Interactive effects of temperature, ocean acidification, and pyrene on *Calanus glacialis* nauplii

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Abstract

Climate change is increasing the earth's temperature and acidifying its ocean. These changes are expected to be more severe in the Arctic region. Higher temperatures in the Arctic Ocean will reduce its sea ice and open it up for shipping and petroleum activity, which will expose the Arctic to polycyclic aromatic hydrocarbons (PAHs) such as pyrene. This study is a part of the Nansen legacy project, and aims to look at the effect of warming, ocean acidification, and pyrene on the nauplii of *Calanus glacialis*. Adult *C. glacialis* females were sampled from the Northern Barents Sea in March of 2021 and brought back to the University of Oslo for the experiments. The eggs from *C. glacialis* were placed in tubes in eight different combinations of Temperature, pH, and Pyrene concentrations. The tubes were checked every day for 1.5 months and the survival and development of the nauplii were noted. It was found that elevated temperatures decreased the development time to nauplii stage 3. Almost no nauplii developed to nauplii stage 3 in the pyrene exposed treatment at lower temperatures possibly due to the narcotic effect of pyrene. Mortality was highest in the pyrene exposed nauplii at the higher temperature. More nauplii reached nauplii 3 at higher temperatures with pyrene than at the lower temperatures. Due to methodological challenges, the effect of future OA could not be concluded. Other studies however point to the nauplii being tolerant to ocean acidification due to genetic expression. Interactive effects between higher temperatures and pyrene toxicity were found, indicating that *C. glacialis* will be exposed to higher PAH toxicity in a future warmer Arctic.

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1. Introduction

1.1 Climate change in the Arctic

The world is currently experiencing an increase in air and water temperature as a result of high anthropogenic CO_2 emissions, with the increase in annual mean temperature in the Arctic region being projected to be twice the amount of the global mean (IPCC 2022, Overland et al 2019). The current goal of halting the annual mean temperature increase by 2°C would result in the annual mean temperature in the Arctic being 4°C higher than the current annual mean temperature (Overland et al 2019). The ocean also acts as a sink for atmospheric CO_2 concentrations, and increased CO_2 will be taken up in the ocean and reduce its pH by reacting with seawater, this process is called ocean acidification (OA) (Doney et al 2009). Ocean acidification will be more prominent in the Arctic as CO_2 absorption in seawater increases with colder temperatures (Pörtner 2008). The ocean acidification process is expected to reduce the ocean's pH by 0.3 - 0.5 by 2100, and as far as 0.77 units in 2300 in a worst-case scenario (Pörtner 2008). The most noticeable effect of a warmer Arctic ocean will be the reduction of sea ice, with the Arctic ocean being projected to be ice-free in September by the end of the century (IPCC 2022). There are large reserves of oil and gas in the Arctic, with sea ice cover currently being one of the major challenges for exploiting these reserves (AMAP 2010, Henderson & Loe 2016). These reserves will in the future with less sea ice be available for the petroleum industry, which will increase the exposure to polycyclic aromatic hydrocarbons (PAHs) in the Arctic ecosystem through produced water and potential oil spills. Less sea ice will also open more sea routes in the Arctic which are expected to reduce the shipping time between Asia and Europe by up to 10 days (Melia et al 2016). These changes are expected to have large impacts on the Arctic ecosystem as a whole, and the organisms that inhabit this region. (IPCC 2022)

1.2 *Calanus glacialis*

Zooplankton is a large and abundant group of organisms that are important for the transfer of energy in all marine habitats. The Arctic ecosystem is no different and here we find three co-existing species of the Calanoid copepod genus *Calanus* that are key components of the Arctic marine food web. These three include the two true Arctic species *Calanus hyperboreus*

and *Calanus glacialis* as well as the boreal species *Calanus finmarchicus (*Figure 1.1). *Calanus glacialis* is mainly distributed in the Arctic fjords and shelf seas. (Falk-Petersen et al 2009).

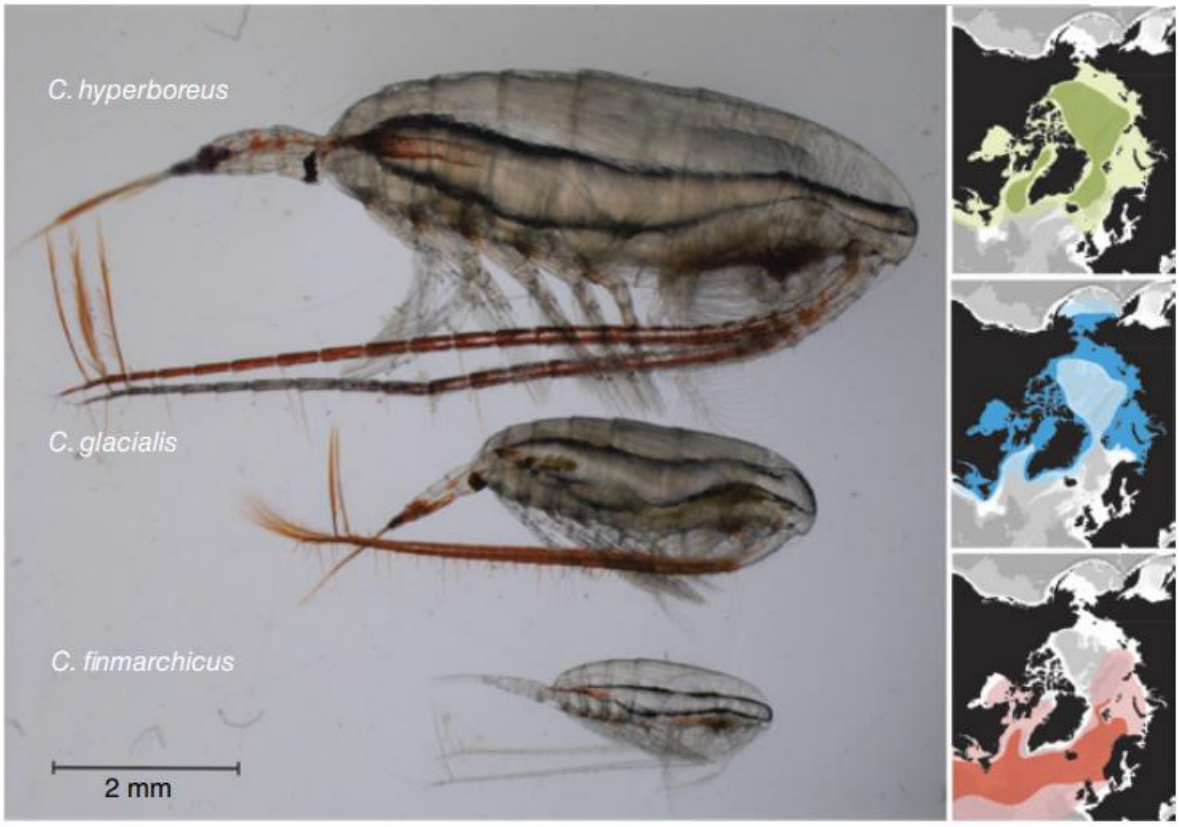

Figure 1.1: Photo of the three main Calanus in the Arctic. The uppermost copepod is Calanus hyperboreus, the one in the middle is Calanus glacialis and the one at the bottom is Calanus finmarchicus. map of the distribution of the 3 Calanus species is shown to the right of the copepods. Photo and credit Malin Daase (Daase et al 2021)

Calanus glacialis is a relatively large copepod (Figure 1.1) and an important prey species for polar cod (*Boreogadus saida*), contributing up to 24-84% of the carbon uptake to this fish which so many depend on in the Arctic marine ecosystem (Bouchard & Fortier 2020). *Calanus glacialis* is also being preyed upon by seabirds, such as the little auk, black-legged kittiwake, and the northern fulmar (Wold et al 2011). *Calanus glacialis* has a 1–3-year life cycle and it mainly develops during the summer/autumn before descending to deeper waters in autumn where it reduces its metabolism to a minimum to survive the long and food-poor winter. (Figure 1.2) (Falk-Petersen et al 2009, Søreide et al 2010). The life cycle of *C. glacialis* consists of 6 nauplii stages (NI-NVI) and 6 copepodite stages (CI-AF/AM) (Søreide et al 2010). Development from NI to CIII occurs in the upper 25m layer of the ocean (Kosobokova 1999). *Calanus glacialis* most likely develop to CIV within one year and then overwinters, before developing to CV during the next year (Falk-Petersen et al 2009). The

main overwintering stages are copepodite stage IV (CIV) and V (CV) (Falk-Petersen et al 2009, Søreide et al 2010). *Calanus glacialis* CV molt into adult males and females during the winter months (Hatlebakk et al 2022, Kosobokova 1999). Males of *C. glacialis* peak in abundance in December-January, while females in January-March (Hatlebakk et al 2022). Before the overwintering, *C. glacialis* prepare by storing large amounts of lipids with a high wax ester content (Falk-Petersen et al 2009.) These large lipid stores are used to survive the winter period when food is scarce and maybe more importantly to invest in early gonad maturation and egg production before the spring bloom in what is termed capital breeding strategy (Varpe et al 2009). In the Arctic region, two algal blooms occur, the first is the ice algal bloom in late April, while the other is the phytoplankton bloom which takes place soon after the sea ice breaks up (Søreide et al 2010). *C. glacialis* will utilize the ice alga bloom to boost gonad maturation and early egg production (Hirche & Kattner 1993, Søreide et al 2010).

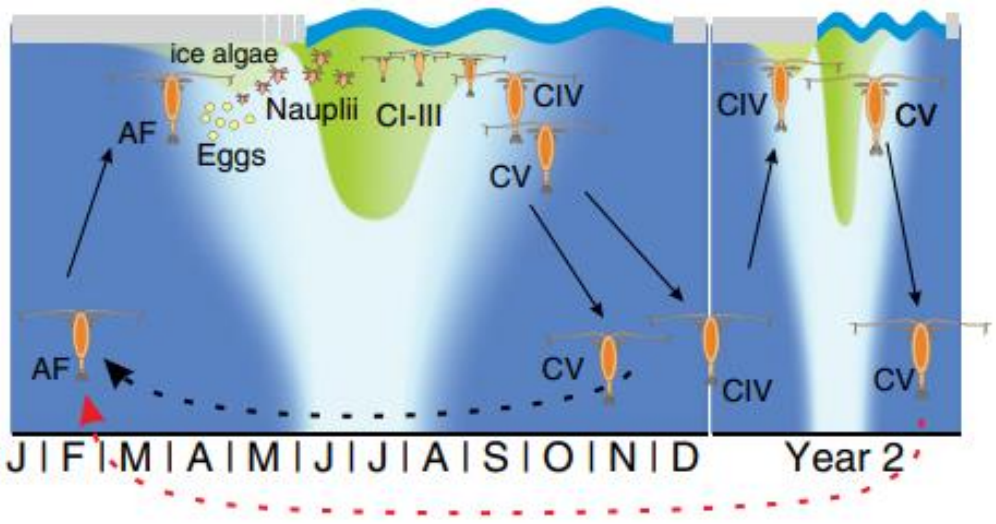

Figure 1.2: Illustration of both the 1- and 2-year life cycles of Calanus glacialis which includes the phytoplankton blooms, ice cover, and light availability throughout the year. Photo source and credit Malin Daase (Daase et al 2021)

1.3 Effect of marine warming on copepods

The range of temperatures in which an organism thrive is called the thermal window, with specific temperature range and amplitude varying between species (Alcaraz et al 2013, Grote et al 2015.) Ectotherms, such as copepods, are in general more responsive to increasing temperatures as they have been shown to have less plasticity in the thermal tolerance (Gunderson & Stillman 2015). It has also been suggested that ectotherms have not evolved

mechanisms to regulate their internal temperature and are therefore dependent on the environmental temperature for optimal development. They are therefore more susceptible to environmental temperature changes outside of this range (Marshall et al 2020) Species inhabiting areas with a larger temperature variability tend to be more tolerant to thermal stress (Sasaki et al 2019). Marine polar regions on the other hand are known to be relatively stable in temperature and marine polar species are sensitive to temperature variations (Peck et al 2004). Another factor increasing the risk of rising temperatures for polar species is that they already occupy the lowest temperature extreme, and cannot migrate to colder areas (Dam 2013). The distribution of *C. glacialis* is strongly correlated to temperature with the copepods being mainly found in areas at <6°C and with seasonal sea ice during the winter period (Carstensen et al 2012). Competition with the temperate *Calanus finmarchicus* may also limit *C. glacialis* southern distribution as *C. finmarchicus* is more adapted to warmer temperatures (Hatlebakk et al 2022). *Calanus glacialis* sampled from the Barents sea have been found to have a thermal optimum for respiration rates at 6°C and at 2.5°C for ingestion rates (Alcaraz et al 2013). *Calanus glacialis* sampled in the Advent-fjorden, Svalbard on the other hand has been found to have increasing respiration and ingestion rates with temperatures up to 10°C, without a clear thermal optimum (Grote et al 2015). *Calanus glacialis* in a controlled experiment showed no up-regulation of heat shock proteins to elevated temperatures (Smolina et al 2015). This is similar to other polar species which lack the biochemical machinery required to deal with warmer temperatures (Dam 2013, Smolina et al 2015). The lack of up-regulation of heat shock proteins in *C. glacialis* indicates that the species is vulnerable to warmer temperatures, which is supported by the distribution of the species (Smolina et al 2015). Higher temperatures have been shown to reduce the grazing rate of *C. finmarchicus* (Dinh et al 2019). Elevated temperatures at 10°C have been shown to cause depressed/delayed gonad maturation in adult *C. hyperboreus* (Hildebrandt et al 2014). Elevated temperatures increase nauplii mortality, too, with the highest mortality at 10°C in *C. glacialis*. (Grenvald et al 2013) Not all effects of higher temperatures are negative. For egg hatching time and nauplii development, warmer sea temperatures are positive for *C. glacialis.* (Grenvald et al 2013).

1.4 OA on copepods

Effects of OA have mainly been studied on calcifying organisms with less focus on non-calcifiers, such as copepods which have a chitinous exoskeleton (Fitzer et al 2012). Kurihara

et al 2004 discussed that reduced pH and increased CO_2 concentration induce metabolic suppression by reducing protein synthesis in organisms (Kurihara et al 2004, and references therein). OA has been found to lower the egg production rate of *Acartia steueri* and *Acartia erythraea*, however, the egg production increased when the copepods were returned to normal seawater (Kurihara et al 2004). OA affects the growth rate of *Tisbe battagliai* with a large reduction in size at maturity (Fitzer et al 2012). Life stage and strategies are factors affecting OA sensitivity as different life stages and species that are exposed to less variable pH concentrations during their lifetime are more sensitive to OA (Kurihara et al 2004, Lewis et al 2013). For instance nauplii in *Acartia tonsa* have shown higher sensitivity to OA than copepodite and adult stages (Cripps et al 2014). Diapausing copepods are exposed to low hemolymphic pH as a part of physiological mechanisms to attain buoyancy during overwintering, whereas non-diapausing copepods had higher hemolymphic pH (Schründer et al 2013). *Calanus glacialis* has similarly been found to have low hemolymphic pH (pH=6) levels during diapause (Freese et al 2015). The ability to survive with such low hemolymphic pH levels has previously been argued as the reason for observed tolerance in *C. glacialis* copepodites (Thor et al 2018a). Nauplii of *C. glacialis* have also been found to be tolerant to reduced pH concentrations predicted for the year 2100 (Bailey et al 2016), with upregulation of several genes involved in pH regulation being linked to exposure and could explain the tolerance (Bailey et al 2017). Other studies have also shown little effect of OA alone on grazing, performance, and egg production (Hildebrandt et al 2014, Hildebrandt et al 2015, Thor et al 2018b). Reduction in metabolic rate in response to pH = 7.87 have been observed in CIV *C. glacialis* populations from Svalbard, but not in CIV from the Disko Bay population in Greenland exposed in the same way. (Thor et al 2018a). No effect was found in CVs in both populations (Thor et al 2018a). Increased metabolic costs for CII-CIII copepodites have also been found, but not in CVs from the *C. glacialis* Svalbard population (Thor et al 2016). These findings indicate that some populations and life stages might be more sensitive to OA than others.

1.5 Effects of PAHs on Copepods

PAHs are a large group of chemicals consisting of two or more benzene rings, produced often from a combustion reaction. Main sources of PAH exposure in the marine environment include exhaust from boats, sewage, runoff from roads, oil spills, and offshore activities

(Hylland 2006). Many of these chemicals are carcinogenic, but other toxicological effects from these compounds include oxidative stress, effects on the immune system, and endocrine disruption. (Hylland 2006). The PAHs are distinguished by molecular weight (Mw): low, intermediate, and heavy molecules and the weight determines the bioavailability of the compound. Low Mw PAHs are known to be toxic, but are highly volatile and have a short half-life in water, and are, therefore, less bioavailable to aquatic organisms. (Bellas & Thor 2007). Heavy PAHs are also toxic but have a low solubility in water and are more associated with sediments. Intermediate PAHs, such as pyrene, are of more concern since they are toxic and water-soluble enough to be bioavailable in the water(Hylland 2006). Pyrene is one of the more dominant PAHs in oil and has relatively low toxicity compared with other PAHs (Barata et al 2005, Nisbet & LaGoy 1992, Toxværd et al 2018b). Pyrene has because of these properties been widely used in toxicity studies on Arctic copepods (Dinh et al 2019, Grenvald et al 2013, Jensen et al 2008, Nørregaard et al 2014, Toxværd et al 2018b). PAHs from weathered crude oil have been shown to bioaccumulate in *Calanus hyperboreus* and lipid-rich individuals are more tolerant to PAH exposure than lipid-poor individuals. (Øverjordet et al 2018). The high lipid contents in the lipid sacs of the *Calanus spp* will absorb lipophilic compounds and offer some protection against toxicity, at least until the lipids are metabolized or a steady state has been reached. (Jager et al 2017). There is also evidence of maternal transfer of PAHs to the offspring of the *C. glacialis* females (Hansen et al 2017). Pyrene is known to have negative effects on hatching success, grazing rate, and size of female copepods (Krause et al 2017), as well as increased mortality. Pyrene has been found to lower the grazing rate of copepods, including *C. glacialis*, possibly due to pyrene having a narcotic effect on the animals (Barata et al 2005, Dinh et al 2019, Jensen et al 2008). Pyrene has also been found to reduce metabolic activity and delay the gonad development in overwintering *C. glacialis* females (Toxværd et al 2018b). The reduced metabolic activity and delayed gonad development further resulted in reduced egg production, but the hatching success was unaffected (Toxværd et al 2018b). Other studies have also shown that the hatching success is unaffected when only eggs of *C. glacialis* and *C. finmarchicus* are exposed, whereas the hatching success of *Calanus hyperboreus*, was lowered in pyrene which can be explained by the two first species having thicker eggshells (Nørregaard et al 2014). *Calanus glacialis* females exposed to low concentrations of crude oil did not show any negative effects, but the nauplii of the exposed females had increased mortality, and nauplii showed deformities (Toxværd et al 2018a).

1.6 Multiple stressors on Copepods

Most experiments on stressors usually include one stressor, however, this is not representative of a field environment where organisms are exposed to a multitude of stressors all at once (Gunderson et al 2016). The effects of these multiple stressors can vary due to interactions between the different stressors (Gunderson et al 2016). These interactions (if any) are classified as being either additive, antagonistic or synergistic (Gunderson et al 2016). Additive effects occur when the effect of two (or more) equals the combined sum of effects. Antagonistic effects occur when the combined effect equals less than the combined sum of effects. Synergistic effects occur when the combined effect equals more than the combined sum of effects (Gunderson et al 2016).

Synergistic effects have been found between marine heatwaves and pyrene on the survival and egg production in a tropical copepod *Centropages velificatus*, with increased detrimental effects when both stressors are combined. (Hernández Ruiz et al 2021). The warmer temperature has been found to increase the toxicity of mercury Hg in copepods (Bai & Wang 2020). Antagonistic effects between OA and warming have been found in temperate species of the Baltic sea which are exposed to variable pH concentrations during their lifetime (Garzke et al 2016).

Hildebrandt et al 2014 found that the metabolic rates of *C. hyperboreus* females were sensitive to a combination of OA and warming. Effects of OA and warming is varies between species having both synergistic and antagonistic effects (Wang et al 2018). Warmer temperatures have been found to affect the toxicity of pyrene somewhat in the Arctic *Calanus*, with negative effects on development. (Grenvald et al 2013). There are however few experiments done on *Calanus glacialis* with multiple stressors. Most experiments have been done with only one stressor, with a few having done two. This means that the potential interactive effects of these stressors remain largely unknown.

1.7 Aims of this study

This study aims to investigate the interactive effects of temperature, ocean acidification, and pyrene on the survival and development of the early life stages of *C. glacialis*. Early life

stages have been chosen since earlier life stages of organisms including copepods be more sensitive to stressors compared to the older and adult stages.

H1: Development times and hatching are faster at 5°C than at 1°C

H2: Survival of the nauplii is higher at 1°C than at 5°C

H3: Ocean acidification alone will not influence the development and survival of the nauplii.

H4: Pyrene has negative effects on the survival and development of the nauplii.

H5: Pyrene and OA have additive/potentiation interactions on the development of the nauplii due to increased energetic costs of dealing with both stressors.

H6: Negative effects of pyrene on survival and development are worse at 5°C.

H7: The three stressors combined will have the greatest negative effect on survival and development.

2. Materials and Method

2.1 Sampling/Cruise

The females *Calanus glacialis* were sampled during the Nansen legacy Q1 cruise on the 9th of March of 2021 onboard RV Kronprins Haakon at station P4, located at N°79,7640, E°33,8356 (Figure 2.1). P4 was at the time of sampling covered in very close drift ice (Figure 2.2).

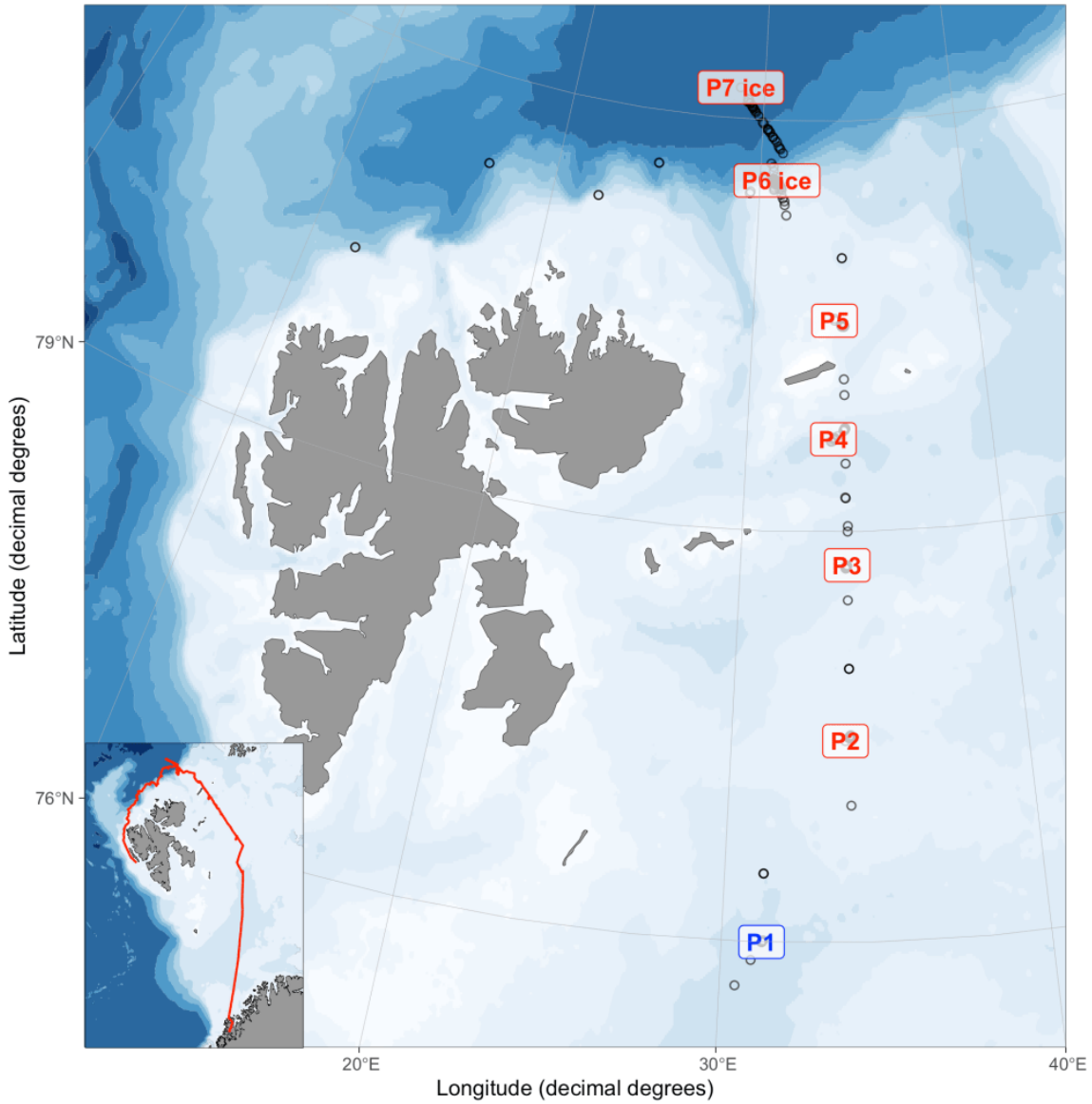

Figure 2.1: Map of the Nansen legacy transect was taken from https://arvenetternansen.com/station-map-seasonal-cruise-q2/ 25.06.2022 15:43. Each sampling location (process station) of the Nansen legacy cruises is shown called P1-7. Sea depth is colored on this map with the continental colored here as white and the deeper sea bluer. The sampling location in our study was P4 which is located on the continental shelf. The smaller map in the lower-left corner shows the route taken during the Q2 cruise (May 2021).

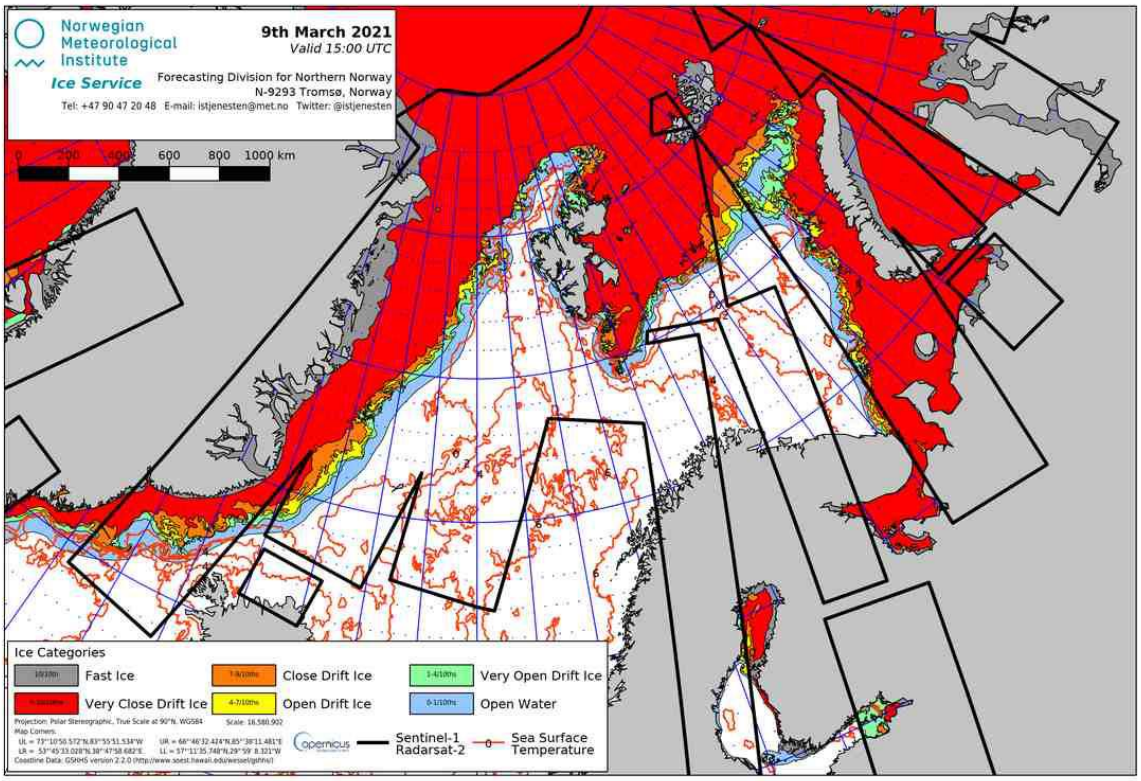

Figure 2.2: Ice cover was at the time of sampling quite extensive with the entire sea east of Svalbard being covered in very close drift. The sampling location P4 was deep within the sea ice at the time of sampling. Photo taken from the Norwegian meteorological institute's cryo service 25.06.2022 15:43 https://cryo.met.no/archive/ice-service/icecharts/quicklooks/2021/20210309/arctic_20210309_col.png .

The copepods were sampled vertically using a Bongo net (hydro-bios, mesh size 180µm and net opening 0.28m^2) from ~20m above the bottom (320) to the surface. The sample was gently transferred to a large 20L bucket containing filtered seawater. This bucket was kept in a room at 1°C when not in use. For detailed identification, the *Calanus* spp. were placed in a 100mm x 15mm Petri dish which was placed in a larger Petri dish 200mm x 15mm containing ice to keep the samples cold.

Female *C. glacialis* were identified to species by their red-pigmented antennas and genital somites to distinguish them from the similar *C. finmarchicus*, which are generally smaller and lack red pigmentation (Choquet et al 2018). 100 Individuals with undamaged antennas and complete fural rami (Figure 2.3) were transferred to 4 1L bottles with 25 individuals in each.

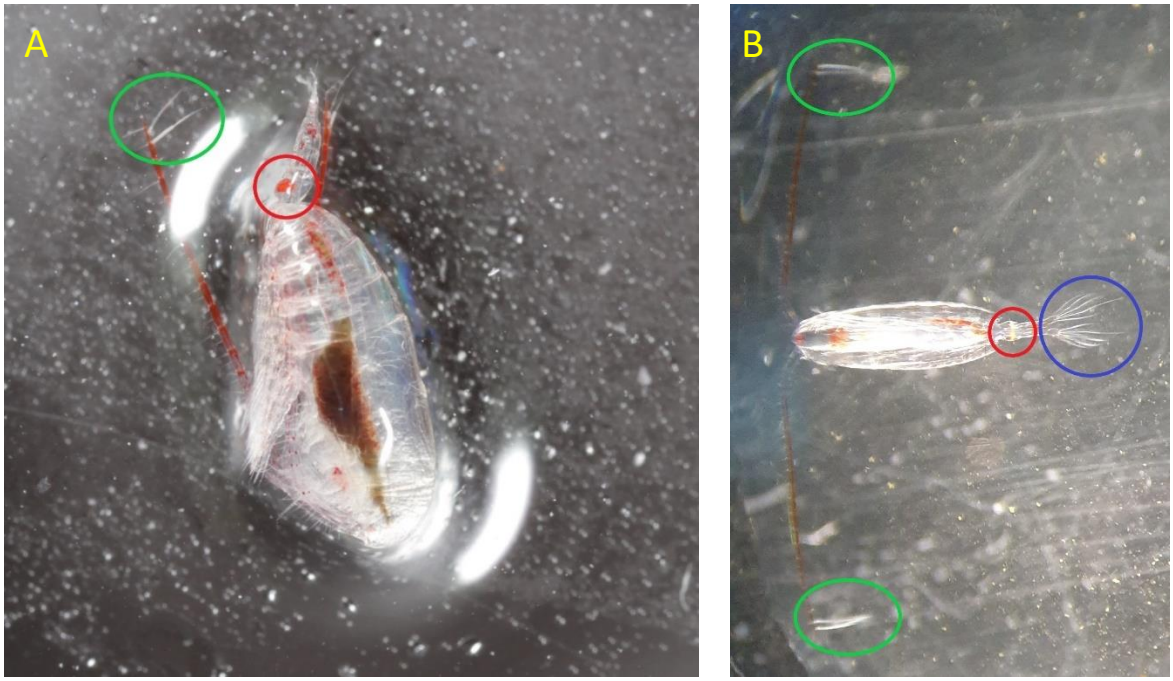

Figure 2.3: A: female C. glacialis with the red genital somites highlighted by the red circle and the spears at one antennule highlighted in green, B: female C glacialis highlighted in the same way as A but with a blue circle highlighting the complete fural rami. Calanus glacialis female A is one of the females from the main experiment, and female B was photographed during the Nansen legacy Q1 cruise. Photo credit Andreas Jortveit

The lid of the bottles were removed to air the bottles every two days to avoid asphyxiation. The bottles containing the female *C. glacialis* were kept in a dark cold room at 1°C until the end of the cruise. The female *C. glacialis* were not fed during this time since they can go long periods of time without food during the winter period.

The seawater used was taken with Niskin bottles from 30mbelow the surface to avoid contamination from the ship. The females were transported from the cruise ending in Tromsø to Oslo on a commercial air flight with the bottles containing the females placed in a zargess box filled with cooling elements and towels to keep the temperature low and disturbance at a minimum during transport. At UiO the bottles with the females were placed in an incubator at 1°C and were left alone for a few weeks to acclimate without food and as little light as possible.

2.2 Experimental set-up

Two levels of each stressor were used. 1°C and 5°C were used to simulate warming. a pH level of 7.55±0.02 was used for the future ocean acidification and untreated filtered seawater

was used for the current pH level. The pyrene exposure was either without pyrene or with 200nM. This gives a total of 8 combinations (figure 2.4)

Freshly filtered seawater was periodically transported from NIVA Solbergstrand to the lab at UiO. A stock solution of 2nM Pyrene was made by dissolving pyrene in acetone. The pyrene concentration of 200nM (40.450µg/L) was prepared by diluting this stock solution with filtered seawater (Dinh et al 2019). New pyrene solutions were made daily to avoid loss of the concentration over time (Grenvald et al 2013, Hjorth et al 2007)

Reduced pH was achieved by bubbling filtered seawater with CO_2 until the pH reached the desired concentration of 7.55±0.02. Stock exposure solutions were made every day and placed in the 1°C incubator for at least 1 hour before use.

Two incubators were used to expose the nauplii to two different temperatures, one at 1°C and the other at 5°C.

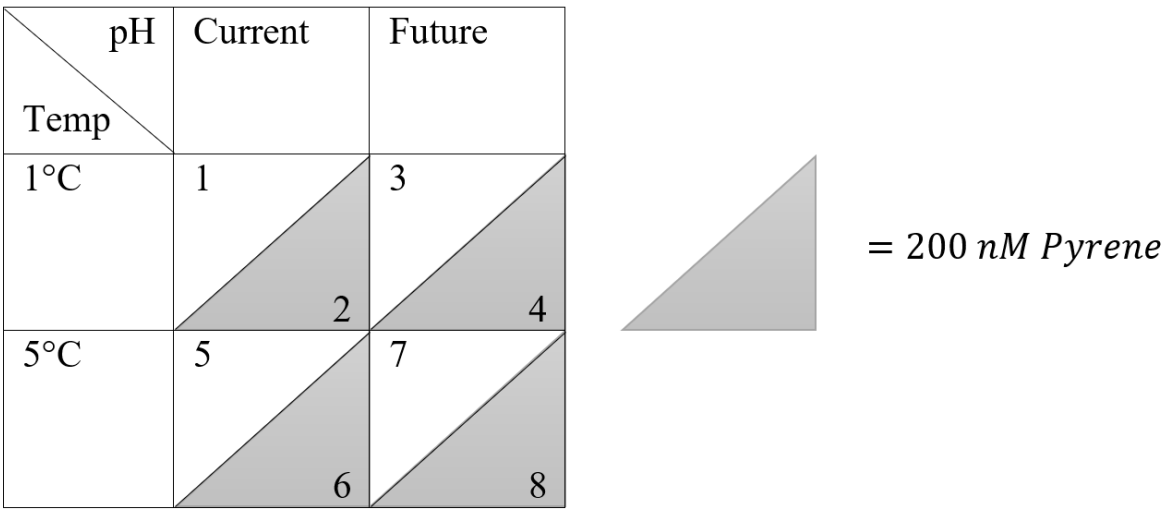

Figure 2.4 illustrative table of the 8 combinations of temperature, pH, and pyrene concentrations used in the multi-stress experiment on the C. glacialis nauplii.

On the 27. April 10 females were taken out of the bottles and placed individually in 250 ml glass bottles and 10 more were placed in glass bottles a few weeks later due to poor egg production. The seawater was not changed for the females for the duration of the experiment, and they were fed with 1 mL of a *Rhodomonas* culture (785 000 cells per mL) 6 days a week. The bottles were filtered through a 50 µm filter 3 days a week from 01.05.2021 until 28.06.2021. The contents of the filter were rinsed into a Petri dish. The fecal pellets and eggs were counted using a stereo microscope. The eggs were kept if the count was 10 or higher. The first two batches were not kept due to them possibly being of unstable quality.

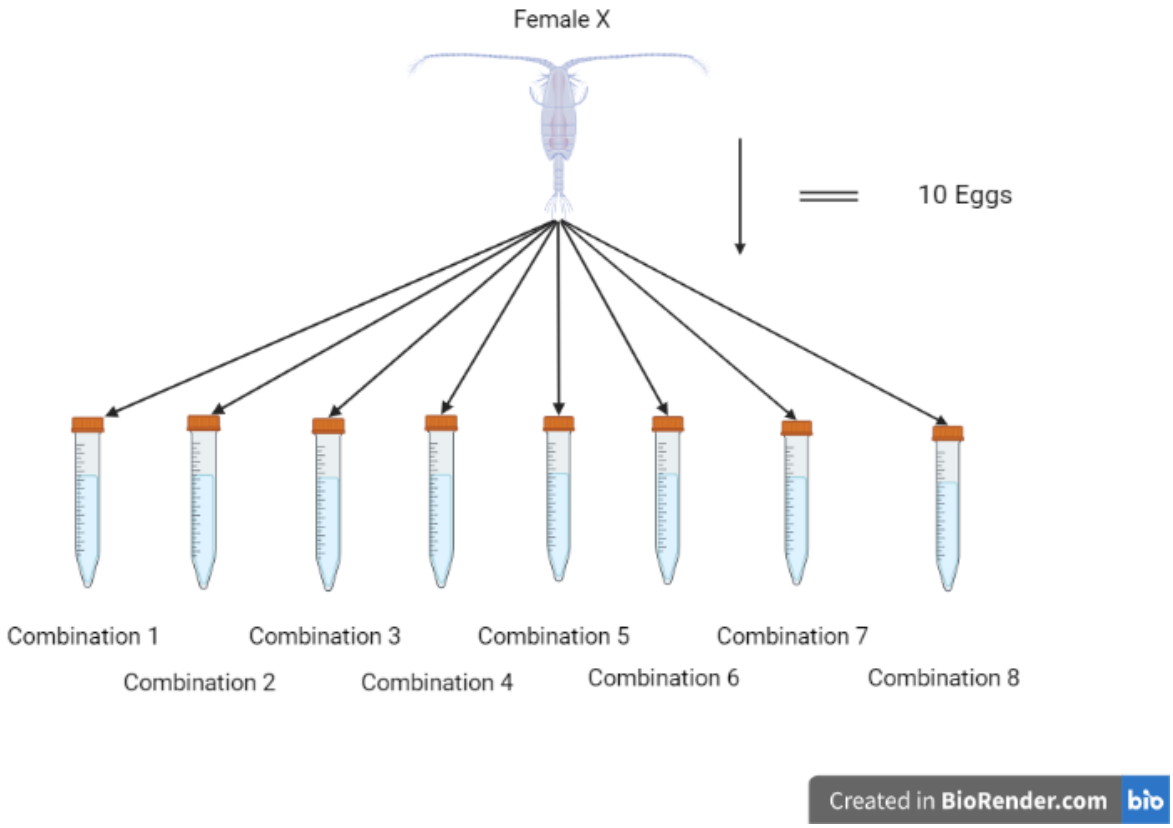

Figure 2.3: An illustration of how the eggs from the females were distributed in the 8 combinations for the multi-stress experiment on C. glacialis nauplii. Note that the copepod used in the illustration is not C. glacialis. Created using Biorender.

Table 2.1: Table of the number of replicates for each combination in the multi-stress experiment on C. glacialis. Each replicate/tube in each combination is from a different female. The number of replicates/tubes is uneven in the different combinations due to the female egg production varying.

Combination	Replicates/tubes
1	10
2	9
3	6
4	8
5	9
6	6
7	7
8	8

10 eggs from each female were placed in a 15 ml tube for each combination (figure 2.3). The contribution of each female to each combination varied due to poor egg production (Table 2.1). The replicates/tubes were placed in racks in the two different incubators and from the

11th of June placed in a water bath to minimize temperature fluctuations. Only the tubes used for the future pH exposures were sealed with parafilm.

The replicates/tubes were checked every day by pouring the contents into a Petri dish, where the egg/nauplii were counted and the development stage was noted using a microscope. The development stages were determined by comparing with the different stages as shown in Hygum et al 2000. Although Hygum et al (2000) presents the morphology of *Calanus finmarchicus*, this is very similar to the morphology of *Calanus glacialis* in the early life stages.

While being checked and the water was changed the tubes and stock solutions were kept in a box filled with ice to keep the temperature down. Only the stock solutions and tubes that were in use were placed in the ice box, the remainder was in the incubators. The smaller Petri dish was placed in another larger petri dish filled with ice as mentioned earlier.

After the contents of the tubes had been placed in the Petri dishes for inspection, new treatment water was added to the tubes, and the eggs/nauplii were transferred back into the tube with a glass pipette. The number of eggs/nauplii were counted and staged during this. Afterward, they were fed ad libitum with several drops from a *Rhodomonas* culture starting from hatching to be certain that food is available when developmental stage 3 is reached. Feeding the nauplii is not necessary until they reach N3 since they lack mouths at N1-N2 and rely on internal energy stores instead (Jung-Madsen et al 2013) Food concentration in the tubes was around 15 188 cells per mL, counted using the Fuchs-Rosenthal method.

This was repeated every day between 31.05.21-15.07.21 and each replicate was ended when all nauplii died, with only a few replicates still alive at the end of the experiment.

2.5 Parafilm test

I noticed that a large number of nauplii seemingly disappeared in the future pH exposures and suspected that the parafilm used on these tubes was interfering. A test was conducted to see if the parafilm used in the future pH exposures had an influence on nauplii survival between 17.01.22-21.01.22. The test was done in the same way as the experiment earlier but had in addition of parafilm as a treatment (Table 2.2). Visual inspection of the parafilm was performed and the number of nauplii stuck to the parafilm was counted.

1) Nauplii from *Calanus hyperboreus* were used as that was available

2) The nauplii used were placed in a common pool before distribution to the treatments.
3) 5 replicates per treatment were used as opposed to the maximum 10 in the main experiment.
4) The experiment was carried out in a cold room at 5°C.
5) Tubes with a combination of pH and pyrene at 1°C and 5°C could not be made due to a lack of available nauplii.

2.6 Data Analysis

All data analysis and visualization were done in R version 4.2.0. Best-fitted models were selected based on AICc values with a delta ≥ 2.0. The dredge function from the MuMIn package (version 1.46.0) was used to test for the AICc values in the different models. A COX proportional hazard model was made using the survival package (version 3.3-1). A Kaplan-Meier survival curve was used to visualize the survival using the ggsurvplot function from the survminer package (version 0.4.9). Although the Kaplan-Meier is a statistical test on its own, it is in our study used only to visualize the survival due to the raw data being unclean and not enough time to fix. Generalized linear mixed effect models were made using the lme4 package (version 1.1-29) with a Poisson distribution (log linked) since the development data contained count data. The batch number was added as a random effect. Five replicates were removed from the analysis of the nauplii 3 count as they contained a starting number of nauplii around 20+ individuals. Three of these were from control exposure at 1°C, current pH, 0 Pyrene, and two from the 5°C, current pH, and 200nM pyrene. Development time to nauplii 3 was defined as the number of days that it took at least one nauplii to develop to nauplii 3. This was done instead of the usual median development time since all nauplii within each replicate that developed to nauplii 3 did so at the same time.

3. Results

3.1 Survival

AICc analysis of the COX proportional hazard model reveals two competing models (AICc = 4738.4 delta = 0.26), and an average model was made (Table 3.1). The nauplii exposed to pyrene at 1°C had the lowest hazard ratio of 0.59 which was significantly lower than 1 (Table 3.1). The hazard ratio of 0.59 means the nauplii exposed to pyrene were 37% less likely to die at any given time than the ones in the control exposure. The nauplii exposed to pyrene at 5°C had a significantly higher hazard ratio of 1.66 ± 0.4 compared to 1 (Table 3.1). The hazard ratio of 1.66 means the nauplii were 62% more likely to die when exposed to pyrene at 5°C than the control. None of the other exposure combinations had a hazard ratio significantly different from 1 (Table 3.1). The overall mortality was high in this study with most nauplii being dead within 20 days (Figure 3.1) and only 5 nauplii out of 683 (99.2%) being alive at the end of the study.

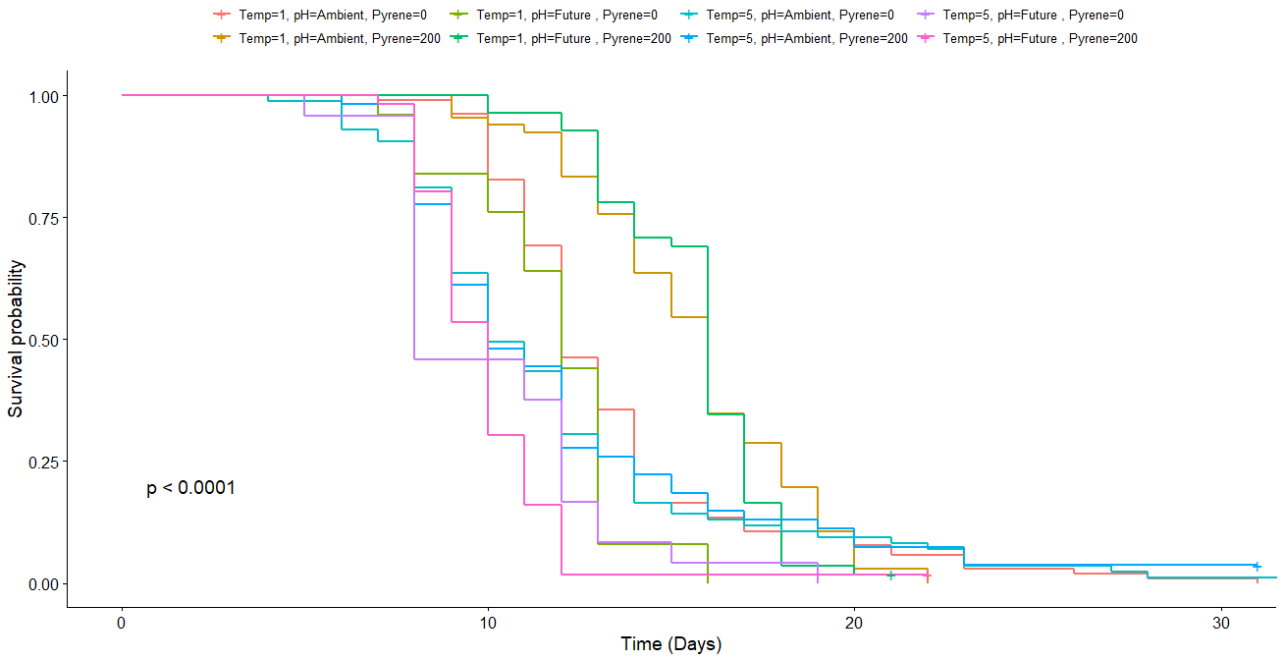

Figure 3.1 Kaplan-Meier survival curves for the different exposure combinations in the multi-stress experiment performed on Calanus glacialis nauplii (see legend for the 7 different treatments and the control). The survival curves show the probability, based on the proportion of nauplii, that are alive at each given time (Days). The p-value below 0.05 indicates that the survival curves are significantly different from each other.

*Table 3.1: Hazard ratios of the average model based on the two best-fitted models explaining the survival in the 7 different treatments compared to control which is set as 1 in the COX proportional hazard model. The average model was chosen due to the delta difference being very low between the two (AICc = 4738.4 delta = 0.26). The two best-fitted models were the full model, and a reduced model without interactive effects between all stressors and pH:Pyrene. * Indicates if the hazard ratio is significantly different from the baseline of 1. Hazard ratios < 1 indicate better survival than the baseline, which is set at 1, while hazard ratios > 1 indicate worse chances of survival. Only pyrene and temp pyrene had a significantly different hazard ratio than the baseline.*

STRESSORS	HAZARD RATIO (LOG)	Z VALUE	PR(>|Z|)	HAZARD RATIO
TEMPERATURE	0.17 ± 0.15	1.110	0.2671	1.18 ± 0.2
PH	0.33 ± 0.25	1.348	0.1778	1.40 ± 0.35
PYRENE	**-0.53 ± 0.17**	**3.217**	**0.0013***	**0.59 ± 0.1***
TEMP:PH	0.43 ± 0.38	1.139	0.2547	1.54 ± 0.59
TEMP:PYRENE	**0.51 ± 0.26**	**1.982**	**0.0475***	**1.66 ± 0.4***
PH:PYRENE	-0.27 ± 0.33	0.813	0.4165	0.77 ± 0.25
TEMP:PH:PYRENE	0.46 ± 0.53	0.872	0.3832	1.6 ± 0.89

3.2 Development

3.2.1 Development stages

The nauplii exposed to 5 °C had a higher number of more advanced stages (nauplii 3 and beyond) compared to the ones exposed to 1°C (Figure 3.2). Across all exposures, most nauplii only reached nauplii 2 with a large reduction in the number of nauplii developing to nauplii 3 (Figure 3.2).

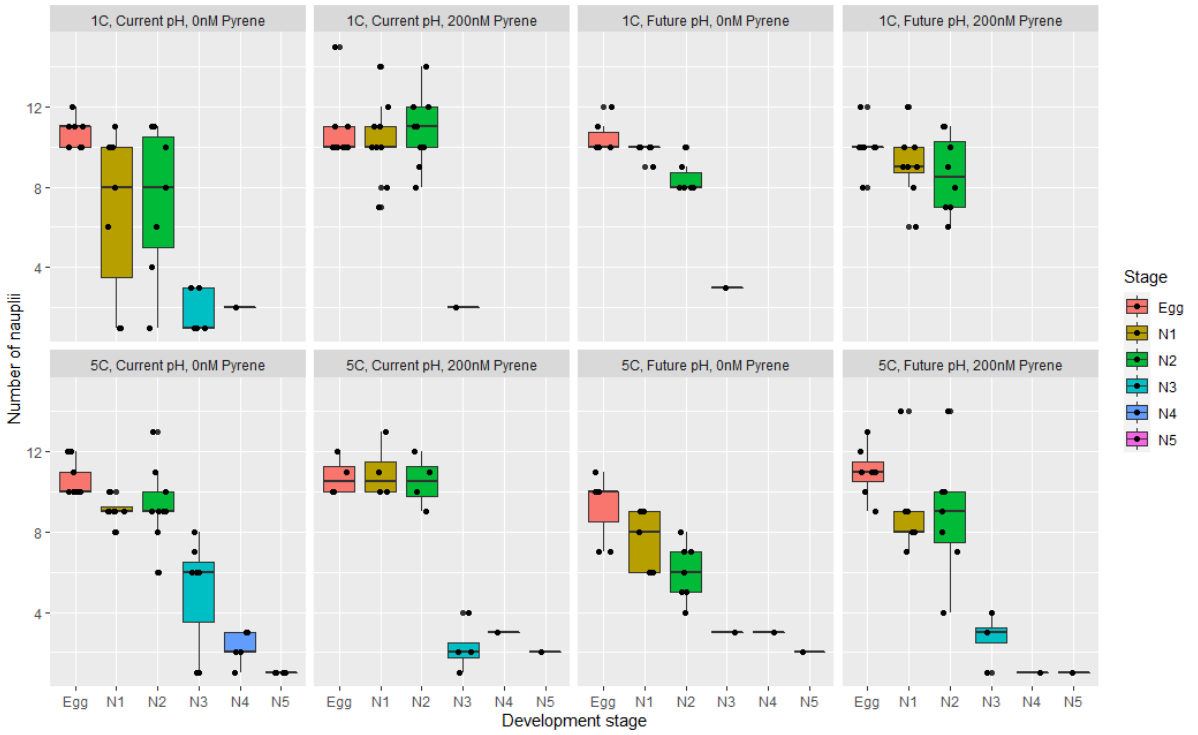

Figure 3.2: The number of C .glacialis nauplii reaching the different developmental stages for the different exposure combinations and control, represented here by a boxplot with the data points scattered within. Most nauplii developed to nauplii 2, although with some exceptions at the future pH exposure, which could be due to the nauplii getting stuck on water droplets on the parafilm. Few nauplii managed to develop to nauplii 3 with even fewer developing to nauplii 4. The sample size in most replicates/tubes shown here was 10 nauplii but some replicates/tubes had more with the highest being 15 nauplii. The boxplot represents the distribution of the data set. The black line in the middle of the box is the median value and is only visible for the exposures with more than one data point. The bottom line of the box is the first quartile (Q1), which is the median of the data points below the median. The upper line of the box is the third quartile (Q3), which is the median of the data points above the median. The whiskers show the distance to the minimum and maximum values (that are not outliers). The data points outside of the box and whiskers are outliers.

3.3.1 Nauplii 3

The number of nauplii that reached development stage 3 was higher at 5 °C than at 1°C. AICc analysis of a full GLMM model identified one model with the best fit being number~temperature+pyrene (AICc=96.6 delta=2.17). The model predicts that at 5°C the number of nauplii that reaches nauplii 3 is 4.7±1.5 which is more than twice as many (123%) than at 1°C (Table 3.2). The model predicts that pyrene on its own reduced the number of nauplii developing to nauplii 3 by roughly 50% (Table 3.2). The model showed no interactive effects of the stressors nor any effect of future pH.

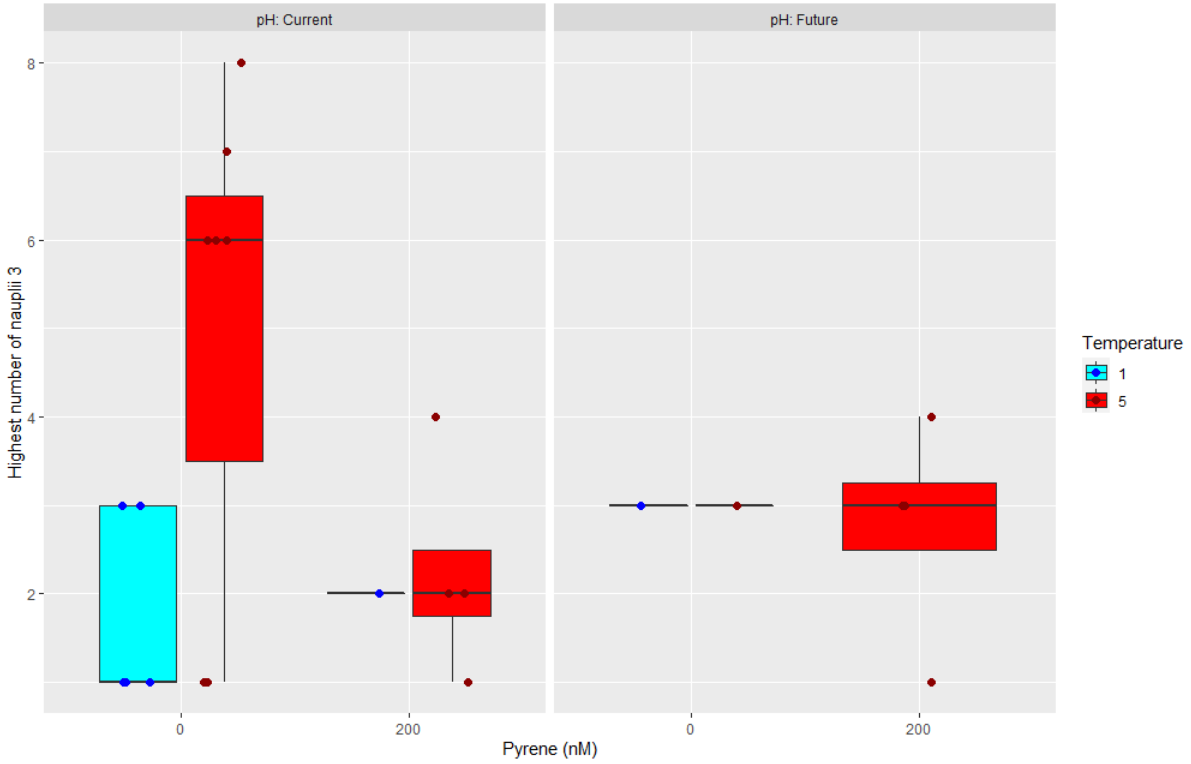

Figure 3.3: Highest number of C. glacialis nauplii that developed to nauplii 3 at the current and future pH exposures with and without pyrene (see legend for temperature). The boxplot represents the distribution of the data set. The black line in the middle of the box is the median value and are only visible for the exposures with more than one data point. The bottom line of the box is the first quartile (Q1), which is the median of the data points below the median. The upper line of the box is the third quartile (Q3), which is the median of the data points above the median. The whiskers show the distance to the minimum and maximum values (that are not outliers). The data points outside of the box and whiskers are outliers.

Table 3.2: Calanus glacialis multi-stress experiment and results from the reduced generalized linear mixed effect model of the number of nauplii that reached N3 in the different combinations. Nauplii~Temperature+Pyrene is the reduced model that had the best fit (AICc=96.6 delta=2.17).

	ESTIMATE (LOG)	Z VALUE	PR(>|Z|)	PREDICTED VALUE
INTERCEPT	0.76 ± 0.27	2.827	0.0047	2.1 ± 0.6
TEMPERATURE	1.54 ± 0.31	2.541	0.0110	4.7 ± 1.5
PYRENE	0.18 ± 0.26	-2.207	0.0273	1.2 ± 0.3

3.3.2 Development time N3

Development time was in general shorter at 5°C compared to 1°C (Figure 3.5) AICc analysis of development time revealed that temperature alone influenced development time (AICc = 125.6 delta= 2.35). The model reveals that higher temperature decreases the development time, from the start of the experiment to N3, from 11 days at 1°C to roughly 8 days at 5°C (Table 3.4).

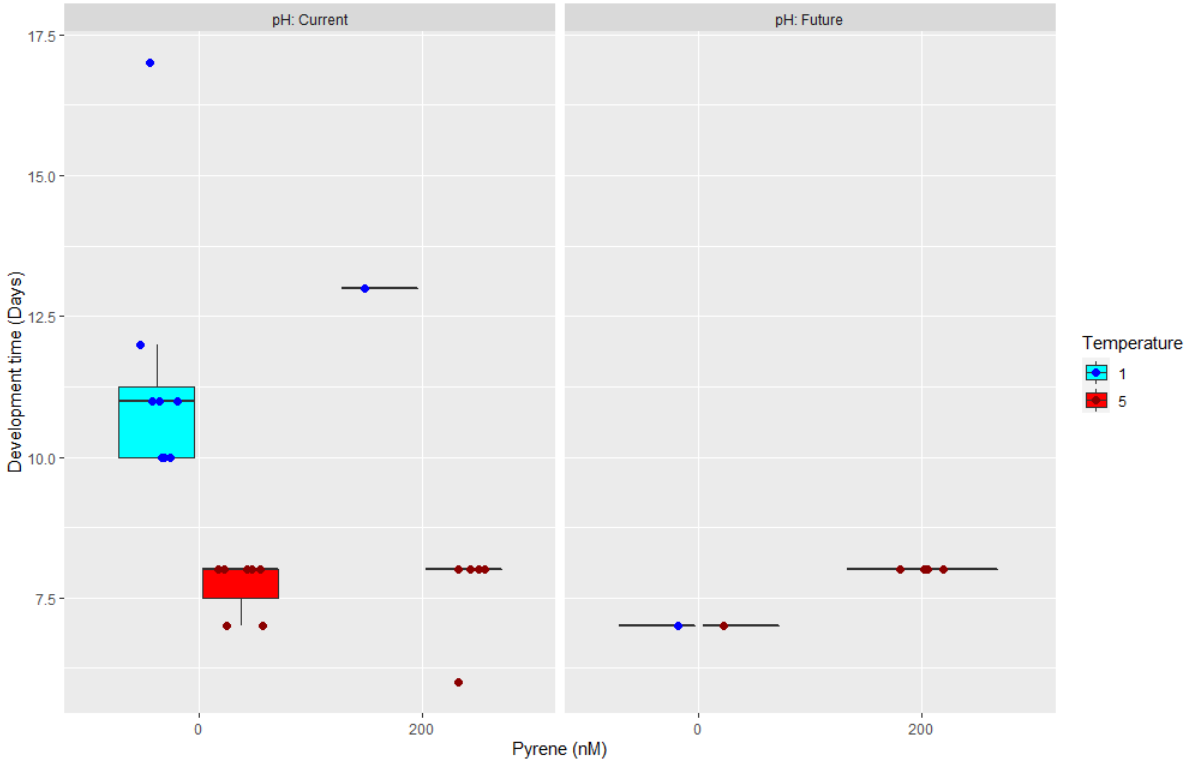

Figure 3.5 Calanus glacialis development time in days from the start of the experiment to N3 at the current and future pH exposures with and without pyrene (see legend for temperature). The boxplot represents the distribution of the data set. The black line in the middle of the box is the median value and are only visible for the exposures with more than one data point. The bottom line of the box is the first quartile (Q1), which is the median of the data points below the median. The upper line of the box is the third quartile (Q3), which is the median of the data points above the median. The whiskers show the distance to the minimum and maximum values (that are not outliers). The data points outside of the box and whiskers are outliers.

Table: 3.4 Calanus glacialis multi-stress experiment and results of the reduced generalized linear mixed effect of the development time from the start of the experiment to N3. Development time~Temperature was the model with the best fit (AICc = 125.6 delta= 2.35).

	ESTIMATE (LOG)	Z VALUE	PR(>|Z|)	PREDICTED TIME (DAYS)
INTERCEPT	2.42 ± 0.09	25.57	< 2e-16	11.2 ± 1.1
TEMPERATURE	2.04 ± 0.13	-2.93	0.00341	7.7 ± 1.0

3.3 Parafilm

The parafilm test revealed that the nauplii could get stuck in water droplets on the parafilm. The mean number of *C. hyperboreus* nauplii that got stuck over a 4-day period was in general higher in the pH-exposed tubes than in the ones without (Figure 3.6). The 1°C 200nM pyrene exposure lost the fewest nauplii with a mean of 0.4±0.25 (Figure 3.6).

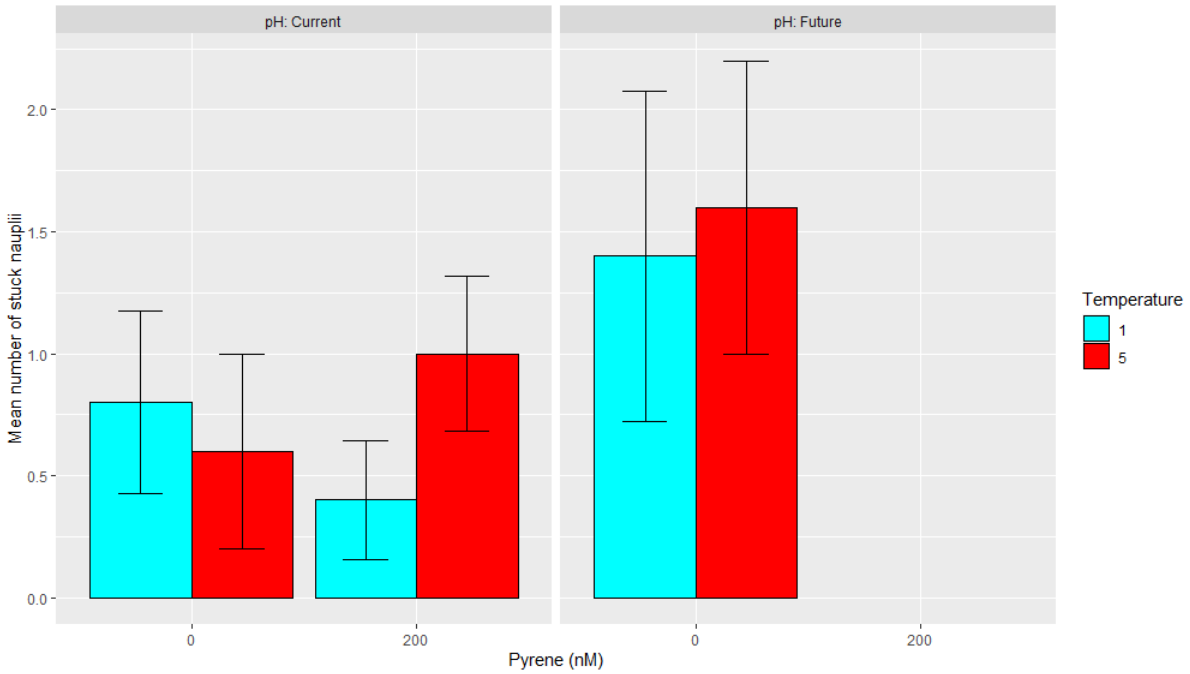

Figure 3.6: The mean number of Calanus hyperboreus nauplii that were observed stuck to parafilm over a 4-day period in the separate parafilm test experiment at the current and future pH exposures with and without pyrene (see legend for temperature). The error bar shows the standard error for each of the different columns.

4. Discussion

4.1 Temperature effects

The faster development to nauplii 3 in our study temperatures fits with other studies (Hirst & Kiørboe 2002) and our expectations (H1). The development time from the start of the experiment to N3 for the *C. glacialis* at the two temperatures were similar to the findings of Grenvald et al (2013). It should be noted that Grenvald et al (2013) calculated the time from hatching while I calculated the time from the start of the experiment with recent (1-3 days) spawned eggs. There was a higher number of nauplii that reached nauplii 3 at the 5°C exposures which is most likely due to development times being faster. The faster development time at 5°C is beneficial for the nauplii due to the later developmental stages being less vulnerable to invertebrate predation (Eiane et al 2002, Eiane & Ohman 2004). Faster development to nauplii 3 is also beneficial due to it reaching the first feeding stage which speeds growth further. Elevated temperatures at 5°C did not increase the mortality in our study, which contradicts our expectations (H2). The study of Grenvald et al (2013) did find an increase in mortality at higher temperatures. In the study by Grenvald et al (2013), elevated temperatures of 5°C and 10°C increased the mortality of the *C. glacialis* nauplii, with the mortality being highest at 10°C. The increased mortality at 5°C was only slightly higher than the mortality at 0°C (Grenvald et al 2013). An explanation for this is that Grenvald et al (2013) had a larger number of nauplii for each replicate (55 ± 16) compared with our 10. Smaller increases in mortality could therefore be more noticeable in their study compared to this study. The faster development and the higher number of nauplii that reached nauplii 3 without a significant decrease in the survival indicate that 5 °C is not stressful for the *C. glacialis* nauplii. This is further supported by the mean summer temperature in the northern Barents sea being around 5 °C (Mohamed et al 2022). The adult *C. glacialis* have been found to stop egg production and descend to deeper depths when temperatures reach ≥5°C (Niehoff & Hirche 2005). This vertical migration is an indication that the adults are stressed by temperatures ≥5°C. The *C. glacialis* nauplii have a peak in abundance in July at the onset of the phytoplankton bloom, where they remain in the warmer upper layer (Hatlebakk et al 2022, Søreide et al 2010). It is, therefore, reasonable to assume that the nauplii might have a better tolerance to temperatures around 5°C. Differences in thermal tolerances at different life stages have been observed in fish (Dahlke et al 2020), as well as

the copepod *Tigriopus californicus* (Tangwancharoen & Burton 2014). The differences in thermal tolerances in fish are due to the later stages having better aerobic capacity due to the cardiorespiratory system being more developed (Dahlke et al 2020). The copepod *Tigriopus californicus* having different thermal tolerances between life stages was argued to be due to the different life stages having different habitats with different temperature ranges (Tangwancharoen & Burton 2014). The nauplii of *C. glacialis* may be more tolerant than the adults to higher temperatures during the summer months since this is beneficial in an active growth phase to speed up development. Higher mortality from higher temperatures has previously been modeled to be somewhat offset by faster development times at higher temperatures in *C. finmarchicus* (Plourde et al 2008).

4.2 Effects of ocean acidification

There were not any noticeable effects of ocean acidification in our experiment on the development time and number of N3 which can be explained by the overall low number of nauplii reaching N3. The low number of nauplii reaching N3 in the future pH exposures can be explained by a confounding factor from the parafilm used and which suggests a bias in the experimental setup. The parafilm issue is however exclusive to the future pH exposures and does not affect the other combinations within the current pH exposures. The additional parafilm test confirms my suspicion that the parafilm interfered with the main experiment. The parafilm test lasted only 4 days, whereas the main experiment lasted around 20 days for most replicates, so the mean number of nauplii getting stuck to the parafilm being higher in the future pH exposure might be somewhat random. The mean number of stuck nauplii being the lowest at 1°C with pyrene could be due to the previously mentioned narcotization making the nauplii less active and thus less likely to get stuck. This is further supported by the Kaplan-Meier survival curves being almost identical for the combinations at 1°C with pyrene and 1°C + Pyrene + Future pH. The hazard ratios were also somewhat high, but not significantly different than 1 due to large variability. I can not conclude anything on the future pH scenario given the bias from the parafilm, which means our expectations (H3, H5, and H7) can not be answered. Seasonal pH levels vary from 8.1 in the surface waters to 7.9 in the deep water (Hildebrandt et al 2015, and references therein). *C. glacialis* nauplii seems to be tolerant to reduced pH predicted for the year 2300, with no effects on the growth of the

nauplii (Bailey et al 2016). Reproduction of *C. glacialis* is shown to be unaffected by ocean acidification with only delayed hatching success observed at extreme pH levels of 6.9 (Weydmann et al 2012). It has been found in other studies that *C. glacialis* is resilient to ocean acidification, with only effects observed at relatively extreme pH levels of pH ≤ 7 (Bailey et al 2016, Hildebrandt et al 2014, Hildebrandt et al 2015, Thor et al 2016, Weydmann et al 2012). It has been found that *C. glacialis* alters its gene expression in response to long-term ocean acidification (Bailey et al 2017). Down-regulation of several universal stress response genes was observed (Bailey et al 2017). The universal stress response involves gene expression of proteins in redox regulation, DNA damage sensing and repair, molecular chaperones, protein degradation, fatty acid/lipid metabolism, and energy metabolism (Bailey et al 2017, Kultz 2005). The downregulation of these stress response genes goes against the upregulation one would expect when exposed to a stressor. Bailey et al (2017) point out however that long-term downregulation of stress genes occurs in other species after a short-term upregulation of stress response genes and is a trait of tolerant species. (Bailey et al 2017, and references therein). A few other genes were upregulated with the most notable being the sodium:proton antiporter (Bailey et al 2017). The sodium:proton antiporter is an important protein in regulating intracellular pH by removing one proton from the cell in a passive process (Bailey et al 2017, and references therein). These findings on the gene expression of the nauplii as well as no strong negative effects of the lower pH indicate that *C. glacialis* is tolerant to reduced ocean pH.

4.3 Pyrene effect

When exposed to pyrene the reduction in the number of N3 could be explained by the overall poorer condition of the nauplii (A. Jortveit, personal observations) preventing further development. The development time to N3 was unaffected by pyrene exposure at 5°C. I did not see any effect of pyrene at the development time, but it should be noted that only two nauplii in one replicate developed to N3 when exposed to pyrene at 1°C. Grenvald et al (2013) found that the development to N3 was prolonged to roughly 20 days when exposed to 100 nM of pyrene at 0°C. It is therefore likely that the reason why I did not see any *C. glacialis* N3 when exposed to pyrene at 1°C is due to the development being delayed. The same study found no delay in development due to pyrene at 5°C which can explain why no effects were found in this study either. The nauplii exposed to pyrene having a lower hazard

ratio is most likely a result of the nauplii dying a few days later than the control. Quantifying at which temperature pyrene had the worst effect is a bit difficult given the different responses to pyrene at the different temperatures. The nauplii exposed to pyrene at 1°C had only one individual develop to N3. This could be an effect of the overall poorer condition of the nauplii at 1°C with pyrene (A. Jortveit, personal observations). Adults of the copepod *Oithona davisae* have been found to recover from narcotization when placed in fresh seawater after exposure (Barata et al 2005). If the *C. glacialis* nauplii however were to recover when removed from the pyrene then the effects of pyrene could be argued to be less severe at 1°C compared to 5°C given that recovering from death is unlikely. Unfortunately, no test was conducted on the nauplii being able to recover from the narcotization by being placed in fresh and clean seawater. The narcotization of the nauplii being more prominent at 1°C with pyrene could be due to pyrene degrading slower at 1°C compared to 5°C (Grenvald et al 2013). The slower degradation could mean that the 1°C exposures had a higher concentration of pyrene before the water was changed and resulted in the narcotic effect being more prominent. No test was conducted on how the pyrene concentration decreased daily and therefore it is not known exactly how much pyrene was left in the tubes before the water got changed. The lower hazard ratio, when exposed to pyrene, could be explained by the narcotic effect delaying death by a few days. Increased mortality due to pyrene could have larger effects on the copepod population as nauplii survival is important for overall recruitment (Plourde et al 2008). I can conclude that the effect of pyrene is overall negative despite the survival probability being higher at 1°C due to the reduced number of nauplii 3, which is most likely due to prolonged development combined with high mortality. These findings support our expectations (H4).

4.4 Interactive effects

No interactive effects between future pH and the other stressors were observed. This goes against our expectation that the other stressors combined with lower pH concentrations could have stronger effects due to the increased energy cost of dealing with all stressors. An explanation for this is that the previously mentioned sodium:proton antiporters regulate the intracellular pH passively and therefore do not consume energy, aside from the production of the protein (Bailey et al 2017). Pyrene is toxic through non-polar narcosis by affecting membrane fluidity (Barata et al 2005, and references therein). If the effect on the membrane

fluidity would affect the sodium:proton antiporter, a membrane protein, then it is possible that pyrene could "activate" negative effects of ocean acidification.

The development times to nauplii were not affected by pyrene at 5°C which is similar to the findings of Grenvald et al (2013). The number of nauplii that developed to N3 was reduced, however, which could be explained by the high hazard ratio being, indicating higher mortality. The narcotic effect observed at 1°C was not as prominent at the 5°C exposure. This could be explained by pyrene being degraded faster at higher temperatures (Grenvald et al 2013). The higher amount of nauplii dying when exposed to pyrene at 5°C fits with the general notion that temperature can increase the toxicity of pollutants (Noyes et al 2009). The higher mortality from pyrene at 5°C indicates stronger lethal effects at higher temperatures, whereas the more narcotic effects at 1°C indicate less lethality at lower temperatures. The same increase in mortality when exposed to pyrene at elevated have been observed with *C. finmarchicus* nauplii (Grenvald et al 2013). The same study however did not find an increase in mortality of pyrene at elevated temperatures in *C. glacialis* nauplii (Grenvald et al 2013). One explanation for this is that *C. glacialis* has a higher lipid content than *C. finmarchicus*. Pyrene and other PAHs are lipophilic. The general higher lipid content in *C. glacialis* could buffer the toxicity of pyrene somewhat by binding the pyrene and making it less bioavailable (Jager et al 2017). Another study has also found that *C. glacialis* upregulates Glutathione-S-transferase (GST) at a higher amount than *C. finmarchicus* (Hansen et al 2011, Hansen et al 2013). Grenvald et al (2013) used pyrene concentrations of 100 nM whereas I used double that amount with 200 nM. This can explain why I saw higher mortality at higher temperatures with pyrene when Grenvald et al (2013) did not. In general, I can see based on previous studies that OA does not affect *C. glacialis* and so will probably not affect this population at the pH levels predicted for the year 2100. Warmer temperatures increase the toxicity in pyrene and indicate that *C. glacialis* will be more vulnerable to PAHs in a warmer future, which support our expectation (H5).

5. Conclusion

This study has shown that elevated temperatures decrease the development time from egg to N3 for *C. glacialis*, resulting in more individuals reaching this developmental stage at the 5°C exposure. This could be beneficial for the copepods due to less predation from invertebrates and by letting them conduct compensatory feeding in response to other stressors. Pyrene had a potential higher narcotic effect at 1°C due to pyrene degrading slower at lower temperatures. Higher narcotic effect and higher mortality due to pyrene exposure reduced the number of nauplii developing to stage 3. Pyrene and warmer temperatures showed interactive effects increasing the mortality when both stressors were combined. The higher toxicity of pyrene also fits with the general notion that higher temperatures increase the toxicity of pollutants. Due to methodological challenges, the effect of future OA could not be concluded. Other studies on ocean acidification show that *C. glacialis* is overall tolerant to pH due to gene expression of sodium:proton antiporters. Our findings indicate that the toxicity of pyrene would be worse in a future warmer Arctic.

6. Future perspectives

One of the biggest challenges in this study was the logistics of handling many replicates which is also one of the major challenges with multiple stressors. The present study had to be limited to only two levels for each of the stressors. The addition of another level to each of the stressors could help to give an even better understanding of the interactive effects.

The temperatures of 5°C used in this study might not be as realistic for a future stressful scenario for the *C. glacialis* given that the mean summer temperature in the northern Barents Sea already reaches 5°C.

I only studied *C. glacialis* in the present study, but *C. hyperboreus* and *C. finmarchicus* both inhabit the Arctic and should also be studied in similar ways. *C. finmarchicus* is especially of note given that it is currently competing with *C. glacialis* as it expands its northern distribution.

Another thing is the high mortality in this study, which meant that few of the *C. glacialis* nauplii managed to develop to more advanced stages. Using a larger number of nauplii could address this issue as then it is more likely that at least some nauplii would develop further. It could also mean that potential long-term effects might go unnoticed.

Other studies would also gain from looking at the gene expression in response to stressors as well as on the survival and development of the species. The gene expression could help with the understanding of whether a species is tolerant to a stressor or have mechanisms to mitigate negative effects.

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