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Effects of produced water components on the early life stages of *Calanus finmarchicus* and *Calanus hyperboreus* reared at different temperatures

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Abstract

Receding sea ice, as a result global warming, increases the marine access to the petroleum resources in the Arctic. Due to the increasing global energy demand, more oil and gas activity is likely to occur. Petroleum activities generate and release produced water into the marine environment. The polycyclic aromatic hydrocarbons (PAHs) are believed to be some of the most toxic compounds in produced water and sub-lethal effects are expected for exposed organisms. The *Calanus finmarchicus* (Gunnerus) and the *C. hyperboreus* (Krøyer) are the copepods dominating the Arctic pelagic ecosystems and the early life stages represent a critical phase among the life stages in these copepods. The present study investigates how hatching, development, growth, and oxygen consumption of eggs and the three first naupliar stages of *C. finmarchicus* and *C. hyperboreus* vary with temperature and reveal the impacts that the produced water-related PAHs have on these relationships. Eggs of *C. finmarchicus* and *C. hyperboreus* were exposed to three different concentrations of the water soluble fraction of 11 selected PAHs (10, 50, 100%, ΣPAH 1.57, 8.17, 15.61 µg L^{-1}, respectively) and raised under three different temperatures (7.5, 10, 12.5°C and 3, 7.5, 10°C, respectively) in a series of laboratory experiments. Time of development was monitored and measurements of oxygen consumption, dry weight, and biometric analysis were conducted for the first four life stages. Hatching time and hatching success were also determined for eggs of both species. Faster hatching and development time were found for eggs and nauplii, respectively, with rising temperature. Oxygen consumption increased with rising temperature and also with increased developmental stage. Additionally, the median length of the N3 nauplii of *C. hyperboreus* seemed to be reduced at 10°C. The results for the PAH exposure did not reveal a clear effect. However, hatching success was high for both species, though eggs of *C. finmarchicus* seemed to be more affected by PAH exposure. The dry weight of *C. hyperboreus* N3 nauplii were significantly lower than unexposed nauplii when exposed to the 100% concentration of the water soluble fraction. Additionally, oxygen consumption of the N2 nauplii of *C. finmarchicus* and the N3 nauplii of *C. hyperboreus* seemed to be more sensitive to temperatures when exposed to PAHs, which could potentially reduce the future recruitment of these copepods. As a consequence, less energy will be probably available for higher trophic levels that rely on the *Calanus* species.

KEY WORDS: *Calanus* • Produced water • PAHs • Temperature • Development • Growth

Sammendrag

Som et resultat av global oppvarming, trekker havisen seg tilbake i Arktis. Dette gir tilgang til de marine petroleumsressursene som finnes her. På grunn av det økende globale energibehovet, er det stor sannsynlighet for at olje- og gassutvinning vil skje i Arktis. Petroleumsaktiviteter genererer og slipper produsert vann ut i det marine miljøet. Polysykliske aromatiske hydrokarboner (PAHer) antas å være de giftigste komponentene i produsert vann og forventes å ha negative effekter på eksponerte organismer. Kopepodene *Calanus finmarchicus* (Gunnerus) og *C. hyperboreus* (Krøyer) dominerer de pelagiske økosystemene i Arktis, hvor de tidlige livsstadiene representerer en kritisk fase hos disse kopepodene. I denne studien ble det undersøkt hvordan klekking av egg, utvikling, vekst og oksygenforbruk varierer med temperatur, og hvordan PAHene i produsert vann påvirker disse forholdene. Egg fra *C. finmarchicus* og *C. hyperboreus* ble eksponert for tre forskjellige konsentrasjoner av den vannløselige fraksjonen av 11 utvalgte PAHer (10, 50, 100%, ΣPAH 1.57, 8.17, 15.61 µg L^{-1}, henholdsvis) og tre ulike temperaturer (7.5, 10, 12.5°C and 3, 7.5, 10°C, henholdsvis) i en serie laboratorieeksperimenter. Måling av utviklingstid, oksygenforbruk og tørrvekt, samt biometriske analyser ble utført for de fire første utviklingsstadiene. Klekketidspunkt og klekkesuksess ble også bestemt for egg fra begge artene. Rask klekketid og utviklingstid ble observert med stigende temperatur for egg og nauplii, henholdsvis. Oksygenforbruket økte ved stigende temperatur, samt for økende utviklingsstadie. Medianlengden for *C. hyperboreus* N3 nauplii syntes å bli redusert ved 10°C. Resultatene for PAH-eksponeringene viste ingen tydelige effekter. Begge artene hadde høy klekkesuksess til tross for eksponering, derimot syntes *C. finmarchicus*-egg å være mer påvirket av PAHene. Tørrvektene for *C. hyperboreus* N3 nauplii eksponert for 100%-konsentrasjonen var betydelig lavere enn ueksponerte nauplii. I tillegg syntes eksponerte N2 *C. finmarchicus* nauplii og N3 *C. hyperboreus* nauplii å være mer sensitive for temperaturer, noe som potensielt kan redusere framtidig rekruttering av disse kopepodene. Som en konsekvens vil mindre energi være tilgjengelig for høyere trofiske nivåer som er avhengige av *Calanus*-artene.

NØKKELORD: *Calanus* • Produsert vann • PAHer • Temperatur • Vekst • Utvikling

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Abbreviations

ANOVA	Analysis of Variance
C1-C5	Copepodite stages 1-5
C L^{-1}	Carbon per liter
DW	Dry weight
GC/MS	Gas chromatography-mass spectrometry
ind.$^{-1}$	per individual
K_{OW}	Octanol-water partitioning coefficient
N1-3	Nauplii stages 1-3
O_2	Molecular oxygen
PAH	Polycyclic Aromatic Hydrocarbons
PW	Produced Water
SD	Standard Deviation
Sm^3 o.e.	Standard cubic Meters of Oil Equivalents
SPE	Solid phase extraction
WSF	Water Soluble Fraction

1 Introduction

The Arctic is a remote area characterised by cold temperatures, a result of the glacial climate, and extreme seasonal variations in solar radiation. Sea ice restricts light penetration and nutrient exchange in the water masses. Large oscillations in ice-cover, water circulation and light regime structure the polar pelagic ecosystems, making the Arctic an area of low species diversity and simple food chains. However, the abundance may be high. Phytoplankton blooms follow the receding ice edge, which limits the biological productions to short periods with ice free waters, in the spring and summer mainly (Dunbar, 1982; Sakshaug et al., 2009). Lipids are central as energy storages in Arctic ecosystems. Low-energy carbohydrates and proteins in ice algae and phytoplankton are converted into high-energy lipids by herbivorous zooplankton such as the calanoid copepods and are rapidly transferred upwards through the food chain, being the major source of energy for large stocks of fish, seabirds and mammals in the Arctic (Falk-Petersen et al., 2009; Falk-Petersen et al., 2007).

1.1 The *Calanus* species

Copepods of the genus *Calanus* dominate the Arctic pelagic ecosystems (Conover, 1988). The Atlantic species *C. finmarchicus* (Gunnerus) dominates the subarctic waters, where a predictable spring bloom occurs between March and May, with highest occurrence in Labrador Sea, northern North Sea and the Norwegian Sea (Planque and Batten, 2000). The related copepods *C. glacialis* (Jaschnov) and *C. hyperboreus* (Krøyer) are considered to be true Arctic species and dominate the water north of the polar front. *C. glacialis* is an Arctic shelf species present in the Barents Sea and the Arctic Ocean, while *C. hyperboreus* occur mainly in the Greenland Sea, Labrador Sea and the Arctic Ocean (Conover, 1988; Hirche and Mumm, 1992). All three species contribute to a large fraction of the total copepod biomass and play a pivotal role in the energy transfer between primary producers and higher trophic levels (Falk-Petersen et al., 2007). This includes many fish species (Runge, 1988), whales (Falk-Petersen et al., 2007) and seabirds, such as the little auk (*Alle alle*), an abundant alcid in the high-Arctic (Mehlum and Gabrielsen, 1993). It forms a direct trophic link to the *Calanus* species, and the *C. hyperboreus* is particularly important in the early chick-feeding period (Mehlum and Gabrielsen, 1993).

1.2 The biology of the *Calanus* species

Both *C. glacialis* and *C. hyperboreus* resemble the *C. finmarchicus* but are larger in body mass and lipid content. All three species differ in feeding, life cycles and reproductive strategies (Conover, 1988). *C. hyperboreus* is the most lipid-rich and has the longest life cycle between the three species with a life span of 2-5 years (Madsen et al., 2001). It reproduces and spawns in the winter prior to the spring bloom, and the production of eggs is entirely dependent on the lipid stores (Henriksen et al., 2012). In contrast, the reproduction of *C. finmarchicus*, which contains less lipid compared to the *C. hyperboreus*, also relies on internal lipids to produce eggs but needs to feed in order to reproduce and complete the oocyte maturation, but may produce 1-3 generations per year (Melle et al., 2014; Swalethorp et al., 2011).

1.2.1 Life cycle of *C. finmarchicus* and *C. hyperboreus*

The life cycle of *C. finmarchicus* and *C. hyperboreus* have some characteristics in common. Eggs hatch as nauplii and a progression of 6 nauplii stages (N1-N6) followed by 5 copepodite stages (C1-C5) occur before moulting into adults (Figure 1.1) (Miller and Tande, 1993). Both species undergo a seasonal migration, where they after a period of active feeding in the surface waters and accumulation of lipid reserves descend to deeper water masses, reduce their metabolic rates and enter a resting state (Hirche, 1996; Hirche, 1997).

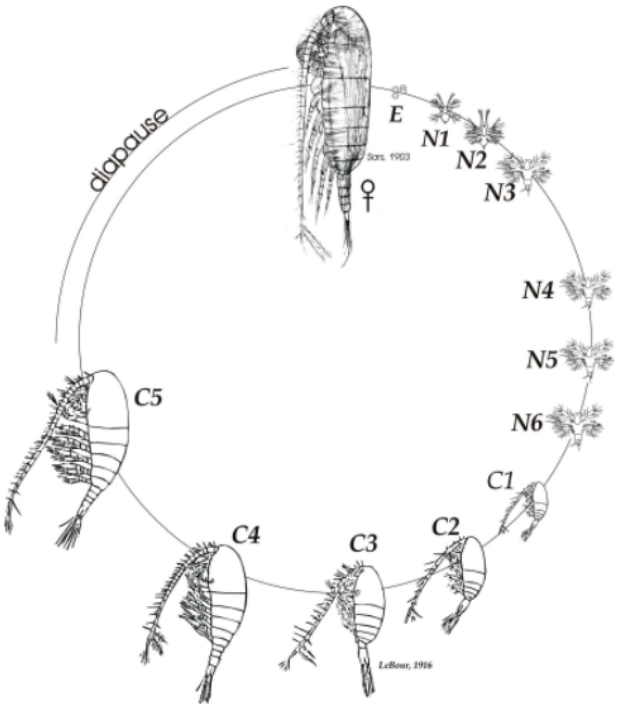

Figure 1.1 The life cycle of a calanoid copepod from egg, followed by 6 naupliar (N1-N6) and 5 copepodite stages (C1-C5) before moulting into adult stage ♀/ ♂ (Baumgartner, 2009).

C. finmarchicus

The C. finmarchicus spend the winter in deep water as the fifth copepodite stage (C5) mainly. They ascend mid-winter and moult to adult stage late winter before the spawning takes place. After mating, females move upward and start spawning in April-May when abundant food becomes available during the spring bloom, and eggs and nauplii are mainly found near the water surface (Hirche, 1996). In Norwegian waters, spawning occurs in March to April and there is time for approximately 2 generations before autumn (Marshall and Orr, 2013; Miller and Tande, 1993). In colder waters, such as off the coasts of northern Norway and Greenland, the life span is mainly one year (Falk-Petersen et al., 2007).

C. hyperboreus

Compared to the *C. finmarchicus*, *C. hyperboreus* matures and spawns earlier. Because it occurs in waters with marked annual and inter-annual variations in ice conditions, *C. hyperboreus* has adapted to different conditions in food availability by having large plasticity in its life strategy (Falk-Petersen et al., 2007). Spawning starts around the beginning of November and is completed by March/April prior to the ice melting and phytoplankton bloom and is fuelled by pre-existing, internal lipid reserves (Falk-Petersen et al., 2009). If primary production is high, the life span of *C. hyperboreus* is 2 years, while under conditions with extensive ice cover the life span varies between 3 and 5 years. Eggs develop rapidly via the naupliar stages to C3, C4 or C5 during the short phytoplankton bloom. After the summer, *C. hyperboreus* has accumulated large lipid reserves and descends to deeper waters and development becomes arrested. C3 is generally the first stage that undergoes diapause *in C. hyperboreus* and moulting into adults occurs over one or more winters and gonads of the adults mature between September and October when food is absent (Falk-Petersen et al., 2007; Hirche and Niehoff, 1996).

1.2.2 Early naupliar growth and development

In terms of morphological characteristics and critical transition stages the nauplii of *C. finmarchicus* and *C. hyperboreus* are similar (Sømme, 1934). Eggs hatch and develop quickly to N1 and N2, two non-feeding stages, where a negative or near zero growth rate is observed. N3 has been suggested to be the first exogenously feeding stage for *Calanus* nauplii (Pedersen et al., 2014), and high growth rates are observed for this stage. The N3 stage is also more prolonged compared to the N1 and N2 stages (Campbell et al., 2001; Marshall and Orr, 2013).

The non-feeding stages rely entirely on maternal energy, and lipids provided with the egg are used for development and respiration (Conover, 1967; Jung-Madsen et al., 2013; Peterson, 2001). The shift in energy source has been reported to be a critical transition stage in the early life stages of copepods and high mortality of the early life stages of nauplii seem to be due to difficulty in the shift of energy sources (Takahashi and Ohno, 1996). The non-feeding stages must therefore develop quickly to the first feeding stage. Over-exploitation of maternal energy may result in energy shortage and prevent the nauplii from developing into N3 (Peterson, 2001).

1.2.3 Relation to the environment

Temperature and food quality and quantity are the most important factors controlling stage development of copepods (Cook et al., 2007). However, developmental rates of nauplii seem to be little affected by variations in food level (Huntley and Lopez, 1992; McLaren, 1978) and have shown to be able to obtain high growth and nearly maximal developmental rates at relatively low food levels (Hygum et al., 2000). Therefore, models using only temperature are frequently used (Cook et al., 2007).

Stage specific developmental rates are described by two rules: the equiproportional rule and the isochronal rule (Peterson, 2001). The rule of equiproportional development, stating that each developmental stage is assumed to occupy the same proportion of time relative to other stages at any constant temperature if food is abundant, is generally assumed when describing the development of copepods (Corkett, 1984; Corkett et al., 1986). This have been shown for *C. finmarchicus* (Campbell et al., 2001), where the relative duration of a given stage was constant for all the experimental temperatures. The isochronal rule states that the stage duration for each stage is the same for all stages (Peterson, 2001). However, this have shown to vary between developmental stages for both *C. finmarchicus* (Campbell et al., 2001) and *C. hyperboreus* (Jung-Madsen et al., 2013). At a given temperature, stage duration increases with developmental stage, with a short duration for the two non-feeding stages (N1 and N2) and a prolonged duration for the first feeding stage (N3).

Previous studies explaining development times as a function of temperature makes use of the Belehradek's temperature function for embryonic development (1935), which assumes that the time from egg to the end of a given life stage is a non-linear function of temperature. Results

from several studies have shown that the developmental time for *C. finmarchicus* decreases with increasing temperature (Campbell et al., 2001; Cook et al., 2007), and this has also been shown for *C. hyperboreus* (Jung-Madsen et al., 2013), when equiproportional development is assumed.

Energetic balance is one of the most important factors regulating the success of copepod populations (Almeda et al., 2011). Because growth and metabolism are closely coupled in living organisms (Ikeda et al., 2001), a metabolic budget may reveal important differences in the cost of maintenance and in the efficiency of food utilisation. Energy demanding processes in an organism are therefore related to the metabolic rate, the rate at which organisms transform energy and matter (Gillooly et al., 2001). Under aerobic condition, respiration and metabolic rates are generally linearly related, because oxygen is used as a reactant in all processes involved. Respiration is therefore frequently used as a proxy for metabolic rate in studies with pelagic copepods (Almeda et al., 2011; Ikeda et al., 2001; Jung-Madsen et al., 2013) and it has been shown that respiration rates increase with larger body size and higher temperatures.

1.2.4 *Calanus* in a warmer Arctic

The Arctic is currently experiencing some of the most rapid and severe climate changes on earth, and major physical, social, economic and ecological changes are expected to occur during the next 100 years (ACIA, 2004). Climate change affects the sea ice coverage and thereby the underwater light climate and availability of nutrients (Tremblay and Gagnon, 2009), factors driving the patterns of plankton succession and the productivity of Arctic marine ecosystems (Falk-Petersen et al., 2007).

Large-scale changes in the biogeography of calanoid copepods have been observed in the North Atlantic Ocean since 1960, with a strong northward extension of warm-water species associated with a decrease in the number of cold-water species (Beaugrand et al., 2002). A warmer Arctic with reduced sea ice cover and new phytoplankton regimes, will most likely favour the *C. finmarchicus*, and will probably be detrimental to the *C. hyperboreus* which is highly adapted to an environment with inter-annual variation in ice cover and algal blooms (Falk-Petersen et al., 2007; Kjellerup et al., 2012). Also, the altered composition of the *Calanus* community will reduce the zooplankton size spectrum to less energy content per individual and

will therefore affect the diet composition and the breeding success of animals that rely on the large and lipid rich *Calanus* species (Falk-Petersen et al., 2007; Wassmann et al., 2011).

Survival of the nauplii is important for the population dynamics and may be sensitive to changes in temperature (Conover, 1967). In years with increased air temperatures, a decrease in sea ice and earlier initiation of the spring plankton bloom have been observed (Hansen et al., 2006; Madsen et al., 2001). For the *C. finmarchicus*, the phytoplankton bloom must match the upward migration from the overwintering depths in order to reproduce successfully (Madsen et al., 2001). It is not known whether *C. finmarchicus* will be able to respond to earlier spring blooms by ascending earlier, but a potential mismatch between these events may result in low reproductive success (Hansen et al., 2003). In contrast, eggs of *C. hyperboreus* are spawned in deep water during winter, prior to the spring bloom (Hirche and Niehoff, 1996). Because of their high lipid-content, the eggs are positively buoyant and float toward the surface (Sømme, 1934), and the nauplii have to survive on the lipids provided by the egg until the spring bloom. When the spring bloom initiates, the nauplii have usually reached the first feeding stage (N3) (Melle and Skjoldal, 1998). Since metabolism increases with increasing temperatures, even small temperature changes may affect survival of the starving *C. hyperboreus* nauplii and a future warmer climate may therefore have large implications for the recruitment of the *C. hyperboreus*.

1.3 Oil production in the Arctic

Receding sea ice cover, as a consequence of global warming, will likely facilitate access to natural resources in the Arctic and expand opportunities for shipping and offshore oil extraction (ACIA, 2004). Oil and natural gas account for approximately 55% of the world´s total energy consumption (IEA, 2016). Most of the Arctic is little explored with respect to petroleum, but the US geological survey has estimated that one quarter of the world´s remaining petroleum resources is expected to be found in the Arctic (USGS, 2008). Because of the increasing energy demand, more oil and gas activity related activities are likely to occur in the Arctic (Peters et al., 2011) and several onshore areas in Russia, Alaska and Canada have already been explored for petroleum resources (USGS, 2008).

Oil and gas activities have been established on the Norwegian Continental Shelf (NCS) over the past 45 years and more than half of the estimated resources remain to be recovered. Despite

the falling oil prices since 2014, drilling and exploration were maintained at a relatively high level (NOG, 2016). For the third year running, oil production rose in 2016 with a production of 232.7 million standard cubic meters of oil equivalents (Sm^3 o.e.), 40.4% of which was oil (Oljedirektoratet, 2017). However, production in the Norwegian and North Sea is expected to decrease in future years but will be offset by increased production in the Barents Sea, reflecting the petroleum exploration results in recent years (NOG, 2016; USGS, 2008).

1.3.1 Produced water

Petroleum activities generate produced water (PW), which is water that has been in contact with geological formations as well as oil in the reservoir. PW is pumped up to the surface with the oil or gas and is treated to separate free oil and is either injected back into the reservoir or discharged overboard (Board et al., 2003). In new fields, PW consists primarily of the water already present in the reservoir, but as the field ages, the quantity of PW increases due to water that is injected to maintain pressure in the reservoir (NOG, 2016). It is permitted as operation discharges and accounts for the largest waste volume from petroleum operations on most offshore platforms (Board et al., 2003; Lee et al., 2011). Estimated release from Norwegian platforms was close to 150 million Sm^3 in 2015 (NOG, 2016). The oil content is regulated and the allowable maximum concentrations vary between region and nation (Board et al., 2003). The official threshold in Norway is 30 mg L^{-1} (NOG, 2016).

PW is a complex mixture of production chemicals added in the separation or production line, dispersed oil droplets, dissolved and particulate organic and inorganic components, such as metals, naturally occurring radioactive substances, alkylphenols, organic acids and polycyclic aromatic hydrocarbons (PAHs) (Lee et al., 2011). Among these, the PAHs are considered to be the most toxic for marine organisms, due to their persistence in the marine environment, and may therefore be used as a proxy for complex oil mixtures. They are defined as hydrocarbons containing two or more fused aromatic rings and are the petroleum hydrocarbons of the greatest environmental concern in the PW. Also, because of variable structures and properties, the PAHs are likely to exert various effects on biological systems (Hylland, 2006).

The PAHs in PW are thought to be highly available to marine organisms (Hylland, 2006), but the exposure of aquatic organisms to hydrocarbons is affected by the portioning of a specific compound. PAHs have a high affinity for organic and particular material, but the relative

partitioning between dissolved and particular fractions varies according to size and the lipophilicity of the component, generally proxied by the octanol-water partitioning coefficient (K_{OW}). Smaller PAHs with a low log K_{OW} are more water-soluble than the heavier PAHs. Dissolved PAHs can be taken up by marine organisms directly from the water by diffusing across gills and the body surface (Board et al., 2003; Hylland, 2006).

1.3.2 Environmental effects

Oil in the sea from anthropogenic sources is considered to be a major environmental problem. Oil can kill or reduce the fitness of organisms and disrupt the structure and function of marine communities and ecosystems (Board et al., 2003). There is also a considerable concern regarding the ocean disposal of PW from petroleum operations due to continuous and increasing discharge volumes, and because the concentrations of the potentially toxic compounds are higher in the PW than the surrounding waters (Lee et al., 2011). However, water column monitoring has shown that PW discharges are rapidly dispersed and diluted by ocean currents after discharge from the platforms (NOG, 2016), and potential biological effects are in general sub-lethal and may potentially affect reproduction, growth and development of exposed organisms (Nørregaard et al., 2014). Also, the content of environmentally hazardous substances will vary between PW from different production fields and region-specific studies are therefore needed to address the risk from different discharges (Lee et al., 2011; NOG, 2016).

The vast majority of the Arctic environment is largely pristine regarding oil hydrocarbons and PAHs, and levels found can usually be attributed to natural sources. Because the Arctic ecosystem differs in several ways from areas further south and because petroleum activity is likely to increase, more knowledge is needed concerning its potential effects in Arctic areas. If exploitation and production are coordinated, it is possible to avoid or reduce potential negative effects on the Arctic marine environment (AMAP, 2010; RCN, 2012).

1.3.3 Effects on *Calanus*

Among the areas were 70% of the undiscovered oil resources are expected to be found, namely the East Greenland Rift Basins, Barents Basins and West Greenland-East Canada (USGS, 2008), are areas were *Calanus spp*. occurs (Conover, 1988). In order to understand how PW

may influence the pelagic production system in these areas, awareness regarding the effect on Arctic zooplankton is decisive (RCN, 2012).

The effects of oil components on zooplankton have been extensively studied. Several studies have been conducted on *C. finmarchicus* and results have shown that components present in PW can modify processes such as development and moulting, metabolism, storage and conversion of fat and defence mechanisms against toxicity and oxidative stress (Hansen et al., 2007; Hansen et al., 2011; Hansen et al., 2008). Narcotisation has been reported as one of the basic modes of action of PAHs in *Calanus* species and it is defined as non-specific disturbance of the cell membrane function caused by lipophilic compounds (Wezel and Opperhuizen, 1995). Disturbance of the membrane function may result in reduced activity and ability to react to stimuli (Barata et al., 2005). This has been observed for the *C. finmarchicus* in several studies, where narcosis and disturbed feeding pattern after pyrene exposure lead to reduced grazing rate (Hjorth and Nielsen, 2011; Nørregaard et al., 2014).

Varying lipid content and potential differences in metabolic activity in different developmental stages within the different species, should be considered when the risk and impacts on marine copepod populations exposed to oil compounds are assessed (Hansen et al., 2011). Because of their lipophilic nature, PAHs bind strongly to storage lipids and cell membranes. This may dilute or postpone any effects in copepods with large fat storage compared to copepods with lower lipid reserves and consequently, an effect will occur faster in copepods with low lipid content (Lotufo, 1998). At present there is little knowledge about the effects of exposure to oil components on early life stages of *Calanus* species. However, since they undergo a series of different developmental stages, where only the last three stages have a high lipid storage, it may be predicted that the early life stages are more sensitive to oil exposure (Hansen et al., 2011). A mesocosm study conducted by Gamble et al. (1987), where natural assemblages of phytoplankton, zooplankton and larval fish from the northern North Sea were exposed to PW concentrations equivalent to 0.5-1 km from the petroleum platform, a noticeable effect was detected on the copepod populations. Early naupliar stages were particularly sensitive to PW and suffered high mortalities. A comparative study conducted to study the effects of PAH exposure on early life stages of *C. finmarchicus* and *C. glacialis* found that both species were affected, but *C. finmarchicus* was more sensitive compared to *C. glacialis*, particularly at increased temperatures. However, eggs of both species hatched successfully in waters contaminated by PAHs, and the high hatching success was thought to be caused by a robust

eggshell acting as a barrier (Grenvald et al., 2013). In contrast, eggs of *C. hyperboreus* are enclosed by a thin and fragile membrane which consist primarily of fatty acids (Jung-Madsen et al., 2013), potentially making them more susceptible to PAH exposure.

In a warmer climate, where access to Arctic oil fields is easier than it is at present, the plankton-based food web may be strongly affected by both increased temperature and petroleum hydrocarbon emissions. Because reduced growth and survival of young life stages will affect population dynamics of copepods, stress from a combination of oil exposure and increased temperatures may potentially have serious effects on zooplankton composition, and thus the ecological function of calanoid copepods in the Arctic marine environment (Hjorth and Nielsen, 2011).

2 Aim of study

Harmful effects from a combination of increasing temperatures and PW components on the early life stages of copepods can have major impacts on the structure and function of marine pelagic ecosystems. Few studies have considered the potential consequences of such environmental changes and chemical emissions on the early life stages of *C. finmarchicus* and *C. hyperboreus*, and while most ecotoxicological data exist on the *C. finmarchicus*, they may not be representative for the *C. hyperboreus*, and a comparative study will therefore be more favourable. It is likely that the *C. hyperboreus* is more susceptible to PW components and elevated temperatures due to its Arctic origin and differences in the egg characteristics when compared to the *C. finmarchicus*.

The aim of this study was therefore to demonstrate how hatching, development, growth and oxygen consumption of egg and the three first naupliar stages of *C. finmarchicus* and *C. hyperboreus* vary with temperature, and reveal whether the WSF of PW-related PAHs have an impact on these relationships. To the authors' knowledge, this is the first study that has been conducted to investigate the combined effects of exposure to temperature and a combination of PAHs on these species. The findings will hopefully provide greater knowledge to the limited information available on the early life stages of *C. finmarchicus* and *C. hyperboreus*.

3 Materials and method

3.1 Experimental setup

This master thesis is a part of the research project "PWC – Arctic: Effects of dispersed oil droplets and produced water components on growth, development and reproduction of Arctic pelagic copepods" founded by Research Council of Norway (project no. 243923/E40). Experimental approaches were based on previous and ongoing research with *C. finmarchicus*.

3.1.1 Laboratory culture of *C. hyperboreus*

Exposure experiments with the Arctic *C. hyperboreus* were performed at the Arctic Station in Disko Island, western Greenland (Figure 3.1), in February 2016. Sampling was conducted from the sea ice in Disko Bay on February 6th, on the coast of Qeqertarsuaq (69.13°N 53.25°W). Mature female copepods were collected with vertical hauls from 180 m depth using a WP3 plankton net (1000 µm) with a closed end container. Net hauls were performed manually at a very low speed to prevent damage to the copepods. The bulk of the zooplankton samples were kept dark at *in situ* temperatures (~0°C) in thermo boxes with seawater. Insulation of the transport containers was necessary to keep the water from freezing. The samples were transported back to the station and copepods were sorted to species level. Ovigerous female *C. hyperboreus* were distributed into clean buckets (10 L) filled with filtered seawater (Sterivex, 0.22 µm filter units). Approximately 40 individuals were maintained in each bucket and kept in the dark at 3°C for egg collection.

Figure 3.1 Location of sampling site and experiments on *C. hyperboreus* in Disko Island, western Greenland (Grenvald et al., 2013).

3.1.2 Laboratory culture of *C. finmarchicus*

Experiments with *C. finmarchicus* were performed at the facilities of NTNU SeaLab in Trondheim, Norway, in November-December 2016. Copepods for the experiments were collected from the continuous in-house culture. The individuals in the culture originate from

copepods collected in the Trondheimsfjord (63°N, 10°E) in 2004 (Hansen et al., 2007). The stock culture is reared in tanks (300 L) with running seawater and fed with a mixture of the microalgae *Isochrysis galbana* (Parke), *Dunaliella tertiolecta* (Bucher) and *Rhodomonas baltica* (Karsten). Approximately 250 female *C. finmarchicus* were transferred from the stock culture to 3 or 4 holding tanks (50 L) for egg collection, fed with *R. baltica* (200 µg C L^{-1}) and kept under regular culture conditions at 10°C for egg collection.

3.2 Experimental system

Eggs of *C. hyperboreus* were exposed to WSF at nominal temperatures 3, 7.5 and 10°C, while exposure experiments on the *C. finmarchicus* eggs were performed at nominal temperatures 7.5, 10 and 12.5°C. Temperatures were controlled by using climate rooms adjusted to the chosen experimental temperature. The experiments were conducted in glass vials (40 mL) marked with corresponding exposure and life stage, which were placed in racks immersed in water baths to decrease temperature variations (Figure 3.2). Throughout the experimental period, the temperature was monitored using a thermometer (sensION 156, P/N 5465069, Hach company, CO, USA).

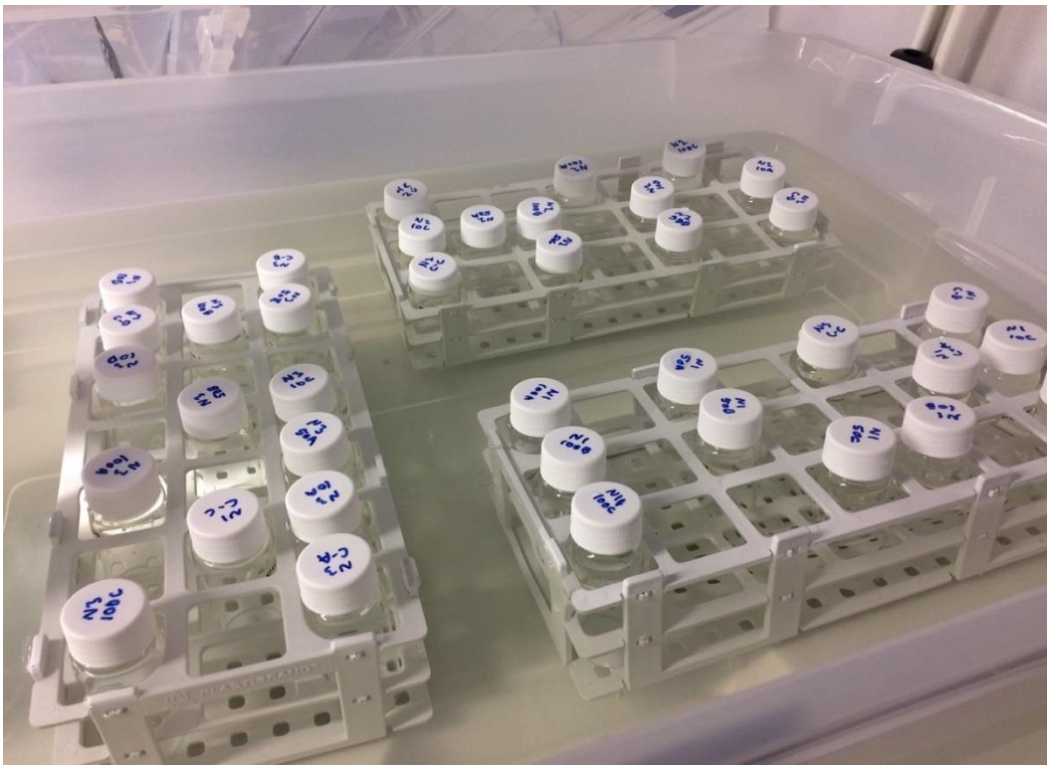

Figure 3.2 Experimental setup for rearing of *C. finmarchicus* and *C. hyperboreus* exposed to WSF at different temperatures. The experiments were conducted in glass vials, marked with corresponding exposure and life stage, placed in racks immersed in water baths to decrease temperature variations.

3.3 Experimental procedure

3.3.1 Generation of exposure solution

The exposure solution, an aqueous solution containing of 11 selected PAHs (Table 3.1), was generated from seawater and a synthetic N-alkane oil spiked with the PAHs (Department of Chemistry and Biochemistry, Florida International University, USA). The selected PAHs are all common components of PW and represent a broad array of lipophilicities which determine the aqueous solubility and bioaccumulation characteristics of the different components.

Table 3.1 The PAHs used to expose eggs of *C. finmarchicus* and *C. hyperboreus* with corresponding log K_{OW} and molecular formula.

ID	Name	Log K_{OW}	Molecular formula
N	Naphthalene	3.30	$C_{10}H_8$
N1	2-Methylnaphthalene	3.79	$C_{11}H_{10}$
N2	2,6-Dimethylnaphthalene	4.24	$C_{12}H_{12}$
F	Fluorene	3.39	$C_{13}H_{10}$
P	Phenanthrene	4.58	$C_{14}H_{10}$
D	Dibenzothiophene	4.37	$C_{12}H_8S$
D1	4-Methyldibenzothiophene	4.86	$C_{13}H_{10}S$
D2	4,6-Dimethyldibenzothiophene	5.33	$C_{14}H_{12}S$
F1	Fluoranthrene	5.19	$C_{16}H_{10}$
Py	Pyrene	5.13	$C_{16}H_{10}$
C	Chrysene	5.78	$C_{18}H_{12}$

The exposure solution was generated at room temperature, filtered and cooled to the experimental temperatures (Figure 3.3). First, a dispersion of the N-alkane oil was produced using a droplet generator system (Nordtug et al., 2011), consisting of four hollows (8 mm) in series connected via nozzles (0.5 mm in diameter). The oil was added through a capillary inlet to the first nozzle from a glass syringe (2.5 mL, SEG, Australia) by an Aladdin syringe pump (0.889 μL min^{-1}) (WPI, Sarasota, FL, USA). Filtered seawater was pumped through the generator system using a Q-SAN metering pump (160 mL min^{-1}) (Fluid Metering Inc., Syosset NY, USA), creating high shear forces downstream the nozzles between each hollow sufficient to break the oil down to homogenous micro droplets and generating an oil dispersion of 5 mg L^{-1} (5 ppm). The dispersion was subsequently led into an overflow chamber to provide

Figure 3.3 Setup of the water soluble fraction generation system. N-alkane oil spiked with 11 selected PAHs was pumped from a glass syringe (A) to the droplet generator (B) where it was forced together with filtered seawater (C) pumped at a flowrate of 160 mL min^{-1} (D), generating a dispersion. The dispersion was kept in the overflow chamber (E) to provide sufficient residence time to assume equilibrium between the PAHs dissolved in the water and in the oil droplets. The dispersion was sucked through two parallel filter units (F) by a pump with a flowrate of 140 mL min^{-1} (G) to ensure only the WSF (H) to come through.

sufficient residence time to assume equilibrium between the PAHs solved in the water and in the oil droplets. Finally, the dispersion was sucked (Q-SAN metering pump, 140 mL min^{-1}) from top to bottom, through two parallel filter holders containing glass wool (10g) and Whatman Grade GF/C and GF/F Glass Microfiber filters (Whatman Ltd., Maidstone, UK; 1.2 μm and 0.7 μm respectively) to remove the oil droplets and ensure only the WSF to pass. The WSF stock was cooled according to the experimental setup and diluted (10, 50 and 100%) prior to exposure of eggs.

3.3.2 Egg sampling and exposure

Eggs from *C. hyperboreus* and *C. finmarchicus* were the starting point of each experiment. For the *C. finmarchicus*, eggs were obtained after 12 hours of spawning. The eggs were collected from the bottom of the tanks using a siphon with a glass tube extension equipped with a terminal suction unit. For the collection of *C. hyperboreus* egg, the eggs were collected after a spawning period of 1-3 days. A ladle and a hose were used for egg collection in the water column and on the water surface. The water was directed through a collecting flask with an internal sieve (mesh size 64 μm) to reduce water volume and trap the eggs, which were further filtered through a funnel with a terminal sieve to further reduce the volume.

Eggs were then transferred to a Petri dish containing a small amount of seawater using a micropipette (20 μL, Eppendorf). For each glass vial 100 *C. hyperboreus* eggs or 200 *C. finmarchicus* eggs were collected. In total 48 vials were used for each temperature experiment: 4 replicates of controls, 10%, 50%, and 100% WSF of PAHs. After all eggs were added, the volume of water in the glass vials was reduced to a fixed amount (4 mL) using a custom-made suction system leaving the eggs in the vials. 35 mL of each exposure solution (10, 50, and 100%) was then added and eggs of *C. finmarchicus* and *C. hyperboreus* were exposed for 24 hours and 48 hours, respectively. Because of the longer embryonic developmental time of *C. hyperboreus* eggs, they were exposed for a longer period to secure that the same embryonic developmental stage in eggs of both species was exposed. After exposure, the eggs were maintained under the selected temperature conditions until the desired developmental stage (N1, N2, and N3) was reached. Animals in filtrated seawater were used as controls. When the nauplii reached the N2 stage, *R. baltica* (200 μg C L^{-1}) was added to each the glass vials to prevent possible low nutritional access and reducing the energy cost in finding food. The

animals were subsequently sampled in four replicates for all exposure concentrations and controls at each developmental stage.

3.3.3 Egg hatching success

Eggs spawned within 12 hours for *C. finmarchicus* and 48 hours for *C. hyperboreus* were transferred to glass vials (8 mL) containing filtered seawater (400 µL). Hatching success experiments were conducted in triplicate for controls and the different exposure concentrations (10, 50, and 100% WSF) at nominal temperatures 7.5, 10 and 12.5°C for *C. finmarchicus* and 3, 7.5 and 10°C for *C. hyperboreus*. Eggs were exposed to WSF (7.5 mL) until given time points: 25, 36, 48 and 61 hours for *C. finmarchicus* and 67, 91 and 115 hours for *C. hyperboreus*. Samples were then terminated using acid Lugol solution. Eggs and nauplii from the exposure vials were subsequently counted and hatching success (%) were determined using equation 3.1, where the number of hatched nauplii was divided by the number of eggs present (hatched + unhatched, n) in the glass vials and multiplied by 100.

$$Hatching\ (\%) = \frac{hatched\ nauplii}{n} \times 100 \qquad [3.1]$$

3.3.4 Stage determination and development time

Animals were sampled when the desired developmental stage was reached. Nauplii stages were determined based on morphological characteristics using a stereo microscope (Leica M80). A stage determination key with close-up pictures and descriptions of the main characteristics (Post-Doc Iurgi Salaverria, Department of Biology, NTNU) helped distinguishing between the different developmental stages. Pictures of eggs and nauplii of *C. finmarchicus* and *C. hyperboreus* are shown in Appendix A. Developmental time was estimated as the time from egg incubation until the sampling of the selected stage.

3.3.5 Oxygen consumption

Approximately 30 eggs and 30 (N1 and N2) or 20 (N3) individuals of the desired developmental stage were collected from the glass vials for measurements of oxygen consumption. Eggs and animals were transferred to micro chambers (100 µL) mounted on a customised cooling block of aluminium placed in a water circulation bath connected to a water cooling bath (Figure 3.4). The dissolved oxygen concentration (mg L^{-1}) in the water of the

micro chambers were monitored by using a fibre-optic oxygen meter (FireStringO2, PyroScience GMbH, Aachen, Germany), which was used with four sensors (ZA7-526-211, ZA7-513-215, ZA7-524-212, ZA7-534-216 – replaced with ZA7-509-214 in the experiments on *C. finmarchicus*). Fibre-optic oxygen sensors (Retractable oxygen micro-sensor, PyroScience, Item Mo. OXR230) continuously measured oxygen concentrations inside the micro chambers and measurements were based on optical detection principles (REDFLASH technology) (del Alamo-Sanza et al., 2014). The instrument was calibrated every sampling day with air-saturated distilled water (Milli-Q) using a one point calibration curve according to the producer's instructions. The air saturated water was prepared by shaking a bottle (500 mL) containing 50% water and 50 % air. Elimination of any air or air bubbles within the chambers was necessary for accurate measurements. Additionally, the chambers had to be filled up to a positive meniscus before capping. The chosen temperature was set and the oxygen content inside the micro chambers was recorded during approximately 1 hour for both *C. hyperboreus* and *C. finmarchicus*.

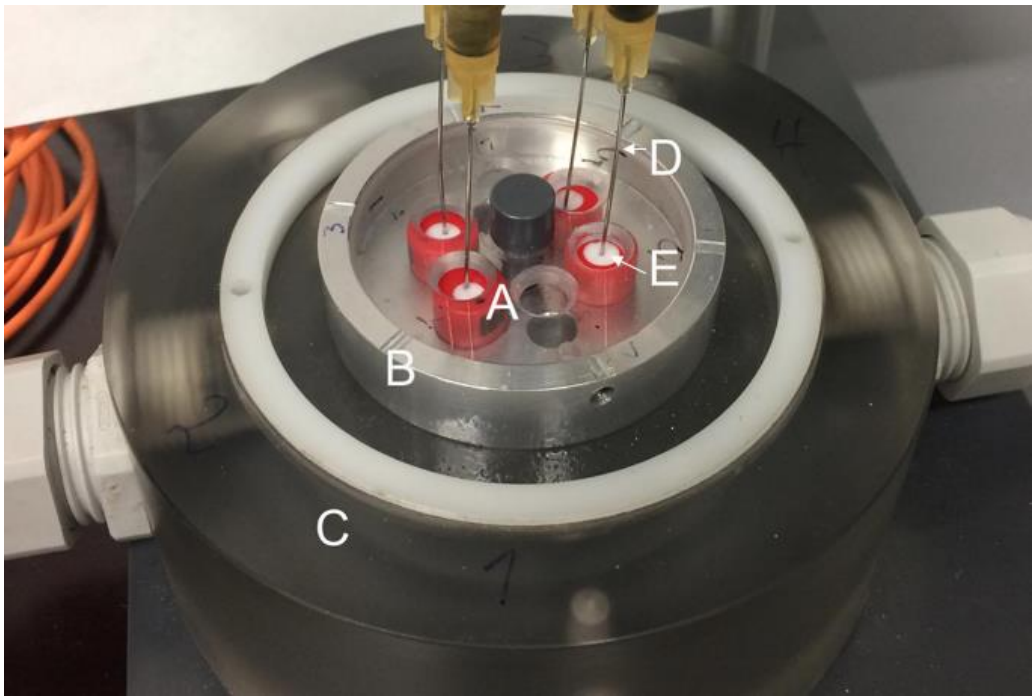

Figure 3.4 Setup for measurement of oxygen consumption. Micro chambers (A) were placed on a cooling block (B) surrounded by a circulating water bath (C) maintaining the chosen experimental temperature. The sensor tip (D) was retracted inside the syringe needle when penetrating the chamber cap septum (E).

Oxygen consumption was determined from regression calculation of the measured oxygen concentration (mg L^{-1}) against time (sec) according to the formula:

$$y = ax + b \quad [3.2]$$

where y is the difference in oxygen concentration over the measuring period (mg L^{-1}), a is the regression coefficient which gives the oxygen reduction rate in the chamber (in mg O_2 L^{-1} min^{-1}), while x is a defined time fraction of the measured period (30 min), and b is the intercept.

The regression coefficient was used to determine oxygen consumption according to:

$$O_2\ consumption\ (\mu g\ O_2\ ind.^{-1}\ h^{-1}) = \frac{a \times V \times 1000 \times 3600}{n} \quad [3.3]$$

where a is the is the regression coefficient, V is the volume of the chamber (L), and n the number of animals in the chamber. To get the measured O_2 unit (mg L^{-1}) in µg L^{-1}, results were multiplied by 1000, and by 3600 to convert to oxygen consumption per hour (h). See Appendix B for details on the regression statistics.

3.3.6 Biometric analysis

Eggs and nauplii from each micro chamber used to measure consumption were placed on a cavity slide for biometric analysis. Images of each individual were captured at a fixed magnification (0.4) with a still-video camera operated by Fire-i software (Unibrain Inc., San Ramon CA, USA). Egg diameter and prosome length of N1, N2, and N3 were conducted manually (Figure 3.5) in the software ImageJ (National Institute of Health, Bethesda MD, USA) with the aid of a graphic tablet (Wacom, Intous3 Co., Ltd, Saitama, Japan), which was calibrated, based on the fixed magnification to count 538 pixels per millimetre.

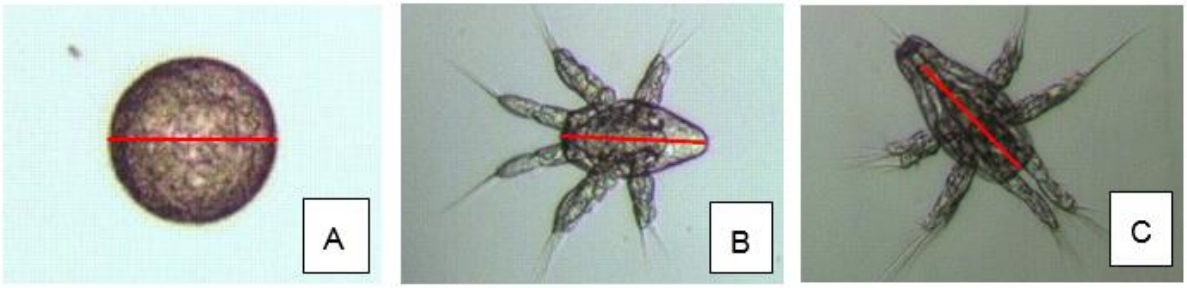

Figure 3.5 Biometric measurements of *C. finmarchicus* and *C. hyperboreus* eggs and nauplii. Red lines indicate diameter of eggs (A), length of prosome of N1 and N2 (B), and N3 (C).

3.3.7 Dry weight

For the analysis of dry weight, the individuals from each sample were transferred to pre-weighed tin capsules (5x9 mm) (Säntis Analytical AG, Teufen, Switzerland) placed in a 96 well plate. To avoid formation of salt crystals, eggs and nauplii were rinsed in an isotonic ammonium format solution (CH_5NO_2) before placing them in the tin capsules. The samples were dried in a heating cabinet (~60°C) for at least 12 hours. Dry weight was determined by subtracting the weight of the empty tin capsules from the weight of the tin capsules containing the samples using a micro weight scale (Mettler Toledo). Dry weight of egg and nauplii were determined according to equation 3.4, where sample weight was divided by the number of animals (n) added to the tin capsule. The results were multiplied by 1000 to get the dry weight (mg) converted to μg.

$$DW\ (\mu g\ ind.^{-1}) = \frac{sample\ (weight)}{n} \times 1000 \qquad [3.4]$$

3.3.8 Chemical analysis of water samples

Water samples of exposure media were collected from controls and different WSFafter 24 and 48 hours for *C. finmarchicus* and *C. hyperboreus*, respectively, and were acidified with diluted hydrochloric acid (15% HCl) to stop microbial degradation of the PAHs in the samples. The PAHs in the stock exposure solutions and collected water samples (volume approximately 700 mL) were extracted by solid-phase extraction (SPE) using an Agilent Bond Elut PPL SPE column performed by SINTEF Materials and Chemistry, Trondheim, according to SINTEF Environmental Technology's standard procedure. Further, the extracts were analysed by gas chromatography-mass spectrometry (GC/MS) using a method based on the US EPA 8270D method to quantify the different PAH components (US EPA, 2014).

3.4 Statistics

Statistical comparisons of the different exposure treatments were performed using Analysis of Variance (ANOVA). The effect of temperature on unexposed eggs, was analysed by using One-Way ANOVA. To check for an interaction effect between temperature and exposure, Two-Way ANOVA analysis was used on exposed nauplii reared under different temperatures. If a significant effect was detected within the main effects, a Holm-Sidak multiple comparison test was run to isolate which the groups that differed from the others. Differences were

considered to be statistically significant if $p < 0.05$. A Shapiro-Wilk test and analysis of data frequency distribution were used to test for normality ($p = 0.05$), while a Brown-Forsythe test and vertical spread of the residuals were used to test for equal variances ($p = 0.05$). If data was not normally distributed nor showed homogeneity of variances, the data was transformed by using log or square root transformation to reduce skewness to the right. ANOVA is not particularly sensitive to non-normality or heterogeneity of variances when applied to a balanced design, but analysis may result in false positives (Type I errors) if the requirements are not met. However, if group sample sizes are approximately equal and large, there is normality and the ratio of the largest group variance is less than three, the ANOVA can be run due to its robustness to the heterogeneity of variances in these circumstances (Laerd Statistics, 2015). Statistical analysis and generation of graphs were performed with the software SigmaPlot 13.0 (Systat Software, Inc). Results are presented as mean ± standard deviation (SD) unless otherwise stated.

4 Results

4.1 Experimental conditions

4.1.1 Temperature

Throughout the experiment, water temperatures were recorded and some deviations from the nominal temperatures were observed (Table 4.1). Measured temperatures are used to describe development of the nauplii, while nominal temperatures are used consistently to describe egg hatching, growth and oxygen consumption.

Table 4.1 Water temperatures (mean ± SD) for each temperature treatment.

C. hyperboreus		*C. finmarchicus*	
Nominal temperature	Measured temperature	Nominal temperature	Measured temperature
3°C	3.5±0.1°C	7.5°C	7.5±0.2°C
7.5°C	7.6±0.3°C	10°C	9.7±0.3°C
10°C	9.3±0.3°C	12.5°C	12.3±0.3°C

4.1.2 Exposure concentration

Stock solutions for the exposure experiments, egg hatching experiments and water samples collected after exposure, were analysed to determine the composition and concentration of the different PAHs (Table 4.2). No water samples were collected from the vials used in the egg hatching experiment. Only minor differences in PAH concentrations were observed for the exposure solutions used to expose *C. finmarchicus* and *C. hyperboreus*, and they were therefore pooled. The 100% WSF concentration of the exposure experiment and the egg hatching experiment had the highest PAH concentration (ΣPAH 15.61 μg L^{-1} and 20.11 μg L^{-1}, respectively), while the 10% exposure solution contained the lowest concentration of PAHs (ΣPAH 1.57 μg L^{-1} and 1.93 μg L^{-1}, respectively). Small amounts were also detected in the controls. The naphthalenes (N, N1 and N2) were the dominating PAHs in the WSF. The measured ΣPAH concentration in the different solutions collected after exposure of eggs, showed an approximately 50% reduction in PAH concentration.

Table 4.2 Measured PAH composition and concentration (μg L^{-1}) (mean ± SD) in the WSF stock solution (exposure solution), the exposure solution collected from the glass vials after 24 and 48 hours for *C. finmarchicus* and *C. hyperboreus*, respectively, and also the WSF stock solution used in the egg hatching experiment of both species. Filtered seawater was used as control. Refer to Table 3.1 for identification of the PAHs.

	Measured concentrations (μg L^{-1})											
	Exposure solution				Collected exposure solution				Exposure solution (egg hatching)			
PAH	Ctrl	10%	50%	100%	Ctrl	10%	50%	100%	Ctrl	10%	50%	100%
N	0.01 ±0.01	0.59 ±0.05	2.97 ±0.19	5.67 ±0.52	0.04 ±0.03	0.34 ±0.09	1.73 ±0.56	3.43 ±1.16	0.01 ±0.00	0.65 ±0.05	3.32 ±0.22	6.60 ±0.57
N1	0.01 ±0.01	0.41 ±0.21	2.24 ±1.13	4.25 ±2.28	0.01 ±0.00	0.20 ±0.15	1.07 ±0.86	2.07 ±1.70	0.01 ±0.01	0.59 ±0.05	3.22 ±0.14	6.44 ±0.39
N2	0.01 ±0.01	0.29 ±0.05	1.52 ±0.25	2.99 ±0.67	0.02 ±0.01	0.14 ±0.05	0.70 ±0.28	1.41 ±0.61	0.01 ±0.01	0.36 ±0.01	1.98 ±0.01	3.91 ±0.02
F	0.00 ±0.00	0.04 ±0.01	0.21 ±0.03	0.39 ±0.06	0.00 ±0.00	0.03 ±0.01	0.12 ±0.04	0.23 ±0.09	0.00 ±0.00	0.05 ±0.01	0.24 ±0.04	0.48 ±0.08
P	0.00 ±0.00	0.17 ±0.04	0.91 ±0.17	1.73 ±0.32	0.01 ±0.00	0.09 ±0.03	0.48 ±0.19	0.96 ±0.39	0.00 ±0.00	0.19 ±0.02	1.04 ±0.09	2.05 ±0.22
D	0.00 ±0.00	0.02 ±0.00	0.09 ±0.01	0.17 ±0.03	0.00 ±0.00	0.01 ±0.00	0.05 ±0.02	0.09 ±0.04	0.05 ±0.05	0.06 ±0.04	0.10 ±0.01	0.15 ±0.07
D1	0.00 ±0.00	0.02 ±0.00	0.10 ±0.01	0.18 ±0.02	0.00 ±0.00	0.01 ±0.00	0.05 ±0.02	0.09 ±0.04	0.00 ±0.00	0.02 ±0.00	0.10 ±0.01	0.21 ±0.01
D2	0.00 ±0.00	0.01 ±0.00	0.06 ±0.02	0.12 ±0.04	0.00 ±0.00	0.01 ±0.00	0.03 ±0.01	0.05 ±0.03	0.00 ±0.00	0.01 ±0.01	0.06 ±0.00	0.12 ±0.00
F1	0.00 ±0.00	0.00 ±0.00	0.01 ±0.01	0.02 ±0.01	0.00 ±0.00	0.00 ±0.00	0.01 ±0.00	0.01 ±0.00	0.00 ±0.00	0.00 ±0.00	0.01 ±0.01	0.02 ±0.00
Py	0.00 ±0.00	0.01 ±0.00	0.03 ±0.02	0.04 ±0.02	0.00 ±0.00	0.01 ±0.00	0.02 ±0.01	0.02 ±0.01	0.00 ±0.00	0.00 ±0.00	0.02 ±0.02	0.05 ±0.00
C	0.00 ±0.00	0.01 ±0.00	0.03 ±0.02	0.05 ±0.02	0.00 ±0.00	0.00 ±0.00	0.01 ±0.01	0.02 ±0.01	0.00 ±0.00	0.00 ±0.00	0.02 ±0.02	0.08 ±0.00
ΣPAH	0.04 ±0.03	1.57 ±0.21	8.17 ±1.26	15.61±3.03	0.10 ±0.05	0.84 ±0.30	4.26 ±1.81	8.40 ±3.65	0.07 ±0.02	1.93 ±0.11	10.10±0.55	20.11±1.37

4.2 Egg hatching

Hatching success (% of eggs present) of both *C. finmarchicus* and *C. hyperboreus* eggs, from incubation of eggs to termination, are plotted against time (days) (Figure 4.1). Hatching time was faster for *C. finmarchicus* eggs compared to eggs of *C. hyperboreus*, but increased water temperatures reduced the egg hatching time of both species. Statistical analysis revealed no significant interaction between temperature and exposure effects on egg hatching of either species at any time point (two-way ANOVA). See Appendix C for more specific data on egg hatching.

No significant effect of both temperature and PAH exposure was observed on the egg hatching after 25 and 67 hours for C. *finmarchicus* and *C. hyperboreus* eggs, respectively. However, after 36 hours, the hatching percentage of *C. finmarchicus* eggs was considerably higher at elevated temperatures, and after two days, an overall highly significant difference in hatching success was detected between 7.5 and 10°C and between 7.5 and 12.5°C ($p < 0.001$, Holm-Sidak). Also, at this time point the hatching percentage at 7.5°C was found to be significantly lower for eggs exposed to 100% WSF compared the treatment groups to the controls ($p = 0.014$). The opposite was observed at 12.5°C, where the 100% exposure treatment significantly increased the hatching percentage when compared to unexposed eggs ($p = 0.032$, Holm-Sidak). In addition, within the 50% exposure treatments, the hatching percentage of *C. finmarchicus* eggs reared at 7.5°C was significantly lower compared to eggs reared at both 10 and 12.5°C ($p = 0.08$, $p = 0.004$, respectively, Holm-Sidak). This was also observed within the 100% exposure treatment, where the hatching percentage of eggs reared at 7.5°C was significantly lower than the hatching percentage in both the 10 and 12.5°C treatment ($p < 0.001$, Holm-Sidak).

At the end of the experiment, no significant differences in hatching success was detected between the different temperatures or exposure treatments. In addition, final hatching success of both *C. finmarchicus* and *C. hyperboreus* eggs was relatively high.

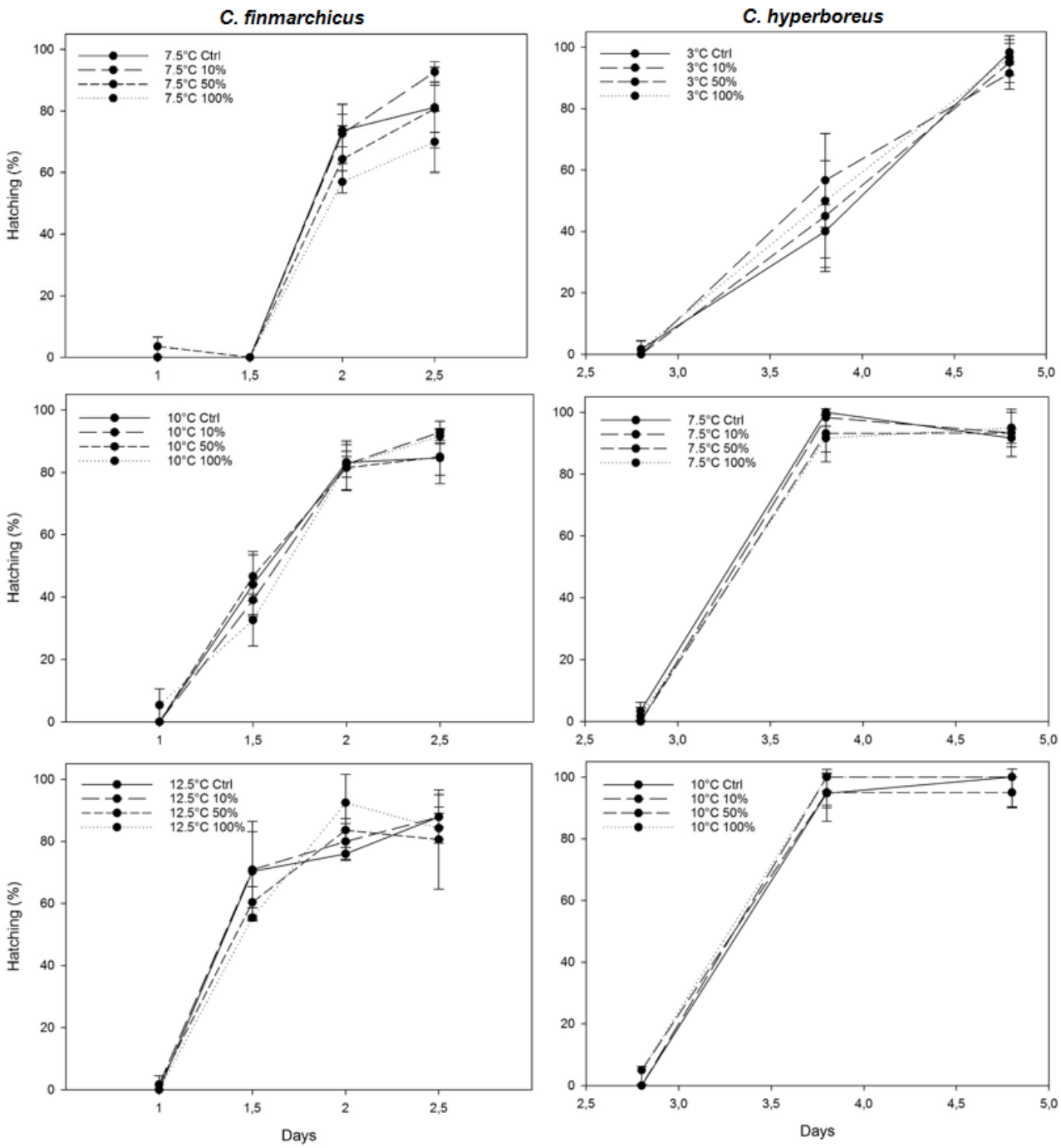

Figure 4.1 Hatching success (%) over time (days) for eggs from *C. finmarchicus* and *C. hyperboreus* exposed to three concentrations of WSF at three different treatment temperatures (mean ± SD).

4.3 Naupliar development

Development time from the incubation of eggs to hatching into the first naupliar stages (N1 to N3) of *C. finmarchicus* and *C. hyperboreus* proved to be temperature dependent (Figure 4.2). For both species, stage duration and developmental time were reduced with increasing temperature for both species. For *C. finmarchicus*, time of development from egg to N3 decreased from 5.1 days at 7.6°C to 3.2 days at 12.3°C. For *C. hyperboreus*, time of development decreased from 14 days at 3.5°C to 9.3 days at 9.3°C. More specific data on stage durations and developmental time for each treatment for both *Calanus* species are given in Appendix D.

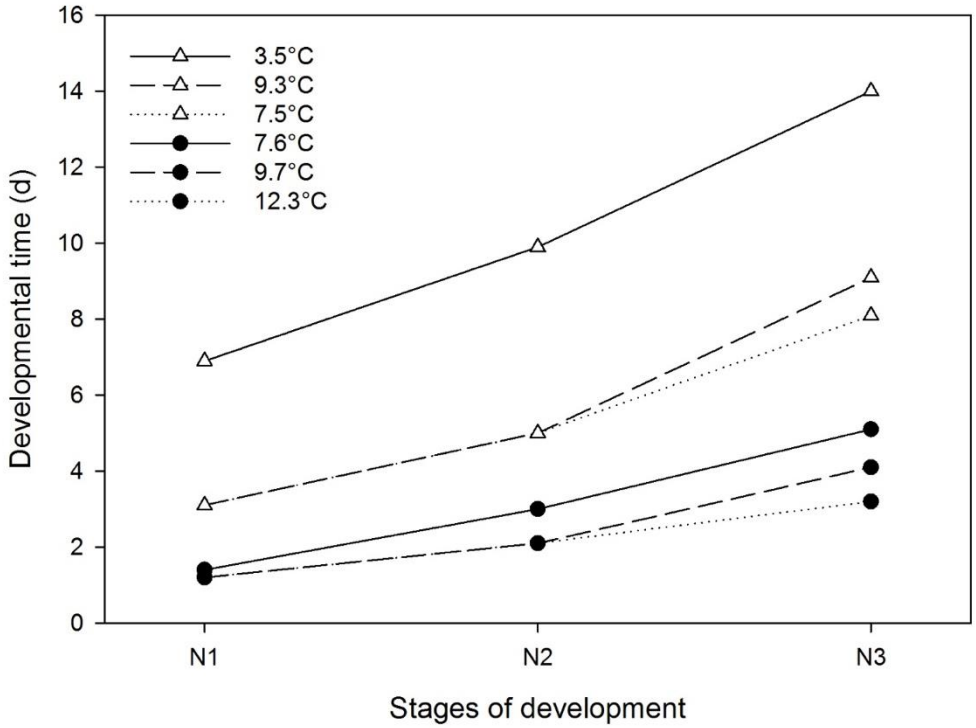

Figure 4.2 Developmental times (days) for *C. hyperboreus* (Δ) and *C. finmarchicus* (•) nauplii at three different temperature treatments for each species. Developmental time equals the time of egg incubation to sampling of the selected developmental stage. N1, N2, and N3 represent each naupliar stage.

4.4 Growth

4.4.1 Egg diameter and naupliar length

Biometric analysis of egg diameter and prosome length revealed a size difference between *C. finmarchicus* (Figure 4.3) and *C. hyperboreus* (Figure 4.4). The diameter of *C. finmarchicus* eggs were smaller compared to the eggs of *C. hyperboreus*. Prosome length of nauplii increased

with developmental stage and *C. hyperboreus* nauplii were larger than *C. finmarchicus* nauplii. No statistically significant interaction between temperature and exposure was detected for either *C. finmarchicus* or *C. hyperboreus* (two-way ANOVA). See Appendix E for more specific data on egg diameter and naupliar length.

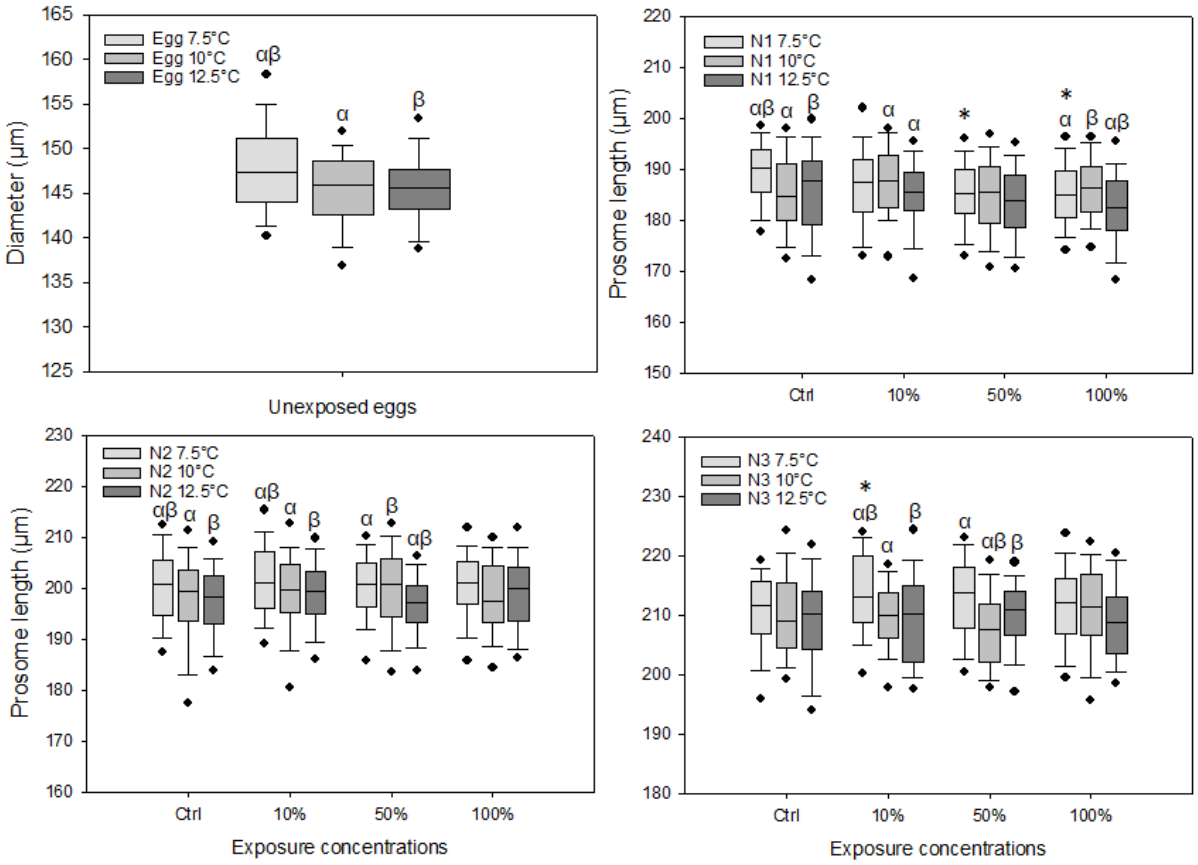

Figure 4.3 ***C. finmarchicus***. Boxplot of diameter of eggs and prosome length (µm) of nauplii stages N1 to N3 in the experimental treatments where eggs were exposed to three different concentrations of WSF (10, 50, and 100%) for 24 hours and reared at three different temperature regimes to developmental stage N1, N2, or N3. The solid horizontal line shows the median, bottom, and top of the box show the 25th and 75th percentiles, respectively. Whiskers extend 1.5 x the interquartile range of the sample, while dots represent the 5th and 95th percentile. Significant differences between temperature treatments within controls or exposure treatments are indicated with α or β, meaning that temperature treatments indicated with α are significantly different, while temperature treatments indicated with β are significantly different. Temperature treatments indicated with αβ means that they are significantly different to the treatment indicated with α and to the treatment indicated with β. Asterisks indicate a significant difference between exposure treatment and control (ANOVA, Holm-Sidak).

For *C. finmarchicus*, increasing temperature reduced the diameter of eggs and a significant difference in egg diameter were detected between eggs reared at 7.5 and 10°C and between eggs reared 7.5 and 12.5°C ($p < 0.001$, $p = 0.001$, Holm-Sidak).

Within the N1 stage, the prosome length of nauplii exposed to the 50% and 100% treatment was lower than that of controls ($p = 0.005$, $p = 0.008$, respectively, Holm-Sidak). When reared at 7.5°C, a highly significant reduction in prosome length was detected for nauplii exposed to 50 and 100% WSF ($p < 0.001$, Holm-Sidak). Within the different exposure treatments, the length of unexposed N1 nauplii reared at 10°C was reduced compared to nauplii reared at 7.5°C ($p < 0.001$) and 12.5°C ($p < 0.001$) (Holm-Sidak), while a significantly reduction in prosome length was observed for N1 nauplii reared at 12.5°C when compared to N1 nauplii reared at 10°C within the 10% exposure treatment ($p = 0.034$). Also, when exposed to 100% WSF, the length of nauplii reared at 12.5°C was significantly shorter than nauplii reared at 7.5°C ($p = 0.042$) and at 10°C ($p = 0.004$) (Holm-Sidak).

A reduced prosome length was also observed for the N2 nauplii within the controls and within the 10 and 50% exposure treatment. The length of unexposed nauplii reared at 7.5°C were significantly longer compared to nauplii reared at 10 and 12.5°C ($p = 0.015$, $p = 0.011$). The same pattern was also observed for nauplii within the 10% exposure treatment ($p = 0.034$, $p = 0.021$), while within the 50% exposure treatment, the prosome length of nauplii reared at 12.5°C were significantly shorter than nauplii reared at 7.5°C ($p = 0.002$) and at 10°C ($p = 0.004$) (Holm-Sidak). For N3 nauplii reared at 7.5°C, the length of the nauplii exposed to 10% WSF were significantly longer compared to the unexposed nauplii ($p = 0.024$, Holm-Sidak). The nauplii reared at 7.5°C were also significantly longer than nauplii reared at 10°C ($p < 0.001$) and at 12.5°C ($p < 0.001$) within the 10% exposure, and nauplii reared at 10°C ($p < 0.001$) within the 50% exposure (Holm-Sidak).

For the *C. hyperboreus*, a large difference in egg size was detected, with a highly significant difference in egg diameter between all temperature treatments ($p < 0.001$, Dunn´s). The N1 nauplii seemed to increase when reared at 10°C within the control and the 50 and 100% exposure treatments. The N1 nauplii reared at 7.5°C were significantly shorter than nauplii reared at 3 and 10°C ($p = 0.033$, $p < 0.001$) within the 50% exposure treatment and were also

significantly shorter than nauplii at 10°C when exposed to 100% WSF (p = 0.030) (Holm-Sidak).

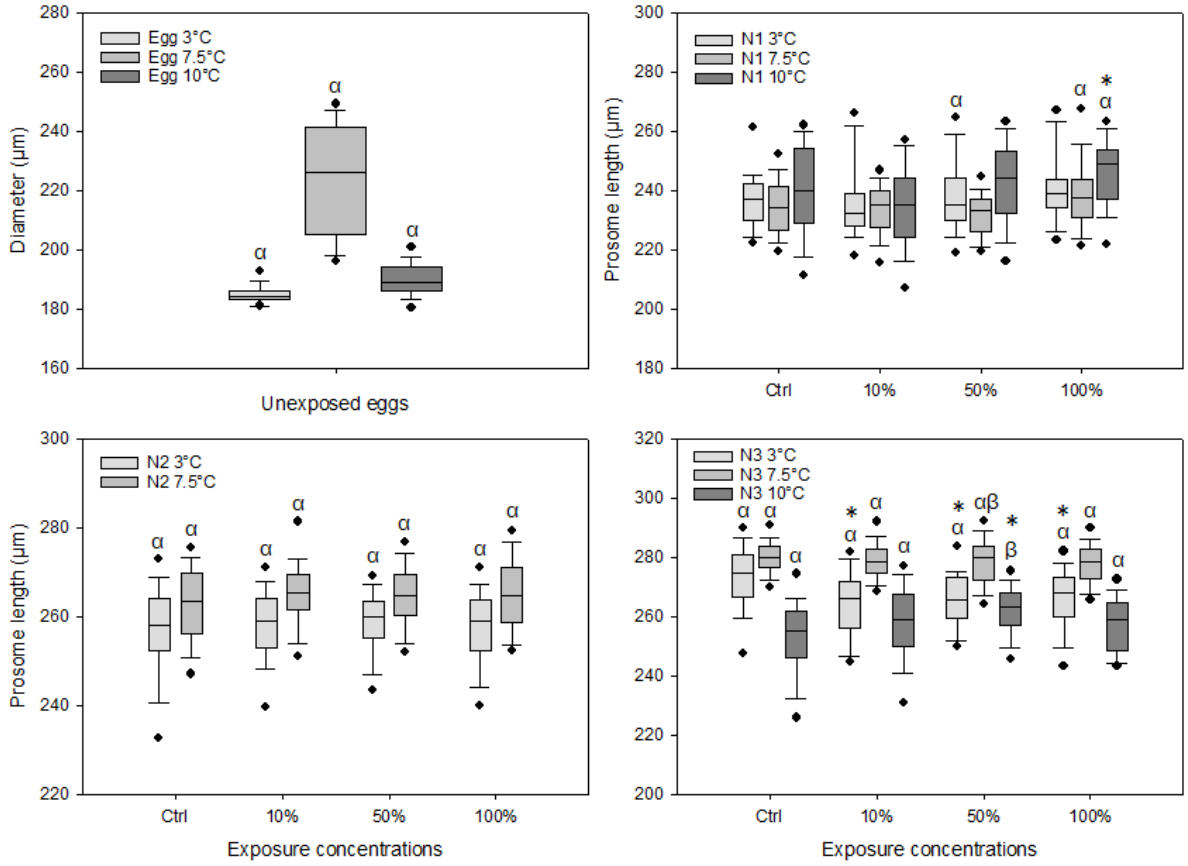

Figure 4.4 ***C. hyperboreus*.** Boxplot of diameter of eggs and prosome length (µm) of nauplii stages N1 to N3 in the experimental treatments where eggs were exposed to three different concentrations of WSF (10, 50, and 100%) for 48 hours and reared at three different temperature regimes to developmental stage N1, N2, or N3. The solid horizontal line shows the median, bottom, and top of the box show the 25th and 75th percentiles, respectively. Whiskers extend 1.5 x the interquartile range of the sample, while dots represent the 5th and 95th percentile. Significant differences between temperature treatments within controls or exposure treatments are indicated with α or β, meaning that temperature treatments indicated with α are significantly different, while temperature treatments indicated with β are significantly different. Temperature treatments indicated with αβ means that they are significantly different to the treatment indicated with α and to the treatment indicated with β. Asterisks indicate a significant difference between exposure treatment and control (ANOVA, Dunn´s, Holm-Sidak).

Within the N2 stage, the length of nauplii in the 3°C treatment was overall significantly shorter than nauplii reared at 7.5°C (p < 0.001) and was also significantly shorter than nauplii reared 7.5°C within the control and the exposed treatments (p < 0.0 01, Holm-Sidak). For the N3

nauplii, the length seemed to increase with increasing temperature, when comparing the 3 and 7.5°C treatments with a highly significant difference within the control and all exposure treatments ($p < 0.001$, Holm-Sidak). However, when reared at 10°C, the prosome length was reduced. This observation was highly significant when comparing the N3 nauplii reared at 10°C to the nauplii at 3 and 7.5°C within the control ($p < 0.001$) and when exposed to the 10% and the 100% WSF ($p < 0.001$) (Holm-Sidak).

4.4.2 Dry weight

For both *C. finmarchicus* and *C. hyperboreus*, the dry weight tended to increase with developmental stage. No statistically significant interaction effect was detected between temperature and exposure on dry weight of the different developmental stages of either *C. finmarchicus* (Figure 4.5) or *C. hyperboreus* (Figure 4.6) (two-way ANOVA).

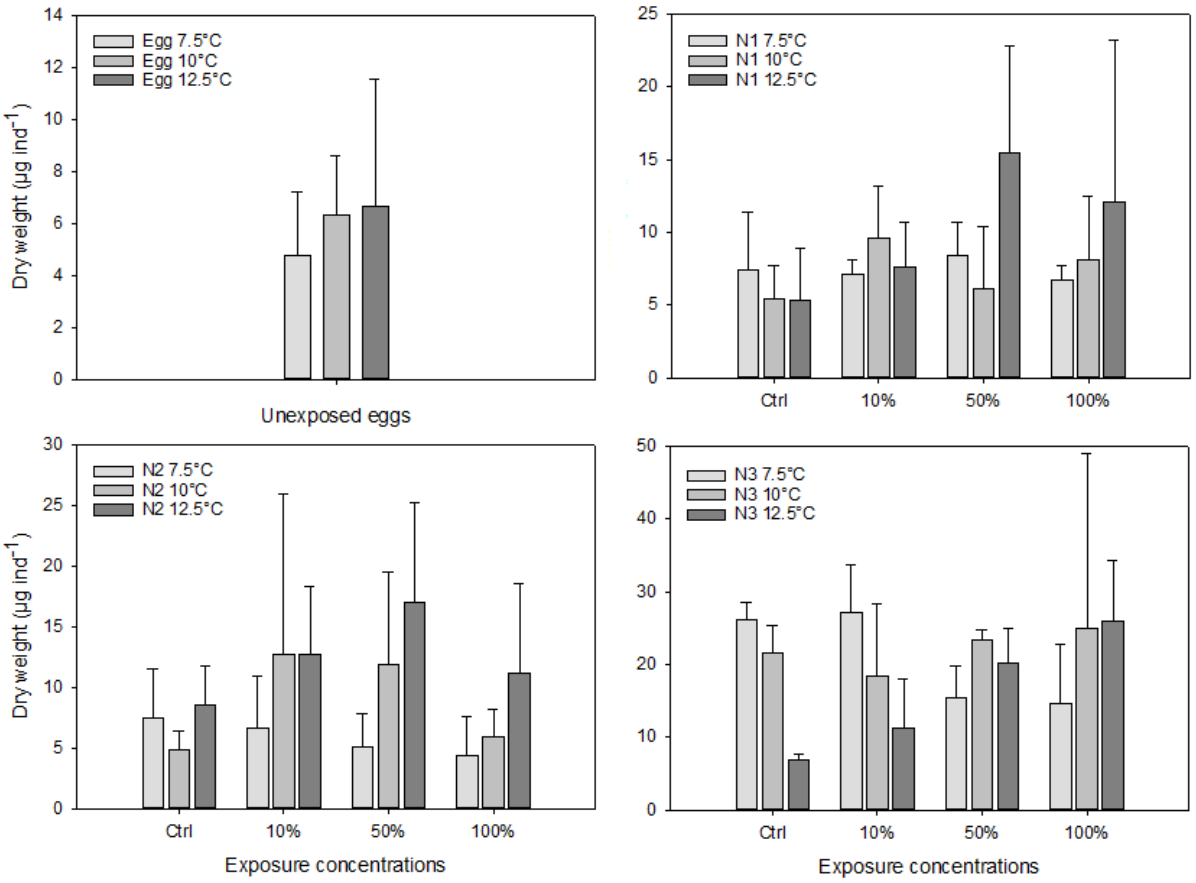

Figure 4.5 *C. finmarchicus*. Dry weight (µg ind.$^{-1}$) of developmental stage egg to N3 (means ± SD). Eggs were exposed to three different concentrations of WSF (10, 50, and 100%) and reared at three temperatures to developmental stage N1, N2, or N3.

For the *C. finmarchicus*, no significant difference was detected between the different temperature or exposure treatments on eggs and the N1 and N3 stage. Within the N2 stage, an overall significantly lower dry weight was detected for nauplii reared at 7.5°C compared to nauplii reared at 12.5°C ($p = 0.024$, Holm-Sidak)

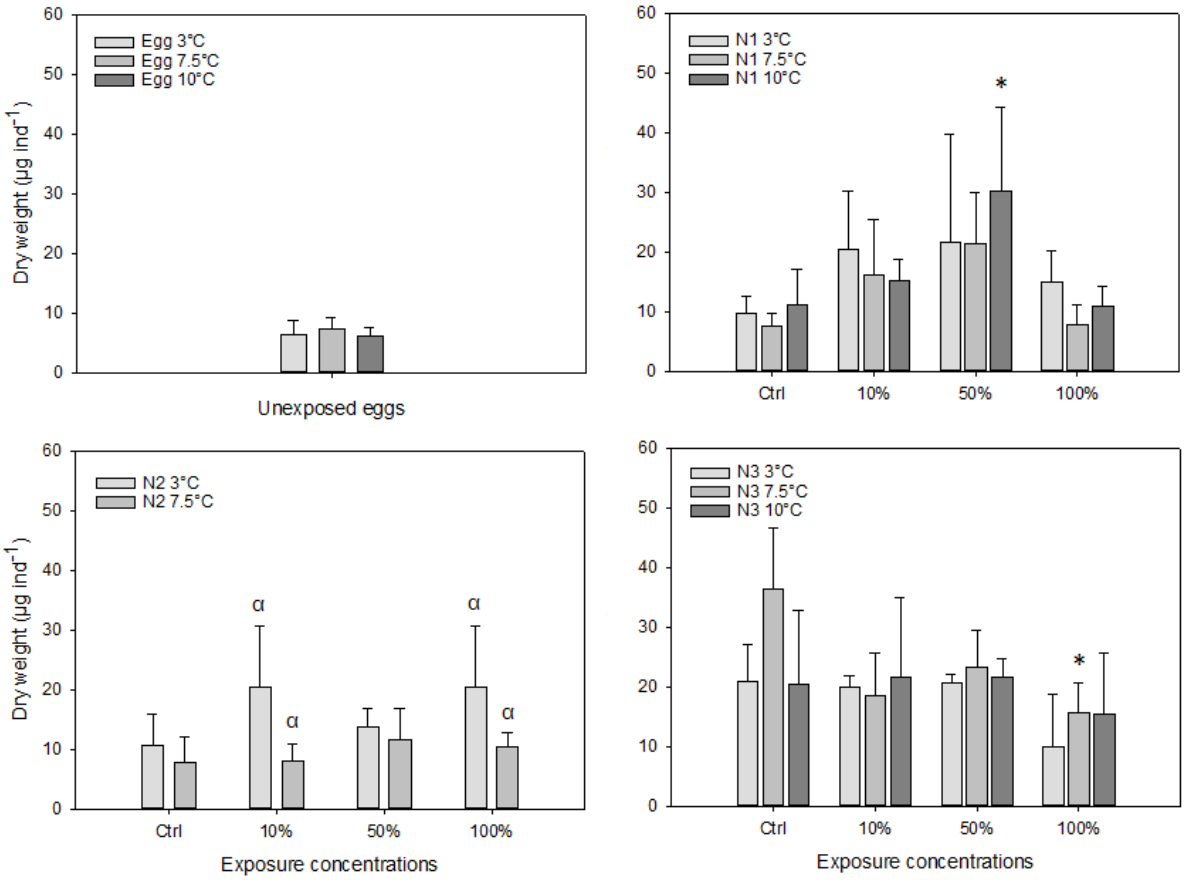

Figure 4.6 *C. hyperboreus*. Dry weight (µg ind.$^{-1}$) of developmental stage egg to N3 (means ± SD). Eggs were exposed to three different concentrations of WSF (10, 50, and 100%) and reared at three temperatures to developmental stage N1, N2, or N3. Significant differences between temperature treatments within controls or exposure treatments are indicated with α or β, meaning that temperature treatments indicated with α are significantly different, while temperature treatments indicated with β are significantly different. Asterisks indicate a significant difference between exposure treatment and control (ANOVA, Dunn´s, Holm-Sidak).

No statistically significant difference was detected between dry weight of *C. hyperboreus* eggs reared at 3, 7.5, and 10°C. Within the N1 stage, an overall higher dry weight was detected for nauplii exposed to 50% WSF when compared to unexposed nauplii ($p = 0.003$, Holm-Sidak). Also, within the 50% exposure treatment, the dry weight of N1 nauplii reared at 10°C was significantly higher than the dry weight of unexposed N1 nauplii ($p = 0.028$, Holm-Sidak).

Within the N2 stage, the dry weight of nauplii reared at 7.5°C was overall significantly lower than the dry weight of nauplii reared at 3°C ($p = 0.005$, Holm-Sidak). Also, dry weight of N2 nauplii reared at 7.5°C was significantly lower when exposed to 10 and 100% WSF ($p = 0.010$, $p = 0.033$, respectively, Holm-Sidak). An overall lower dry weight was detected for N3 nauplii exposed to 100% WSF for the N3 nauplii ($p = 0.020$, Holm-Sidak). In addition, dry weight of N3 nauplii exposed to 100% WSF at 7.5°C was significantly lower than dry weight of unexposed N3 nauplii reared at the same temperature ($p = 0.027$, Holm-Sidak).

4.5 Oxygen consumption

The change in oxygen consumption per individual tended to increase with increasing temperature for both *C. finmarchicus* (Figure 4.7) and *C. hyperboreus* (Figure 4.8). No interaction between temperature and exposure was detected within the developmental stages for either *C. finmarchicus* or *C. hyperboreus* (two-way ANOVA).

Oxygen consumption of *C. finmarchicus* eggs reared at 7.5 and 12.5°C was significantly higher compared to eggs reared at 10°C ($p = 0.019$, $p = 0.031$, Holm-Sidak). Oxygen consumption of N1 nauplii did not differ significantly between any of the temperature treatments or WSF exposure treatments.

Within the N2 stage of *C. finmarchicus*, the increase in metabolic rate with increasing temperature was highly significant for all temperature treatments ($p < 0.001$, Holm-Sidak). Within the control treatment the oxygen consumption of nauplii reared at 7.5°C was significantly lower compared to nauplii reared at 10 and 12.5°C ($p = 0.042$, $p = 0.001$, respectively, Holm-Sidak). This was also observed for nauplii exposed to 10% WSF ($p = 0.029$, $p < 0.001$, Holm-Sidak). However, when exposed to 50 and 100% WSF, a significant increase in metabolic rate was detected for all temperatures. Within the 50% exposure, the oxygen consumption increased significantly for N2 nauplii reared at 10°C when compared with N2 nauplii reared at 7.5°C ($p = 0.004$), and for N2 nauplii reared at 12.5°C when compared to N2 nauplii in the 10°C treatment ($p = 0.034$) (Holm-Sidak). Within the 100%, a significant increase in oxygen consumption was observed for N2 nauplii reared at 10°C when compared to nauplii in the 7.5°C treatment ($p = 0.031$), and for nauplii reared at 12.5°C when compared to nauplii reared at 10°C ($p = 0.027$). Within both the 50 and 100% exposure treatment, the increase in

metabolic rate for nauplii reared at 12.5°C were highly significant when compared to nauplii reared at 7.5°C ($p < 0.001$, Holm-Sidak).

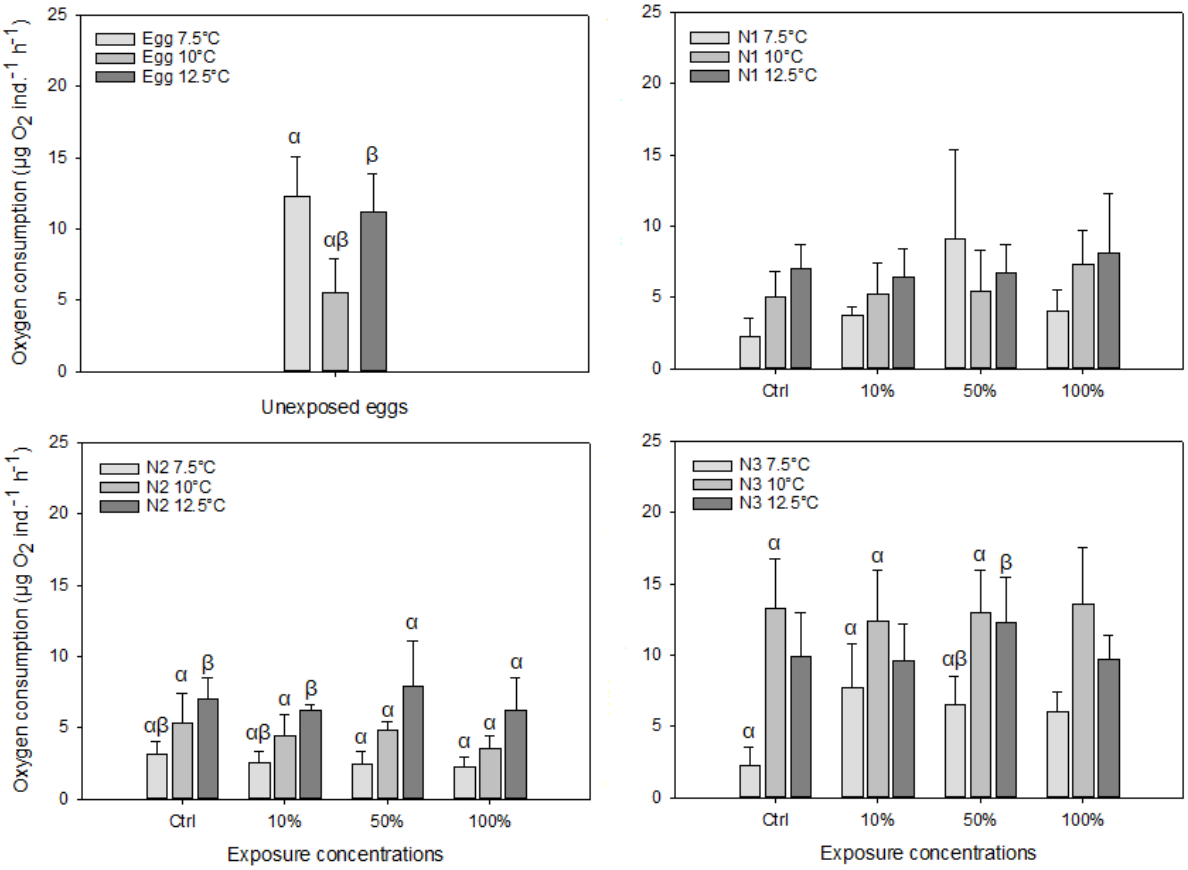

Figure 4.7 C. finmarchicus. Oxygen consumption (µg O_2 mg ind.$^{-1}$ h^{-1}) in developmental stage egg to N3 (means ± SD). Eggs were exposed to three different concentrations of WSF (10, 50, and 100%) and reared at three temperatures to developmental stage N1, N2, or N3. Significant differences between temperature treatments within controls or exposure treatments are indicated with α or β, meaning that temperature treatments indicated with α are significantly different, while temperature treatments indicated with β are significantly different. Temperature treatments indicated with αβ means that they are significantly different to the treatment indicated with α and to the treatment indicated with β (ANOVA, Holm-Sidak).

Overall, a significant increase in respiration was observed for N3 nauplii of *C. finmarchicus* reared at 12.5°C when compared to 10°C ($p = 0.010$) and 7.5°C ($p = 0.008$) (Holm-Sidak). The increased oxygen consumption observed for 10°C was highly significant when compared to N3 nauplii reared at 7.5°C ($p < 0.001$, Holm-Sidak). Within the different exposure treatments, analysis revealed a significant metabolic increase for nauplii reared at 10°C when compared to 7.5°C within the control treatment ($p = 0.023$), and within the 10% ($p = 0.015$) and 50% ($p =$

0.003) exposure treatments (Holm-Sidak). The increased respiration for nauplii reared at 12.5°C was also significantly higher when compared to nauplii in the 7.5°C treatment when exposed to 50% WSF ($p = 0.006$, Holm-Sidak). No significant increase in oxygen consumption was observed within the 100% exposure.

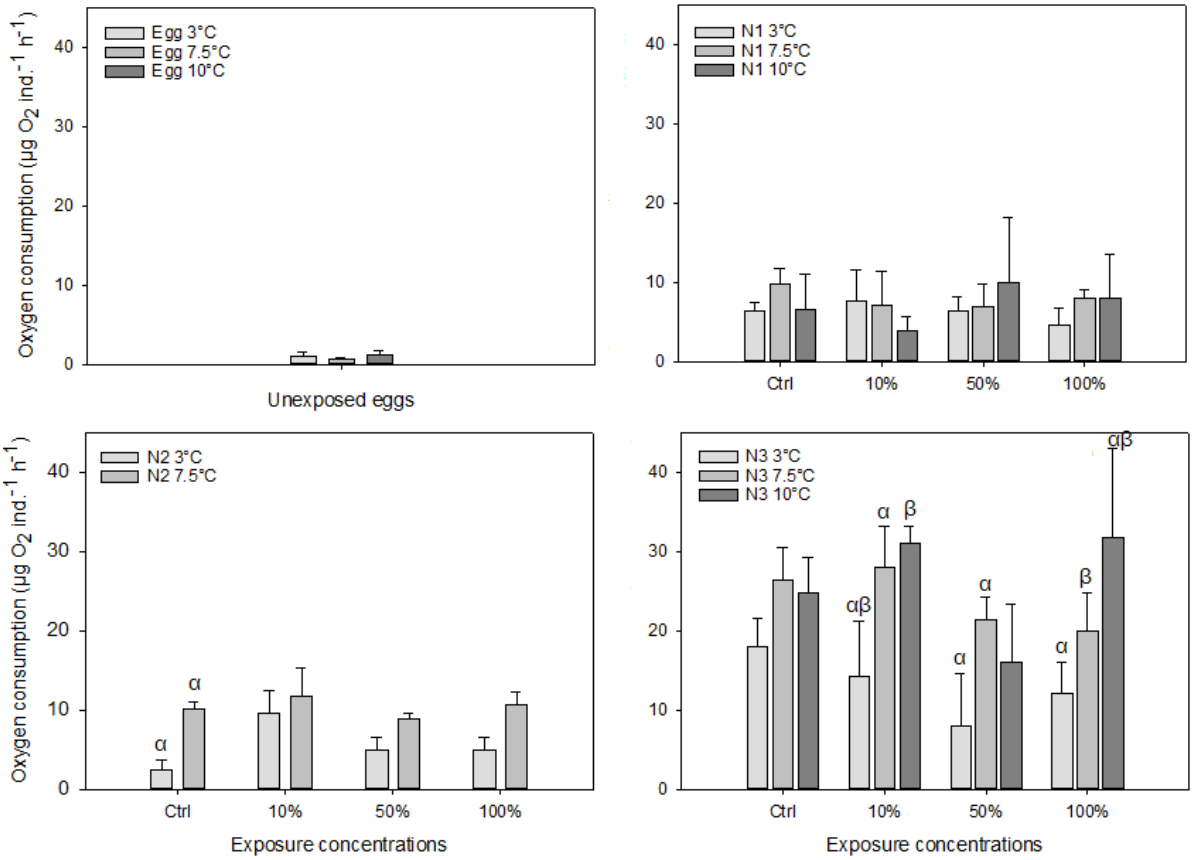

Figure 4.8 ***C. hyperboreus*.** Oxygen consumption (µg O_2 mg ind.$^{-1}$ h^{-1}) in developmental stage egg to N3 (mean ± SD). Eggs were exposed to three different concentrations of WSF (10, 50, and 100%) and reared at three temperatures to developmental stage N1, N2, or N3. Significant differences between temperature treatments within controls or exposure treatments are indicated with α or β, meaning that temperature treatments indicated with α are significantly different, while temperature treatments indicated with β are significantly different. Temperature treatments indicated with αβ means that they are significantly different to the treatment indicated with α and to the treatment indicated with β. (ANOVA, Holm-Sidak).

For the *C. hyperboreus*, no significant difference in the oxygen consumption of eggs and N1 stage was observed. For the N2 stage, an overall significant increase in respiration was observed for nauplii reared at 7.5°C when compared to 3°C ($p = 0.001$) and a significant difference between these temperatures was also observed in the control treatment ($p = 0.002$)

(Holm-Sidak). No significant difference was observed for the different exposure concentrations when compared to the control. Within the N3 stage, there was an overall significant increase in oxygen consumption with a highly significant difference for both 7.5 and 10°C when compared to nauplii reared at 3°C ($p < 0.001$, Holm-Sidak). Overall, there was also a significantly lower respiration rate of nauplii exposed to 50% WSF when compared to unexposed nauplii ($p = 0.034$, Holm-Sidak).

No significant difference in respiration rate was observed for nauplii within the control treatment. However, a significant increase in oxygen consumption was observed for both 7.5 and 10°C when compared to nauplii reared at 3°C within the 10% exposure treatment ($p = 0.014$, $p = 0.004$, respectively, Holm-Sidak). Also, the difference between 7.5 and 3°C was also significant within the 50% exposure treatment ($p = 0.024$, Holm-Sidak). For nauplii exposed to 100% WSF, statistical analysis also revealed a significant increase in respiration rate for nauplii reared at 10°C when compared to 7.5°C ($p = 0.037$), and the increased metabolic rate for nauplii reared at 10°C were highly significant compared to 3°C ($p < 0.001$, Holm-Sidak).

5 Discussion

The main objective of this study was to investigate the effect of exposure to the WSF of PW-related PAHs on the early life stages of *C. finmarchicus* and *C. hyperboreus* at different temperature regimes. The findings demonstrated a relatively clear temperature effect on egg hatching, development and oxygen consumption of both species, where egg hatching time and developmental time of the nauplii were reduced with increasing temperature, while an increase in oxygen consumption was observed. No clear impacts of temperature on dry weight, diameter of eggs and naupliar prosome length were detected, but the median length of the N3 stage of *C. hyperboreus* seemed to be reduced when reared at 10°C. However, the results did not reveal a clear effect of PAH exposure. Although eggs of *C. finmarchicus* seemed to be more affected by PAH exposure, due to the lower hatching percent compared to hatching of *C. hyperboreus* eggs, the hatching success of both species was high despite PAH exposure. Also, the temperature sensitivity of the metabolic rate of N2 nauplii of *C. finmarchicus* and N3 nauplii of *C. hyperboreus* increased when exposed to PAHs.

5.1 Experimental conditions

PAHs are considered to be the most toxic components in PW and were therefore used as a proxy for PW in this study. Other studies that have addressed the effect of PAH exposure on early life stages of *Calanus* copepods have only investigated the effects of single PAHs, mainly pyrene (Grenvald et al., 2013; Hjorth and Nielsen, 2011; Jensen et al., 2008; Nørregaard et al., 2014) and the potential additive toxic effect that may arise from a combination of PAHs (Barata et al., 2005) have therefore not been considered. In the present study, eggs of *C. finmarchicus* and *C. hyperboreus* were exposed to three different concentrations of the WSF of 11 PW-related PAHs to simulate an environmentally relevant exposure to PW.

Analysis of the PAH composition of the exposure solutions revealed the highest occurrence of the naphthalenes (N, N1, and N2), the smallest compound among the PAHs assessed in this study. This can be explained by the relatively low log K_{OW} of these naphtalenes making them more easily dissolved in water compared to larger PAHs with a higher log K_{OW}. Measurements of PAH concentrations in the exposure solution after exposing eggs showed an approximately 50% reduction in total PAH concentration, which may indicate that the PAHs were accumulated in the eggs. However, some of the PAHs, such as the naphthalenes, could also

been lost due to evaporation, because of the higher volatility of these compounds. Evaporation can also lead to spreading by air and might explain why naphthalenes were detected in the controls, albeit at low concentrations. Because the total PAH concentration was higher in the control solution collected after exposure (ΣPAH 0.10 μg L^{-1}) compared to the stock solution before exposure (ΣPAH 0.04 μg L^{-1}) it is uncertain if any PAHs were accumulated in the eggs. The concentrations were however low, and minor effects can probably be excluded.

It is not known how the PW will spread and dilute in Arctic waters (AMAP, 2007). However, monitoring the concentrations of PW in the North Sea has been included in the quantitative risk assessment of the DREAM model (Johnsen and Frost, 2011) since the late 90s, and the concentration of total PAH may vary from 40 to 3000 μg L^{-1} (Neff et al., 2011). In the present study the measured PAH concentrations were in the range of 0.04 and 20.11 μg L^{-1} (Table 4.2), likely making the results of this experiment relevant for copepod populations in the Arctic. In addition, the characteristics of PAHs at lower temperature should be investigated further, due to the potential persistence of these compounds in cold water leading to longer exposure periods of marine organisms to PAHs.

The treatment temperatures used in the present study corresponded to the preferred water temperature for *C. finmarchicus* in the North Sea (7.5 - 9°C) (Jónasdóttir and Koski, 2011) and for *C. hyperboreus*, which experiences a wide range of temperatures, from -1.8°C in the Arctic Ocean to 8°C in the Gulf of Maine (Conover and Corner, 1968; Hirche and Niehoff, 1996). According to the Intergovernmental Panel on Climate Change the warming predicted for the Arctic is ~3 - 4°C until 2050 and the treatment temperatures used in the present study are therefore relevant for copepods in an area where some of the strongest warming trends are found (IPCC, 2014).

5.2 Hatching success

The shorter hatching time of eggs with increasing temperature observed for both *C. finmarchicus* and *C. hyperboreus* (Figure 4.1) indicates a clear temperature effect on hatching time and is consistent with previous studies on copepod hatching (Campbell et al., 2001; Grenvald et al., 2013; Jung-Madsen et al., 2013). The present study also shows that eggs of *C. finmarchicus* and *C. hyperboreus* can successfully hatch after exposure to PAHs commonly

present in PW. This has also been observed in other studies, but only after exposure to pyrene alone (Grenvald et al., 2013; Jensen et al., 2008; Nørregaard et al., 2014).

Grenvald et al. (2013) argued that the efficient hatching of *C. finmarchicus* and *C. glacialis* eggs despite PAHs exposure, is caused by the eggshell acting as a barrier against environmental stress factors. However, *C. hyperboreus* eggs are not enclosed by a hard shell but by a thin membrane consisting of fatty acids (Jung-Madsen et al., 2013), and may therefore be more susceptible to environmental stressors. It should be mentioned that the *C. hyperboreus* eggs in the study conducted by Nørregaard et al. (2014) originated from females exposed to pyrene and were hatched in uncontaminated seawater. The authors predicted that a larger effect could be expected with continued PAH exposure, which would be the case if eggs were hatched in an environment with continued supply of produced water. However, in the present *C. hyperboreus* eggs were exposed to PAHs and hatched in contaminated water, but no effects of exposure were detected. Also, a higher hatching percent was observed for the *C. hyperboreus* eggs compared to eggs of *C. finmarchicus* and could be explained by the higher lipid content in these eggs, which may delay any toxic effects of the PAHs. This may indicate that eggs of *C. finmarchicus* are more sensitive to PAH exposure than eggs of *C. hyperboreus*.

5.3 Development

Development times for both *C. finmarchicus* and *C. hyperboreus* were reduced with increasing temperature (Figure 4.2) and are consistent with earlier studies on nauplii development (Campbell et al., 2001; Cook et al., 2007; Jung-Madsen et al., 2013). Previous studies on stage duration of *C. hyperboreus* (Conover, 1967; Jung-Madsen et al., 2013) have only recorded the development times at 5°C, but the pattern in these studies fits well with the observations of the present study where a shorter stage duration was observed with increasing temperatures. The development for both species was not isochronal over the temperature ranges, meaning that the stage duration for the different nauplii were not of equal length. However, the relative duration of a given stage was more or less constant over all temperature treatments, supporting the assumption of an equiproportional growth (Campbell et al., 2001). As commonly observed, the two non-feeding stages were of short. No further study on development after sampling of the N3 stage was conducted, and a precise estimate of the N3 stage was therefore not possible in this study. Nevertheless, the extended sampling period of the N3 nauplii supports the assumption of a non-isochronal development rate for both *C. finmarchicus* and *C. hyperboreus*

(Campbell et al., 2001; Jung-Madsen et al., 2013). The prolonged duration of the first feeding stage is thought to be related to the time it takes for the nauplii to recuperate weight loss during the two non-feeding stages (Marshall and Orr, 2013).

Greater dissimilarities in development time were observed with increasing developmental stages between the different temperature treatments (Figure 4.2). For the *C. finmarchicus*, only 1.2 days differed between 12.5 and 7.5°C within naupliar stage N1, while the corresponding difference was 1.9 days at developmental stage N3. The dissimilarities were even greater for *C. hyperboreus*, where the difference between 10 and 3°C was 3.8 days for the N1 nauplii and 4.9 days for developmental stage N3. However, the various developmental times at different temperatures could be considered as a key adaption to the highly variable environment of these species. Because sufficient amounts of resources are needed to induce molting (Miller et al., 1977), a rise in respiration with elevated temperatures could lower the amount of energy available to support development. Therefore, the nauplii must develop quickly to avoid depletion of the maternal resources before reaching the first feeding stage.

5.4 Metabolic rates

Metabolic rates, measured as oxygen consumption, tended to increase with temperature for both *C. finmarchicus* (Figure 4.7) and *C. hyperboreus* (Figure 4.8). Despite exposure to PAHs, the N1 and N2 stage of both *C. finmarchicus* and *C. hyperboreus* had relatively similar metabolic rates within a given treatment temperature. The respiration rate is known to increase when nauplii switch to exogenous feeding (Pörtner et al., 2010) and this was also observed for the N3 stage of both species in this study. Similar results have previously been reported for the early life stages of *C. finmarchicus* (Buraas, 2015), where oxygen consumption was measured for nauplii reared under different temperature regimes.

Regarding the metabolic rate of the eggs, temperature had only a significant effect on the eggs of *C. finmarchicus*, while no effect of neither temperature nor PAH exposure was observed on the N1 stage of both species. No observed temperature effect on eggs and the N1 stage of *C. hyperboreus* could be explained by the fact that *C. hyperboreus* eggs are spawned in deep water (Sømme, 1934) and may encounter water masses with different temperatures when ascending to the surface, necessitating that eggs and nauplii are less sensitive to temperature. There were no clear effects on the metabolic rate of nauplii related to PAH exposure. However, for the N2

stage of *C. finmarchicus*, the sensitivity to higher temperatures seemed to increase when exposed to higher concentrations of the WSF. A similar pattern was also observed for oxygen consumption of the *C. hyperboreus* N3 stage, where a significant temperature effect was observed only within the exposed treatments. Particularly, when exposed to the 100% WSF concentration, the difference in oxygen consumption of nauplii reared at 3 and 10°C was highly significant. This could imply that the effect after PAH exposure of egg on the metabolic rate is delayed and could be explained by the higher lipid content in the *C. hyperboreus* which can act as a buffer for any toxic effects of the PAHs (Lotufo, 1998).

Prosser (1961) distinguished the metabolic rate of animals between "standard", "routine" and "active" metabolism and is described by Ikeda et al. (2001). It was not possible to measure swimming activity during the measurements of oxygen consumption in the present study, but some activity was observed, probably resulting in an intermediate between the "standard" and "routine" metabolic rate for the non-feeding stages. Regardless, increased metabolism will either result in reduced or elevated performance and depends on whether the increased energy demand can be sustained by energy intake (Stumpp et al., 2011). The non-feeding N2 stage of *C. finmarchicus* seemed to be more sensitive to higher temperatures when eggs were exposed to increasing WSF concentrations. Since this life stage only have a finite amount of energy available, a rise in respiration might lower the amount of energy available before developing into the first feeding stage (Pedersen et al., 2014). In contrast, although the N3 stage of *C. hyperboreus* seemed to be more sensitive to elevated temperature within the highest exposure concentration, it may be able to support the elevated respiration through feeding. However, the shift to exogenous feeding demands the N3 nauplii to start active feeding and therefore, swimming activity is likely to increase. The result will be an increased metabolic demand.

5.5 Growth of nauplii

C. finmarchicus and *C. hyperboreus* are morphologically very similar (Appendix A) but differ in size (Figure 4.3 and 4.4). Several studies have reported size classes for the copepodite stages of *Calanus* species, but data on size distribution of nauplii are scarce, and to the authors' knowledge only a few studies have established size classes for some of the naupliar stages of *C. finmarchicus* and *C. hyperboreus* (Campbell et al., 2001; Hygum et al., 2000; Jung-Madsen et al., 2013; Sømme, 1934). In comparison with the nauplii of *C. finmarchicus*, *C. hyperboreus*

nauplii are, as expected, larger than the *C. finmarchicus* nauplii. Egg diameter is also larger for *C. hyperboreus* eggs.

Within a given developmental stage, there were small variations in the median length of the nauplii, indicating only minor effects on nauplii growth after egg exposure to PAHs. Previous studies have also shown that temperature does not affect the size of copepod nauplii until the last naupliar or first copepodite stage (Campbell et al., 2001; Cook et al., 2007). Unexposed eggs of *C. hyperboreus* reared under 7.5°C had a significantly larger median diameter compared to eggs reared at 3 and 10°C and there were also large variations between the eggs within the 7.5°C treatment. In addition, the N2 and N3 nauplii developed from these eggs had a larger median length. It should be noted that eggs used in the 7.5°C experiment originated from females collected in Disko Bay, Greenland, in February 2017 and shipped to SeaLab, Trondheim for PAH exposure. In contrast, *C. hyperboreus* eggs reared at 3 and 10°C were collected from ovigerous females at the same location but 1 year earlier. Since size variation has been found among nauplii from different populations (Daase et al., 2011), comparing size distribution between populations from different environments may be challenging. The naupliar length of *C. finmarchicus* in this study appeared to be larger compared to the measurements done by Hygum et al. (2000) who also used nauplii from wild caught females. Additionally, when comparing the length measurements in this study with the measurements done by Jung-Madsen et al. (2013) on *C. hyperboreus* nauplii from Disko Bay, the prosome length of the N2 and N3 stages appeared to be smaller in this study.

No consistent differences in dry weight of *C. finmarchicus* between the different temperature treatments were observed. Also, the variations in dry weight of the N1 and N2 nauplii of both *C. finmarchicus* and *C. hyperboreus* were somehow unexpected. A negative zero or zero growth rate is usually observed for these developmental stages (Campbell et al., 2001), but in this study, the dry weight of both N1 and N2 appeared to be higher than the dry weight of the eggs (discussed later). Campbell et al. (2001) found that body weights are inversely related to temperature, but dry weight of nauplii within the N2 stage was significantly higher when reared at 12°C when compared to nauplii developed at 7.5°C. However, dry weight of *C. hyperboreus* nauplii seemed to be reduced with increasing temperature when exposed to PAHs, and a significantly lower dry weight were detected for N2 nauplii exposed to 10 and 100% at 7.5°C. For the N3 developmental stage of *C. hyperboreus*, dry weight of nauplii exposed to the 100% concentration of WSF was significantly lower than unexposed nauplii. The variation observed

within the N3 stage of *C. finmarchicus*, and also for the unexposed N3 *C. hyperboreus* nauplii reared at 7.5°C could be explained by the later sampling time of some of these groups, resulting in a larger growth of the individuals in these groups due to feeding.

5.6 Implications for Arctic ecosystems

Early life stages of copepods are the most numerous metazoans on the planet (Turner, 2004) and changes in naupliar growth survival will affect the population dynamics, and thereby also the food availability for higher trophic levels. The shift in energy source from endogenous to exogenous sources has been reported as a critical phase in the naupliar development and may explain some of the high mortality rate observed among early life stages of copepods (Pedersen et al., 2014).

The increased sensitivity to temperature when exposed to higher PAH concentrations, may lead to a depletion of the maternal energy before nauplii reaches the first feeding stage. This was observed for the N2 stage of *C. finmarchicus* and could be explained by the lower lipid content of *C. finmarchicus* nauplii compared to the *C. hyperboreus* nauplii, leading a faster induction of toxic effects by the PAHs.

It is also important that food is available when the nauplius reaches the first feeding stage. Plourde et al. (2003) showed that a warmer climate will lead to shorter winter-spawning for *C. hyperboreus*, possibly result in a mismatch between the development of the first feeding stage and the phytoplankton spring bloom. Since developmental time is reduced with increasing temperature, the *C. hyperboreus* nauplii may reach the N3 stage before the initiation of the spring bloom. However, an earlier spring bloom is also expected to occur in the Arctic due to a warmer climate and trends towards earlier phytoplankton blooms have already been observed in several areas in the Arctic (Kahru et al., 2011). Therefore, even though the N3 stage of *C. hyperboreus* seemed to be more sensitive to higher temperatures compared to the two non-feeding stages, the increased metabolic rate may be counteracted by feeding.

The temperature-dependent model developed by Huntley et al. (1992) predicts that increasing environmental temperature will decrease the body size of copepods. Because of the warming currently occurring in the Arctic, less energy will probably be available in the pelagic food web to sustain growth at higher trophic levels that rely on the lipid rich *Calanus* species.

Additionally, considering the increased sensitivity to temperature observed for nauplii developed from eggs exposed to petroleum related PAHs, expansion of petroleum activity into the Arctic will most likely increase the external stress that these organisms are already facing due to increasing temperatures in an area where currently some of the most rapid and severe climate change on Earth occurs.

5.7 Methodological reflections

The experiments conducted in the present study depended on carefully handling of small sized animals, and therefore, obtained results could easily be affected by both methodical and personal errors. During the experiment, animals got stuck in the pipettes and disappeared during measurement of oxygen consumption or when transferred between different measurement devises. Also, due to movement of the nauplii during the biometric measurements and difficulty in separating the individuals, some nauplii were measured more than once. It was therefore difficult to perform accurate measurements and imprecise number of animals resulted in altered means and high standard deviations, representing an uncertainty that may have affected the results in this study. Additionally, even though the animals were rinsed in ammonium formate solution, formation of salt crystals may still have occurred in the samples. Because of the small sizes of the animals, crystal formation in the samples will constitute a considerable percentage of the total dry weight and could to some degree explain the unexpected dry weight of some of the samples observed in this study. Therefore, the result must be interpreted with care. The metabolic rates of poikilotherms are known to be ultimately governed by body mass (Ikeda, 1985), but due to the inconclusive results for the dry weight of both *C. finmarchicus* and *C. hyperboreus* in the present study, the oxygen consumption was based on the number of animals in the measuring chamber. Basing the model on number of animals, instead of the dry weight may reduce the correlation of the oxygen consumption due to variations in body size among the nauplii being measured.

The present study used the approach of laboratory *in vivo* experiments as tools to study the impacts of PAH exposure on the development in the early life stages of *C. finmarchicus* and *C. hyperboreus* at different temperature regimes. Laboratory *in vivo* experiments permit close monitoring during the rearing of animals and allow well-controlled environmental conditions (e.g. temperature, food). Further, these conditions can be maintained constant throughout the experiment. Such experiments are therefore a fundamental tool for understanding the effects

of environmental variables on marine zooplankton. However, caution is required when extrapolating laboratory results to the field (Almeda et al., 2011) because the conditions in the laboratory may induce stress on the individuals or change the behaviour compared to that in the natural environment (Harris et al., 2000). Compared to the individuals used in the exposure experiment on *C. hyperboreus* which originated from wild-caught females, individuals used in the experiment on *C. finmarchicus* have been maintained at the research facilities of NTNU SeaLab for approximately 13 years and are therefore well adapted to laboratory conditions. Also, *C. finmarchicus* were exposed to the WSF of PAHs for 24 hours, while *C. hyperboreus* were exposed for 48 hours, making a direct comparison difficult. Nevertheless, handled with the necessary caution, the results from the present study should be regarded as valuable new information in a research field of great economic and environmental concern.

5.8 Conclusions

The present study indicates that the early life stages of *C. finmarchicus* and *C. hyperboreus* are more affected by temperature than by short term exposure to environmentally relevant concentrations of 11 PAHs. Increasing temperatures lead to reduced developmental time for embryo and nauplius, while the oxygen consumption increases. Although this study did not provide any significant effects on growth for the *C. finmarchicus* nauplii, the significant increased metabolic rate of the N2 nauplii can cause energy depletion for this non-feeding stage. The increased temperature sensitivity observed for the N3 stage of *C. hyperboreus* could potentially be compensated by increased feeding. The results did not reveal a clear effect of PAH exposure but the increased sensitivity of temperature on the metabolic rate of both species when exposed to PAHs shows that the impacts of PW have to be assessed in the context of climate change. Additionally, the reduced growth of exposed *C. hyperboreus* N3 nauplii could potentially reduce the energy availability for higher trophic levels.

These finding expand the limited knowledge regarding the early life stages of *C. finmarchicus* and *C. hyperboreus* and the results may benefit the management and the industry in minimising the risk of environmental impacts from the ongoing expansion of offshore petroleum activities in the Arctic.

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Appendix A

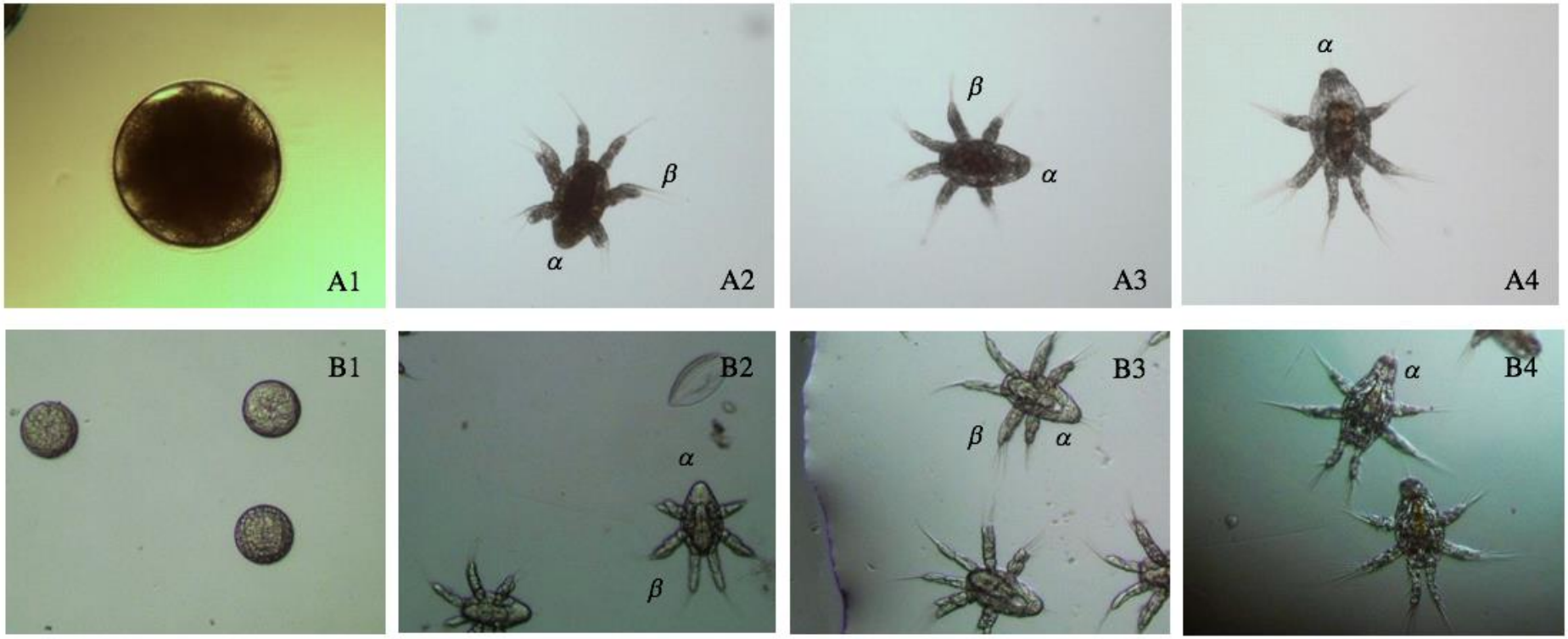

Figure A1 Developmental stages in *C. hyperboreus* (A) and *C. finmarchicus* (B). **1**: Eggs; diameter before hatching is about 145 µm for the *C. finmarchicus* (Marshall and Orr, 2013) and 270 µm for the *C. hyperboreus* (Sømme, 1934). **2**: N1; two thin, relatively short, widely separated spines on the posterior end of the body (α), three thin setae on the extremities on each antennule (β). **3**: N2; slightly more elongated body shape, two thin, long, and closely gathered spines on the posterior end of the body (α), antennule strucutre similar to N1 (β). **4**: N3; two pairs of short spines and one pair of long spines on the posterior end of the body (α), red matter inside the gut (algae) indicates start of exogenous feeding.

Appendix B

Table B1 ***C. finmarchicus***. Regression statistics on oxygen consumption. Regression coefficient (mg O_2/L min^{-1}) with R-squared values for replicates (n) in the different WSF concentrations and treatment temperatures for all development stages.

	C. finmarchicus																										
	7.5°C									10°C									12.5°C								
	Egg									**Egg**									**Egg**								
	n		a (10^a)		R^2					n		a (10^a)		R^2					n		a (10^a)		R^2				
	4		10.20		0.99					4		4.57		0.88					4		9.28		0.94				
	N1			**N2**			**N3**			**N1**			**N2**			**N3**			**N1**			**N2**			**N3**		
	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2
Ctrl	4	3.15	0.85	4	2.65	0.83	4	4.27	0.80	4	4.21	0.84	4	4.47	0.81	4	3.72	0.95	4	5.86	0.80	4	5.85	0.86	4	5.49	0.86
10%	4	7.55	0.88	4	2.15	0.76	4	3.63	0.87	4	4.38	0.67	4	3.67	0.81	4	6.90	0.90	4	5.36	0.82	4	5.21	0.81	4	5.36	0.79
50%	4	3.38	0.82	4	2.08	0.71	4	3.33	0.85	4	4.53	0.74	4	4.02	0.84	4	7.22	0.95	4	5.59	0.88	4	6.62	0.90	4	6.84	0.91
100%	4	7.07	0.95	4	1.91	0.70	4	5.05	0.92	4	6.07	0.88	4	2.97	0.79	4	7.55	0.97	4	6.72	0.84	4	5.21	0.76	4	5.37	0.86

Table B2 ***C. hyperboreus***. Regression statistics on oxygen consumption. Regression coefficient (mg O_2/L min^{-1}) with R-squared values for replicates (n) in the different WSF concentrations and treatment temperatures for all development stages.

	C. hyperboreus																								
	3°C									7.5°C									10°C						
	Egg									**Egg**									**Egg**						
	n	a (10^a)	R^2							n	a (10^a)	R^2							n	a (10^a)	R^2				
	4	0.78	0.40							4	0.51	0.30							4	0.98	0.63				
	N1			**N2**			**N3**			**N1**			**N2**			**N3**			**N1**			**N2**			**N3**	
	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2	n	a (10^a)	R^2
Ctrl	4	5.32	0.94	4	7.96	0.94	3	9.27	0.96	3	8.15	0.79	3	8.35	0.96	3	14.68	0.98	4	5.48	0.79	-	-	-	3	12.39	0.97
10%	4	6.33	0.86	4	4.07	0.94	3	7.09	0.74	3	5.93	0.81	3	9.83	0.94	3	15.52	0.99	4	3.20	0.85	-	-	-	3	17.24	0.99
50%	4	5.25	0.87	4	4.07	0.94	3	3.99	0.90	3	5.68	0.88	3	7.45	0.97	3	11.89	0.97	4	8.26	0.89	-	-	-	3	8.85	0.92
100%	4	3.81	0.82	4	6.12	0.87	4	6.00	0.96	3	6.63	0.92	3	8.89	0.95	3	11.10	0.98	3	6.68	0.92	-	-	-	3	17.66	0.96

Appendix C

Table C1 Egg hatching success (%) for *C. finmarchicus* and *C. hyperboreus* (mean ± SD).

		C. finmarchicus						*C. hyperboreus*		
		25 h	36 h	48 h	61 h			67 h	91 h	115 h
7.5°C	Ctrl	0	0	74 ± 5	81 ± 13	3°C	Ctrl	2 ± 3	40 ± 9	98 ± 3
	10%	0	0	72 ± 10	93 ± 3		10%	0 ± 0	57 ± 15	91 ± 3
	50%	4 ± 3	0	64 ± 11	81 ± 8		50%	0 ± 0	45 ± 18	95 ± 7
	100%	0	0	57 ± 4	70 ± 10		100%	0 ± 3	50 ± 22	97 ± 6
10°C	Ctrl	0	44 ± 10	83 ± 2	85 ± 5	7.5°C	Ctrl	3 ± 3	100	92 ± 3
	10%	0	39 ± 5	83 ± 4	93 ± 3		10%	0 ± 0	98 ± 3	93 ± 8
	50%	0	47 ± 8	81 ± 7	85 ± 9		50%	0 ± 0 (2)	93 ± 6	93 ± 8
	100%	5 ± 5	32 ± 8	82 ± 8	92 ± 2		100%	0 ± 3	92 ± 8	95 ± 5
12.5°C	Ctrl	0	70 ± 16	76 ± 2	88 ± 3	10°C	Ctrl	0 ± 0	93 ± 8	100
	10%	2 ± 3	71 ± 12	80 ± 6	88 ± 7		10%	0 ± 0 (2)	97 ± 8	96 ± 6
	50%	2 ± 3	60 ± 5	84 ± 4	81 ± 16		50%	3 ± 3	95 ± 5	95 ± 5
	100%	0	55 ± 1	92 ± 9	84 ± 5		100%	3 ± 3	98 ± 3	100

Appendix D

Table D1 Summary of temperature conditions and developmental time in all nauplii experiments conducted on *C. finmarchicus* and *C. hyperboreus*.

C. finmarchicus				*C. hyperboreus*			
Temperature (°C)	Sampling stage	Development time (days)	Stage duration (days)	Temperature (°C)	Sampling stage	Development time (days)	Stage duration (days)
7.5	N1	1.4	1.6	3.5	N1	6.9	3
	N2	3	2.1		N2	9.9	4.1
	N3	5.1	-		N3	14	-
9.7	N1	1.2	0.9	7.6	N1	3.1	1.9
	N2	2.1	2		N2	5	3.1
	N3	4.1	-		N3	8.1	-
12.3	N1	1.2	0.9	9.3	N1	3.1	1.9
	N2	2.1	1.5		N2	5	4.1
	N3	3.2	-		N3	9.1	-

Appendix E

Table E1 ***C. finmarchicus***. Box plot values for egg diameter and naupliar length (µm). n is sample size. M: median. UQ: upper quartile. LQ: lower quartile.

		E	N1				N2				N3			
			Ctrl	10%	50%	100%	Ctrl	10%	50%	100%	Ctrl	10%	50%	100%
7.5°C	**n**	121	103	82	93	84	111	115	111	107	62	72	80	76
	Max	155	197	196	193	194	210	211	208	208	218	223	222	221
	UQ	151	194	192	190	190	205	207	205	205	216	220	218	216
	M	147	190	188	185	185	201	201	201	201	211	213	213	212
	LQ	144	186	181	181	181	195	196	196	197	207	209	208	207
	Min	141	180	175	175	177	190	192	192	190	201	205	203	201
10°C	**n**	112	68	98	69	87	110	75	118	100	66	81	78	73
	Max	150	196	197	194	194	208	208	210	208	220	217	217	220
	UQ	149	191	193	190	190	203	205	206	204	215	214	212	217
	M	146	184	188	185	185	199	199	201	197	209	210	207	212
	LQ	143	180	182	179	181	193	195	194	193	204	206	202	207
	Min	139	174	180	174	177	183	188	188	189	201	203	199	199
12.5°C	**n**	73	72	73	89	87	113	116	122	112	71	76	66	66
	Max	151	196	193	192	195	206	208	204	207	219	219	217	219
	UQ	148	192	190	189	191	203	203	201	204	214	215	214	213
	M	146	188	185	184	186	198	199	197	200	210	210	211	209
	LQ	143	179	182	178	181	193	195	193	193	204	202	206	203
	Min	140	173	174	173	178	187	190	189	188	196	199	202	200

Table E2 ***C. hyperboreus.*** Box plot values for egg diameter and naupliar length (µm). n is sample size. M: median. UQ: upper quartile. LQ: lower quartile.

		E	N1				N2				N3			
			Ctrl	10%	50%	100%	Ctrl	10%	50%	100%	Ctrl	10%	50%	100%
3°C	**n**	73	93	100	89	102	112	109	106	76	50	53	60	70
	Max	189	245	261	259	263	268	267	267	267	286	279	275	278
	UQ	186	242	239	244	244	264	264	263	264	281	272	273	273
	M	184	237	232	235	239	258	259	259	259	274	266	265	268
	LQ	183	230	228	230	234	252	253	252	252	267	256	259	260
	Min	181	224	224	224	226	241	248	244	244	259	247	252	250
7.5°C	**n**	82	63	47	70	52	89	82	83	84	53	57	54	59
	Max	246	247	244	240	255	273	274	274	276	286	287	289	286
	UQ	241	242	239	237	244	267	270	269	271	284	283	284	282
	M	226	234	235	233	237	263	264	264	265	280	279	279	279
	LQ	205	226	228	226	231	256	260	260	258	276	274	273	272
	Min	197	222	221	221	224	251	254	254	254	272	270	267	268
10°C	**n**	105	106	79	101	68	-	-	-	-	43	57	52	49
	Max	197	260	255	260	261	-	-	-	-	266	274	273	269
	UQ	194	254	244	253	254	-	-	-	-	262	268	268	265
	M	189	240	235	244	249	-	-	-	-	255	259	263	259
	LQ	186	229	224	232	237	-	-	-	-	246	250	257	249
	Min	183	217	216	222	231	-	-	-	-	232	241	247	244