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Combined effects of elevated CO_2 and food limitation on early life stages of *Clanus finmarchicus*

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Marine Coastal Development
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Front page potho of *C .finmarchicus* by Dag Altin

ABSTRACT

The early life stages of *Calanus finmarchicus* were exposed to two levels of pCO_2, 380 ppm (control) and 2080 ppm (elevated CO_2) and two different food concentrations, ~ 600 µg C/L (high food concentration) and ~150 µg C/L (food limited). Carbon and nitrogen analysis were performed at the first feeding stage (nauplii stage 3), and the last nauplii stage before moulting to copepodit (nauplii stage 6). The elevated CO_2 and food limitation had a significant negative additive effect, when combined, on carbon and nitrogen content on nauplii stage 3. In nauplii stage 6, elevated CO_2 had no significant effect, but food limitation had a strong negative effect on both carbon and nitrogen content, indicating that food limitation may over masks the effects of elevated CO_2 in the later exogenous feeding stages. The C:N ratio was not significant effected by either elevated CO_2 nor food limitation. Nauplii that received high food concentration and elevated CO_2 combined were not significantly different in carbon and nitrogen content compared to nauplii in the control treatment (high food concentration, 380 ppm), indicating that *C. finmarchicus* may be able to compensate for higher energy demand due to elevated CO_2, by increasing the feeding rate. The hatching success and the naupliiar survival were not affected by either elevated CO_2 or food limitation. Results in the present study support previous findings that *C. finmarchicus* may be relative robust to direct effect of elevated CO_2 predicted in the medium-term future (2000 ppm), but changes in food availability or food quality, due to ocean acidification and increasing temperatures, can have large effect on carbon and nitrogen content in *C. finmarchicus* nauplii

FIGURE LIST

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1. INTRODUCTION

1.1 The copepod *Calanus finmarchicus*

Copepods are a group of small crustaceans common all over the world, both in freshwater and in the sea (Bliss, 1982). In the North Atlantic Ocean copepods of the genus *Calanus* might very well be the most important genus, due to its high abundance (Conover, 1988, Planque and Batten, 2000). Both in the North Sea, the Norwegian Sea and Norwegian fjords, *C. finmarchicus* is the most abundant of the *Calanus* species (Williams and Lindley, 1980, Planque and Batten, 2000) and may constitute up to 70 percent of the mesozooplankton biomass in these waters (Williams and Lindley, 1980). The annual production of this single species has been estimated to 74 mill, tons in the Nordic Sea (Aksnes and Blindheim, 1996).

The annual life cycle of *C. finmarchicus* includes six nauplii stages (N1-N6), five copepodit stages (C1-C5) and one adult stage (Maar et al., 2013). During late summer and autumn, adults and individuals of C5 migrate down to deep water, conducting diapause throughout the winter (Conover, 1988, Heath et al., 1999). In February adults migrate up to the surface waters, where females release their eggs (Heath, 1999). Egg production is closely related to food supply (Hirche et al., 1997), and peaks during the phytoplankton bloom (Diel and Tande, 1992, Plourde and Runge, 1993, Niehoff et al., 2000). The eggs hatch after ~ 24 hours and the nauplii stages are concentrated in upper layers of the water column (Conover, 1988), where the access to food is high. The first two nauplii stages are non-feeding, using their lipid storages to rapidly develop to nauplii stage 3, where they start feeding (Maar et al., 2013). Their main diet consist of diatoms (Kiørboe and Nielsen, 1994, Starr et al., 1999, Søreide et al., 2008), but they have been found to feed on dinoflagellates, ciliates (Leiknes et al., 2014) and microzooplankton as well (Ohman and Runge, 1994). During spring and summer, *C. finmarchicus* use energy for somatic growth, as well as storing energy in form of wax ester and triacylglycerols (Lee et al., 1970). The extensive lipid storage makes it possible for *C. finmarchicus* to survive the diapause (Lee et al., 1970, Hirche, 1996b), and is crucial during the energy-demanding processes of gonadogenesis and oogenesis (Hirche, 1996a). Because of their high abundance and energy rich lipid sack, *C. finmachicus* is an important species in the ecosystem (Ji et al., 2012). Many fish species, like Atlantic mackerel (*Scomber scombrus),* blue whiting (*Micromesistius poutassou),* Norwegian spring-spawning herring *(Clupea harengus)* (Prokopchuk and Sentyabov, 2006), Salmon (*Salmo salar*) (Olsen et al., 2004),

Cod (*Gadus morhua*), and Haddock (*Melanogrammus aeglefinus)* (Gaard and Reinert, 2002) are dependent on both egg, nauplii and copepodit stages of *Calanus* spp. Other marine animals are also dependent of this species, like bowhead whales (Laidre et al., 2007) and seabirds (Karnovsky et al., 2003). In addition to its importance in the ecosystem, *Calanus* spp. have gained increasing attention as a source for human utlilization. Due to their lipid storage, containing high amounts of marine fatty acids, *Calanus* spp. are now used to in production of omega-3 capsules for dietry supplements (Larsen et al., 2011). For the same reason, the species have been considered as a promising source of marine fatty acids for fish feed (Olsen et al., 2004, Bogevik et al., 2009, Colombo-Hixson et al., 2013) to the expanding fish farming industry (Olsen et al., 2004).

1.2 Ocean acidification

During the last two centuries there have been an increase in emission of atmospheric CO_2 due to combustion of fossil fuels, which have lead to global warming (Solomon et al., 2007). The mean global surface temperature has increased by 0.76°C during the past 150 years, and it is predicted to rise by additional 1.1°C-6.4°C (Solomon et al., 2007). The ocean act as a buffer for the climate change, and has taken up approximately 30% of the emitted CO_2 during the last 200 years and has thereby slowed down the global heating process (Sabine et al., 2004). However, the increased uptake of atmospheric CO_2 in the ocean alter the chemical properties in the ocean, making it more acidic (Caldeira and Wickett, 2003), a phenomenon called ocean acidification. The dissolved atmospheric CO_2 taken up by the ocean reacts with H_2O forming carbonic acid (H_2CO_3), which dissociates to bicarbonate- (HCO_3^-) and hydrogen (H^+) ions. The elevated concentration of H^+ causes the pH to drop, resulting in a more acidic marine environment. With higher concentrations of H^+ the carbonate ion (CO_3^{2-}) concentrations will drop because the H^+ binds to it and forms HCO_3^- (se equation 1) (Doney et al., 2009).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \qquad (1)$$

Estimates suggest that the surface water will experience an approximately 50% reduction of carbonate ions by 2100 (Brewer, 1997). Since pre-industrial time, atmospheric CO_2 has increased from 280 ppm to nearly 384 ppm in 2007 (Solomon et al., 2007). Models and observations indicate that this change has led to a drop in the pH by 0.1 units and it is expected to continue dropping 0.4 units by year 2100 and 0.7 units by year 2300 (Caldeira

and Wickett, 2003). Such a large change in ocean pH has not been experienced on Earth during the last 300 million years (Caldeira and Wickett, 2003).

1.3. Biological effect of ocean acidification

In general, marine organisms with calcium carbonate structures are assumed to be most sensitive to ocean acidification (Caldeira and Wickett, 2003, Kroeker et al., 2010). When the carbonate chemistry shifts in the oceans, there will be less carbonate ions (CO_3^{2-}) available for calcifying organisms (Gattuso and Buddemeier, 2000, Orr et al., 2005), making it more energy demanding to form calcium carbonate ($CaCO_3$) (Orr et al., 2005, Guinotte and Fabry, 2008). A study by Ries et al. (2009) showed that 10 out of the 18 studied species had a reduction in rates of net calcification due to ocean acidification. Since copepodes have a chitinous exoskeleton rather than an aragonite or calcite shell, *C. finmarchicus* are assumed to be less vulnerable to ocean acidification than calcifying organisms (Fitzer et al., 2012). However, it is evident that ocean acidification may effect a widespread of marine organisms, not only the calcifying organisms. In 2010, Kroeker with colleagues gathered information from previous studies done on marine organisms and ocean acidification, and found negative effects on survival, growth and reproduction among several different marine taxonomic groups. The sensitivity of the organisms varied among the different taxa, and furthermore between the different life stages within the species (Kroeker et al., 2010). A review by Ross et al. (2011) examined studies done on marine larvae of different taxonomic groups of invertebrates, and concluded that they were generally sensitive to the chemical changes associated with ocean acidification.

1.3.1 Acid-base regulation

Ocean acidification has the potential to interfere with the physiological processes of marine organisms, particularly through disturbance of the extracellular acid base balance (Widdicombe and Spicer, 2008). A change in intracellular pH can affect protein synthesis, ion regulation, cell volume control and metabolism (Whiteley, 2011). Marine organisms have different mechanisms to cope with changes in pH (Whiteley, 2011). During short-term exposure, animals may choose metabolic suppression where energy consuming activities like protein synthesis are shut down, and the animal hibernates until the end of the transient acidification event (Fabry et al., 2008). As a more long-term strategy, aquatic animals may use active transport of ions to maintain a stable pH in the intra- and extracellular

compartments. The ion transport occur in membranes separating the intra and extracellular compartments, and over epithelial membranes separating the animals from the water (Pörtner et al., 2004). The ion transport is driven by a bosolateral Na^+/K^+-ATPase, where intra- and extracellular HCO_3^- is exchanged for Cl^- and intra and extracellular H^+ for Na^+ (Henry and Wheatly, 1992, Wheatly and Henry, 1992, Pörtner et al., 2004). This process requires energy, and it has been estimated that this activity may constitute up to 40 % of total energy expenditure in sea urchin development (Leong and Manahan, 1997). Energy normally invested in growth, development and reproduction may be transferred to maintenance processes such as acid-base regulation and may potentially have a great impact on the species survival, distribution and abundance (Pörtner et al., 2004, Whiteley, 2011)

1.4 Copepods and Ocean acidification

Studies on the effect of ocean acidification on copepods have so far mostly been short-term exposures and mainly focused on egg production and hatching success in different species. Kurihara et al. (2004b) found that the egg production declined when females of the copepod *Acartia steueri* was exposed to +10 000 ppm for 8 days, but the survival rate of the adult females were not affected. A study on *Calanus glacialis* found that the CO_2-enriched seawater (~ 6000 ppm) reduced the hatching success and delayed the hatching, but did not affect the egg production (Weydmann et al., 2012). Similar trends were observed in a study done on *C. finmarchicus* where egg production and carbon- and nitrogen content were not affected in adult females, but only 4% of the eggs hatched when exposed to pCO_2 ~ 8000 ppm (Mayor et al., 2007). Another study by Kurihara et al. (2004a) also demonstrated that the nauplii of the copepod *Acartia erythraea* was more sensitive to elevated CO_2 (+ 5000, + 10 000 ppm) than the eggs. A study by Pedersen et al. (2014) found that the hatching success of *C. finmarchicus* where not affected, but the survival rate of hatched nauplii were negative effected when exposed to 8800 ppm. Further, the authors found no significant effect on mortality in nauplii stage ≥3 when exposed to 8800 ppm CO_2, but a delay in ontogenetic development.

Only a few multi-generation studies examining the effect of elevated CO_2 have been conducted so far. A multi-generation study by Kurihara and Ishimatsu (2008) on *Acartia tsuensis* found no effect of increased CO_2 (2380 ppm) on survival, development rate, body size, egg production or hatching rate. However, a study by Fitzer et al. (2012) suggest that elevated CO_2, may drive shifts in life history strategies, favouring smaller brood sizes,

females and possibly later maturing females. These suggestions were based on a observed effect of elevated CO_2 (~ 550 ppm) when the copepod *Tisbe battagliai* where exposed for three generations.

1.4.1 Respiration

Beside growth and reproduction, metabolism is expected to be affected when the acid-base balance is disturbed due to ocean acidification. Previous studies on marine organisms have observed a variety of effects. A study on the blue mussel *Mytilus edulis* found a parabolic increase in routine metabolic rates with increasing CO_2 concentrations, when exposed the animals were exposed to pCO_2 (up to 4000 ppm) for 2 moths (Thomsen and Melzner, 2010). A study on larva of the sea urchin *Strongylocentrotus purpuratus,* showed that respiration rate increased with 100% when exposed to elevated CO_2. In the control treatment, 78-80% of the energy available was used for growth, while 39-45% was used for growth in the CO_2 exposed treatment (Stumpp et al., 2011). Relatively few studies conduct respiration measurements when investigating the effects of ocean acidification on copepods. A study on the copepod *Centropages tenuiremis* showed that lower pH led to higher respiration rate, and that the animals compensated for the higher energy use by consuming more food (Li and Gao, 2012). This suggests that some copepods may increase their respiration and feeding rates to balance the energy cost associated with ocean acidification. However, in the later study *Centropages tenuiremis* where only exposed for elevated CO_2 for 4 days. A study done on *Calanus glacialis* and *Calanus hyperboreus* showed that elevated CO_2 concentrations (3000 µatm) had no effect on respiration rate when exposed for several months (Hildebrandt et al., 2014).

1.4.2 The importance of food availability

It has been stated that food limitation has a great effect on *Calanus* spp. (Mullin and Brooks, 1970, Breteler and Gonzalez, 1982, Campbell et al., 2001). Campbell et al. (2001) found that food limitation (25- and 50 µg C/L) had a negative effect on growth and development rate, and led to more variability in stage duration in *C. finmarchicus* nauplii. They found that the C:N ratio decreased in restricted feed regimes, and that food limitation had a negative effect on the carbon content, indicating that food availability has a strong effect on the lipid storage. A study on *C. glacialis* showed that starved nauplii developed slower and had higher mortality than nauplii feed with algae of high food quality when maintained under stable temperatures (Daase et al., 2011). A study done by Hygum et al. (2000) showed that nauplii

of *C. finmarchicus* may attain high growth and nearly maximum development rates at relatively low food concentrations (50 µg C/L), and suggested that nauplii are less dependent on food supply than the copepodid stages. The combined effects of elevated CO_2 and food limitation in copepods are yet to be determined. A study by Melzner et al. (2011) demonstrated that food limitation and elevated CO_2 had an additive negative effect on the shell length growth of the blue mussel (*Mytilus edulis*) when low food supply and elevated CO_2 were combined. In a review done by Ross et al. (2001), the need for studies on food supply combined with elevated CO_2 was addressed. They suggested that if an exogenous food supply is limited, it might interact with elevated CO_2 levels and determine the response of the organism.

1.5 Aim of the study

An increasing awareness regarding ecosystems response to ocean acidification and knowledge on how key species will respond to elevated CO_2 is needed (Doney et al., 2009, Dupont et al., 2010). As *C. finmarchicus* is a key species in its ecosystem it is thus essential to gain knowledge on how this species will respond to elevated CO_2 concentrations. The results from this study seek to contribute to a better understanding of the overall effects of ocean acidification on *C. finmarchicus.* As Ross et al. (2001) suggested, nauplii and larva of marine organisms might be more vulnerable to elevated CO_2 then adults and food supply might interact with elevated CO_2 to determine the organism's response. The aim of the present study was to investigate the combined effects of food limitation and elevated CO_2 in the nauplii stages of *C. finmarchicus*. It was hypothesised that nauplii treated with elevated CO_2 may have a higher energy demand to maintain a stable internal pH- environment and that nauplii receiving high food concentrations would be able to compensate for the higher energy demand by increase feeding rate, whereas nauplii treated with elevated CO_2 and low food concentrations will not. This would be reflected in lower carbon and nitrogen content of the animals treated with elevated CO_2 and limited feed. Further, I gain to measure the respiration rate of *C. finmarchicus* to detect possibly higher energy demand due to elevated CO_2.

2. MATERIAL AND METHOD

2.1 Experiment set up

The experiment was carried out at NTNU Center of Fisheries and Aquaculture (SeaLab) in December 2013. Eggs were harvested from females cultivated at SeaLab, and transferred to cell strainers (BD Falcon™, USA) which were distributed randomly between twelve 90 L tanks for exposure (see figure 1). The cell strainers were 1.4 cm high, 2.5 cm diameter and had a volume of 6.8 ml. Five cell strainers floated on the surface in each exposure tank, where three were used for respirometry measurements, one for carbon and nitrogen analyses and one for algae concentration measurements. The cell strainers intended for respirometry and algae measurements contained 20 eggs each. The cell strainers reserved for carbon and nitrogen analyses contained between 55 and 140 eggs. There were two feeding levels, 150 µgC/L and 600 µgC/L. The animals were feed unicellular algae *Rhodomonas baltica*, which were cultivated at SeaLab. Two levels of CO_2 were applied, 380 ppm (control) and 2080 ppm. Seawater from ~ 80 meters depth in Trondheimsfjorden was filtrated and kept in reservoirs tanks for maturation and temperature regulations before CO_2-equilibiration. CO_2-enriched air used for the high CO_2 concentration was made by a custom-built gas mixing system (HTK Hamburg GmbH) which used compressed air and 100% CO_2 to create CO_2-enriched air. The air was mixed with the treated seawater using an aquarium diffuser (Wooden air-stone, Aqua medic) in two different equilibrium columns to create the two different CO_2-concentrations. Water from the equilibrium columns entered the tanks at two inlets. Approximately 70 % of the incoming water was at the inlet near the surface and the remaining (approximately 30 %) at the inlet in the bottom of the tanks. This contributed to mixing of the water within the tanks. The outgoing water left the tanks by an overflow outlet at the water surface. The exchange rate of the water was 90 L /day. A submersible aquarium pump (Aquarium Systems NEWA Micro-Jet MC 450) in each tank created flow, ensuring vigorous mixing of the water and water exchange within the cell strainers that were placed on the surface.

There were four treatments; Control (high food concentrations and 380 ppm), Limited feed (low food concentrations and 380 ppm), CO_2 (high food concentrations and 2080 ppm) and CO_2, Limited feed (low food concentrations and 2080 ppm) There were three replicates of each treatment. The experiment was done in a temperature controlled room set to 7.1°C, and with water temperature at 10°C, and a 12:12 (light:dark) light regime.

2.2 Water parameters

The water parameters pH, total alkalinity, water temperature and salinity was measured daily during the experiment.

2.2.1 Measuring pH_{tot}

The pH_{tot} (total power of hydrogen) was measured in all tanks daily by using a method described by Dickson et al. (2007). The method involves adding a dye, m-cresol purple solution, followed by measuring the ratio between the absorbance maxima of the base (I^{2-}) and acid (HI^{-}) that forms of the added dye. (See equation 2). The wavelength used to record the colour dye was 578 nm and 434 nm, while 730 nm was used to correct for the background.

$$HI^{-}(aq) = H^{+}(aq) + I^{2-}(aq) \qquad \text{(equation 2)}$$

Water from the surface was collected from each tank in 50 ml glass bottles. The bottles were rinsed three times in the tank water prior to water sampling. The glass bottles were placed in a water bath with a temperature set to 25°C. When the temperature of the water samples reached 25°C, the bottles were taken out one by one for measurement. A pipette was used to transfer water from the bottles to a glass cuvette, which was used for the spectrophotometry. The cuvette was carefully wiped clean before placed in the spectrophotometer, and the absorbance at the different wave length was recorded. A solution of m-cresol purple (5µl) was added to the water sample, and the absorbance was measured again. The pH_{tot} was determined using equation 3 and 4. (Dickson et al., 2007)

$$pH = 8.0056 + \log\left(\frac{\left(\frac{A_1}{A_2} - 0.00691\right)}{\left(2.222 - \left(\frac{A_1}{A_2} x\ 0.1331\right)\right)}\right) \qquad \text{(equation 3)}$$

where

$$\frac{A1}{A2} = \frac{(A578cresol - A578blank - (A730cresol - A730blank)}{(A434cresol - A434blank - (A730cresol - A730blank)} \qquad \text{(equation 4)}$$

To calculate the actual pH in the tanks at 10°C the free software package “CO2-SYS” version 2.1 (Pierrot et al., 2006) was applied.

2.2.2 Total alkalinity

The total alkalinity (A_t) was measured each day in one of the tanks (A1, control), assuming identical alkalinity in all tanks. The water sample was collected the by same procedure as for the pH_{tot} and incubated in water bath at 25°C. Titrab TIM860 Titration Manager (Radiometer Analytival SAS; Villeurbanne, France) and PHC2001-8 pH electrode was used for the measurements and certified seawater with known A_t was used to calibrate the system. Approximately 25 ml of sampled water was weighted (Precisa 180A), and the exact weight was typed in to the program (SW-ALK-TAO-2009). The titration solution (0.02 M HCl) was kept in the water bath at a stable temperature of 25°C. The Titration Manager calculated automatically the A_t by using equation 5.

$$M_{At} = c_{HCl} \times \frac{v_{HCl}}{m_{sw}} \qquad \text{(equation 5)}$$

Where

C_{HCl} = morality of the HCl solution

V_{HCl}= ml of HCl solution sonsumed

m_{sw} = mass (g) of seawater sample

2.2.3 Salinity

A H_2Ocean ATC salinity refractometer was used to determine the salinity in one of the tanks (A1, control), assuming identical salinity in all tanks. Certified seawater was used to calibrate the instrument. The measurements were done in the same room and in the same light conditions each time to ensure consistent measurements. The water sample was collected the same way as for the pH_{tot}.

2.2.4 Temperature

The temperature was measured by using a certified glass thermometer (VWR Precicion Thermometer, accuracy ± 0.3°C). Immersion heaters were placed in each tank regulating the water to 10°C.

2.3 Feed

The *C. finmarchicus* were feed unicellular *R. baltica*. Two stock solutions of algae were prepared daily and adjusted to reach a target consentration of 600- or 150 µg C /L in the experimental tanks. The algae feed was pumped from the two stock solutions by a multi-channelled peristaltic dosing pump (Watson-Marlow 520S) to the tanks through tubes attached just above the water surface, near the incoming water from the equilibrium columns, so that the feed was evenly distributed in the tank. The feeding was started three days post egg harvesting by adding 500 ml seawater with corresponding algae concentrations, to achieve the desired target concentrations of feed in the tanks. At this point, the multi-channelled peristaltic dosing pump was started to maintain the target feed concentrations in the tanks.

2.3.1 Algae concentration measurements

One of the cell strainers was reserved for algae measurements only and will hereby be referred to as the algaecup. Water was sampled out from the algaecup with a modified pipette with sive mesh (45 µm) glued on the end (2 ml), and diluted with filtrated seawater (7 ml) before analysis. The mesh prevented animals getting in to the water samples. After the mixing, the water was transferred to 25ml beaker and the algae concentration was measured by using Coulter Counter (Beckman Multisizer™3 Coulter Counter ®). To prevent water from the outside of the algaecup to be drawn in to the inside of the algaecup when the sample was taken, and thereby alter the algal density, only a small sample volume was used. The dilution of the sample was necessary to obtain large enough volume for the Coulter Counter.

The Coulter Counter measured the algae concentration by recording changes in the electrical resistance of the aperture and small changes in the ionic current through the aperture. This produces a pulse that the Coulter Counter can use to count and measure the size of the particles in the water (Graham, 2003). The Coulter Counter was programmed to take out 1 ml of the water sample per sample.

2.4 Handling *Calanus finmarchicus*

Females (1000 individuals) were collected from the *C. finmarchicus* culture cultivated at SeaLab and randomly divided between two polyethylene buckets (50 L) containing 500 individuals each. One group was incubated in 50 L buckets with control water and the other group was incubated in 50 L buckets of CO_2-enriched water. The water was collected from the tanks that later were used in the experiment, and was added *R. baltica* algae to reach the target concentration of 600 µg C/L. The females were incubated for 12 hours, before they were carefully transferred to temporary buckets. The remaining water was sieved trough a 64 µm sieve and the egg were collected on the sieve. The eggs were then transferred to a petri dish and a mouth pipet was used to place the eggs in the cell strainers. The cell strainers were kept in a glass bowl with seawater, and were randomly distributed between the tanks containing the same CO_2-level. The eggs were collected over three days, and the same procedure was repeated each time. After collecting the eggs, the females were carefully put back in the 50 L buckets and a new supply of algae was added. The first four tanks (A1, A2, A3 and A4) was one day older than B1, B2, B3 and B4, and two days older than C1, C2, C3 and C4.

The eggs were placed in 40 µm cell strainers. The cell strainers were 1.4 cm high, 2.5 cm diameter and had a volume of 6.8 ml. Each tank housed five cell strainers, which four contained 20 eggs and one which contained between 55 and 140 eggs. The latter was used for carbon and nitrogen analyses. Three of the cell strainers containing 20 eggs were used for respirometry during the experiments while the last one was reserved for algae measurements. After the animals reached nauplii stage 3, the 40 µm cell strainers were replaced with 100 µm, to achieve better water exchange. A parafim rim was also mounted on top of the cell strainers to ensure that no animals could escape. The cell strainers floated on the water surface and were restricted by a floating ring (20 cm diameter) to ensure movement and promote circulation between the water in the strainers and the tank, see figure 1.

During the experiment the cell strainers was exchanged two times. When exchanging the cell strainers, the latter were moved from the tank in a petri dish, to ensure that the animals were fully submerged at all times. The animals were flushed over to a new petri dish using tank water, and a mouth pipette was used to place them in to the new cell strainers. During this procedure, the number of live and dead animals was noted.

FIGURE 1: Picture form experiment tanks. **Left**; the five cell strainers with a parafim on top floating on the water surface restricted by the floating ring (20 cm diameter). **Right**; four of the experiment tanks with cell strainers and floating rings placed on the water surface.

2.5 Analyses

2.5.1 Carbon and nitrogen analyses

Animals for carbon and nitrogen analyses were taken at nauplii stage 3 (between 44 and 60 animals) and at nauplii stage 6 (between 6 and 30 animals). Destilled water mixed with 29.22 g NaCl (58.44 g/mol) was used to prevent algae and bacteria to contaminate the carbon results. The animals were washed three times in the saltwater solution (1000 mOsm NaCl) and transferred to 5x9 mm tin capsules (Mikro Kemi AB, Sweden) by using a mouth pipet. The tin cup was stored at -20°C during the experiment and dried at 60°C in 24 hours before being sent to SINTEF Center of Fisheries and Aquaculture (SeaLab) for analysis in a CHN Elemental Analyser 1106 (Carlo Erba Instruments, Italy)

In order to calculate µg C / L of algae in the experimental tanks, samples of *R. baltica* ware analysed for carbon content as well. A stock solution was set up with air supply to avoid sedimentation, in the the dark at 10°C for about 12 hours. This was done to in order to reproduce the conditions during the experiment period. The stock solution was diluted by filtered seawater (10 ml stock solution, 90 ml filtered seawater) and cell density was determined by using the Coulter Counter. The solution was then vacuum filtrated (50ml) using a vacuum pump (Vacuumbrand GMBH, Wertheim Germany) and collected on a standard filter (Whatman® glass microfiber filters, binder free, Grade GF/C, 24 mm, 1.2 µm) and folded in to individuals tin capsules, same as used for the nauplii. There were three replicates.

2.5.2 Respirometry

The O_2 concentrations in the resirometry were measured using a fiberoptic oxygen meter (FireSting O_2, PyroScience GmbH). Emitted light signals (620 nm) from the fiberoptic cables created oxygen-dependent luminescence that was detected by the sensor in the near infrared band (NIR, 760-790 nm). When oxygen level is low, the NIR luminescence is high, and when oxygen level is high, the NIR luminescence is low. Oxygen sensitive patches were used in order to measure the O_2 level in a closed system. These patches were coated with REDFLASH dye, enabling the fiberoptic cables to detect the signals through glass.
Four 2 ml glass bottles were used for the respirometric measurements. Oxygen sensitive patches were placed at the bottom of the bottles, and a screw cap was used to close the system. The bottles were placed in holes drilled in Plexiglas. On the backside of the Plexiglas smaller holes was drilled for the fiberoptic cables, so that they were located in the centre of the oxygen sensitive patches (se figure 2). The system was placed in a water bath at 10°C. The measurements were done over 24 hours with lights on. Before each measurement the system was calibrated by using calibration mode 1-Point in air saturated water. This mode used air saturated water to determine the air calibration value, and the 0% calibration value was taken from the Sensor Code (FireSting O_2, user manual). In this experiment filtrated water from the thanks was used for calibration of air saturated water, by vigorously shaking a bottle of air and filtrated water a few minutes so that the water became air saturated. The water was then added in all respirometry glass bottles, and each channel calibrated at 10°C.

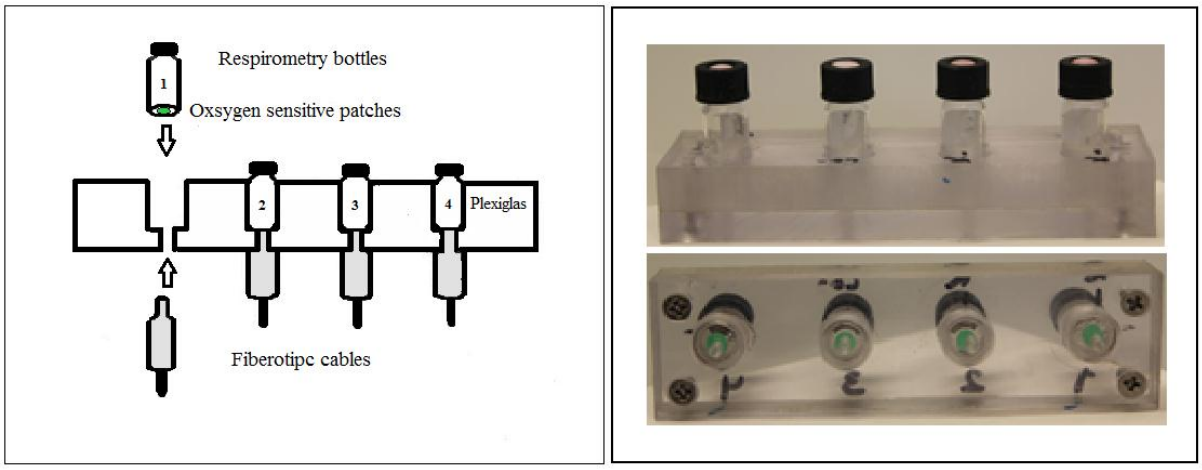

FIGURE 2: Respirometry setup. **Left**; illustration from side. Fiberoptic cables (grey) placed in drilled holes in the Plexiglas. Respirometry bottles (numbers 1 to 4) placed in drilled holes on top of the Plexiglas. Oxygen sensitive patches (green) placed in the bottom of the respirometry bottle. **Right top**; picture from side. Respirometry bottles placed in drilled holes in the Plexiglas. **Right down**; picture from underneath. Respirometry bottles placed in the drilled holes and the green sensitive patches are displayed. Small drilled holes in the Plexiglas in the center of the oxygen sensitive patches for the fiberoptic cables.

The respirometry measurements were preformed on nauplii stage 1-2, 3 and 6. Animals were collected from one of the cell strainers. Tank water was used to incubate the animals during the measurement, and was filtrated through 0.22 and 0.20 µm filters prior use. The animals were rinsed using the filtered water prior to the respirometry, to reduce metabolic contribution from algae and bacteria during the measurements. The rinsing procedure was preformed three times, by transferring the animals to a Petri dish containing filtrated water by mouth pipet. After rinsing, the animals were transferred to the respirometry glass bottles and the number of animals in each bottle was noted.

2.6 Data analysis

Microsoft Office Excel (2003) was applied to process the data before statistical analyzes and to calculate average and standard deviation.

2.6.1 Calculation of hatching success and naupliiar survival

The hatching success was calculated based on observed non-hatched eggs (out of 20), two days after egg laying, when one of the cell strainers were taken out for the first respiration measurement in nauplii stage 1-2. Animals missing were assumed hatched but dead. The survival rate was calculated based on observed living and dead animals at the time the cell strainers was taken out for respirometry measurement at nauplii stage 1-2, 3 and 6 and when the cell strainers was exchanged in nauplii stage 3. Animals missing were assumed dead. The survival rate at nauplii stage 1-2 and 6 was calculated by percent survival out of 20 animals. For the nauplii stage 3, percent survival was calculated from two cell strainers in each tank (20 animals in each cell strainer) and the average percentage was calculated from these two. The control- and the CO_2-treatment were terminated at day 15, when the first C1 appeared. The limited feed- and the CO2 and Limited feed combined-treatment was terminated eight days later (when the first C1 appeared) due to slower development rate in the groups received restricted feeding.

2.6.2 Nitrogen content

The sample size for nitrogen analysis was quite small, and to ensure that the nitrogen values were reliable the detection limit was calculated. This was done by calculating the standard deviation of the six blank samples, and multiply by two (Gabriels, 1970). Values below the detection limit was exclude and replaced by the mean of the two other replicates. Two values were below the detection limit, on in the CO_2 and Limited feed combined-treatment in nauplii stage 3, and the other in the Limited feed-treatment in nauplii stage 6.

2.6.3 Calculation of respiration rate

Values from respirometry measurements were used to generate a graph of the O_2 consumption over time. For the measurements including *C. finmarchucs*, the first 30 min of the graph was excluded, to ensure that increase respiration due to stress of the animals was not included, and the last part was excluded if O_2 consecration had decreased more than 10%. For the measurement done on blank samples, irregular values was excluded. The slope of the graph was calculated by linear regression in Microsoft office Excel (2003). The slope was then divided by number of individuals and corrected for blank/background respiration.

2.6.3 Statistics

PERMANOVA (Permutational multivariate analysis of variance) was used to test for significant differences in carbon and nitrogen content, C:N ratio and hatching success, and was conducted in R version 3.0.2 (R Core Team, 2013) using the packages “vegan” and “permute”. PERMANOVA is a multivariate analyse that tests the simultaneous response of one or more variables to one or more factors. The only assumption is that the observation units are exchangeable under a true null hypothesis. (Anderson, 2001, McArdle and Anderson, 2001). When results of the PERMANOVA tests were found to have significant differences, *post hoc* analysis using Tukey was performed in SPSS Statistics software for Windows, Version 21.0. (Armonk, NY: IBM Corp.). Repeated measures ANOVA was used to test significant differences in survival of the nauplii in SPSS. Al the assumptions associated with the different statistical tests were satisfied. Figures presented in present study were produced in SigmaPlot version 10.0 (Systat Software, San Jose, CA).

3. RESULTS

3.1 Water parameters and feeding concentrations

The pH_{tot}, salinity, temperature and total alkalinity measured during the experiment was used to calculated the *in situ* pH_{tot}, pCO_2, omega Calsitt and omega Argonitt (table 1 and table 2). The average temperature was 10.0°C in the Control- and CO_2-treatment and 9.9°C in the Limited feed- and CO_2 and Limited feed combined -treatment. In all treatments, the average alkalinity and salinity was 2431 and 33.56 respectively. The average pCO_2 in Control- and Limited feed-treatment was 484 ppm and 485 ppm respectively. For the CO_2- and CO_2 and Limited feed combined-treatment the average pCO_2 were 2292 ppm and 2516 ppm respectively. The Control- and Limited feed-treatment had an average *in situ* pH_{tot} of 7.99. The average *in situ* pH_{tot} of the CO_2-treatment were 7.32, and the average *in situ* pH_{tot} of CO_2 and Limited feed combined-treatment were 7.36.

TABLE 1: Water parameters (mean±SD) in experimental tanks during the experiment. The measured parameters included *in situ* temperature (Temp), pH_{tot} measured at 25°C ($pH_{25°C}$), Total alkalinity (A_T) and salinity.

Treatment	Temp. (°C)	$pH_{25°C}$	A_T	Salinity
Control	10±0.06	7.77±0.03	2431±161	33.56±0.17
Limited feed	9.94±0.05	7.77±0.02	-	-
CO_2	10±0.08	7.15±0.04	-	-
CO_2, Limited feed	9.94±0.06	7.19±0.03	-	-

TABLE 2: Water parameters (mean±SD) in the experimental tanks during the experiment calculated based on measured temperature, pH_{tot}, total alkalinity and salinity. The calculated water parameters included *in situ* pH_{tot} ($pH_{in\ situ}$), partial pressure of CO_2 (pCO_2), omega Calsitt (Ω Ca) and omega Argonitt (Ω Ar)

Treatment	$pH_{in\ situ}$	pCO_2 (ppm)	Ω Ca	Ω Ar
Control	7.99±0.03	484.69±48.09	2.93±0.34	1.86±0.21
Limited feed	7.99±0.01	485.88±38.76	2.96±0.23	1.88±0.15
CO_2	7.32±0.04	2516.39±322.43	0.69±0.07	0.44±0.04
CO_2, Limited feed	7.36±0.03	2292.94±230.01	0.77±0.07	0.48±0.04

The average algal density in experimental tanks is presented in figure 3. The algal density (mean±SD) in the two treatments with high food concentration, the Control- and CO_2-treatment, was 621± 151.80 µg C/L and 628±99.47 µg C/L, respectively. The algal density (mean±SD) in the two treatments with low food concentration, the Limited feed- and CO_2 and Limited feed combined-treatment, was 160±25.33 µg C/L and 146±30.10 µg C/L, respectively. There was significant difference in algal density in the two treatments with limited feed (p=0.009). The average algal density was 8.7 % less in the CO_2 and Limited feed combined-treatment compared to the algal density in the Limited feed-treatment. The algal density in the treatments with high food concentrations increased over time, whereas the two limited feed treatments where relatively stable. Daily variation in the algal density was observed in all treatments.

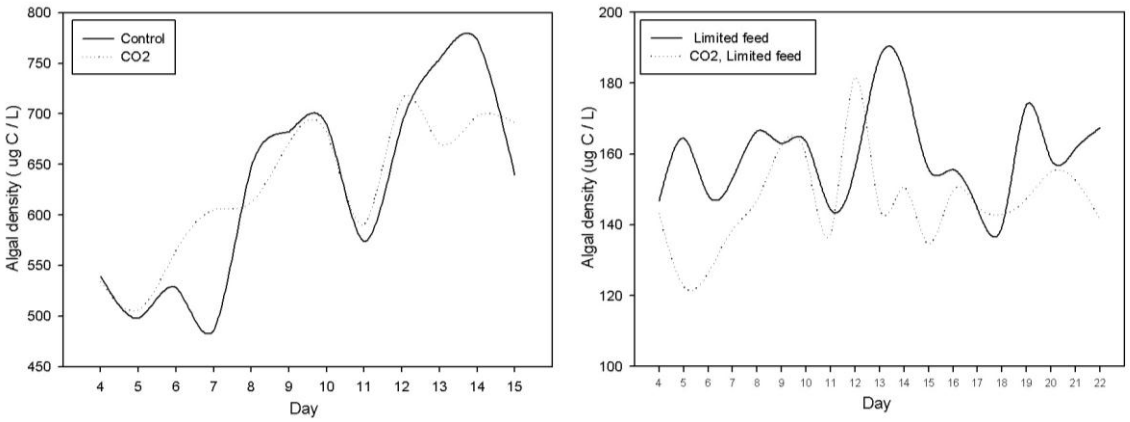

FIGURE 3: Mean algal density (µg C /L) over time (days) in the experimental treatments based on daily algae measurements. Three replicates in each treatment. **Left:** algal density in the two treatments with high food concentration, Control (solid line) and CO_2 (dashed line). **Right:** algal density in the two treatments with limited feed, Limited feed (solid line) and CO_2, Limited feed (dashed line).

3.2 Hatching success and naupliiar survival

The hatcing success (mean±SD) in the Control-, Limited feed-, CO_2- and CO_2 and Limited feed combined-treatment was 88.3±5.8 %, 85±0.0 % 81,7±2.9 % and 86.7± 2.9 % respectively. There were no significant differences in the htacing sucsees between the different treatments.

Figure 4 presentes the naupliiar survival (percent) during the experiment. There were no significant differencens in survival between the different treatments. The percent survival (mean±SD) of animals in nauplii stage 1-2, 3 and 6 in the Control-treatment was 80.00±5.00, 72.50±2.50 and 73.33±7.64 respectively. The percent survival (mean±SD) of animals in nauplii stage 1-2, 3 and 6 in the Limited feed-treatment was 76.67±10.41, 78.33±8.78 and 58.33±12.58 respectively. The percent survival (mean±SD) of animals in nauplii stage 1-2, 3 and 6 in the CO_2-treatment was 63.33±17.56, 70.00±11.46 and 66.67±11.55 respectively. And the percent survival (mean±SD) of animals in nauplii stage 1-2, 3 and 6 in the CO_2 and Limited feed combined-treatment was 71.67±5.77, 70.00±7.50 and 56.67±16.07 respectively.

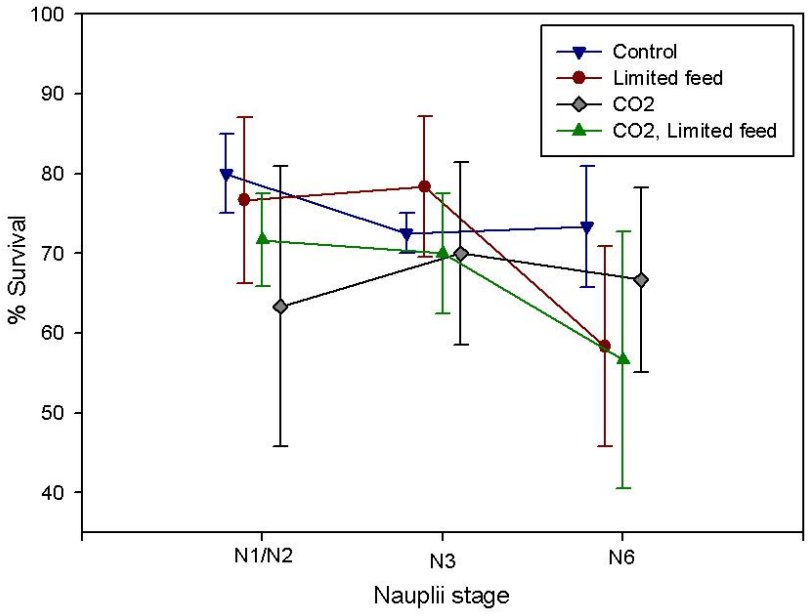

FIGURE 4: Percent survival of animals in nauplii stage 1-2 (N1/N2), 3 (N3) and 6 (N6) in the different treatments; control (blue, high feed, low pCO_2), Limited feed (red, low feed, low pCO_2), CO_2 (grey and black, high feed, high pCO_2), and CO_2, Limited feed (green, low feed, low pCO_2). The graph show mean (points), standard deviation bars (whiskers) and intergroup trend line (line between points).

3.4 Carbon and nitrogen content in the nauplii

Carbon content of nauplii stage 3 and 6 are presented in figure 5, in terms of µg C per individual. The carbon content (mean±SD) of nauplii stage 3 in the Control- , Limited feed-, CO_2- and CO_2 and Limited feed combined-treatment was 0.19±0.03, 0.16±0.01, 0.17±0.02 and 0.12±0.02 respectively. The carbon content (mean±SD) of nauplii stage 6 in the Control- , Limited feed-, CO_2- and CO_2 and Limited feed combined-treatment was 01.04±0.08, 0.43±0.09, 0.95±0.06 and 0.46±0.08 respectively. Feeding level had a significant effect on carbon content in both nauplii stages (see table 5). Elevated CO_2 had a near significant effect on carbon content in nauplii stage 3, and no significant effect on carbon content in nauplii stage 6 (see table 5). In nauplii stage 3, there were significant lower carbon content in animals that recived elevated CO_2 and limited feed combined, compared to animals in the control-treatment (p=0.022). The average carbon content of animals that recived elevated CO_2 and limited feed combined was 36.84 % lower compared to the average carbon content of animlas in the Control-treatment.

In nauplii stage 6, there was a significant lower carbon content in animals treated with limited feed alone and in animals treated with elevated CO_2 and limited feed combined, compared to animals in the Control-and CO_2-treatment (p = >0.001). The average carbon content of animals in the Limited feed-treatment were 58.38% and 54.47 % lower compared to the average carbon content of animals in the Control-and CO_2-treatment. The average carbon content of animals in the CO_2 and Limited feed combined-treatment were 55.43 % and 51.25 % lower compared to the average carbon content of animals in the Control- and CO_2 treatment.

TABLE 3: PERMANOVA analysis performed on carbon content, nitrogen and C:N ratio in nauplii stage 3 and 6. Significant differences between the treatments are indicated using asterisk.

PERMANOVA		Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Carbon Content N3	CO2	1	0.023481	0.023481	4.0116	0.17454	0.054
	Feed	1	0.057116	0.057116	9.7579	0.42455	**0.008****
	CO2:Feed	1	0.007111	0.007111	1.2149	0.05286	0.310
	Residuals	8	0.046826	0.005853		0.34806	
Carbon Content N6	CO2	1	0.00144	0.00144	0.315	0.00307	0.583
	Feed	1	0.42944	0.42944	93.507	0.91167	**0.002****
	CO2:Feed	1	0.00342	0.00342	0.746	0.00727	0.468
	Residuals	8	0.03674	0.00459		0.07800	
Nitrogen Content N3	CO2	1	0.42728	0.42728	6.2278	0.24665	**0.038***
	Feed	1	0.068287	0.068287	9.9531	0.39419	**0.008****
	CO2:Feed	1	0.007330	0.007330	1.0684	0.04232	0.323
	Residuals	8	0.054887	0.006861		0.31684	
Nitrogen Content N6	CO2	1	0.00738	0.00738	0.5222	0.01309	0.497
	Feed	1	0.42540	0.42540	30.1166	0.75493	**0.001***
	CO2:Feed	1	0.01772	0.01772	1.2544	0.03144	0.282
	Residuals	8	0.11300	0.1413		0.20054	
C:N Ratio N3	CO2	1	0.001442	0.001442	0.34506	0.04049	0.599
	Feed	1	0.000126	0.000126	0.03024	0.00355	0.939
	CO2:Feed	1	0.000617	0.000617	0.14767	0.01733	0.714
	Residuals	8	0.033422	0.0041777		0.93864	
C:N Ratio N6	CO2	1	0.001077	0.001077	0.28217	0.02460	0.617
	Feed	1	0.000947	0.000947	0.24816	0.02163	0.614
	CO2:Feed	1	0.0011233	0.0011233	2.94238	0.25647	0.122
	Residuals	8	0.030541	0.0038176		0.69731	

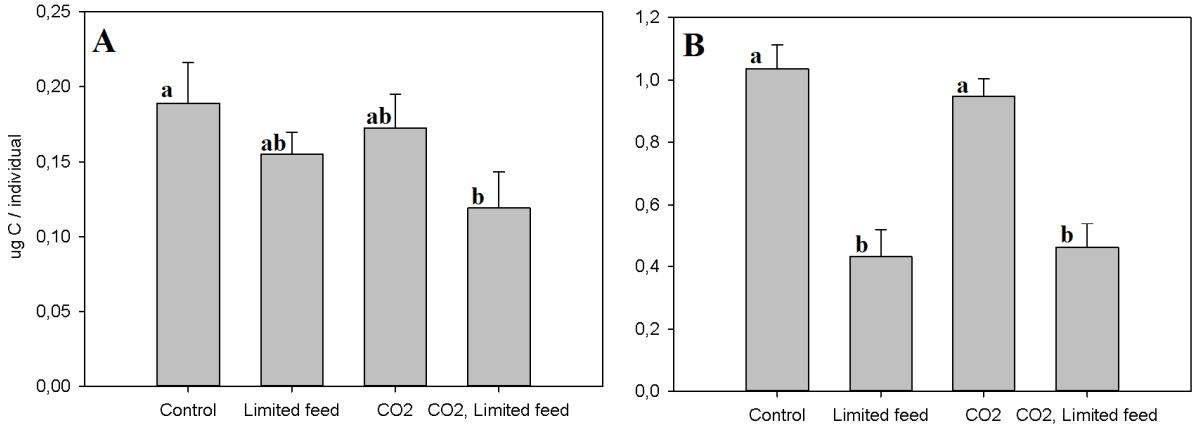

FIGURE 5: Carbon content (µg C /individual) in the different treatments; Control (high feed, low pCO_2), Limited feed (low feed, low pCO_2), CO_2 (high feed, high pCO_2), and CO_2, Limited feed (low feed, high pCO_2). Bars indicate mean±SD (n=3). Different letters denotes significant differences. **A**) Carbon content of nauplii stage 3. **B**) Carbon content of nauplii stage 6.

Nitrogen content of nauplii stage 3 and 6 are presented in figure 6, in terms of µg N per individual. The nitrogen content (mean±SD) of nauplii stage 3 in the Control-, Limited feed-, CO_2- , and CO_2 and Limited feed combind-treatment was 0.037±0.003, 0.029±0.005, 0.031±0.003 and 0.021±0.005 respectively. The nitrogen content (mean±SD) of nauplii stage 6 in the Control-, Limited feed-, CO_2- , and CO_2 and Limited feed combined-treatment was 0.15±0.04, 0.06±0.02, 0.13±0.02 and 0.07±0.02 respectively. Feeding level had a singificant effect on nitrogen content in both nauplii stages. Elevated CO_2 had a significant effect on nitrogen content in nauplii stage 3, but not in nauplii stage 6. (see table 5). In nauplii stage 3, there were significantly lower nitrogen content in animals that recived elevated CO_2 and limited feed combined, compared to animals in the control-treatment (p=0.009). The average nitrogen content of animals in the CO_2 and Limited feed combind-treatement were 42.07 % lower compared to the average nitrogen content of animals in the Control-treatment.

In nauplii stage 6, there were a singifcantly lower nitrogen content in animals that recived limited feed alone and that recived elevated CO_2 and limited feed combined, compared to animals in the Control-treamtnet (p=0.006 and p=0.014 respectively). There were significant lower nitrogen content in animals treated with limited feed alone, compared to animals treated with elevated CO_2 alone (p=0.034). The average nitrogen content of animals in the Limited feed-treatment were 63,87 % lower compared to the average nitrogen content of animals in the Control-treatment, and 56.46 % lower compared to the average nitrogen content of animals in the CO_2-treatment. The average nitrogen content of animals in the CO_2 and

Limited feed combined-treatment was 55.68 % lower compared to the average nitrogen content of animals in the Control-treatment, and 46.60% compared to the average nitrogen content of animals in the CO_2-treatment.

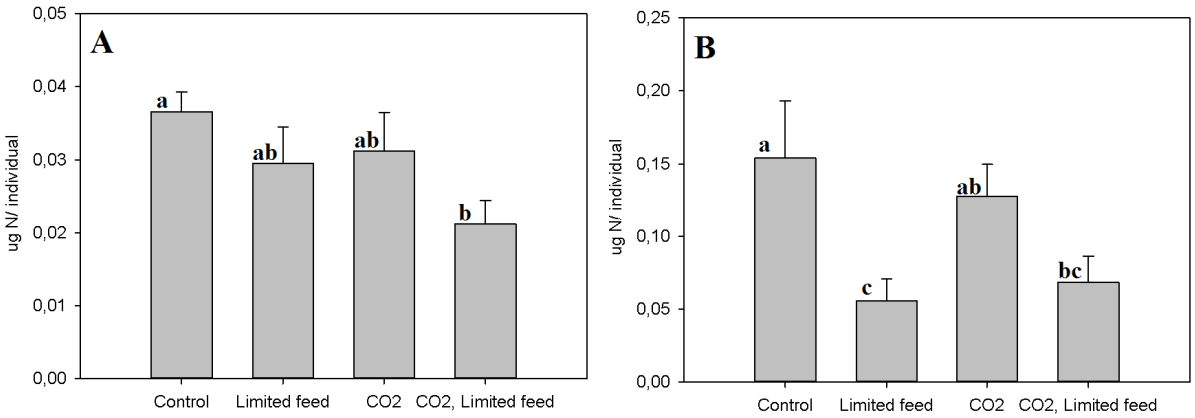

FIGURE 6: Nitrogen content (µg N /individual) in the different treatments; Control (high feed, low pCO_2), Limited feed (low feed, low pCO_2), CO_2 (high feed, high pCO_2), and CO_2, Limited feed (low feed, high pCO_2). Bars indicate mean±SD (n=3). Different letters denotes significant differences. **A**) Nitrogen content of nauplii stage 3. **B**) Nitrogen content of nauplii stage 6.

Figure 7 present the C:N ratio in nauplii stage 3 and 6 in terms of µg C / µg N per individual. There were no significant effect of CO_2 nor feed concentration on C:N ratio in nauplii stage 3 and 6. The C:N ratio (mean±SD) of nauplii stage 3 in the Control- , Limited feed-, CO_2-, and CO_2 and Limited feed combined-treatment was 5.15±0.46, 5.32±0.44, 5.60±1.23 and 5.40±0.33 respectively. The C:N ratio (mean±SD) of nauplii stage 6 in the Control- , Limited feed-, CO_2-, and CO_2 and Limited feed combined-treatment was 6.93±1.21, 8.06±0.64, 7.52±0.98 and 6.87±0.61 respectively.

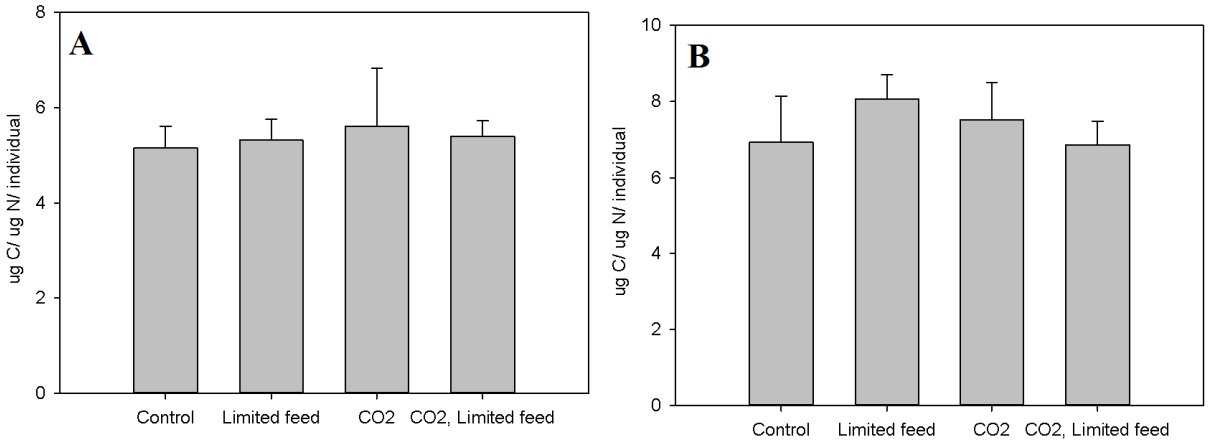

FIGURE 7: C:N ratio (µg C / µg N/ individual) in the different treatments; Control (high feed, low pCO_2), Limited feed (low feed, low pCO_2), CO_2 (high feed, high pCO_2), and CO_2, Limited feed (low feed, high pCO_2). Bars indicate mean±SD (n=3). Different letters denotes significant differences. **A**) C:N ratio of nauplii stage 3. **B**) C:N ratio of nauplii stage 6.

3.5 Respiration

The measured oxygen consumption increased with increasing nauplii stage (figure 8). During the experiment, the O_2 consumption of the blank samples increased in accordance with the increase in O_2 consumption of the animals (figure 8 B). The O_2 consumption in the blank samples were in some cases close to the O_2 consumption values observed in measurements done with animals and in two cases, the O_2 consumption in the blank sample was even greater (Limited feed- and CO_2 and Limited feed combined- treatment in nauplii stage 6). When the measurements for O_2 consumption for the different stages were corrected for the O_2 consumption in the blank samples, these two values become negative (see figure 8 C). When corrected for the average O_2 consumptions of the blank samples corresponding to the measurements done in same respirometry bottle and with the same fiberoptic cable was done (data not showing) even more of the values turned negative (al nauplii stage 1-2 measurements). As a consequence of these results no further analysis were done.

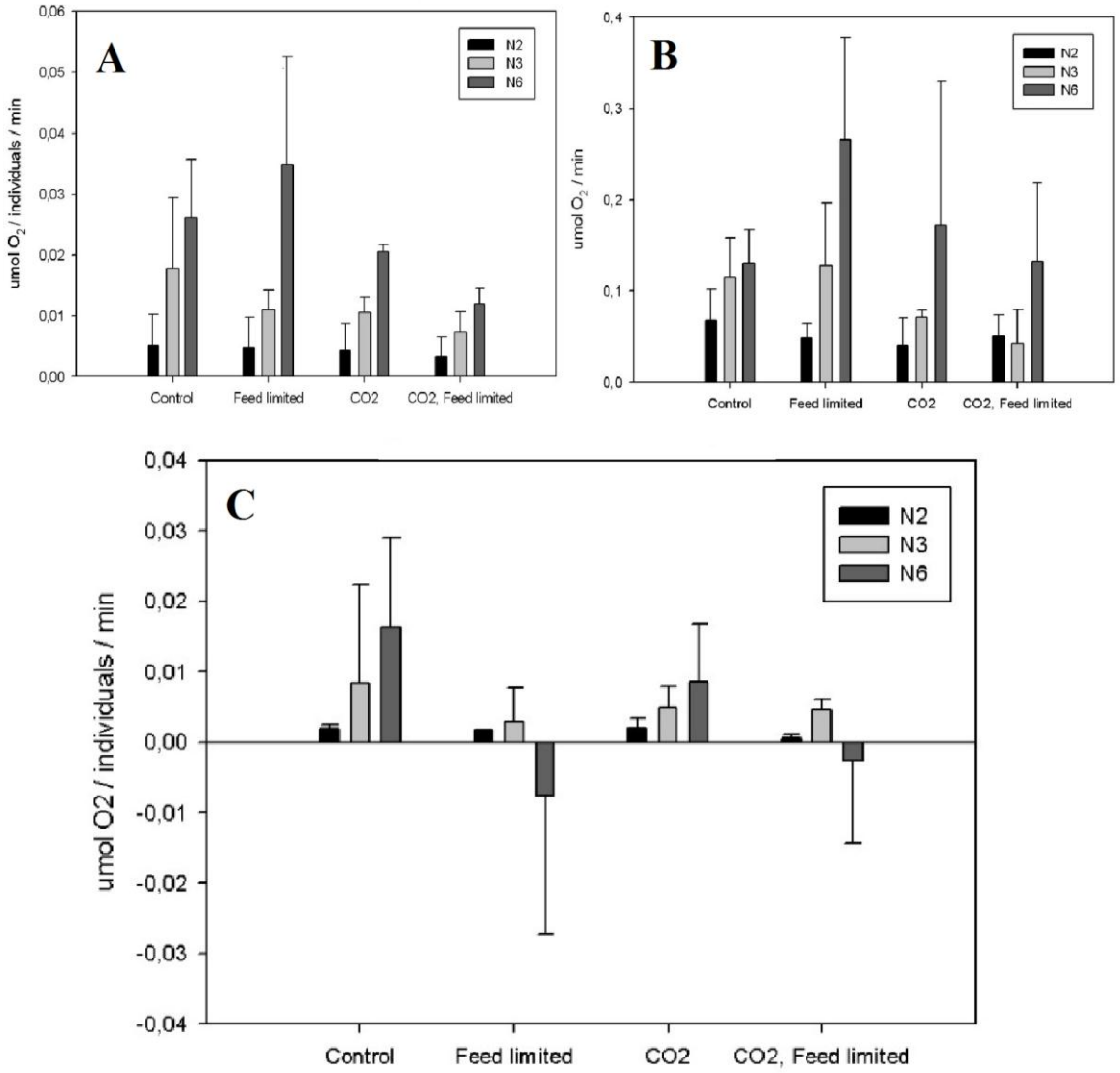

FIGURE 8: Respirometry measurement done on nauplii stage 1-2, (N1/N2, black), 3 (N3, light gray) and 6 (N6, dark gray) in the different treatments; Control (high feed, low pCO_2), Limited feed (low feed, low pCO_2), CO_2 (high feed, high pCO_2), and CO_2, Limited feed (low feed, high pCO_2). The bars indicate mean±SD (n=3) **A)** O_2 consumption of measurements done with animals (µmol O_2/individuals/min) not correlated for blank samples. **B)** O_2 consumption in blank samples (µmol O_2/min) measured at same time as animals. **C)** O_2 consumption of animals (µmol O_2/individuals/min) corrected for blank samples measured at same time with corresponding respirometry bottles and fiberoptic cables.

4. DISCUSSION

4.1 Hatching success and naupliiar survival

The feed concentration and CO_2 concentration was found to have no significant effect on hatching success or naupiiar survival in present study. Campbell et al. (2001) showed that food concentrations as low as 20µg C/L had no effect on survival of *C. finmarchus*. In the same study the investigators found that the critical concentration for 90 % maximum development rate was about 70 µg C/L. Compared to this, the feeding levels used in present study were much greater (~600 µg C/L and ~150 µg C/L), and the absence of significant reduction of hatching success and nupliiar survival is therefore consistent with Campbell et al. (2001). Previous studies on the effect of elevated CO_2 have found that hatching success and survival in copepods is generally quite robust to elevated CO_2. The hatching success and naupliiar survival of *C. glacialis* were found to be negatively affected by elevated CO_2 of approximately 7000 ppm (Weydmann et al., 2012) and similar effects were found when *C. finmarchicus* were exposed to 8000 ppm CO_2 (Mayor et al., 2007). The present study is consistent with this and further supports the impression from previous findings that *C. finmarchicus* may be relative robust to elevated CO_2 levels which are predicted in medium-term future (~2000 ppm) in terms of both hatching success and survival (Pedersen et al., 2013, Pedersen et al., 2014)

4.2 Carbon and nitrogen content

4.2.1 Carbon and nitrogen content in nauplii stage 3

The carbon and nitrogen contents in the nauplii stage 3 generally showed the same overall pattern in the different experimental treatments. Elevated CO_2 and limited feeding alone had no effect (a non-significant reduction in carbon and nitrogen content was observed), but a additive negative effect of elevated CO_2 and limited feed combined was found (36.84% reduction in carbon content and 42.07% reduction in nitrogen content compared to the control). An effect of combined exposer to elevated CO_2 and limited feed, similar to that observed in the present study, was found on the blue mussel *Mytilus edulis*. Here, the shell length growth was additive negative effected when exposed to elevated CO_2 combined with low food supply. (Melzner et al., 2011). A additive negative effect of food limitation and elevated CO_2 has not been demonstrated in *Calanus* spp. prior to present study, and clearly

shows that ocean acidification might have a more severe effect when interacting with low food availability.

A study by Håkedal (2013) found a tendency of lower body mass and decreased length in *C. finmarchicus* (C5) that had developed at 2080 and 3080 ppm. However, a study on adult and late copepodit stages of *C. galcialic* and *C. hyperboreus* found no effect on body mass (dry weight, carbon and nitrogen content) when animas where exposed to 3000 ppm (Hildebrandt et al., 2014). It is possible that the additive negative effect of elevated CO_2 and limited feed combined, on the observed carbon and nitrogen content at the nauplii stage 3 in present study, largely reflects a negative effect accumulated during the previous non-feeding life stages (N1/N2). A study by Pedersen et al. (2014) found a negative effect on naupliiar survival, but no effect on hatching success of *C. finmarchicus* when eggs were exposed to 8800 ppm CO_2, indicating that the nauplii stages may be more sensitive to elevated CO_2 than eggs. Further they found that the survival of the non-feeding stages (N1-N2) were negatively affected by the elevated CO_2 level, whereas nauplii stage ≤ 3 was not. This indicates that non-feeding stages of *C. finmarchicus* may be the most sensitive life stages towards elevated CO_2. Because these stages only rely on energy stores from the egg for metabolism and somatic growth (Campbell et al., 2001), the non-feeding stages might be particularly sensitive to elevated CO_2, whereas the later stages might be able to compensate for higher energy demands by increasing the feeding rate (Li and Gao, 2012). This might also explain the non-significant reduction of carbon and nitrogen content observed in present study for the nauplii that received high food concentrations and elevated CO_2 combined, where the nauplii may have been able to increase the feeding rate due to high food concentrations, in contrast to the nauplii exposed to elevated CO_2 and limited feed combined.

As Li and Gao (2012) demonstrated the copepod *Centropages tenuiremis* was able to compensate for higher energy use due to elevated CO_2 by increasing the feeding rate. Saba et al. (2012) demonstrated similar findings in a study where the feeding rate of adult Antarctic krill (*Euphausia superba*) increased 3.5 times when exposed to ~ 672 ppm. A study done on juvenile blue mussel (*Mytilus edulis*) found that the growth and calcification chiefly depended on food supply, with only minor impacts of pCO_2 up to 3350 ppm (Thomsen et al., 2013). They concluded that benthic stages tolerated high ambient pCO_2 when food supply was abundant. This seems to be the case in present study as well, were nauplii treated with elevated CO_2 and high food concentrations had no significant decrease in carbon and nitrogen

content compared to nauplii in the control treatment. This support the hypothesis that nauplii of *C. finmarchicus* achieving high food concentrations combined with elevated CO_2 were able to compensate for the higher energy demand associated to elevated CO_2, by increasing the feeding rate.

4.2.2 Carbon and nitrogen content nauplii in stage 6

Both carbon and nitrogen content was significant reduced by food limitation in nauplii stage 6. Nauplii that received a low food concentration (both limited feed alone, and elevated CO_2 and limited feed combined) had significantly lower carbon (58% and 55% respectively) and nitrogen content (64% and 56% respectively) compared to nauplii in the control treatment.

In contrast to nauplii stage 3, no significant effect on carbon and nitrogen content by elevated CO_2 was found at nauplii stage 6. This strengthens the hypothesis that early life stages of *C. finmarchicus* may be more sensitive to elevated CO_2 than the later stages and is consistent with previous findings (Pedersen et al., 2014). As the *C. finmarchicus* grew to nauplii stage 6, the food limitation in the two limited feed treatments (limited feed alone and elevated CO_2 and limited feed combined) seem to become increasingly severe. Food limitation caused a strong reduction in carbon and nitrogen content and may therefore have masked any possible negative effect of elevated CO_2.

Previous studies have found that food limitations have a strong effect on growth in the copepodit stages in copepods. Richardson and Verheye (1999) found that food limitation caused a decline in growth rate in the copepodit stages of *Calanus agulhensis* and *Calanoides carinatus*. Mullin and Brooks (1970) found that dry weight of *C. helgolandicus* (C4) was directly correlated with the concentrations of food available during growth. However, the nauplii stages of *C. finmarchicus* has been found to be less dependent on food supply than the copepodits stages and can obtain high growth rate and a nearly maximal development rate at relatively low food levels (~ 50 µg C/L) (Hygum et al., 2000). The same trend was found in a study by Campbell et al. (2001), where the effect of food limitation on *C. finmarchicus* nauplii was smaller compared to the copepodit stages. However, the investigators observed a decrease in development rate and carbon and nitrogen growth in the nauplii stages, indicating that food limitation also effects these stages, but not to the same extent as in copepodites. Since the present study only investigate the effect of food limitation on nauplii stage, we can

not conclude that nauplii stages are less dependent of food supply that the copepodit stages, however it is evident that food limitation have an effect on nauplii stages of *C. finmarchicus*.

The increased negative effect of food limitation on nauplii stage 6, compared to nauplii stage 3 indicates that the food limitation have larger effect on older stages. Campbell et al. (2001) found that the effect of food limitation was more severe among the copepodites than in the nauplii stage. As nauplii stage 6 is the final nauplii stage before moulting to copepodit, it is therefore reasonable that food limitation had a larger effect on this stage compared to the nauplii stage 3, where the animals just have started feeding, and the effect of food limitation is less severe.

2.4.3 C:N ratio

There was no significant effect of elevated CO_2 or feed limitation on the C:N ratio in either of the nauplii stages. This is in agreement with previous studies, as Campbell et al. (2001) found that the carbon and nitrogen growth displayed a equal proportional decrease with food limitation in the nauplii stages, resulting in an unaffected C:N ratio. However, Campbell et al. (2001) found that the C:N ratio was largely affected in the copepodit stages, where carbon growth rates was more reduced than nitrogen growth rate, leading to a decreases in C:N ratio.

4.3 Evaluation of experimental methods

There was a significant difference between the algal density in the two treatment with limited feed, where the CO_2 and limited feed combined-treatment had 8.7 % lower algal density compared to the Limited feed-treatment. The differences in algal density might be a possible explanation for the observed differences in carbon and nitrogen content in the analyses of the nauplii stage 3. However, if the differences in algal density was the cause of differences in carbon and nitrogen content, we should expect the carbon and nitrogen content in the nauplii stage 6 to have similar trends. Here, the carbon and nitrogen content of nauplii stage 6 in the two treatments with limited feed were approximately similar. We therefore assume that the differences in algal density did not affected the results in any great extent.

Nauplii taken out for carbon and nitrogen analysis were not properly stage determined due to time constraints. Thus, some of the nauplii analyzed may have been younger or older than the target nauplii stage. Especially for the N6 analyses, there might have been individuals of N5

included in the analyses in the two limited feed treatments (Limited feed- and CO_2 and Limited feed combined- treatment), due to the later and more variable development rate of the animals in the feed limited treatments. This might potentially have caused a bias towards smaller individuals in the treatments with limited feed. In addition, the sample sizes for nitrogen analyses were small due to limited number of animals, and some of the results had to be excluded because they were under the detection limit. However, it seems that the result gave a clear indication on how nitrogen content is affected by food concentrations and elevated CO_2 despite the small sample size.

The number of animals used to calculate the hatching success and the naupliiar survival was small, also due to limited number of animals/eggs. Investigating the hatching success and naupliiar survival was not the main goal for this thesis and was only intended as supplementary information. The small sample size caused large standard deviations, and this made it difficult to detect potential significant differences when applying statistical analysis. If the main goal was to investigate hatching success and naupliiar survival, a larger sample size would have be desirable. However, previous studies on *C. finmarchicus* have shown that the food concentrations and elevated CO_2 concentrations applied in present study have no effect on hatching success or survival of nauplii (Campbell et al., 2001, Pedersen et al., 2013, Pedersen et al., 2014), and it is therefore likely that even with larger sample sizes the results would have been the same.

The eggs used in present study were harvested from cultivated animals, breed under near optimal conditions. This may have led to a higher tolerance of the eggs and nauplii, due to greater energy stores in the "yolk sac". As Campbell et al. (2001) demonstrated, food limitation had a great effect on the lipid store in the copepodit stages and since the lipid store are imported in the reproduction process (Hirche, 1996a) the egg quality might have been poorer if the adult was breed under same conditions as in present study. It can therefore not be excluded that the effects of elevated CO_2 and food limitation observed in present study might have been more severe if the experiment had been run for generations.

4.4. Main findings in ecological context

Results from the present study, and previous studies (e.g. Pedersen et al., 2013) indicate that elevated CO_2 concentrations predicted in medium-term future may not pose direct threat for survival of *C. finnarmchicus*. However, ocean acidification and increasing ocean temperatures can lead to changes in phytoplankton community (Hare et al., 2007, Feng et al., 2009), decrease of annual primary production (Gröger et al., 2012) and decreasing the food quality by changes in the fatty acid composition (Rossoll et al., 2012). As increasing temperature and ocean acidification occur together (Solomon et al., 2007), future climate changes might therefore have a indirect effect on *C. finmarchicus*. Since lipid storage of *C. finmarchicus* are strongly affected by food limitation (Campbell et al., 2001), changes in food availability and/or food quality might thus lead to poorer egg production and/or poorer quality of the egg. It may be speculated that reduced egg quality might lead to further reduced CO_2 resilience. As Rossoll et al. (2012) demonstrated, both egg production and growth was negatively affected in the copepode *Acertia tonsa* when feed the diatom *Thalasssiosira pseudonana*, both bred under elevated CO_2 (750 ppm) condition. Further, as Fitzer et al. (2012) suggested, increase in pCO_2 (~ 550 ppm) might result shifts in life history stages of the copepode *Tisbe battagliai* that favour smaller brood size, females and later maturing females. These levels of pCO_2 are relatively small compared to pCO_2 used in present study, indication that elevated CO_2 might have a larger effect when combined with other stressors such as food limitation, and when exposed over several generations. Changes in growth and reproduction could also have a large impact further up in the food chain since *C. finmarchicus* are an important food source for many marine organisms (Ji et al., 2012).

4.5 Respirometry

How the respiromentry measurements were corrected for blank (background respiration) had a large influence on the outcome of the results. The method used in present study for respirometry measurement was therefore not optimal and further improvements are needed. Due to these uncertainties it was concluded that the results from the respirometry measurements could not be trusted.

4.5.1 Contamination of bacteria

Even though the water used for calibration and measurements was filtered to remove bacteria before use, there might have been inoculation from bacteria in the air, causing a decline in O_2

level in the blank samples. The respirometry bottles were only flushed with filtrated water from the tanks between each measurement making it possible for bacteria to survive and grow on the walls of the bottles between the measurements. Over time, the bacteria content in the vials might have grown, resulting in increased O_2 consumption rate over time. The blank samples were measured continuously during one hour, while *C. finmarchicus* samples were measured during 24 hours. The bacterial respiration in the blank samples might have changed during the next 24 hours and thereby altered the slope of the blank samples. This was suggested by Pomeroy et al. (1994) which showed that bacteria had different respiration rates over time. If the respiration rate of the bacteria was not constant (as assumed), the O_2 consumption slope generated form the blank samples might have been misleading.

4.5.2 Experimental design

The problem with the repirometry measurements could also be related to the setup. If there were leakage in the screw cap in the respirometry bottles, gas exchange with the surrounding water migth have occurred. This would potentially lead to O_2 diffusing into the bottles as the concentration drop due to respiration.

Even though the system was calibrated each time, there might have been small drifts in the system, accumulating during the experiments and leading to greater error in the measurements over time. An unsuccessful calibration might also have lead to the observed problems. During measurements different signal strength in the ficeroptic cables was observed. This might have been due to variation in how centered the fiberoptic cables was on the oxygen sensitive patches, and could have resulted in large variance between the measurements, making it more difficult to correct for the background respiration.

Most likely several of the mentioned factors may have contributed and caused the respirometry measurements to be unsuccessful. Bacteria contamination combined with drifts in the system and incorrect calibration are probably the essential factors to improve in the future. Previous studies have successfully used the Firesting O2, READFLASH system to measure respiration rate in many different fish species (e.g. Couturier et al., 2013, Rummer et al., 2013) but, to the authors knowledge, never successfully on copepods.

5. CONCLUSION

Elevated CO_2 and food limitation had an additive negative effect on carbon and nitrogen content in nauplii stage 3, probably due to higher sensitivity of elevated CO_2 in the non-feeding stages. As the nauplii developed to the nauplii stage 6, the limited feed conditions became increasingly severe and masked the possible effect of elevated CO_2. This, together with the non- significant effect of elevated CO_2 on hatching success and naupliiar survival, indicates that *C. finmarchius* may be robust to elevated CO_2 predicted in medium-term future (2000 ppm), and thus support previous finding. However, changes in the food availability or quality due to climate changes can have a indirect effect on *C. finmarchicus*, leading to reduce in lipide storage which is important in the reproduction process. Further research is needed to evaluate the effects on several life stages of *C. finmarchicus* when food availability and/or quality changes due to ocean acidification.

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