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Effects on Scope for Growth due to elevated carbon dioxide in the copepod Calanus finmarchicus

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Master Thesis

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Ole Jacob Håkedal

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THE NORWEGIAN UNIVERSITY OF SCIENCE AND TECHNOLOGY

Abstract

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Master

Effects on Scope for Growth due to elevated carbon dioxide in the copepod *Calanus finmarchicus*

by Ole Jacob HÅKEDAL

The calanoid copepod *Calanus finmarchicus* is a keystone species in the North Atlantic. Adverse effects of ocean acidification on this species could have major implications for ecosystem structure and function, as well as socio-economic impacts on fisheries. Cohorts of *Calanus finmarchicus* exposed to simulated ocean acidification environments were followed during the course of two consecutive generations. The time of development into the different molting stages were monitored. While the more moderate CO_2 concentration (1080 ppm) did not seem to affect the development rate, a slower development into molting stages were found in the highest exposure groups (2080 - 3080 ppm CO_2), compared to a control treatment group (380 ppm CO_2). Measurements of oxygen consumption and feeding rate in sub adult individuals (copepodite stage C5) from the second generation of continuously exposed *C. finmarchicus* were integrated into calculations of the overall energy balance (Scope for Growth) of the animals. Together with biometric measurements that were performed, these results points to an energy depletion due chronic exposure to elevated CO_2. *Calanus finmarchicus* seems to be tolerant to a more moderate CO_2 elevation (1080 ppm). No signs of adaptation to the treatments were detected over the course of two generations.

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Abbreviations

μ**atm**	**m**icro **a**tmospheres
μ**g C L**$^{-1}$	**m**icrogram **C**arbon per **L**iter
A	**A**bsorbance
ANOVA	**A**nalysis **O**f **V**ariance
ATP	**A**denosine **t**ri**p**hosphate
C 1-5	**C**opepodite stage **1** - **5**
CO$_2$	**C**arbon **d**ioxide
CO$_3^{2-}$	**C**arbonate
C$_T$	**T**otal inorganic **C**arbonic species
DIC	**D**issolved **I**norganic **C**arbon
DW	**D**ry **W**eight
F	**F**iltering rate
F1	**F**irst filial generation
F2	**S**econd filial generation
H$_F$	**F**ree **H**ydrogen ions
H$_T$	**T**otal **H**ydrogen ions
H$_2$**CO**$_3$	**C**arbonic acid
HCO$_3^-$	**B**icarbonate
I	**I**ngestion rate
L h^{-1}	**L**iters per **h**our
MS-222	**T**ricaine methanesulfonate
N 1-6	**N**auplii stages **1** - **6**
O$_2$	molecular **O**xygen
pg C cell^{-1}	**p**icogram **C**arbon per cell
pH	**p**ower of **H**ydrogen

pH$_T$	**T**otal **p**ower of **H**ydrogen
ppm	**p**arts **p**er **m**illion
rpm	**r**ounds **p**er **m**inute
SD	**S**tandard **D**eviation
SfG	**S**cope **f**or **G**rowth
T$_A$	**T**otal **A**lkalinity
TukeyHSD	**T**ukey's **H**onestly **S**ignificant **D**ifference test

Chapter 1

Introduction

1.1 Ocean Acidification

The atmospheric CO_2 over the last 650,000 years has been determined by the analysis of Antarctic ice cores. During this time and until the industrial revolution the concentration of this trace gas has varied between the range of 180 and 300 parts per million (ppm) Siegenthaler et al. [2005]. Today the global mean atmospheric CO_2 is close to 400 ppm Conway and Tans [2012]. Human activities releases large quantities of CO_2 into the atmosphere, mainly by burning of fossil fuel, cement production, and agricultural practices such as burning of forest to produce crop- and pasture lands. The CO_2 released by human activities is refereed to as anthropogenic CO_2, and has resulted in a rate of increase that may not have been experienced for millions of years Doney et al. [2009].

In discussions of the impact of anthropogenic CO_2 emissions a lot of focus has been directed upon the radiative forcing of CO_2 and the warming of the Earth‘s climate Houghton et al. [1996]. The last few decades has revealed another major impact of CO_2 emissions called ocean acidification. This is commonly refereed to as the decrease in ocean pH due to uptake of anthropogenic CO_2 Zeebe [2012].

The oceans and the atmosphere exchange large amounts of CO_2. As the partial pressure of gaseous CO_2(g) in the atmosphere rises the concentration of CO_2(aq)in the oceans rises proportionally. This is due to a thermodynamic equilibrium that follows Henry‘s law Gattuso and Hansson [2011].

In water dissolved CO_2 occurs in three main inorganic forms: free aqueous carbon dioxide ($CO_{2(aq)}$), bicarbonate ion (HCO_3^-), and carbonate ion (CO_3^{2-}). Carbonic acid (H_2CO_3) is a minor form that constitute less than 0.3% of [$CO_{2(aq)}$] (brackets denote concentrations). The sum of [H_2CO_3] and [$CO_{2(aq)}$] is therefore commonly denoted as [CO_2] Gattuso and Hansson [2011].

The dissolved carbonate species are related by the following equations 1.1 and 1.2.

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+; K_1^* \tag{1.1}$$

$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+; K_2^* \tag{1.2}$$

The proportion of the carbonate species are determined by their stoichiometric dissociation constants (K_1^* and K_2^*), which are a function of pH, temperature, salinity, and surface pressure. This is illustrated in a Bjerrum plot shown in figure 1.1. At typical surface seawater with pH measured at the total pH scale (pH_T) of 8.2 the speciation of [CO_2], [HCO_3^-], and [CO_3^{2-}] is respectively 0.5%, 89%, and 10.5% Gattuso and Hansson [2011].

Because of the buffer capacity in the seawater the sum of the inorganic carbonic species (C_T) does not change proportionally to that in the atmosphere. The Revelle factor describes the relative change in seawater $CO_{2(aq)}$ to the relative change of C_T in equilibrium with atmospheric $CO_{2(g)}$. A doubling in atmospheric [$CO_{2(g)}$] increases C_T only by 10% Gattuso and Hansson [2011].

The carbonate buffering system is the process in which the relatively stable pH are maintained in the oceans. It relies on the availability of carbonate ions from which the supply mainly are from the slow process of geological erosion Widdicombe and Spicer [2008].

1.1.1 Future predictions

The athmospheric CO_2 is currently rising due to natural and anthropogenic causes. There exists a broad scientific agreement that the contribution to the rate of increase by human activities are substantial. Several estimates has been constructed to elucidate

the future levels of atmospheric CO_2, each by using different scenarios for future CO_2 emissions Solomon et al. [2007].

Since the industrial revolution the oceans have absorbed $\frac{1}{3}$ of the CO_2 emmited by human activities, and this has been estimated to have reduced the pH by 0.1 units, which approximately corresponds to a 30% increase in hydrogen ions Fabry et al. [2008].

Based upon a "buisness as usual" model it has been estimated that the level of atmospheric CO_2, due to its equilibration with surface waters will result in a decrease in pH by 0.3 units within 100 to 150 years Caldeira and Wickett [2003].

From models of further rise in CO_2 it has been estimated that the atmospheric level may exceed 1900 ppm by the year 2300, and that this would result in a pH drop of 0.77 units in surface oceans Caldeira and Wickett [2003].

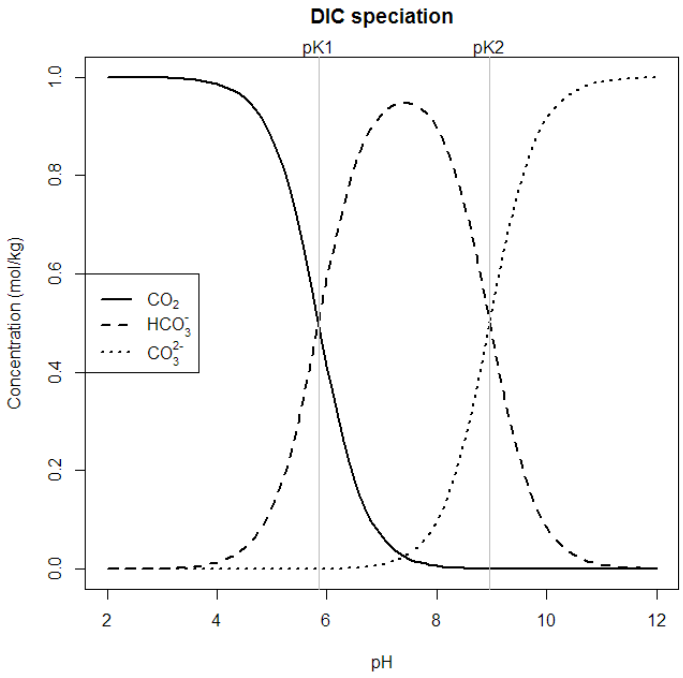

FIGURE 1.1: The concentration of carbonate species as a function of pH_T (temperature = 0°C, salinity = 35‰) illustrated in a Bjerrum plot. (The figure was made in *R version 3.0.0 (2013-04-03)* using the *seacarb* package, from Zeebe and Wolf-Gladrow [2001])

1.1.2 Marine life in high CO_2 environments

All organisms expend metabolic energy in maintaining a cellular pH level to ensure that biochemical processes operate efficiently Raven et al. [2005].

Studies of elevated CO_2 effects on marine organisms range from short time acute exposures to high concentrations, medium term exposures over weeks, to more realistically approaches using predicted CO_2 concentrations in long time studies on the scale of months. These different approaches have reveled valuable information concerning responses in marine organisms. Short time approaches can elucidate mechanistic modes of action for CO_2 toxicity (hypercapnia), while medium or long term studies may reveal more relevant information about population level responses Whiteley [2011].

Several studies have revealed adverse effects on marine life due to elevated CO_2 at levels that are within projections for the near future. The majority of the species studied have shown negative effects, but broad variations of responses to ocean acidification have also been found Whiteley [2011]. The variation has been linked to the wide variety of processes that are affected by this change in the abiotic environment, such as calcium carbonate dissolution rates, calcification rates, growth rates and development Kroeker et al. [2010].

Most studies on ocean acidification have focused on calcifying organism, since the increased concentration of CO_2 has been found to affect the formation and dissolution of calcium carbonate structures. Among organisms studied there has been found results indicating adverse effects in calcifying algae Kuffner et al. [2007], corals Gagnon [2013], mollusks Gazeau et al. [2007], echinoderms Kurihara and Shirayama [2004], Stumpp et al. [2011], and crustaceans Kurihara et al. [2008], due to elevated CO_2 conditions.

The negative effects have been to a large degree explained by a decreased solubility state for calcium carbonate (aragonite and calcite) in CO_2 enriched seawater. It has been demonstrated that different calcifying organisms do not respond uniformly to ocean acidification. Variation in responses between calcifying organisms (individuals or species) have been explained by differences in the ability for pH control near calcification sites. Some are found to be better equipped to cope with ocean acidification Kroeker et al. [2010].

1.1.3 Susceptibility to high CO_2 in marine organisms

Physiological effects of elevated CO_2 exposure are mediated through low pH in the ambient water, and through diffusion of CO_2 into organisms. Water breathers depend on CO_2 excretion through gills or other structures that are in close contact with the water, and this becomes more difficult as the carbonate chemistry changes due to increased CO_2. A result of this is a build-up of CO_2 in the oxygen carrying fluids (blood or haemolymph) and subsequently a decrease in pH (acidosis) Pörtner et al. [2004]. Since CO_2 easily diffuses through biological membranes this also results in a decreased pH inside cells Whiteley [2011].

Cellular acidosis may disrupt biological processes including iono-regulation, protein synthesis, and metabolism. Lowered pH also affects the oxygen transport and delivery to tissues by influencing the conformation, and oxygen affinity in respiratory pigments (Bohr effect). Organisms are therefore equipped with buffering mechanisms to adjust the acid-base equilibria in their body fluids Pörtner et al. [2004].

To buffer the extracellular fluid in order to maintain homeostasis, the organisms depend mainly on the inward transport of bicarbonate ions from the seawater in exchange from an outward transport of calcium ions. After the hydration reaction of CO_2, which is catalyzed by carbonic anhydrase enzymes, an outward transport of hydrogen ions is exchanged for sodium ions. This is an example of acid-base regulation and iono regulation sharing the same mechanisms Whiteley [2011].

Tolerance to high CO_2 environments is linked to the ability to compensate for altered extracellular acid-base status. Animals with a powerful ionic regulatory apparatus are more adapted to cope with ocean acidification Pörtner et al. [2004]. The exchange of ions across cellular membranes is driven by baso-lateral Na^+/K^+-ATPase enzymes and is thereby energy demanding. It has been estimated that up to 40% of total energy expenditure are associated with active ion transport Whiteley [2011].

The ability to cope with increased extracellular hypercapnia is therefore linked to the metabolic capacity of marine animals. Active organism have to adjust fluctuating extracellular conditions by active cellular processes, and are thus more adapted, compared to less metabolic active organisms to elevated CO_2 Melzner et al. [2009]. Individuals and taxa that are poorer equipped with respect to the capacity for ion regulation are

more susceptible for adverse effects. Early developmental stages (eggs, sperm, larvae), and juveniles are found to be more vulnerable compared adult individuals to elevated CO_2 Pörtner et al. [2005].

Invertebrates have generally a lower regulatory capacity for ion exchange compared to vertebrates, and are generally thought to be less tolerant to elevated CO_2 compared to vertebrates Pörtner et al. [2005]. Among crustaceans responses show a large degree of variation, and some are considered to be relatively tolerant to elevated CO_2 due to their high ion-regulatory capacity, but there is a general lack of data on long term effects Melzner et al. [2009].

1.1.4 Long term effects of elevated CO_2

The majority of investigations of CO_2 effects are short time exposure studies, and have not implemented the continuum between time- and concentration dependent effects Pörtner et al. [2005].

The reduced capacity for oxygen transport caused by acidosis can be counteracted by enhanced ventilation. However because the distribution of CO_2 between the water and the organisms is close to equilibrium, the release of CO_2 due to increased ventilation is limited. Water breathing organisms must therefore compensate for pH disturbance caused by CO_2 mainly by the previously described mechanisms, involving trans-epithelial ion exchanges Pörtner et al. [2005].

When the partial pressure of CO_2 in the seawater rise, the levels of dissolved CO_2 in intra and extracellular spaces of organisms will rise. This is due to increased diffusion, and will continue until a new value is reached that is sufficient to restore CO_2 excretion Fabry et al. [2008]. This will be accompanied by a shift in metabolism. Such processes may not be life threatening for individuals, but may affect slow processes like growth and reproduction. At long time-scales this could potentially have negative effects on higher biological levels Pörtner et al. [2005].

Ocean acidification may result in reduced marine biodiversity through loss of species that are sensitive to changes in CO_2 and pH. If sensitivity is a function of the taxonomic group, taxonomic diversity may be reduced. Loss or reduced function of keystone species

could reduce habitat complexity and biological regulation mechanisms Widdicombe and Spicer [2008].

In their long term exposure study, Kurihara et al. [2008], using predicted future CO_2 levels, found effects on antennae growth, moulting frequency, and mortality in the marine shrimp *Palaemon pacificus*. In this study impacts of CO_2 were manifested after several weeks of exposure, indicating a decreased ability for acid-base regulation over time. Their results also pointed towards a change in the feeding rate of the shrimps, although these results were not statistically significant. The authors pointed out that the whole process of digestion, nutrient absorption from the gut, and nutrient assimilation could have been depressed, affecting the energetics of the animals Kurihara et al. [2008].

Calcium carbonate is incorporated as a strengthening component in the chitionous cuticle (exoskeleton) of crustaceans Neues et al. [2007]. The calcification of the exoskeleton in crustaceans is considered as being less vulnerable to ocean acidification compared to organisms such as molluscs and echinoderms. This is due to a dominant proportion of a more stable form of calcium carbonate mineral (calcite) Whiteley [2011]. The calcification site is also protected by an outer layer of the shell (epicuticula). It is however unclear whether the balance between calcification and dissolution may be adversely affected, especially in long term exposure situations Whiteley [2011].

There is limited information about the sensitivity in zooplankton for future CO_2 conditions, partly reflecting the complexity in working with these organisms. Most investigations have been performed focusing on effects on calcification in species such as pteropods and foraminifera. Less is known about the effects of ocean acidification on growth, reproduction, and grazing rates in zooplankton, especially in non-calcifying species Gattuso and Hansson [2011].

In a study by Fitzer et al. [2012] the copepod *Tisbe battagliai*, when exposed to elevated CO_2 in the course of three generations, showed responses of reduced growth and reproductive performance. The levels of CO_2 used in their study (<600 μatm)are within what is expected for the relatively near future.

1.1.5 Assessment of energy balance: Scope for Growth

The growth of an organism involves physiological, cellular and biochemical activities that are related to factors within the organism's environment. By analyzing growth, one can therefore obtain information related to environmental factors which organisms are exposed to. Direct measurements of growth are difficult to obtain, but an estimate of the energy available for growth and reproduction is more easily derived Navarro et al. [2006]. An estimate for this energy is given by the Scope for Growth (SfG) index, developed by Warren and Dav1s [1967].

SfG is estimated by subtracting the energy utilized in respiration and excretion from the total amount of energy absorbed from food. A positive value for SfG is interpreted as an excess of energy being available for growth and reproduction. A negative SfG is obtained when the energy expenditure in organisms exceeds the energy absorbed from food, and means that the organism must rely on its body reserves for maintaining basic life processes Navarro et al. [2006].

Since SfG provides an instantaneous measure of the energy status of an individual, is sensitive and gives a quantitative response that can be related to tissue chemistry it has been used as a biomarker of effect in studies of environmental stressors such as temperature, salinity and pollutants such as pesticides Verslycke et al. [2004], and other chemical contaminants Widdows et al. [1995].

It has also been employed as a measure of effects due to elevated CO_2 conditions. Stumpp et al. [2011] found a decrease in SfG in larvae of the sea urchin *Strongylocentrotus purpuratus*at elevated CO_2 exposure, at a pH of 7.7.

1.2 *Calanus finmarcicus* (Gunnerus, 1770)

The marine copepod *Calanus finmarcicus* is considered to be a keystone species in the North-Atlantic ecosystem. As a mainly herbivorous zooplankton it contributes to the pivotal role of providing energy transfer from primary producers to higher throphic positions of the food web Runge [1988]. The importance of *Calanus finmarcicus* can be illustrated its contribution to the total copepod biomass, which has been estimated to

be as high as 70% to 90% in some areas Planque and Batten [2000]. An illustration of the life cycle of *Calanus finmarcicus* is shown in figure 1.2

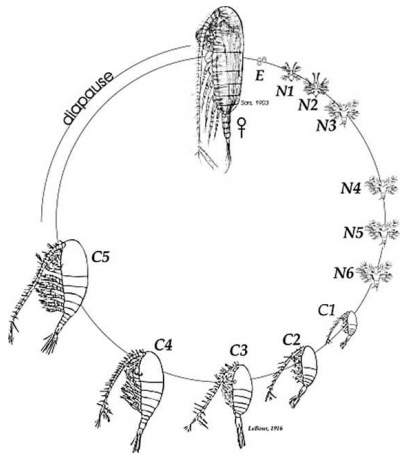

FIGURE 1.2: Illustration of the life cycle of *Calanus finmarcicus*. 6 naupili stages are followed by 5 copepodite stages, before turning into adulthood. The figure also illustrates the relative body sizes and relative duration between each molt. Individuals in the stage C5 that have acquired adequate lipid reserves may descend into deep waters for overwintering (diapause) Bagøien et al. [2012]. (The figure was made by Baumgartner [2009] at Woods Hole Oceanographic Institution)

Biological activity is one of the major drivers for changing the CO_2 conditions in the surface oceans Kerrison et al. [2011], and the fecal pellets produced by Calanoid copepods can dominate the vertical transport of carbon in some areas Mayor et al. [2012]. They thereby sequester a large portion of the CO_2 captured by photosyntetic biomass production into deeper seas.

1.2.1 Effects on *Calanus finmarcicus* due to ocean acidification

It has been documented a reduction in total calanus biomass of 70% since 1960, and a change in biogeographical distribution of *Calanus finmarcicus* in the North Sea and Northeast Atlantic Beaugrand et al. [2002]. While the underlying mechanism for these changes remains unclear, the ongoing warming of the surface sea temperature has been ascribed to as an possible explanation Edwards et al. [2008]. Since the ocean acidification phenomenon happens at the same time as the warming of the climate, it can not be ruled out as being a contributing factor Mayor et al. [2012].

Some results have indicated that adult *Calanus finmarchicus* may be relatively robust to near future CO_2 conditions, but adverse effects has been found in eggs and earlier developmental stages in combination with realistic scenarios of future warming in Calanus species Mayor et al. [2012].

1.2.2 The present study

In this study we investigated effects of three elevated CO_2 conditions on two consequtive generations of *Calanus finmarchicus*. The species of study was chosen based on its key position in the marine ecosystem, and the lack of data on its response to future ocean acidification predictions Mayor et al. [2007]. *Calanus finmarchicus* has a relatively short generation time, and are therefor suitable in investigation of potential long term effects on full life cycle of development due to environmental stressors.

A negative effect on this specie has the potential to have a major impact on ecosystem structure and function, and could also have negative socio-economic impacts as it represents an important food source for several fish species that are harvested for human consumption Runge [1988].

The effects of elevated CO_2 has been found to affect the same parameters as changed temperature conditions Pörtner et al. [2005]. One possible effect could be a change in developmental rate, as this has been found in studies of this species Campbell et al. [2001]. This may be important as the development of *C.finmarchicus* is tuned to match algae blooms Bagøien et al. [2012].

1.3 Aims of the study

The management of economically important species is currently moving towards an ecosystem based approach. Among other things, this requires an understanding of interactions with abiotic components such as CO_2 Bagøien et al. [2012]. This project aims to provide some insight that may be useful in population model studies to elucidate potential ecological and socio-economic impacts of future CO_2 conditions.

The objective of the study was to investigate long term effects of different future CO_2 scenarios on growth and development of *C. finmarchicus*. Since growth and development

involves energy demanding processes, we investigated potential effects on feeding and oxygen consumption in a sub adult developmental stage (C5). Feeding and oxygen consumption data were used in calculation of the overall energy balance (Scope for Growth).

Chapter 2

Materials and Methods

2.1 Experimental setup

The experiments described in this thesis were conducted at NTNU Center of Fisheries and Aqua-culture (SeaLab), Trondheim. Starting from early November 2011 and lasted until late April 2012.

As a participant in a collaborative group involved in the project I will present the main experiment, and the experiments that I were directly involved in. This includes determination of stage development in the first generation, measurements of feeding and oxygen consumtion in moulting stage C5 of the second generation of the cultured copepods, and overall monitoring of the exposure conditions.

An experimental setup customized for exposure of *Calanus finmarchicus* to elevated CO_2 concentrations were installed in a temperature controlled room at SeaLab zoo-physiology laboratory. Twelve cylindro-conical-shaped polystyrene tanks (90L) were divided into tree replicates of the following treatments: Air (control, approximately 380 ppm CO_2), 1080 ppm CO_2, 2080 ppm CO_2, and 3080 ppm CO_2. Four tanks receiving one of each treatment were installed together, making three separate blocks differently located in the room. Relatively large tank volumes were chosen to approach a microcosm-scale situation for the animals.

Pure CO_2 gas provided by pressure flask and ambient air provided by the central compressed air distribution at SeaLab was mixed to produce different levels of CO_2-enriched

air. The composition of the gas mixtures were controlled by a custom developed gas mixing system (HTK Hamburg®GmbH). There were separate channels for each of the four treatment concentrations.

Filtered natural sea water was used in the experiment. This was pumped from the Trondheim fjord at approximately 80 meter depth and collected in reservoir tanks for temperature regulation and maturation of the water.

In four equilibrating columns, one for each treatment, the gas entered the water in form of small bubbles provided by an aquarium diffuser (Wooden air-stone, Aqua medic) located at the bottom of the columns. An inner tube collected the bubbles and as water entered this tube from the top a counter current system ensured effective gas transfer into the water by diffusion.

The water from the equilibrating columns were distributed to the experimental tanks from the top and also from the bottom apex positions in order to prevented stagnated water. CO_2-enriched air was also mixed directly in the experimental tanks, by the same principle as in the equilibrating columns. This prevented loss of CO_2 gas due to air-water exchange to decrease the concentrations in the experimental water.

The water outlet was located at the top of the tanks, and the flow rate was sat at approximately 3.75 liters hour^{-1} to reach total change of water in 24 hour intervals. To prevent animals from escaping the outlet was covered by a filtering cloth (nylon mesh).

The tanks were without lids, therefore to compensate for carbon dioxide loss by air-water gas exchange; the CO_2-enriched air was adjusted at a slightly higher level at the gas mixer. To approach the target CO_2 concentrations, pH, temperature and total alkalinity were measured. CO_2 concentrations were calculated by using the open source software CO2SYS-v2.1.xls Pelletier et al. [2007].

The light setting was at a 12 hour light and 12 hour dark cycle. To prevent stress due to sharp light Mauchline [1998] the lamps in the room was covered with dark fabric, providing a dim light condition.

2.2 Experimental conditions

2.2.1 pH measurements

A colorimetric method for pH measurement was employed as described by Dickson [1993]. This method provides values based on the total pH scale. The equilibrium constant and pH value of the reference materials were also determined based on this scale (TRIS buffer).

The definition of the total pH scale is given by the equations:

$$[H^+]_T = [H^+]_F(1 + (S_T/K_S)) \tag{2.1}$$

$$[H^+]_T \approx [H^+]_F + [HSO_4^-] \tag{2.2}$$

Where $[H+]_F$ is the free hydrogen ion concentration, included hydrated forms, S_T is the total sulfate concentration, K_S is the second dissociation constant of sulfuric acid and $[HSO_4^-]$ represents the hydrogen sulfate ion concentration Dickson [1993].

The method for measurement is based upon the dissolution of a sulfonephthalein indicator dye (m-cresol purple). The second dissociation of this diprotic acid is described by the following reaction.

$$HI^-_{(aq)} = H^+_{(aq)} + I^{2-}_{(aq)} \tag{2.3}$$

Where I represents the indicator dye. The principle for pH_T determination is based upon the different absorption spectra for the different forms of the dye. The total ion concentration can be determined by the following formula.

$$pH_T = pK(HI^-) + log10\frac{[I^{2-}]}{[HI^-]} \tag{2.4}$$

The following procedure was employed. Water samples were collected into glass bottles (50 ml) with a sealing cap preventing air pockets. The samples were placed into a water bath set at 25°C. After temperature equilibration water was transferred into a gas-tight

glass cuvette. The samples absorbance was read specrophotometrically at the wavelengts 578, 434 and 730 nm. Then m-cresol purple solution (50 μl) was added into the cuvette containing the sample, and the absorbance was read at the same wavelengths. The following formula as described by Dickson [1993] was employed for the calculations:

$$pH = 8.0056 + Log10[\frac{(A1/A2 - 0.00691)}{2.222 - (A1/A2 * 0.1331)}] \tag{2.5}$$

where A1/A2 = $\frac{(A578cresol - A578blank) - (A730cresol - A730blank)}{(A434cresol - A434blank) - (A730cresol - A730blank)}$, and A represents the absorption value at the representative wavelength Dickson [1993]. The pH was also monitored by a handheld potentiometric pH-meter. This was performed because it was less time consuming and allowed a more regular testing of this water parameter.

2.2.2 Temperature measurements

A certified glass thermometer was used for regularly temperature measurements (VWR®Precicion Thermometer, accuracy ± 0.3 °C). In addition was the built in thermometer in the potentiometric pH meter used for these measurements.

2.2.3 Total alkalinity measurements

Measurements of total alkalinity (T_A) were performed using an automatic potentiometric titratior (Radiometer Analytical SAS), according to a method as described by Xiaowan et al. [2009]. The measurements were performed in one of the replicate tanks for the control treatment at a regular schedule.

2.2.4 Feeding conditions

The copepods were given a sole diet of the cryptophyte microalgae *Rhodomonas baltica* (strain C/95). The alga was provided as a stock solution of known concentration. A continuous addition of algae into the different experimental tanks was provided by a multi-channeled peristaltic dosing pump (Watson-Marlow 520S).

The inflow of algae in the tanks was located near the top and in close proximity to the inflow of the water from the equilibration columns. This ensured a good circulation and an even distribution of algae in the tanks.

The amount of algae given to the copepods was determined based on a previous feeding experiment, and the target concentration was $200 \mu g C L^{-1}$. This concentration was determined to prevent *ad libitum* (in excess) supply of food from potentially mask energetic effects due to the CO_2 exposure.

The alga level in the tanks was measured with a coulter counter (Beckman Multisizer^{TM}3 Coulter Counter®). This was performed on a regularly basis during the experiment(daily or every second day). The algae concentrations (cells ml^{-1}) were determined based upon the integration of number of particles counted v.s. particle diameter (μm) in the range of 5.363 to 9.561 μm. This range gave a clear peak indicating presence of *R. baltica* cells. The integrations were based upon the graph displayed from three consecutive measurements performed by the coulter.

The results from the cell counts were transformed into carbon equivalents (μg C L^{-1}) based on a linear relationship ($R^2 = 0.976$) between algae cell density and the carbon biomass of *R. baltica* cultivated at SeaLab using the same nutrient media and the same algae strain Skogstad [2010]. Based on this an estimate for carbon mass were calculated (45 pg C $cell^{-1}$). The estimate was close to a value reported elsewhere for *R. baltica* Båmstedt et al. [1999].

Due to the observation that there was a difference in algae concentration between some of the tanks, attempts were made to adjust the alga supply during the time of the second generation. This was performed by providing a series of dilutions of the algae stock to the tanks with higher concentrations. No such actions were performed during the time of the first generation of the copepods.

2.2.5 The animals

The animals that were used in the experiment were obtained from a culture of *Calanus finmarchicus*. The cultured animals originate from the Trondheim fjord, and has been kept stable since 2004 at SINTEF / NTNU SeaLab Saage et al. [2008].

They were maintained in 300 Liter tanks and the development of the animals were kept under surveillance until there was found a relatively large amount of female animals with visible eggs. Adult animals (mostly females) were separated from the other developmental stages by carefully sieving through an appropriate sized mesh, and transferred into new containers (50 liter) containing normal seawater for egg laying.

To increase the egg production they were fed *ad libitum*. After 24 hours eggs were collected by sieving of the bottom content of the containers. The amount of eggs in the batch was estimated by mixing 1 liter of water containing the eggs, and counting the egg-content in a 10 mL sub-sample. The eggs were evenly distributed into the experimental tanks, with approximately 2050 eggs in each tank. The median egg-laying period was noted as 12 hour after the incubation of the gravid females.

2.2.6 Sampling procedure

The sampling procedure was based upon previous studies of copepods, e.g. Campbell et al. [2001], were a large cohort of eggs laid in a short time interval were followed during the development to adult copepods. Samples were taken to determine the stage composition of each cohort over time.

The method assumes that the initial cohort is large enough that the stage composition is not affected by the sampling, and that the size of each sample is large enough to represent the culture Hu et al. [2007].

The sampling of animals was performed by attaching a 500 ml measuring cup at the end of a pvc pipe. This sampler was then put into the tanks and gently stirred by a rotating motion from the bottom and upwards three times before the sampling volume was taken out. This action was performed in order to approximate an even distribution of the animals, and thereby obtain representative samples. The sampling volume was 500 ml when the animals consisted of mostly nauplii stages. The volume was increased to 1500 ml as copepodite stages became more dominant.

The sampling volume was poured onto a funnel with a sieve collecting the content. The content was then transferred along with seawater into 8 ml glass vials and preserved by adding 16μl of Lugol's solution (phytofix).

Samples were taken at frequent intervals and from where the stages of development were determined. The sampling intervals were based upon the works of Campbell et al. [2001], and were most frequent the first days of the experiment in order to get individuals of the first short lasting developmental stages.

The first sampling was performed 36 hours after the midpoint of the egg laying period and the following samples was taken at 8 hours intervals the first three days of the experiment. The interval was then increased to 12 h, and this interval lasted until copepodites were observed in the tanks (after 10 days). Thereafter the sampling was performed once a day for 10 days, every second day for 10 days, and every fourth day for the rest of the generation period.

When the animals consisted mostly of adult individuals, we started the preparation for the culturing of the second generation. Due to the cannibalistic tendency documented in calanoid copepods Bonnet et al. [2004], the adults were separated from their eggs in order to secure the second generation. We also wanted to reduce the impact of variable developed animals that could make it difficult to distinguish between the two generations. In addition, available eggs and nauplii could potentially represent an extra energy source and thereby mask CO_2 related effects.

Adult animals were transferred for egg laying in separate 50 liter containers containing water from their respective treatment tanks. The procedure was similar as for the first generation.

2.2.7 Stage determination

The preserved samples for the first generation animals were stored in a cold room for approximately 11 month before examination of the content. In most cases the animals were well preserved, and the stage specific characteristics were visible. The stage determination was performed by examining the animals under a microscope (Leica M 205 C). The morphological characteristics of the different stages that was focused upon was based upon literature descriptions Marshall and Orr [1972], Mauchline [1998], and a stage determination key with close up pictures and a short description of the most prominent characteristics worked out by one of our co-workers (Researcher Iurgi Imanol Salaverria-Zabalegui, Department of Biology, NTNU).

2.2.8 Determination of median development time and stage durations

The samples representing the evolving stage structure of the tank population were used to estimate the median development time (MDT), which is defined as the time from the midpoint in the egglaying period and until 50 percent of the cohort has reached a specific developmental stage Landry [1983].

The fraction of the cohort that had molted past a specific stage at each sampling time was plotted against the time that has passed since the midpoint of the egg-laying period. MDT values were determined by the coefficients of a least square linear regression performed on the linear portion of the plot (excluding the tails < 0.05, > 0.95), similar to the procedure described by Campbell et al. [2001], except that a larger portion of the plot were included to get a better fit.

In a few cases the fraction became less than one at a sampling time where it at an earlier time had been determined that the cohort was completely molted passed that specific stage. This had an strong influence on the regression model and made a poor linear fit. Therefore were regression performed in the time interval limited to the first point in time where the fraction equaled one.

2.3 Feeding and oxygen consumption in CO_2 exposed copepodides (C5), second generation

The measurements of feeding rate and oxygen consumption were conducted on individual stage 5 copepodites (C5) from the F2 generation. The experiments were performed over 7 days with each day covering one of the three replicate tank series. The replicate tanks were covered two times during the experiment. In order to reduce potential variation in feeding and respiration performance due to dial rhythms Mauchline [1998] the measurements were performed at approximately the same time of the day.

2.3.1 Grazing measurements

A volume (3L) was sampled from each experimental tank. To get a representative random selection the sampling procedure was performed by applying the same technique

as in the stage development experiment, however due to few animals in the target developmental stage a larger sampling volume were applied.

Every day of the experiment 3 sub-replicate animals from each of the treatment tanks in one of the treatment series were transferred into glass bottle grazing chambers (50 ml), with one animal in each chamber (12 animals). This gave measurements on a total of 72 animals covering all replicate tanks two times.

Identification of stage 5 copepodites was performed under a dissection microscope. All handling of animals and the set-up of the experiment were conducted inside a temperature controlled room set at 10°C. The bottles were pre filled with control- or CO_2 treated water and algae content from each respective tank.

To measure the change in algae content, one bottle representing each treatment contained water and algae from the respective tank without animals. This gave 4 bottles representing each tank, and a total of 16 bottles per replicate treatment series. To prevent air from entering the bottles and possible loss of treatment conditions, glass caps were mounted onto a positive meniscus, squeezing the excess water out.

After sealing, the 16 bottles were mounted onto a motor driven wheel rotating at approximately 1 round per minute (RPM) to keep the algae cells in suspension, as described by Huntley et al. [1983]. Constant temperature during the grazing period was ensured by rotating the wheel in a controlled water bath inside a polystyrene insulated box. Stable temperature was confirmed by measurements before and after the incubation by a calibrated glass thermometer. The setup is illustrated in figure 2.1

To prevent algae growth only dim light was used during the animal handling, while the majority of the incubation period was performed in the dark. After the incubation period (19.64±0.73 hours) the animals were transferred to respiration chambers for determination of oxygen consumption.

Algae content was determined using a Coulter counter as described in the **feeding condition section** 2.2.4.

Filtration rates, defined as *the volume of water swept clear due to grazing by the copepods* and ingestion rates were calculated using methods described by Frost [1972] and Marin et al. [1986]. The filtration rate, F, were given by:

$$F(\text{ml copepod}^{-1}\text{h}^{-1}) = \frac{V * g}{N} \tag{2.6}$$

Where V is the volume (ml) of the grazing chamber, g is the grazing constant, and N is the number of copepods in the grazing chamber. The constant, g, was calculated from:

$$g = k\frac{(lnC2^* - lnC1^*)}{(t_2 - t_1)} \tag{2.7}$$

Where k, is the algae growth constant given by:

$$k = \frac{(lnC2 - lnC1)}{(t2 - t1)} \tag{2.8}$$

Where $C1$ and $C2$ are the initial and final algae concentrations (cells ml^{-1}) in the control chamber without copepods. $C1^*$ and $C2^*$ are the initial and final algae concentrations in the experimental grazing chambers and $t_2 - t_1$ the time elapsed during the experiment.

In our experiment the algae growth was limited due to dark conditions. To test this $C2$ values were compared with algae counts from the respective experimental tanks measured at the same day, but at a different time of the day than the grazing experiments, by setting these values as $C1$ in the calculations. The k values from this check were considered as being close to zero (0.005 ± 0.006). It was therefore assumed that no significant alga growth had occurred. The grazing coefficient, g, were calculated assuming that $k = 0$ as described by Marin et al. [1986], and Huntley et al. [1983] for similar situations (i.e., $C1 = C1^*$).

The ingestion rate, I, is given by:

$$I(\text{cells copepod}^{-1}\text{h}^{-1}) = F * C \tag{2.9}$$

Where C is, according to Frost [1972] the mean cell concentration, calculated from:

$$C = \frac{C1 * [e^{(k-g)(t2-t1)} - 1]}{(t_2 - t_1) * (k - g)} \tag{2.10}$$

Again, due to the assumption of no algae growth during the time of incubation, the ingestion rates in our experiment were calculated, following Marin et al. [1986], by:

$$I = F * C1 \tag{2.11}$$

Samples were preserved for later determination of the fecal pellet production; however these were later discharged as they were in a varying degree degraded at the time of inspection.

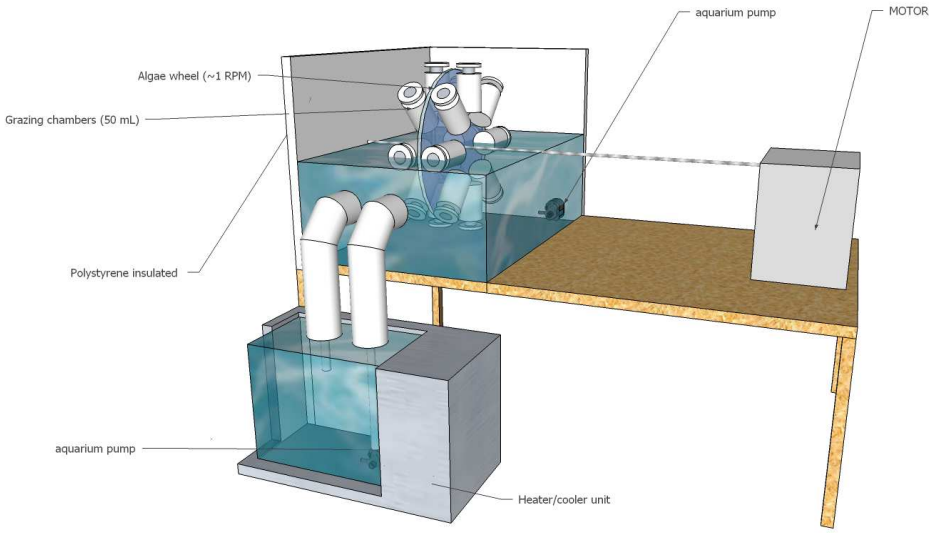

FIGURE 2.1: Illustration of the custom made setup for the grazing experiment. The grazing chambers (50 ml sealed bottles) were attached to a wheel (30 cm diameter) with 8 chambers on either side. The wheel was driven by a peristaltic pump motor adjusted to turn the wheel at approximately 1 RPM. The wheel was half way submerged in a temperature controlled water bath. The water level was held constant by overflow. The inflow was supplied by an aquarium pump from an adjusted heater/cooler unit set at 9.7°C. Circulation of water was provided by another aquarium pump inside the polystyrene insulated box. A lid (not shown), prevented temperature flux in the air in which the grazing chambers was exposed to half of the incubation time (approximately 20 hours). The setup was installed inside a chilling room set at 10°C. The wheel was made by product designer Ola Flatvad at Ula Jern, Trondheim. Modification and assembly were performed at the workshop at NTNU, SeaLab. Trondheim (The figure is made in Google SketchUp 8)

2.3.2 Respirometry

The three sub-replicate animals from each experimental regime were transferred directly from the 50 mL bottles into individual glass respirometry chambers (2 mL). The chambers had been tested by filling them with nitrogen saturated water; measurements reviled no rise in oxygen content confirming that they were gas tight.

Before the transfer of animals, the chambers were pre filled with control or CO_2 treated water. The water was taken from the grazing chambers to approximate similar situations as they experienced in the grazing experiment. Only water from the respective treatment grazing chambers that did not contain animals was used in this pre filling. One exception to this was on the first day of measurement (A series), where the respirometry chambers were pre filled with content from the respective grazing chambers.

A large plastic pipette was used to transfer the animals. The animals were washed by first pipetting them into a small dish containing the appropriate water in order to dilute eventual differences in algae concentrations. This was to approximate similar algae situation between the sub replicates during the respirometry.

All air bubbles were eliminated by mounting the chamber cap onto a positive meniscus, squeezing out excess water. The respirometry chambers were mounted onto a customized acrylic plate and placed into a controlled water bath inside a heater/cooler unit. The time of the animal transfer were noted as the start of incubation.

The oxygen measurements were performed using a fiber-optic oxygen meter (Fibox 3 LCD trace, Precision Sensing GmbH). Calibrated according to the device manual. The oxygen content in the water was detected by illumination and optical reading of an oxygen sensitive patch mounted inside the respiration chambers. Small pieces of silicone tube were attached to the glass vials in order to center the probe at the location of the oxygen sensitive patch.

The oxygen content in the chambers was recorded approximately every 60 minutes during the incubation period (approximately 8 hours). The time from start of incubation and until the first oxygen reading varied between the chambers due to the preparation time for subsequent chambers. The first readings were performed at approximately one hour after preparation of the last chamber. This was to prevent readings during a sharp decrease in oxygen level previously observed in copepode respirometry due to handling

stress Marshall and Orr [1972]. Approximately 10 readings were performed with 0.5 second intervals, from which an average were used as a measurement point. All readings were performed during a linear decrease in oxygen concentration. The respirometry was terminated before the oxygen level was reduced by 40 percent of the first measured value.

Oxygen consumed by the animals was determined based upon the least square regression of oxygen concentration (mg/l) vs incubation time (hours). The regression coefficients were used to determine the reduction in oxygen due to respiration by the following formula:

$$O_2 consumption(\mu g O_2 * mg DW^{-1} * hour^{-1}) = \frac{(a(b+a)) - (ac(bc+ac)) * V * 1000}{DW} \tag{2.12}$$

Where a and b refers to the regression coefficients in the formula for the regression line in the form:

$Y = ax + b$

The coefficients from the no animal control chamber (ac and bc) was withdrawn to remove the influence in oxygen consumption due to algae and bacteria respiration, V is the volume of the chambers (2 ml), and DW is the dry weight (mg) of the copepods. Results were multiplied by 1000 to get the measured O_2 unit (mg/L) in to μg/L.

After the oxygen measurements the animals were inspected for movements as sign for vitality and any visible signs of damage that could have influenced their respiration rate. They were sedated by adding a small amount of MS-222 (Finquel, Agent Chemical Laboratories Inc), and photographed (Sony DFW-SX900 color digital camera) under a dissection microscope (LEICA MZ 12_5). The animals were then dipped in distilled water to remove salt, before they were transferred into pre weighted tin capsules placed inside a 96 well plate. The animals were stored in a freezer (- 20°C).

All animals were identified as being in stage C5 prior to the incubation, and all were confirmed to be alive and intact at the end of experiments. There were some evidence that 4 of the animals had gone through a molt during the incubation. Based upon examination of the photographs and one incident were a shed skin were found in the grazing chamber 2.2, one animal from the 380 ppm, one from the 1080 ppm, and 2 from

the 3080 ppm CO_2 group were identified as adults. All obtained values were included in the analysis.

FIGURE 2.2: Picture of a shed shell found in the grazing chamber after the experiment, this belonged to an individual in the 2080 ppm CO_2 treatment group. Three others were identified as being adults by examination of the pictures; one from the 380 ppm, and two from the 3080 ppm CO_2 treatment group

2.3.3 Biometry

The photographs were used for length and area measurements, that was utilized for volume calculations by a method described by Miller et al. [2000]. A calibrated software (Image J, National Institutes of Health, Bethesda MD, USA) made use of the pixel count on a computer screen to read the measurements in millimeters. The calibration was based on the known magnification in the photographs.

Length measurements were performed by superimposing a line from the anterior dome of the head to the posterior-most extent of the last thoracic segment. The length of the oil-sac were performed by superimposing a line from the visible part of the anterior to the posterior of the oil containing membrane 2.3. The area was estimated by tracing an outline of the oil-sac on the computer screen. All length and area measurements were performed three times from which an average value was used for cylindrical tube volume approximations by the following formula.

$$V = \frac{\pi A^2}{4L} \tag{2.13}$$

Where A is the projected area, and L is the length of the principal axis of the oil sac Miller et al. [2000].

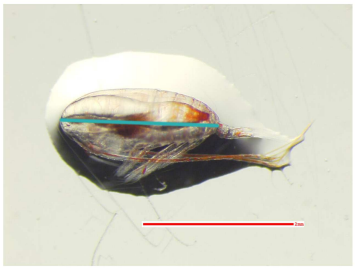

FIGURE 2.3: Length measurement were performed by superimposing a line on the animal as shown by the green line. The same was performed on the visible oil-sac. In addition were the outline area of the oil-sac traced in order to estimate the volume.

2.3.4 Dry weight determination

The animals were dried in a heating cabinet at 60°C for 24 hours as described by Williams and Robins [1982]. The weight were determined on a micro-scale weight (Mettler Toledo, checked monthly, at 10 mg the acceptable deviation is 0.0008 mg). To reduce potential gaining of weight due to air humidity, the samples were stored in a desiccator between each measurement.

Two control weights were performed with one hour in the heating cabinet between each weighing in order to confirm the stability of the values. Dry weights were determined by subtracting the weight of the tin capsules.

2.3.5 Energy equivalents and SfG calculation

It was assumed that the carbon content in our strain of *R.baltica* were 45 percent of dry weight Kiørboe et al. [1985]. Due to the lack of egestion and excretion data the assimilated food was assumed to be 80 percent of the dry weight normalized ingestion value, as this is considered a general assimilation efficiency level for herbivorous feeding copepods Båmstedt et al. [1999]. The energy assimilated from the algae ingested was based upon assumed content of protein, lipids, and carbohydrate as respectively 50, 10, and 20 percent of dry weight Båmstedt et al. [1999], with energy equivalents of 24.0, 39.5 and 17.5 kJ g^{-1} for protein lipid, and carbohydrate respectively Hoegh-Guldberg and Emlet [1997].

The energy used in aerobic respiration were calculated into energy equivalents assuming 484 μJ nmol^{-1} O_2, according to Stumpp et al. [2011].

SfG was calculated by:

$$SfG(Joule * mgDW^{-1} * hour^{-1}) = A - R \tag{2.14}$$

Where A is the assimilated energy, and R is the energy lost due to respiration.

2.4 Statistics

The Median development times for the different moulting stages were analysed by one-way ANOVA. Any significant results were analysed with TukeyHSD in order to assess which treatment group that differ.

The measurements and estimates of dry weight, volume of the oil sac, body length, filtering rate, ingestion rate, oxygen consumption and Scope for Growth were analysed by a three factor nested ANOVA . Significant differences between treatment groups were extracted by setting the control group as a treatment contrast in the analysis.

All sorting of data into appropriate datasets were performed in *Microsoft excel (version 2010)*, while the analysis were performed in *R version 3.0.0 (2013-04-03)*, R Core Team [2013]. The statistical method, and R packages used for analysis of the results from measurements on stage C5 from the second generation are described in Appendix A. The significance level for all tests were set at 0.05.

Chapter 3

Results

3.1 Experimental conditions

3.1.1 Water parameters

pH, temperature and total alkalinity measured during the first and second generation are listed in table 3.1 and 3.2 respectively. These values were used to calculate the respective pCO_2 (μatm) also included in the tables. The calculated pCO_2 values differed from the aim concentrations for all the treatments, but are refereed to as their aim values in this text.

TABLE 3.1: Water parameters (mean$\pm SD$) calculated from samples taken from all experimental tanks across the duration of **first generation** of the copepods

Treatment	pH_T	Temp (°C)	A_T	pCO_2 (μatm)
380 ppm	8.00±0.01	9.75±0.15	2269.44±21.78	437.14±16.94
1080 ppm	7.64±0.03	9.89±0.15		1102.34±76.61
2080 ppm	7.33±0.06	9.87±0.15		2306.78±303.17
3080 ppm	7.15±0.03	9.68±0.12		3501.99±261.86

TABLE 3.2: Water parameters (mean±SD) calculated from samples taken from all experimental tanks across the duration of **second generation** of the copepods

Treatment	pH_T	Temp (°C)	A_T	pCO_2 (μatm)
380 ppm	8.02±0.01	9.79±0.10	2245.07±16.36	421.25±16.34
1080 ppm	7.66±0.05	9.94±0.10		1051.89±110.73
2080 ppm	7.39±0.04	9.94±0.09		2019.99±190.04
3080 ppm	7.16±0.08	9.79±0.09		3481.81±542.13

3.1.2 Feeding conditions

The algae concentration μC L^{-1} of the different treatments for each measurement (mean±SD, n=3) are shown in figure 3.1. There were some differences in the concentrations between the different treatment groups. While no differences were found at the time of the nauplii stages, the highest exposure group had a significantly higher algae concentration than the control treatment (P=0.007), later into the experiment where the majority of the animals consisted of copepodites (day 20 to day 76 in the first generation). An approximate estimate for the duration of the nauplii stages was derived from the estimated MDT value of development stage C1, while the rest of the duration were assumed to be mainly composed of copepodites.

The same trend seemed to be present in the second generation. Here were also a significant higher algae concentration found in the highest CO_2 treatment group (P=0.027), in the assumed duration where copepodites dominated the stage compositions (day 100 to day 160). Means and standard deviation over all measurements are shown in figure 3.2.

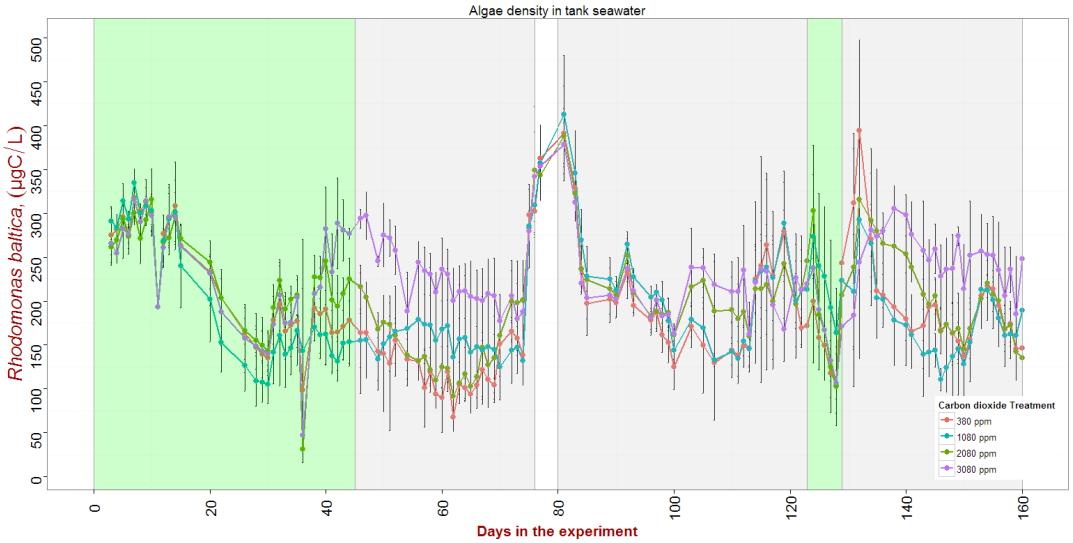

FIGURE 3.1: Concentration of *Rhodomonas baltica* μC L^{-1} during the experiment (mean$\pm SD$, n=3). vertical lines represents the duration of the first and the second generation of the studied *C. finmarchicus*. Green areas represents the duration of the sampled development data from the first generation (left), and the duration of the feeding and oxygen consumption measurements of C5 copepodites in the second generation (right).

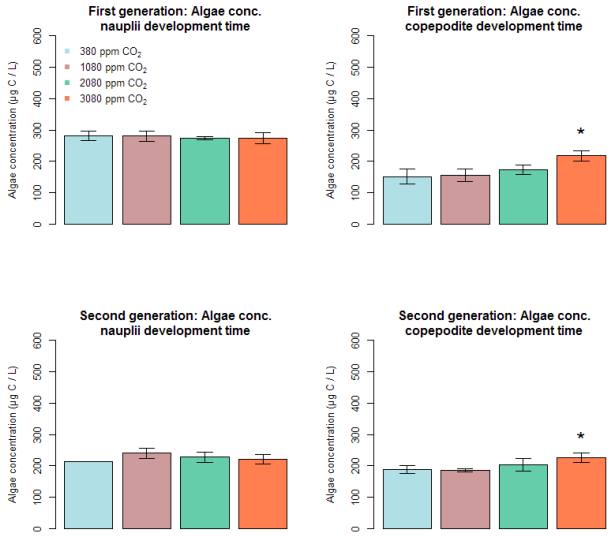

FIGURE 3.2: Algae concentrations (mean$\pm SD$, n=3) in the treatment groups during the approximate nauplii development time (day 0 to day 20)(left), and copepodite development times (day 20 to day 76)(right), for the first (top), and second (bottom) generations (nauplii: day 80 to day 100, and copepodites: day 100 to day 160). No differences were detected during the development of nauplii stages. There were found a significant difference in the algae concentrations during the copepodite development times for both the first ($F_{3,8}$=7.389 P= 0.011) ,and the second generation ($F_{3,8}$= 5.087 P=0.029). The highest CO_2 treatment had a significantly higher concentration compared to the control treatment in the first (P=0.007) and the second generation(P=0.027), determined by multiple comparisons of means: Dunnett Contrasts. No other treatment groups differed significantly from control.

3.2 Stage development of the first generation of exposed *C. finmarchicus*

3.2.1 Naupliar median development time

The estimated median development times for the nauplii stages (N1 to N6) from the first generation of the studied *C.finmarchicus* are shown in figure 3.3.

The time for 50% of the cohort to develop past the N5 stage was found to be on average 2.42 days later for the 3080 ppm CO_2 than the control treatment group. A near significant difference was found for the development past this stage ($F_{3,8}$=3.918, P=0.054) between the treatment groups, and a significant difference between 380 ppm CO_2 and 3080 ppm CO_2 was detected (P=0.043).

The development past the stage N6 were significantly different between the treatments ($F_{3,8}$=4.781, P=0.034), with a significant difference between the control and 2080 ppm CO_2 group(P=0.031). The MDT for this stage was on average 2.50 days later in the 2080 ppm CO_2 than in the control group

The results points to a later time before molting into the last few nauplii developmental stages in the highest two CO_2 treatments.

3.2.2 Copepodite median development time

The estimated median development times for the copepodite stages (C1 to C5) from the first generation of the studied *C. finmarchicus* are shown in figure 3.3. The results were analyzed the same way as for nauplii stages. A significant difference in MDT was detected between the treatments in stage C4 ($F_{3,8}$=5.361, P=0.0257). In Tukey HSD differences were found between the treatments 380 ppm CO_2 and 2080 ppm CO_2 (P=0.019). The time for 50% of cohort to develop past stage C4 was on average 4.14 days later in the 2080 ppm CO_2 than in control.

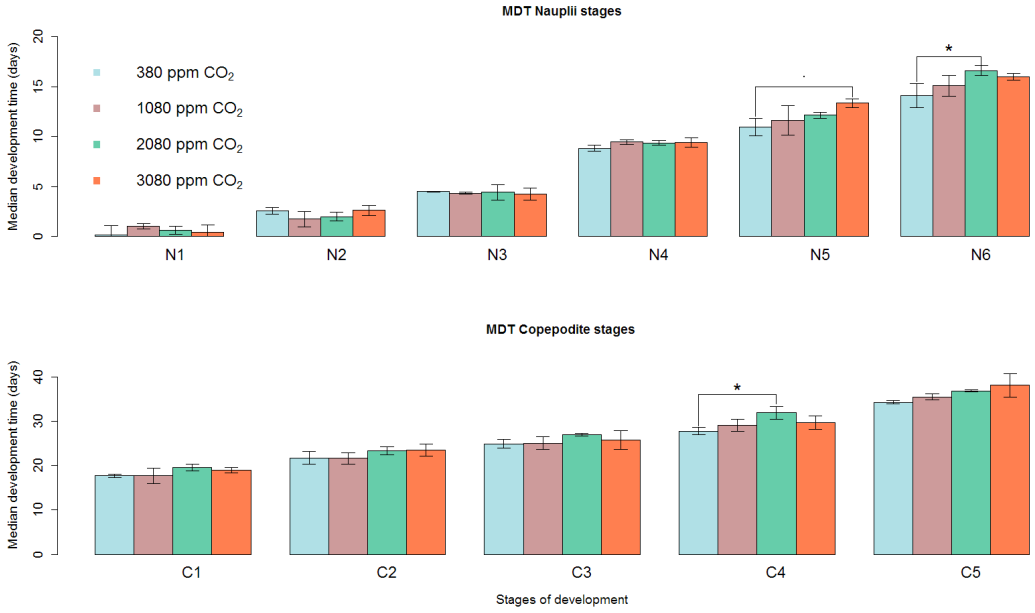

FIGURE 3.3: Median development times for the nauplii stages (top), and copepodite stages (bottom), (means$\pm SD$, n=3). A later MDT was detected in the nauplii stages N5 and N6 for the highest CO_2 treatment groups than the control group, only the result from the N6 stage were statistically significant. In copepodite stages a significant later MDT value was detected for the 2080 ppm treatment group compared to control.

3.3 Energy balance of CO_2 exposed C5 copepodites (second generation)

3.3.1 Impact on body mass, length, and energy stores

The measurements of dry weight, body length, and the calculated estimate of their oil-sac volumes are summarized in table 3.3.

Compared to control (380 ppm CO_2) the mean dry weight were higher in the lowest CO_2 group, while the higher concentration groups had a lower mean dry weight. The difference in dry weight between the treatments were not found to be significant ($F_{3,8}$=2.97, P=0.097).

The volume of the oil-sac were significantly larger in the 1080 ppm CO_2 group (P=0.023). In the 3080 ppm CO_2 group the mean volume of the oil sac were smaller compared to control, this was found to be close to significant (P=0.0504).

The mean of the measured body lengths were shorter in all CO_2 treatments than in control, this was not found to be significant ($F_{3,8}$=3.68, P=0.063).

TABLE 3.3: Dry weight, body length and estimated volume of oil sac(means and standard deviations) in the stage C5 copepodites from all experimental tanks across the duration of the grazing and oxygen consumption measurements of the second generation of CO_2 exposed *C.finmarchicus*.

Treatment (CO_2)	Dry weight (mg)	Body length (mm)	Volume oil sac (mm^3)
380 ppm	0.193±0.056	2.229±0.235	0.082±0.048
1080 ppm	0.199±0.084	2.110±0.167	0.108±0.079
2080 ppm	0.183±0.046	2.140±0.139	0.080±0.045
3080 ppm	0.136±0.042	2.080±0.211	0.041±0.026

3.3.2 Impact on filtering and ingestion rates

Filtering (or clearance) rate (ml copepod^{-1} hour^{-1}) for the animals in the different treatments are shown in figure 3.4 (means±SD, n=3).

The mean filtering rate were higher in the 1080 ppm CO_2 group and lower in the 2080 and 3080 ppm CO_2 groups than in the control group, following the same trend as for the dry weight, volume of oil sac and to a degree also the body length measurements. The treatments were however not found to have an significant impact on the filtering performance ($F_{3,8}$= 3.80, P=0.058).

The Ingestion rates were calculated using the estimated filtering rate values. Estimated cells eaten were transformed into carbon equivalents and normalized by their dry weights. A significant difference were found between the treatments ($F_{3,8}$=4.526,P=0.039), and a significant higher ingestion rate was found in the 1080 ppm CO_2 group compared to the control treatment (P=0.011).

Negative filtering rates and therefore also ingestion rates were obtained in one of the animals from the 380 ppm , one animal in the 1080 ppm, and in three of the animals in the 3080 ppm CO_2 group. All values were included in the analysis.

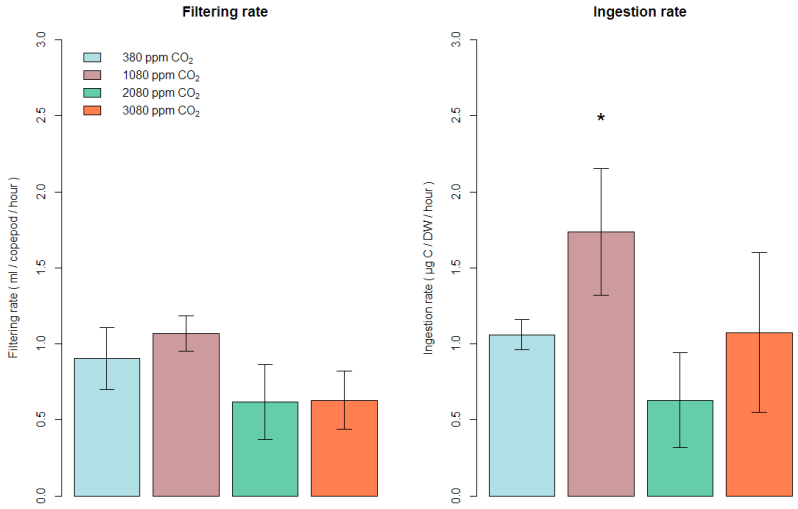

FIGURE 3.4: Filtering rates (ml copepod^{-1} hour^{-1}) (left) and ingestion rates (μg C DW^{-1} hour^{-1})(right) in the different treatment groups (means$\pm SD$, n=3). No significant differences were detected in the filtering rates, but the ingestion rate was found to be significantly higher in the 1080 ppm CO_2 group.

3.3.3 Impact on aerobic metabolic rates

Oxygen consumption rates (μg O^2 mg DW^{-1} hour^{-1}) are shown in figure 3.5. The mean oxygen consumption increased with increasing CO_2 concentrations. No significant differences between the treatment groups were detected ($F_{3,8}$=3.35 ,P=0.076).

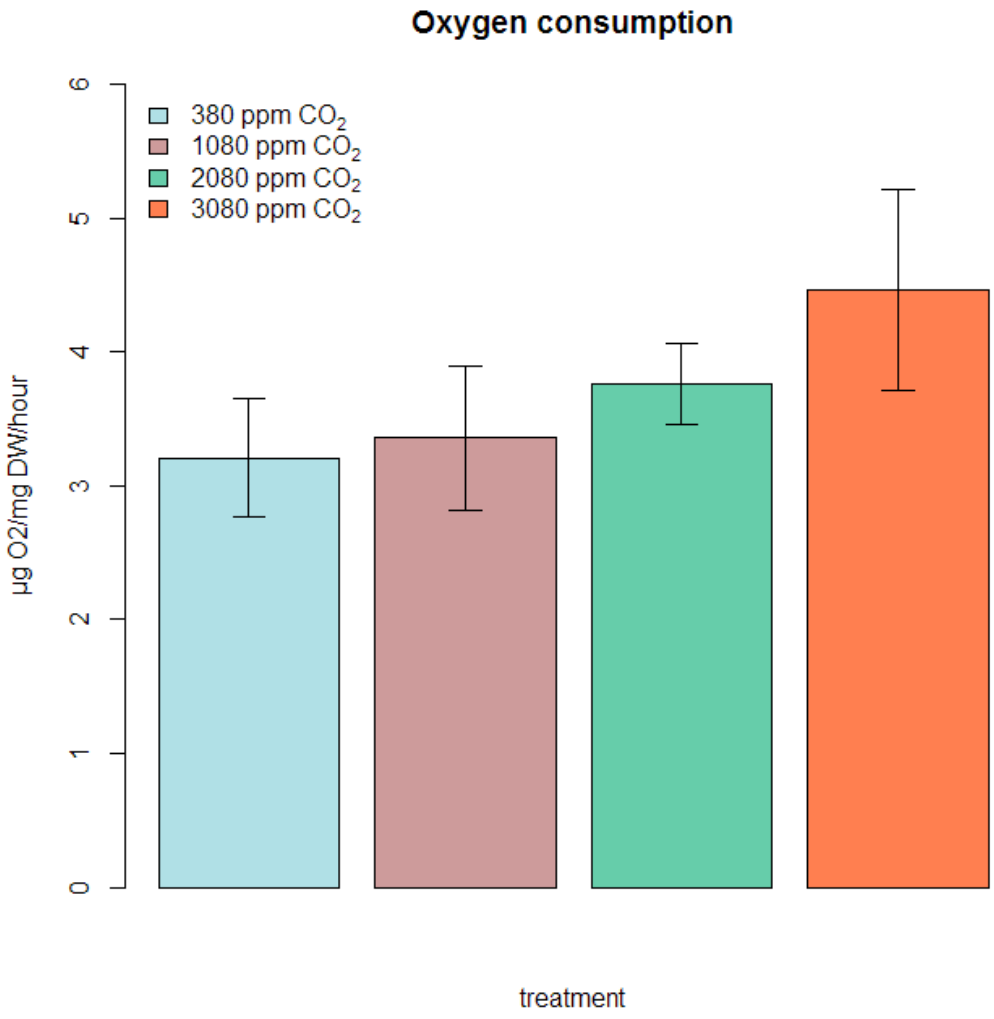

FIGURE 3.5: Oxygen consumption (μg O^2 mg DW^{-1} $hour^{-1}$),in *C. finmarchicus* stage C5,(means$\pm SD$, n=3). The mean values display a concentration dependent increase in oxygen consumption. No significant differences were detected.

3.3.4 Impact on Scope for Growth (SfG)

The result of SfG (Joule mg DW^{-1} $hour^{-1}$) estimated using energy intake through feeding and respiratory energy loss versus dry weight of the copepods are shown in figure 3.6. A negative SfG was detected in the control group as well as in the 2080 and 3080 ppm CO_2 groups. This indicates a depletion in energy reserves in *C. finmarchicus* measured in those groups.

A positive SfG was detected in the 1080ppm group. The difference between the treatment groups were significant ($F_{3,8}$=21.72, P=0.0003), and all elevated CO_2 concentrations were significantly different from the control treatment, 1080 ppm (P=0.0001), 2080 ppm (P=0.0272) and 3080 ppm (P=0.0179) .

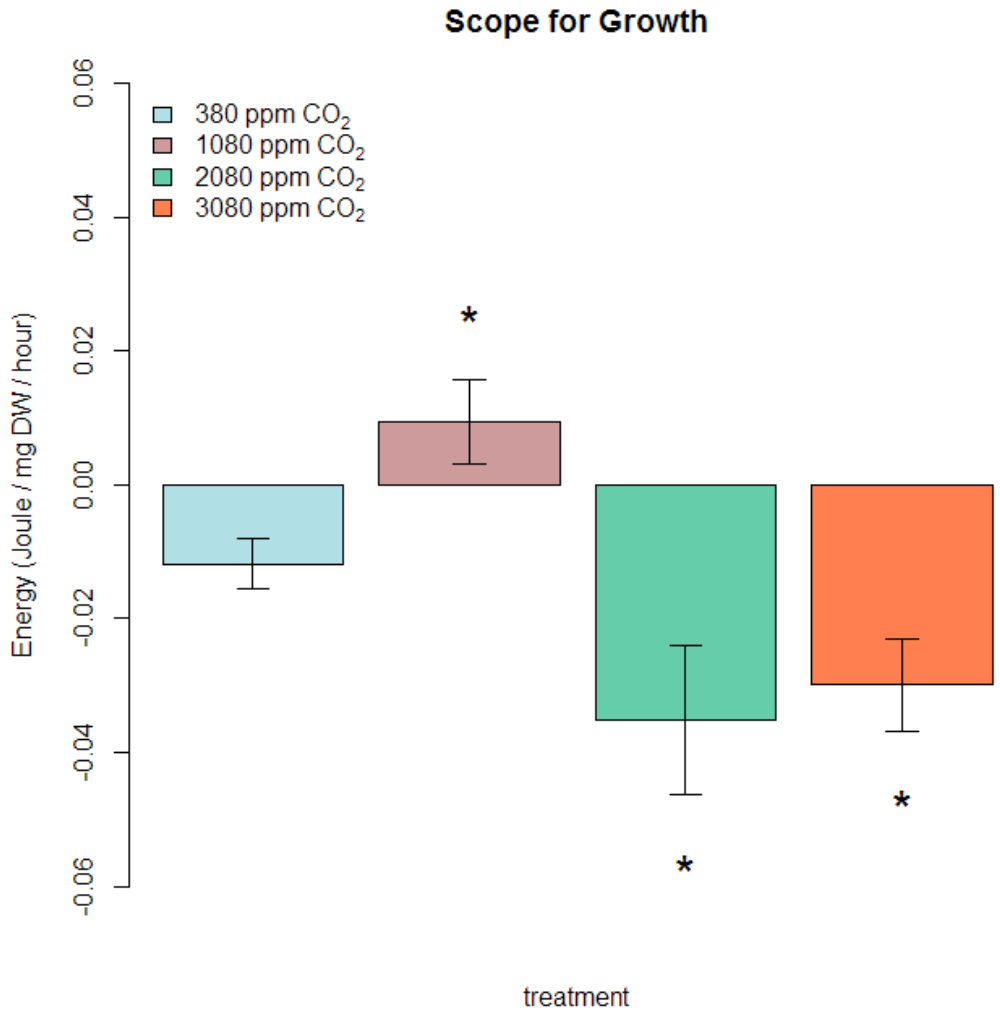

FIGURE 3.6: Scope for Growth (Joule mg DW^{-1} $hour^{-1}$), in *C. finmarchicus*, (means$\pm SD$, n=3). All elevated CO_2 treatment groups differed significantly from the control treatment. A higher SfG was obtained in the 1080ppm group (P=0.0001), and a lower SfG was obtained in the 2080 ppm (P=0.0272) and 3080 (P=0.0179) ppm CO_2 groups.

Chapter 4

Discussion

4.1 Energetic effects of elevated CO_2 on stage C5 *C.finmarchicus*

The different measurements and calculated estimates performed in this experiment indicates a general pattern of responses to the treatments. The animals exposed to the lowest elevated CO_2 treatment seemed to perform better than those exposed to higher concentrations. In some of the measurements this treatment group even seemed to perform better than the control group. Indicating a tolerance for moderate elevation in CO_2 in this development stage of *C.finmarchicus*. The higher concentrations of CO_2 seemed to have a more negative impact on the measured and estimated parameters in the animals.

Even though not all of the tested parameters showed a statistical difference compared to control in this experiment, they all seemed to follow the same pattern, indicating an overall adverse effect with respect to energy balance in animals in the 2080 ppm and 3080 ppm CO_2 groups.

4.1.1 Feeding rate

The filtering rates and ingestion rates obtained were within values previously reported for *C. finmarchicus* Mauchline [1998], Mayor et al. [2006]. Some of the filtering values were negative. This may represent an artifact as has been obtained in other feeding

experiments, and can occur when the net growth of feeding cells is greater in an experimental bottle relative to control and may have resulted in an underestimation of the ingestion rates Mayor et al. [2006]. In 3 of the 5 obtained negative values the animals were also identified as being adults after the experiment. The process of molting may have influenced their feeding performance.

A higher feeding rate has previously been demonstrated as a response to elevated CO_2 exposure in copepods. In a short term exposure study Li and Gao [2012] found that the feeding rate increased in the calanoid copepod *Centropages tenuiremis* at a moderate elevated CO_2 (pH 7.8). They suggested that this may be explained by an increase in food acquisition to compensate for a higher energy demand for maintainance of intracellular acid-base balance, evident from an increased aerobic metabolic rate.

In the results from our experiment it was also found a significantly higher ingestion rate in the 1080 ppm CO_2 group, and may demonstrate an increased food demand for compensatory processes due to the exposure. However it was not found any clear evidence for extra energy expenditure in this group from the result of the SfG estimates. Animals from the 1080 ppm CO_2 group group had also a significantly larger volume of their oil-sac indicating a good capacity for accumulating nutrients to form energy stores. This may indicate an excess of energy in spite of the potential stress due to this CO_2 treatment.

All though not significant the results may point to a lower filtering and ingestion rate in the two highest CO_2 treatment groups compared to the control. The feeding rate in *C. finmarchicus* has been found to correlate with the amount of food available Mayor et al. [2006], and it may therefore be expected that the feeding rate increased in the highest exposure group since these tanks had a significantly higher algae density. It may also be expected that these animals would increase their food acquisition in order to cope with the stress elicited by the CO_2 exposure Li and Gao [2012]. The results obtained in our experiment may indicate the contrary to be the case for the highest two CO_2 treatment groups. One possible explanation for this may be that the stress adversely affected their ability to obtain food due to an exceeded time and/or concentration dependent threshold for CO_2 toxicity Pörtner et al. [2005].

A decreased feeding rate in crustaceans has been reported as a response to toxic exposures. In a study of the crustacean mysid *Neomysis integer* Verslycke et al. [2004] found

a significant decrease in feeding rate as a response to high chlorpyrifos concentrations. A suppressed feeding has also been found as a response to acidified seawater in three species of bivalve mollusks at a pH value of 7 Bamber [1990]. In sea urchin larvae exposed to simulated ocean acidification Stumpp et al. [2011] found a reduction in feeding rate in a high pCO_2 treatment. They explained this as a possible inhibition of growth in their ciliated feeding structures due to the exposure.

Copepods may be more tolerant against calcification-related effects of CO_2 exposure. This is because they possess a chitinous exoskeleton rather than a aragonite or calcite shell Fitzer et al. [2012]. A potential for CO_2 exposure to impair the growth of mineralized chitinous exoskeleton structures has however been found in other crustaceans Whiteley [2011]. Among the large variation in mineral composition in different crustaceans a more soluble calcium carbonate component (amorphous calcium carbonate) has been found Neues et al. [2007]. This mineral has been suggested to act as a source for bicarbonate to acid-base buffering Whiteley [2011].

The formation of calcium carbonate is thought to depend on maintainance on an more alkaline pH in the exoskeleton compartment relative to the heamolymph. A lower pH in this compartment due to elevated influx of dissolved CO_2 may influence the precipitation of calsium carbonate. It is also possible that post-moult calcification could be disrupted since this process is dependent on a large uptake of calcium and bicarbonate ions from the seawater. This ion influx is sensitive to an increase in the concentration of external hydrogen ions because this reduces the bicarbonate uptake Whiteley [2011].

Post-moult delay has been observed in the crab *Callinectes sapidus* as a response to hypercapnia, explained by an impaired uptake of external bicarbonate Cameron and Wood [1985]. A shortening of antennae was observed in the shrimp *Palaemon pacificus*, and this was attributed to a dissolution of calcium carbonate stores, followed by a disruption of acid-base homeostasis after long term CO_2 exposure Kurihara et al. [2008].

As a filter feeder *C. finmarchicus* is dependent upon the movement of mouth parts to set up water currents for obtaining food items Marshall and Orr [1972]. If an impaired structure or function of components in the filtering apparatus is a consequence of high CO_2 exposure, it could potentially reduce the food acquisition in the animals.

A lower feeding rate may also partly explain the observed increase in the algae concentrations in the highest CO_2 treatment tanks. However, this increase may also have been caused by a lower density in the tanks due to mortality. Lethal effects of of the treatments were not measured, but by visual inspection of the tanks there were signs of a lower animal density in the highest two CO_2 treatment tanks.

The CO_2 concentrations had been determined based upon their possible sub-lethal effects. The highest concentration (3080 ppm) did not cause decreased survival in eggs or nauplii stages in an earlier study performed at SeaLab on animals obtained from the same original culture as was used in our experiment Våge [2011]. This study was however more short time based, and lethal effects due to a longer lasting exposure situation may not be ruled out.

Exposure of adult *C. finmarchicus* to concentrations as high as 5000 to 8000 ppm CO_2 did not reduce their survival in a study by Mayor et al. [2007].

If the feeding rate is altered as some of our findings indicates, it may have serious consequences for the balance of phytoplankton production and zooplankton grazing in marine ecosystems, at similar environmental conditions.

4.1.2 Aerobic metabolic rate

The oxygen consumption measured is close to previously reported values in stage C5 of *C. finmarchicus* Marshall and Orr [1972]. While the mean values points to an CO_2 concentration dependent increase in the dry weight normalized oxygen consumption rate, no significant differences were found between the treatments. A higher energy utilization may be a result from increased cellular regulatory activity to maintain proper acid-base conditions.

The dry weights are likely to have influenced the observed pattern as seen in the mean values, since the oxygen consumption is related to the mass of the animal. Copepod respiration rate has been found to increase with increasing body weight Almeda et al. [2011].

4.1.3 Scope for Growth

Sub-lethal processes involved in reproduction and development has been considered to be particularly vulnerable to ocean acidification effects Fitzer et al. [2012]. Calculations of SfG provides an estimate for the available energy for such processes,and may therefor serve as a useful biomarker to access effects from CO_2 exposure. It is important to investigate such effects since they may have the potential to alter the balance in marine ecosystems through reduced fitness in individuals and populations Fitzer et al. [2012].

The significant results of a lower SfG in the two highest CO_2 treatments points to a depletion of their energy reserves. The estimated lower oil-sac volumes for these animals may also support this to be the case. They also showed a tendency for a lower body mass and length in these measurements despite a higher level of food available.

The egg production and hatching success of the developing females in the cohorts were studied by sets of separate experiments performed by Tagliati [2013]. She found a significantly lower rate of eggs produced in the highest CO_2 treatment, and a tendency for a concentration dependent decrease in hatching success between the treatments were observed. Her results thereby supports the present indication of a reduction in the available energy for reproduction at the highest CO_2 treatments in this experiment. A reduced hatching success may serve as an explanation for the observed lower animal density in those treatment tanks.

The excretion of the animals were not measured in this experiment, and a different pattern may have been reviled if this had been performed. The lack of incorporation of excretion values may have lead to an overestimation of SfG Stumpp et al. [2011].

Potential stress during the incubation in small containers may have influenced the feeding behavour and oxygen consumption results Mauchline [1998]. The volume of the respiration champbers were small, and an potential stress from interactions with the chamber walls may have elevated their respiratory rate, and given a decrease in the SfG values. This may explain the negative SfG found in the control group.

Copepods are regarded as being mainly ammonotelic animals, that means that their main excretion product is ammonia. Ammonia is known to have toxic properties, and its speciation is dependent on pH and temperature. Dependent on the animal density and the replacement of the experimental water the ammonia production may therefore

have elicited a stress upon the animals. Ionized ammonia (NH_4^+) (or ammonium) is lower at low pH, and has been found to be less toxic, compared to the unionized form (NH_3) Sullivan and Ritacco [1985]. The lower pH may therefore have played a protective role against this potential stress factor, and may have resulted in a better energetic performance in the 1080 ppm CO_2 treatment group (personal communication, Reseacher S.A. Pedersen, Department of Biology, NTNU). The levels of ammonia were unfortunately not measured in our system.

It should also be noted that the values of the energy equivalents were based upon some assumptions on the carbon content and nutrient composition in the *Rhodomonas baltica*, as well as metabolic energy utilization in form of oxygen, that were not measured for this particular case, but based upon literature values.

4.1.4 Development time in long term elevated CO_2 exposed *C.finmarchicus*

The results points to a longer median development time for the highest CO_2 treatment group, at least for some of the developmental stages. The results from the energetics measurements points to a energy depletion in those groups and delayed development may be a result of the energetic cost to cope with the stress of elevated CO_2 exposure.

The development and the process of moulting is energy demanding Mauchline [1998]. It is therefor likely that a depletion of energy reserves could result in a slower stage development rate as has been observed in this experiment. Similar results were obtain from the study of the development rate in the second generation, where a significant slower development were found in the highest CO_2 exposure group in the stages N5, N6 and C1 , compared to the control treatment Gustavson [2013]. No obvious differences between the generations with respect to stage development rate were detected.

Shorter intervals between each sample may have resulted in a better estimate for the development time for some of the stages. The median development time for stage N1 (estimate for egg duration) were probably poorly estimated due to a too long time before the first samplings. Because of the sampling was terminated before all animals had been developed into adults an estimate of the generation time was not possible to obtain. A more optimal procedure may have been to monitor the development more closely during the experiment, to ensure that samples near critical times was taken.

The size of the starting cohort (approximately 2050 eggs) may have been in the short range to reach an adequate density and sample sizes large enough to meet the assumptions for the method Hu et al. [2007]. Based on this the MDT values may only be regarded as a relative measure to detect treatment effects, and not an estimate of the actual development times of the animals.

To investigate responses to environmental changes it is important to place the exposure levels in a context with regional and seasonal projections, and with respect to current environmental conditions in the habitat Byrne [2011]. The highest CO_2 concentration used in this experiment may exceed the highest projections for the year 2300 with respect to atmospheric level under modeled "business-as-usual" scenarios Caldeira and Wickett [2003]. However such concentrations may represent a "worst case scenario" Mayor et al. [2007] and may also occur locally e.g. due to leakage from man made or natural CO_2 storage reservoirs Hawkes et al. [2005].

Since the main periods of egg production and growth of *C. finmarchicus* is tuned to match algae development, a change may result in a mismatch it timing between trophic levels. This may lead to changes in structure and/or function of the marine ecosystem, e.g in the Norwegian sea in which *C. finmarchicus* has been regarded as the most important zooplankton species Bagøien et al. [2012].

4.1.5 Conclusion

The results presented in this thesis points to energy depletion in *Calanus finmarchicus* due to continuously exposure to 2080- and 3080 ppm CO_2 over the course of two generations. As the estimated Scope for Growth were negative, and also significantly lower compared to the control treatment.

Adverse effects due to energy depletion in those treatments may have been manifested in reduced amount of energy stores as indicated by their close to significantly smaller oil-sac volumes. Although not significant the presented results may also have revealed a tendency for lower body mass as measured by their dry weights, and reduced growth indicated by the body length measurements, as compared to control.

The findings of slower development rate as were observed in some of the molting stages. And the lower reproductive output found in the adult females Tagliati [2013] cultured

in the 3080 ppm CO_2 treatment, supports presented the findings since these processes are influenced by the energy balance in the copepods.

Energy depletion is likely caused by decreased food intake, and/or increased metabolic expenditure, as indicated by some of the feeding and oxygen consumption measurements. This response may have been a result of an allocation of energy to fuel processes related to maintenance of proper acid-base homeostasis challenged by hypercapnia.

C. finmarchicus seemed to be more tolerant to exposure to 1080 ppm CO_2, and no indication of adverse effects on the energy balance was found at this level of exposure. In this treatment group the animals were found to have available energy reserves for growth and reproduction. This is supported by larger estimated oil sac volumes, and a tendency for higher dry weight values compared to the control treatment. Eventual extra energy demand for acid-base regulation due to elevated CO_2 was likely compensated for by increased energy intake through feeding in this treatment group.

No indication of increased adaptation to the exposures over two consecutive generations was found.

The present results may contribute to the work of elucidate the potential response of *C. finmarchicus* to the ongoing elevation of ocean CO_2. They may also serve as baseline data for further studies of effects of CO_2 combined with other environmental stress factors such as increased temperature or pollutants.

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Appendix A

Statistical analysis of energetic measurements on copepodite stage C5

The primary interest was to investigate whether there were any effects of the CO_2 treatments on the measured and calculated parameters that indicates possible impacts on the animals energy balance. Individual sampling days and readings as well as the individual experimental tanks may have contributed to variability with respect to measurements that could potentially have masked the ability to detect an impact of treatments (e.g. different age of copepods, available food or temperature conditions).

The data were therefore analyzed by applying a balanced three factor nested ANOVA as described by Logan [2011]. The different parameters had been measured by three separate readings on two different days of sampling from each of three individual experimental tanks per one of four different treatments.

The treatments represent a fixed factor (Factor A). The replicate experimental tanks represent a random nesting factor (Factor B), and are the replicate for the treatment effects. The day of sampling represents an additional random nesting factor (Factor C). The measurements of triplicate *C.finmarchicus* are the units of replication for the day of sampling.

The following describes the analysis of the dry weight measurements and illustrates the method whereby all the parameters were analyzed in this experiment (bodylength, volume oil sac, filtering rate, ingestion rate, oxygen consumption and Scope for Growth).

The following table A illustrates the conseptulised hierachial structure of the dataset of the dryweight measurements and the nesting order of the factors. Treatment 1 is the control treatment, 2, 3 and 4 is the increasing CO_2 treatment concentrations. Tank 1 to 3 represents the replicated tanks, and day is a factor with 2 levels and represents the day of the sampling for the experiment.

			replicate	1	2	3
Treatment	Tank	Day				
1	1	1		0.2070	0.2194	0.1053
		2		0.2199	0.2604	0.1642
	2	1		0.1993	0.1900	0.1681
		2		0.1401	0.2600	0.2084
	3	1		0.1350	0.2715	0.2487
		2		0.2126	0.2026	0.0582
2	1	1		0.2507	0.1232	0.1310
		2		0.0869	0.1269	0.2205
	2	1		0.1723	0.2513	0.3124
		2		0.2453	0.2555	0.2849
	3	1		0.3032	0.3247	0.1261
		2		0.0429	0.1306	0.1908
3	1	1		0.1710	0.1915	0.1804
		2		0.1586	0.1609	0.2270
	2	1		0.1915	0.2385	0.1705
		2		0.2540	0.0821	0.1813
	3	1		0.2304	0.2376	0.1921
		2		0.1742	0.1704	0.0875
4	1	1		0.0723	0.1390	0.0870
		2		0.0914	0.1606	0.0801
	2	1		0.1826	0.1584	0.1154
		2		0.2213	0.1633	0.1221
	3	1		0.1701	0.1094	0.1757
		2		0.1493	0.1573	0.0923

The total sum of squares was calculated by A.1

$$SST = \sum y^2 - \frac{[\sum y]^2}{72} \tag{A.1}$$

$SST = 2.560002 - 12.797^2/72 = 0.285513$

Where y is the individual measured values of the dry weight from all off the 72 *C.finmarchicus* examined in the experiment.

The sum of squares for the treatments, SSA, was calculated as the sum of the four treatments . Each were the sum of 18 numbers (3 sub replicates x 2 sampling days x 3 replicate tanks). The subtotal square was therefore divided by 18 before the correction factor were subtracted A.2 and A.3.

$$SSA = \frac{\sum T^2}{18} - \frac{[\sum y]^2}{72} \tag{A.2}$$

$$SSA = \frac{3.4707^2 + 3.5792^2 + 3.2995^2 + 2.4476^2}{18} - \frac{12.797^2}{72} = 0.04405972 \tag{A.3}$$

The sum of square for the difference between the tanks was calculated by A.4:

$$\frac{sum(tapply(DRY, list(Treatment, tank), sum))^2}{6} = 2.358103 \tag{A.4}$$

where "DRY,list(Treatment,tank),sum" is the R code that gave the sum of all the values within each of the 12 tanks. The sum was divided by 6 because each value was the sum of 3 measured values x 2 sampling days.

The sums of squares for the sampling days were similarly calculated A.5. Dividied by three because each value is the sum of three values from the sub replicated *C.finmarchicus*.

$$\frac{sum(tapply(DRY, list(Treatment, tank, day), sum)^2)}{3} = 2.403678 \tag{A.5}$$

The corrected sums of squares for each of the nested factors were calculated using the value from the next scale above in the model hierarchy.

$$SS_{TANKS} = \frac{\sum TANKS^2}{6} - \frac{\sum Treatment^2}{18} \tag{A.6}$$

$SS_{TANKS} = 2.358103 - 2.318549 = 0.039554$

$$SS_{DAYS} = \frac{\sum DAYS^2}{3} - \frac{\sum TANKS^2}{6} \tag{A.7}$$

$SS_{DAYS} = 2.403678 - 2.358103 = 0.045575$

$$SS_{Calanus} = \frac{\sum Calanus^2}{1} - \frac{\sum DAYS^2}{3} \tag{A.8}$$

$SS_{Calanus} = 2.560002 - 2.403678 = 0.156324$

The values were used to fill in the following ANOVA table.

Source	SS	d.f	MS	F	Critical F
Treatment	0.04405972	3	0.01468657	2.970434	4.066181
Tanks in treatments	0.039554	8	0.00494425	1.301832	2.848565
Days in tanks	0.045575	12	0.003797917	1.166168	1.960121
Calanus in days	0.156324	48	0.00325675		
Total	0.285513	71			

From this table we can see that the F test for treatment effect: $\frac{0.01468657}{0.00494425} = 2.970434$ were not significant, ($P = 0.09698285$). The same conclusion can be drawn for differences between tanks within treatments:$\frac{0.00494425}{0.003797917} = 1.301832$, ($P = 0.3281844$), and days within tanks:$\frac{0.003797917}{0.00325675} = 1.166168$, ($P = 0.3335043$).

Linear models were fitted to test the null hypothesis of no effect due to the treatments, and no added variance due to tanks within treatments and sampling days within tanks within treatments.

The control treatment (380ppm) were defined as a treatment contrast to assess any significant result in the elevated CO_2 treatments. Any significant results between the treatments were ignored if the overall results were not significant (e.g. TREAT: 380ppm vs 3080ppm in test of effect on dry weight A.1).

A.1 ANOVA tables

The following tables shows the summary statistics of possible effects of the treatments on dry weight A.1 , volume of the oil sac A.2, body length A.3, filtering rate A.4, ingestion rate A.5, oxygen consumption A.6, and Scope for Growth A.7.

TABLE A.1: Dry weight (n.s.)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	0.04	0.01	2.97	0.0970
TREAT: 380ppm vs 1080ppm	1	0.01	0.01	2.16	0.1796
TREAT: 380ppm vs 2080ppm	1	0.00	0.00	0.87	0.3788
TREAT: 380ppm vs 3080ppm	1	0.03	0.03	5.88	0.0415 *
Residuals	8	0.04	0.00		
Residuals	12	0.05	0.00		
Residuals1	48	0.16	0.00		

TABLE A.2: Volume of oil sac

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	0.04	0.01	4.87	0.0326 *
TREAT: 380ppm vs 1080ppm	1	0.02	0.02	7.91	0.0228 *
TREAT: 380ppm vs 2080ppm	1	0.00	0.00	1.42	0.2668
TREAT: 380ppm vs 3080ppm	1	0.01	0.01	5.29	0.0504 .
Residuals	8	0.02	0.00		
Residuals	12	0.03	0.00		
Residuals1	48	0.14	0.00		

TABLE A.3: Body length (n.s.)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	0.22	0.07	3.68	0.0625
TREAT: 380ppm vs 1080ppm	1	0.02	0.02	1.02	0.3411
TREAT: 380ppm vs 2080ppm	1	0.00	0.00	0.12	0.7424
TREAT: 380ppm vs 3080ppm	1	0.20	0.20	9.89	0.0137 *
Residuals	8	0.16	0.02		
Residuals	12	0.36	0.03		
Residuals1	48	1.82	0.04		

Table A.4: Filtering rate (n.s)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	2.61	0.87	3.80	0.0582
TREAT: 380ppm vs 1080ppm	1	1.66	1.66	7.24	0.0275 *
TREAT: 380ppm vs 2080ppm	1	0.27	0.27	1.18	0.3097
TREAT: 380ppm vs 3080ppm	1	0.68	0.68	2.98	0.1224
Residuals	8	1.83	0.23		
Residuals	12	6.57	0.55		
Residuals1	48	24.35	0.51		

Table A.5: Ingestion rate (dry weight specific)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	11.33	3.78	4.53	0.0390 *
TREAT: 380ppm vs 1080ppm	1	9.02	9.02	10.81	0.0111 *
TREAT: 380ppm vs 2080ppm	1	2.31	2.31	2.77	0.1348
TREAT: 380ppm vs 3080ppm	1	0.00	0.00	0.00	0.9606
Residuals	8	6.68	0.83		
Residuals	12	25.38	2.12		
Residuals1	48	60.02	1.25		

Table A.6: Oxygen consumption rate (dry weight specific) (n.s.)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	17.13	5.71	3.35	0.0764
TREAT: 380ppm vs 1080ppm	1	2.79	2.79	1.64	0.2368
TREAT: 380ppm vs 2080ppm	1	0.07	0.07	0.04	0.8418
TREAT: 380ppm vs 3080ppm	1	14.26	14.26	8.36	0.0202 *
Residuals	8	13.65	1.71		
Residuals	12	20.35	1.70		
Residuals1	48	78.99	1.65		

TABLE A.7: Scope for Growth (energy / mg DW / hour)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TREAT	3	0.02	0.01	21.72	0.0003 *
TREAT: 380ppm vs 1080ppm	1	0.02	0.02	49.07	0.0001 *
TREAT: 380ppm vs 2080ppm	1	0.00	0.00	7.27	0.0272 *
TREAT: 380ppm vs 3080ppm	1	0.00	0.00	8.81	0.0179 *
Residuals	8	0.00	0.00		
Residuals	12	0.03	0.00		
Residuals1	48	0.07	0.00		

The variance components were extracted after fitting a linear mixed effect model to the data, using the VarCorr function in the nlme package. This function calculates the variances, standard deviations of random factors in such models. The output were used to calculate the percentage of the contribution of added variance due to different tanks within treatments and due to different days within tanks within the treatments. The results are summarised in table A.1.

TABLE A.8: Percent contribution to variance from random factors

	Dry weight	vol.Oil-sac	Body length	
Tanks	5.27	0.00	0.00	
Days	4.97	0.45	0.00	
	Filtering rate	Ingestion rate	Oxygen consumption	Scope for Growth
Tanks	0.00	0.00	0.10	0.00
Days	0.00	8.59	1.00	3.87

A generalized mixed effect model procedure was performed using the lmer package. It should be noted that the calculations of these p values may be suspect Logan [2011].A generalized mixed effect modeling procedure was performed, using the lmer package. Since this did not produce F-ratios for the treatment effects, a sampling distribution generated on the estimated parameters by a Markov Chain Monte Carlo technique was examined, as described by Logan [2011].The mcmcsamp function were used to extract an p value for the overall treatment effect. Since this method may not have been rectified

yet, the p values based on this method may be suspect. I have therefore only mentioned them as a point to potential effects that may be interesting to examine.

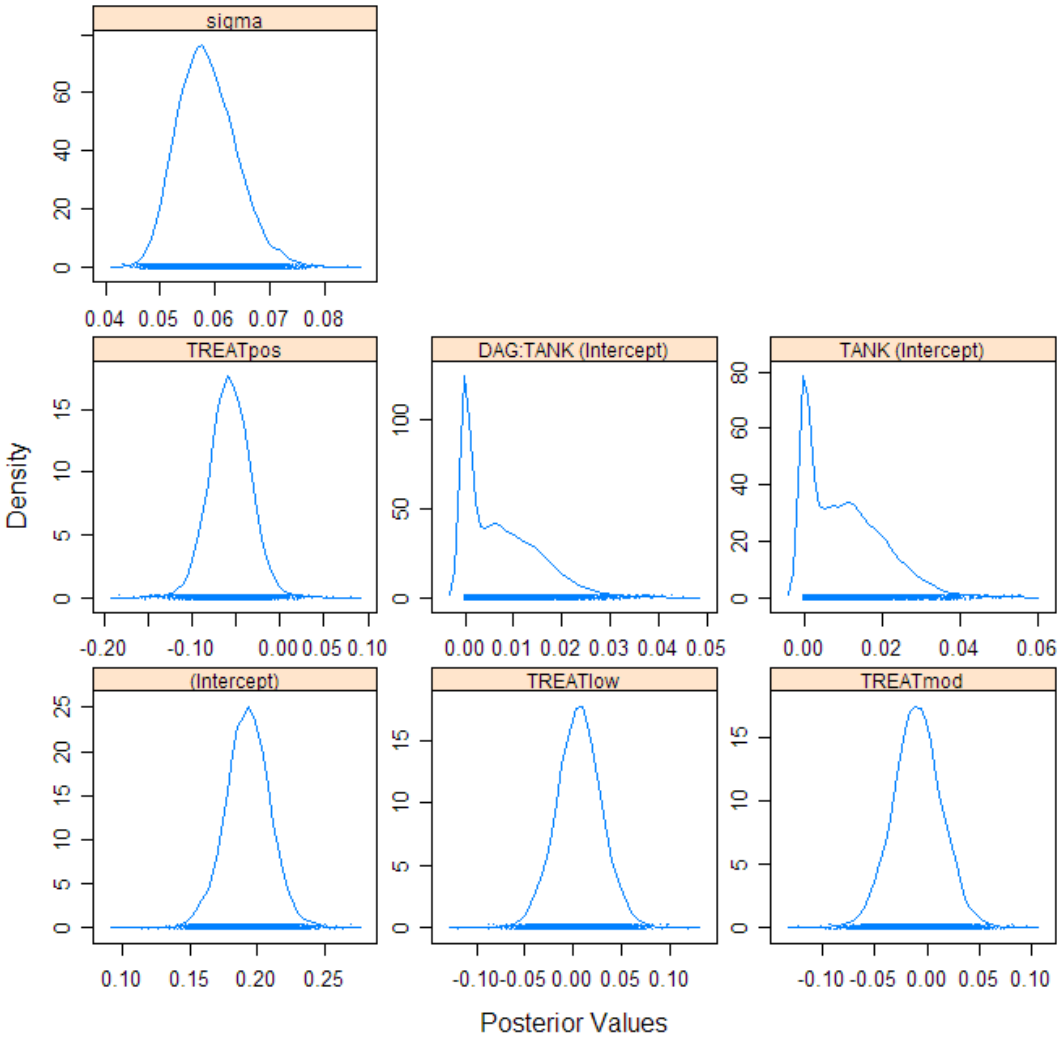

FIGURE A.1: Sampling distributions of parameter estimates for the dry weight measurements generated by 10000 samples using a Markow chain Monte Carlo technique.

TABLE A.9: Summary of p-values for differences between CO_2 treatments

$p-value$ p-value (MCMC)		
Dry Weight	0.0970	0.0387 *
Vol. Oil-sac	0.0326 *	0.0226 *
Body length	0.0625	0.1605
Filtering rate	0.0582 .	0.2014
Ingestion rate	0.0390 *	0.0826
Oxygen consumption	0.0764	0.0535 .
Scope for Growth	0.0003 *	0.009 *

A.2 boxplots

Assumptions for normality and homogeneity of variance were assessed by inspecting boxplots. Although some deviations from these assumptions may have occurred I decided to proceed and relied on the robustness of parametric tests for balanced designs Logan [2011]. The boxplots for the fixed effects are shown below. These are based upon generated datasets with means within each tank as values, as they represents the replicates for the treatment effects.

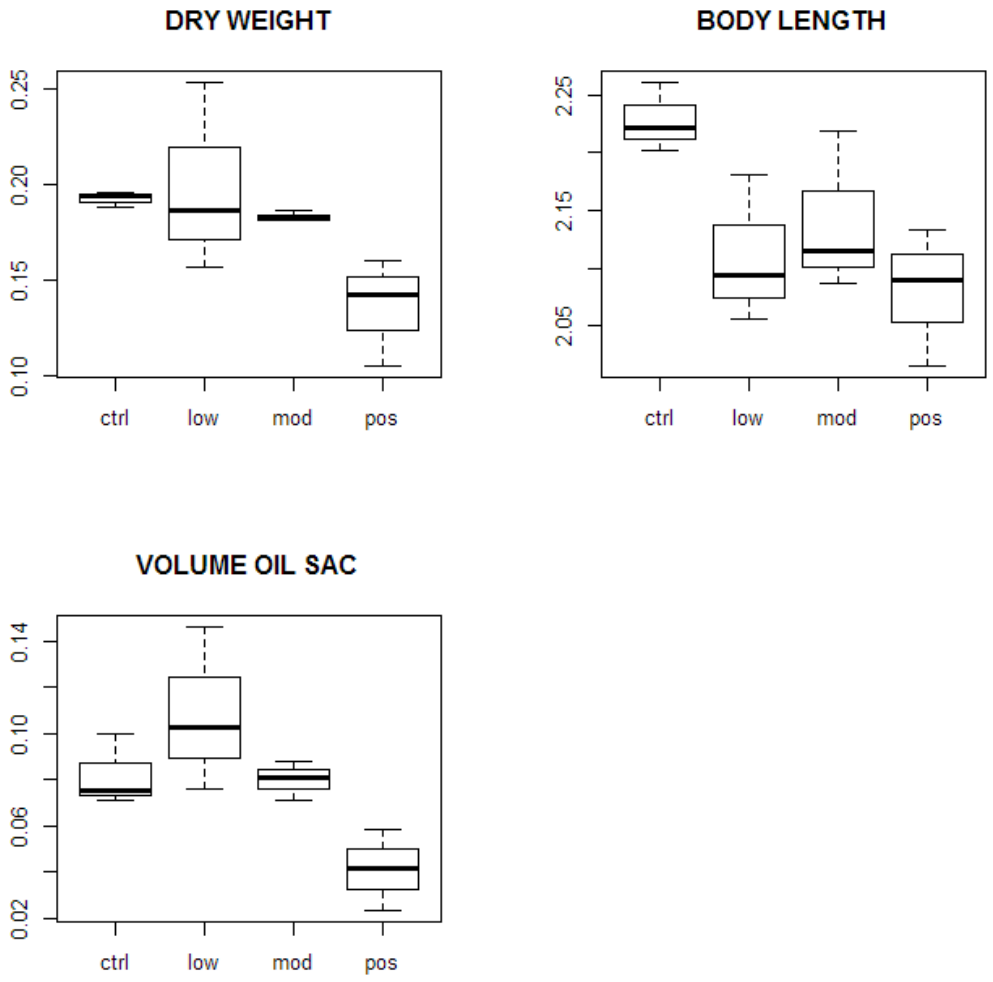

FIGURE A.2: Caption for box1

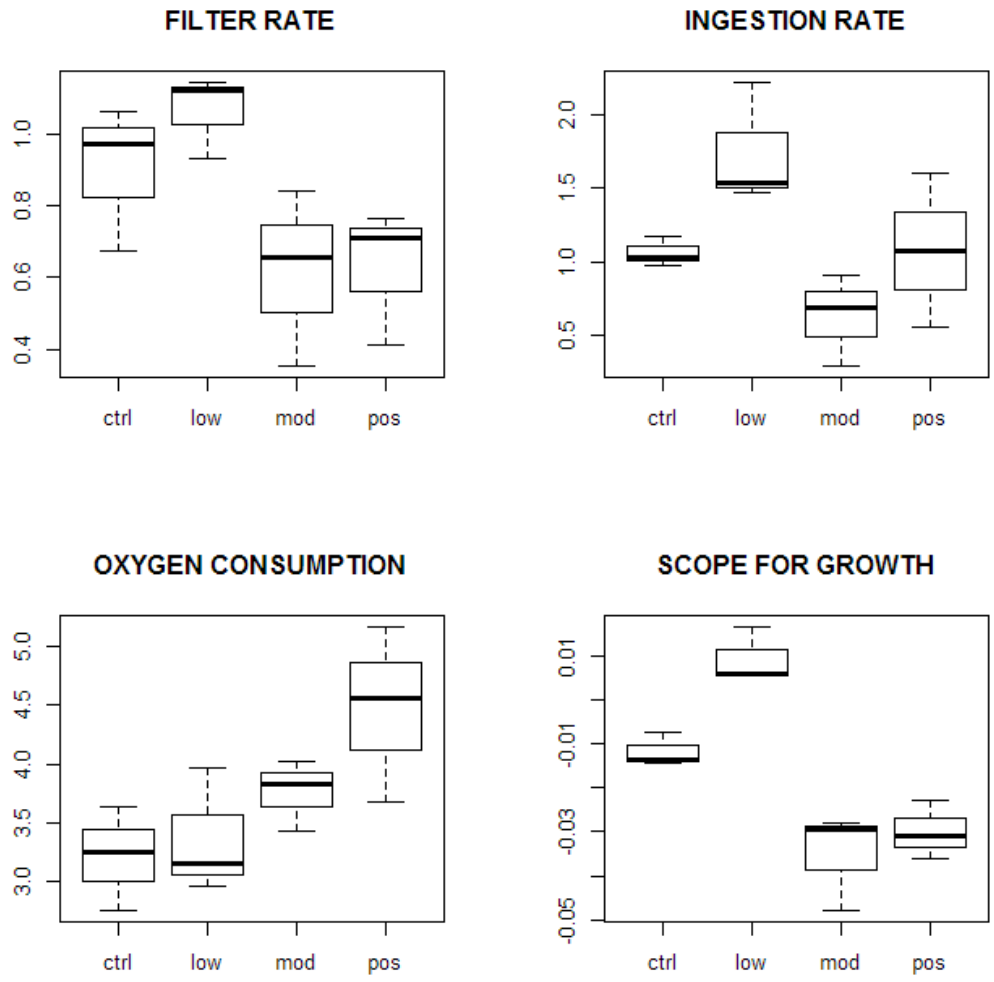

FIGURE A.3: Caption for box2