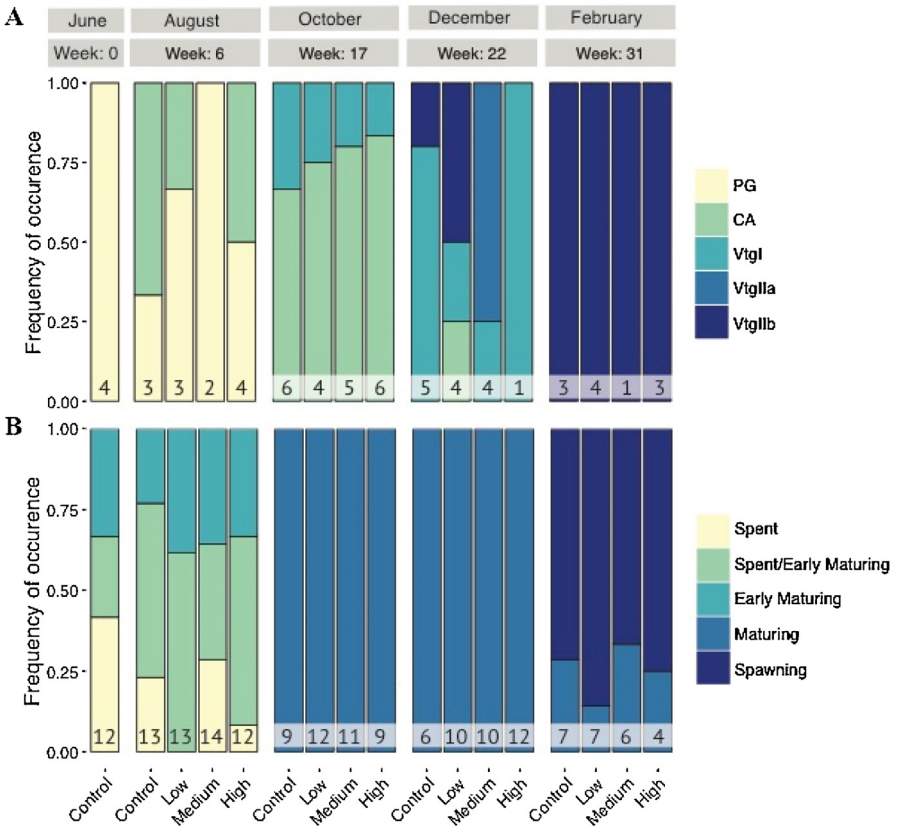

Fig. 4. Histological analysis of gonadal maturity stages in maturing (A) female and (B) male polar cod over a period of gonadal development from June to February. The frequency of occurrence of each maturity stage, represented by different colors, in each treatment group after 0, 6, 17, 22, and 31 weeks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

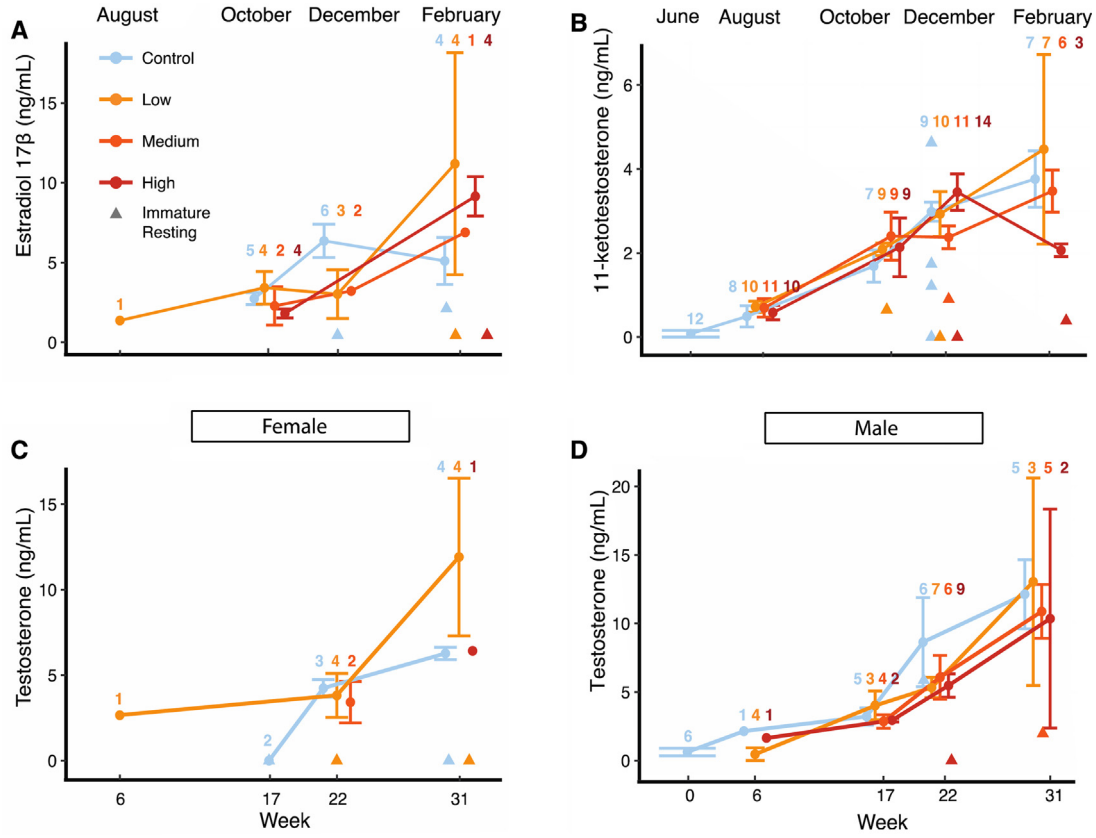

Fig. 5. Plasma concentrations displayed by mean ± standard error of (A) estradiol-17β in maturing female polar cod; (B) 11-ketotestosterone in maturing male polar cod; (C) testosterone in maturing female polar cod; (D) testosterone in maturing male polar cod after 0, 6,17, 22 and 31 weeks of exposure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

3.3.3. Plasma steroid concentrations

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

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

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

3.3.4. Sperm quality

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

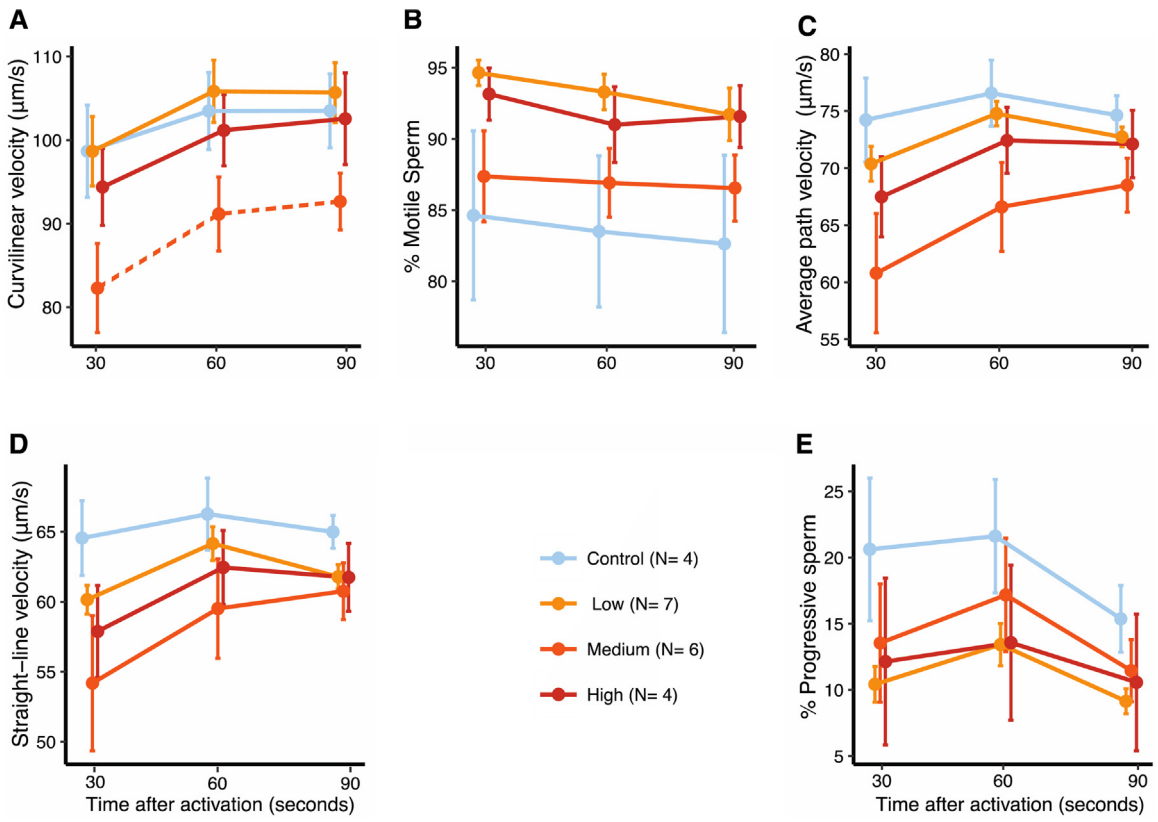

Fig. 6. Sperm motility measurements at 30, 60, and 90 s after activation from male fish stripped in week 31 (February 3rd) displayed by mean ± standard error: (A) Curvilinear velocity; (B) Percentage motile sperm; (C) Average path velocity of sperm; (D) Straight-line velocity; (E) Percentage progressive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

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

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

4. Discussion

4.1. Effect on exposure indices

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

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

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

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

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

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

4.2. Effects on reproductive parameters

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

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

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

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

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

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

4.3. Conclusion and outlook

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

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

Ethics statement

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

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Appendix A. Supplementary data

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

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SUPPLEMENTARY INFORMATION

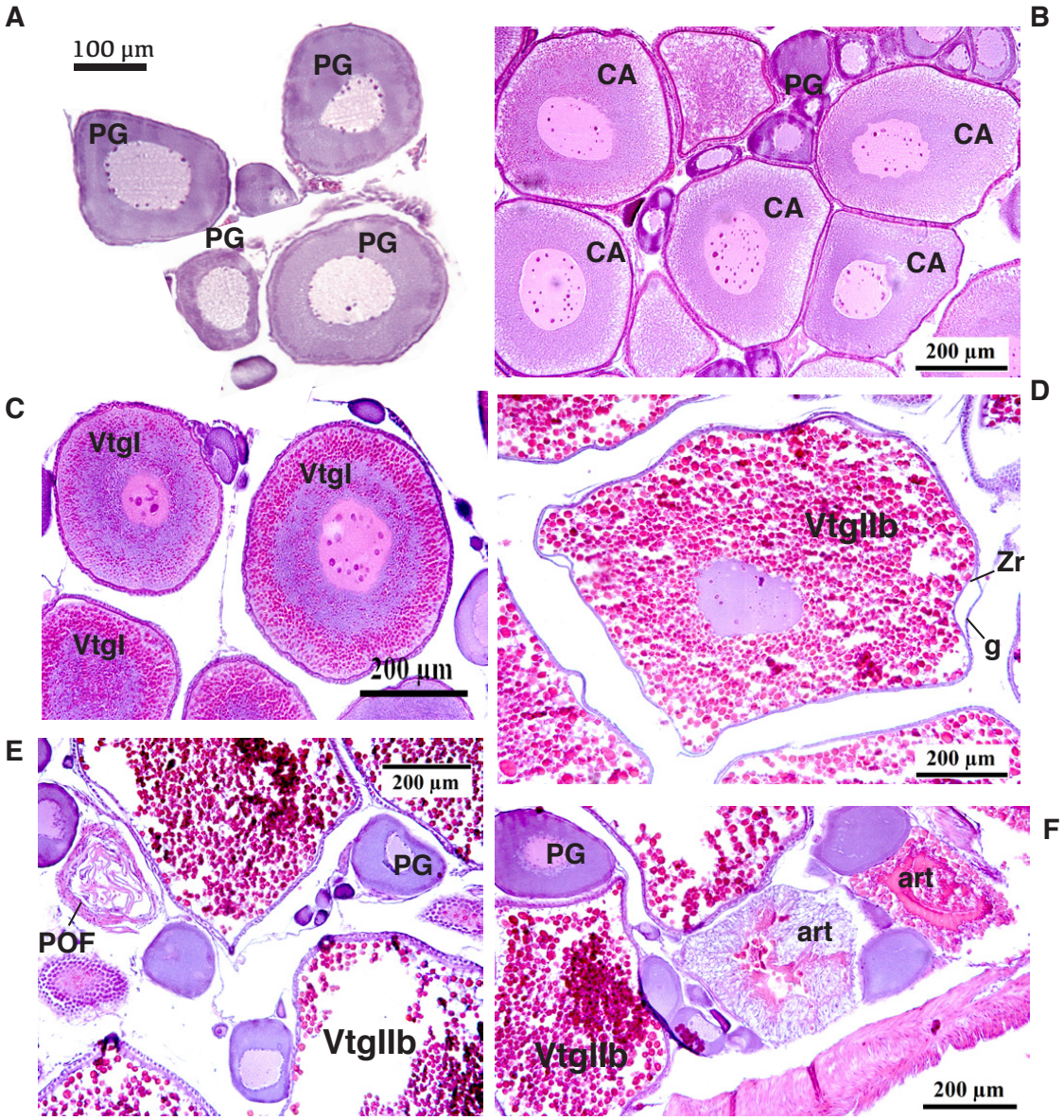

Figure A.1 Photomicrographs of ovarian histology, illustrating select stages of oogenesis in polar cod: (A) Early stage ovary with primary growth (PG) oocytes; (B) Developing ovary with cortical alveolar (CA) oocytes;(C) Vitellogenesis I (Vtg I) oocytes include inclusion of yolk globules in the outer periphery of the oocyte (D) Vitellogenesis IIb (Vtg IIb) oocyte with yolk globules filling cytoplasm and evident egg shell, *zona radiata* (Zr) and follicle cell layer (g) around oocyte periphery; (E) Postovulatory follicle complex (POF) as evidence of previous spawning in maturing female; (F) Atretic oocytes (art) together with PG and Vtg II oocytes in maturing ovary.

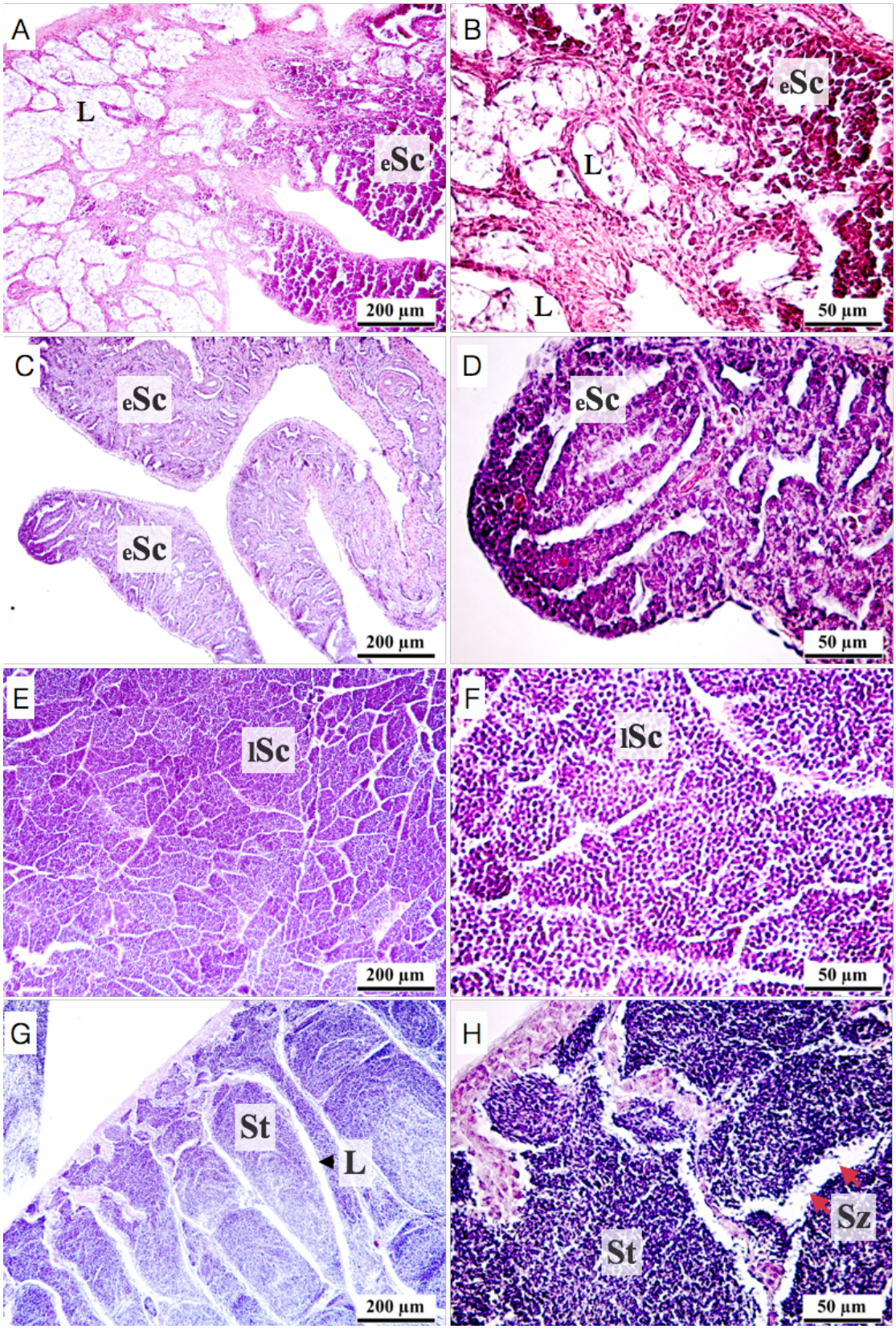

Figure A.2 Photomicrographs of testicular development in polar cod: (A) Spent portion of testis next testis with empty lobules (L) and early stage spermatocytes (eSc); (B) Higher magnification of spent and early stage spermatocytes in resting male; (C) Early maturing testis full of early spermatocytes; (D) Higher magnification of early maturing testis with spermatocytes (eSc); (E) Maturing males with late spermatocytes (lSc); (F) Higher

magnification of maturing males with late spermatocytes (lSc); (G) Spawning testis with spermatid (St); (H) Spawning testis at higher magnification with mature spermatozoa (Sz) in sperm ducts.

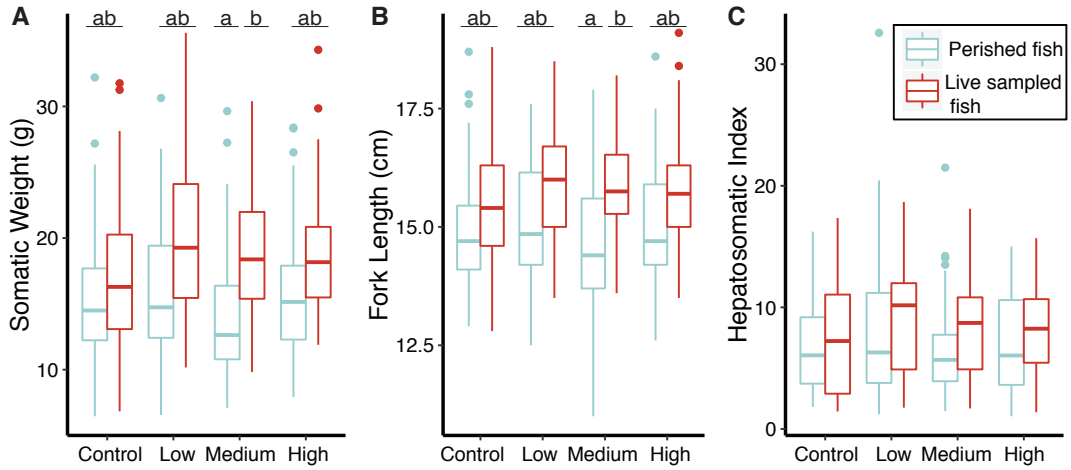

Figure A.3 Plots comparing (A) somatic weight; (B) fork length; (C) hepatosomatic index of the polar cod that perished during the experimental period and polar cod that were sampled out during the crude oil exposure period from 30th of June to 3rd of February. Boxplots represent the median (horizontal line), 1st and 3rd quartile (box), non-outlier range (whisker), outlier (points) of the data. Low, medium and high crude oil exposure treatments and control groups are spread along the x-axis. Significance between the perished and sampled fish in each treatment groups is signified by the lowercase letters above the distinguished group ($p<0.05$).

Table A1. Summary of cross reactivity for the steroids Estradiol 17 B (E_2), Testosterone (T) and 11-ketotestosterone (11KT) as given by Frantzen et al. (2004) and 11KT cross reactivity is given by Johnsen et al. (2013).

Compound	Cross reactivity (%)		
	E_2-AS	T-AS	11KT-AS
1,3,5(10)-estratriene-3,17β-diol (estradiol-17β; E_2)	100	<0.1	<0.1
3-hydroxy-1,3,5(10)-estratrien-17-one (estrone)	5.6		
1,3,5(10)-estratriene-3,16α,17 β-diol (estriol)	1.2		
17β-hydroxy-4-androsten-3-one (testosterone; T)	0.65	100	3.1
11β-17,21-trihydroxy-4-pregnene-3,20-dione (cortisol)	0.37	<0.1	<0.1
4-androstene-3,17-dione (androstenedione)	<0.1	7	<0.1
4-androstene-3,11,17-trione (adrenosterone)	<0.1	<0.5	0.9
17β-hydroxy-4-androstene-3, 11-dione (11-ketotestosterone; 11KT)	<0.1	6.6	100
11β,17β-dihydroxy-4-androsten-3-one (11β-hydroxytestosterone)	<0.1	5.8	2.3
17β-hydroxy-5α -androstan-3-one (5α-dihydrotestosterone)	<0.1	60.1	1.1
17β-hydroxy-5β -androstan-3-one (5β-dihydrotestosterone)		11.6	0.2
17α,20β-dihydroxy-4-pregnen-3-one (17,20β -P)	<0.1	<0.1	<0.1
5α-androstane-3β,17β -diol (17β-dihydroandrosterone)	<0.1	13.8	<0.1
17-hydroxy-4-pregnene-3,20-dione (17-hydroxyprogesterone)	<0.1	<0.1	<0.1
4-pregnene-3,20-dione (progesterone)	<0.1		<0.1
17,21-dihydroxy-4-pregnene-3,20-dione (cortisone)	<0.1	<0.1	
11β-hydroxy-4-androstene-3,17-dione (11β-hydroxyandrostenedione)		<0.5	<0.1
3β-hydroxy-5-pregnen-20-one (pregnenolone)			<0.1

Table A2. Morphometric summary of polar cod sampled over the course of 31 weeks of exposure to different crude oil doses (0.11, 0.57 and 1.14 µg crude oil/g fish/day) and controls. The number of female and male fish sampled over the five sampling events from week 0 in June to week 31 in February in each treatment is further divided by percentage of fish maturing in each treatment group as determined by histology. Somatic weight, fork length and hepatosomatic index (HSI) for mature fish pool was calculated. All values are mean ± SD.

			Fish Sampled				Maturing fish morphometrics		
Week	Date	Treatment	♀	♂	Total	% Maturing	Somatic wt (g)	Fork length (cm)	HSI (%)
0	June	Control	4	12	16		14.1 ± 2.6	15.1 ± 0.9	2.5 ± 0.7
6	August	Control	3	13	16		13.1 ± 5.9	14.5 ± 0.9	3.4 ± 1.1
		Low	3	13	16		14.7 ± 3.2	15. 1 ± 0.8	3.6 ± 1.2
		Medium	2	14	16		16.1 ± 3.1	15.5 ± 0.8	3.7 ± 1.5
		High	4	12	16		15.9 ± 3.7	15.3 ± 0.9	4.2 ± 1.6
17	October	Control	7	9	16	94%	19.6 ± 4.5	15.7 ± 1.0	9.5 ± 2.3
		Low	4	12	16	100%	20.0 ± 5.7	15.8 ± 1.2	9.1 ± 2.5
		Medium	5	11	16	100%	19.6 ± 5.0	16.1 ± 1.3	8.7 ± 1.2
		High	6	10	16	94%	19.0 ± 3.4	15.7 ± 0.8	7.3 ± 1.7
22	December	Control	6	10	16	69%	21.2 ± 4.5	16.2 ± 1.0	13.1 ± 2.0
		Low	5	11	16	88%	23.0 ± 4.5	16.2 ± 0.9	12.1 ± 1.9
		Medium	4	12	16	88%	20.3 ± 4.8	16.1 ± 1.0	10.9 ± 3.8
		High	1	15	16	81%	19.6 ± 2.9	15.7 ± 0.7	11.5 ± 1.8
31	February	Control	4	8	12	83%	23.8 ± 4.8	17.0 ± 1.0	11.8 ± 2.8
		Low	5	7	12	92%	23.2 ± 5.4	16.7 ± 0.9	13.0 ± 3.0
		Medium	1	7	8	88%	21.1 ± 3.8	16.3 ± 1.0	11.8 ± 2.9
		High	4	5	9	78%	25.8 ± 7.0	17.6 ± 1.4	12.3 ± 3.0

Paper 2

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Growth and metabolism of adult polar cod (*Boreogadus saida*) in response to dietary crude oil

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ABSTRACT

The increasing human presence in the Arctic shelf seas, with the expansion of oil and gas industries and maritime shipping, poses a risk for Arctic marine organisms such as the key species polar cod (*Boreogadus saida*). The impact of dietary crude oil on growth and metabolism of polar cod was investigated in the early spring (March–April) when individuals are expected to be in a vulnerable physiological state with poor energy stores. Adult polar cod were exposed dietarily to three doses of Kobbe crude oil during an eight weeks period and followed by two weeks of depuration. Significant dose-responses in exposure biomarkers (hepatic ethoxyresorufine-O-deethylase [EROD] activity and 1-OH phenanthrene metabolites in bile) indicated that polycyclic aromatic hydrocarbons (PAHs) were bioavailable. Condition indices (i.e. Fulton's condition factor, hepatosomatic index), growth, whole body respiration, and total lipid content in the liver were monitored over the course of the experiment. The majority of females were immature, while a few had spawned during the season and showed low hepatic lipid content during the experiment. In contrast, males were all, except for one immature individual, in a post-spawning stage and had larger hepatic energy stores than females. Most specimens, independent of sex, showed a loss in weight, that was exacerbated by exposure to crude oil and low hepatic liver lipids. Furthermore, females exposed to crude oil showed a significant elevation of oxygen consumption compared to controls, although not dose-dependent. This study highlights the importance of the energy status of individuals for their response to a crude oil exposure.

1. Introduction

Climate variability and global warming have changed and will continue to change the Arctic, most notably seen in the abrupt decline in Arctic sea ice extent and thickness (Barber et al., 2015). In parallel with these changes, anthropogenic activities including oil and gas exploration, maritime shipping, and tourism are all predicted to increase (Smith and Stephenson, 2013), posing a risk to arctic marine organisms.

The sensitivity of polar cod (*Boreogadus saida*), a key fish species in the Arctic marine ecosystem (reviewed by Mueter et al., 2016), to petroleum related compounds, has been investigated intensively in the past two decades (Christiansen and George, 1995, Nahrgang et al., 2010a, 2010b, 2010c, Geraudie et al., 2014, Andersen et al., 2015, 2015b, Bender et al., 2016, Vieweg et al., 2018, Bender et al., 2018). Early life stages have shown a high sensitivity to very low levels of a crude oil water-soluble fraction (WSF) (Nahrgang et al., 2016), while adult specimens are considered more robust when exposed to low environmentally relevant concentrations of dietary crude oil (Bender et al., 2016; Vieweg et al., 2018). Thus far, only few studies have investigated the effects of crude oil on energy homeostasis and associated physiological processes in polar cod (Christiansen et al., 2010; Vieweg et al., 2018, Bender et al., 2018). Crude oil and related contaminants, such as polycyclic aromatic hydrocarbons (PAHs) have been shown to affect growth (e.g. Gravato and Guilhermino, 2009; Kerambrun et al., 2012; Claireaux et al., 2013; Sandrini-Neto et al., 2016) and metabolism (Davoodi and Claireaux, 2007; Christiansen et al., 2010; Klinger et al., 2015) in fish. The mechanisms behind these effects can be multiple, including increased energy costs from detoxification metabolism

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and toxicity (Klinger et al., 2015), behavioral changes leading to reduced nutrient assimilation (Moles and Rice, 1983; Christiansen and George, 1995), and toxicant induced alterations in nutrient assimilation (Saborido-Rey et al., 2007). Adult polar cod have previously been shown to exhibit altered growth performance when exposed to dietary crude oil (Christiansen and George, 1995) and a depression in routine metabolism following an exposure to the WSF of crude oil (Christiansen et al., 2010).

The Arctic is characterized by a strong seasonality in light availability, profoundly affecting biological activity and basic physiological processes in arctic marine ecosystems (Berge et al., 2015). The vast majority of experimental studies on adult polar cod have dealt with specimens during the late summer/fall concurrent with gonadal maturation (Hop et al., 1995, Hop and Graham, 1995, Christiansen and George, 1995, Nahrgang et al., 2010b, 2010c, Christiansen et al., 2010, Bender et al., 2018). Therefore a marked need exists to determine the physiological trade-offs and sensitivity to contaminant exposure during the late winter/spring season, which also coincides to a post-spawning stage for mature individuals. Polar cod invest important amounts of energy into reproduction (Hop et al., 1995) and may be highly susceptible to post-spawning mortality. Exposure to crude oil related compounds may have consequences for post-spawning survival and be directly relevant to population level effects. The present study aimed therefore at investigating the effects of dietary crude oil exposure on growth, lipid class composition, and routine metabolic rate in adult specimens in the early spring. At this time, energy levels in polar cod are expected to be significantly reduced after reproduction and following a period of low food availability during the dark winter months. The primary hypothesis of the present study was that the exposure to crude oil might lead to a reallocation of energy from somatic growth towards detoxification, and lead to an increase in oxygen consumption. The dietary route of exposure was chosen as it allows for accurate control of the dose of contaminant given to the test organism. Furthermore, although it is in general less studied for petroleum products, it may constitute an important pathway for long-term toxicity (Agersted et al., 2018).

2. Materials and methods

2.1. Sampling and acclimation period

Polar cod were caught in Rijpfjorden (Svalbard) with a Campelen bottom trawl attached to a fish-lift (Holst and McDonald 2000), onboard R/V *Helmer Hanssen* and were transferred to the experimental facilities at the University Centre in Svalbard (Norway) in mid January 2012. Upon arrival, polar cod were kept in acclimation until early March in two 700L tanks under running 25 µm filtered seawater and constant darkness. Temperature loggers (HOBO onset) recorded continuously (19/01/2012–25/05-2012) air temperature (4.1 ± 0.2 °C), water temperature (6.9 ± 1.0 °C), and salinity (28.6 ± 1.3 psµ) in the acclimation tanks. During acclimation, polar cod were fed every 3 days with aquaculture feed AgloNorse TROFI AS, Tromsø, Norway (protein 59%; fat 18–20%; ash 10%; fibres 1%; moisture 8–9%; PUFA n-3 2.4%; PUFA n-6 2.6%). During acclimation, feeding was done by giving food in excess to the tanks. One week before exposure start, fishes (n = 36 per treatment) were randomly transferred to exposure tanks (200L) placed in the same room as the acclimation tanks and containing 5 µm filtrated seawater. During transfer, each fish was anesthetized with metacain (1 mg/L seawater), tagged (Floy Fish Dangler Tags), and total length and body weight were recorded to the nearest 0.1 mm and 0.1 g.

2.2. Preparation of the food

The treatments consisted of aquaculture feed pellets hydrated with 0.77 g water per g dry pellets, and blended with 0.1, 1, and 5 mg Kobbe crude oil per gram food wet weight, for the low, medium, and high treatments, respectively. For the control group, the feed pellets were hydrated but crude oil was not added. Individually tagged syringes were prepared in advance with food mixture corresponding to 4% body wet weight of each specific fish and stored at −80 °C. The choice of the crude oil doses was selected based on literature review of similar experiments that employed dietary crude oil exposure or PAH mixture exposure and that showed alterations at physiological levels. In particular the study by Christiansen and George (1995) showed alterations in growth performances. Our levels correspond to a range one order of magnitude lower to one order of magnitude higher than those used in Christiansen and George (1995).

2.3. Experimental design

The experiment started in March and consisted of four treatments (4 tanks, n = 34 per tank) with fish exposed once a week to crude oil contaminated feed (control, low, medium, and high doses) during eight weeks and followed by two weeks (one feeding) of recovery. During the recovery, all specimens received the same uncontaminated feed as that of the control group during the exposure period. Once a week, on the day of feeding, a batch of syringes were thawed and fish were force fed 4% body weight using 1 ml Luer-lokk syringes (BD Plastipak™). The feeding took maximum 20 s per fish. Force feeding was chosen to control crude oil dose and avoid confounding effects of differential feeding behavior on growth response (Christiansen and George, 1995; Saborido-Rey et al., 2007). Upon force-feeding, fish were transferred to new tanks containing fresh seawater (5 µm filtered) that had been equilibrating to room temperature during 24 h. In addition, 80% of the water of the experimental tanks was changed every second day. Water temperature (5.9 ± 0.7 °C) and pH (7.9 ± 0.1) in the semi-static experimental tanks were monitored daily over the course of the experiment using a handheld WTW multimeter.

Polar cod were sampled at exposure start (holding tanks, n = 14), and after one and after eight weeks of exposure (experimental tanks, n = 12 per treatment per timepoint). A final sampling point for recovery consisted in 10 additional specimens sampled per treatment (ten weeks). Total length, total weight (TW), gonad weight (GW) and liver weight (LW) were recorded. The liver samples were snap frozen in liquid nitrogen, and stored at −80 °C for further analyses. At the start (week zero) and end (week eight) of the exposure period, a portion of gonad tissue was fixed in 4% neutral buffered formaldehyde for histological analysis. Otoliths were collected for age analysis.

Hepato- and gonadosomatic indices (HSI and GSI, respectively) were determined using the following equations:

$$GSI\,(\%) = \frac{GW}{(\mathrm{TW} - (\mathrm{GW} + \mathrm{LW}))} \times 100$$

$$HSI\,(\%) = \frac{LW}{(\mathrm{TW} - (\mathrm{GW} + \mathrm{LW}))} \times 100$$

The specific growth rate (SGR, % per d) was based on records of initial (i, at tagging) and final (f, at sampling) TW records, using the following equation:

$$SGR\ (\%\ per\ d) = \frac{(\ln \mathrm{TW_f} - \ln \mathrm{TW_i})}{\text{Time in days}} \times 100$$

2.4. Respirometry

Whole body respiration was measured on polar cod after two, four, six, eight, and ten weeks (n = 8 per treatments) using an automated intermittent flow through respirometer equipped with eight chambers (volume of 573 ml) (Loligo® Systems, Denmark). The oxygen consumption was measured using a polymer optical fiber dipping probe. Measurement were always performed on the day prior to feeding, i.e. six days after the previous feeding, in order to limit the effects of

specific dynamic action (SDA). The chambers were placed in individual tanks containing filtered seawater equilibrated to room temperature (5.9 ± 0.7 °C). When possible, the same individuals, identified by tags, were used each time; however, due to some mortality, different fish were used at the end of the experiment. Fish were weighed and placed in individual chambers. The automated respiration consisted in 7-min cycles of closed respirometry and flushing. Prior to the experiment, eight fish from the holding tank were placed in the chambers and oxygen consumption was recorded during 24 h to evaluate the time necessary to reach the routine metabolism (Fig. S1). The oxygen consumption decreased typically exponentially over the course of the first 3 h and the average oxygen consumption between 2.5 h and 3 h was used for the data analysis for the experimental fish.

2.5. Age estimation

Polar cod age (years) was based on otolith readings: for small transparent otoliths, white winter rings were counted in sub-surface light with a Leica M205 C stereo microscope and a Planapo 1.0 × objective lens (Gjøsæter and Ajiad, 1994); for all larger otoliths, cross sectioning with a scalpel blade and counting the rings under polarised light was necessary.

2.6. EROD activity

Liver samples were homogenized in a phosphate buffer (0.1M, pH 7.4) using a precellys bead-beater and centrifuged 9000 g during 30 min (S9 fraction). EROD activity was measured according to Eggens and Galgani (1992). The reaction mix consisted of 10 µl microsomal fraction in 100 mM of Tris-phosphate buffer (pH 7.4), ethoxyresorufin 46 µM as substrate in a final volume of 230 µl. Reaction started by adding 0.25 mM NADPH in the microwells. The resorufin production was measured in four replicates during 20 min at room temperature with a Biosynergy H1 plate reader at 544/584 nm excitation/emission wavelengths, respectively. A resorufin standard curve (0–2 µM) was used for determination of the reaction rates in pmol of resorufin produced $min^{-1}mg^{-1}$ of total protein (S9 fraction).

2.7. Histology

The fixed gonad samples were routinely processed by dehydration and embedded in paraffin wax in a Shandon Citadel 1000 (Micron AS, Moss, Norway). Embedded tissues were sectioned at 5 µm thickness in a Leitz RM 2255 microtome, stained with hematoxylin/eosin, and examined under a Leica Wild M10 dissecting scope with a Leica DFC295 camera for maturity status and indications of previous spawning. For each fish, six replicate slices were prepared and viewed under 40 × and 80 × magnification. Characterization of the gonadal development was based on Brown-Peterson et al. (2011) with 5 categories (immature, developing, spawning capable, regressing, regenerating) for females (N = 27), and for males (N = 34). The presence of late vitellogenic (Vtg3) atretic residual oocytes was interpreted as evidence that specimens had spawned in the present season.

2.8. Lipid analysis

Lipids composition was analyzed on liver of 10 individuals per treatment after eight weeks of exposure. The lipids of the liver samples were extracted and the different lipid classes were separated by Solid Phase Extraction (SPE) prior to analysis of fatty acids by gas chromatography. The total lipids of liver samples were extracted by a modified Folch method with chloroform/methanol (2:1 v/v) (Folch et al., 1957).

The lipid extract was separated into major lipid classes by a SPE procedure adapted from the Kaluzny et al. (1985), using aminopropyl bonded phase columns to separate lipid mixtures into individual classes. Briefly, 0.5 ml of lipid extract (approximately 8 mg lipid) was loaded in a 500 mg aminopropyl modified silica minicolumn (Macherey-nagel gmbh & co. Germany), which had been previously activated with 4 ml of hexane. Neutral lipid (Triacylglycerol's and cholesterol, NL), free fatty acid (FFA), and phosphatidylcholine/phosphatidylethanolamine (PC/PE) were sequentially eluted with 7 ml of chloroform/isopropanol (2:1 v/v), 5 ml of 2% acetic acid in diethyl ether, and 10 ml of methanol. The eluates were collected in 15 ml thick-walled glass tubes with Teflon lined screw caps, which contained nonadecanoic acid (19:0) as internal standard. The phosphatidylserine/phosphatidylinositol (PS/PI) fraction was obtained by opening the column and collecting all of the stationary phase directly to the test tubes. All the eluates were dried by nitrogen gas and the fatty acids were analyzed by gas chromatography with a flame ionization detector (GC-FID). Prior to analysis on GC-FID, all SPE fractions from the liver samples were methylated with 2.5 M dry HCl in methanol (HPLC-grade, Merck in Oslo, Norway) to obtain fatty acid methyl esters (FAME) that was analyzed on gas chromatograph according to Meier et al. (2006).

2.9. PAH analyses in feed

Analyses of PAHs in the fish feed were carried out by Akvaplan-niva (accredited for the methods). Three replicate feed samples per dose were analyzed. Each sample was thoroughly grounded and homogenized prior to analyses. Samples were weighed and a potassium hydroxide-methanol solution and an internal standard-mix of deuterated PAHs were added. The solution was boiled with reflux for 4 h (saponification), before filtration and extraction with pentane. Samples were purified using gel permeation chromatography (GPC), with dichloromethane as a mobile phase. Samples were filtrated and further purified by solid phase extraction (SPE). Analyses were performed using a GC-MSD (Agilent 7890 GC with split/splitless injector, Aglient 7683 and Agilent 5975C, mass spectrometer with EI ion source). Blind samples were run in parallel to all samples, and proficiency test samples (Quasimeme, Netherlands) were used as control samples. The limit of detection (LOD) was determined from analyses of a series of blank samples, processed along with real samples, and calculated as: LOD = (blank average) + 3 × (blank standard deviation). For the calculation of sum PAHs, values below detection limit were not considered.

2.10. Biliary 1-OH phenanthrene metabolite

1-OH phenanthrene was analyzed according to Nechev et al. (unpublished) on bile samples from the experimental fish collected at week zero, one and eight weeks of exposure. Briefly, 1-OH phenanthrene was extracted from bile samples through enzymatic hydrolysis. Bile samples were freeze dried overnight and 40 µl of water was added to each sample. Samples were incubated for 1 h at 37 °C with β-Glucuronidase/aryl sulfatase (5 µl) and an internal standard (5 µL triphenylamin in methanol, 160 ng/ml) was added. After incubation, 750 µl of methanol were added and centrifuged for 10 min at 13000 g and supernatants were collected. Extracts were analyzed using a HPLC Agilent 1200 Series equipped with a fluorescence detector FLD Agilent 1200 Series G1321A. Separation of the compounds was performed in a C18 column (Eclipse XDB-C18, 150 × 4.6 mm; 5 µm particle size; Agilent, USA) heated to 35 °C. The injected volume was 25 µl. The initial composition of the mobile phase was 40:60 acetonitrile:water (vv) and a linear gradient to 100% acetonitrile was programmed in 30 min, with a final hold of 5 min. Initial conditions were reached in 1 min and maintained for 2 min before the next run. The total run time was 38 min with a flow rate of 1 ml/min 1-OH phenanthrene was detected at its optimal excitation/emission wavelength pair 256/378 nm (1-OH-phenanthrene). Samples of bile in the medium group at eight weeks exposure were lost during extraction and are thus not analyzed.

2.11. Statistical analyses

All statistical analyses were conducted with R 3.1.1 (R Core Team 2014). As our data fell outside a normal distribution, non-parametric Kruskal-Wallis tests by ranks were employed to investigate differences between the sexes and crude oil treatments at each time point on the continuous factors of age, morphometrics, EROD activity, PAH bile metabolite concentrations, SGR, and oxygen consumption. Length and sex were tested as covariates of response variables to account for initial difference between treatment groups at tagging and inherent differences in physiology between sexes. When significant results were encountered, a post hoc pairwise Dunn's test using rank sums was performed between the control and crude oil treatments. These tests were done in conjunction with a Benjamini-Hochberg adjustment on p-values to account for potential errors arising from multiple comparisons (Benjamini and Hochberg, 1995). Correlation tests were performed using the Spearman method for PAH levels, HSI, SGR, and liver lipid levels. Linear models were used to explore the relationship between SGR and HSI in relation to crude oil treatment, sex, and length for fish after one week of exposure. Comparisons were considered significantly different than the control when $p \leq 0.05$ level. Values are reported as mean ± standard deviation (SD).

3. Results

3.1. Dietary doses of PAHs, levels of 1-OH phenanthrene in the bile and EROD activity

Polar cod from the low, medium, and high treatment were exposed weekly to 4, 40, and 200 µg crude oil/g fish, respectively (Table 1). This weekly dietary dose corresponded to a sum 26 PAHs (Σ26PAHs) in the feed of 0.004, 0.06, 0.4, and 2.4 µg 26 PAHs/g fish/week in the control, low, medium, and high treatments, respectively. The levels of PAHs measured in the feed were significantly correlated to the nominal crude oil doses ($R^2 = 0.97$, $p < 0.001$), indicating that the crude oil was homogenously mixed in the feed. Typical for crude oil, the most abundant PAHs in the feed were the low molecular weight naphthalenes with predominance of substituted compounds in the order C3 > C2 > C1, followed by substituted phenanthrenes (Table 2). All 26 PAHs analyzed in the feed were above detection limit in the highest exposed feed, except for indeno(1,2,3cd)pyrene (Table 2).

Levels of 1-OH-phenanthrene in polar cod bile (Fig. 1A), and EROD activity (Fig. 1B) increased in a dose-dependent manner after one week of exposure and remained at similar levels after eight weeks of exposure regardless of fish sex or length.

3.2. Fish morphometrics, gonadal maturation stages and mortality during the exposure

Although polar cod were collected and randomly distributed to the experimental tanks, at tagging the average fork length and total weight from the fish in the "low" group were significantly higher compared to medium and control groups ($p = 0.03$ and 0.05 for length and weight, respectively) (Table S1). At subsequent sampling times, however, the fork length and total weight were no longer significantly different among treatments and sexes. Overall, the sex ratio was biased towards males in the medium and high treatments, unfortunately affecting the sex balance in the high treatment at eight weeks exposure (n = 1 female). Sex and length were included as covariate in response analysis to account for potential bias by the initial conditions. Specimens were between two and four years old.

The sexual maturity status of polar cod varied between gender and sampling time, but not with oil treatment. Female specimens showed low GSI (1–3%) throughout the experiment, and the majority (n = 19 out of 27 analyzed) were immature, i.e. they had never spawned (Fig. 2 and Table S2). Specimens categorized as "regressing" (n = 6) showed late vitellogenic (Vtg3) atretic residual oocytes that suggested spawning during the season. Two of these regressing females showed residual oocytes at more advanced atretic stages, a potential indication of spawning that occurred during the previous season. Thus, these two specimens may have belonged to the regenerating category. Finally, two females were in a regenerating stage with late-stage atretic residual oocytes (i.e. had skipped the present reproductive cycle). Regressing females were not significantly different in length or total weight compared to immature or regenerating females, however and although not significant, they showed a slightly reduced HSI ($p = 0.15$) and reduced lipid concentration in the liver ($p = 0.09$, Table S2).

Males showed significantly higher GSI (11–17%) than females at week zero ($p = 0.01$) and week one ($p < 0.001$) and compared to GSI in males sampled after eight and ten weeks ($p < 0.01$). Histological analysis at week zero indicated specimens in an early post-spawning stage (regressing) at exposure start (Fig. 2) with discontinuous germinal epithelium throughout the testis, no active spermatogenesis, and residual spermatozoa in lobule lumens and sperm ducts. After eight weeks of exposure, the GSI of males had decreased to levels similar to that of females (below 2%, Table S2). Except for one immature specimen in the medium treatment, all males were in a late regressing or regenerating stage (Fig. 2).

During the experiment, some mortality occurred in the low (n = 2 at three weeks) and high (n = 1 after three days, and n = 4 at three weeks) treatments. Furthermore, one individual was removed from the high treatment in the sixth week due to the appearance of finrot (disintegration of caudal fin). Mortality occurred only in males. Except for two specimens of the high treatment (death at three weeks), all mortalities occurred among the specimens used in respirometry experiments, although several days after the respirometry handling. The number of polar cod left for the last sampling time (respiration individuals at ten weeks) was reduced to eight, nine and five in the low, medium, and high treatments, respectively.

3.3. Lipid class composition in the liver

Total liver lipid content and lipid class composition was studied on ten of the twelve specimens sampled from each treatment after 8 weeks of exposure to dietary crude oil. The total lipid content of the liver was correlated to the HSI ($R^2 = 0.62$, $p < 0.001$). Furthermore, males had significantly higher liver lipid content (329 ± 17 mg/g liver wwt) than females (212 ± 32 mg/g liver wwt, $p = 0.002$, Table 3) with little

Table 1
Crude oil nominal concentrations, and measured concentrations of sum of 26 PAHs in the feed (µg/g feed wwt) and as weekly doses in the fish (µg/g fish/week or µg/fish/week). Data represent mean ± standard deviation.

Treatments	Crude oil nominal doses		Sum 26 PAHs		
	Feed mg/g wwt	Fish dose µg/g fish/week	Measured concentration in feed, µg/g wwt	Fish dose µg/g fish/week	Fish dose µg/fish/week
Control	0	0	0.08 ± 0.01	0.003	0.05 ± 0.01
Low	0.1	4	1.6 ± 0.04	0.06	1.1 ± 0.4
Medium	1	40	11.7 ± 0.5	0.5	7.0 ± 2.1
High	5	200	64.4 ± 3.8	2.6	41.4 ± 8.0

Table 2
Concentration of 26 PAHs (µg/kg wet weight) and their sum (µg/g wwt) in the diet fed polar cod (*Boreogadus saida*) in the control, low (0.1 mg crude oil/g feed), medium (1 mg crude oil/g feed) and high (5 mg crude oil/g feed) treatments. For the determination of the sum PAHs, values below the limit of detection (LOD) were not considered. Data represent mean ± standard deviation.

	Control	Low	Medium	High
Naphthalene	< 5.8	85.7 ± 4.0	674.2 ± 27.5	3673.3 ± 163.8
C1-Naphthalene	< 10	208.2 ± 12.1	1684.5 ± 92.2	9325.3 ± 723.3
C2-Naphthalene	< 13	295.6 ± 11.7	2428.9 ± 107.4	13367.7 ± 634.0
C3-Naphthalene	78.4 ± 13.7	701.9 ± 20.7	4819.5 ± 214.9	26505.0 ± 1650.4
Acenaphthylene	0.8 ± 0.1	0.7 ± 0.01	1.3 ± 0.2	4.2 ± 0.5
Acenaphthene	< 1.1	3.1 ± 0.4	23.8 ± 1.7	135.6 ± 11.5
Fluorene	< 0.8	9.0 ± 1.0	67.5 ± 1.9	369.2 ± 31.1
Dibenzothiophene	< 0.5	3.6 ± 0.2	27.1 ± 1.1	151.4 ± 2.5
C1-Dibenzothiophene	< 1.5	8.6 ± 0.2	61.8 ± 5.7	361.0 ± 35.9
C2-dibenzothiophene	< 4.5	16.8 ± 0.6	125.6 ± 6.6	656.0 ± 39.3
C3-dibenzothiophene	< 4.1	17.5 ± 0.6	136.8 ± 5.6	716.2 ± 30.7
Antracene	< 0.3	0.4 ± 0.1	0.5 ± 0.1	2.9 ± 0.3
Phenanthrene	< 3.0	21.6 ± 0.5	183.1 ± 5.5	996.5 ± 28.5
C1-Anthr/Phenanthrene	< 8.6	45.5 ± 1.6	343.9 ± 25.6	1959.6 ± 85.7
C2-Anthr/Phenanthrene	< 7.4	73.4 ± 2.1	602.4 ± 19.2	3305.6 ± 178.8
C3-Anthr/Phenanthrene	< 4.7	62.1 ± 7.0	473.3 ± 26.8	2692.7 ± 282.2
FLuoranthene	< 2.0	< 2.0	3.2 ± 0.5	17.6 ± 9.1
Pyrene	< 3.3	< 3.3	6.7 ± 3.1	34.5 ± 9.5
Benzo(a)anthracene	< 0.5	< 0.5	2.3 ± 0.3	16.5 ± 5.2
Chrysene	< 0.6	0.8 ± 0.02	6.9 ± 0.2	44.1 ± 3.3
Benzo(b)fluoranthene	< 0.7	< 0.7	1.2 ± 0.4	10.4 ± 9.7
Benzo(k)fluoranthene	< 0.2	< 0.2	< 0.2	3.7 ± 3.1
Benzo(a)pyrene	< 0.3	< 0.3	0.6 ± 0.2	6.8 ± 6.1
Indeno(1,2,3-cd)pyrene	< 0.7	< 0.7	< 0.7	< 0.7
Benzo(ghi)perylene	< 0.6	< 0.6	< 0.6	5.7 ± 4.8
Dibenzo(a,h)anthracene	< 0.26	< 0.26	< 0.26	1.60 ± 1.5
SUM 26 PAHs, µg/g	0.08 ± 0.01	1.6 ± 0.04	11.7 ± 0.5	64.4 ± 3.8

variation correlated with length ($p = 0.52$). For both sexes, the neutral lipids (NL) accounted usually for more than 90% of the lipid classes. The NL were totally dominated by storage lipids, triacylglycerols. The polar membrane lipids contributed with less than 7% (PC/PE [3–5%], PS/PI [2%]) of the total lipids, and FFA with 1.5% of the total lipids. In females, there was generally a high variability in liver lipid content, with some specimens (n = 3) showing extreme low values (41–61 mg/g liver wwt). In general, females in a regressing stage showed lower lipid content than specimens in an immature or regenerating stage (Table S2). Unfortunately, two specimens with extreme low values were represented in the control group, which resulted in lower average lipid levels in the control group (average of 120 ± 40 mg/g liver wet weight) compared to the other groups (> 243 ± 54 mg/g liver wet weight), and thus erroneously suggesting an increasing trend in lipid content with crude oil dose. This also led to differences in lipid classes distribution (e.g. average of 70% NL) in the control group compared to the other treatments (> 90% NL). In males, there were no significant differences between treatments.

3.4. Specific growth rates (SGR)

Males and female polar cod showed no significant differences in

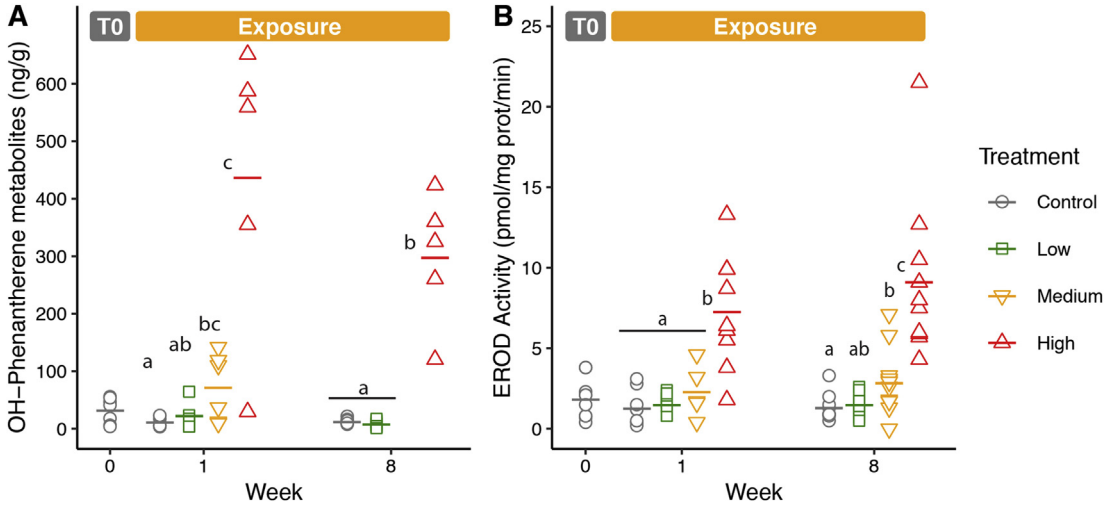

Fig. 1. (A) 1-OH phenanthrene metabolites (ng/g bile dw) in polar cod bile, and (B) EROD activity (pmol/min/mg protein) in polar cod liver, at exposure start (zero weeks), and after one and eight weeks of exposure. Bile metabolite samples in the medium group at eight weeks were not available (NA). Plots show individual data points distinguished by shape and color for each treatment group, treatment group means are represented with a dash (−). Different letters (a, b, c) indicate significant differences (Kruskal Wallis test, $p < 0.01$) among treatments for each time point. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

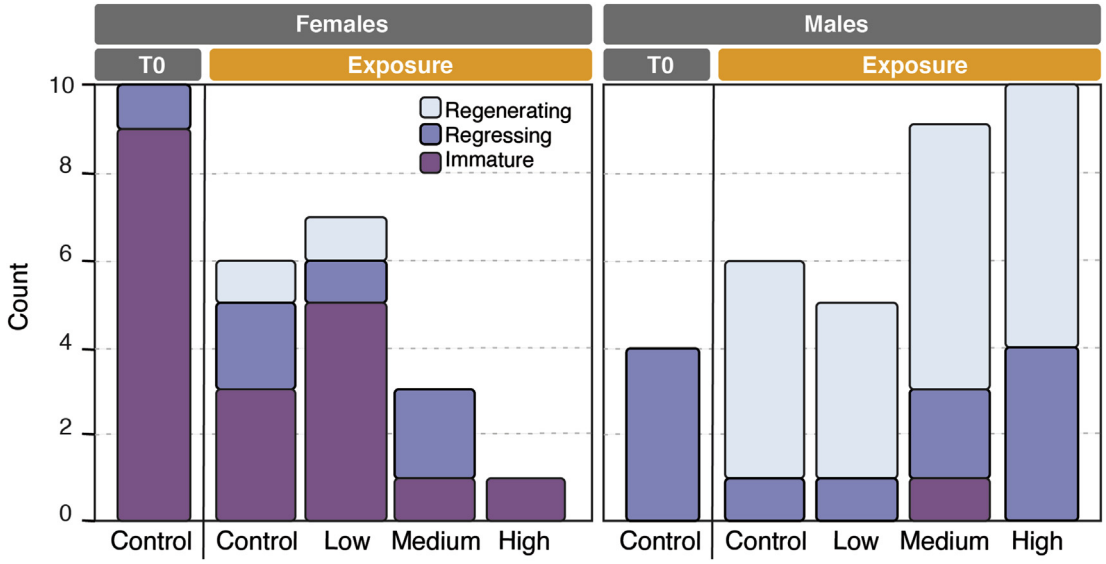

Fig. 2. Sexual maturity of polar cod at exposure start (T0) and after eight weeks of exposure, based on histological examination of gonads. Only three maturity stages were identified (immature, regressing, and regenerating). Bars are representing counts.

SGR over the course of the experiment and with regard to dose. Following one week of exposure, also corresponding to the first dietary dose, a significant decrease in SGR was observed with increasing oil exposure (Kruskal-Wallis, p = 0.03). Interestingly, SGR was lowest in individuals that presented a reduced HSI (Fig. 3). This relationship increased in strength with increasing dose (R^2 = 0.2, p = 0.23 in the low treatment and increased to R^2 = 0.63, p < 0.001 in the high treatment). The negative interaction between crude oil treatment and HSI on SGR was strongest in the high oil treatment group (p = 0.017) regardless of fish sex (p = 0.81) or length (p = 0.38).

After eight weeks of exposure, the SGR was no longer dose-dependent (Fig. 4). Specimens sampled after ten weeks i.e. eight weeks of exposure and two weeks of recovery, showed a negative SGR, and females also exhibited a tendency to a dose-dependent reduction in SGR, although not significant (p = 0.44). These specimens were also those used for respirometry every second week, thus subjected to additional handling stress over the course of the experiment.

3.5. Whole body oxygen consumption

Oxygen consumption was, in general, elevated in oil-exposed females (min-max range 72.5–202.3 mg O_2/kg fish/hr) compared to controls (min-max range 61.8–102.9 mg O_2/kg fish/hr) after four weeks of exposure (Fig. S2). This increased oxygen consumption was however not dose-dependent. In males, oxygen consumption was elevated in the medium group (min-max range 112.5–226.2 mg O_2/kg fish/hr) compared to the other treatments (min-max range 43.1–131.5 mg O_2/kg fish/hr). Although not significant, this group was characterized by the smallest average total weight, condition factor, and GSI.

4. Discussion

4.1. Uptake and bioavailability of dietary crude oil

The determination of 1-OH-phenantrene metabolites in the bile and EROD activity were used as biomarkers of exposure to PAHs. As indicated by the presence of 1-OH-phenantrene metabolites in the bile and the levels of EROD activity, metabolism of PAHs was already taking place following a single dose of crude oil (first week of exposure) in both females and males. Furthermore, the dietary exposure remained dose-dependent throughout the exposure period. Dietary oil compounds ingested weekly during eight weeks in the present study were

Table 3
Lipid content (mg/g liver wet weight) and lipid class distribution (% distribution of the fatty acids in the different lipid classes) in polar cod liver following eight weeks of exposure. Out of the four control group females, two had atretic vitellogenic oocytes and extreme low levels (< 61 mg/g liver wet weight) of liver lipids. Males and females showed significant differences in total lipid levels (Kruskal-Wallis test, p = 0.002). NL; neutral lipid, PC/PE; phosphatidylcholine/phosphatidylethanolamine, PS/PI; phosphatidylserine/phosphatidylinositol, FFA; free fatty acid.

	Control			Low			Medium			High		
Females	(n = 4)			(n = 6)			(n = 3)			(n = 1)		
Lipid (mg/g)	120	±	40	244	±	58	243	±	54	292		
Lipid class distribution (%)												
NL	70.2	±	17.0	91.6	±	2.5	92.1	±	2.7	93.6		
PC/PE	19.9	±	11.3	5.2	±	1.5	4.4	±	2.0	3.8		
PS/PI	7.1	±	4.6	2.1	±	0.8	1.9	±	0.5	0.9		
FFA	2.8	±	1.2	1.2	±	0.3	1.7	±	0.3	1.6		
Males	(n = 5)			(n = 4)			(n = 7)			(n = 9)		
Lipid (mg/g)	363	±	21	345	±	78	322	±	25	308	±	28
Lipid class distribution (%)												
NL	95.7	±	0.2	93.6	±	2.0	94.9	±	0.5	94.4	±	0.5
PC/PE	2.3	±	0.1	3.9	±	1.5	2.4	±	0.3	3.4	±	0.4
PS/PI	0.5	±	0.1	1.2	±	0.5	1.1	±	0.2	1.0	±	0.2
FFA	1.5	±	0.1	1.3	±	0.2	1.6	±	0.1	1.2	±	0.2

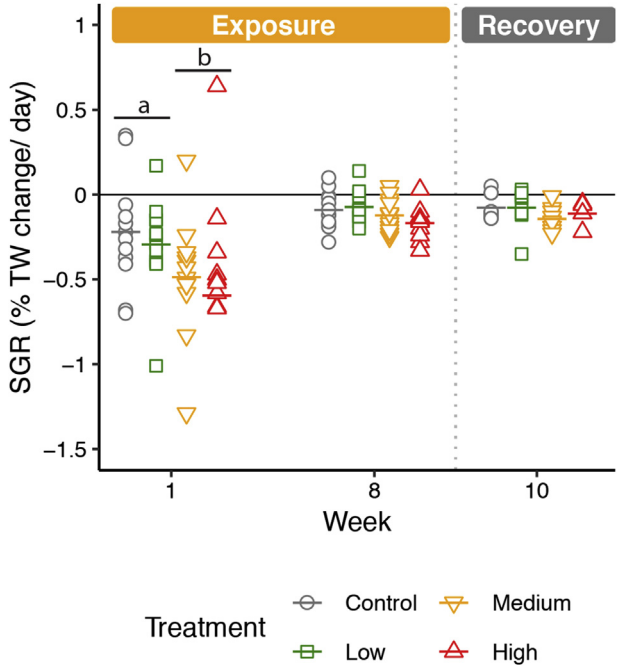

Fig. 3. The interaction of HSI (%) and treatment on the total weight specific growth rate (% TW per day) of mixed sex fish in the first week of exposure. Results from linear models and 95% confidence intervals are plotted for each treatment group with data points representing individual fish.

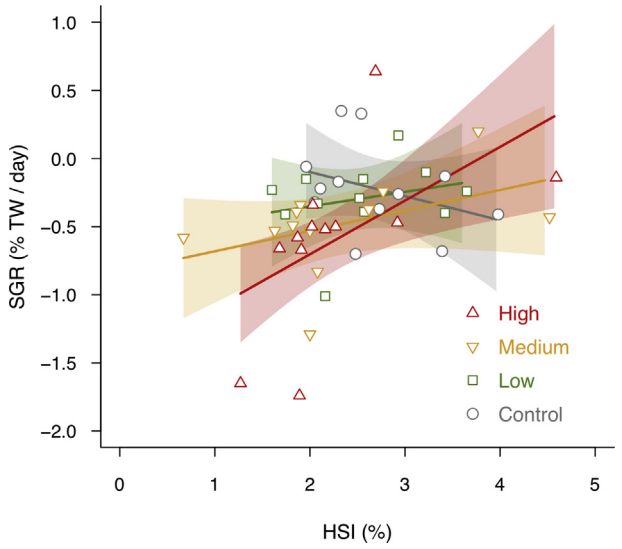

Fig. 4. Specific growth rate (SGR, % TW per day) of mixed females and males between the period from tagging to one, eight and ten weeks. Plots show individual data points distinguished by shape and color for each treatment group, treatment group means are represented with a dash (−). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

most likely bioavailable for the entire experimental period (ten weeks), including the last two weeks of depuration where polar cod were force-fed uncontaminated feed. For instance, Bakke et al. (2016) showed that a single dietary dose of phenanthrene and benzo(a)pyrene (0.40 ± 0.12 µg/g and 1.15 ± 0.36 µg/g fish for phenanthrene and benzo(a)pyrene, respectively) was retained in the tissues for at least 30 days, even for not covalently bound metabolites.

It is important to note that the responses observed in exposed polar cod of the present study cannot be solely attributed to the PAH fraction but rather a complex mixture of several thousands of unidentified petroleum compounds from the unresolved complex mixture (UCM). The UCM contains highly bioaccumulative and potentially toxic substances, and for which the toxicokinetics and toxicodynamics are largely unknown (Scarlett et al., 2007; Melbye et al., 2009; Petersen et al., 2017).

The crude oil doses used in the present study (4–200 µg crude oil/g fish/week) were in the same range as previous dietary studies on polar cod (George et al., 1995; Bender et al., 2016; Vieweg et al., 2018) that also showed the induction of hepatic EROD activity at their highest doses (George et al., 1995; Vieweg et al., 2018). In comparison with dietary studies on different fish species, our doses were similar to those of Bratberg et al. (2013) for cod (*Gadus morhua*) and were considered environmentally relevant. It is however important to highlight that the exposure method is not reflecting realistic environmental exposure, that would have required exposure of live feed to dispersed oil. The dietary crude oil taken-up by polar cod in this experiment was thus not represented by a realistic fraction composed of potential metabolites produced by living prey items. Furthermore, all compounds present in the whole crude oil were ingested by polar cod including fractions that may not have been bioavailable to the fish through exposure to live feed in the natural environment.

4.2. Baseline physiological status, SGR, and routine metabolic rate in control specimens

The study design suffered from an unexpected strong divergence in the physiological state of females and males that forced a sex-specific data analysis for certain parameters such as lipid composition. Accounting for these initial conditions by including physiological covariates such as sex and length allowed for a thorough investigation of possible effects of crude oil exposure on physiological endpoints. Even though some of the parameters did not show significant effects ($p > 0.05$), pronounced trends are discussed hereafter.

The majority of the female polar cod in the present study were immature and had thus never spawned before. By contrast, the histological analysis of the male gonads suggested that males had been spawning capable and most likely spawned earlier in the season, and advanced from a post-spawning (regressing) stage at the exposure start towards a resting (regenerating) stage at the end of the ten week experimental period concurrent with a significant decrease in GSI. This was further supported by the GSI at the exposure start that were lower than values known for ripe males in January (> 30% in e.g. Hop et al., 1995; Nahrgang et al., 2014), and in the known spawning timeframe (January–March) of polar cod populations of the Barents Sea (Hop and Gjøsæter, 2013). The disparity in maturity stage between sexes may be explained by males reaching sexual maturity at a younger age than females (Hop and Gjøsæter, 2013; Nahrgang et al., 2014). However, this hypothesis was not verified based on the otolith readings in the present study. In general, the very low HSI (75th percentile = 3.4% all treatments combined) indicated that the specimens were in a weak physiological state. No previous studies have shown such low HSI levels in this species for any season (Nahrgang et al. 2010a, 2014, Bender et al., 2016; Vieweg et al., 2018). The hepatic lipid levels in our post-spawning males were half those reported in males in the fall and early winter (Hop et al., 1995, 1997), suggesting an important allocation to reproduction. Females in the present study showed even lower levels of hepatic lipid content than males. In particular, the few females that were in a post-spawning stage had less than half the total lipid levels observed in males, and storage lipids (NL) represented as little as 20% of the total lipid class composition.

Specific growth rates were in general lower than rates reported in the same species elsewhere (Hop et al., 1997; Laurel et al., 2016, 2017). Although comparison to other studies may be difficult due to different factors (e.g. feed type, age, size range, temperature, and handling stress), SGR in polar cod fed to satiation have been shown to range between 0.5% and 1.5% wwt/day depending on size (Hop et al., 1995;

Laurel et al., 2016). In the present study, SGR levels were negative in most individuals. Our weekly feed rations (4% body wwt/week) were similar to maintenance levels reported by Hop et al. (1997). However, our study was conducted at higher temperatures than in Hop et al. (1997) (ca 6 °C instead of 0 °C), and our metabolic rates in control specimens (e.g. 92.0 ± 12.1 mg O_2/kg fish/hr for mean ± SE at ten weeks) were elevated compared to levels (51.03 ± 6.27 mg O_2/kg fish/hr) reported in Hop and Graham (1995). Given the elevated metabolic costs at increased temperatures, the rations given in the present study were insufficient to reach a positive growth in weight. The weight loss could be further rationalized by the particular weak physiological state of our specimens in early spring, as indicated by the reduced hepatic lipid levels. Finally, the weekly force-feeding most likely represented an additional handling stress that affected growth performance (e.g. McCormick et al., 1998; Barton, 2002; Jentoft et al., 2005). The specimens in the present study were therefore under sub-optimal conditions for growth including elevated temperatures, reduced feed ration, and low energy reserves.

4.3. Effect of crude oil on total wet weight alterations, and routine metabolic rate

The deleterious effect of crude oil or petroleum related compounds on fish growth has been shown previously in polar cod (Christiansen and George, 1995, Bender et al., 2018), as well as in other fish species (Al-Yakoob et al., 1996; Moles and Norcross, 1998; Kerambrun et al., 2012; Claireaux et al., 2013; Sandrini-Neto et al., 2016). In sexually developing polar cod, Christiansen and George (1995) found a reduction in weight gain when exposed to crude oil contaminated feed at levels (ca 2.1–2.6 µg crude oil/g fish/day) in the lower range of this study (0.6–28 µg crude oil/g fish/day). In the present study, there was a seemingly rapid (following the first dietary dose) and dose-dependent loss in weight, especially in individuals with an initial low condition (see section 4.2., and Fig. 3), suggesting an increased energy trade-off between somatic growth, and potential detoxification metabolism in individuals with reduced energy stores. Handling stress from the force-feeding may as well have been an aggravating factor on growth performance (McCormick et al., 1998). Indeed, fish were fed by hand during the acclimation period, and the first force-feeding event corresponded to the start of the exposure, one week following transfer to experimental tanks and tagging.

Another hypothesis that cannot be ruled out is the alteration of feed assimilation and/or conversion, in crude oil exposed groups, leading to a decrease in energy intake. A reduction in digestive function from crude oil exposure was suggested for river otters (*Lontra canadensis*) (Ormseth and Ben-David, 2000), and juvenile turbot (*Scophthalmus maximus*) (Saborido-Rey et al., 2007). It is not possible to discriminate the mechanisms leading to an accelerated loss in weight, but the effects in the high oil treatment are likely due to a combination of several factors such as an increased energy demand due to handling stress and detoxification metabolism, and alteration in digestive function.

The dose-dependent increase in weight loss after one week of exposure seemed to be offset over the eight week exposure period in all oil treatments. Similarly, Bender et al. (2018) found a transient depression in growth in polar cod acutely exposed to dispersed oil, followed by a period of increased growth in exposed individuals compared to controls. While the mechanisms could not be explained, a temporary reduction in feeding activity in exposed fish or potentially compensatory mechanisms for growth were suggested. In the present study, force feeding allowed for control of the feed intake by each individual. Thus, changes in feeding regime or appetite (Christiansen and George, 1995) could not explain the accelerated weight loss in oil exposed individuals, nor the following reduction in weight loss. Ali et al. (2003) suggested that behavioral adjustments (e.g. reduction in locomotion and metabolic costs) and changes in growth efficiency may play a role in growth compensation. Also, an habituation to the force-feeding over the following seven weeks may also have attenuated the combined effects of the exposure and stress on growth observed during the first sampling point (McCormick et al., 1998). Fish that were monitored for growth following the final two weeks of depuration (tenth experimental week) had also been used in respirometry measurements every second week during the entire experimental period, and had thus undergone additional handling stress. The worsening effect of handling stress was again marked on the health of these individuals with the increased incidence of mortality in males from the oil treatments and the trend to a dose-dependent reduction in weight loss in females.

The consistency in oxygen consumption levels found from week to week in both sexes suggested that these specimens had reached a steady state, and had adjusted their routine metabolic rate within the first weeks of exposures. Females exposed to crude oil showed a dose-independent, but elevated oxygen consumption, suggesting a threshold response to an elevated energy demand. This elevated oxygen consumption may correspond to the so-called “resistance” phase in the conceptual model of the general adaptation syndrome developed by Selye (1973). At equal feed intake and considering the increased trend in weight loss with dose, it can be hypothesized that females of the high treatment may have had a more important energy trade-off compared to the low and medium treatment females. On the contrary, Christiansen et al. (2010) showed a decrease in routine metabolic rate in polar cod exposed to the crude oil WSF both acutely and for the following four weeks. In this case, the depression in oxygen consumption from acutely exposed specimens was mostly attributed to an immediate response associated with behavioral changes (e.g. immobility). The depression of long-term (four weeks) exposed individuals could not be explained, but was suggested to be related to a crude oil induced alteration in digestion or assimilation by Klinger et al. (2015), resulting in decreased SDA and associated metabolic rates relative to controls.

Male polar cod showed in general no significant alterations of neither weight loss nor routine metabolic rate with dose. The elevated metabolic rate found in males from the medium group at all time points studied, could be explained by a lower body mass (mean 13 ± 2 g wwt) compared to the other groups (mean 14 ± 4 g wwt) (Table S1). Mass specific oxygen consumption increases with decreasing body weight in fish, thus suggesting that the increased routine metabolic rate in the medium group was a size artefact rather than the effect of the crude oil exposure. Although, males seemed more robust to the exposure than females, exposure may alter active metabolic rate and thus the metabolic scope for activity, even though their minimum energy demands were maintained. Such effects were shown in common sole (*Solea solea*) exposed to fuel oil (Davoodi and Claireaux, 2007). Furthermore, mortality during the experiment was solely observed in male specimens thus suggesting a sex-specific sensitivity.

Post-spawning survival is believed to be linked to remaining energy reserves and the capability of polar cod to resume feeding (Hop et al., 1995). This feature is highly important, especially in females for which fecundity is limited by body size, and are thus dependent on growing larger and reproducing over several winters to maximize fecundity (Nahrgang et al., 2014). Polar cod shows a high-energy investment in reproduction, compared to other gadids, with a total body weight loss of 30–50% through gonadal development and spawning (Hop et al., 1995). Post-spawning survival is thus dependent on optimal conditions to resume feeding and acquiring new energy stores. The additional stress from exposure to petroleum may thus divert already low energy reserves to detoxification metabolism and away from growth, potentially leading to a significant reduction in condition or even death. While previous studies have investigated the sensitivity of polar cod to low exposure doses during gonadal maturation and concluded with a certain robustness (Bender et al., 2016, 2018), the low-energy status of females in spring, potentially related to spawning, may be a more critical time period during the year, where initial low conditions of these specimens could have important consequences on their capacity to cope with additional stress factors such as pollutants. Females were also

suggested to be more at risk due to their indiscriminate feeding behavior when offered both contaminated and uncontaminated feed (Christiansen and George, 1995). The present study, does not allow drawing firm conclusions on the true risk implied in this hypothesis, and requires future work.

The two weeks of recovery where fish were fed clean feed did not show any changes in SGR compared to specimens exposed during eight weeks. The two weeks window may have been too short to highlight any significant physiological changes in the organisms or the endpoints measured were simply not sensitive enough to highlight any recovery. For instance, restoration of baseline levels within two weeks following crude oil exposure has been previously found in the same species but for molecular and cellular biomarkers (Nahrgang et al., 2010c; Andersen et al., 2015.

5. Conclusion

The present study revealed a negative impact of crude oil exposure on growth performance on adult polar cod with low condition in the early spring. The differential physiological states of both sexes in terms of liver lipid content and maturity status, influenced their response to crude oil exposure, with females increasing their routine metabolic rate, and mortality only observed in males. The present study suggests that hepatic storage lipids are a critical factor for growth of adult polar cod, especially when exposed to additional stressors such as dietary crude oil. Dietary levels of crude oil as low as 4 µg crude oil per g fish per fish led to reduced SGR in specimens with low HSI and this effect increased with increasing crude oil dose. The present study further stresses the importance of investigating the sensitivity to oil exposure of specimens in a post-spawning state. It also calls for caution in study designs that involve a significant amount of animal handling, as this can have important consequences on data quality and conclusions drawn.

Ethical statement

All work was performed according to and within the regulations enforced by the Norwegian Animal welfare authorities. The R/V Helmer Hanssen is owned by the University of Tromsø, which has all the necessary authorization from the Norwegian Fisheries Directorate to use a bottom trawl to collect fish for scientific purposes. Permission to carry out this experiment was granted by the Norwegian Animal Welfare Authority in 2012 (ID 4377).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.04.082.

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SUPPLEMENTARY INFO

Table S1. Overview of polar cod age (years, min-max range), fork length (mean ±SD, cm), total weight (mean ±SD, g), Fulton's condition factor K (mean ± SD, %), GSI (mean ± SD, %), and HSI (mean ± SD, %) per treatment (control, low, medium, and high) and for all organisms before exposure start (tagging) and at each sampling time (zero, one, eight and ten weeks). F for females and M for males. During tagging and transfer to the different treatments, sex was unknown. Letters show significant differences (Dunn's Test, $p<0.05$) between treatments for each sex and time point. Numbers in bold show significant differences (Kruskal Wallis Test, $p<0.05$) between sexes for each treatment and time point.

Time	Treatment	N	Age (years)	Fork length (cm)	Total weight (g)	K	GSI	HSI
Tagging	Control	34		13.7 ± 1.0[a]	15.0 ± 3.0[a,b]			
	Low	34		14.6 ± 1.4[b]	17.8 ± 5.7[b]			
	Medium*	33		13.7 ± 1.1[a]	14.5 ± 3.4[a]			
	High	34		14.0 ± 1.4[a,b]	16.1 ± 3.5[a,b]			

Time (weeks)		F	M	F	M	F	M	F	M	F	M	F	M	F	M
0	Control	10	4	2-3	2	14.9 ±1.8	14.3 ±2.1	16.2 ±6.7	17.1 ±8.4	0.45 ±0.02	0.47 ±0.07	**2.6 ±0.8**	**11.8 ±7.9**	2.2 ±0.9	1.8 ±0.8
1	Control	5	7	2-3	2-3	13.8 ±0.6	13.7 ±1.2	13.3 ±1.5	14.3 ±3.5	0.48 ±0.08	0.47 ±0.05	**2.1 ±0.6**	**14.3 ±4.5**	2.7 ±0.6	2.6 ±0.7[a]
	Low	6	6	2-4	2-3	15.2 ±0.8	15.1 ±1.2	17.2 ±9.2	20.0 ±5.4	0.44 ±0.02	0.48 ±0.03	**3.5 ±1.5**	**16.3 ±3.1**	2.5 ±0.8	2.6 ±0.6[a]
	Medium	6	6	2-4	2-3	14.3 ±1.0	13.9 ±1.4	13.6 ±2.4	14.9 ±5.3	0.45 ±0.07	0.45 ±0.04	**2.3 ±0.5**	**17.3 ±7.5**	**2.8 ±1.1**	**1.8 ±0.6[b]**
	High	4	8	2-4	2-4	15.6 ±2.7	14.3 ±0.5	18.1 ±6.8	15.5 ±2.2	0.45 ±0.08	0.46 ±0.04	**2.8 ±0.8**	**14.5 ±3.4**	2.7 ±1.4	2.1 ±0.3[a,b]
8	Control	6	6	2-3	2-3	14.5 ±1.0	14.1 ±0.8	15.2 ±2.8	15.0 ±2.0	0.48 ±0.03	0.51 ±0.02	1.7 ±0.3	1.2 ±0.7[a,b]	**2.0 ±0.4**	**3.5 ±0.7**
	Low	7	5	2-4	2-3	14.4 ±1.5	14.4 ±0.8	16.5 ±5.4	16.1 ±3.2	0.51 ±0.05	0.51 ±0.03	1.5 ±0.3	1.2 ±0.5[a,b]	2.7 ±0.7	3.4 ±1.2
	Medium	3	9	2-3	2-3	14.5 ±1.7	13.2 ±1.1	14.8 ±2.3	12.2 ±2.7	0.48 ±0.08	0.50 ±0.03	1.7 ±0.04	0.9 ±0.2[a]	2.7 ±0.3	3.2 ±0.9
	High	1	11	2-3	3	14.2	13.7 ±1.0	15.2	13.6 ±2.6	0.51	0.50 ±0.04	1.2	2.1 ±2.6[b]	3.3	3.5 ±0.8
10†	Control	4	6	2-3	2-3	15.0 ±1.4	13.4 ±1.1	15.5 ±3.8	13.0 ±3.6	0.48 ±0.04	0.51 ±0.05	**1.4 ±0.3**	**0.8 ±0.3**	3.0 ±0.9	2.6 ±1.0
	Low	3	5	3	2-3	15.1 ±0.9	14.5 ±1.4	18.9 ±3.5	16.0 ±4.9	0.52 ±0.02	0.49 ±0.04	**1.5 ±0.1**	**1.0 ±0.2**	3.2 ±1.4	2.2 ±1.3
	Medium	3	6	2	2	13.9 ±2.0	13.7 ±0.7	13.2 ±2.0	12.8 ±2.4	0.47 ±0.02	0.48 ±0.03	0.7 ±0.4	0.7 ±0.2	2.3 ±0.6	2.7 ±0.7
	High	2	3	n.a	2-3	13.9 ±0.1	13.4 ±0.4	14.0 ±0.7	13.0 ±2.2	0.50 ±0.007	0.51 ±0.05	2.0 ±1.6	1.3 ±0.7	2.6 ±0.4	3.9 ±0.9

* one fish had jumped out before experiment start.

† specimens at 10 weeks were individuals used in respirometry

Table S2. GSI (mean ± SD, %), HSI (mean ± SD, %), and lipid content (mean ± SD, mg/g liver wet weight) in female and male polar cod according to sexual maturation stages after eight weeks of exposure. Statistical significance (Kruskal-Wallis test, p-values<0.05) between reproductive stages are in bold.

	n	GSI (%)			n	HSI (%)			n	Lipid content (mg/g)		
Female												
Immature	10	1.53	±	0.26	10	2.73	±	0.58	8	252.5	±	113.2
Regressing	5	1.66	±	0.23	4	1.92	±	0.44	5	122.8	±	89.8
Regenerating	2	1.79	±	0.08	2	2.51	±	0.62	1		331	
Male												
Immature	1		**0.78**		1		3.28		1		376	
Regressing	8	**2.49**	**±**	**2.84**	8	**3.66**	**±**	**0.71**	8	336.9	±	96.1
Regenerating	21	**1.03**	**±**	**0.27**	21	**3.33**	**±**	**0.93**	17	326.6	±	85.2

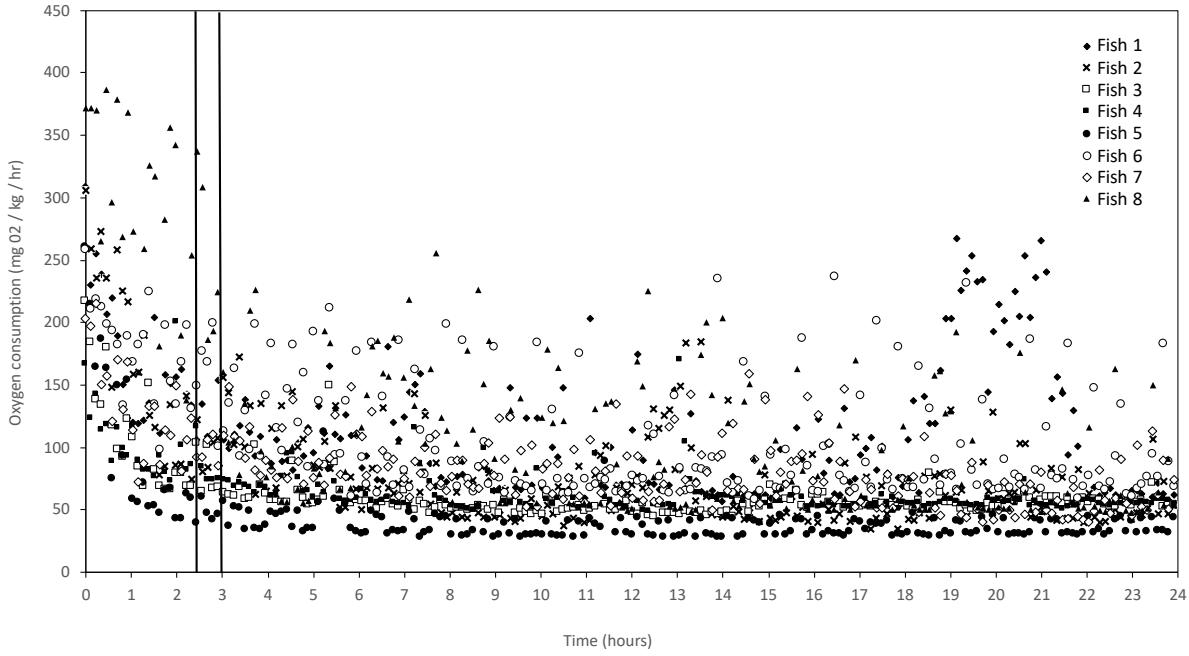

Figure S1. A 24 hour cycle of the whole body respiration of eight polar cod during the acclimation period. The elevated respiration in the first two hours is due to the stress of handling and the new environment in the respirometry chambers. Lines at 2.5 hours and 3 hours show the time in between which respiration data was used and averaged for experimental results.

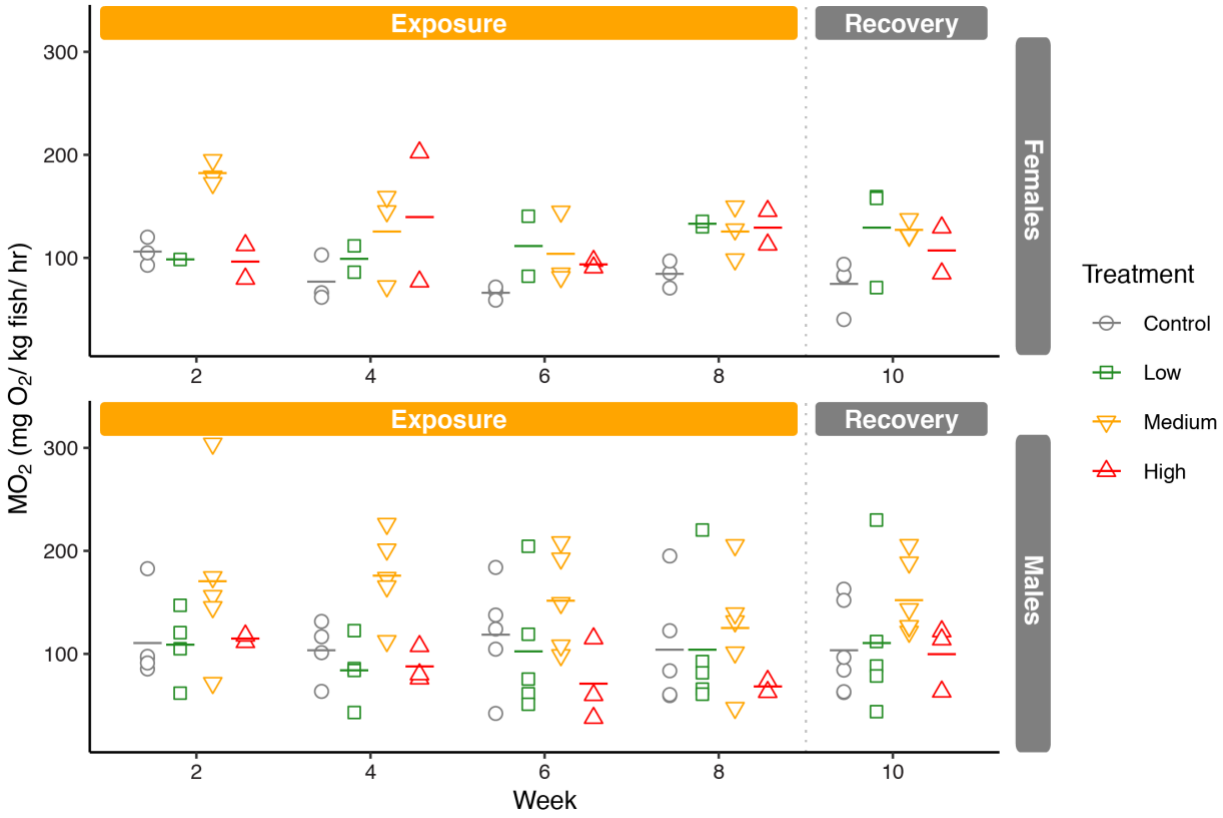

Figure S2. Mass specific oxygen consumption (mg O_2 per kg fish per hour) of female and male polar cod measured in the same specimens with two weeks intervals (11-17 days) over the course of the experiment, i.e. eight weeks of exposure to dietary crude oil (2-8 weeks) and following two weeks of recovery (10). Plots show individual data points with the average (rectangle).

Paper 3

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Effects of acute exposure to dispersed oil and burned oil residue on long-term survival, growth, and reproductive development in polar cod (*Boreogadus saida*)

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ABSTRACT

The present study investigates the potential long-term physiological effects on maturing polar cod (*Boreogadus saida*), an Arctic key species, after an acute exposure (48 h) to environmentally realistic concentrations of either mechanically dispersed oil (MDO), chemically dispersed oil (CDO) or burned oil residues (BO) (N = 58–60 per treatment). Following exposure, fish were monitored in a common tank supplied with clean water for a seven-month period coinciding with the period of reproductive development. Females exposed to BO residues were more frequently found in an earlier phase of gonadal maturation compared to unexposed females while no effects of different oil spill response (OSR) actions were seen in the reproductive development of males. Mechanically and chemically dispersed oil induced a transient short-term reduction in growth in the first week post-exposure. Overall, no significant long-term effects of exposure were seen in growth or mortality. Ultimately, this study provides information for the assessment of population consequences of different OSR actions as part of a net environmental benefit analysis.

1. Introduction

Increasing anthropogenic activities related to petroleum in the Arctic elevates the risk of acute and chronic oil spills. Conditions inherent in the Arctic, e.g. weather, sea ice, limited infrastructure, remoteness, and lack of precedent for responses in areas under multinational governance, place specific requirements on operations including eventual oil spill response (OSR) (Harsem et al., 2011). Recent OSR actions have stressed the need for OSR decision making that is site- and situation-specific to adequately consider physical factors and environmental and societal resources at risk (Beyer et al., 2016; Fingas, 2016).

Polycyclic aromatic hydrocarbons (PAHs) are one group of toxic compounds in petroleum, some of which are known to exhibit carcinogenic, genotoxic, and endocrine disrupting properties, even at low concentrations (Kime, 1995; Horng et al., 2010; Vignet et al., 2014). However, a large portion of the water-soluble fraction of crude oil is made up of poorly characterized mixtures of organic compounds, also classified as unresolved complex mixtures (UCM) (Melbye et al., 2009), with toxicological effects difficult to discern (Booth et al., 2007, 2008).

Mechanical recovery of spilled oil is often the first choice in an OSR and risk mitigation, however, chemical dispersants and *in situ* burning may be better suited in ice infested waters of the Arctic (Fritt-Rasmussen et al., 2015). Use of chemical dispersants as an OSR action is intended to increase the biodegradation potential of petroleum by forming oil-surfactant micelles (Lessard and Demarco, 2000). The use of chemical dispersants thereby enhances the oil concentration in the water column for a time whilst reducing the surface slick, the risk for encapsulating the oil slick into the sea ice (Brandvik et al., 2006), and the exposure potential for birds and marine mammals (Fingas, 2011). Increased concentrations of small oil droplets in the water column may lead to increased bioavailability of PAHs (Ramachandran et al., 2004; Milinkovitch et al., 2012), which has been linked to adverse effects on behavior, growth, reproduction, and survival in several fish species (Gulec et al., 1997; Yamada et al., 2003; Milinkovitch et al., 2011; Wu et al., 2012; Yu et al., 2015; Nwaizuzu et al., 2016).

In situ burning is a countermeasure technology that was first implemented in 1958 along the ice-covered Mackenzie River in Northwest

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Canada and since then has been used operationally in ice free areas during the 1989 Exxon Valdez oil spill in Alaska and extensively in the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (Beyer et al., 2016; Buist, 2004; Hunt, 2009). Burning is a quickly implemented OSR action that requires little infrastructure and, most significantly, is effective, leaving approximately 10% of the original hydrocarbon load (Guenette and Sveum, 1995; Buist, 2004; Buist et al., 2013; Fingas, 2016). Burning creates a burned oil residue (BO) that may sink depending on oil characteristics (Fritt-Rasmussen et al., 2015). A limited number of studies have investigated the potential effects of BO on biota and agreement on the low acute toxicity of BO applications has been reached (for reviews see Buist, 2004; Buist et al., 2013; Fingas, 2016). Compared to untreated and chemically dispersed oil, burned oil was found to be less acutely toxic in rainbow trout (*Onchoryncus mykiss*), three-spined stickleback (*Gasterosteus aculeatus*) (Blenkinsopp et al., 1996), Australian bass (*Macquaria novemaculeata*) (Cohen and Nugegoda, 2000, 2006), amphipods and snails (Gulec and Holdway, 1999), and in copepods (Faksness et al., 2012). However, the long-term effects of acute exposure to burned oil residues has yet to be examined.

Polar cod (*Boreogadus saida*) is an endemic key species with a pan Arctic distribution (Bradstreet and Cross, 1982) that has been used as a sentinel species in Arctic environments to understand the effects and mechanisms of petroleum exposure (Christiansen and George, 1995; Jonsson et al., 2009; Andersen et al., 2015; Nahrgang et al., 2010, 2016; Bender et al., 2016). The geographical distribution of polar cod overlaps with oil and gas fields and transport areas in all Arctic seas and fish can therefore encounter petroleum products (Bailly, 2008; Bird et al., 2008; David et al., 2015). Gonadal development in polar cod begins in early autumn and final maturation and spawning takes place from January to March (Nahrgang et al., 2014; Mueter et al., 2016). Gonadal investment in polar cod is high with up to 87% of liver reserves metabolized during reproductive development (Hop et al., 1995). Throughout the active gametogenesis period, male fish undergo more rapid development of gonadal tissue and an earlier increase in plasma sex steroid concentrations than female fish (Bender et al., 2016).

Acute toxicity data (e.g. LC_{50}) using model organisms represents the majority of existing data on the effects of OSR actions while actual oil spills present the challenge of confounding factors (e.g. other sources of pollution or climate and fishing driven pressure), indirect effects, and missing background data when interpreting effects on biota (Beyer et al., 2016; Peterson et al., 2003). Expanding potential effects from short-term responses of biomarkers after acute exposure to a predictive indicator of long-term effects is challenging (Forbes et al., 2006; Claireaux et al., 2013). The objective of this study was to investigate long-term resilience of adult polar cod exposed to mechanically dispersed oil (MDO), chemically dispersed oil (CDO) or BO. To link acute exposure to long-term effects, survival, growth, and reproductive investment in polar cod were monitored for seven months after an acute (48 h) exposure. Simulated wave and current energy in open exposure tanks ensured that acute exposures set up was dynamic whereby organisms were exposed to the whole dispersed oil solution (dissolved fractions and oil droplets) (Milinkovitch et al., 2011; Frantzen et al., 2015, 2016). We hypothesized that addition of chemical dispersants would increase the exposure of polar cod to crude oil resulting in reductions in growth and reduced investment in reproductive development compared to exposure to MDO and BO treatments. Growth and reproductive development are physiological endpoints that can provide an integrative measure of the effects of pollutants on whole organisms and are important to consider when evaluating the potential risks to populations. The aim of this study is to generate sound information on the sensitivity of adult polar cod to support the net environmental benefit analysis (NEBA) of OSR actions in the Arctic marine system.

2. Material and methods

2.1. Fish collection and husbandry

Wild polar cod were collected in Svalbard fjords in September 2014 by bottom trawl during a cruise aboard the RV Helmer Hanssen and thereafter transported to the Akvaplan-niva marine laboratory in Tromsø, Norway. The fish were reared in a single common 5000 L tank for an eight-month acclimation and maintenance period and hand fed twice a week on a commercial marine fish feed (ration equal to 4% body weight per feeding; Skretting, 3–4 mm dry pellets). The light regime was maintained on a simulated Svalbard light throughout acclimation, exposure and post-exposure periods. The seawater temperature in the tank followed the annual variation of Grøtsundet, the fjord outside the marine laboratory where seawater was collected from 50 m depth, with a high of 8.6 ± 0.1 °C in September and low of 3.74 ± 0.02 °C in February and yearly average of 6.2 ± 0.1 °C. Oxygen saturation was keep above 90% for acclimation and post-exposure period. On the 19th of May 2015, all fish (n = 310) were anesthetized (Metacaine at 0.08 g/L seawater) and received a passive integrated transponder tag (Trovan®) inserted intraperitoneally with no mortality or negative effects observed in the first weeks post tagging.

2.2. Exposure design

Exposure to MDO, CDO or BO commenced in late June 2015, and growth was followed over a seven-month period (i.e. until January 2016) coincident with the active vitellogenesis/spermatogenesis period of polar cod (Bender et al., 2016). Goliat (Kobbe) crude oil, a sweet light crude oil with a density of 0.83 kg/L, an °API gravity of 40.3 and a sulphur content of 0.14% wet weight (Eni Norge, 2015) was used for the MDO and CDO treatments. For preparing BO, 40 L of Goliat (Kobbe) crude oil was added at the surface of a tank containing approximately 200 L sea water. The oil was set on fire and burned for 3 min until the fire died out, leaving approximately 4 L burned residue in the tank (i.e. ~90% of the fresh oil volume was burned off). The set-up consisted of four treatments and three replicates per treatment; control (no oil, Ctrl), MDO or CDO (premixed with dispersant FINASOL®; 5% w/w) and BO residue. The BO concentration equaled 10% of the nominal oil concentration used in the mechanically and chemically dispersed oil treatments (i.e. 90% burned off in BO compares to MDO and CDO). To be able to directly compare between dispersed oil toxicity (MDO, CDO) and BO toxicity, the same exposure protocol was used for all treatments.

The dispersant mixtures were generated according to Frantzen et al. (2015, 2016) following the protocol developed by Cedre, France for the DISCOBIOL project (e.g. Milinkovitch et al., 2011). Briefly, the oil treatments (MDO and CDO; nominal concentration of 67 mg/L) or BO (nominal concentration of 6.7 mg/L) were introduced to individual 120 L exposure tanks through a funnel fixed at the surface. A pump in the bottom of each tank provided continuous mixing energy in all tanks. In order to ensure a homogeneous exposure mixture in the tanks and to allow some weathering of the oil to take place prior to exposure start, water and oil/oil premixed with dispersant/BO were mixed for 24 h before the introduction of fish to the system. The water system was static and oil exposures were conducted for 48 h after the introduction of the animals (water temperature 6.4 ± 0.3 °C; O_2 saturation was held > 80% with aide of aerators). In total, 236 specimens were exposed from the 26th to the 28th of June 2015 with each replicate exposure tank (n = 3 per treatment) containing 18–20 fish.

2.2.1. THC and PAHs in seawater

In order to monitor exposure concentrations, water samples (approximately 1 L) were taken from all exposure tanks (n = 3 per treatment) at the beginning of the experiment (t 0 h), after 24 h (t 24 h), and at the end of the 48 h exposure (t 48 h). Determination of total

hydrocarbon content (THC; n-C10 – n-C35) was performed on isooctane extracts by Gas Chromatography-Flame Ionization Detector (GC-FID). Each sample extract was analyzed on the GC simultaneously with control solutions (reference oil EDC95/11; Norwegian Environment Agency M-408/2015) of three known concentrations of THC, covering the concentration range of the sample extracts (Frantzen et al., 2016). Analysis of 26 PAHs (16 Environmental Protection Agency [EPA] priority parent PAHs and C1−C3-alkylated naphthalenes, phenanthrenes and dibenzothiophenes) concentrations was performed by GC–Mass Spectrometry (GC–MS) operated in selected ion monitoring mode. Single PAH concentrations were calculated by quantification of altered deuterated standards added prior to extraction, and development of a pre-determined calibration curve of five PAH-standards at different concentrations (Frantzen et al., 2016). The measured THC and PAH concentrations represent dissolved components as well as oil droplets. In the determination of Σ26 PAH concentrations, single components with values below the limit of detection (LOD) were assigned a value of zero. Due to a technical instrument failure, water samples from t0h at the start of the exposure gave unreliable results and were excluded from further analysis.

2.3. Post-exposure monitoring and final sampling

The 48 h exposure period was followed by a 48 h recovery period in 500 L flow-through tanks and subsequent growth registration (T1; see paragraph below) before the fish were transferred back to the common 5000 L rearing flow-through tank. The common tank ensured identical post-exposure rearing conditions for all treatment and replicate groups. The fish were fasted two days prior to exposure start, during the 48 h exposure period, and two days prior to every growth measurement. Fish were, however, offered food immediately following exposure in clean water tanks even though it was less than 48 h before the growth checkpoint.

Mortality was recorded daily over the entire experiment. Growth was recorded at monthly intervals by first anesthetizing, then measuring the total weight (± 0.01 g) and total length (± 0.1 cm) at the following time points: T0 (May 19th, pit tagging), T1 (June 30th, 2 days post-exposure), T2 (July 30th), T3 (September 3rd), T4 (October 5th), T5 (November 3rd), T6 (December 9th), and T7 (January 5th). An additional group of “unexposed” polar cod was included in the common rearing tank which consisted of the remaining acclimation fish that fell below (Unexp. 1) and above (Unexp. 2) the desired intermediate size range and were therefore not included the exposure experiment (n = 74). These additional unexposed fish provided a control for experimental handling stress related to the exposure with growth measurements undertaken at T0, T2-T7 (excluded from T1 due to logistical limitations).

On the 5th of January, all remaining experimental fish and the unexposed fish were sacrificed by a sharp blow to the head and the following measurements were collected: total length (± 0.1 cm), total weight (± 0.01 g wet weight [wwt]), sex, gonad weight (± 0.01 g wwt), liver weight (± 0.01 g wwt) and somatic weight (empty carcass weight, ± 0.01 g wwt). The middle section of the testis and ovaries were fixed in a buffered formaldehyde solution (4%) for later histological analysis. Otoliths were collected for age determination and read under a dissection microscope (Leica M205C).

Specific growth rate (SGR) for individual fish for the entire experimental period was determined according to the equation:

$$SGR = [(\ln {}_tW_2 - \ln {}_tW_1)t^{-1}]100$$

Where SRG is % increase in body weight per day. ${}_tW_1$ and ${}_tW_2$ are the total weights of the fish recorded at times 1 and 2 respectively, and t is the number of days between weighting events.

Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated according to the following equations:

$$GSI = (\text{gonad weight/somatic weight})*100$$

$$HSI = (\text{liver weight/somatic weight})*100$$

Condition factor for the different time points (T0-T7) was calculated:

$$CF = (W/L^3)*100$$

Where W is total weight in g and L is the total length in cm.

2.4. Histological analysis

Briefly, gonad tissues were rinsed of buffered formalin, dehydrated in a series of 70% ethanol baths and embedded in paraffin wax (Aldrich, USA) overnight using Histo-clear® as a clearing agent in a Shandon Citadel 1000 (Micron AS, Moss, Norway). Tissues were then embedded into paraffin and sliced at 5 μm (females) and 3 μm (males) thickness, using a Leica RM 2255 microtome before being stained with haematoxylin and eosin. Two slides were prepared for each fish. Gonad maturity stages in females were classified using the development stage of oocytes within the respective categories of immature, resting, and early and advanced stages of maturation. Immature and resting females had only primary growth (PG) oocytes while maturing females had vitellogenic oocytes present. Resting females were identified by the presence of residual oocytes from previous spawning events with otherwise only PG oocytes. Maturing females exhibited different phases of oocyte development with varying extents of vitellogenin derived oil droplets in the oocyte cytoplasm (Fig. 4ab). Oil droplets were present but filling less than ½ of the cytoplasm in early maturing females while advanced maturing females had oocytes completely filled or nearly filled with oil droplets. Abnormal oocyte development was noted with regard to the location of cortical alveolar vesicles and oil droplet within the oocyte. Oocyte diameter (n ≥ 6 oocytes per female) was counted for oocytes in the most advanced cohort using the image processing software (Leica DFC 295 camera attached to a Leica DM 2000 LED microscope and Leica analysis software) and then averaged for each female. Oocyte stage frequency disruption was determined by classifying all oocytes with a nucleus in an area of 20 mm^2 placed randomly on the tissue slice. Frequency counts were averaged over both replicate slides. Presence of residue oocytes was noted and relative frequencies of atretic oocytes were semi-quantified using a 0–3 scale ranging from 0 (0% of oocytes were atretic); 1 (1–2%); 2 (3–20%); and 3 (20–30% of oocytes were atretic) for each female. Male testes were classified into the four different maturity stages of immature, resting, and maturing with either late spermatocytes stage I (Figure S1a) or with late spermatocytes stage II dominating (Figure S1b). Immature males were identified as those with testis containing only early stage spermatocytes (Figure S1c) and a low GSI while resting males had spent testis containing portions of early stage spermatocytes with otherwise empty lumen space (Figure S1d).

2.5. Statistical analysis

All statistical analyses were performed with R 3.1.1 (R Development Core Team, 2014). A Levene's test was used to test for normality and homogeneity of variance. When homogeneity criteria were met, a one-way analysis of variance (ANOVA) was run, and when a significant treatment effect was found, the Tukey's HSD post hoc for unequal sample sizes was used to distinguish differences between treatment groups. In cases where homogeneity criteria were not met, a nonparametric Kruskal Wallis ANOVA was used, followed by a multiple comparison of mean rank of all group tests. Difference in SGR variance was tested using an F-test. Maturity stage frequency distributions were tested using a Fishers exact test with the null hypothesis that all treatments have similar maturity stage distributions. With a significant Fishers exact test result, a *chi* squared test was run comparing all treatment groups and control against one another. A probability level of

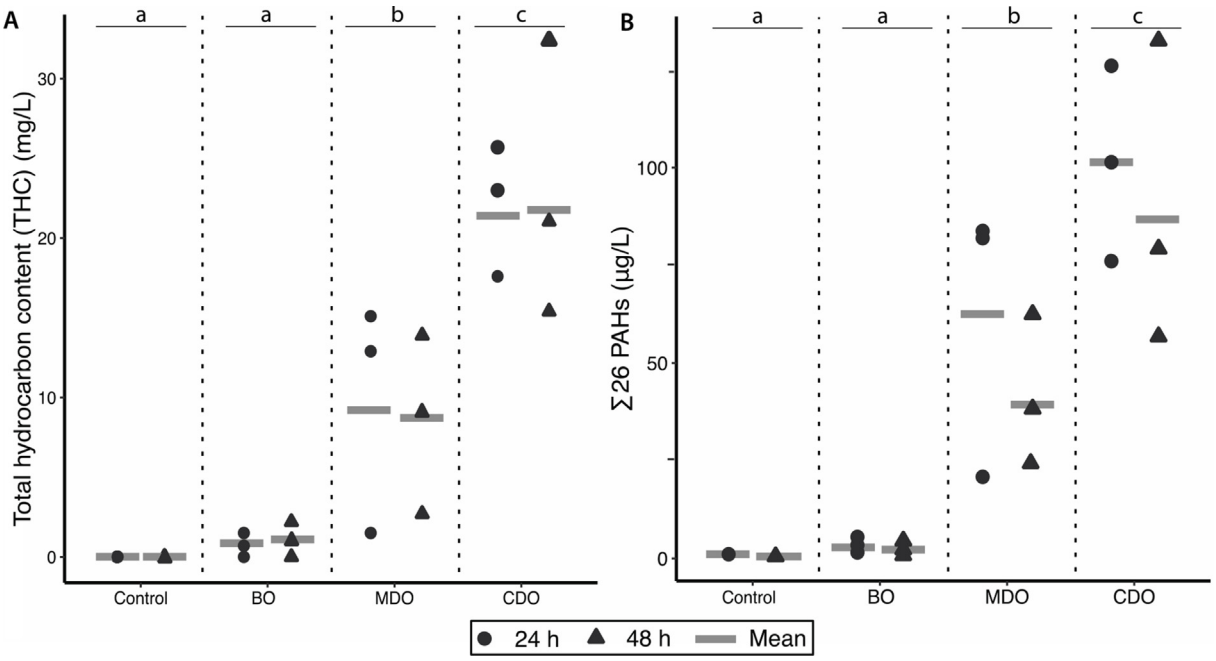

Fig. 1. (a) THC and (b) Σ26 PAH concentrations at t24 (circles) and t48 (triangles) for all treatment groups with mean concentrations illustrated by black bars. Treatment mean concentrations that do not share a letter are significantly different ($p < 0.01$).

$p \leq 0.05$ was considered significant for all tests. All values are presented as mean ± standard error of the mean (SE).

3. Results

3.1. Water chemistry

The total hydrocarbon content (THC) in water samples from the control treatment was not detectable throughout the exposure period, and remained relative stabile throughout the last 24 h of exposure with values of 0.9 ± 0.5, 9.2 ± 3.7, 22.5 ± 3.7 mg/L in BO, MDO and CDO, respectively (Fig. 1a). Average Σ26 PAH concentrations were highest after 24 h in all treatments and thereafter decreased by ca. 20% at 48 h (Fig. 1b, Table S1). Highest Σ26 PAH concentrations were found the CDO treatment (101.5 ± 14.3 µg/L) at 24 h followed by the MDO (62.4 ± 20.7 µg/L), BO (3.5 ± 1.2 µg/L) and Ctrl (1.05 ± 0.0 µg/L) treatments. Dominating PAHs (> 98% of Σ26 PAH) in all treatments (BO, MDO and CDO) were parent and alkylated naphthalenes, phenanthrene/anthracenes and dibenzothiophenes, whereas only parent and C1, C2-naphthalenes were detected in the Control. In BO treatment, the only high molecular weight PAHs measured above detection limits were benzo(*b*)fluoranthene (0.03 µg/L) and benzo(k)fluoranthene (0.01 µg/L), and these concentrations were comparable to measured concentrations in MDO and CDO (0.01–0.10 µg/L and 0.01–0.013 µg/L, respectively; Table S1). Acenapthylene was the only PAH with a higher concentration in BO (0.01 µg/L) compared to MDO and CDO (< 0.004–0.005 µg/L) (Table S1).

3.2. The initial fish population

Fish initially part of the exposure experiment ranged in size from 12.0 to 59.0 g total weight (mean 34.7 ± 0.6 SE), 12.0–22.0 cm length (mean 17.3 ± 0.1 SE) and age ranged between 2 and 6 years (mean 4.5 ± 0.1 SE) at T0 (Table 1). Fish used for the exposure experiment were all selected from the intermediate size group of the collected fish (size range 24.0–47.5 g) with no significant difference in size between any of the groups (Ctrl, BO, MDO, CDO). The remaining unexposed polar cod were not included in any of the treatment groups and were classified by size as they exhibited a bimodal size range that was significantly smaller (Unexp1; size range 12–32 g) and bigger (Unexp2; size range 38–59 g) than the exposed fish.

3.3. Mortality

Mortality was observed after the first month post collection, and in the period February/March 2015 following the natural spawning period before exposure took place (data not shown), after which mortality subsided. Fish were otherwise in good condition throughout the acclimation, exposure and post-exposure monitoring period. No mortality was registered in any treatments tanks during the 48 h exposure period. Mortality was, however, observed during the post exposure period for all treatments independent of exposure. Mortality was most prevalent in the first month post-exposure (T1-T2 [June 30th – July 30th]) with 8–12% mortality occurring in all oil treatments and control. The mortality rate steadied to between 2 and 5% per month until the final sampling in January for all treatments and control with no statistical difference in cumulative mortality (Fig. 2). The group of larger unexposed fish (Unexp 2.) exhibited the highest cumulative mortality (32%).

3.4. Specific growth rate

In general, there was a great variation in SGR within all treatment groups throughout the experiment ranging from −2.5 to 3.5% change in body weight per day. Overall, growth rates (mean ± SE) were lowest after tagging and during exposure (T0-T1 [May 19th - June 30th], (−0.01–0.15% increase in body weight per day) and highest in the consecutive time period (T1-T2 [June 30th –July 30th]), 0.47–0.73% increase in body weight per day). In the period from tagging to immediately after exposure (T0-T1), significant treatment effects on growth rates were observed with high rates in the BO compared to lower growth rates in the MDO ($p < 0.01$) and CDO treatments ($p < 0.01$) (Fig. 3). In the following period (T1-T2) growth rates in the BO treatment were significantly reduced only when compared to the CDO treatment ($p < 0.01$). No significant differences in SGR were seen between any treatment groups or unexposed fish for the entire period (T0-T7 [May 19th – Jan 5th]) or for any other growth periods beyond the first two periods. Female and male SGR were not significantly

Table 1
Summary of polar cod sampled in January after a 7-month monitoring period following 48 h exposure to *in situ* burned oil residues [BO], mechanically dispersed oil [MDO], and chemically dispersed oil [CDO] treatment, and a control group. Unexposed fish have size distributions which fall outside the intermediate range included in the exposure experiment. Age, as determined by otoliths, total length, total weight, hepatosomatic index (HSI), and condition factor were calculated for all fish. All values are mean ± SE.

Treatment	Number of fish sampled							
	Females	Males	Total	Age	Length (cm)	Total Weight (g)	HSI (%)	Condition factor
Control	27	20	48	4.7 ± 0.1	20.0 ± 0.2	56.5 ± 2.0	9.3 ± 0.3	0.53
BO	26	22	49	4.7 ± 0.1	19.8 ± 0.2	56.4 ± 2.4	9.3 ± 0.3	0.53
MDO	20	19	40	4.4 ± 0.1	19.7 ± 0.2	54.3 ± 2.1	9.5 ± 0.4	0.53
CDO	25	18	46	4.5 ± 0.1	19.9 ± 0.2	56.2 ± 2.1	9.6 ± 0.3	0.54
Unexp. 1	12	18	30	3.8 ± 0.2	17.5 ± 0.2	38.0 ± 1.8	8.5 ± 0.3	0.55
Unexp. 2	17	6	23	4.6 ± 0.2	22.3 ± 0.3	80.0 ± 2.8	9.4 ± 0.4	0.54

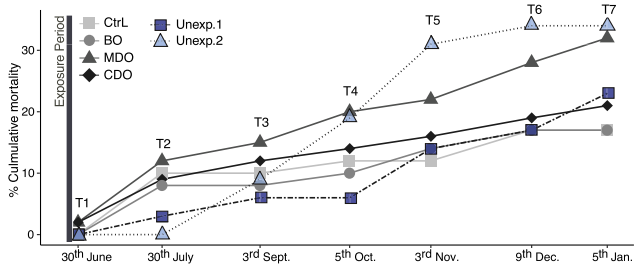

Fig. 2. Cumulative mortality (% of overall mortality) of polar cod during the course of the exposure and post exposure period (June 2015–January 2016) for each treatment group. No significant difference in % mortality was found between treatment groups, control or unexposed groups.

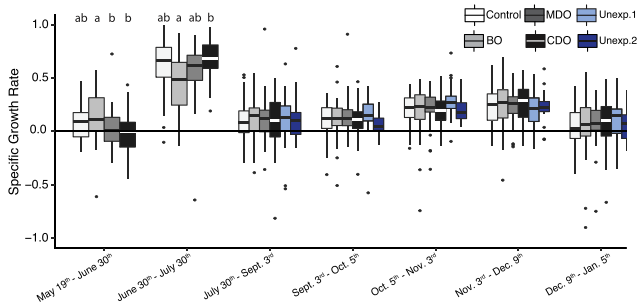

Fig. 3. Specific growth rate (SGR; % change in body weight per day) of different post-exposure time periods (n = 40–49 per treatment [Unexp. n is 23–30 fish]). The box ranges from the first (Q1) to the third quartile (Q3) of the data and represents the interquartile range (IQR). The line across the box indicates the median while the extreme data (outliers) outside Q1 − 1.5 × IQR and Q3 + 1.5 × IQR are displayed as individual points. Limits of displayed data are only for SGR values from 1 to −1 excluding values outside that range. Different letters indicate significant differences between treatment groups.

different at any time period, therefore both sexes were pooled for statistical analysis.

3.5. Condition factor and hepatosomatic index

At T0 (May 19th), males (exposed and unexposed combined) had a significantly higher condition factor compared to females at 0.68 ± 0.0 and 0.64 ± 0.0, respectively. At T1 (June 30th), the condition factor was significantly higher in females in the BO treatment (0.66 ± 0.0) compared to females in the other groups (control [0.62 ± 0.0], MDO [0.62 ± 0.0] and CDO [0.60 ± 0.0]). At no other time point were there significant differences found between any of the treatment groups (including control) or sex. Furthermore, no significant difference in age, HSI, or condition factor was seen between any treatment or sex at the end of the experimental period in January (Table 1).

3.6. Reproductive development

3.6.1. Females

Histological analyses revealed that 56% of female fish had spawned previously as determined by presence of residual oocytes, while 22% exhibited first time maturation with no evidence of previous spawning and the remaining specimens were immature (6%) or resting (16%). From the maturing females, 68% revealed a leading oocyte cohort that had reached the vitellogenic stage II (Vtg II) and were categorized as advanced maturing with mean oocyte diameter of 547 ± 8 µm, a centrally placed nucleus and the cytoplasm filled with vitellogenin derived oil droplets (Fig. 4). In 32% of maturing females, however, the most advanced oocyte cohort was in an early vitellogenetic stage (Vtg I) and was thus categorized as early maturing with an oocyte diameter of 446 ± 11 µm and vitellogenin derived yolk droplets only at the periphery of the cytoplasm and persisting cortical alveolar vesicles, often in combination with atresia (Fig. 4). Abnormal oocyte development, characterized by partial inclusion of cortical alveolar vesicles into the cytoplasm, non-radial yolk globule orientation around nucleus, and few oocytes in the most advanced oocyte cohort, was observed in 35% of early maturing females with no statistical significance of treatment. Significant differences in gonadal maturity stage was observed in the BO exposed females exhibited by a lower percentage of advanced maturing (35%) and higher percentage of early maturing females (38%) compared to other treatment groups (mean percentage in advanced maturing stage was 61%) ($p = 0.042$) and when tested against the control group only, the significance increased (X-squared = 7.99, df = 2, p-value = 0.018) (Fig. 5a). No significant differences were found between treatments in mean oocyte diameter, the relative number of oocytes in the leading cohort, presence of residual oocytes or frequency of atresic oocytes. However, significantly greater variation in oocyte diameter was observed in early maturing females in the BO treatment (443.5 ± 42 µm, n = 7) compared to the control (409.0 ± 10.7 µm, $p = 0.015$, n = 5).

Gonadosomatic index (GSI) in females ranged between 0.6 and 11.5 with no significant difference between any of the treatments (Fig. 5b). GSI (Mean ± SE) for immature, resting, advanced maturing and early maturing females was (0.9 ± 0.0), (2.6 ± 0.8), (5.7 ± 0.3) and (3.6 ± 0.3), respectively. Advanced maturing female GSI was significantly higher than all other maturity stages ($p < 0.001$).

3.6.2. Males

Testis development appeared normal for males in all treatments with no significant difference in the frequency of occurrence of different maturity stages among the treatments (Fig. 6a). GSI in males at the end of the experiment (T7) ranged between 0.0 and 33.3 with no significant difference between any of the treatments (Fig. 6b). Immature and resting fish made up 5.9% and 2.9% of the sampled males respectively

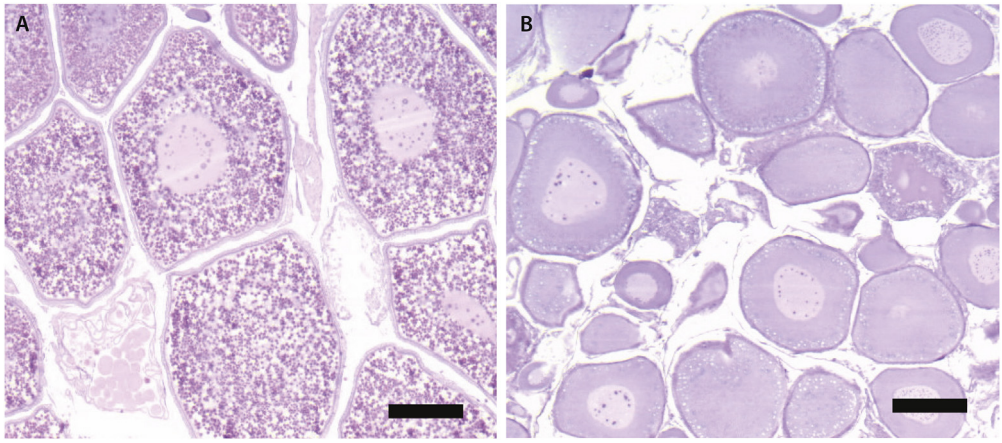

Fig. 4. Histological representation of an (a) early maturing female with cortical alveoli vesicles and early signs of vitellogenesis with yolk globules present in oocyte periphery; (b) advanced maturing female with vitellogenic oocytes. Scale bare is 200 μm in both pictures.

while 53.9% of males were in an early stage of maturation (late Sc I) and 37.3% of the males were in a later stage of development (late Sc II) (see Fig S1. for maturity stage representations). Immature and resting males had a low mean GSI (2.0 ± 1.6 and 2.0 ± 0.9, respectively). Maturing males with late spermatocytes stage I had a lower GSI (15.8 ± 0.8) compared to those with more developed late spermatocytes stage II (22.6 ± 1.0).

4. Discussion

4.1. Exposure to dispersed oil and burned oil residue

The present study simulates conditions in which dispersant (CDO treatment) or *in situ* burning (BO treatment) might be used to combat an oil spill in Arctic waters in comparison to no action (MDO treatment). THC and PAH water concentrations in both MDO and CDO reflected environmentally realistic concentrations reported from experimental field trials and dispersant operations during actual oil spills (i.e. THC concentrations of 30–50 mg/L below the spill just after treatment before decreasing to < 1–10 mg/L, and ΣPAH concentrations of 6–115 μg/L the first days or weeks after accidental oil spills) (Law, 1978; Humphrey et al., 1987; Lunel et al., 1995; Short and Harris, 1996; Kingston, 1999; Reddy and Quinn, 1999; Lessard and Demarco, 2000; Sammarco et al., 2013). Reports of hydrocarbon concentrations in seawater after *in situ* burning operations are scarce. PAH and THC levels in the present study are below seawater concentrations measured after experimentally spilled and burned oil in the Newfoundland Oil Burn Experiment (3.78 μg/L Σ16 EPA PAHs) (Daykin et al., 1994), and above THC concentration from an oil spill simulation and test burning experiment in the Barents Sea (13 μg/L) (Brandvik et al., 2010).

The overall THC and Σ26 PAH concentrations in the Ctrl, MDO and CDO treatments were in agreement with previous experiments using the same nominal oil concentrations and exposure set-up as in the present study (Frantzen et al., 2015, 2016), and confirms that the addition of chemical dispersant increases the efficiency of the dispersion process leading to significantly elevated THC and PAH concentrations in CDO compared to MDO. Measured BO concentrations were 8 ± 2% of the measured MDO concentrations, indicating that mechanical dispersion of BO into the water column was equally efficient as for oil. In the present study, an identical exposure protocol was used for all

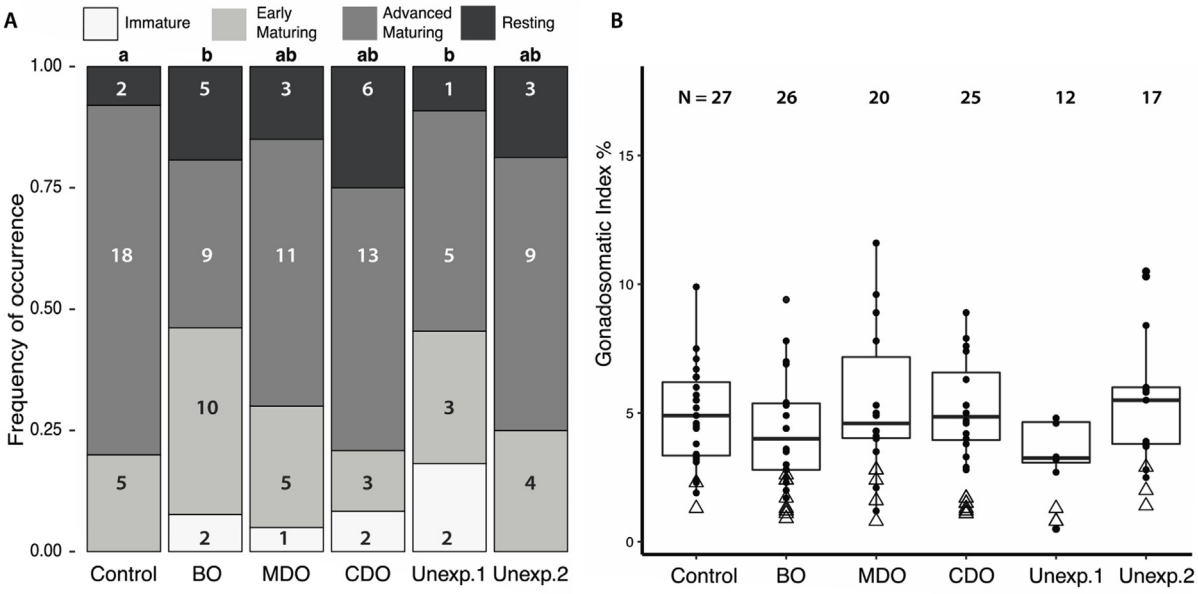

Fig. 5. (A) Maturity stage frequency distribution of females from all treatments; (B) boxplots of GSI of female fish in different treatment groups, maturing females are plotted in the boxplots and immature and resting females are indicated at triangles. Different letters above treatment groups indicate significant differences between treatment groups, those with letters in common are not significantly different from one another. The number in each box refers to the number of fish in that category.

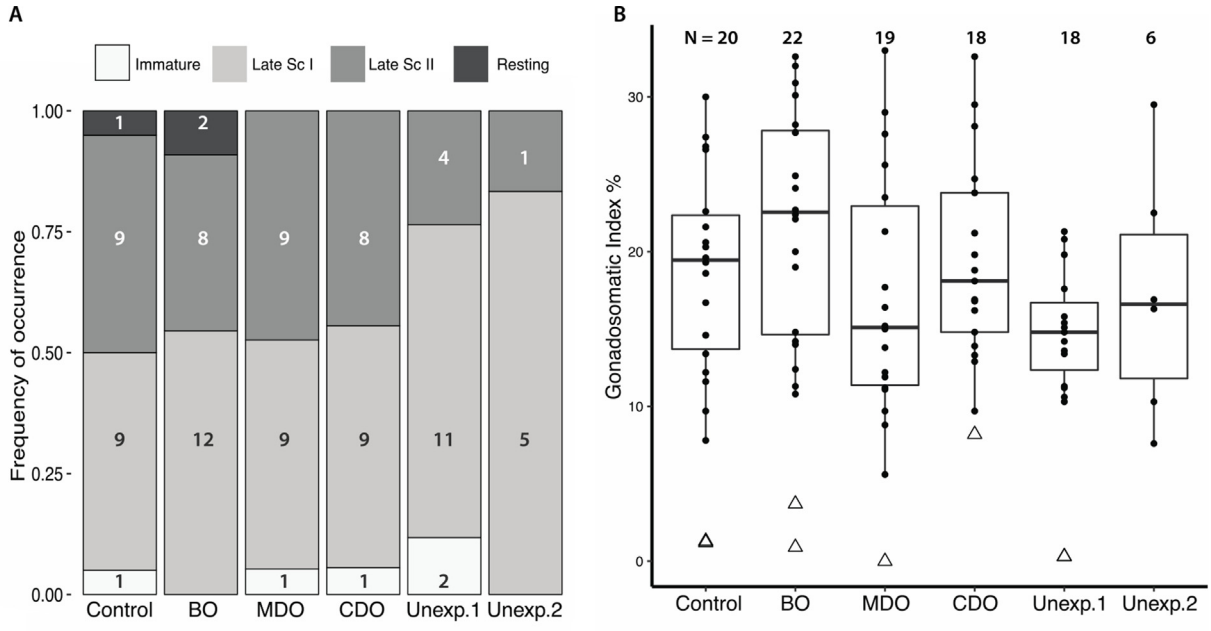

Fig. 6. (A) Maturity stage frequency distribution of males from all treatments; (B) boxplot of GSI of male fish in different treatment groups, maturing males are plotted in the boxplots and immature and resting males are indicated at triangles. No significant differences were found between treatments groups. The number in each box refers to the number of fish in that category.

treatments to allow for direct comparison of effects between the oil spill response measures investigated. Energy was added to the seawater to simulate a dynamic exposure with wave energy for the period of 4 tidal systems (48 h) (Merlin, 2005; Milinkovitch et al., 2011), and the measured concentrations of hydrocarbons represented both the water-soluble fraction as well as BO residue particles/dispersed oil droplets. Adding mixing energy to simulate wave action to the BO residue exposure dispersing it in the water column is, however, novel as previous studies have exposed organisms only to the burned oil WSF (Faksness et al., 2012; Gulec and Holdway, 1999), and reported measurements are taken only of seawater hydrocarbon concentrations underneath burned areas (Brandvik et al., 2010).

Forming of short-term temporary oil slicks, variation in oil adherence to equipment and mixing by fish movements between replicate tanks may be a source of the individual variability in THC and PAHs concentrations between replicate water samples, and the increased PAH/THC concentration at T48h compared to T24h observed in two individual tanks (one MDO and one CDO tank, respectively). Inter- and intra-tank variations did however not influence the overall significant difference in THC/PAH concentrations between the OSR actions investigated. Low concentrations of naphthalene measured in the control water may be considered elevated background levels with no potential toxic effects to biota (Molvær et al., 1997) and are evidence of the ubiquity of PAHs, especially naphthalene, one of the most abundant PAHs in the marine environment (Latimer and Zheng, 2003).

4.2. Physiological and reproductive effects

No relationship was found between treatment and mortality. The sustained mortality rate in seen in all groups (both exposed and unexposed) is most likely due to the post spawning physiological state of the mature fish as confirmed by the presence of residual oocytes in 56% of females. Handling stress at the beginning of the experiment could have induced higher mortality at this early time point. The mortality rate seen in this experiment (~24%) was lower than the mortality observed (~56%) in a long-term crude oil exposure on adult feral polar cod held in captivity (Bender et al., 2016). Fish were in a good state of health as evidenced by an unanimously high condition factor and HSI in all treatment groups at the final sampling in January, although the HSI values reported for fish in the present study (8.5–9.6%) were lower than for polar cod of a similar size held in captivity at the same time of year (10.9–13.1%) (Bender et al., 2016). Fish in the latter study were fed a natural diet of *Calanus* sp. zooplankton whereas commercial feed was used in the present study and this difference in diet may have influenced the HSI. Higher condition factor in males compared to females at the start of the experiment is most likely due to the difference in the timing of reproductive investment, where males start gonadal investment earlier in the season than females (Hop et al., 1995; Nahrgang et al., 2014).

Growth rates observed in polar cod of the present study were within reported ranges from previous studies of mature fish (Jensen et al., 1991; Hop et al., 1997). Furthermore, the observed trends in growth rate did not indicate significant long-term effects by any of the OSR actions. The transient decrease in SGR for the MDO and the CDO treatments compared to BO treatment, may however, be due to a transient appetite depression in these two groups in the first days following the exposure. Low feeding activity was visually observed at this time. No effect of crude oil exposure on appetite has been observed in polar cod previously; however, exposure to crude oil contaminated food did lead to reduced growth in exposed fish (Christiansen and George, 1995). The SGR in July (T1-T2) was highest (0.6 ± 0.0% body weight change per day) in all treatment groups compared to all other periods (0.1–0.2% change per day) and may reflect some compensatory growth following handling and fasting during exposure (Ali et al., 2003). Reduced feeding and growth was also observed in African catfish (*Claris gariepinus*) for two months following exposure to crude oil and dispersed crude oil (Nwaizuzu et al., 2016).

Females likely to spawn in the coming winter season were in the late maturing (Vg II) stage with a GSI around 5.7 ± 0.3 while it is unclear when or if the females in the early maturing stage would spawn. The timing of spawning from other laboratory polar cod populations in an analogous reproductive stage suggests that the late maturing females would be ready to spawn in March (Bender et al., 2016). The high frequency of early maturing females may be an evidence of stress resulting in reduced investment into reproductive development (Rideout et al., 2005; Kime, 1995). However, with only a single histological

sampling point it is not possible to resolve if the females in the early maturation stage initiated vitellogenesis at the same time as females in the late maturing phase and then paused further development or if vitellogenesis was ongoing at a reduced pace. Nevertheless, abnormal oocyte development observed in some early maturing females (i.e. nonconforming yolk globule orientation) may suggest that vitellogenesis was interrupted and that these oocytes may soon be reabsorbed through atresia (Rideout et al., 2005). Reabsorbing vitellogenic oocytes result in a lower fecundity and have been observed in Atlantic cod under environmental stressors like low temperature, poor nutritional, and pollution (Rideout et al., 2005). However, no increased incidence of atresia was observed in early maturing females at sampling. The increased frequency of early maturing females in the BO exposure group could indicate a reduced population fecundity compared to the unexposed and control groups. The large variation in oocyte size of early maturing females exposed to BO treatment may be early signs of reabsorption of vitellogenic oocytes or of some other disruption of oogenesis. PAHs have endocrine disrupting properties with potential to impair vitellogenesis in fish (Hylland, 2006; Aruwke and Goksøyr, 2003). Despite low tissue PAH concentrations, reproductive impairment was seen in Gulf killifish two months after the Deepwater Horizon oil spill (Whitehead et al., 2012). Similarly, depressed plasma 17β-estradiol concentrations were seen in dolly varden and yellowfin sol after the Exxon Valdez oil spill (Sol et al., 2000).

Although the overall THC/PAH concentration in BO was an order of magnitude lower than in MDO and CDO, differences in physical characteristics of the BO may have altered the exposure route and time exposed to the BO treatment and thus enhanced the toxicity of the BO residue compared to MDO and CDO. Burned oil residues have increased viscosity and stickiness compared to crude oils (Fritt-Rasmussen et al., 2015; Fingas, 2016). The size of oil droplets and BO particles were not measured in this experiment; however, BO particles were most likely larger than MDO and CDO oil droplets as they could be observed with the naked eye as “black dots” in the water column during the exposure. In contrast to BO particles, mechanically and chemically dispersed oil droplets are generally found to be in the size of $\leq 100\,\mu m$ and 10–50 μm, respectively (Lessard and Demarco, 2000; Lewis and Daling, 2001), and could not be observed by eye. The BO residue may have clogged gills, adhered to skin, and/or been ingested and stick to the digestive tract of the fish and thereby increased the exposure time to PAHs and other compounds (including UCM) present in the BO residue compared to MDO and CDO. For instance, burned residues can be enriched in high molecular weight PAHs, pyrogenic PAHs, and metals (Buist, 2004; Shigenaka et al., 2015; Fingas, 2016). The UCM profiles of burned oil residues from the DWH burns exhibited an altered shape and lacked the more volatile *n*-alkanes when compared to the fresh unburned oil (Stout and Payne, 2016). These compounds were not quantified in the present study despite their potential contribution to adverse effects. Other studies investigating acute toxicity of BO residues have found non-toxic or little effects on snails and amphipods at concentrations below 1.46 mg/L THC or 5.83 μg/L total PAHs when exposed for 24 h (Gulec and Holdway, 1999). No additional effect of the WSF after burning on *Calanus* spp. when exposed for 96 h at concentrations less than 1 mg/L THC compared to the WSF prior to burning (Faksness et al., 2012). Australian bass exposed to burned oil WAF for four days did express EROD activity levels, a biomarker of PAH exposure, similar to levels in fish exposed to mechanically dispersed oil WAF but significantly lower than fish exposed chemically dispersed WAF (Cohen et al., 2006) However, knowledge on the toxicokinetics of BO compared to other treatments is still lacking and there is a strong uncertainty when it comes to toxicity of UCM.

Gonadal investment occurred earlier in males compared to females in accordance with other studies investigating polar cod reproductive development (Bender et al., 2016; Nahrgang et al., 2014). No effect of any treatment on the timing, structure, or investment in male reproductive development indicated the relative resilience of this sex. Male polar cod invest less energy in reproductive development compared to females (Hop et al., 1997), which may allow for greater tolerance to xenobiotic exposure during the reproductive development period. Inclusion of the unexposed fish into the experimental design provided additional information on background physiological change due to size differences. The smaller unexposed fish (Unexp. 1) were generally younger and less likely to mature in the current season, with an increased prevalence of immature individuals and lower HSI compared to their larger unexposed counterparts (Unexp. 2). Maturing individuals in Unexp. 1 had generally lower GSI values than maturing fish in larger size categories emphasising the importance of size in reproductive output (Nahrgang et al., 2014). The Unexp. 2 fish were of a similar age and larger size (both length and weight) than fish included in the exposure experiment but exhibited a higher mortality rate and no immature individuals further supporting the hypothesis that mortality is related to previous spawning events.

4.3. Conclusion and outlook

The transient effects observed on growth rate in the present study did not affect overall growth and survival of the polar cod during this 7-month experiment, demonstrating the robustness of adult polar cod. Early life stages of polar cod however have demonstrated increased sensitivity to crude oil (Nahrgang et al., 2016) and therefore are a major source of uncertainty when assessing population level impacts of a potential spill and response actions. The decreased frequency of maturing females exposed to the BO treatment is of importance with regard to potential reductions in population fecundity (Spromberg and Meador, 2006) and may reveal a sensitivity of polar cod when exposed to dispersed BO residues from this OSR countermeasure. This effect observed on the potential reproductive output of female polar cod exposed to BO is not explained by the relatively low THC and PAH levels measured in the BO treatment, therefore other hydrocarbon compounds, the UCM, physical properties and toxicokinetics of the BO residue warrants further investigation.

With increasing anthropogenic activity in the Arctic, polar cod are at risk for exposure to petroleum and OSR actions through accidental spills. The purpose of a NEBA is to aid in the decision making of an OSR and evaluate the environmental effects of an action or combination of actions *a priori*. However, no long-term effects on polar cod survival and growth were observed under acute dynamic exposure conditions to BO, MDO or CDO. Observed effects were overall limited. The physiological effects of BO need further investigation, including exposure method validation and additional chemical analysis. The reduction of overall oil by ~90% with *in-situ* burning will reduce the oil volume and the potential for organisms to come into contact with the oil and may still be a viable option despite the potential adverse effects observed in this study. Overall, this study demonstrates the robustness of the adult life stage of polar cod to a variety of OSR actions. The final endpoints of reproduction, such as fecundity, fertilization success and survival and fitness of offspring of exposed polar cod, were not included in the present study, however these endpoints would provide valuable information on ecosystem sensitive for the NEBA in the Arctic marine system. This study provides new evidence to aid in OSR decision making on the sensitivities of the Arctic key species polar cod.

Ethics statement

Permission to carry out this experiment was granted by the Norwegian Animal Welfare Authority in 2015 (ID 7851).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.marenvres.2018.09.005.

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Supplementary Information

Figure SI 1. (A) Testis from male polar cod undergoing maturation with late stage spermatocytes (Late Sc I); (B) Testis undergoing maturation with late stage spermatocytes and spermatid (Late Sc II); (C) Testis in an immature or early development stage; (D) testis in a resting stage with large open lumens and residual spermatid from previous spawning events. Scale bars represent 100 µm.

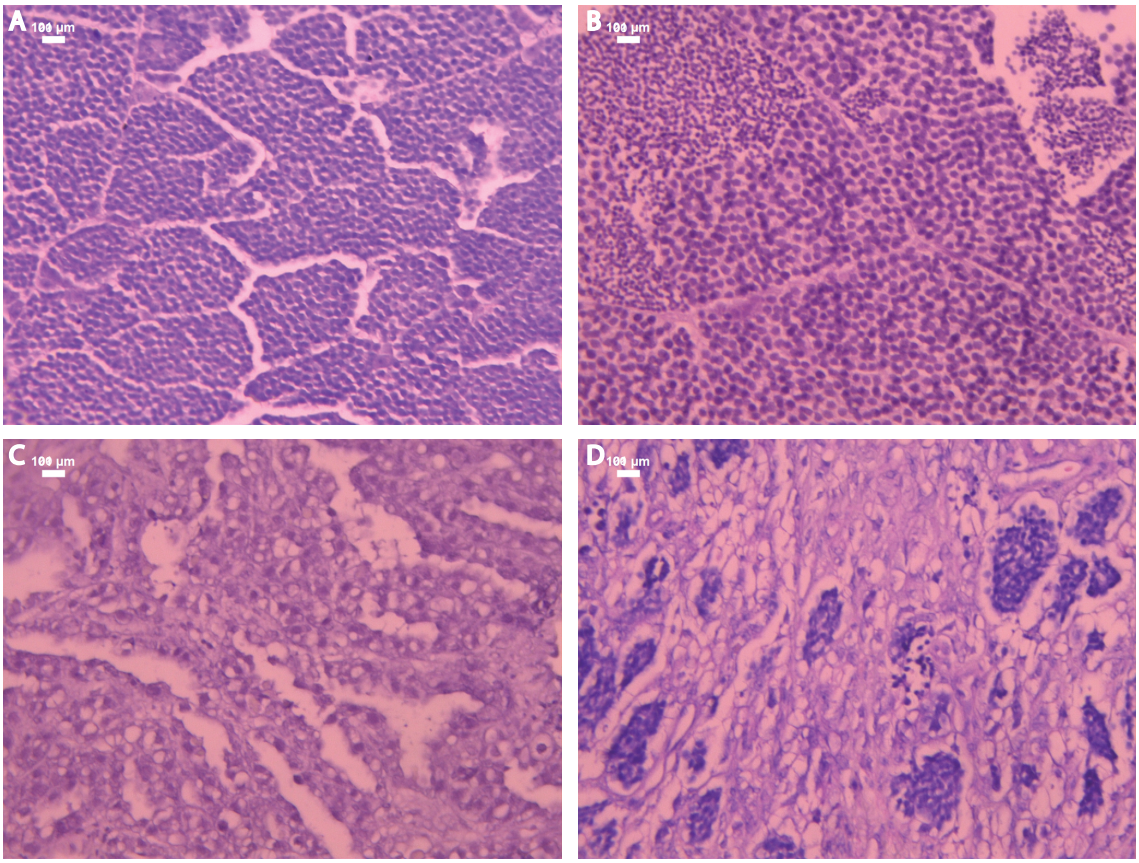

Table SI. Mean concentrations of 26 PAHs, Σ16 EPA PAHs and Σ26PAHs (µg/L water ± SEM), and total hydrocarbon concentration (THC, mg/ L water ± SEM) in water at 24 hours and 48 hours into exposure for each treatment (control, *In situ* burned oil residues [BO], mechanically dispersed oil [MDO], and chemically dispersed oil [CDO] treatment). Three replicates were analyzed for each treatment group. Values under limit of detection (LOD) are not included in Σ26PAH calculations. Values with no SE are singular replicates that exceeded the LOD.

	24 hour				48 hour			
PAH Composition	Control	BO	MDO	CDO	Control	BO	MDO	CDO
Naphthalene	0.28 ± 0.01	0.31	1.26 ± 0.30	1.11 ± 0.16	0.21 ± 0.005	0.25 ± 0.01	0.89 ± 0.31	0.74 ± 0.16
C1-Naphthalene	0.45 ± 0.02	0.54 ± 0.01	3.18 ± 0.13	3.15 ± 0.45	0.37 ± 0.02	0.43 ± 0.02	2.36 ± 0.46	2.41 ± 0.43
C2-Naphthalene	0.32 ± 0.02	0.50 ± 0.03	9.46 ± 2.46	12.36 ± 2.16	0.29 ± 0.02	0.44 ± 0.04	5.23 ± 0.82	9.09 ± 2.43
C3-Naphthalene	< 0.572	0.63 ± 0.04	22.85 ± 8.70	34.99 ± 5.11	< 0.572	< 0.572	13.03 ± 4.17	27.94 ± 7.66
Phenanthrene	< 0.090	0.10 ± 0.00	2.01 ± 0.68	2.92 ± 0.42	< 0.090	< 0.090	1.13 ± 0.28	2.19 ± 0.53
Antracene	< 0.009	< 0.009	0.03 ± 0.01	0.02 ± 0.00	< 0.009	< 0.009	0.015 ± 0.001	0.14 ± 0.12
C1-Phenanthrene/anthracene	< 0.146	0.26 ± 0.04	4.02 ± 1.59	6.68 ± 0.88	< 0.146	0.18	2.77 ± 0.96	6.05 ± 1.53
C2-Phenanthrene/anthracene	< 0.257	0.75 ± 0.30	7.24 ± 2.90	14.36 ± 2.10	< 0.257	0.59 ± 0.25	5.82 ± 2.06	13.89 ± 3.11
C3-Phenanthrene/anthracene	< 0.169	0.90 ± 0.32	6.42 ± 2.53	13.56 ± 1.80	< 0.169	1.08 ± 0.40	5.59 ± 1.96	15.17 ± 3.61
Dibenzothiophene	< 0.007	< 0.007	< 0.007	0.01 ± 0.00	< 0.007	0.01 ± 0.001	0.20 ± 0.05	0.36 ± 0.08
C1-Dibenzothiophene	< 0.025	0.41 ± 0.01	0.63 ± 0.25	1.14 ± 0.11	< 0.025	0.04 ± 0.01	0.45 ± 0.14	1.03 ± 0.27
C2-dibenzothiophene	< 0.051	0.21 ± 0.08	2.12 ± 0.83	4.82 ± 0.36	< 0.051	0.19 ± 0.08	1.76 ± 0.62	4.56 ± 1.12
C3-dibenzothiophene	< 0.041	0.29 ± 0.11	2.17 ± 0.86	4.64 ± 0.60	< 0.041	0.34 ± 0.14	1.90 ± 0.70	4.91 ± 1.09
Acenaphthylene	< 0.004	0.01 ± 0.00	0.004 ± 0.00	< 0.004	< 0.004	0.009 ± 0.001	< 0.004	0.004 ± 0.00
Acenaphthene	< 0.013	< 0.013	0.05 ± 0.01	0.06 ± 0.01	< 0.013	< 0.013	0.038 ± 0.006	0.05 ± 0.01
Fluorine	< 0.044	< 0.044	0.28 ± 0.07	0.37 ± 0.05	< 0.044	< 0.044	0.172 ± 0.22	0.27 ± 0.05
Fluoranthene	< 0.040	< 0.040	0.18 ± 0.00	0.25 ± 0.02	< 0.040	< 0.040	0.10 ± 0.03	0.22 ± 0.04
Pyrene	< 0.200	< 0.200	< 0.200	0.28 ± 0.06	< 0.200	< 0.200	< 0.200	0.34
Benzo(a)anthracene	< 0.025	< 0.025	0.05 ± 0.00	0.07 ± 0.01	< 0.025	< 0.025	0.044 ± 0.01	0.08 ± 0.01
Chrysene	< 0.053	< 0.053	0.16 ± 0.00	0.22 ± 0.03	< 0.053	< 0.053	0.13 ± 0.03	0.23 ± 0.05
Benzo(b)fluoranthene	< 0.018	0.03	0.06 ± 0.00	0.01 ± 0.01	< 0.018	0.028	0.04 ± 0.01	0.10 ± 0.01
Benzo(k)fluoranthene	< 0.007	0.01	0.01 ± 0.00	0.001 ± 0.00	< 0.007	< 0.007	0.01	0.013± 0.002
Benzo(a)pyrene	< 0.034	< 0.034	< 0.034	0.04	< 0.034	< 0.034	< 0.034	0.045
Indeno(1,2,3-cd)pyrene	< 0.011	< 0.011	< 0.011	< 0.011	< 0.011	< 0.011	< 0.011	< 0.011
Benzo(ghi)perylene	< 0.029	< 0.029	< 0.029	< 0.029	< 0.029	< 0.029	< 0.029	0.037
∑ 16 EPA, µg/L:	0.28 ± 0.01	0.40 ± 0.05	3.95 ± 0.62	5.32 ± 0.80	0.21 ± 0.01	0.27 ± 0.01	2.69 ± 0.28	4.50 ± 1.04
∑26 PAHs, µg/L:	1.05 ± 0.02	3.49 ± 1.23	62.36 ± 20.71	101.45 ± 14.33	0.88 ± 0.03	2.71 ± 0.56	41.60 ± 11.21	89.56 ± 22.19
THC, mg/ kg water	0.00 ± 0.00	0.73 ± 0.43	9.82 ± 4.22	22.1 ± 2.37	0.00 ± 0.00	1.05 ± 0.63	8.55 ± 3.23	22.98 ± 4.99

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Paper 4

INTERACTIVE EFFECTS OF CRUDE OIL POLLUTION AND WARMING ON POLAR COD EARLY LIFE STAGES

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ABSTRACT

Spawning areas of the key Arctic forage fish, Polar cod (*Boreogadus saida*), are "hotspots of warming". Concurrently, reduced sea ice has enabled increases in both human activity and the risk of petroleum pollution in these regions. In an *ex-situ* multifactorial experiment, we show a more severe physiological sensitivity of polar cod early life stages to the water-soluble fraction of crude oil in conjunction with a 2.3ºC increase in water temperature. Higher incubation temperatures elicited responses in mRNA expression of biotransformation and stress-related genes in embryos, decreased the duration of embryogenesis, and led to increased larval growth rates. Embryogenic exposure to low levels of crude oil (equating to maximum initial concentrations of 240 ng/L total polycyclic aromatic hydrocarbons in the water) led to reductions in egg buoyancy and cardiac activity, higher mortality in larval stages, deformities of the jaws, eyes, and spin and edema, lower incidence of feeding and swim bladder inflation, and slower growth. The interaction of increased temperature and crude oil exposure resulted in higher rates of malformation and lower survival of larvae. In the future, a warmer, more active Arctic may threaten the sensitive early life stages of this key circumpolar forage fish.

Keywords (6-10 words): Arctic, polar cod, early life stage, climate change, egg buoyancy, physiology, deformities, synergy, multiple stressors

INTRODUCTION

The Arctic is undergoing profound environmental change with declining sea ice, higher surface temperatures, changes in community structure, and increased human activity (IPCC, 2019; Carmack et al. 2015). Climate change and environmental pollution can act synergistically to challenge an organism or population beyond the degree of any single stressor (Crain et al. 2008). Climate change has been seen to amplify the adverse effects of exposure to low levels of pollutants in Arctic wildlife (Borgå, 2019), which may have the potential for population-level effects. Identifying and quantifying the cumulative effects of anthropogenic stressors on organisms, populations, and ecosystems enables more informed societal, political, and economic decision-making (Cotè et al. 2016; Chapin et al. 2000).

One such species experiencing environmental stress is polar cod (*Boreogadus saida*), an endemic circumpolar Arctic species with a large population biomass, high energy content, high trophic connectivity, and high societal value despite a limited commercial harvest (Steiner et al. 2019; Kortsch et al. 2015; Harter et al. 2013;). Polar cod stocks in the Barents Sea, Iceland-East Greenland waters, parts of the Bering Sea, Canadian Arctic Archipelago, and in Disko Bay (Greenland) have been in decline and retreating northward with increased water temperatures (Steiner et al. 2019 and references within; ICES, 2018; Eriksen et al. 2015; Astthorsson, 2015; Divoky et al. 2015; Hansen et al. 2012). Climate-driven changes in life-history traits have already been observed in mature polar cod (Nahrgang et al. 2014). However, fish early life stages (ELSs) are more vulnerable, owing to physical and biological factors (Pörtner and Farrell, 2008; Pollino and Holdway, 2002). To ensure survival and a strong year class, pelagic eggs and larvae must maintain their position in the water column through buoyancy control and swim bladder inflation, be able to forage when yolk reserves are exhausted and be able to grow quickly and build up reserves for overwintering while avoiding

63 predation (Miller and Kendall, 2009). The positively buoyant embryos and larvae of polar cod
64 have limited mobility and a shallow vertical distribution (Bouchard et al. 2017). These factors
65 put fish ELSs at a higher risk for direct exposure to increased sea surface temperatures and
66 pollution events in surface waters (Geoffroy et al. 2015). Additionally, fish ELSs are generally
67 more sensitive than juveniles or adults to temperatures outside their thermal window (Pörtner
68 and Farrell, 2008) and toxicants (Petersen and Kristensen 1998; Pollino and Holdway, 2002).
69 Sensitivity is attributed to the relatively underdeveloped organs and lower metabolism during
70 early ontogeny (Jung et al. 2015; Ingvarsdottir et al. 2012; Paine et al. 1992), and a large surface
71 area to volume ratio compared to adults (Miller and Kendall, 2009). Exposure of fish eggs and
72 larvae to increased temperature can increase enzyme activity, increased metabolic rate, and
73 accelerate development while decreasing the efficiency of metabolism and reducing the energy
74 available for development (Dahlke et al, 2018; Polotis et al. 2017). Furthermore, elevated
75 temperature decreases the duration of embryogenesis, reduces size and survival of hatchlings
76 (Hansen and Falk-Petersen 2001), increases larval growth rate (O´Dea et al. 2019), and
77 increases the frequency of deformities in polar cod (Graham and Hop, 1995) and many other
78 fish species (Réalis- Doyelle et al. 2016, 2018; Wargelius et al. 2005).
79
80 Few direct observations have been made of the ice-covered waters where polar cod spawn
81 during the winter months (Mueter et al. 2016; Sakuri et al. 1998). However, models derived
82 from the back-calculated drift of larvae agree with field collections of maturing females and
83 identified spawning areas of the European Arctic in Northwest Barents and Kara Sea (Eriksen
84 et al. 2019). Remarkably, these spawning areas are in a “hotspot of global warming” (Lind et
85 al. 2018), where the loss of sea ice and warming are occurring more rapidly (Screen and
86 Simmonds, 2010). Warmer Atlantic water masses (>2.5°C) are predicted to replace the cooler
87 Arctic waters (<1°C) before the end of the century (Lind et al. 2018). Similarly, climate models

using the IPCC business-as-usual RCP 4.5 climate change scenario predict a 1°C increase in sea surface temperature in the next 50 years around Svalbard (Hanssen-Bauer et al. 2018).

Concurrently with rising sea surface temperatures, declining sea ice extent has facilitated ship traffic in the Arctic region to increase by 60% since 2012 (Champine et al. 2019).Especially in the Barents Sea and along the Northern Sea Route and Northwest passage route (Eguíluz et al. 2016), both of which traverse polar cod spawning areas (Eriksen et al. 2019; AMSA Report 2009). These activities increase the risk of petroleum pollution (Congressional Research Service, 2018). Exposure to petroleum pollution in developing fish is hypothesized to act through multiple pathways causing developmental toxicity and sublethal effects that persist to later life stages even at very low concentrations (Laurel et al. 2019; Mager et al. 2014, Hicken et al. 2011; Carls and Meador, 2009; Carls et al. 1999). Temperature affects biotransformation and elimination processes in an organism (Andersen et al. 2015). Exposure to crude oil triggered gene pathways involved in biotransformation, stress response, lipid metabolism, and ion regulation in Atlantic haddock ELSs (Sørhus et al. 2017). On the organismal level, crude oil exposure has resulted in reductions in larval size, defects of the heart, increased incidence of edema and deformities, reduced growth, and reduced survival in many fish species (Pasparakis et al. 2016; Incardona et al. 2015; Carls et al. 1999), including polar cod (Laurel et al. 2019; Nahrgang et al. 2016).

Most studies investigating the cumulative effects of temperature and crude oil exposure on ELS of fish have focused on warm water species, such as Mahi-mahi (*Coryphaena hippurus*), especially in the wake of the Deepwater Gulf Horizon oil spill in 2010. Greater metabolic demands and higher frequency of deformities, altered cardiac function, and changes in embryo buoyancy were reported as synergistic effects of these two stressors (Perrichon et al. 2018;

113 Pasparakis et al. 2016). Increased temperature may increase the metabolic demand leaving
114 limited energetic reserves for detoxification processes in fish ELSs (Pasparakis et al. 2016) and
115 increasing the potentially toxic effects of petroleum exposure. Exposure to acidified ocean
116 conditions led to a narrowing of the embryonic thermal window in polar cod (Dahlke et al.
117 2018).
118
119 In light of the environmental change and pollution risk present in the Arctic (Borgå, 2019) and
120 the vulnerability of fish ELSs to environmental stressors, a holistic investigation on the effects
121 on the health of sensitive species in the changing Arctic was warranted. We conducted an *ex-*
122 *situ* experiment where we exposed polar cod ELS to increased temperatures and crude oil
123 pollution at environmentally realistic levels starting after egg fertilization and continuing into
124 exogenous feeding larval stages for 170 days.
125
126 Experimental conditions were designed to mimic an oil spill at three concentrations of the
127 water-soluble fraction of crude oil across two thermal conditions, 0.5°C (contemporary) and
128 2.8°C (future, year 2100). We hypothesized that (a) water temperature would affect the
129 concentration of petroleum compounds in the water, bioaccumulation in embryos, and the rate
130 of development; (b) exposure to crude oil would negatively affect development,
131 osmoregulation, growth, and survival in early life stages; and (c) simultaneous exposure to
132 crude oil and warmer water temperatures would act synergistically, leading to more severe
133 physiological outcomes than any singular stressor. We investigated several vital consequences
134 of petroleum exposure for polar cod ELSs including bioaccumulation, biotransformation, the
135 molecular stress response, egg buoyancy, cardiac activity, growth, survival, developmental
136 rate, and deformities. Endpoints were compared across crude oil concentrations and

temperature groups to ascertain the possible additive, antagonistic, or synergistic effects at given ontogenic stages.

METHODS

The collection of wild polar cod and experimentation with feeding larval stages was carried out with approval from the Norwegian Fisheries Department (17/14068) and The Norwegian Food Safety Authority (17/243813), respectively.

BROODSTOCK COLLECTION AND HUSBANDRY

Mature polar cod were captured south of Northeast Land, Svalbard (78° 55´N, 23° 40´E) using a bottom trawl fitted with a live fish box (Holst and McDonald 2000) aboard the RV *Helmer Hanssen* in November of 2017. Fish were kept on deck in four 400L flow-through, covered seawater tanks during the eight-day transport to the Biological Station in Tromsø. Broodfish were acclimated to the lab in a 4000L tank under a natural daylight cycle of 69°N at 2.3-2.5°C, fed to satiation with frozen wild *Calanus* zooplankton (Calanus AS) and treated with Halamid® disinfectant solution (0.01 g/L) as needed. Daily checks of fish readiness, done by attempting to strip spawn anesthetized females, were made to determine the optimal spawning time in the week before the start of the experiment.

OIL EXPOSURE PREPARATIONS AND CHEMICAL ANALYSIS

Seawater was contaminated by contact with oiled gravel to form the water-soluble fraction of crude oil (Nahrgang et al. 2016; Carls et al. 1999; Incardona et al. 2015). Gravel was cleaned before oiling by first sieving (4-8 mm diameter), soaking in 1M HCl for two hours, overnight immersion in 90% ethanol, and then dried at 60°C. Gravel was then mixed with fresh Kobbe crude oil (provided by SINTEF) at concentrations of 0, 0.19, 0.75 and 3 g crude oil/ kg gravel (corresponding to control, low, medium and high treatments, respectively) and set to dry at -4°C for 72 h. Polyvinyl chloride (PVC) cylinders (n=8, 1 m tall) were loaded with 11 kg of oiled gravel (or clean control gravel) with two cylinders for each oiled gravel concentration.

Cylinders were further divided into two temperature groups, such that each temperature group contained one of each of the four oiled gravel concentrations. Cylinders were capped with aquarium filter floss to catch any oil droplets and sealed to prevent overflow. Seawater, filtered at 20 µm and UV cleaned at 34 PSU, was cooled to either 0.5 ± 0.4°C or 2.8 ± 0.27°C (now referred to as "warm" and "cold" treatments, respectively) and fed from two 300 L header tanks to ensure stable temperatures. The cooled seawater was flushed up through the gravel at 100 L/ h for 76 h to allow removal of the most volatile oil compounds such as the acutely toxic BTEX (benzene, toluene, ethylbenzene, and xylene) compounds. Cylinders were prepared in advance of the experiment start and were frozen at -20°C after initial flushing. Immediately before the start of exposure, cylinders were thawed, and outflow from each cylinder was distributed into four conical incubators (25 L capacity; flow at 25 L/h, constant aeration); one cylinder was used per oil/ temperature combination (n=8) (Fig. S1). The experiment included 32 incubators. Incubators were randomly distributed with regard to crude oil exposure within the two temperature treatments. Temperature and light levels were logged every minute in two random incubators at each temperature group (Fig. S2), and oxygen was monitored bi-weekly (>98 % of saturation). The light cycle in the experimental room mimicked that of 80°N.

EXPERIMENTAL SETUP

In January 2018, mature Polar cod were randomly selected from the broodstock in the lab, sedated (MS 222, 50 mg/ L), and strip spawned. Visually viable eggs from 49 females and milt from 21 males were collected. All milt samples were pooled 1:1. Eggs from each female were divided into two groups of the same volume and pooled 1:1. Fertilization of each egg group was conducted within a temperature-controlled water bath (either 0.5°C or 2.8°C) by adding half of the milt pool and 3 L of seawater. After 10 minutes, egg groups were rinsed of milt and distributed across 16 incubators (28 mL/incubator) corresponding to the fertilization temperature. Each incubator held 8988 ± 1066 eggs at the start of the experiment. Developing

187 embryos were followed for 170 days post fertilization (dpf); however, this work presents
188 endpoint measurements from the egg development to first feeding within each temperature
189 group (52 dpf at 2.8°C and 76 dpf at 0.5°C). The developmental progress of embryos was
190 tracked daily by imaging three eggs or larvae from two additional incubators held at each
191 corresponding temperature. Embryos were photographed at multiple magnifications using a
192 Leica M205 C stereomicroscope and camera (Leica, MC 170 HD). Sampling events were
193 performed at similar developmental stages in the two temperature groups based on the stage
194 determined from eggs and larvae in the additional incubators. Staging of embryogenesis
195 (cleavage stage, blastulation, gastrulation, and organogenesis) and of the larval stages (yolk
196 sac larvae and preflexion/feeding larvae) is described in greater detail in Table S1. Collection
197 and enumeration of dead or dying eggs or larvae from the bottom of all incubators was done
198 every 24 h. Instantaneous mortality rates (M) were calculated for the developmental stages at
199 each temperature group according to the development of the control embryos or larvae from
200 the additional incubators using the equation:

201 $$\mathrm{M} = \frac{\ln N_t - \ln N_o}{-t} \quad (1)$$

202 where No is the abundance at the start of the developmental period, Nt is the abundance at the
203 end of the developmental period, and t is the number of days spent in the developmental period
204 (Houde, 1989). Abundance was determined using a total tally of dead, sampled, and alive
205 larvae over the entire experiment. Mortality rates from the preflexion period only accounted
206 for the larvae that perished up until day 52 at 2.8°C and day 76 at 0.5°C.
207
208 Enriched rotifers (5 ind/ml density in incubators) and green water (Nanochloropsis, Reed
209 Mariculture) were introduced to the incubators when jaw formation was observed at each
210 temperature group (35 dpf and 56 dpf at the warmer and colder treatments, respectively; Fig.

S2). Larvae were weaned onto Artemia nauplii (2 ind/ ml density in incubators) as their intestines began to curl (47 dpf and 60 dpf at the warmer and colder treatments, respectively).

ANALYTICAL CHEMISTRY

WATER COLLECTION AND POLYCYCLIC AROMATIC HYDROCARBON (PAH) EXTRACTION

Water samples for chemical analysis were taken directly from the cylinder outflow at the start of the experiment (day 0) and days 4, 10 (warm treatment only), 18, 28 (warm treatment only), and 56 for each temperature and crude oil concentration (n=8 total samples). Samples were acidified (15% HCl) and stored in the dark at 4°C until analysis. The organic phase was extracted from the water by first transferring the entire sample to separatory funnels and rinsing sample bottles with dichloromethane (DCM, 60-90 mL depending on sample size). Surrogate internal standards (25.08 ng naphthalene-***d***8, 5.00 ng phenanthrene-***d***10, 4.86 ng chrysene-***d***12 and 5.08 ng perylene-***d***12) were added to each sample. Different sample phases were allowed to separate after shaking, and the organic phase was transferred to a flask with sodium sulfate. This was repeated two more times before the organic phases were combined and concentrated using a TurboVap® evaporator. Before analysis, recovery internal standards (fluorene-d10 and acenaphthene-d10) were added to samples. A laboratory blank (MilliQ water) was included with each sample set.

EMBRYO BODY BURDEN AND PAH EXTRACTION

On day 4 and day 18, viable eggs (n=25) were sampled from each incubator and combined with another incubator of the same treatment such that each unique treatment was represented by two samples of 50 eggs. Samples were immediately frozen at -80°C until analysis. Upon analysis, samples were weighed within 0.01 mg accuracy and extracted following a method developed by Sørensen and colleagues (2016). Briefly, surrogate internal standards (same as for water samples) and sodium sulfate were added before samples were homogenized with *n*-

hexane-DCM using a disperser (IKA 10 basic ULTRA-TURRAX®), vortexed and centrifuged. Supernatants containing the organic extracts were collected and cleaned using solid-phase extraction with silica (500 mg, Agilent Bond Elut SI, Agilent Technologies, USA), and eluted with DCM in *n*-hexane (1:9, v/v, 6 mL). Purified extracts were then concentrated on a heat block (40°C) under a gentle N^2 steam. Prior to analysis, recovery internal standards (same as for water samples) were added to the purified extracts. A laboratory blank (MilliQ water) was included with each sample set.

PAH IDENTIFICATION AND QUANTIFICATION

Samples were analyzed by an Agilent 7890 gas chromatograph (GC) coupled with an Agilent 7010B triple quadrupole mass spectrometer fitted with an EI source (70eV) and collision cell (Agilent Technologies, Santa Clara, CA, USA). Two GC-columns were coupled in series through a purged ultimate union. Samples (1µL) were injected at 310°C splitless and carried by high purity helium at a constant flow. The temperature started at 40°C and increased in intervals until reaching 330°C when the first column was back-flushed. N_2 was used as collision gas and helium was used as a quench gas. Target PAHs were identified by two unique multiple reaction monitoring transitions and quantified by the most intense peak (Sørensen et al. 2016). Alkyl PAH clusters were determined by multiple reaction monitoring using transitions from the molecular ion, as described by Sørensen and colleagues (2016b). Parent PAH compounds were quantified by quadratic regression of a 12-level calibration curve (0.01-250 ng/mL), while alkyl PAH groups were quantified by the response factor calculated for a methyl-substituted PAH reference compound (Sørensen et al. 2016b). Standards were run for each of the 12 sample injections. The level of detection is reported in Table S2 for each PAH and ranged from 0.0001 -1.75 ng/L (mean 0.143 ng/L). Concentrations are described using total PAHs (tPAHs) as clusters of alkylated homologs. The bioaccumulation of compounds internally is compound, species, and life stage specific and can be measured through the

bioconcentration factor (BCF) to provide a more direct link to potential toxic responses (Meador et al. 2008). The BCF was calculated using PAH levels in the water and the mean embryo PAH levels at day 4 and day 18 using the following equation:

$$\mathrm{BCF} = \frac{PAH\ concentration_{embryos}}{PAH\ concentration_{water}} \quad (2)$$

Predicted BCF values were calculated for comparison purposes using a log K_{ow} (n-Octanol/Water Partition Coefficient) equation: (Veith et al. 1979)

$$log\ BCF = 0.85 * log\ Kow - 0.7$$

TWO-DIMENSIONAL GAS CHROMATOGRAPH - MASS SPECTROMETRY

Water samples from the high and control treatment at day 0, 4, and 10 at both temperatures were analyzed by an Agilent 7890B gas chromatograph coupled with an Agilent 7250 quadrupole time of flight mass spectrometer fitted with an EI source and collision cell (Agilent Technologies, Santa Clara, CA, USA). Samples (1µL) were injected at 250 °C splitless. The carrier gas was high purity helium at constant flow (1 mL/min). First dimension separation was achieved using an Agilent J&W DB-1MS column (30 m × 0.25 mm x 0.25 µm). A Zoex ZX2 cryogenic modulator was used to trap and transfer continuous fractions from the first to the second-dimension columns. The hot jet pulse was constantly +50 °C offset from the oven temperature with a pulse of 350 ms. Second dimension separation was achieved using an Agilent J&W DB-17MS column (1.5 m × 0.25 mm x 0.25 µm). The oven temperature was kept at 90 °C for 1 min, then ramped to 300 °C by 2 °C/min and held for 5 minutes. The transfer line temperature was 300°C and the ion source temperature was 230 °C. The source was operated at 70 eV, scan speed was 50 Hz and scan range 50-450 m/z. Data were collected in Agilent Masshunter and processed using GCImage®.

EMBRYOGENIC ENDPOINTS

GENE EXPRESSION

Prior to 50% hatch in both temperature groups (day 28 at 2.8°C and day 48 at 0.5°C), 100 eggs were randomly sampled out of each incubator and frozen at -80°C for gene expression analysis. Quantitative reverse-transcription PCR was performed for 4 genes (*cyp1a, cyp1c1, hsp8,* and *hsp70*) on RNA extracted from embryos after homogenization in Trizol buffer and the addition of chloroform. RNA was extracted and cleaned using an RNeasey mini kit, with additional DNAase treatment. Between 706 – 4195 ng/µl RNA was extracted from each sample (mean 2868 ng/µl RNA) with 500 ng of RNA used to reverse transcribe to cDNA. Control genes were validated by non-significant treatment effect on cycle threshold values. Relative fold change was calculated using the efficiency-adjusted ΔΔCt method (Pfaffl 2001) from 3 control genes (*b actin, elf1a,* and *rpl4*) and geometric means of all control genes are reported.

EGG SINKING SPEED

Twenty eggs were randomly sampled out of each incubator prior to peak hatch in both temperature groups (day 29 at 2.8°C and day 56 at 0.5°C). Eggs were transferred with a minimum amount of water to a 100 mL glass graduated cylinder filled with diluted seawater (28 ‰) at either 0.5°C or 2.8°C and the time it took for the egg to sink 5.5 cm was recorded. The time and distance that eggs descended in the water column were converted into a sinking speed (cm/s). Further conversion of sinking speed to specific egg density was performed using Stoke's Law of fluid dynamics in the following equation:

$$\text{Egg density } \left(\frac{\text{g}}{\text{ml}}\right) = \frac{18\,\mu * \text{sinking speed } \left(\frac{\text{cm}}{\text{s}}\right)}{g d^2} + \rho w$$

Where the kinetic velocity of seawater (µ) at 28 ‰ was extracted from table 25 in Riley and Skirrow (1975) based on temperature, g is the acceleration due to gravity, an average egg

diameter (d) of 1.63 mm measured from a sub-sample of eggs, and ρw is the density of seawater (Vesilind et al. 1994; Laurel et al. 2010).

SAMPLING OF YOLK SAC LARVAE

The proportion of larvae to eggs was followed over time by taking a sample from the incubator and enumerating eggs and larvae during the period where both were observed simultaneously in the incubators. Early in the hatching period of both temperature groups, 20-30 newly hatched yolk sac larvae were randomly sampled out of each incubator, sedated, and photographed at 1.6x magnification. Total length (to the nearest 0.01 mm), yolk sac area (traced from picture, in mm^2), and presence of yolk sac edema were assessed from pictures for each individual larva using ImageJ (Rasband, 2015).

CARDIAC ACTIVITY

Newly hatched yolk sac larvae were sampled from each incubator at 30-31 dpf for 2.8°C and 48-49 dpf for 0.5°C (n=3 / incubator). Larvae were placed laterally on the left side in a watch glass with 500µl of seawater set on a stage thermally controlled by a circulating cooling bath calibrated at 2.8°C or 0.5°C. Each larva was video recorded for one minute at 4x magnification under a stereomicroscope. Heart rate was determined by counting heartbeats within a 30 second time window using a manual counter. Arrhythmia was calculated using the number of frames between beats for a 20 second period and the standard deviation between the first seven beats recorded. All analysis was done blindly in reference to crude oil treatment and all recordings were analyzed by the same observer.

SAMPLING OF FEEDING LARVAE

Following confirmation of yolk sac absorption and initiation of first feeding in the additional control incubator at each temperature (day 52 at 2.8°C and day 76 at 0.5°C), 30 larvae were randomly sampled from each incubator, anesthetized, and photographed at 1.6x magnification.

Information on larval length (ImageJ), swim bladder inflation (absence/presence), feeding success (scored from 0-3: with 0 being an empty stomach, 1 containing a single food particle, 2 containing multiple food particles and 3 being a full stomach) and incidence of deformities was extracted from the images for each larva. Prevalence of deformities of the eyes, jaws, spine, and pericardial edema was quantified using absence (0)/ presence (1) for each larva. An index was calculated for each incubator using the number of fish with a given condition divided by the total number of fish sampled from each incubator (Wassenberg and Digulio, 2004). The specific growth rate was calculated using incubator-averaged length measurements taken from pictures of yolk sac larvae following hatch (L_o) and feeding larvae (L_t) of each temperature group and accounting for the period of time between the time points (t) in the following equation:

$$\text{Specific Growth Rate (\% length } day^{-1}) = 100 * \frac{\ln L_t - \ln L_o}{t} \quad (3)$$

STATISTICAL ANALYSIS

Statistical analysis to assess the effect of increased temperature, crude oil exposure and the interaction of these two stressors was performed in R (R Core Team, 2018) using the `nlme ´ package (Pinheiro et al. 2020). Water chemistry results were modeled using a simple linear model and a three-way interaction term, including time and treatments, and a Pearsons correlation was run on initial concentrations. A linear mixed effect model (lme) was run for single measures in each incubator (e.g., survival, body burden, gene expression, growth rate and deformity scores). A generalized least squares linear model (gls) was used in instances when measurements were made on single organisms within an incubator (e.g., embryo sinking speed, length and yolk sac measurements, and cardiac activity measurements). The gls model with compound symmetric correlation within the incubator assumes constant correlation between the eggs/larvae in the incubator but not between incubators and takes into account the

355 possible inequality of variance in the observations (Pinheiro and Bates, 2000). Temperature
356 and crude oil treatments were always treated as factors in models to reveal nonlinear trends in
357 concentration response. To test the statistical significance of possible interactions between the
358 stressors of temperature and crude oil treatment we used log-likelihood ratio tests to compare
359 model with all fixed effect interaction against models omitting the interaction (Zuur et al.
360 2009). An ANOVA was run on the best model fit and reported together with the best fit model
361 in the text of each figure. Model coefficients were extracted from models with significant
362 interactions to assess whether interactions acted antagonistic or synergistic for each treatment
363 combination (Folt et al. 1999). An interaction coefficient <0 was categorized as congruent with
364 stressor antagonism, a coefficient >0 was congruent with stressor synergism. Responses that
365 did not have significant interaction terms when significant effects were present in both
366 temperature and crude oil treatments revealed additive effects. The normality of model
367 residuals was checked and non-normally distributed data (e.g., length and egg sinking speed)
368 were log-transformed. Treatment effects from all models are reported in the Supplementary
369 Information (Table S5), best model parameters and ANOVA results (F-values and p-values)
370 are displayed in each figure. “Statistical significant” is not stated in the text in an effort to
371 reduce the dichotomous interpretation of responses, rather, model outputs are reported and
372 trends are discussed in terms of biological significance (Wasserstein et al. 2019; Amrhein et
373 al. 2017). Correlations were run for feeding larvae biomorphometrics and deformities using
374 individual paired assessments and the non-parametric Spearman method. All effects reported
375 in the results are where p-values <0.05 for reading clarity. Values are displayed as mean ± 1
376 SEM, and an arbitrary significance level was set at p-values <0.05.
377

RESULTS

TEMPERATURE AND CRUDE OIL EXPOSURE

From fertilization to first feeding, temperature in the two temperature groups differed by approximately 2.3ºC with the warm group at 2.79 ± 0.27°C (hereafter referred to as 2.8°C or 'warm' treatment) and cold group at 0.54 ± 0.4°C (hereafter referred to as 0.5°C or 'cold' treatment). Following the flushing of the oiled rock cylinders and start of the exposure, tPAH concentrations (calculated from 44 parent and alkylated PAHs) were generally low (<1 part per billion), correlated with crude oil concentration in gravel ($R(6)=0.93$, p-value<0.01), and declined over time (Fig. 1, Table 1, Table S2.). The highest concentrations of tPAHs were measured at the start (day 0) in the warm, high oil treatment (237 ng/L tPAHs) followed by the cold, high oil treatment (140 ng/L tPAHs). For the hatching period (day 28 in warm and day 56 in cold incubated treatments), all treatments had reached tPAH levels ~1 ng/L except for the highest crude oil exposure at both temperature groups (4-5 ng/L tPAHs). While no particulate oil was observed in the effluent nor was any sheen visible on the surface of the incubators, analysis of water samples taken from the warm, high exposure effluent by comprehensive two-dimensional GCxGC-MS analysis of the exposure effluent showed a typical crude oil profile of the exposure media. This indicates the presence of 'bulk' oil in the exposure system, likely in the form of microdroplets (Fig. S3). In addition to the PAHs and alkylated homologues, several petroleum compound groups were observed in the extracts, including *n*-alkanes, branched and cyclic aliphatics, as well as a complex set of monoaromatic compounds in this mostly qualitative analysis (Fig. S3). While steadily decreasing, the oil-like profile remained until day 10. No 'bulk' oil profile was observed in the cold treatment, although tPAH concentrations were similar enough to the warm treatment that the bulk oil profile should

have been detected if present. The presence of bulk oil in the warm treatment is also supported by the elevated concentration of higher molecular weight PAHs (12.98 ng/L; Table S2).

EFFECTS ON EMBRYOS

Accumulation of tPAHs in embryos was elevated in the warm, high oil treatment (Fig. 2, Table S3ab). The highest levels of tPAHs in embryos were measured in the warm, high oil treatment (843.0 ± 193.45 ng/g ww (mean ± SEM)) at day 4 (Table 1); accumulated tPAH levels in this treatment decreased by 60% over a two-week period (337.7 ± 32.7 ng/g ww tPAHs at day 18). Embryos in the cold, high oil treatment accumulated 459.4 ± 24.5 ng/g ww tPAHs by day 4 and these levels were elevated by 6% on day 18 (489.3 ± 6.9 ng/g ww). Bioconcentration factors (BCFs) calculated for naphthalenes, phenanthrene (including alkylates), and pyrene are similar across temperatures and crude oil exposure concentrations. Yet, at day 18 the high oil, cold group had consistently higher BCFs of alkylated phenanthrenes and pyrene (Fig. S4, Table S4).

During the period of fertilization through gastrulation, no difference in mortality was seen in temperature groups or crude oil treatments with mean instantaneous mortality rates with a range of 0.013-0.020 day^{-1} (Fig. S5). During organogenesis, the mortality rates decreased for all treatments (0.0008 - 0.0057 day^{-1}; Fig. S5); however, exposure to high oil and warmer water temperature negatively affected survival in embryos in an additive manner.

Synergistic effects of the warm, high oil dose were measured for both *cyp1a* (Fig. 3a), with a maximum relative fold change of 52, and for *cyp1c1* (Fig. S6a), with a maximum relative fold change at 7.6. The effects of oil exposure and temperature were measured in *hsp 70* (Fig. 3b) with a maximum relative fold change of 1.3, and no effects were observed in *hsp 8* (Fig. S6b).

426 Exposure to crude oil resulted in increased density of embryos by as much as 0.002 g/ml in the
427 cold, high oil treatment (97% faster sinking speed in the dilute seawater medium) compared to
428 the cold control group when calculated using the average of four incubator means (Fig. 4). In
429 the warm group, the high oil treatment embryos sank 14% faster (0.001 g/ml heavier) than
430 control embryos. The effects of oil and temperature were seen in embryo density with the
431 strongest effects observed in the cold water incubated embryos. Cold reared embryos and those
432 exposed to the medium and high oil treatments had higher densities.

433 EFFECTS ON YOLK SAC LARVAE

434 The warm incubated embryos hatched earlier and over a shorter duration (day 27 - 32)
435 compared to the cold group (day 43-65; Fig. S2). In the yolk sac stage, warm temperature and
436 high crude oil exposure acted synergistically on larval mortality (Fig. 5a). Adverse effects of
437 oil treatment and temperature were observed in yolk sac larval length with no strong interaction
438 (Fig. 6a). On average, the warm, control yolk sac larvae had 60% greater yolk sac area (Fig.
439 S7) and were 20% shorter relative to the cold, control yolk sac larvae (Fig. 6a). Embryos in the
440 cold, high oil treatment hatched at a reduced length (5.55 ± 0.03 mm) compared to the control
441 larvae of the same temperature (6.01 ± 0.03 mm), but no such trend was seen at warm treatment
442 (4.81 ± 0.04 mm and 4.45 ± 0.05 mm for control and high treatment, respectively). Yolk sac
443 area was only affected by temperature (Fig. S7). Temperature affected the development of
444 embryos in a nonlinear fashion with the cold reared group developing more slowly in the later
445 phases of embryogenesis development (Fig. S8), hatching with well-developed jaws, eyes, and
446 hindguts and using up yolk reserves quickly before exogenously feeding.
447
448 Heart rate in newly hatched larvae decreased with increasing exposure to crude oil (Fig. 6b).
449 In the high oil treatments, heart rate averaged 28% and 18% lower than in the warm and cold
450 control larvae, respectively. Cardiac arrhythmia, measured as inter-beat variability, increased

in warm reared larvae and those exposed to the highest oil treatment (Fig. 6c). Higher incidences of yolk sac edema were seen in high oil treatment larvae in both temperature groups with up to 62% of yolk sac larvae afflicted in the cold, high oil exposed larvae (Fig. 6d). A higher prevalence of yolk sac edema was exhibited in the unexposed cold reared larvae compared to the unexposed warm group.

EFFECTS ON FEEDING LARVAE

As larvae began exogenous feeding, synergistic effects of increased temperature and crude oil exposure were evident on survival and spinal and jaw deformities (Fig. 7). The length of feeding larvae decreased with exposure to the medium and high oil treatments and exposure to the warm temperature (Fig. 7a). The specific growth rate was negatively affected by high oil exposure and positively affected by temperature. In the warm group, the highest growth rate was seen in the medium oil treatment ($1.07 \pm 0.17\%$ of length day^{-1}) and the lowest rates were seen in the low and high oil treatments (0.6 - 0.7% of length day^{-1}) (Fig. 7b). Growth rates in the unexposed fish were 92% higher in the warmer treatment ($0.99 \pm 0.16\%$ of length day^{-1}) compared to colder treatment ($0.52 \pm 0.03\%$ of length day^{-1}) using group means. Swim bladder inflation (Fig. 7c) and feeding activity (Fig. 7d) were negatively affected by crude oil exposure at the high and medium treatments but were similar across temperature groups.

Synergistic effects of increased temperature and crude oil exposure were observed in the prevalence of jaw and spinal deformities and pericardial edema in feeding larvae (Fig. 7e-f). An antagonistic effect of the combined stressors was observed in the prevalence of pericardial edema (Fig. 7e). Larvae from the warm group exhibited a higher incidence of all deformities at lower crude oil concentrations than larvae in the colder group (Fig.7h-j). Pericardial edema, as well as eye (Fig. S9) and jaw deformities, were present in nearly 100% of larvae exposed to high oil treatment regardless of temperature. In contrast, the medium oil treatment, warm reared

larvae had 20% or higher incidence of all deformities including spinal deformities compared to the colder group at the same crude oil treatment. Endpoints investigated in feeding larvae were highly correlated (Fig. 8). While effects of temperature and crude oil exposure have been reported at length, this analysis reveals that physiological endpoints such as length, feeding, and swim bladder inflation are negatively correlated with the presence of deformities. Jaw and eye deformities are very highly positively correlated (R>0.75), while spinal deformities have the weakest correlations with the other deformities (R ranges from 025-0.5).

DISCUSSION

KEY EFFECTS OF OIL EXPOSURE AND WARMER TEMPERATURE

Exposure to low levels of the water-soluble fraction of crude oil during embryonic development led to effects at the sublethal and lethal level in polar cod embryos and larvae, and these effects were further amplified by an increase of 2.3°C in water temperature. Interaction effects of temperature and crude oil exposure were observed in 64% (14 of 22) of the responses measured and spread across all developmental stages from embryos to feeding larvae and at multiple levels of biological organization from gene expression to rates of deformities and mortality. Within the interaction effects, 43% (6 of 14) of these response interactions found to be strong ($p<0.05$) synergistic effects, most notably in the survival of yolk sac and feeding larval stages. Synergism is hypothesized to be the most likely outcome of simultaneous exposure to multiple stressors, as the increased stressor intensity will likely overcome compensatory mechanisms in the organisms (Gunderson et al, 2016). Multiple stressors, in the case of synergism, have a larger impact on the organism than the sum of the individual stressors (*i.e.*, an additive response) and contribute to a realistic understanding of the biological responses of organisms in a more complex environment.

In the water of the warm, high oil treatment bulk oil was present, likely due to the relatively low viscosity, low pour point, and low asphaltenic content, and thus lower retention of the fresh Goliat Kobbe crude oil in the gravel cylinders (Sorheim and Moldestad, 2008; Sørensen et al. 2014). This same phenomenon was not observed in the cold, high oil treatment and so we proceed cautiously with our analysis of potential synergistic effects. As evidenced by the two-dimensional gas chromatography analysis, we see that using standard PAH quantification techniques in a whole crude oil exposure scenario risks underestimating the contribution and the mechanistic understanding of the effects of semi-polar, polar, and monoaromatic compounds, which have been demonstrated to be of toxicological significance and worthy of additional investigation (Meador and Nahrgang 2019; Sørensen et al. 2019). The presence of oil droplets in the system alone may not be linked to increased toxicity as ELS studies have shown embryos to be mainly affected by the WSF of crude oil (Sørensen et al., 2017; Carls et al., 2008). However, crude oil droplets, when present, adhere to the chorion of polar cod embryos (Laurel et al. 2019) and may increase the internal concentrations of PAHs, especially larger and alkylated homologues (Sørensen et al. 2017) but this is observed at higher nominal oil concentrations (<900 µg/L; Laurel et al. 2019). We observed the opposite trend with higher BCFs of larger and more alkylated PAHs in the cold group compared to the warm incubated embryos, evidence that oil droplet were likely not altering bioavailability and that elimination of PAHs was occurring. K_{ow} predicted BCFs were lower than calculated BCFs for most PAHs and may b explained by our dynamic exposure design which did not allow for a steady state to establish and the high lipid content of polar cod eggs (11-17% total lipids; Laurel et al. 2018) not accurately predicted by the K_{ow} metric.

The upregulation of genes encoding for *cycp1a* and *cycp1c1* indicates that crude oil-derived compounds were biologically available (Carls et al. 1999) and that biotransformation

mechanisms are in place early in polar cod embryogenesis, as is seen in Atlantic cod embryos (Olsvik et al. 2012). The warm, high oil-treated embryos prior to hatch (day 28) exhibited a robust up-regulation of biotransformation genes *cyp1a* and *cyp1c1*, evidence for metabolism of PAHs and an explanation for the decreased body burden from day 4 to Day 18. However, this is an interpolation between the two sampling points, and further experimentation would need to confirm a cause-effect relationship. While synergistic effects of warm water and high oil treatment were seen in molecular responses, there were no interactions observed between the effects of temperature and crude oil exposure on early embryonic survival.

TEMPERATURE AS A STRESSOR?

Lower embryonic survival and premature hatching of the warm reared larvae, determined by the smaller size and lack of facial structures like jaws or advanced eye and digestive system development, compared to the cold reared larvae, corroborates the high thermal sensitivity of polar cod (Laurel et al. 2018). Development times reported in this study agree with other polar cod experiments and observations around the Arctic (Laurel et al. 2018; Nahrgang et al. 2016; Kent et al. 2016; Graham and Hop, 1995; Sakurai et al. 1998; Altukhov, 1981; Aronovich et al. 1975). Maximum polar cod egg survival is between 0 - 1.5°C with a reduction in egg survival seen at 3.0-3.5°C (Laurel et al. 2018; Dahlke et al. 2018), confirming that the study presents data from the higher end of the thermal window for polar cod embryos at 2.8°C. Premature hatching with increased temperature is a phenomenon also seen in polar cod in Alaskan waters and Atlantic cod (Laurel et al. 2018; Jordann et al. 2006). The heat shock response gene (*hsp 70*) induced in the warm treated embryos indicated a greater stress response and not an advanced developmental stage with an intrinsically greater molecular ability for responding to stress. The larger size and more advanced developmental stage at hatch in the cold-reared larvae resulted in exogenous feeding shortly after hatch. Hatching at smaller sizes

549 may leave warm reared larvae more vulnerable to predation and less fit to forage in the wild
550 (Porter and Bailey, 2007). However, larval growth rates were higher in the warm reared larvae
551 and the prevalence of swim bladder inflation and feeding rates did not differ between the
552 temperature groups.
553
554 Field studies consistently report temperature as the main driver of polar cod ELS abundance,
555 with less fish found in warmer waters (Bouchard et al. 2017; Marsh et al. 2019; Huserbråten et
556 al. 2019), and suggest a wider thermal tolerance (-1.7 - 6.5°C) for spawning areas and larvae
557 occurrence (Eriksen et al. 2015). For example, hatching in ice-free areas as warm as 3.9 ºC had
558 led to a high abundance of polar cod in the North Water Polynya in Northern Baffin Bay
559 (Fortier et al. 2006), although incubation temperatures may have varied prior to sampling. In
560 fact, we observed higher larval growth rates in warmer temperatures, which potentially place
561 surviving, more tolerant individuals on better survival trajectories after hatch. Growth and
562 survival rates are both stage-specific and temperature-dependent, even within the larval stage
563 of polar cod (Koekner et al. 2018). Accelerated larval growth is likely associated with higher
564 energetic demands and may increase the vulnerability of growing larval to alteration in food
565 quality and abundance (Bouchard and Fortier, 2020; Laurel et al. 2018), a predicted change in
566 the Arctic ecosystem with ongoing warming (Weydmann et al. 2014). Biophysical modeling
567 of Barents Sea polar cod ELS have already observed the northward retreat of the western
568 spawning location and further forecast an "imminent recruitment collapse" in polar cod ELS
569 with continued sea-ice reduction and increased temperature (Huserbråten et al. 2019).
570
571 *EMBRYO BUOYANCY AS AN ECOLOGICALLY IMPORTANT EFFECT*
572 The adverse effect of crude oil exposure on embryo buoyancy, measured as embryo sinking
573 speed in less saline water and converted to egg density, may have implications on early life

574 survival, horizontal and vertical dispersal of ELSs (Myksvoll et al. 2014), and, ultimately, year-
575 class abundance (Sundby and Kristiansen, 2015). Similar reductions in buoyancy were
576 observed in Mahi-mahi embryos exposed to crude oil and higher temperatures and was
577 suggested to be adaptive as embryos actively avoid adverse conditions at the surface
578 (Pasparakis et al. 2017). While a mechanistic understanding of changes in egg buoyancy is
579 outside the scope of this study, exposure to crude oil has likely disrupted osmoregulation of
580 the normally hyperosmotic, buoyant eggs. In haddock, embryonic exposure to crude oil caused
581 differential expression of pathway related to osmoregulation (Sørhus et al. 2017). While
582 depletion of yolk reserves or altered lipid metabolism would likely alter buoyancy in embryos,
583 yolk sac area measurements in larvae shortly after hatch did not statistically differ with crude
584 oil exposure. Differences between the temperature groups may be explained by changes in
585 body structure (*i.e.,* greater length at hatch in the cold reared group) as proteins are the heaviest
586 component of embryos (Sundby and Kristiansen, 2015). Notedly, in the warm treatment, the
587 magnitude of the effect of crude oil exposure on embryo buoyancy was dampened and may
588 suggest a protective effect of increased temperature.
589
590 *TOLERANCE AND DEFORMITIES*
591 Subtle stress levels are hypothesized to lead to more antagonist effects while synergism is more
592 common at high-stress levels (Lange and Marshall, 2017); however, our experimental results
593 did reveal a single statistically significant antagonist interaction between the two stressor types
594 at the high oil treatment level (83% afflicted) in the prevalence of pericardial edema response
595 in feeding larvae. At this point in their development as exogenous feeding larvae, it is possible
596 that the warm, high oil group were tolerant to the multiple stressor scenario with the more
597 robust individuals remaining after the periods of elevated mortality in the yolk sac and feeding
598 larval periods (Gunderson et al, 2016). Even with higher rates of deformities (*i.e.*, jaw, eye,

599 spinal) at lower oil exposures, warm treated fish were as able to feed and reached similar
600 developmental milestones, such as swim bladder inflation, as cold reared larvae exposed to oil.
601 Higher mortality rates in the warm treatment could be driving selection for more robust, albeit
602 deformed larvae in these treatments. The high incidence of craniofacial deformities for polar
603 cod exposed to low concentrations of crude oil has been observed in other studies (Nahrgang
604 et al. 2016; Laurel et al. 2019). The reduction in heart rate and increased heart arrhythmia are
605 a well-studied response to petroleum exposure in fish ELSs and occur at relatively low levels
606 of total petroleum hydrocarbons (mg/L concentrations; Incardona et al. 2015). Alteration of
607 cardiac functions lead to many of the observed deformities (Incardona et al. 2004). Deformities
608 likely hamper the swimming and foraging ability in larvae resulting in the high correlation
609 between feeding success and presence of deformities. The timing of the loss of severely
610 deformed larvae matches temperature corrected starvation windows determined
611 experimentally for polar cod larvae (35-50 days post hatch; Laurel et al. 2018) and, thus,
612 mortality is an indirect effect of crude oil exposure.
613
614 *THE RELEVANCE OF CRUDE OIL EXPOSURE LEVELS*
615 The tPAHs measured on day 0 in the highest crude oil exposure treatments at both temperatures
616 are above what is considered hazardous for fish ELS following an oil spill (>100 ng/L; Hodson,
617 2017); notably, tPAH levels fell below that threshold within the first 4 days of exposure.
618 Concentrations of tPAHs polar cod embryos were exposed to in the present study (5-237 ng/L)
619 are within the lower range of tPAHs measured in waters in the years following Exxon Valdez
620 Oil Spill in Prince William Sound, Alaska (129 - 126635 ng/L; Boehm et al. 2007) and within
621 the low range of concentrations measured after Deepwater Horizon Oil Spill (Echols et al.
622 2015). Caution in interpretation and cause/effect relations should be exhibited as this study has
623 only measured and compared the concentration of 44 PAHs, which make up a very small

624 percentage of the total hydrocarbons of the WSF of crude oil. The compound composition can
625 vary between crude oils further weakening the comparative strength across studies.
626
627 *ECOLOGICAL RELEVANCE AND IMPLICATIONS*
628 Global environmental change is not limited to increased water temperature and acute pollution
629 events, as this study presents. Reductions in sea ice extent, ocean acidification, increasing
630 freshwater input, southern species moving northward to alter community structures and other
631 pollution issues like microplastic all stand to affect polar cod (Dahlke et al. 2018; Bouchard
632 and Fortier, 2011; Christiansen, 2017; McNicholl et al. 2018; Kühn et al. 2018) with possible
633 cascading effects to the entire Arctic marine ecosystem. The reductionist approach to the
634 present work explored the physiological resilience of this species at its most vulnerable life
635 stage. Studies on juvenile and mature polar cod report higher thermal preferences (Laurel et al.
636 2016) and robust physiological response to chronic and acute crude oil pollution (Bender et al.
637 2016, 2018). Further investigation in the chronic physiological effects of subtle multi-stressor
638 exposure on growth, energy use, and behavior would further this research and aid in addressing
639 the possible long-term toxic responses and population-level effects.
640
641 *CONCLUSION*
642 A 2.3ºC increase in temperature resulted in synergistic effects of crude oil exposure, especially
643 on larval survival and on the frequency of deformities. The warm, high oil treatment likely
644 altered the exposure from the water-soluble fraction to one containing bulk oil microdroplets
645 detected only through two-dimensional gas chromatography; conversely, increased
646 bioavailability is not seen using calculated BCFs. Crude oil exposed embryos exhibited
647 reduced buoyancy, and larvae were afflicted with deformities of the jaws, eyes, and spine and
648 a reduced heart rate. Despite the expectation that increases in temperature and low levels of

crude oil exposure would result in minimal responses (O´Dea et al. 2019; Liess et al. 2016), the magnitude of the effects observed in this study is large, further supporting the vulnerability of polar cod ELS to environmental stressors such as increased temperature and petroleum pollution. Under a multi-stressor scenario, polar cod will likely be at a disadvantage in the changing Arctic environment and antagonistic interactive effects will, unfortunately, challenge our predictive models and ability to protect sensitive species.

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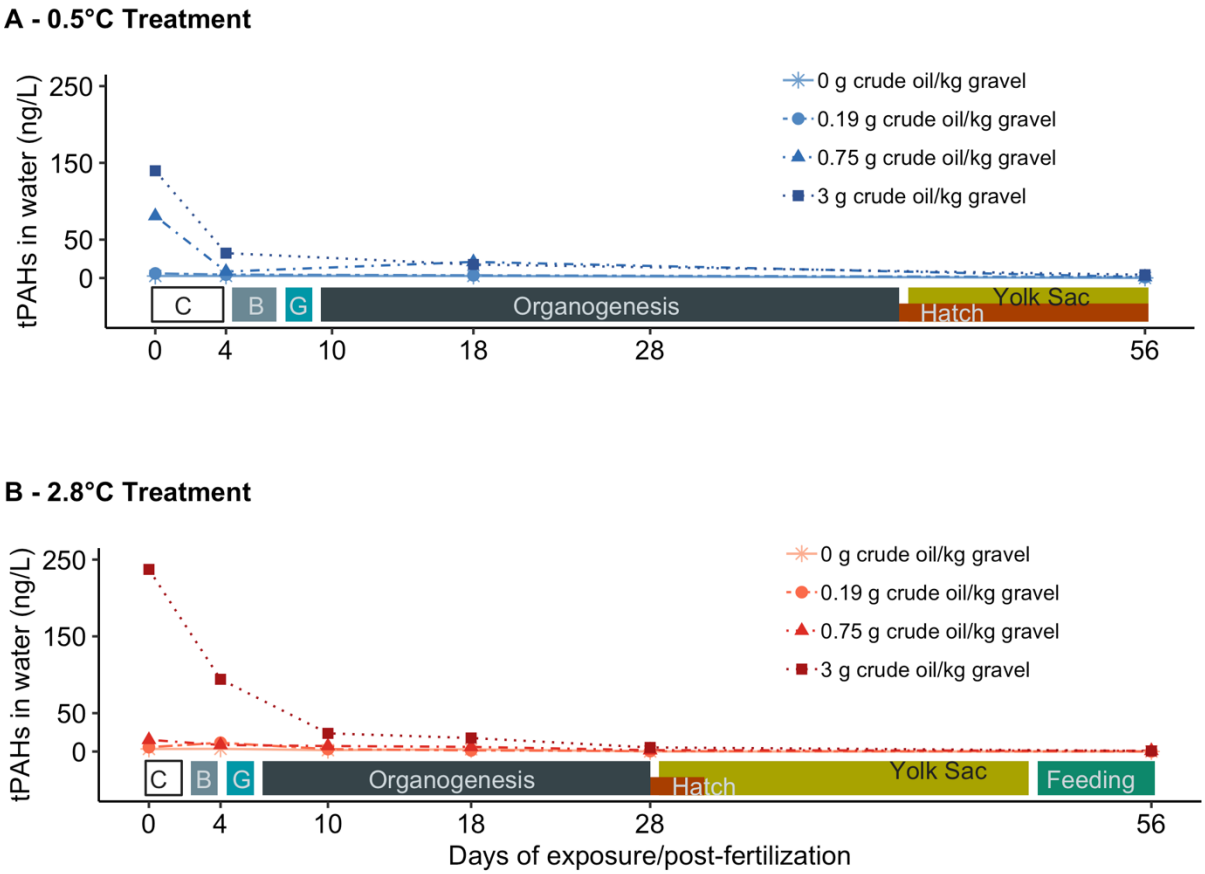

Figure 1. The concentration of tPAHs (ng/L) over time in the cooler, 0.5°C temperature group (top) and the warmer, 2.8°C temperature group (bottom). Different concentrations of crude oil and gravel are distinguished by color, shape and line type. The best linear model *lm(tPAH ~ Time + factor(Temperature) + factor(Oil Treatment)* reported the effects of time (F-value=6.09, p-value=0.019), temperature (F-value = 0.0211, p-value = 0.885) and oil treatment (F-value=4.5034, p-value=0.009). The developmental timeline of embryos and larvae in each temperature group are displayed in each panel. Early embryogenesis is divided into cleavage stage (C), blastulation (B), and gastrulation (G).

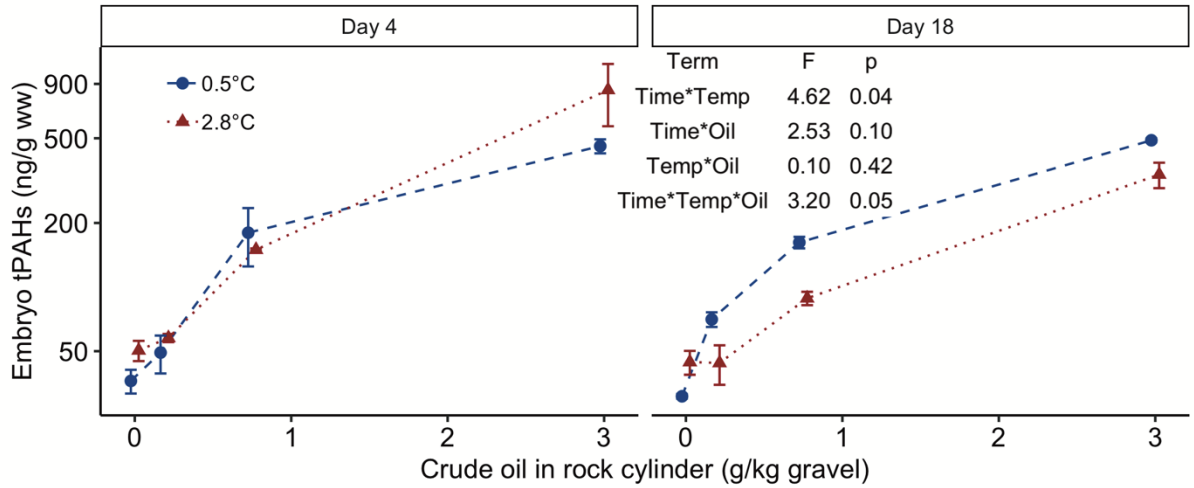

Figure 2. The effect of temperature and WSF crude oil exposure on tPAH levels (ng/g ww) in embryos on day 4 (left) and day 18 (right) displayed as treatment means (± SEM depicted as bars, each treatment is represented by two independent pools of two incubators) plotted on a log scale. Colors, symbols, and line types distinguish the temperature groups. The best linear mixed effect (lme) model was lme(tPAH ~ time*factor(temperature)*factor(oil treatment) and ANOVA f-values and p-values for the interactions are displayed. Embryos were at different development stages during the sampling; Day 4: blastulation (2.8°C) and late cleavage stage (0.5°C); Day 18: Mid organogenesis (2.8°C) and early organogenesis (0.5°C).

Table 1. Quantification of exposure through total polycyclic aromatic hydrocarbons (tPAHs, Σ PAH 44) in water at start of experiment and in embryos on day 4 and day 18 (concentrations are mean (± SEM) of 2 independent pools per oil treatment).

Crude Oil Exposure	Rearing Temperature	Initial tPAH concentration in water (ng/L)	Embryo tPAHs (ng/g ww)	
			Day 4	Day 18
Control 0 g crude oil/kg gravel	0.5°C	2.60	36.24 ± 3.28	30.69 ± 0.54
	2.8°C	3.34	50.40 ± 3.88	44.45 ± 4.04
Low 0.19 g crude oil/kg gravel	0.5°C	5.99	49.23 ± 7.04	70.52 ± 3.94
	2.8°C	5.65	57.69 ± 1.79	44.01 ± 6.55
Medium 0.75 g crude oil/kg gravel	0.5°C	80.78	180.01 ± 38.90	162.07 ± 7.10
	2.8°C	15.22	149.78 ± 1.50	88.60 ± 4.53
High 3 g crude oil/kg gravel	0.5°C	139.70	459.40 ± 24.50	489.30 ± 6.92
	2.8°C	237.26	842.96 ± 193.45	337.70 ± 32.68

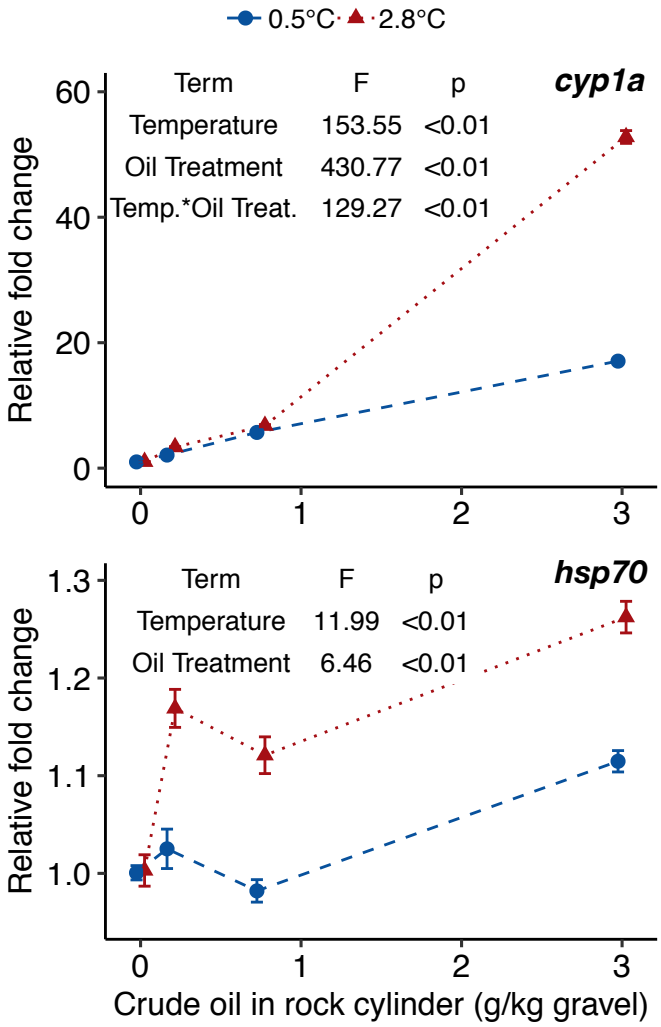

Figure 3. The effect of temperature and WSF crude oil exposure on gene expression of (A) *cyp1a* and (B) *hsp 70* measured by qRT PCR in embryos just prior to hatch (day 28 at 2.8°C and day 48 at 0.5°C). Data is displayed as treatment means (± SEM depicted as bars, each treatment is represented by four independent incubator pools of 50 embryos each). Colors and symbols indicate temperature groups and dashed and dotted lines represent the linear mixed effect (lme) model trend line for each temperature group. The terms of the best linear mixed effect model for each gene and the ANOVA F-values and p-values are displayed in each panel.

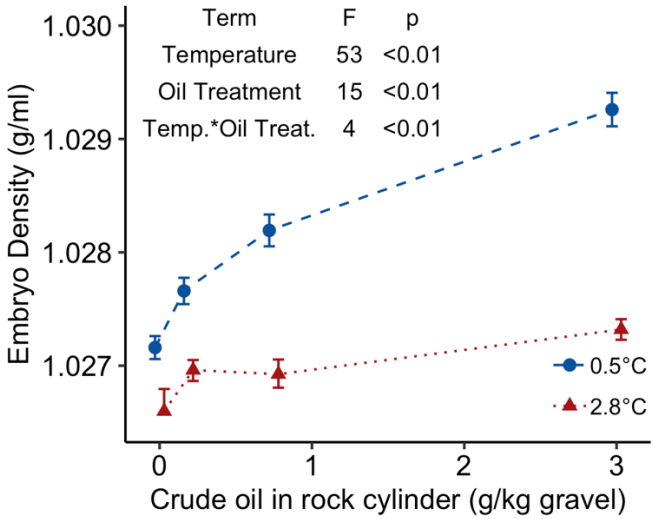

Figure 4. The effect of temperature and WSF CO exposure on embryo density before peak hatch in the respective temperature groups (day 29 in 2.8°C and day 56 in 0.5°C) displayed as treatment means (± SEM depicted as bars, each point includes mean values from four incubators, each with 20 embryos). Colors and shapes distinguish temperature groups. Fitted dashed lines are the results of a generalized least squares (gls) model using incubator as a random factor. The terms of the best gls model for embryo density and the ANOVA test F-values and p-values are displayed.

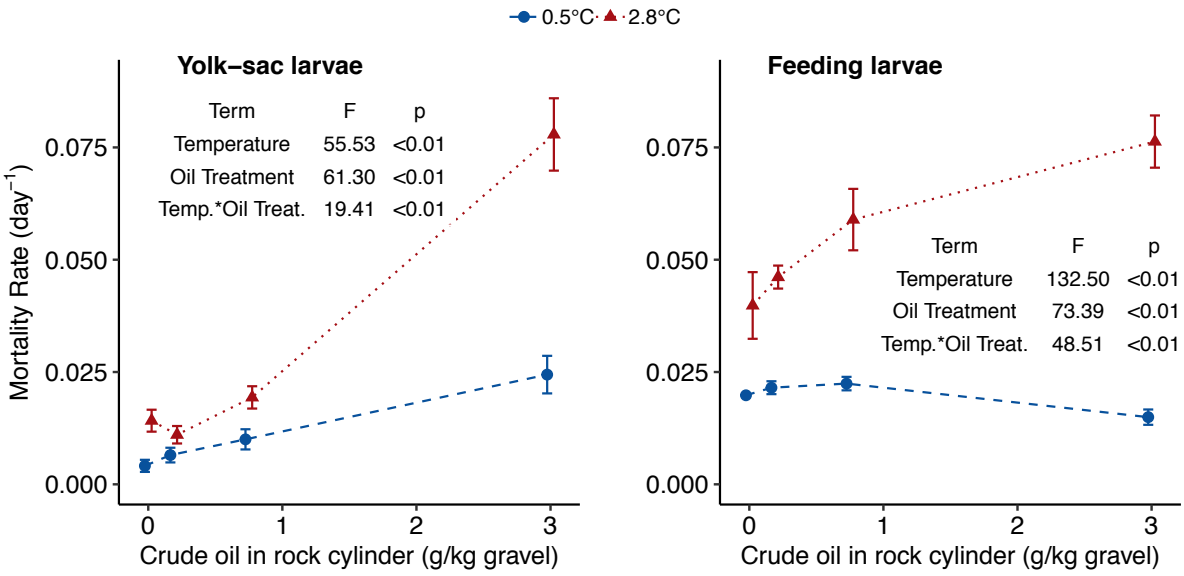

Figure 5. The effect of temperature and crude oil exposure on instantaneous mortality rates for yolk-sac larvae (left) and feeding larvae (right). Data is displayed as treatment means (± SEM depicted as bars, each treatment is represented by four independent incubators). Colors and symbols indicate temperature groups and dashed and dotted lines represent the trend line for each temperature group. The terms of the best lme models for each gene are displayed in the respective panels with the associated ANOVA test F-values and p-values.

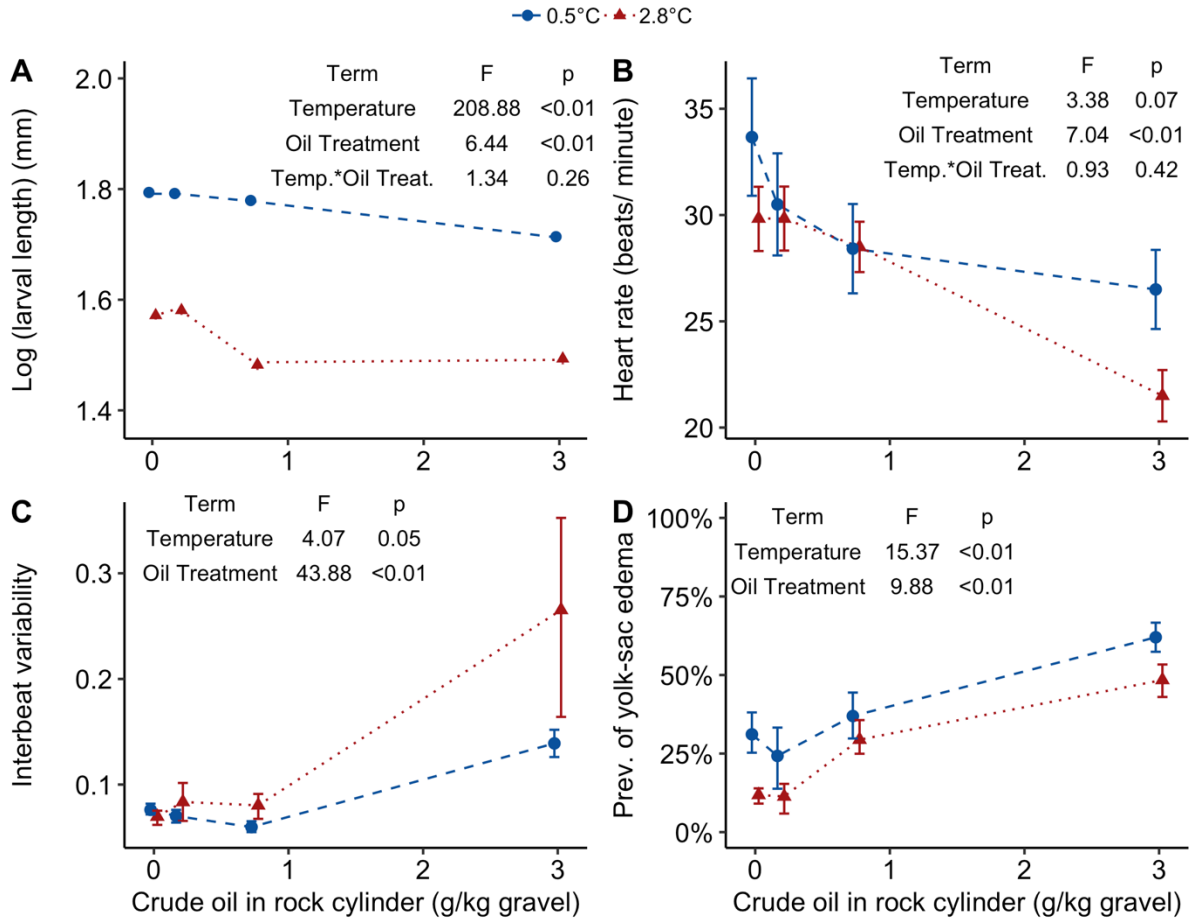

Figure 6. The effects of temperature and WSF crude oil exposure on yolk sac larvae: (A) log (length (mm)) at day 28 for 2.8°C and day 50 for 0.5°C displayed as incubator means (± SEM depicted as bars, each point includes 20-30 larvae); **(B) heart rate** and **(C) cardiac arrhythmia** at day 30-31 for 2.8°C and Day 48-49 for 0.5°C displayed as interbeat variability; and **(D) the prevalence of yolk sac edema** at day 28 for 2.8°C and day 50 for 0.5°C. Fitted dashed lines are the results of gls models using incubator as a random factor for panel A-C. For all panels, colors, line types, and shapes distinguish temperature groups and treatment means are plotted (± SEM depicted as bars, each point includes four incubator means calculated from 30 larvae (panel A) or three larvae each (panel B and C)). Panel D presents treatment means (± SEM depicted as bars, each point includes deformity scores (i.e. percentage of larvae afflicted) from four incubators) and the fitted line is a result of the lme model using incubator as a random factor. The terms of the best gls model for larval length, heart rate and arrhythmia and the best lme model for prevalence of yolk-sac edema are displayed in the respective panels with the ANOVA test F-values and p-values.

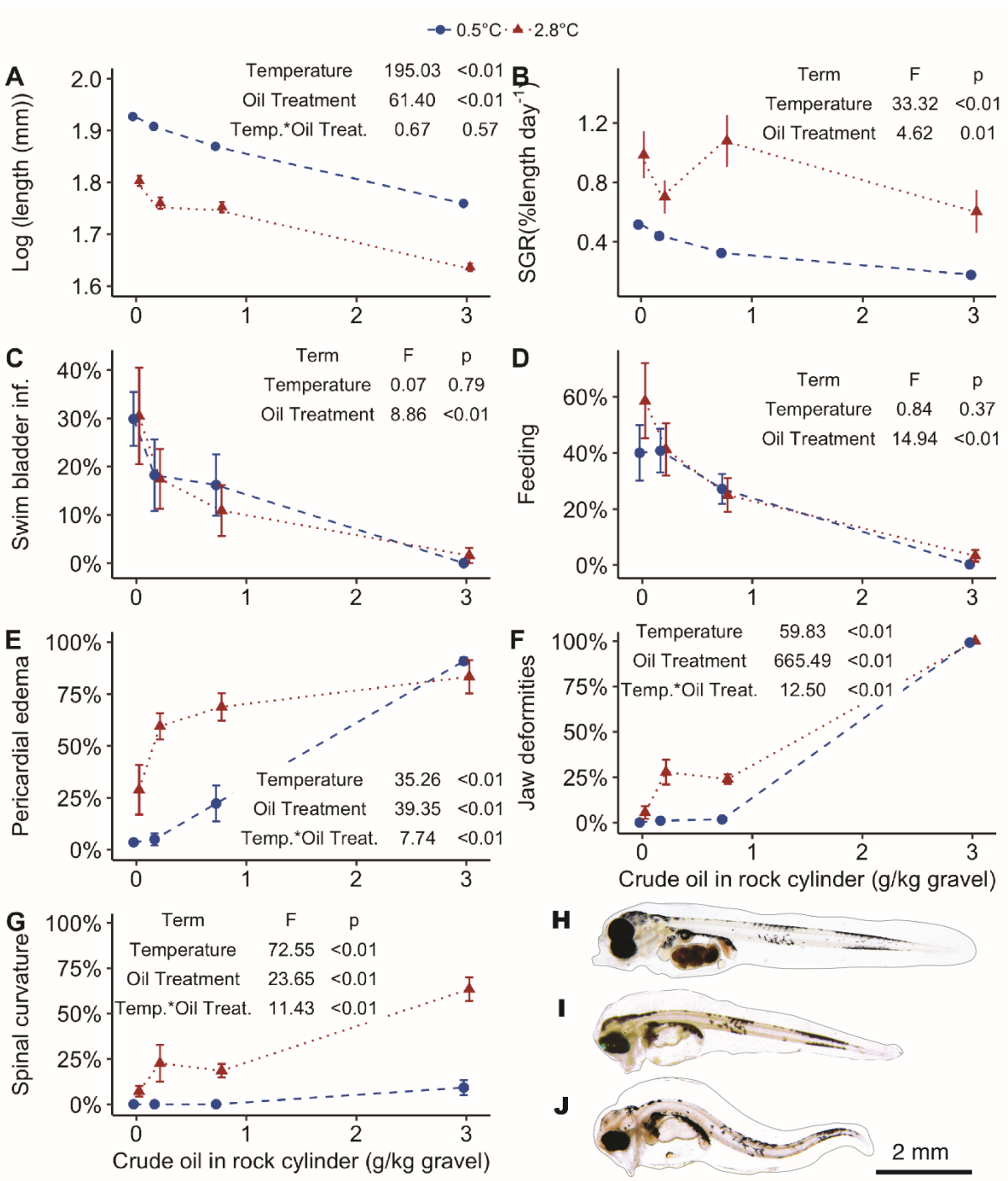

Figure 7. The effects of temperature and WSF crude oil exposure on exogenously feeding larvae (day 52 for 2.8°C and day 76 for 0.5°C) morphometrics, development and phenotype: **(A) log (length (mm))** of larvae represented as incubator means (± SEM depicted as bars, each point includes 30 larvae). **(B) The log of the specific growth rate (% length day^{-1})** displayed as CO treatment means (± SEM depicted as bars, each point includes four incubator means from 30 larvae). Fitted dashed lines are the result of a gls model using incubator as a random factor. The terms of the best gls model for larval length and SGR are displayed in the respective panels with the ANOVA test F-values and p-values. The prevalence of **(C) swim bladder inflation**; **(D) feeding**; **(E) pericardial edema**; **(F) jaw deformities**; and **(G) spinal curvature** is represented as treatment means (± SEM depicted as bars, each point includes 4 incubator deformity scores). Fitted dashed lines are the results of lme model using incubator as a random factor. The terms of the best lme models for each parameters is displayed in the respective panels with the associated ANOVA test F-values and p-values. For all panels, colors, shapes, and line types distinguish temperature groups. Typical phenotypes of exogenously feeding larva exposed to **(H) Control oil treatment in the cold group**; **(I) Cold, high oil treatment**; and **(J) warm, high oil treatment** are displayed in the lower right.

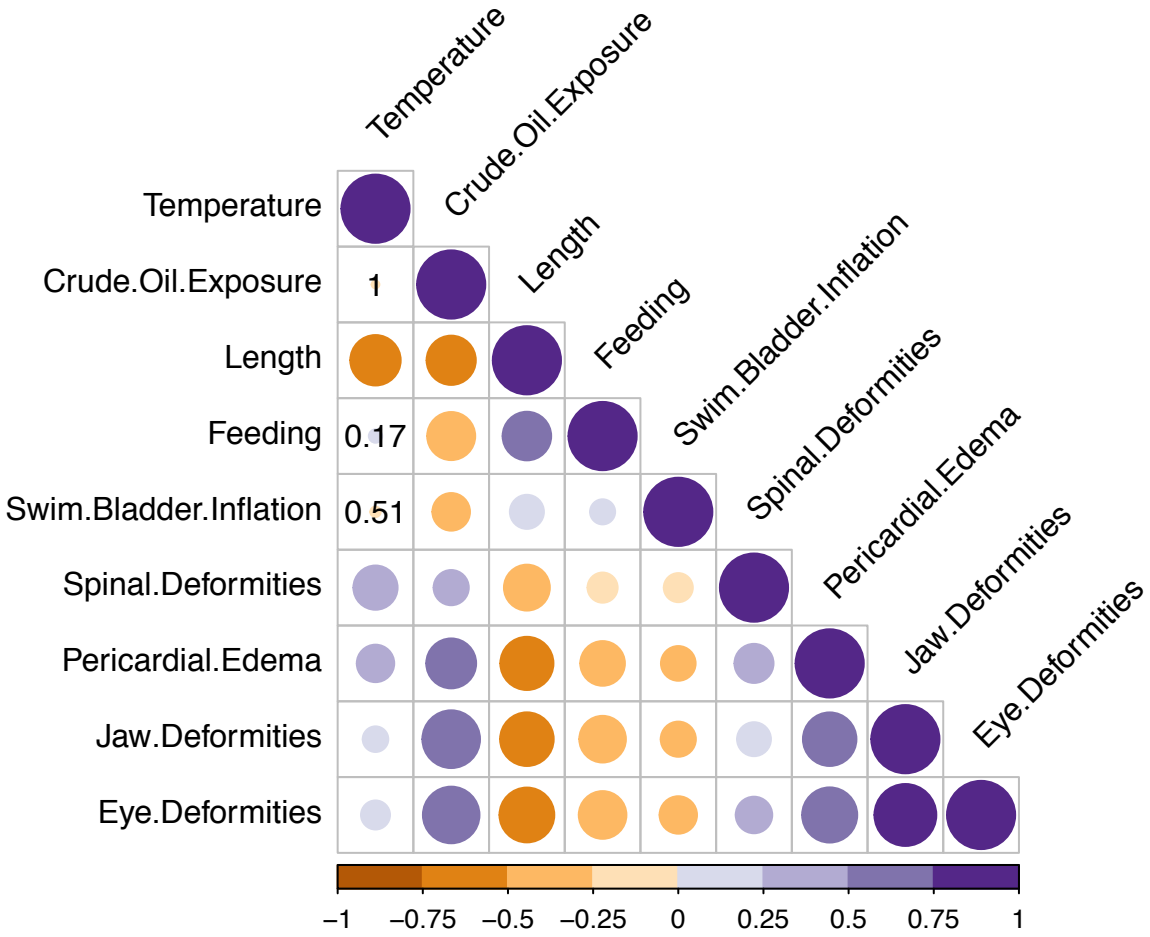

Figure 8. Correlation matrix calculated with the non-parametric Spearman method for treatment, biomorphometrics and deformity correlations in individual feeding larvae from all treatments (n=809 larvae) for day 52 for 2.8°C and day 76 for 0.5°C. Dot size and color indicate strength and direction of the correlation. P-values are displayed for correlations which have a value >0.01.

SUPPLEMENTARY INFORMATION

Table S1. Explanation of stages used for polar cod embryos and larvae in the present work. Degree day duration for each stage in each temperature group is calculated in the right columns.

Stage	Phenology	Description	Degree days for stage duration	
			0.5°C	2.8°C
Cleavage Stage		Initiated with fertilization, synchronous cell division until 128 cell stage	0 - 2	0 - 8.4
Blastulation		Blastodisc present on top of yolk	2 - 3.5	8.4 - 11.2
Gastrulation		Appearance of germ ring; graduated stages of epiboly; end of gastrulation identified with 50% epiboly	3.5 - 4.5	11.2 - 16.8
Organogenesis		Formation of visible notochord and somites; eyes become pigmented; heart beating visible; embryos move inside chorion; hatch glands appear; 100% tail curl; body pigmentation visible	4.5 – 31.0*	16.8 - 84.0*
Hatch		The event of embryos exiting chorion, hatch period defined by first observation on unperturbed hatching in incubator and ends with last viable eggs hatching while at incubator surface	21 - 32.5	78.4 - 89.6
Yolk Sac		Free larvae with yolk sac; undeveloped digestive tract, jaws, eyes, and face; finfold present; swim bladder inflation, begin feeding on rotifers and artemia nauplii	31 - 35.5	84.0 - 137.2
Exogenous feeding larvae**		Yolk sac absorbed; exogenously feeding on artemia; straighten notochord; no separate medial fins; caudal fin rays begin to form; fully developed jaws, eyes, and face; more pigmentation in eyes and body	35.5 - 86	137.2 - 324.8

Based of *G. morhau* stages in Hall et al. 2004; Miller and Kendall 2009;

* Organogenesis stageduration calculated until peak hatch at each temperature

**Exogenous feeding stage duration based off data not presented in this study

Table S2. Concentrations of the 44 PAH analytes measured in the water at the start of the exposure (Day 0) for each temperature and crude oil treatment group. Lower limit of detection (LOD) is noted for each analyte in the first column.

Analyte (ng/L)	LOD	0.5°C				2.8°C			
		Control	Low	Medium	High	Control	Low	Medium	High
Benzothiophene	0.07	0.10	0.13	0.18	0.21	0.14	0.10	0.12	0.15
Naphthalene	0.39	0.81	0.74	1.80	14.27	0.74	0.89	1.37	12.39
C1-NAP	0.05	0.10	0.27	1.90	16.84	0.09	0.53	1.34	16.47
C2-NAP	1.13	<LOD	<LOD	2.09	12.46	<LOD	<LOD	1.43	12.19
C3-NAP	0.02	0.05	0.41	6.04	20.10	0.06	0.53	1.52	31.62
C4-NAP	0.04	0.07	0.67	16.93	23.88	0.12	0.57	2.06	64.19
Biphenyl	0.13	0.24	0.26	0.68	4.00	0.21	0.40	0.67	3.16
Acenaphthylene	0.10	0.10	<LOD	0.40	1.25	0.11	0.13	0.23	1.98
Acenapthene	0.04	0.05	<LOD	0.17	0.57	0.05	<LOD	0.10	0.56
Dibenzofuran	0.15	0.19	0.21	0.61	2.87	0.20	0.29	0.49	2.41
Fluorene	0.12	0.14	0.20	0.73	3.78	0.15	0.28	0.54	3.06
C1-FLU	0.01	0.01	0.05	0.55	1.56	0.02	0.07	0.17	2.32
C2-FLU	0.01	0.02	0.14	3.36	3.28	0.03	0.10	0.34	9.85
C3-FLU	0.06	<LOD	0.07	2.27	1.08	<LOD	<LOD	0.13	5.20
Phenanthrene	0.14	0.24	0.72	4.54	14.22	0.82	0.84	1.89	15.41
Anthracene	0.88	<LOD	<LOD	0.97	<LOD	<LOD	<LOD	<LOD	2.71
C1-PHE	0.01	0.02	0.15	2.79	4.36	0.02	0.13	0.45	9.72
C2-PHE	0.25	<LOD	0.29	3.98	3.10	<LOD	0.26	0.59	6.48
C3-PHE	0.01	0.01	0.35	8.46	2.67	0.02	0.12	0.40	10.01
C4-PHE	0.12	<LOD	0.31	8.74	1.81	<LOD	<LOD	0.21	9.10
Dibenzothiophene	0.11	0.11	0.18	1.31	2.05	0.13	0.21	0.36	4.27
C1-DBT	1.76	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
C2-DBT	0.01	0.01	0.02	0.23	0.18	0.01	0.01	0.03	0.36
C3-DBT	0.01	<LOD	0.01	0.34	0.11	<LOD	0.01	0.02	0.40
C4-DBT	0.00	<LOD	0.01	0.25	0.06	0.00	0.00	0.01	0.26
Fluoranthene	0.22	0.23	0.35	0.79	0.76	0.25	<LOD	0.24	1.02
Pyrene	0.02	0.10	0.11	1.20	0.95	0.16	0.08	0.17	1.70
C1-FLA/PYR	0.01	0.01	0.10	1.50	1.08	0.02	0.04	0.16	2.18
C2-FLA/PYR	0.01	<LOD	0.07	1.06	0.44	<LOD	0.02	0.05	1.38
C3-FLA/PYR	0.01	<LOD	0.06	1.03	0.35	0.01	0.01	0.04	1.12
Benz[a]anthracene	0.06	<LOD	<LOD	0.78	0.11	<LOD	<LOD	<LOD	0.79
Chrysene	0.00	<LOD	0.05	2.67	0.92	<LOD	<LOD	0.07	2.76
C1-CHR	0.01	<LOD	0.04	0.61	0.19	<LOD	<LOD	0.03	0.70
C2-CHR	0.03	<LOD	<LOD	0.08	<LOD	<LOD	<LOD	<LOD	0.09
C3-CHR	0.01	<LOD	0.01	0.08	0.02	<LOD	0.02	<LOD	0.09
C4-CHR	0.02	<LOD	<LOD	0.07	<LOD	<LOD	<LOD	<LOD	0.08
Benzo[b]fluoranthene	0.03	<LOD	<LOD	0.35	0.11	<LOD	<LOD	<LOD	0.29
Benzo[k]fluoranthene	0.00	<LOD	<LOD	0.25	<LOD	<LOD	<LOD	<LOD	0.21
Benzo[e]pyrene	0.00	<LOD	<LOD	0.46	0.07	<LOD	<LOD	<LOD	0.37
Benzo[a]pyrene	0.00	<LOD	<LOD	0.28	<LOD	<LOD	<LOD	<LOD	<LOD
Perylene	0.00	<LOD	<LOD	0.12	<LOD	<LOD	<LOD	<LOD	0.06
Indeno[1,2,3-cd]pyrene	0.07	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Dibenz[ah]anthracene	0.18	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Benzo[ghi]perylene	0.00	<LOD	<LOD	0.11	<LOD	<LOD	<LOD	<LOD	0.11
Sum PAH		2.60	5.99	80.78	139.70	3.34	5.65	15.22	237.26
Sum NAP		1.03	2.10	28.76	87.55	1.01	2.52	7.72	136.86
Sum 2-3rings PAH		1.23	3.09	40.57	47.16	1.89	2.95	6.74	87.42
Sum 4-6rings PAH		0.34	0.81	11.45	4.99	0.44	0.17	0.76	12.98

Table S3a. Concentrations of the 44 PAH analytes measured in embryos at Day 4 for each temperature and crude oil treatment group.

		Day 4							
PAH Analytes ng/g ww	**LOD**	**0.5°C**				**2.8°C**			
		0 g / kg	**0.19 g / kg**	**0.75 g / kg**	**3 g / kg**	**0 g / kg**	**0.19 g / kg**	**0.75 g / kg**	**3 g / kg**
Benzothiophene	0.068	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Naphthalene	0.273	10.95 ± 0.41	8.07 ± 0.33	11.26 ± 0.48	13.31 ± 0.41	12.75 ± 0.92	6.60 ± 0.57	11.90 ± 2.12	23.53 ± 0.05
C1-NAP	0.018	1.65 ± 0.07	1.37 ± 0.06	3.15 ± 0.08	14.83 ± 0.23	1.77 ± 0.18	1.23 ± 0.06	3.59 ± 0.23	14.49 ± 1.09
C2-NAP	0.015	0.58 ± 0.02	1.40 ± 0.20	4.89 ± 0.20	17.04 ± 1.86	0.66 ± 0.07	1.59 ± 0.07	4.47 ± 0.10	17.35 ± 0.87
C3-NAP	0.013	1.18 ± 0.09	2.82 ± 0.30	9.49 ± 0.55	49.65 ± 3.72	1.44 ± 0.11	3.73 ± 0.04	8.83 ± 0.18	56.08 ± 2.32
C4-NAP	0.024	1.71 ± 0.02	3.92 ± 0.71	16.53 ± 2.87	86.06 ± 3.10	2.03 ± 0.23	5.03 ± 0.32	13.40 ± 0.26	115.10 ± 7.80
Biphenyl	0.152	<LOD	<LOD	4.74 ± 0.20	7.09 ± 0.87	<LOD	<LOD	4.11 ± 0.72	8.33 ± 0.15
Acenaphthylene	0.049	<LOD	<LOD	<LOD	3.18 ± 0.38	<LOD	<LOD	<LOD	3.34 ± 0.15
Acenapthene	1.161	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Dibenzofuran	0.058	1.61 ± 0.16	2.24 ± 0.19	5.42 ± 0.13	11.39 ± 0.92	1.88 ± 0.14	2.11 ± 0.04	4.77 ± 0.29	12.80 ± 0.13
Fluorene	0.070	<LOD	<LOD	3.76 ± 0.15	6.72 ± 0.36	<LOD	1.27 ± 0.04	3.32 ± 0.72	7.68 ± 0.43
C1-FLU	0.002	0.17 ± 0.03	0.38 ± 0.06	1.44 ± 0.21	4.65 ± 0.13	0.19 ± 0.01	0.48 ± 0.01	1.02 ± 0.03	6.09 ± 0.65
C2-FLU	0.005	0.59 ± 0.01	0.94 ± 0.11	8.71 ± 3.82	18.34 ± 0.26	0.64 ± 0.09	1.07 ± 0.01	2.77 ± 0.00	39.37 ± 13.53
C3-FLU	0.016	0.28 ± 0.00	0.58 ± 0.07	6.70 ± 3.22	9.57 ± 0.11	<LOD	0.85 ± 0.12	2.12 ± 0.05	26.55 ± 11.11
Phenanthrene	0.506	<LOD	9.72 ± 1.75	31.15 ± 3.87	64.85 ± 2.03	13.65 ± 0.00	11.33 ± 0.44	23.97 ± 2.88	98.33 ± 15.56
Anthracene	2.401	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
C1-PHE	0.003	0.21 ± 0.02	1.07 ± 0.12	8.01 ± 2.34	30.96 ± 1.08	0.24 ± 0.00	1.66 ± 0.09	3.85 ± 0.06	50.26 ± 11.61
C2-PHE	0.014	0.35 ± 0.03	1.09 ± 0.10	8.55 ± 2.46	29.20 ± 2.49	0.40 ± 0.02	1.47 ± 0.05	6.96 ± 0.31	59.65 ± 13.51
C3-PHE	0.023	1.71 ± 0.00	2.02 ± 0.04	14.47 ± 5.14	22.24 ± 2.05	1.72 ± 0.17	2.91 ± 0.03	11.09 ± 0.78	79.18 ± 34.56
C4-PHE	0.155	9.19 ± 1.70	5.04 ± 0.01	15.17 ± 3.61	13.52 ± 1.49	11.94 ± 0.35	5.72 ± 0.38	15.70 ± 0.66	58.28 ± 23.91
Dibenzothiophene	0.040	<LOD	<LOD	2.67 ± 0.87	13.68 ± 0.53	<LOD	0.55 ± 0.01	2.23 ± 0.30	25.61 ± 7.71
C1-DBT	0.002	0.06 ± 0.01	0.14 ± 0.01	1.71 ± 0.86	3.68 ± 0.16	0.08 ± 0.00	0.167 ± 0.00	0.57 ± 0.01	10.72 ± 4.69
C2-DBT	0.001	0.03 ± 0.01	0.08 ± 0.01	1.12 ± 0.56	2.18 ± 0.25	0.02 ± 0.00	0.11 ± 0.00	0.47 ± 0.04	7.64 ± 3.32

C3-DBT	0.001	0.05 ± 0.01	0.06 ± 0.00	1.22 ± 0.69	0.95 ± 0.14	0.03 ± 0.00	0.091 ± 0.00	0.38 ± 0.03	8.05 ± 4.90
C4-DBT	0.031	2.27 ± 0.65	1.27 ± 0.03	2.46 ± 0.13	2.03 ± 0.34	3.30 ± 0.21	1.22 ± 0.11	2.85 ± 0.35	9.02 ± 3.14
Fluoranthene	0.108	<LOD	<LOD	2.43 ± 0.00	5.63 ± 0.43	<LOD	<LOD	2.89 ± 0.28	7.77 ± 0.23
Pyrene	0.102	<LOD	<LOD	5.28 ± 0.00	9.07 ± 1.03	<LOD	<LOD	3.16 ± 0.08	17.55 ± 3.45
C1-FLA/PYR	0.004	0.34 ± 0.02	0.36 ± 0.01	1.91 ± 0.58	6.06 ± 0.25	0.38 ± 0.03	0.48 ± 0.01	1.54 ± 0.04	11.62 ± 2.73
C2-FLA/PYR	0.047	1.50 ± 0.28	0.81 ± 0.01	2.37 ± 0.68	3.07 ± 0.19	1.93 ± 0.04	0.99 ± 0.10	2.31 ± 0.09	12.13 ± 4.99
C3-FLA/PYR	0.010	0.84 ± 0.07	0.63 ± 0.02	1.98 ± 0.65	1.12 ± 0.10	1.07 ± 0.13	0.62 ± 0.02	1.32 ± 0.06	10.65 ± 6.10
Benz[a]anthracene	0.000	0.82 ± 0.15	1.29 ± 0.39	5.09 ± 1.58	7.95 ± 0.78	0.99 ± 0.25	1.69 ± 0.52	3.41 ± 0.41	20.19 ± 6.68
Chrysene	0.622	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	12.01 ± 0.00	<LOD
C1-CHR	0.001	0.10 ± 0.03	0.15 ± 0.04	0.76 ± 0.36	0.65 ± 0.06	0.07 ± 0.01	0.21 ± 0.06	0.38 ± 0.02	4.32 ± 2.49
C2-CHR	0.001	0.01 ± 0.00	<LOD	0.15 ± 0.00	0.02 ± 0.00	<LOD	<LOD	0.02 ± 0.00	0.63 ± 0.42
C3-CHR	0.001	0.03 ± 0.00	<LOD	0.20 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.86 ± 0.55
C4-CHR	0.001	0.02 ± 0.00	<LOD	0.13 ± 0.00	<LOD	<LOD	<LOD	<LOD	0.51 ± 0.34
Benzo[b]fluoranthene	0.000	<LOD	0.68 ± 0.33	0.41 ± 0.19	0.42 ± 0.06	<LOD	0.84 ± 0.46	0.29 ± 0.06	1.03 ± 0.43
Benzo[k]fluoranthene	0.031	<LOD	<LOD	<LOD	<LOD	<LOD	2.07 ± 0.00	<LOD	<LOD
Benzo[e]pyrene	0.000	<LOD	0.22 ± 0.15	0.20 ± 0.13	0.16 ± 0.01	<LOD	0.42 ± 0.26	0.05 ± 0.01	1.50 ± 0.10
Benzo[a]pyrene	0.405	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Perylene	0.000	<LOD	<LOD	0.64 ± 0.37	<LOD	<LOD	0.21 ± 0.00	<LOD	<LOD
Indeno[1,2,3-cd]pyrene	0.311	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Dibenz[ah]anthracene	0.617	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Benzo[ghi]perylene	0.187	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sum PAH		36.24 ± 3.28	49.23 ± 7.04	180.01 ± 38.90	459.40 ± 24.50	50.40 ± 3.88	57.69 ± 1.79	149.78 ± 1.50	842.96 ± 193.45
Sum NAP		16.06 ± 0.57	17.57 ± 1.59	45.32 ± 3.21	180.90 ± 8.10	18.65 ± 1.51	18.18 ± 1.06	42.19 ± 1.82	226.57 ± 3.44
Sum 2-3rings PAH		16.38 ± 2.62	27.01 ± 4.13	117.29 ± 28.20	244.20 ± 13.60	27.27 ± 5.28	31.80 ± 0.57	86.20 ± 4.10	510.88 ± 148.76
Sum 4-6rings PAH		3.80 ± 0.08	4.64 ± 1.31	17.40 ± 7.44	34.27 ± 2.86	4.48 ± 0.11	7.70 ± 1.30	21.39 ± 4.44	105.51 ± 41.24

Table S3b. Concentrations of the 44 PAH analytes measured in embryos at Day18 for each temperature and crude oil treatment group.

		Day 18							
PAH Analyte ng/g ww	**LOD**	**0.5°C**				**2.8°C**			
		0 g / kg	**0.19 g / kg**	**0.75 g / kg**	**3 g / kg**	**0 g / kg**	**0.19 g / kg**	**0.75 g / kg**	**3 g / kg**
Benzothiophene	0.068	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Naphthalene	0.273	9.18 ± 0.37	11.02 ± 0.28	14.77 ± 0.95	9.35 ± 0.95	10.64 ± 0.11	8.02 ± 0.13	8.92 ± 0.02	10.40 ± 2.31
C1-NAP	0.018	1.30 ± 0.08	1.72 ± 0.07	2.96 ± 0.07	6.21 ± 0.10	1.39 ± 0.02	1.20 ± 0.01	1.92 ± 0.10	5.30 ± 0.50
C2-NAP	0.015	0.52 ± 0.06	1.28 ± 0.03	2.48 ± 0.11	8.49 ± 0.35	0.66 ± 0.03	1.02 ± 0.06	2.01 ± 0.11	6.48 ± 0.30
C3-NAP	0.013	0.99 ± 0.01	3.81 ± 0.03	7.90 ± 0.20	32.36 ± 1.19	1.44 ± 0.07	3.20 ± 0.25	5.84 ± 0.26	23.61 ± 1.39
C4-NAP	0.024	1.77 ± 0.32	7.59 ± 0.06	16.12 ± 1.04	104.70 ± 2.70	2.08 ± 0.25	6.14 ± 0.56	12.80 ± 0.81	85.55 ± 0.94
Biphenyl	0.152	<LOD	<LOD	<LOD	4.13 ± 0.00	<LOD	<LOD	2.27 ± 0.00	3.42 ± 0.50
Acenaphthylene	0.049	<LOD	<LOD	<LOD	2.23 ± 0.11	<LOD	<LOD	<LOD	1.65 ± 0.09
Acenapthene	1.161	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Dibenzofuran	0.058	1.77 ± 0.17	2.37 ± 0.03	3.80 ± 0.17	6.57 ± 0.19	1.55 ± 0.01	1.94 ± 0.17	2.55 ± 0.08	4.88 ± 0.42
Fluorene	0.070	<LOD	1.53 ± 0.00	2.70 ± 0.43	2.54 ± 0.06	<LOD	<LOD	1.80 ± 0.09	2.40 ± 0.31
C1-FLU	0.002	0.17 ± 0.04	0.51 ± 0.02	0.78 ± 0.02	2.23 ± 0.06	0.16 ± 0.02	0.34 ± 0.03	0.57 ± 0.05	1.76 ± 0.03
C2-FLU	0.005	0.85 ± 0.28	2.13 ± 0.13	3.20 ± 0.32	18.49 ± 0.41	0.79 ± 0.24	0.92 ± 0.07	2.56 ± 0.28	13.41 ± 0.27
C3-FLU	0.016	<LOD	1.46 ± 0.10	2.61 ± 0.20	16.49 ± 0.53	0.49 ± 0.13	0.74 ± 0.00	1.44 ± 0.07	10.02 ± 0.29
Phenanthrene	0.506	<LOD	12.13 ± 0.95	22.94 ± 2.62	38.51 ± 0.19	<LOD	<LOD	15.80 ± 0.32	30.59 ± 2.55
Anthracene	2.401	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
C1-PHE	0.003	0.32 ± 0.11	2.74 ± 0.37	4.98 ± 0.30	29.60 ± 0.46	0.29 ± 0.10	1.12 ± 0.14	2.82 ± 0.26	19.04 ± 0.25
C2-PHE	0.014	0.41 ± 0.10	2.69 ± 0.31	9.28 ± 0.11	46.10 ± 0.66	0.59 ± 0.16	0.88 ± 0.02	2.98 ± 0.31	25.69 ± 4.83
C3-PHE	0.023	1.59 ± 0.24	4.83 ± 1.02	15.45 ± 0.69	46.51 ± 1.07	2.90 ± 0.10	1.71 ± 0.04	4.35 ± 0.67	22.37 ± 5.00
C4-PHE	0.155	6.37 ± 1.07	6.68 ± 0.69	20.72 ± 2.74	22.35 ± 0.17	11.09 ± 1.45	6.49 ± 0.60	8.81 ± 2.25	15.69 ± 4.90
Dibenzothiophene	0.040	<LOD	<LOD	2.48 ± 0.11	15.30 ± 1.05	<LOD	<LOD	1.96 ± 0.12	10.28 ± 0.62
C1-DBT	0.002	0.05 ± 0.01	0.42 ± 0.12	0.71 ± 0.00	3.20 ± 0.05	0.09 ± 0.00	0.17 ± 0.02	0.50 ± 0.02	2.22 ± 0.05
C2-DBT	0.001	0.03 ± 0.00	0.27 ± 0.09	0.69 ± 0.03	3.55 ± 0.02	0.06± 0.02	0.08 ± 0.01	0.31 ± 0.03	2.21 ± 0.51

C3-DBT	0.001	0.06 ± 0.02	0.33 ± 0.13	0.60 ± 0.01	1.92 ± 0.03	0.11 ± 0.05	0.05 ± 0.00	0.24 ± 0.02	1.19 ± 0.23
C4-DBT	0.031	1.42 ± 0.60	1.35 ± 0.15	3.49 ± 0.68	1.84 ± 0.14	2.15 ± 0.171	1.70 ± 0.12	1.60 ± 0.45	1.70 ± 0.75
Fluoranthene	0.108	<LOD	2.07 ± 0.00	3.88 ± 0.28	7.82 ± 0.14	<LOD	<LOD	2.45 ± 0.00	6.87 ± 1.08
Pyrene	0.102	<LOD	<LOD	4.16 ± 0.01	12.64 ± 1.13	<LOD	<LOD	1.84 ± 0.00	8.93 ± 1.63
C1-FLA/PYR	0.004	0.34 ± 0.04	0.70 ± 0.10	2.60 ± 0.02	15.01 ± 0.24	0.50 ± 0.17	0.28 ± 0.01	1.04 ± 0.01	5.53 ± 0.60
C2-FLA/PYR	0.047	1.03 ± 0.17	0.10 ± 0.15	3.62 ± 0.48	7.60 ± 0.01	1.77 ± 0.34	<LOD	1.29 ± 0.00	2.73 ± 0.63
C3-FLA/PYR	0.010	0.85 ± 0.06	0.86 ± 0.12	2.08 ± 0.21	2.73 ± 0.02	1.13 ± 0.25	0.57 ± 0.04	0.74 ± 0.08	1.27 ± 0.26
Benz[a]anthracene	0.000	0.77 ± 0.12	1.86 ± 0.27	5.32 ± 0.02	16.35 ± 0.19	1.65 ± 0.12	1.12 ± 0.12	2.69 ± 0.34	11.06 ± 1.98
Chrysene	0.622	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
C1-CHR	0.001	0.08 ± 0.03	0.25 ± 0.07	0.76 ± 0.02	2.36 ± 0.03	0.21 ± 0.07	0.05 ± 0.00	0.12 ± 0.01	0.71 ± 0.06
C2-CHR	0.001	<LOD	<LOD	0.02 ± 0.00	0.07 ± 0.00	<LOD	<LOD	0.02 ± 0.00	0.03 ± 0.00
C3-CHR	0.001	<LOD	<LOD	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	<LOD	<LOD	<LOD
C4-CHR	0.001	<LOD	<LOD	0.02 ± 0.00	0.02 ± 0.00	<LOD	<LOD	<LOD	<LOD
Benzo[b]fluoranthene	0.000	0.24 ± 0.01	0.31 ± 0.06	0.59 ± 0.00	0.91 ± 0.03	1.73 ± 0.00	<LOD	<LOD	0.26 ± 0.04
Benzo[k]fluoranthene	0.031	<LOD	<LOD	<LOD	0.53 ± 0.00	0.96 ± 0.00	<LOD	<LOD	<LOD
Benzo[e]pyrene	0.000	0.08 ± 0.05	0.16 ± 0.04	0.29 ± 0.06	0.75 ± 0.03	0.46 ± 0.07	0.01 ± 0.00	0.17 ± 0.00	0.44 ± 0.01
Benzo[a]pyrene	0.405	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Perylene	0.000	<LOD	0.23 ± 0.11	<LOD	0.08 ± 0.00	<LOD	<LOD	0.08 ± 0.00	<LOD
Indeno[1,2,3-cd]pyrene	0.311	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Dibenz[ah]anthracene	0.617	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Benzo[ghi]perylene	0.187	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sum PAH		30.69 ± 0.54	70.52 ± 3.94	162.07 ± 7.10	489.30 ± 6.92	44.45 ± 4.04	44.01 ± 6.55	88.60 ± 4.53	337.70 ± 32.68
Sum NAP		13.76 ± 0.83	25.42 ± 0.21	44.23 ± 0.32	161.10 ± 3.36	16.21 ± 0.49	19.58 ± 0.73	31.50 ± 1.30	131.33 ± 5.44
Sum 2-3rings PAH		13.44 ± 0.38	38.66 ± 3.53	94.42 ± 6.55	261.56 ± 4.36	20.25 ± 2.99	21.97 ± 5.33	49.40 ± 3.45	168.52 ± 21.05
Sum 4-6rings PAH		3.49 ± 0.08	6.44 ± 0.21	23.42 ± 0.91	66.60 ± 0.77	7.99 ± 0.57	2.46 ± 0.49	7.72 ± 2.37	37.85 ± 6.19

Table S4. Bioconcentration factor in high oil treatment embryos calculated from initial measured water concentrations (Day 0). All values are reduced by a factor of 1000 for the simplicity of representation ($x10^3$).

Analyte (x 10^3)	**0.5°C**		**2.8°C**	
	Day 4	**Day 18**	**Day 4**	**Day 18**
Benzothiophene	<LOD	<LOD	<LOD	<LOD
Naphthalene	2915.05	3248.59	3985.70	4054.45
C1-NAP	5322.90	3456.93	3062.35	3091.82
C2-NAP	9604.20	5781.31	5281.50	5186.32
C3-NAP	16243.98	22819.48	9412.52	18482.53
C4-NAP	13921.02	52384.45	4992.92	43232.02
Biphenyl	7775.26	4456.18	9821.93	4420.88
Acenaphthylene	12010.79	9911.10	7923.79	8904.98
Acenapthene	<LOD	<LOD	<LOD	<LOD
Dibenzofuran	17437.77	9942.66	20338.17	9512.54
Fluorene	9919.64	4196.30	8706.63	4833.94
C1-FLU	19640.71	18460.21	11919.88	15476.78
C2-FLU	20642.25	66158.33	9803.40	42117.50
C3-FLU	22574.78	112200.72	9518.31	50698.79
Phenanthrene	23225.07	25128.03	29796.61	20548.28
Anthracene	<LOD	<LOD	<LOD	<LOD
C1-PHE	32873.04	109241.05	17059.89	68408.38
C2-PHE	23230.24	73890.92	13050.50	36708.88
C3-PHE	19807.24	90278.39	9993.35	24617.96
C4-PHE	19197.64	73883.61	8237.94	25709.87
Dibenzothiophene	28298.93	43694.17	13964.60	30386.58
C1-DBT	<LOD	<LOD	<LOD	<LOD
C2-DBT	28696.44	76553.42	29364.07	38170.67
C3-DBT	18749.99	59286.48	27396.52	22028.19
C4-DBT	81008.04	119983.29	48568.27	60043.32
Fluoranthene	13259.69	19710.43	9618.72	19678.76
Pyrene	21364.35	57165.11	11729.06	36658.79
C1-FLA/PYR	13141.03	93059.89	6613.74	19536.49
C2-FLA/PYR	15266.41	84834.14	10324.37	20199.89
C3-FLA/PYR	7401.78	45712.63	11984.83	10129.11
Benz[a]anthracene	<LOD	<LOD	28136.53	<LOD
Chrysene	<LOD	<LOD	12471.09	<LOD
C1-CHR	8027.55	65946.81	7934.29	9666.32
C2-CHR	<LOD	<LOD	7587.90	<LOD
C3-CHR	1986.52	<LOD	7156.82	2215.68
C4-CHR	<LOD	<LOD	7167.68	<LOD
Benzo[b]fluoranthene	6280.19	<LOD	3432.07	<LOD
Benzo[k]fluoranthene	<LOD	<LOD	0.00	<LOD
Benzo[e]pyrene	<LOD	<LOD	4346.64	<LOD
Benzo[a]pyrene	<LOD	<LOD	0.00	<LOD
Perylene	<LOD	<LOD	<LOD	<LOD
Indeno[1,2,3-cd]pyrene	<LOD	<LOD	<LOD	<LOD
Dibenz[ah]anthracene	<LOD	<LOD	<LOD	<LOD
Benzo[ghi]perylene	<LOD	<LOD	<LOD	<LOD

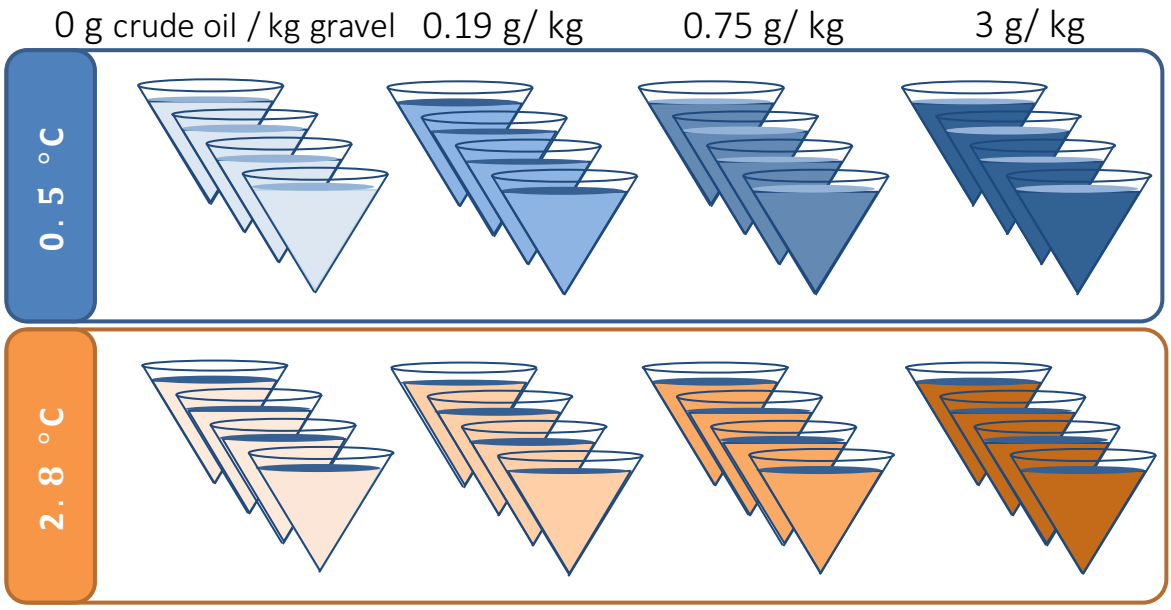

Figure S1. Experimental design with temperature treatments and crude oil exposure levels in groups with four replicate incubators, each incubator contains 8988 ± 1066 eggs at the start of the experiment.

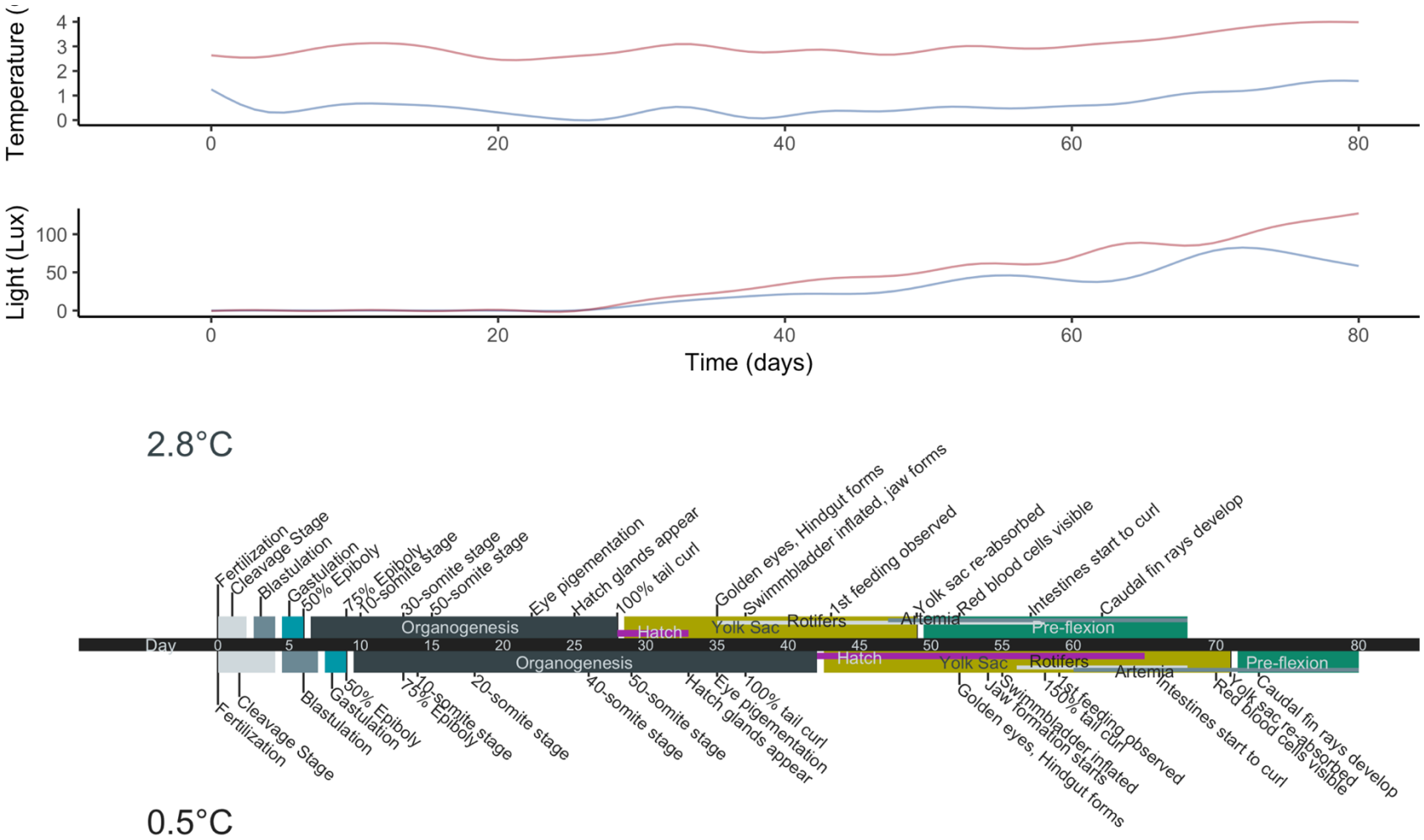

Figure S2. Timeline of the stage and key developmental events of polar cod early life stages reared at 2.8°C (top) and 0.5°C (bottom) for the full period of the study including data not presented in the manuscript. Feeding schemes are presented as small bars to the periphery of the stage bar at each temperature group. Stages were based off G. mohua staging presented in Hall et al. 2004.

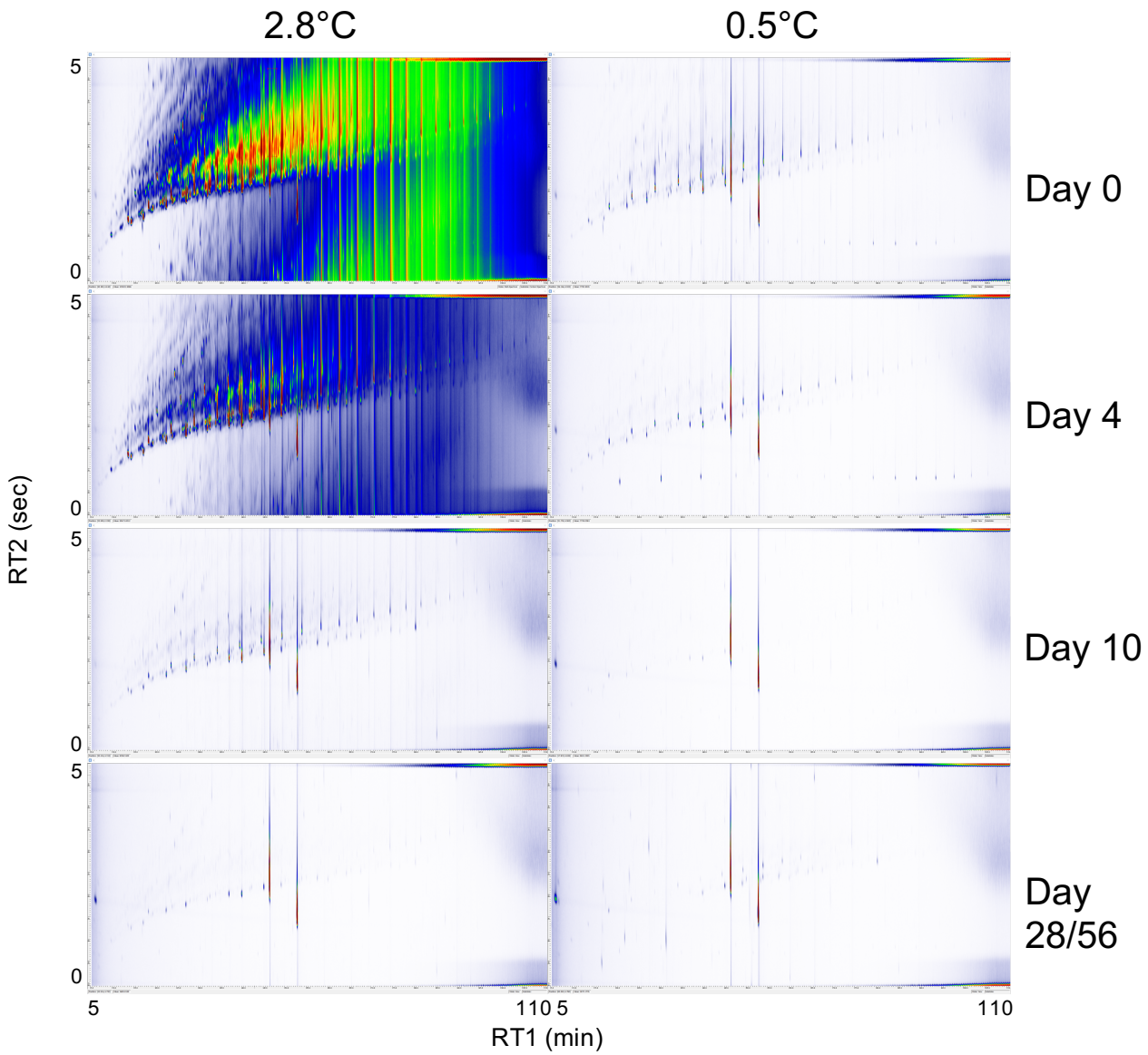

Figure S3. Two dimensional gas chromatography (GCxGC-MS) total ion chromatograms for water samples from the high oil treatments at four different timepoints. The two major peaks visible in all the chromatograms are internal standards. Color scheme is the relative signal strength from cool to warm.

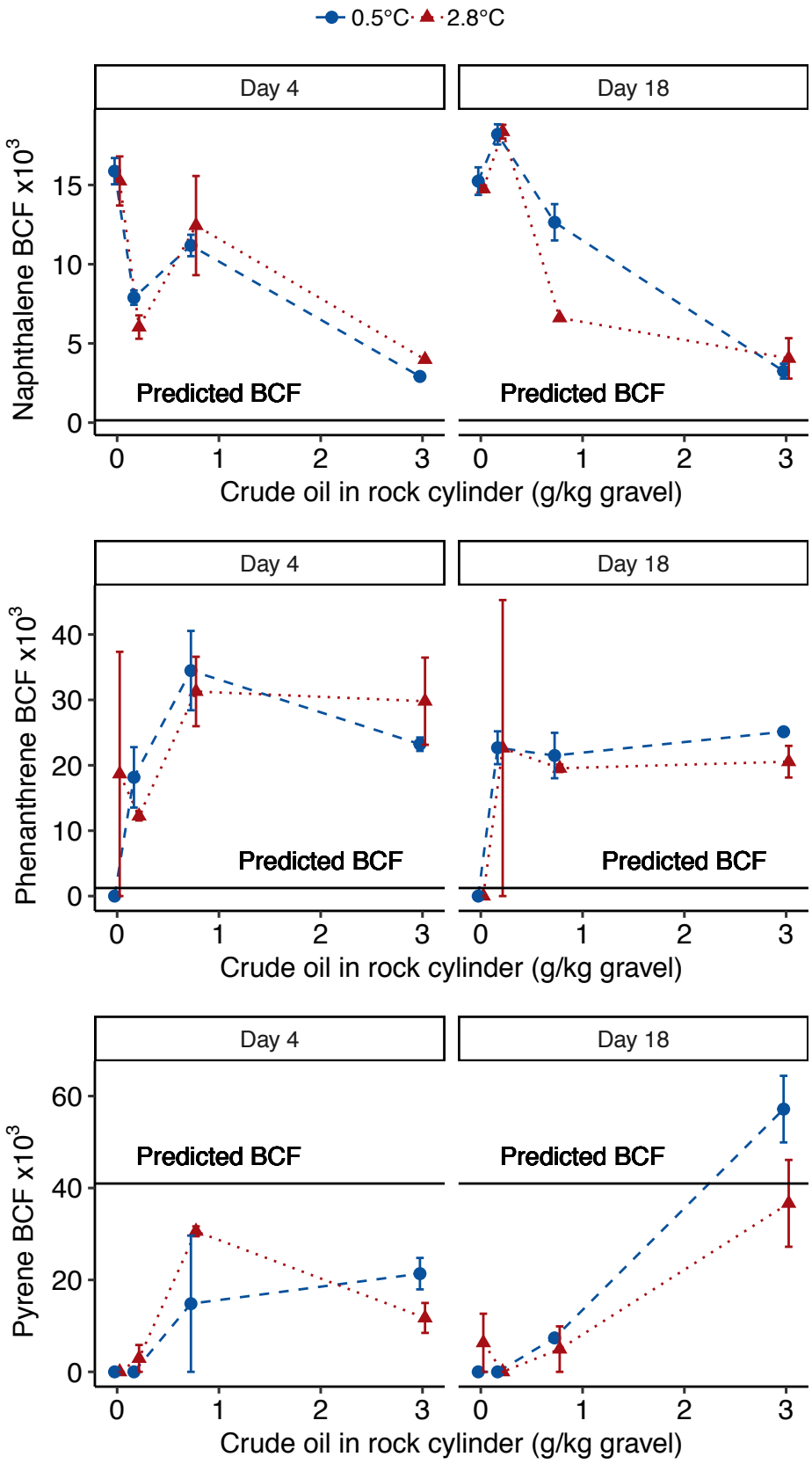

Figure S4. Bioconcentration factors calculated for Naphthalene, Phenanthrene and Pyrene at Day 4 and Day 18. Data is displayed as treatment means (± SEM depicted as bars, each treatment is represented by two independent incubator pools). Colors and symbols indicate temperature groups and dashed and dotted lines represent the trend line for each temperature group. Predicted BCF values using the log K_{ow} equation (log BCF= 0.85* log K_{ow} -0.7) are also displayed for comparison purposes.

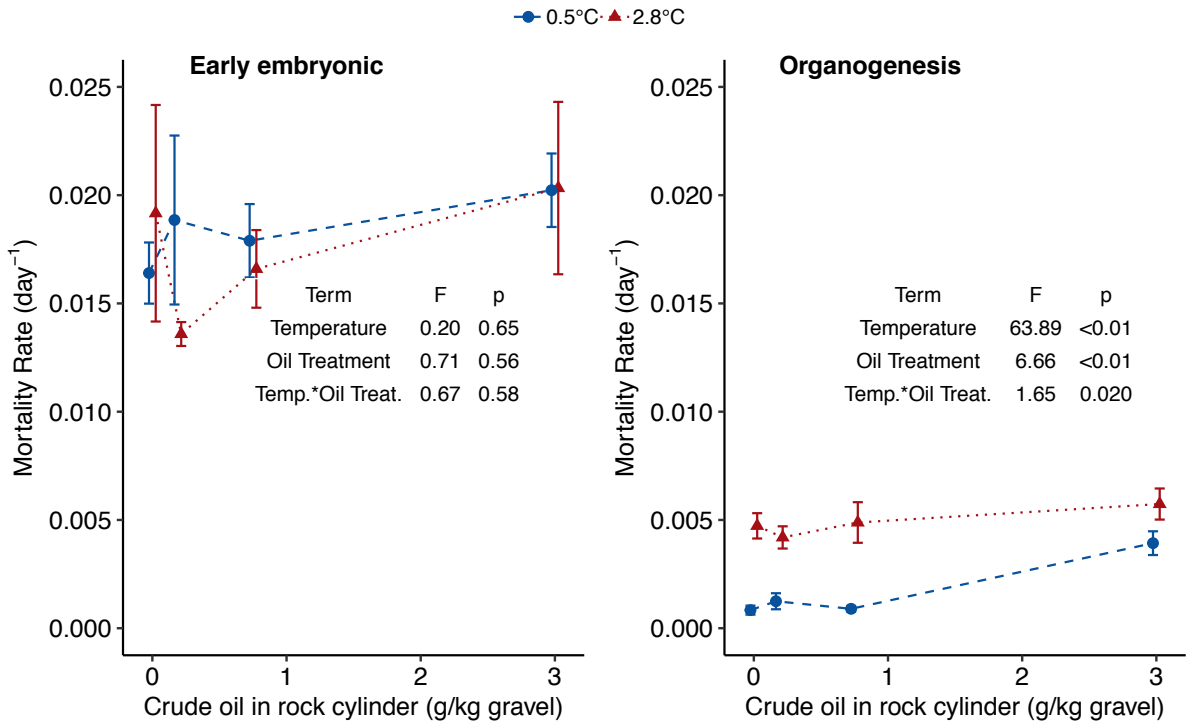

Figure S5. The effect of temperature and crude oil exposure on instantaneous mortality rates for early embryogenesis (left) and late embryogenesis / organogenesis (right). Data is displayed as treatment means (± SEM depicted as bars, each treatment is represented by four independent incubators). Colors and symbols indicate temperature groups and dashed and dotted lines represent the trend line for each temperature group. The terms of the best lme models for each gene are displayed in the respective panels with the associated ANOVA test F-values and p-values.

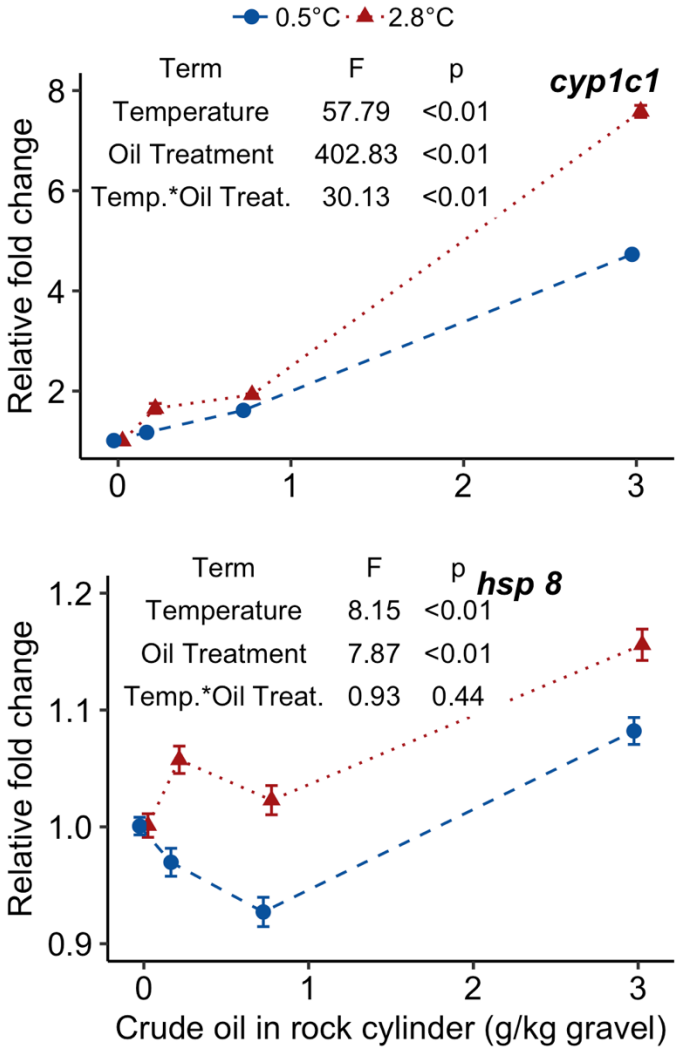

Figure S6. The effect of temperature and crude oil exposure on gene expression of (A) *cyp1c1* and (B) *hsp 8* measured by qRT PCR in embryos just prior to hatch (day 28 at 2.8°C and day 48 at 0.5°C). Data is displayed as treatment means (± SEM depicted as bars, each treatment is represented by four independent incubators). Colors and symbols indicate temperature groups and dashed and dotted lines represent the trend line for each temperature group. The terms of the best lme models for each gene are displayed in the respective panels with the associated ANOVA test F-values and p-values.

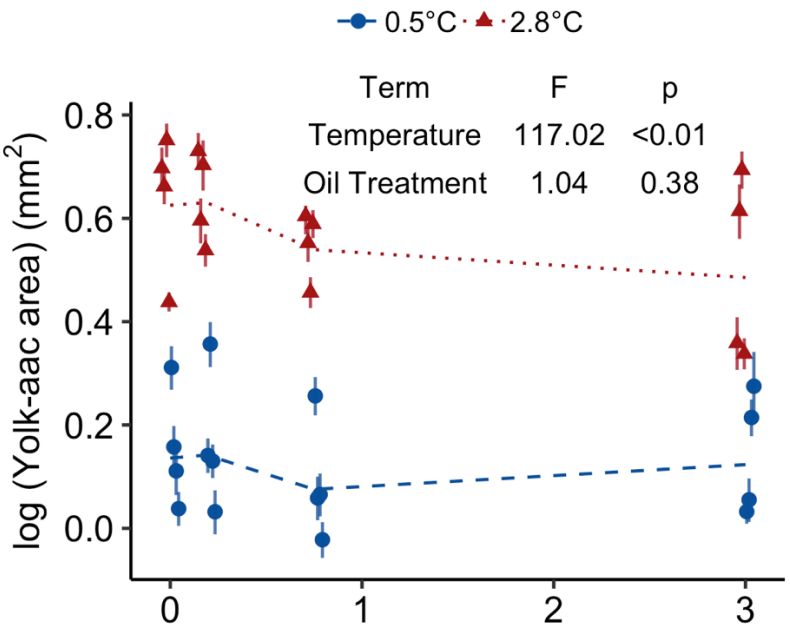

Figure S7. The effect of temperature and crude oil exposure on a log scale (yolk sac area) in yolk sac larvae at day 28 for 2.8°C and day 50 for 0.5°C displayed as incubator means (± SEM depicted as bars, each point includes 20-30 larvae). Colors, symbols, and line type distinguish the temperature groups. The terms of the best gls model for yolk-sac area is displayed with the associated ANOVA test F-values and p-values.

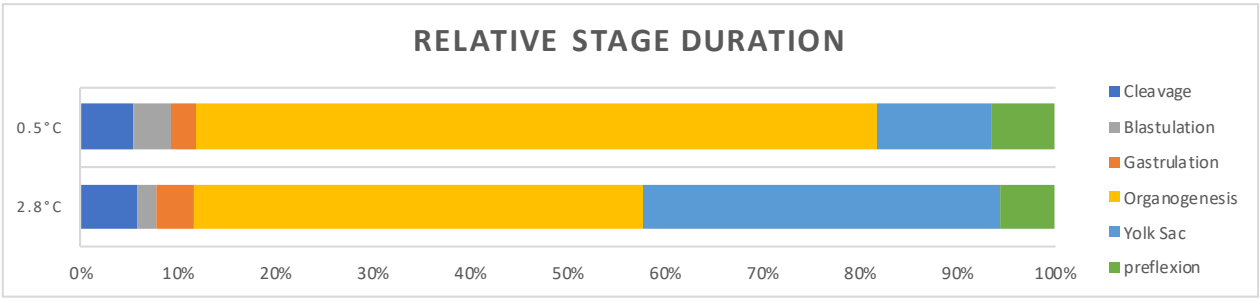

Fig S8. Relative stage duration for both temperature groups over the period of development presented in this study.

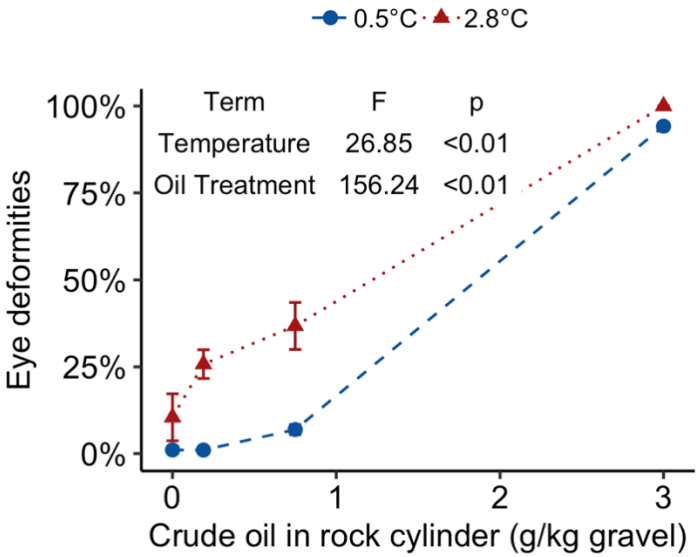

Figure S9. The effect of temperature and crude oil exposure on incidence of jaw deformities in the exogenous feeding larvae. Treatment means (± SEM depicted as bars, each point includes 4 incubator scores) are overlaid on fitted lines produced from the lme model. Model outputs and p-values are displayed in the upper left of each panel. Colors, symbols, and line type distinguish the temperature groups. The terms of the best lme model for prevalence of eye deformities is displayed with the associated ANOVA test F-values and p-values.

Table S5. Best model summaries for all statistical models used. Model terms with p-values < 0.05 are in bold.

Water Chemistry	**Type of Model Additive**	**Model:**	**lm(tPAH ~ Time + factor(Temperature) + factor(Treatment)**			
Coefficients:	Estimate	Std. Error	t	value		
(Intercept)	17.493	15.436	1.133	0.265		
Time	**-0.745**	**0.302**	**-2.470**	**0.019**		
Temperature	-1.806	12.422	-0.145	0.885		
Low Oil Treatment	1.754	17.213	0.102	0.919		
Medium Oil Treatment	13.143	17.213	0.764	0.450		
High Oil Treatment	**55.291**	**17.213**	**3.212**	**0.003**		
Body Burden	**Interaction**	**Model:**	**lme(tPAH ~ Time * factor(Temperature) * factor(Treatment)**			
Coefficients:	Estimate	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	37.826	94.075	15	0.402	0.693	
Low Oil Treatment	5.328	132.899	15	0.040	0.969	
Medium Oil Treatment	147.309	132.899	15	1.108	0.285	
High Oil Treatment	**413.021**	**132.899**	**15**	**3.108**	**0.007**	
Temperature	14.274	132.899	15	0.107	0.916	
Time	-0.396	7.207	15	-0.055	0.957	
Low Oil Treatment : Temperature	4.171	187.948	15	0.022	0.983	
Medium Oil Treatment : Temperature	-32.159	187.948	15	-0.171	0.866	antagonistic
High Oil Treatment : Temperature	**522.199**	**187.948**	**15**	**2.778**	**0.014**	**synergistic**
Low Oil Treatment : Time	1.917	10.193	15	0.188	0.853	synergistic
Medium Oil Treatment : Time	-0.885	10.193	15	-0.087	0.932	antagonistic
High Oil Treatment : Time	2.531	10.193	15	0.248	0.807	synergistic
Temperature : Time	-0.029	10.193	15	-0.003	0.998	antagonistic
Low Oil Treatment : Temperature : Time	-2.469	14.415	15	-0.171	0.866	antagonistic
Medium Oil Treatment : Temperature : Time	-3.059	14.415	15	-0.212	0.835	antagonistic
High Oil Treatment : Temperature : Time	**-38.196**	**14.415**	**15**	**-2.650**	**0.018**	**antagonistic**
Mortality Rates (Early Embryonic Period)	**Interaction**	**Model:**	**lme(Instantous mortality rate of early embryonic phase ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.016	0.003	24	5.653	0.000	
Low Oil Treatment	0.002	0.004	24	0.597	0.556	
Medium Oil Treatment	0.001	0.004	24	0.364	0.719	
High Oil Treatment	0.004	0.004	24	0.932	0.361	
Temperature	0.003	0.004	24	0.673	0.507	
Low Oil Treatment : Temperature	-0.008	0.006	24	-1.383	0.179	antagonistic
Medium Oil Treatment : Temperature	-0.004	0.006	24	-0.700	0.491	antagonistic
High Oil Treatment : Temperature	-0.003	0.006	24	-0.459	0.651	antagonistic

Table S5 continued. Best model summaries for all statistical models used. Model terms with p-values < 0.05 are in bold.

Mortality Rates (Organogenesis Period)	**Interaction**	**Model:**	**lme(Instantous mortality rate of organogenesis phase ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.001	0.001	24	1.494	0.148	
Low Oil Treatment	0.000	0.001	24	0.519	0.609	
Medium Oil Treatment	0.000	0.001	24	0.076	0.940	
High Oil Treatment	**0.003**	**0.001**	**24**	**3.913**	**0.001**	
Temperature	**0.004**	**0.001**	**24**	**4.925**	**0.000**	
Low Oil Treatment : Temperature	-0.001	0.001	24	-0.845	0.406	antagonistic
Medium Oil Treatment : Temperature	0.000	0.001	24	0.085	0.933	synergistic
High Oil Treatment : Temperature	-0.002	0.001	24	-1.865	0.074	antagonistic
Mortality Rates (Yolk-Sac Period)	**Interaction**	**Model:**	**lme(Instantous Mortality rate of yolk-sac phase ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.004	0.004	24	1.119	0.274	
Low Oil Treatment	0.002	0.005	24	0.462	0.648	
Medium Oil Treatment	0.006	0.005	24	1.137	0.267	
High Oil Treatment	**0.020**	**0.005**	**24**	**3.912**	**0.001**	
Temperature	0.010	0.005	24	1.937	0.065	
Low Oil Treatment : Temperature	-0.006	0.007	24	-0.753	0.459	antagonistic
Medium Oil Treatment : Temperature	-0.001	0.007	24	-0.098	0.923	antagonistic
High Oil Treatment : Temperature	**0.043**	**0.007**	**24**	**5.911**	**0.000**	**synergistic**
Mortality Rates (Feeding Larvae)	**Interaction**	**Model:**	**lme(Instantous Mortality rate of feeding phase ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.005	0.003	24	1.566	0.130	
Low Oil Treatment	-0.002	0.004	24	-0.368	0.716	
Medium Oil Treatment	0.002	0.004	24	0.532	0.599	
High Oil Treatment	0.007	0.004	24	1.557	0.133	
Temperature	0.009	0.004	24	2.027	0.054	
Low Oil Treatment : Temperature	-0.003	0.006	24	-0.466	0.645	antagonistic
Medium Oil Treatment : Temperature	0.006	0.006	24	1.050	0.304	synergistic
High Oil Treatment : Temperature	**0.060**	**0.006**	**24**	**9.962**	**0.000**	**synergistic**
Gene Expression (*cyp1a*)	**Interaction**	**Model:**	**lme(Relative fold change of *cyp1a* ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	1.001	1.087	24	0.921	0.366	
Low Oil Treatment	1.090	1.537	24	0.709	0.485	
Medium Oil Treatment	**4.699**	**1.537**	**24**	**3.057**	**0.005**	
High Oil Treatment	**16.067**	**1.537**	**24**	**10.453**	**0.000**	
Temperature	0.022	1.537	24	0.014	0.989	
Low Oil Treatment : Temperature	1.254	2.174	24	0.577	0.570	synergistic

Table S5 continued. Best model summaries for all statistical models used. Model terms with p-values < 0.05 are in bold.

Medium Oil Treatment : Temperature	1.049	2.174	24	0.483	0.634	synergistic
High Oil Treatment : Temperature	**35.704**	**2.174**	**24**	**16.425**	**0.000**	**synergistic**
Gene Expression (*hsp70*)	**Additive**	**Model:**	**lme(Relative fold change of *hsp70* ~ factor(Temperature) + factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	
(Intercept)	1.9477	0.0349	27	55.771	0.000	
Low Oil Treatment	**0.0952**	**0.0442**	**27**	**2.156**	**0.040**	
Medium Oil Treatment	0.0497	0.0442	27	1.125	0.270	
High Oil Treatment	**0.1868**	**0.0442**	**27**	**4.228**	**0.000**	
Temperature	**0.1082**	**0.0312**	**27**	**3.462**	**0.002**	
Gene Expression (*hsp8*)	**Interaction**	**Model:**	**lme(Relative fold change of *hsp8* ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	1.001	0.032	24	31.352	0.000	
Low Oil Treatment	-0.031	0.045	24	-0.686	0.500	
Medium Oil Treatment	-0.073	0.045	24	-1.628	0.117	
High Oil Treatment	0.081	0.045	24	1.803	0.084	
Temperature	0.001	0.045	24	0.011	0.991	
Low Oil Treatment : Temperature	0.087	0.064	24	1.366	0.185	synergistic
Medium Oil Treatment : Temperature	0.095	0.064	24	1.491	0.149	synergistic
High Oil Treatment : Temperature	0.073	0.064	24	1.148	0.262	synergistic
Gene Expression (*cyp1c1*)	**Interaction**	**Model:**	**lme(Relative fold change of *cyp1c1* ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	1.011	0.169	24	5.976	0.000	
Low Oil Treatment	0.163	0.239	24	0.682	0.502	
Medium Oil Treatment	**0.603**	**0.239**	**24**	**2.523**	**0.019**	
High Oil Treatment	**3.718**	**0.239**	**24**	**15.545**	**0.000**	
Temperature	-0.009	0.239	24	-0.039	0.970	
Low Oil Treatment : Temperature	0.489	0.338	24	1.445	0.161	synergistic
Medium Oil Treatment : Temperature	0.321	0.338	24	0.948	0.353	synergistic
High Oil Treatment : Temperature	**2.864**	**0.338**	**24**	**8.467**	**0.000**	**synergistic**
Embryo Density	**Interaction**	**Model:**	**gls(Embryo Density^0.25 ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	t-value	p-value	interaction type	
(Intercept)	1.007	0.000	5890.072	0.000		
Low Oil Treatment	0.000	0.000	0.506	0.613		
Medium Oil Treatment	0.000	0.000	1.047	0.295		
High Oil Treatment	**0.001**	**0.000**	**2.127**	**0.034**		
Temperature	**0.001**	**0.000**	**4.428**	**0.000**		
Low Oil Treatment : Temperature	0.000	0.000	0.726	0.468	synergistic	
Medium Oil Treatment : Temperature	0.000	0.000	0.232	0.817	synergistic	

Table S5 continued. Best model summaries for all statistical models used. Model terms with p-values < 0.05 are in bold.

High Oil Treatment : Temperature	0.000	0.000	0.624	0.533	synergistic
Log(length of yolk-sac larvae)	**Interaction**	**Model:**	**gls(Log(length) ~ factor(Temperature) * factor(Treatment))**		
Coefficients:	Value	Std.Error	t-value	p-value	interaction type
(Intercept)	1.791	0.023	78.221	0.000	
Low Oil Treatment	0.000	0.032	-0.006	0.995	
Medium Oil Treatment	-0.014	0.032	-0.432	0.666	
High Oil Treatment	**-0.079**	**0.032**	**-2.453**	**0.014**	
Temperature	**-0.222**	**0.032**	**-6.834**	**0.000**	
Low Oil Treatment : Temperature	0.001	0.046	0.023	0.982	synergistic
Medium Oil Treatment : Temperature	0.014	0.046	0.315	0.753	synergistic
High Oil Treatment : Temperature	-0.069	0.046	-1.502	0.134	antagonistic
Log(yolk-sac area)	**Additive**	**Model:**	**gls(Log(yolk-sac area) ~ factor(Temperature) + factor(Treatment))**		
Coefficients:	Value	Std.Error	t-value	p-value	
(Intercept)	0.155	0.046	3.337	0.001	
Low Oil Treatment	-0.075	0.059	-1.280	0.201	
Medium Oil Treatment	0.005	0.059	0.085	0.933	
High Oil Treatment	-0.073	0.059	-1.238	0.216	
Temperature	**0.451**	**0.042**	**10.817**	**0.000**	
Heart Rate in yolk-sac larvae	**Interaction**	**Model:**	**gls(Heart Rate ~ factor(Temperature) * factor(Treatment))**		
Coefficients:	Value	Std.Error	t-value	p-value	interaction type
(Intercept)	33.667	1.786	18.852	0.000	
Low Oil Treatment	-3.167	2.526	-1.254	0.213	
Medium Oil Treatment	**-5.250**	**2.526**	**-2.079**	**0.041**	
High Oil Treatment	**-7.167**	**2.526**	**-2.838**	**0.006**	
Temperature	-3.831	2.589	-1.480	0.143	
Low Oil Treatment : Temperature	3.164	3.617	0.875	0.384	synergistic
Medium Oil Treatment : Temperature	3.914	3.617	1.082	0.282	synergistic
High Oil Treatment : Temperature	-1.169	3.617	-0.323	0.747	antagonistic
Heart Arrhthymia	**Additive**	**Model:**	**gls(Interbeat variability ~ factor(Temperature) + factor(Treatment))**		
Coefficients:	Value	Std.Error	t-value	p-value	
(Intercept)	-2.701	0.071	-37.901	0.000	
Low Oil Treatment	-0.046	0.089	-0.517	0.606	
Medium Oil Treatment	-0.108	0.093	-1.165	0.247	
High Oil Treatment	**0.749**	**0.089**	**8.463**	**0.000**	
Temperature	**0.126**	**0.063**	**2.018**	**0.047**	
Yolk-sac edema	**Additive**	**Model:**	**lme(prevelance of yolk-sac edema ~ factor(Temperature) + factor(Treatment))**		
Coefficients:	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.282	0.047	27	6.003	0.000

Table S5 continued. Best model summaries for all statistical models used. Model terms with p-values < 0.05 are in bold.

Low Oil Treatment	-0.045	0.062	27	-0.736	0.468
Medium Oil Treatment	**0.125**	**0.058**	**27**	**2.157**	**0.040**
High Oil Treatment	**0.335**	**0.059**	**27**	**5.638**	**0.000**
Temperature	**-0.133**	**0.042**	**27**	**-3.144**	**0.004**
Log(length of feeding larvae)	**Interaction**	**Model:**	**gls(Log(length) ~ factor(Temperature) * factor(Treatment))**		
Coefficients:	Value	Std.Error	t-value	p-value	interaction type
(Intercept)	1.926	0.013	148.510	0.000	
Low Oil Treatment	**-0.168**	**0.018**	**-9.225**	**0.000**	
Medium Oil Treatment	-0.020	0.018	-1.108	0.268	
High Oil Treatment	**-0.058**	**0.018**	**-3.188**	**0.002**	
Temperature	**-0.123**	**0.019**	**-6.636**	**0.000**	
Low Oil Treatment : Temperature	0.000	0.026	0.016	0.987	synergistic
Medium Oil Treatment : Temperature	-0.030	0.026	-1.127	0.260	antagonistic
High Oil Treatment : Temperature	0.002	0.026	0.080	0.936	synergistic
Specific Growth Rate	**Additive**	**Model:**	**gls(SGR ~ factor(Temperature) + factor(Treatment))**		
Coefficients:	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.516	0.090	26	5.763	0.000
Low Oil Treatment	-0.179	0.113	26	-1.585	0.125
Medium Oil Treatment	-0.029	0.117	26	-0.247	0.807
High Oil Treatment	**-0.361**	**0.113**	**26**	**-3.197**	**0.004**
Temperature	**0.469**	**0.081**	**26**	**5.772**	**0.000**
Swim Bladder Inflation	**Additive**	**Model:**	**lme(prevelance of swim bladder inflation ~ factor(Temperature) + factor(Treatment))**		
Coefficients:	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.307	0.046	27	6.728	0.000
Low Oil Treatment	**-0.124**	**0.060**	**27**	**-2.066**	**0.049**
Medium Oil Treatment	**-0.169**	**0.056**	**27**	**-3.007**	**0.006**
High Oil Treatment	**-0.294**	**0.058**	**27**	**-5.092**	**0.000**
Temperature	-0.011	0.041	27	-0.270	0.790
Feeding (Stomach Fullness)	**Additive**	**Model:**	**lme(Incidence of feeding ~ factor(Temperature) + factor(Treatment))**		
Coefficients:	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.469	0.060	27	7.808	0.000
Low Oil Treatment	-0.080	0.079	27	-1.013	0.320
Medium Oil Treatment	**-0.237**	**0.074**	**27**	**-3.205**	**0.004**
High Oil Treatment	**-0.475**	**0.076**	**27**	**-6.264**	**0.000**
Temperature	0.049	0.054	27	0.914	0.369
Pericardial Edema	**Interaction**	**Model:**	**lme(prevelance of pericardial edema ~ factor(Temperature) * factor(Treatment))**		
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.036	0.070	24	0.513	0.612	

Table S5 continued. Best model summaries for all statistical models used. Model terms with p-values < 0.05 are in bold.

Low Oil Treatment	0.014	0.098	24	0.145	0.886	
Medium Oil Treatment	0.187	0.098	24	1.902	0.069	
High Oil Treatment	**0.873**	**0.098**	**24**	**8.878**	**0.000**	
Temperature	**0.253**	**0.098**	**24**	**2.577**	**0.017**	
Low Oil Treatment : Temperature	0.292	0.145	24	2.016	0.055	synergist
Medium Oil Treatment : Temperature	0.212	0.136	24	1.567	0.130	synergist
High Oil Treatment : Temperature	**-0.328**	**0.139**	**24**	**-2.361**	**0.027**	**antagonist**
Eye Deformities	**Additive**	**Model:**	**lme(prevelance of eye deformities ~ factor(Temperature) + factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	
(Intercept)	-0.030	0.038	27	-0.799	0.431	
Low Oil Treatment	0.071	0.049	27	1.440	0.161	
Medium Oil Treatment	**0.168**	**0.046**	**27**	**3.615**	**0.001**	
High Oil Treatment	**0.913**	**0.048**	**27**	**19.139**	**0.000**	
Temperature	**0.176**	**0.034**	**27**	**5.181**	**0.000**	
Jaw Deformities	**Interaction**	**Model:**	**lme(prevelance of jaw deformities ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.000	0.025	24	0.000	1.000	
Low Oil Treatment	0.010	0.035	24	0.286	0.778	
Medium Oil Treatment	0.017	0.035	24	0.484	0.633	
High Oil Treatment	**0.992**	**0.035**	**24**	**28.321**	**0.000**	
Temperature	0.055	0.035	24	1.569	0.130	
Low Oil Treatment : Temperature	**0.213**	**0.052**	**24**	**4.129**	**0.000**	**synergist**
Medium Oil Treatment : Temperature	**0.167**	**0.048**	**24**	**3.462**	**0.002**	**synergist**
High Oil Treatment : Temperature	-0.047	0.050	24	-0.941	0.356	antagonist
Spinal Deformities	**Interaction**	**Model:**	**lme(prevelance of spin deformities ~ factor(Temperature) * factor(Treatment))**			
Coefficients:	Value	Std.Error	DF	t-value	p-value	interaction type
(Intercept)	0.000	0.042	24	0.000	1.000	
Low Oil Treatment	0.000	0.060	24	0.000	1.000	
Medium Oil Treatment	0.000	0.060	24	0.000	1.000	
High Oil Treatment	0.092	0.060	24	1.534	0.138	
Temperature	0.072	0.060	24	1.198	0.243	
Low Oil Treatment : Temperature	0.155	0.088	24	1.757	0.092	synergist
Medium Oil Treatment : Temperature	0.114	0.082	24	1.381	0.180	synergist
High Oil Treatment : Temperature	**0.471**	**0.085**	**24**	**5.573**	**0.000**	**synergist**