NTNU – Trondheim
Norwegian University of
Science and Technology

Interaction Effects of Ocean Acidification and Warming on the Marine Copepod *Calanus finmarchicus*

Andreas Wæhre

Marine Coastal Development
Submission date: May 2014
Supervisor: Bjørn Munro Jenssen, IBI
Co-supervisor: Sindre Andre Pedersen, IBI

Norwegian University of Science and Technology
Department of Biology

ACKNOWLEDGMENTS

This master thesis has been written at the Department of Biology, at the Norwegian University of Science and Technology (NTNU), Trondheim. The experiment was conducted at NTNU Center of Fisheries and Aquaculture (SeaLab) in collaboration with the master students Ida Soltvedt Johannessen, Anja Elise Hanssen and Elisabeth Sivertsen, from December 2012 to January 2013. Thank you all for your excellent collaboration in all laboratory work and for sharing information and ideas during the writing process.

I would like to thank my supervisor, Dr. Sindre Andre Pedersen for his guidance throughout the process from planning of the experiment to the end of the writing process and letting me be a part of this interesting project. Thanks to Research Scientist Dag Altin, Biotrix for showing me how to perform biometric analysis and Anders Johny Olsen for providing sampling equipment.

Special thanks to my girlfriend Margrete for keeping my spirits high during challenging periods of this thesis and believing in

Abstract

Ocean acidification and concurring warming of the oceans poses an ongoing threat to marine organisms, but little is known about their combined effects. The boreal calanoid copepod *Calanus finmarchicus* is a key stone species in North Atlantic Ocean and adverse effects of concurring OA and warming could have major impacts for ecosystem structure and function. A 2x2 cross factorial experiment with two levels of CO_2-concentration (390 (ambient) and 2080ppm (acidified)), and two levels of temperature (11°C (ambient) and 14°C (warming)) were set up to study possible interactive (synergistic/ antagonistic) effects between these two factors. The results show that interactions between the two stressors induced a positive synergistic effect on the development rate (only significant at the C1-stage). Also, a significant reduction in dry weight (1,24 fold) and lipid content (1,56 fold) in animals exposed to warming and acidification combined, suggests that *C. finmarchicus* could be sensitive towards ocean acidification predicted to occur by year 2300, in combination with increasing temperature. However, acidification also displays an antagonistic effect by reducing the negative effect of warming on the dry weight and lipid content of the animals. The complex interactions induced by combining warming and acidification highlight the importance of looking at multiple environmental factors simultaneously, as this approach might reveal biological responses previously unsuspected.

Sammendrag

Havforsuring og en samtidig oppvarming av havene er en pågående trussel for marine organismer, men det finnes lite kunnskap om hvilke effekter disse vil ha sammen. Den boreale calaniode kopepoden *Calanus finmarchicus* er en nøkkelart i Nord-Atlanteren og betydelig effekter av havforsurning og en samtidig oppvarming kan få store konsekvenser for strukturen og funksjonen til økosystemer. En 2x2 kryss-faktor eksperiment med to nivå av CO_2-konsentrajon (390 (nåtid) og 2080ppm (forsuring)) og to nivå av temperatur (11°C (nåtid) og 14°C (oppvarming)) ble satt opp for å studere mulige interaksjons-effekter (synergistisk/ antagonistisk) mellom de to faktorene. Resultatene viser at en interaksjon mellom de to stressorene førte til en positiv synergistisk effekt på utviklingsraten (bare signifikant ved C1-stadiet). Sammen med en significant reduksjon i tørrvekt (1,24 gange) og fett-innhold (1,56 gange) i behandlingen hvor dyr ble eksponert for både forsuring og oppvarming samtidig, antyder dette at *C. finmarchicus* kan være sårbar for havforsuring som er estimert til å skje fram til år 2300, kombinert med økende temperatur. Men, forsuring førte også til en antagonistisk effekt ved å redusere den negative effekten av økende temperatur på tørrvekt og fettinnhold i dyrene. De komplekse interaksjonene forårsaket av forsuring og økt temperatur belyser viktigheten av å se på flere miljøfaktorer samtidig, ettersom denne metoden kan påvise biologiske effekter som ikke har kunnet blitt påvist tidligere.

Table of contents

ACKNOWLEDGMENTS iii
ABSTRACT v
SAMMENDRAG vii
LIST OF FIGURES xi
LIST OF TABLES xv
ABBREVIATIONS xvii
1 INTRODUCTION 1
1.1 OCEAN ACIDIFICATION 1
1.2 PHYSIOLOGICAL PRINCIPLES OF CO_2 AND TEMPERATURE EFFECTS 3
1.2.1 Physiological effects of exposure to high CO_2 levels 4
1.2.2 Physiological effects of predicted future levels of CO2 4
1.2.3 Physiological effects of increasing temperature 5
1.2.4 Physiological effect of concurrent warming and ocean acidification 5
1.3 *CALANUS FINMACHICUS* 6
1.3.1 Life cycle and development of Calanus finmarchicus 7
1.4 PRESENT STUDIES OF *CALANUS FINMARCHICUS* 8
1.5 AIM OF STUDY 9
2 MATERIALS AND METHODS 11
2.1 EXPOSURE REGIMES 11
2.2 EXPERIMENTAL DESIGN AND SETUP 12
2.2.1 Controlling the CO_2-concentrations 12
2.2.2 Controlling temperature 15
2.3 EXPERIMENTAL CONDITIONS 15
2.3.1 pH measurements 15
2.3.2 Total alkalinity determination 16
2.3.3 Temperature measurements 16
2.3.4 Salinity measurements 16
2.3.5 Feed conditions 17
2.4 ANIMALS 18
2.4.1 Collecting eggs for cohort of C. finmarchicus 18
2.4.2 Sampling to determine median development time 19
2.4.3 Stage determination 20
2.4.4 Determination of median development time 21
2.4.5 Proportional duration 21
2.4.6 Sampling animals for biometric, and dry weight analysis 22
2.4.7 Biometric measurements 22
2.4.8 Dry weight 23
2.4.9 Neutral red staining 23
2.5 STATISTICS 24
3 RESULTS 25
3.1 EXPERIMENTAL CONDITIONS. 25
3.2 ALGAE CONCENTRATION 26
3.3 MORTALITY 27
3.4 DEVELOPMENT RATE 29

3.5 PROPORTIONAL DURATION 32
3.6 DRY WEIGHT OF *C. FINMARCHICUS* 33
3.7 LIPID-SAC VOLUME OF *C. FINMARCHICUS* 34
4 DISCUSSION 35
4.1 EVALUATING EXPOSURE CONDITIONS 35
4.2 METHOD ASSESSMENT 36
4.3 MORTALITY 37
4.4 DEVELOPMENT RATE 38
4.4.1 Effects of temperature on the development rate 38
4.4.2 Effects of acidification on the development rate 39
4.4.3 Effects of concurrent warming and acidification 39
4.4.4 Proportional duration 41
4.5 LIPID CONTENT AND DRY WEIGHT 41
4.6 ECOLOGICAL IMPLICATIONS 43
5 CONCLUSION AND FUTURE WORK 45
6 REFERENCES 47
APPENDIX A – LINEAR REGRESSION DATA .....
APPENDIX B – STAGE DETERMINATION DATA .....
APPENDIX C – BIOMETRY DATA .....

List of Figures

Figure 1.1: Illustrating the effect of temperature (T), pressure (P) and salinity (S) of the abundance of dissolved inorganic carbon (DIC) species as a function of pH………..……………………..………………………………………………........ 2

Figure 1.1: Simlified life cycle of *Calanus finmarchicus*. The reproductive stage (adult) release the eggs, which hatch into the first nauplii stage (NI). From NI to NII they subsist of their maternal lipid sac, before they start feeding at the prolonged NIII stage. After NVI they moult to the fist copepodite stage where they develop the characteristic antennae and store up lipids before they diapause as C5 stage……………............................... 8

Figure 2.1: Illustaration of an equilibrium column with one internal cylinder mounted inside of an external cylinder. As water entered the external cylinder through the top of the column, a submerged pump lifted the water from the external to the internal cylinder, creating a downward movement of water. CO_2 gas was mixed in the water through an air stone at the bottom of the internal cylinder, creating a counter current system and efficient mixing of CO_2 and water. Water leaved through two holes located at the base of the internal cylinder to the external cylinder and led to the exposure tanks……………..........…………………………… 12

Figure 2.2a): A illustration of a cross section of an exposure tank and a temperature controller box. A controller box (1) was installed with a feed line providing power (2). A PID temperature controller installed in the box, regulated the temperature in the exposure tank by connecting a temperature sensor (6) and a heater (8) submerged in the internal cylinder of the equilibrium system. Water from the primary equilibrium columns (blue lines (4)) entered through two inlets. One at the top, in connection to the algae feed (green line (3)) to ensure good distribution of the feed, and one at the bottom. The secondary equilibrium column was mounted inside the exposure tank. A submerged pump (5) transferred water from the tank to the secondary equilibrium column and creating a downward movement and a recycling loop of water (red lines). CO_2 gas was mixed in the water through an air stone (9) at the bottom of the column, which created counter-current system and efficient mixing of CO_2 and water. Water leaved the column at the bottom through filtering mesh (dashed lines), which prevented animals to enter the system. Waste water (brown arrow (7)) exited the system through an overflow outlet at the surface level…………………………………………………………………………………………….. 13

Figure 2.2b): The spatial arrangement of the replicate groups, equilibrium columns (EC1-4) and the temperature controllers (T1-4). T1-3 regulated temperature in each replicate group while T4 regulated temperature in the primary equilibrium columns.......... 13

Figure 2.3a): Illustration of stirrer used to ensure uniform distribution on the tank prior to sampling.......... 19

Figure 2.3b): Sampling tube used during sampling for N3 and C1- animals. By pulling the line the plunger closes the tube and a water sample is obtained........ 19

Figure 2.3c): Filtration system with a filter mesh attached to a screw cap at the outlet of the filter tube. The water is filtrated through and the animals retains in the filter, which is removed and preserved.......... 19

Figure 3.1: Development in measured algae concentration (ug C L^{-1}) in the different treatments during the exposure period (mean ± SD), n=3......... 25

Figure 3.2: Animals colored using neutral red staining. Figure 2a show the result from the verification of the method where dead animals (white coloration) were easily discriminated from live animals (red coloration). Figure 2b and c show animals sampled during the experiment and shows examples of how coloration varied among different live animals.......... 26

Figure 3.3: The mean values (mean ± SD, n = 3 replicate tanks) of Median Development Time (MDT) for Nauplii 3-stage of *C. finmarchicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments ($p< 0.05$).......... 28

Figure 3.4: The mean values (mean ± SD, n = 3 replicate tanks) of Median Development Time (MDT) for copepodite 1-stage of *C. finmarchicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments ($p< 0.05$).......... 29

Figure 3.5: The development index score in the different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm), 26,3 days from mid egg-laying point (mean ± SD, n = 3 replicate tanks). A high index-score indicates fast development. Different letters denoted significant differences between treatments ($p< 0.05$).......... 30

Figure 3.6: Mean values (mean ± SD, n = 3 replicate tanks) of the proportional duration (MDT C1-stage/MDT N3-stage) between N3 and C1 stage *C. finmachicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments ($p < 0.05$)..... 31

Figure 3.7: Mean values (mean ± SD, n = 3 replicate tanks) of dry weight (mg) of C5-stage *C. finmarchicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments ($p < 0.05$)..... 32

Figure 3.8: Mean values (mean ± SD, n = 3 replicate tanks) of lipid-sac volume as percentage of whole body of C5-stage *C. finmachicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Values are calculated from biometric values calculated from 8 x 3 animals from each treatment. Different letters denoted significant differences between treatments ($p < 0.05$).. 33

Figure 4.1: Theoretic predictive model showing the relationship between fitness, growth and metabolism under different environmental conditions (temperature and CO_2). An increases scope for growth as an effect of both warming and acidification combined can be achieve because both factors induces an increased metabolism (shift to the right on the metabolic curve) till the point of metabolic depression. Acidification can then lead to a positive effect (under non-limiting energy conditions, green dots) or a negative effect (under limiting energy conditions, red dots) on growth rates depending on the baseline metabolism.. 39

List of tables

Table 1.1: Experimental setup with 2x 2 factorial design resulting in four treatments. Replicate groups and their spatial arrangement is also included………………………………………………………………………………………….. 10

Table 3.1: Water parameters (pH_{tot} temperature, alkalinity and salinity) for the different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm) during the experiment (mean ±SD). The values are the overall means of three replicate tanks for the duration of the experiment. The pCO_2 (ppm), $pH_{tot,\ in\ situ,}$ ΩCa and ΩAr values, are values corrected for in situ experimental temperature……….. 24

Table 3.2: Water parameters (pH_{tot}, temperature, alkalinity and salinity) for the different treatments: of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm) during the incubation period were animals moulted from C4 into C5 stage. The values are mean (mean ±SD) of the three replicate buckets where the animals were incubated. The pCO_2 (ppm), $pH_{tot,\ in\ situ}$, ΩCa and ΩAr values, are values corrected for in situ experimental temperature………………………………….... 24

Table 3.3: Statistical results derived from PERMANOVA tests. Temp= temperature, d.f= degrees of freedom, SS= sum of squares, MS= mean sum of squares, *= interaction term. Significant results in bold…………… 27

Table A.1 Linear regressions for the cumulative stage proportions for N3 and C1-stage. The coefficients of the linear equation y=ax+b, and the R^2 –values to describe the linear fit, are included………………………………………………..

Table B.1 Raw data of the stage determination. Time is days after mid-egg laying point and volume of the samples is included. Data in bold is excluded from the analysis because of few animals in the samples (<5 animals). The table continues on subsequent pages…………………………………………

Table C.1 Data obtain from biometric analysis and the calculated values of volumes of lipid sac, body volume and lipid sac %……………………………………………….

Abbreviations

μatm	Micro atmospheres
μm	Micro meter
μg C/L	Microgram Carbon per Liter
C1-5	Copepodite stage 1-5
ΩAr	Aragonite saturation
ΩCa	Calcite saturation
CO_3^{2-}	Carbonate
H_2CO_3	Carbonic acid
HCO_3^-	Bicarbonate
K_1^* and K_2^*	Stoichiometric equilibrium constants
CO_2	Carbon dioxide
CT_{max}	Critical Thermal Maximum
D	Development time
d.f	degree of freedom
DIC	Dissolved Inorganic Carbon
H^+	Hydrogen ion
LSD post-hoc	Least Significant Difference post-hoc
MDT	Median Development Time
MS	Mean sum of Squares
N 1-6	Nauplii stages 1-6
OA	Ocean acidification
P	Pressure
PERMANOVA	Permutational Multivariate Analysis of Variance
Pg C $cell^{-1}$	Pictogram Carbon per cell
pH	Power of Hydrogen
pH_{tot}	total hydrogen ion concentration pH scale
Ppm	Parts per million
ppt	parts per thousand
R^2	Coefficient of determination
S	Salinity

SD	Standard Deviation
SPV	Stage Specific Value
SS	Sum of Squares
T	Temperature
T_A	Total Alkalinity
TO	Temperature Optimum

1 Introduction

1.1 Ocean acidification

An increase of atmospheric carbon dioxide (CO_2) from a preindustrial level of 280 ppm, up to a present day level of 380 ppm is mainly due to anthropogenic activities. The increased concentration of CO_2, which by far exceeds the natural range over the last 650,000 years is primarily due to fossil fuel burning and to a lesser extent cement production and altered land use (Solomon, 2007, Zeebe, 2012). Further, model predictions for the future suggest that by year 2300, atmospheric CO_2 concentration may reach 1900 ppm (Caldeira and Wickett, 2005).

When atmospheric concentration of CO_2 (g) rises, equilibrium is reached between air and water, through the carbonate system, thus rising concentration of dissolved CO_2 in seawater. Dissolved CO_2 in seawater reacts with water and creates carbonate species described by the following equations:

$$CO_2 + H_2O \leftrightharpoons HCO_3^- + H^+ \quad (1.1)$$

$$HCO_3^- \leftrightharpoons CO_3^{2-} + H^+ \quad (1.2)$$

For the description of the carbonate system in seawater, stoichiometric equilibrium constants are used, which are related to concentrations:

$$K_1^* = \frac{[HCO_3^-][H^+]}{[CO_2]} \quad (1.3)$$

$$K_2^* = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} \quad (1.4)$$

where the stoichiometric equilibrium constants (K_1^* and K_2^*) are dependent on temperature, pressure and salinity according to figure 1.1 (Zeebe and Wolf-Gladrow, 2001). The carbonate system is largely responsible for controlling the pH of seawater. When CO_2 dissolves in water, carbonic acid (H_2CO_3) is formed, which quickly dissociates into bicarbonate (HCO_3^-) and hydrogen ions (H^+) (equation 1.1). Hydrogen ions can then react with carbonate (CO_3^{2-}) to form bicarbonate (equation 1.2), constituting a buffer to prevent changes in pH of seawater. Net effect of adding CO_2 to water is increased concentrations of carbonic acid, bicarbonate and hydrogen ions, thus decreasing the pH (pH= $-\log [H^+]$) (Fabry et al., 2008).

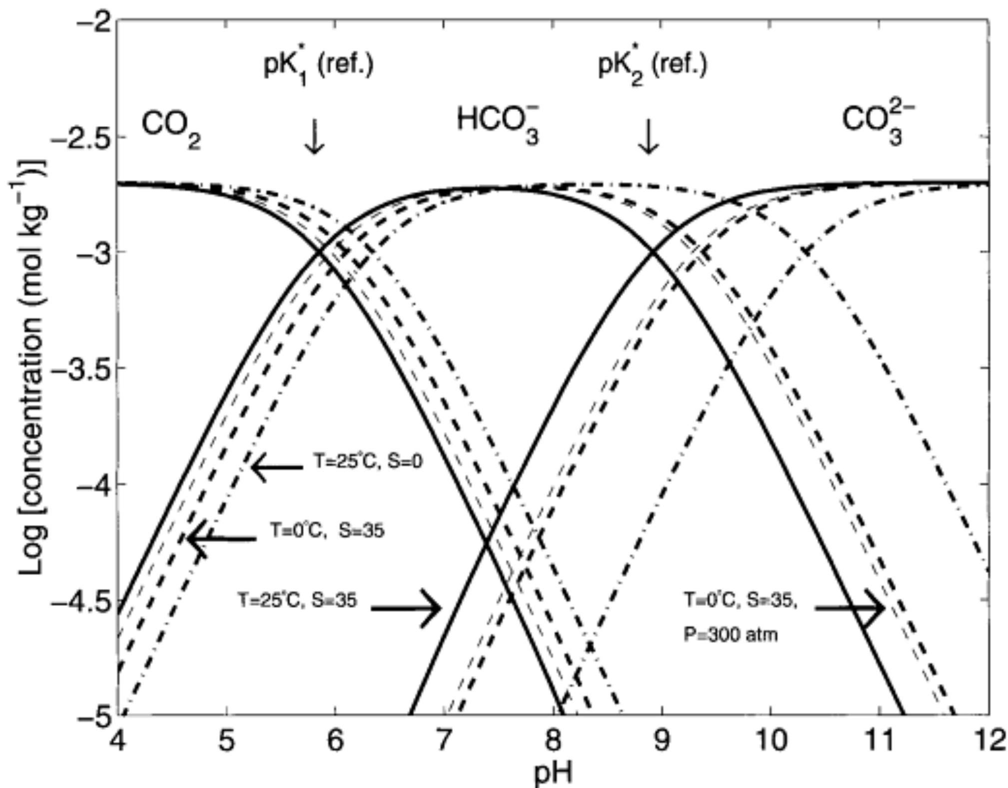

Figure 1.1: Illustrating the effect of temperature (T), pressure (P) and salinity (S) of the abundance of dissolved inorganic carbon (DIC) species as a function of pH (Zeebe and Wolf-Gladrow, 2001).

The world oceans also constitute a buffer-system of atmospheric CO_2, by absorbing about one third of the discharged CO_2 since 1750 (Sabine et al., 2004, Zeebe, 2012), which has caused a decrease of surface-ocean pH by 0.1 units (from 8.2 to 8.1). This phenomenon is referred to as ocean acidification (OA), which derives from this lowering of seawater pH due to increased emissions of anthropogenic CO_2 (Zeebe, 2012). Surface-ocean pH has been relatively stable during the past 2 million years (Hönisch et al., 2009). Thus, the relatively rapid decrease of ocean surface pH and its impact on marine fauna and ecosystem processes

has been of increasing concern to the scientific community (Fabry et al., 2008, Gibson et al., 2011).

The current trend of increasing atmospheric CO_2 is accompanied by a simultaneous increase in ocean temperature. During the 20th century, the global oceans average temperature increased by 0.74°C (Solomon, 2007). Future scenario models have estimated a further increase by at least 2-4°C by year 2050 to 2100 (Solomon, 2007). Global warming has already changed species specific geographical distributions and created increased risk of local extinctions of species (Parmesan and Yohe, 2003). North Atlantic copepods have shown a strong bio-geographical shift with a northward extension of more than 10° latitudes of warm water species associated with a decrease in the number of colder-water species (Beaugrand et al., 2002).

Similar to thermal stress, CO_2 effects may have an effect on different organization levels, from molecular and cellular levels, up to whole organism functioning and thus also visible effects on an ecosystem level (Pörtner, 2008). Effects of increased temperature and CO_2-concentration influence each other (Hoegh ‐ Guldberg, 2005, Pörtner et al., 2005) and it is therefore important to investigate potential interaction effects of these stressors.

1.2 Physiological principles of CO_2 and temperature effects

Knowledge of the molecular and biochemical mechanisms affected by increased levels of temperature and CO_2-concentration is crucial to understand the effects visible at an ecosystem level and to predict how ocean acidification may influence marine ecosystems. Effects of CO_2 depend on both concentration and time scale (Pörtner, 2008). Future scenarios of anthropogenic ocean acidification involves relatively low levels of CO_2 and therefore the effects are long term rather than acute. However, studying acute effects of elevated CO_2-concentration is important to identify the mechanisms and regulatory pathways responding to CO_2 (Pörtner et al., 2005). Studies of calcifying organisms has previously been favoured because of concerns over calcium dissolution as an effect of acidification (Anthony et al., 2008, Hofmann et al., 2008). Copepods possess an exoskeleton of chitin, and are therefor thought to be more robust against calcification-related effects of acidification than calcifying organisms with aragonite or calcite shells (Fitzer et al., 2012).

1.2.1 Physiological effects of exposure to high CO_2 levels

As high concentrations of CO_2 accumulate in the extracellular fluids, including blood and haemolymph (a state named hyercapnia), homeostatic processes will work to maintain homeostasis. Electroneutral ion exchange across gill epithelia is the most significant process for pH-regulaton, where bicarbonate (HCO_3^-) from external seawater is exchanged for Cl^- and H^+ is exchanged for Na^+ (Taylor, 1992). Haemolymph proteins (mainly haemocyanin) and bicarbonate ions buffers the pH in the haemolymph to prevent disruption of haemolymph acid-base status.

The most comprehensive data on physiological effects of hyercapnia is provided by studies of a non-calcifying worm *Sipunculuc nudus*. Metabolic depression, (Pörtner et al., 1998) reduced rates of tissue acid-base regulation (Pörtner et al., 2000), and reduced rates of protein synthesis (Langenbuch et al., 2006) are key effects as extracellular acid-base status is disrupted. Effects of CO_2 are by far unilateral and depend on species, life history and ontogenetic age within species (Fabry et al., 2008). Strong iono- and osmoregulators are more likely to be less vulnerable to ocean acidification, at least in shorter term, because of their well developed ion exchange mechanisms (Pörtner, 2008). Increase levels of CO_2 is also thought to be more damaging to larval and juvenile, than adult stages, of several marine animals (Kurihara, 2008, Gibson et al., 2011).

1.2.2 Physiological effects of predicted future levels of CO2

Exposure to more realistic exposure regimes projected to occur in near future, is more relevant to biological changes created by OA. Predicted future levels of CO_2 is to low to create acute effects in animals, thus, studies of crustaceans have concentrated on alterations in compensatory capacities over time (Pörtner, 2008). Acid-base regulation is likely to be metabolically expensive over weeks to months (Whiteley, 2011). It has been estimated that 2.8 to 40% of the total energy expenditure may be used by Na^+/K^+-ATPase activity, which drives the ion exchange mechanism to regulate the pH (Leong and Manahan, 1997). Integrated bioassays, such as scope for growth provide measurements of energy status of an organism, which is based on the concept that energy in excess of what is required to maintain normal maintenance will be available for growth and reproduction (Navarro et al., 2006). By

calculating scope for growth, Stumpp et al. (2011) found that the sea urchin larvae *Strongylocentrotus purpuratus* used 39-45% of available energy for somatic growth raised under high CO_2, while control larvae allocated 78-80% of the available energy into somatic growth. Changes in metabolic turnover and specific allocation of energy is ultimately linked to effects at an eco-system level (McKenney Jr, 1998, De Coen, 1999).

1.2.3 Physiological effects of increasing temperature

Temperature is considered to be the most important environmental factor controlling invertebrates` growth, reproduction, development, recruitment dynamics and species distributions (Pechenik, 1987, O'Connor et al., 2007). The response of different biological processes to temperature displays a positive effect, until reaching a temperature optimum (TO) (e.g. temperature induce an up-regulation of metabolism) (Peck and Prothero-Thomas, 2002)). Further increases in temperature lead to the critical thermal maximum (CT_{max}, (Somero, 2010), before a drop in the velocity of the process in question (Frederich and Pörtner, 2000, Deutsch et al., 2008, Dell et al., 2011). Thus, knowing species thermal window of performance is crucial to understand the effect of increased temperature (Pörtner and Knust, 2007). Negative effects from +2-4°C warming, within near-future projections have been widely reported (Bartolini et al., 2013).

1.2.4 Physiological effect of concurrent warming and ocean acidification

Predicting potential impacts of climate change and OA on marine biota poses a significant challenge for integrative marine biology and ecology (Harley et al., 2006, Przeslawski et al., 2008). As benthic marine invertebrates live in a multi-stressor world, the need to consider concurrent effects likes warming and acidification has become increasingly important. This is reflected in the recent increase in cross-factorial studies of development of marine vertebrates where different levels of these stressors are tested in all combinations (Byrne and Przeslawski, 2013). A combination of concurrent warming and acidification can produce a simple additive effect (no interaction between the two factors) or complex interactive effects with synergic effects (increased stress greater than the sum of the individual stressor effects) or antagonistic effects (decreased stress greater than the sum of the individual stressor effects) on biological processes. In many species, exposure of embryos, larvae and/or juveniles to warming and acidification have resulted in additive negative effects, where no interactions

between the concurrent factors have been observed. While antagonistic effects also have been commonly found in studies, synergistic effects have been less commonly reported (Byrne and Przeslawski, 2013).

Elevated CO_2-concentration can enhance the sensitivity of organisms to thermal extremes by reducing tissue function capacity, with those involved in oxygen supply (may cause hypoxia) being a vital one (Pörtner et al., 2005, Metzger et al., 2007). Hypercapnia have been shown to cause a narrowing of the thermal window, making organisms more sensitive to extreme temperatures. Conversely, exposure to thermal extremes may enhance sensitivity to elevated CO_2 levels (Pörtner, 2008). Both hypercapnia and hypoxia cause a decrease in an organisms capability to regulate pH (Pörtner, 2008). However, effects of concurrent OA and warming is dependent on the species in question, as great disparity in sensitivity towards these stressors exists between species. A 3°C warming and a decreased pH (pH ~7.5) caused significant synergistic effects on total egg and nauplii production of *Acartia bifilosa* (Vehmaa et al., 2012), while elevated temperature (ΔT=2-4°C) and decreased pH (pH ~7.7) did not affect egg hatching rates in *C. helgolandicus* (Mayor, 2012). Thus, to model predictions of ecosystem it is crucial to investigate potential effects of stressors in various species in various habitats, where keystone species should be targeted.

1.3 *Calanus finmachicus*

The marine copepod *Calanus finmarchicus* is a representative of the family Calanidae and was first scientifically described by Johan Ernst Gunnerus in 1770. The species is temperate-boreal and found mainly in the sub-Atlantic water masses. The distribution ranges from the North Altantic drift from the west coast of U.S.A, and as far as beyond 70°N (Conover, 1988) and they have been found to dominate the standing stock of zooplankton in the North Sea (Bagøien et al., 2012). It is considered to be a keystone species, both ecologically and economically because of its abundance and function in the marine food chain. During winter *C. finmarchicus* diapause at depth of 200-300m, before migrating to the surface in March/April. The animals almost exclusively overwinter as stage CV and moults to adults during this vertical migration in spring (Marshall and Orr, 1955).

In spring, *C. finmarchicus* may constitute up to 70% of all zooplankton, playing a vital role in energy transfer from primary production and higher levels in the marine food chain (Runge, 1988). Later stages of the species have large lipid stores, which may constitute up to 50% of dryweight biomass. This is why commercially important species like cod (*Gadus morhua*), herring (*Clupea harengus*), mackerel (*Scomber scombrus* and Haddock *(Melanogrammus aeglefinus*) are dependente on *C. finmarchicus* as their main food resource, particularly during their larval stages (Kane, 1984, Kaartvedt, 2000).

1.3.1 Life cycle and development of *Calanus finmarchicus*

The life cycle of *C. finmarchicus* consists of twelve stages, six nauplar stages, five copepodite stages and a final reproductive stage (adults) (see figure 1.2). Like all crustaceans, the animals exhibit a stepwise growth by performing a process called moulting, where their chitin-based exoskeleton is shed and a new one is formed (Mauchline, 1998). It has been stated that *C. finmarchicus* has an equiproportional development (Corkett et al., 1986), i.e. that the relative duration of a given stage (i.e. stage duration/egg duration) is constant to other stages at a constant temperature. Campbell et al. (2001) also found that the relative duration was the same at different temperatures (4, 8 and 12°C). However, stage duration is not constant over all development stages. The first exogenous feeding stage of *C. finmarchicus* (N3) is prolonged, relative to the two first non-feeding stages (N1 and N2). The duration of N4 to N6 is found to be similar and copepodite stage duration increases with increasing development stage (Campbell et al., 2001). Development time (D) as a function of temperature (T) under high food conditions can be expressed by the equation;

$$D = a(T - \alpha)^b \qquad (1.5)$$

where a, b and α are constants (Belehradek, 1935).

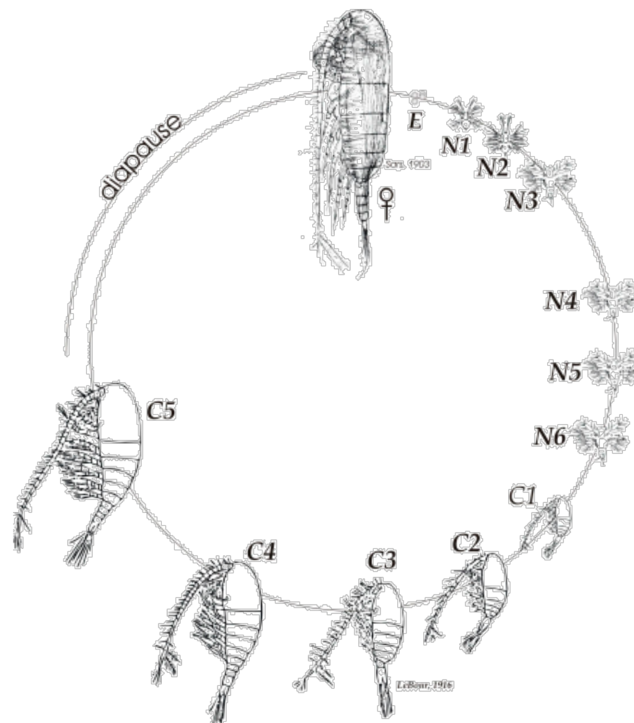

Figure 1.2: Simlified life cycle of *Calanus finmarchicus*. The reproductive stage (adult) release the eggs, which hatch into the first nauplii stage (N1). From N1 to N2 they subsist of their maternal lipid sac, before they start feeding at the prolonged N3 stage. After N4 they moult to the fist copepodite stage where they develop the characteristic antennae and store up lipids before they diapause as C5 stage.

1.4 Present studies of *Calanus finmarchicus*

Although research on effects of both CO_2-concentration and temperature has been studied separately, studies of interactive effects are rare. This study is the first to examine the interactive effects of CO_2-concentration and temperature over a complete life cycle (excluding reproduction) on *C. finmarchicus*. In a previous study by Mayor (2007) the growth of adult female *C. finmarchicus* was not affected by exposure to a high level of CO_2 (8000 ppm), but a significant reduction of the hatching success was observed. Less severe acidification scenarios, which are more realistic to occur as a result of future emissions of CO_2 to the atmosphere (1900ppm by year 2300), has suggested that *C. helgolandicus* (congeneric species distributed more southerly) is robust towards OA (Mayor, 2012). The results in the studies by (Mayor, 2007, Mayor, 2012) are based on short-term exposure to CO_2. Medium-term (one generation) laboratory studies indicate that the highest CO_2 level expected by the year 2300 ($CO_2 \leq 2000$ppm) may not directly affect survival or development rate of *C. finmarchicus* (Gustavson, 2013, Pedersen et al., 2013). However, energy depletion

in *C. finmarchicus* exposed to 2080ppm over the course of two generations has previously been observed (Håkedal, 2013).

A temperature optimum between 6 and 10.6°C has been demonstrated for *C. finmarchicus*, but the species has a relatively broad tolerance interval (Helaouët and Beaugrand, 2007). Rearing under laboratory conditions at three different temperatures (4, 8, and 12°C) showed an inverse relation between temperature and body size (length and weight). However, growth rate also increased with increasing temperature which resulted in a decreased development time (from egg to C5-stage) (Campbell et al., 2001).

1.5 Aim of study

The aim of the study was to investigate potential interactive effects (synergistic/antagonistic) on morality, development, dry weight and biometry of these stressors in one generation of *C. finmarchicus*. The study will provide knowledge about how this species will respond to ocean acidification projected to occur within year 2300, where warming of the ocean is a concurrent stressor. The result might also be useful in population predictive models to estimate potential ecological and socio-economical impact of future OA and warming as separate, but also as concurrent factors.

2 Materials and methods

The experiment was conducted at NTNU Center of Fisheries and Aquaculture, Sealab, Trondheim from December 2012 to January 2013. Cohorts of Calanus finmarchicus were cultivated under four different treatments from egg to C5-stage. Two different regimes of CO_2 (380 ppm and 2080 ppm) and two temperatures (11°C and 14°C) were combined in a fully crossed design, to give the treatments shown in table 1, all treatments with three replicates each. The experimental setup consisted of a flow-through system with 12 experimental tanks. A gas mixing system provided the CO_2-gas to the mesocosms, and the gas flow was adjusted to obtain correct CO_2-consentration in each individual tank. Custom-made temperature-regulators provided the different temperatures for the experiment. The animals used to initiate the experiment were obtained from a continuous culture maintained at Sealab.

Table 2.1: Experimental setup with 2x 2 factorial design resulting in four treatments. Replicate groups and their spatial arrangement are also included.

Treatment	CO_2 (ppm)	Temperature (°C)	Replicate A	Replicate B	Replicate C
Ambient	380	11	A1	B1	C1
Warming	380	14	A2	B2	C2
Warming*Acidified	2080	14	A3	B3	C3
Acidified	2080	11	A4	B4	C4

2.1 Exposure regimes

The elevated CO_2-regime (2080 ppm) was selected according to a worst case, near-future, scenario using a logistic CO_2-emission pathway to estimate CO_2-concentration by the year 2300 (Caldeira and Wickett, 2005). A temperature increase of 3°C was selected from near-future scenarios with an estimated increase in global ocean temperature by at least 2-4°C, by 2050 to 2100 (Solomon, 2007)

2.2 Experimental design and setup

Twelve mesocosms (90 L conical-shaped polystyrene tanks) placed in a temperature-controlled room at 10°C constituted the experimental setup. These tanks were arranged in three separate blocks, where the different treatments were represented in different locations within the climate room, according to figure 2.2. The CO_2-gas was provided to the tanks by a gas-mixing system (HTK Hamburg GmbH, Germany) by enriching ambient air, provided by the central compressed air distribution system, with pure CO_2-gas (100%CO2, Mapcon, Yara ProXair). Each tank had a separate channel connected to the gas-mixing system to allow manual regulation of the flow of the CO_2 –enriched air.

2.2.1 Controlling the CO_2-concentrations

The seawater used in this experiment was collected from the Trondheimsfjorden at approximately 70 m depth and filtrated to remove particulate matter. CO_2-enriched air was then added to natural seawater, using four equilibrium-columns (one for each treatment). These equilibrium-columns consisted of two cylinders, one internal and one external. The internal cylinder housed a wooden aeration stone (Aqua Medic GmbH, Germany), at the base, through which CO_2-enriched air was added to the ambient water. The water was added to the external cylinder at the top of the columns and a water pump (Micro-Jet MC450, aquarium systems) created lifted water from the external to the internal cylinder. This creates a downward water stream in the internal cylinder and a counter-current with the rising CO_2-boubbls from the aeration stone, providing optimum conditions for the equilibrium process. The equilibrated water exited the external cylinder through holes at the base of the internal cylinder. An illustration of a equilibrium column is given in figure 2.1.

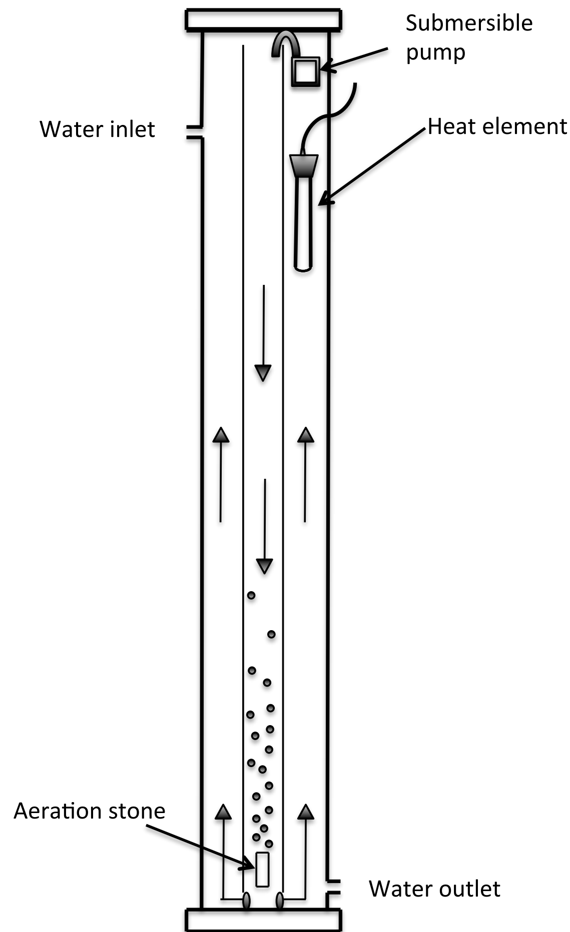

Figure 2.1: Illustaration of an equilibrium column with one internal cylinder mounted inside of an external cylinder. As water entered the external cylinder through the top of the column, a submerged pump lifted the water from the external to the internal cylinder, creating a downward movement of water. CO_2 gas was mixed in the water through an air stone at the bottom of the internal cylinder, creating a counter current system and efficient mixing of CO_2 and water. Water leaved through two holes located at the base of the internal cylinder to the external cylinder and led to the exposure tanks.

To compensate for loss of CO_2 through water-air diffusion, and to add stability, CO_2-enriched air was also added directly to each exposure tank through a secondary equilibration system. The same principle as for the primary equilibration system applied for the secondary system. An aeration stone mixed the CO_2 enriched air into ambient water and a water pump created a downward flow from the external cylinder (the 90 L experimental tank), to the internal cylinder. The water from the internal cylinder exited through a mesh-covered hole (120 µm) at the bottom of the cylinder. The mesh provided a barrier to prevent animals from entering the equilibrium system. Equilibrated seawater from the primary equilibrium-columns entered the tanks through two inlets, on at the bottom at the tank, and one on the top of the tank. The outlet was at the top at the tank to complete the flow through system. The seawater exchange

rate was approximately 90 L per day. A cross-section illustration of an exposure tank is given in figure 2.2a, and the spatial arrangement of the tanks is illustrated in figure 2.2b.

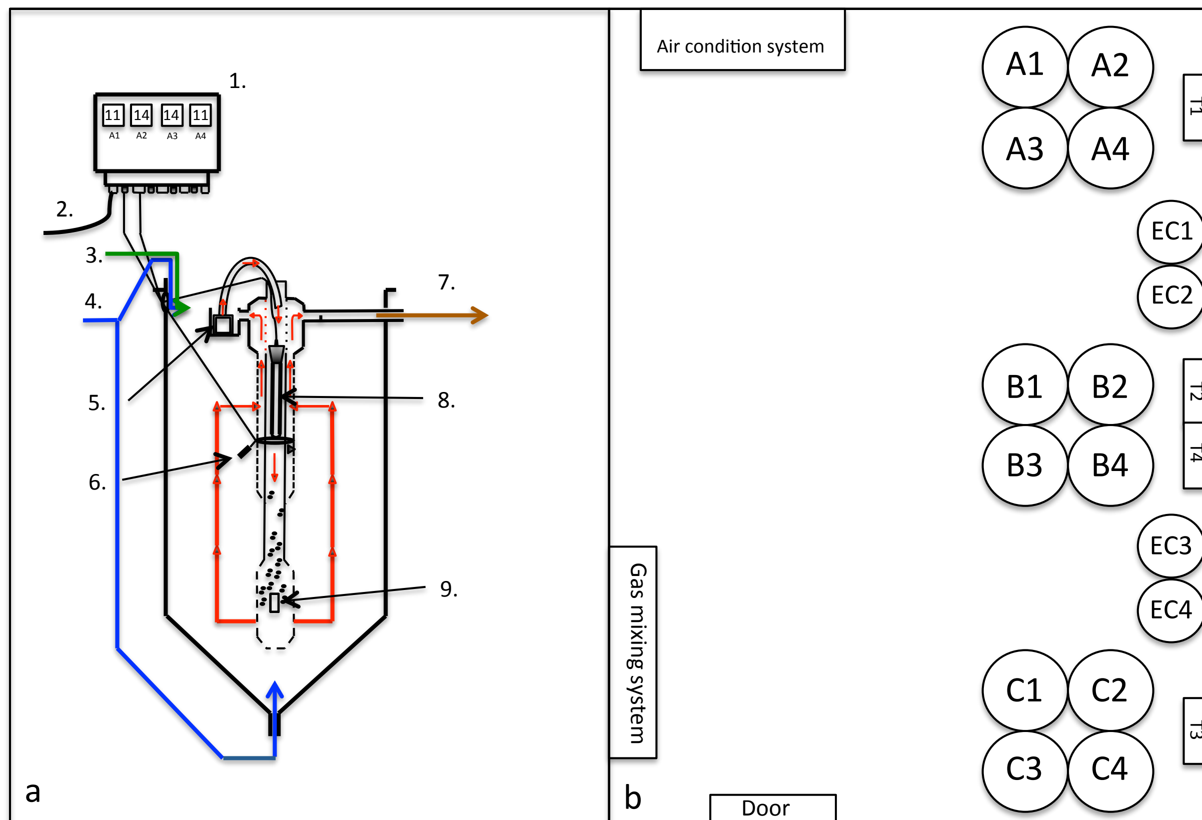

Figure 2.2: a) A illustration of a cross section of an exposure tank and a temperature controller box. A controller box (1) was installed with a feed line providing power (2). A PID temperature controller installed in the box, regulated the temperature in the exposure tank by connecting a temperature sensor (6) and a heater (8) submerged in the internal cylinder of the equilibrium system. Water from the primary equilibrium columns (blue lines (4)) entered through two inlets. One at the top, in connection to the algae feed (green line (3)) to ensure good distribution of the feed, and one at the bottom. The secondary equilibrium column was mounted inside the exposure tank. A submerged pump (5) transferred water from the tank to the secondary equilibrium column and creating a downward movement and a recycling loop of water (red lines). CO_2 gas was mixed in the water through an air stone (9) at the bottom of the column, which created counter-current system and efficient mixing of CO_2 and water. Water leaved the column at the bottom through filtering mesh (dashed lines), which prevented animals to enter the system. Waste water (brown arrow (7)) exited the system through an overflow outlet at the surface level. **b)** The spatial arrangement of the replicate groups, equilibrium columns (EC1-4) and the temperature controllers (T1-4). T1-3 regulated temperature in each replicate group while T4 regulated temperature in the primary equilibrium columns.

As the experimental tanks were not closed to the ambient air, CO_2 was continuously lost to the atmosphere due to a CO_2–gradient between water and air. To ensure correct

concentrations of CO_2 the pH, temperature, total alkalinity and salinity were measured each day and used to calculate the CO_2-concentrations, using the program CO2SYS-v2.1.xls (Pelletier et al., 2007).

2.2.2 Controlling temperature

To reach targeted temperatures of 11 and 14°C, temperature control units (incorporating four SYL-2372 PID temperature control units (Auber instruments, Alpharetta, Georgia, USA)), were constructed. Three units were assembled to control temperature to each replicate (A, B and C) and one unit to control the temperature in the equilibrium-columns. To each of the four units, four temperature controllers were integrated. A temperature sensor (Platinum RTD. Pt 100-element) placed in the middle of the tanks gave feedback on the water temperature to the temperature controller. The controller uses an auto-tune function to control a heat element (Newatt Eco Therm Aquarium Heater, 50W), which heats the water up to a chosen temperature (11 or 14°C). This ensures accuracy within one degree. The heater was placed 10 cm below the water level in the inner column on the tanks.

The temperature was also controlled in the equilibrium columns to pre-heat the water before entering the inlet to the exposure tanks. This was done only for the 14°C exposed tanks, as the difference between the room temperature (10°C) and the targeted temperature (11°C) were only 1°C. Prior to the experiment, each temperature controller was calibrated to 0°C, by using a ice-water mix (1:1).

2.3 Experimental Conditions

2.3.1 pH measurements

The pH in the exposure tanks was measured each day during the experiment with a spectrophotometric method according to Dickson et al. (2007), with some modifications. This method determines the pH of seawater on the total hydrogen ion concentration pH scale (pH_{tot}). The method utilizes the pH-dependent colouration of the indicator dye *m*-cresol purple, an indicator dye. *m*-cresol have different forms (I^{2-}, and HI^{-}) and the dissolution ratio between this forms decides the coloration of the sample.

Water samples were collected just beneath the water surface of the experimental tanks with 50

ml glass bottles. The bottles were closed with caps to eliminate air bubbles in the samples. They were then incubated in a water bath to 25°C before pipetted into a 3,7 ml gastight glass-cuvette with a Teflon top. The cuvette was carefully mixed and the outside was cleaned. The absorption of the blank sample was measured by a spectrophotometer (Cary 50 Bio UV-visible Spectrophotometer, Varian Inc., Mulgrave, Australia) at three different wavelengths (434, 578 and 730 nm).

The pH of the seawater and *m*-cresol purple was calculated from the absorption values of the samples with and without *m*-cresol purple according to Dickson et al. (2007).

2.3.2 Total alkalinity determination

Total alkalinity (A_t) was measured each day by using an automated open-cell titration method (Xiaowan Tao, 2009), with minor modifications. Before measurements, the method was evaluated by analysing Standard seawater (Scripps Institution of Oceanography, La Jolla, CA, USA) with known salinity and A_t. A water sample from a random tank was incubated at 25°C in a water bath before titrated with a TitraLab TIM860 Titration Manager, with a PHC2001-8 ph electrode (Radiometer Analytical SAS, Villeurbanne, France). The sample was titrated with 0.1 M hydrochloric acid (HCl) with 0.68 M NaCl. Approximately 25 ml of the water sample was weighted by using a Precisa® 180A Precision Balance, (Precisa Gravimetrics AG, Aldingen, Germany), as an indirect measure of the volume of the sample. A magnet stirrer stirred the sample during the titration.

2.3.3 Temperature measurements

The temperature was measured once a day, using a glass thermometer (VXR®Precicion Thermometer), with an accuracy of +/- 0.3°C. The thermometer was placed between the wall of the tanks and the internal equilibrium column of the tank, where it was left to equilibrate with the ambient water temperature. The thermometer was rinsed with fresh water between each tank to avoid transfer of copepods between treatments.

2.3.4 Salinity measurements

Salinity of the water was measured by using a refractometer (H_2Ocean ATC salinity refractometer), which measures salinity of seawater within the range of 20-40 ppt. Before salinity of a sample was determined, the refractometer was calibrated by using standard seawater. Salinity was measured each day during the exposed period.

2.3.5 Feed conditions

During the experiment the copepods were fed with three different algae; *Isochrysis galbana, Dunalella teriolecta* and *Rhodomonas baltica*. *I. galbana* is considered to be to small to constitute as significant food resource to *C. finmarchicus* (Båmstedt et al., 1999). However, since this combination of algae have been proved successful in previous experiments (Hansen et al., 2007), this algae was also included in the mixture. Other studies has also pointed out the potential importance of small cells in the diet of *Calanus spp* (Meyer et al., 2002)

The algae were continuously added to the different experimental tanks with a twelve channel peristaltic pump (Watson-Marlow 520S). The algae suspension was delivered to the tanks using twelve silicone tubes just beneath the water surface, in connection to the inflow of water, to ensure a good distribution of algae in the tanks.

The targeted concentration of algae was 250 μg C L^{-1}. *R. baltica* and *D. teriolecta* constituted 90% of the targeted amount and contributed with 88.8 and 11.1% of the carbon, respectively. The remaining 10% of the targeted amount came from *I. galbana.* The targeted concentration was selected to prevent excess of food in the tanks and to give a mild limitation of the animals´ food supply. The targeted concentration was set to 250 μg C L^{-1} based on previous feeding experiments.

For determination of algae concentration, samples of seawater was collected from the experimental tanks by first placing a filter cup (64 μm mesh) into the tanks to prevent copepods in samples. Then, a 25 ml cup was almost filled up with water from inside of the filter cup. The filter cup was rinsed with distilled seawater between tanks. The water samples were then placed on a stirring table to prevent algae sinking to the bottom of the cup, before algae concentration was measured with a coulter counter (Beckman Militisizer™ 3 Coulter Counter®). The coulter counter determines the algae concentration (# cells mL^{-1}) by counting particles and their diameter (μm) on three subsamples from each sample, resulting in three curves representing the distribution of algae size in the sample. The average of these curves were used and the area under the particles in size range from 5.363 to 9.561 μm were calculated.

Number of cells (# cells mL^{-1}) was transformed to carbon equivalents (μg C L^{-1}) by using the carbon content of the three algae. Carbon analysis of the algae was performed after the experiment. Stock solution of *R. blatica, D.tertiolecte* and *I. galbana* were incubated for 12

hours in experimental conditions (10°C), before algae concentration were determined by a coulter counter. A known number of cells were then diluted to 100 mL with filtrated seawater and filtrated by using a vacuum pump (Vacuumbrand GMBH, str 4, Wertheim Germany) onto a 1,2 μm glass microfiber filter (Whatman®, Grade GF/ C, 24mm). The filters with algae were then placed in tin capsules (SANTIS analytical 5x9 mm) and dried in a heater cabinet (Termarks TS4115) for 24 hours prior to carbon analysis. The algae were then analysed in a CHN Elemental Analyser 1106 (Carlo Erba Instruments, Italy) performed at SINTEF aquaculture and fisheries, Trondheim.

2.4 Animals

2.4.1 Collecting eggs for cohort of *C. finmarchicus*

Adult copepods from the culture of *C. finmarchicus* were transferred to eight 50 L polyethylene tanks (500 animals/ tank). These tanks were filled almost full with seawater with a temperature of 10°C. After incubation for 24 hours the water in the tanks were reduced to approximately 3 L and the remaining water poured over a filter cup (200 μm pore size) and collected in a glass bowl. The adult copepods remained in the filter cup, while the water, together with the eggs, was collected in the glass bowl. This water was filtrated again with a filter cup with a pore size of 46 μm, to collect the eggs. Eggs from all of the eight tanks were pooled and diluted to a volume of 2 L in a measuring cylinder. The cylinder was gently inverted to obtain a homogenous mixture and a subsample of 10 mL was counted under a Leica M205 C stereomicroscope (Leica Microsystem, Wetzlar, Germany). The concentration was found to be 290 eggs/ 10mL, which gave a total yield of 58.000 eggs. 2 L homogenous mixture was evenly divided to twelve beaker glasses, one to each experimental tank, by adding small subsamples to the beakers in several sequences until each beaker glass was filed with 166 mL of the homogenous mixture. An estimate of 4800 eggs in each beaker represented the starting concentration in each tank in the start of the experiment.

Before the eggs were released into the experimental tanks, they were acclimated to the experimental conditions. Eggs for treatment with ambient CO_2 concentration (treatment ambient and warming) were washed thee times with water gathered from their respective

experimental tanks. This was done by carefully submerging a 46 μm pore sized filter cup with eggs into three separate glass bowls filled with water from the experimental tanks. After the washing procedure the eggs were transferred to a glass bottle (500 mL), one to each tank, with a waterproof screw cap. This procedure was also followed with the eggs for treatments with elevated CO_2-concentration (treatment acidified and acidified*warming). The glass bottles were then submerged into their respective tanks to equilibrate to the temperature of the ambient water. After a few hours, when the temperature had equilibrated, the eggs were released into the tanks.

2.4.2 Sampling to determine median development time

Three stages were targeted for sampling, nauplii 3 (N3), Copepodite 1 (C1), and copepodite 5 (C5). Sampling of the various stages was conducted according to the expected duration of stages (Campbell et al., 2001). An interval of 8 hours was adopted during sampling of the N3-animals. The sampling was ended when 100% of animals in samples had reached the targeted stage. During sampling for C1-animals an interval of 12 hours was choosen. Late in the C1-stage the interval was increased to 24 hours. A custom-made 200 ml sampling tube was used to sample N3 and C1 animals (figure 2.3b). Targeted C5 animals were only sampled at one point, after 23,3 days, with a 2x 1 L beaker giving a total of 2 L sample from each tank, this procedure was selected due to lack of suitable sampling equipment and time to perform the sampling. Prior to the sampling a stirring – procedure was carried out to ensure uniform distribution of animals in the tanks. A circular custom- made PVC plate was carefully pushed up and down the water column four times before animals was sampled (see figure 2.3a). To ensure a representative measure of the stage distribution in the tanks, several subsamples were taken in different locations in the tank, to give the required volume of sample. The total volume of samples increased as the densities in the tanks decreased. Subsamples were transferred to a 1 L glass bottle. Here the animals were stained with neutral red staining, a method more closely described in chapter 2.4.9. The sample was then filtered through a custom-made filter system with a filter attached to outlet to collect the stained animals (see figure 2.3c). The filter with the animals was then preserved with 4% formaldehyde (Apotekerproduksjon AS, Oslo, with phosphate buffer) in 8 mL glass bottle and stored at 4°C in the dark.

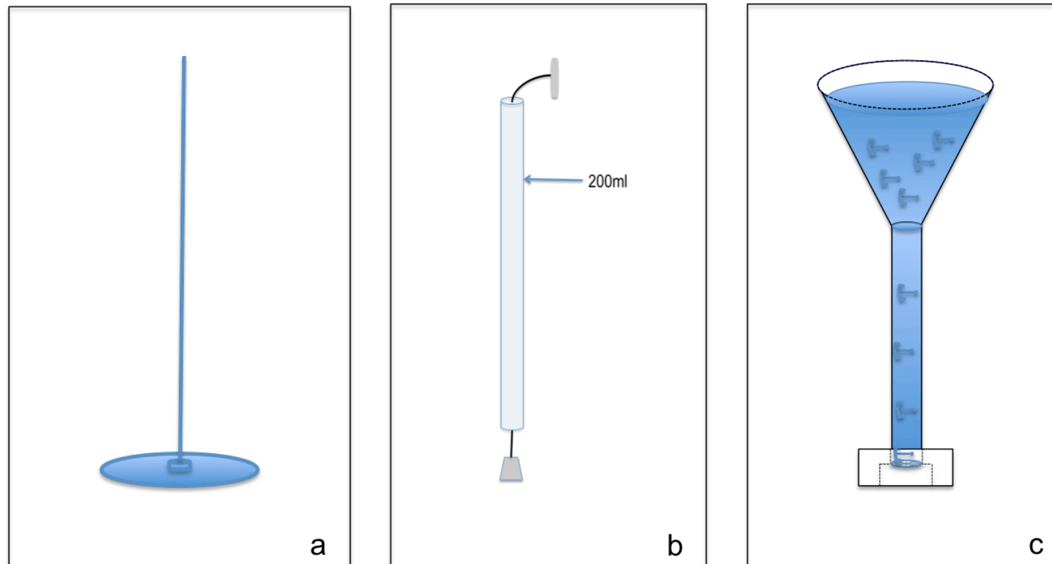

Figure 2.3: a) Illustration of stirrer used to ensure uniform distribution on the tank prior to sampling. **b)** Sampling tube used during sampling for N3 and C1- animals. By pulling the line the plunger closes the tube and a water sample is obtained. **c)** Filtration system with a filter mesh attached to a screw cap at the outlet of the filter tube. The water is filtrated through and the animals retains in the filter, which is removed and preserved.

2.4.3 Stage determination

To assess the stage distribution in the experimental tanks, stage determination was performed under a Leica M205 C stereomicroscope (Leica Microsystem, Wetzlar, Germany). First, the 4% formaldehyde - solution preserving the sample was filtered out using a 64 μm mesh. Then, the animals were rinsed in a phosphate buffer solution three times in separate stages, to remove excess formalin. The washed animals were then washed out of the filter using phosphate buffer, and collected in a glass well. All steps were done thoroughly to prevent loss of animals. The identification of stages was done according to Marshall (1972) and Mauchline (1998). Stages N1 and N2 were identified by the number of spines of the posterior end of the bodies. The number of appendages and the number of posterior spines identified stages N3 to N6. The copepodites (and adults) were identified by counting the number of segments of the tail and body.

After stage identification the animals was transferred back to their 5 mL glass bottle by using a funnel and added 4% formalin before they were stored in dark at 4°C.

2.4.4 Determination of median development time

Median development time (MDT) is defined as the time from the midpoint of the egg-laying period and until 50% of the cohort has reached a specific developmental stage (Landry, 1975). The cumulative fraction of the cohort, which had moulted to, or past, a targeted stage, was calculated using the stage distribution measured in the tanks. This fraction was plotted against the time from mid egg-laying point, which was set at 12 hours from start of incubation (i.e the median age of the collected eggs). Tail values were excluded from the sigmoidal cumulative fraction plots (<0.15, >0.85), to fit a linear curve and a least square regression determined MDT values (Campbell et al., 2001).

Samples with few animals (n<5) were assumed not to be representative and therefore excluded from the regression. Also, it is implied that a proportion of a cohort that has reached a certain stage, will increase over time. Thus, when fractions of a specific stage became smaller than a fraction determined at an earlier time, it was excluded from the regression.

To assess stage development differences 26,3 days after mid egg-laying point, an index based on the observed stage distribution, was used to estimate the development rate in the different treatments. The harmonic average stage in the collected samples was calculated by multiplying the number of animals within each stage with their relative stage duration (stage duration/duration C1-adult) at 12,5°C (mean of 11 and 14°C). Reference MDT values (Campbell et al., 2001) for N6 to adult were used to calculate the relative duration of C1 to adult. This number gives a stage-specific value (SPV). The sum of all SPVs was divided by the total number of animals in the sample, which gave the harmonic average stage in the sample (which indicates the development speed). To avoid potential contribution from 2nd generation animals all nauplii stages were excluded from this analysis.

2.4.5 Proportional duration

The proportional duration between C1 and N3-stage of *C. finmarchicus* was calculated by dividing the MDT-values provided at C1-stage with the MDT-values obtain from the N3-stage (MDT C1-stage/MDT N3-stage).

2.4.6 Sampling animals for biometric, and dry weight analysis

Sub-adult copepodites (C5) were collected for determination of biometric characters and dry weight. To ensure that the measured animals were as similar as possible with regards to ontogenetic development stage within the animals (C5+48 hours), approximately 200 copepodite 4-stage (C4) animals were identified under a microscope (Leica MZ 125) and incubated for 24 hours in 5 L polyethylene buckets in water collected from their respective tanks. After the incubation period, all animals, which had moulted into C5, were transferred to a 1L bucket and then incubated for another 48 hours. After incubation, eight C5-animals were sedated with tricaine methanesulfonate (FinQuel, 15 g/L in sea water, Argent Laboratories, Redmond WA, USA) and transferred to a petri dish to confirm the stage and take pictures. The pictures were taken using a digital video camera (Sony DWF-sx900, Sony Corporation, Tokyo, Japan), by using Fire-i software (Unibrain Inc., San Ramon CA, USA), before transferred separately to pre-weighted tin cup and stored in a freezing room at -18°C. This operation was followed for all treatments. The *C. finmarchicus* exposed to 14°C had a faster development than animals exposed to 11°C. Sampling for animals exposed to 11°C was therefore done four days after 14°C animals had finished their incubation period. The water in the buckets where the animals were incubated was exchanged every 24 hours with water from their respective tanks. To ensure correct treatment water parameters was analysed each day during the incubation period.

2.4.7 Biometric measurements

Pictures of 96 sampled animals (8 individuals from each treatment, with three replicates) were measured with respect to area and length of whole body and the lipid-sac by using the software program Image J (National Institute of Health, Bethesda MD, USA). Previous to photography of the sampled animals a picture of a reference ruler was taken. This made it possible to calibrate Image J to known pixel magnification of the pictures. By drawing a line from the anterior part of the head to the last segment of the thorax, the length of the whole body was measured. To calculate the area of the body a trace line was drawn around the body. The drawing was done on a pen-on-screen graphical tablet (Wacom Intous3, Wacom CO., LDT., Saitama, Japan). These values was calculated according to Miller et al, (Miller et al., 2000). The same method was used to calculate the lipid-sac. Using these values the percentage of lipid-sac volume to total volume of body was calculated.

2.4.8 Dry weight

After photography of 96 C5 animals was taken, for the purpose of biometric measurements, they were placed separately in empty tin capsules (SANTIS analytical 5x9mm). They where then dried in a heating cabinet (Termarks TS4115) at 60°C for 48 hours. The weight of the tin cups was measured on a micro scale (Mettler Toledo UMX2, ©Mettler-Toledo International Inc) before and after the animals was placed in the cups. The difference in weight before and after animals were placed in the cups was considered to represent the dry weight.

2.4.9 Neutral red staining

Neutral red staining is a method for differentiating live and dead marine zooplankton (Elliott and Tang, 2009). Neutral red staining is based on the ability of viable cells to incorporate and bind the dye in the lysosomes and therefore appear red (Repetto et al., 2008). Prior to the experiment the method of neutral red staining to assess mortality on *C. finmarchicus* was verified. A known amount of dead animals (killed by exposing animals to 50°C for 5 min, according to Elliott and Tang (2009), was stained together with the same amount of live animals and assessed under a microscope after incubation. (Leica MZ 125). This test produced satisfying results.

After subsamples were collected from the experimental tanks and transferred to 1 L glass bottles, 1,5 µL neutral red stock was added to every 1 mL of sample (0,9 mL neutral red to 600 mL sample) according to previous experiments with copepods (Elliott and Tang, 2009). The neutral red stock was made prior to the experiment by adding 1 g neutral red powder (Sigma-Alderichemie GmdH) to 100 mL deionized water, and slowly stirred overnight with a magnet stirrer to completely dissolve the powder. After adding neutral red stock to the sample, glass bottles were carefully stirred to completely dissolve the neutral red in the sample. The sample was then incubated for 1 hour before filtrated with the custom made filtration system.

2.5 Statistics

Permutational multivariate analysis of variance (PERMANOVA) tests were used to run two way analysis of the effect of CO_2-concentration and temperature or any interaction between the two factors. PERMANOVA is a permutational MANOVA procedure (Anderson, 2001, McArdle and Anderson, 2001) which uses the similarity or dissimilarity matrix of choice (in this case Bray-Curtis distance). This method avoids assumptions of distributions like normality by using permutation to generate null hypothesis distribution for its pseudo-F statistics. Significant findings were followed by LSD post-hoc tests. All pseudo-F statistics and p values given in parenthesis in the results represent the results from the respective PERMANOVA tests. All p values given, without pseudo-F values, represent the outcome of the subsequent LSD post hoc tests. All data passed the Levene`s test of equality of error variance before running LSD post-hoc.

All PERMANOVA analyses were carried out by the statistical program R (ver. 2.15.1), The R Foundation, Austria). The package VEGAN was downloaded and the function “adonis” used to run PERMANOVA tests. All LSD post-hoc test were performed by using the statistical package SPSS (IMB® SPSS® Statistics, Version 20). The significant level for all tests were set to 0.05

3 Results

3.1 Experimental conditions.

The experimental conditions measured during the exposure period (pH_t, temperature, alkalinity and salinity) are presented in table 3.1. Table 3.2 shows the conditions during the incubation period where C4 animals moulted into C5 stage.

Table 3.1: Water parameters (pH_{tot} temperature, alkalinity and salinity) for the different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm) during the experiment (mean ±SD). The values are the overall means of three replicate tanks for the duration of the experiment. The pCO_2 (ppm), $pH_{tot,\ in\ situ,}$ ΩCa and ΩAr values, are values corrected for in situ experimental temperature.

Treatment	$pH_{tot,\ 25^{\circ}C}$	Temperature (°C)	Alkalinity	Salinity (ppt)	pCO_2 (μatm)	$pH_{tot,\ in\ situ}$	ΩCa	Ωar
Ambient	7,78±0,02	11,07±0,1	2244,9±11,8	33,6±0,4	470,9±67,4	7,99±0,02	2,82±0,01	1,79±0,01
Warming	7,80±0,02	14,00±0,2			508,3±77,0	7,96±0,03	2,98±0,03	1,90±0,02
Warming* acidified	7,25±0,02	14,00±0,1			2118,4±296,5	7,38±0,03	0,88±0,01	0,56±0,01
Acidified	7,20±0,03	11,13±0,2			2160,5±333,6	7,37±0,03	0,76±0,02	0,48±0,01

Table 3.2: Water parameters (pH_{tot}, temperature, alkalinity and salinity) for the different treatments: of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm) during the incubation period were animals moulted from C4 into C5 stage. The values are mean (mean ±SD) of the three replicate buckets where the animals were incubated. The pCO_2 (ppm), $pH_{tot,\ in\ situ}$, ΩCa and ΩAr values, are values corrected for in situ experimental temperature. Animals treated at elevated temperature (warming and warming*acidified) were sampled at day 27 for incubation. Animals treated at ambient temperature (ambient and acidified) were sampled at day 31 for incubation.

Treatment	$pH_{tot,25^{\circ}C}$	Temperature (°C)	Alkalinity	Salinity (ppt)	pCO_2 (μatm)	$pH_{tot,\ in\ situ}$	ΩCa	ΩAr
Warming	7,70±0,03	13,8±0,4	2238,3±4,5	33,5±0,0	593,7 ±42,9	7,88±0,03	2,27±0,00	1,44±0,00
Warming* Acidified	7,23±0,06	13,6±0,6			754,8 ±183,6	7,80±0,08	2,38±0,04	1,52±0,03
Ambient	7,68±0,03	11,2±0,3	2233,7±5,1	33,3±0,2	2176,0±342,6	7,36±0,06	0,84±0,06	0,53±0,04
Acidified	7,26±0,03	11,2±0,2			1809,8±153,2	7,43±0,03	0,86±0,04	0,55±0,03

3.2 Algae concentration

The algae concentration (µg C L^{-1}) in the different treatments during the experiment is shown in figure 3.1. The algae concentration showed a similar increase from day 3 to day 10 in all treatments. After this, the concentration declined in all treatments, but the treatments with elevated temperature (14°C) consistently showed a lower algae concentration throughout the rest of the experiment compared to the treatments with ambient temperature (11°C).

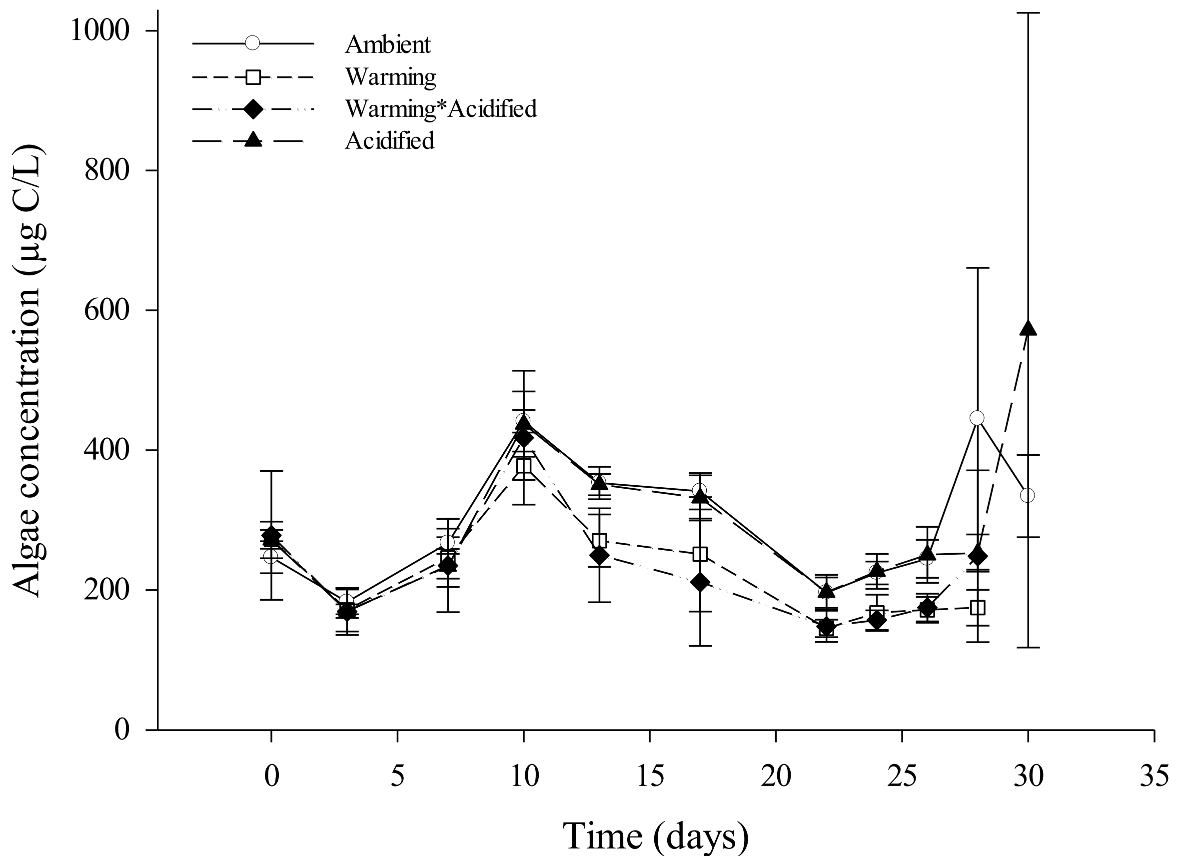

Figure 3.1. Development in measured algae concentration (ug C L^{-1}) in the different treatments during the exposure period (mean ± SD), n=3.

3.3 Mortality

During verification of the neutral red staining method it proved easy to discriminate between live and dead animals (see figure 3.2a). However, animals sampled and stained during the experiment (both nauplii and copoepodites) were quite variable with regards to coloration intensity (see figure 3.2b and c). Consequently, mortality as an endpoint had to be abandoned from the experiment.

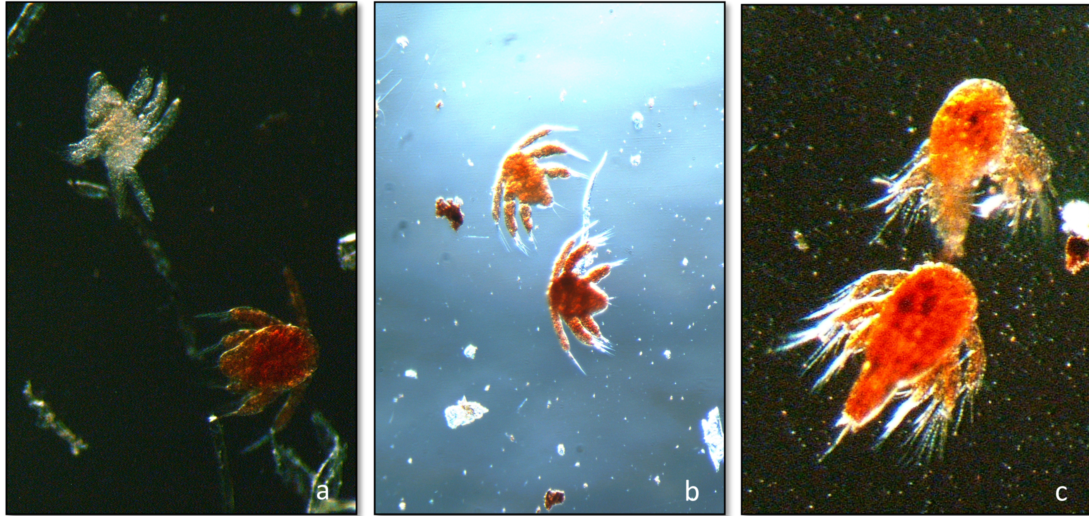

Figure 3.2: Animals colored using neutral red staining. Figure 2a show animals stained during the verification of the method where dead animals (white coloration) could easily be discriminated from live animals (red coloration). Figure 2b and c show animals sampled during the experiment and shows examples of how coloration intensities varied among different live animals.

Table 3.3: Statistical results derived from PERMANOVA tests. Temp= temperature, d.f= degrees of freedom, SS= sum of squares, MS= mean sum of squares, *= interaction term. Significant results in bold.

MDT N3-stage						
	d.f	SS	MS	F	R^2	*p*
CO_2	1	0,00000108	0,00000108	0,0159	0,00058	0,913
Temp	**1**	**0,0126301**	**0,0126301**	**18,7005**	**0,67538**	**0,004**
CO_2*Temp	1	0,0006569	0,0006569	0,9726	0,03513	0,349
Residuals	8	0,0006754	0,0006754		0,28892	
MDT C1-stage						
	d.f	SS	MS	F	R^2	*p*
CO_2	1	0,0006579	0,0006579	1,954	0,02814	0,211
Temp	**1**	**0,0160449**	**0,0160449**	**47,662**	**0,68634**	**0,001**
CO_2 *Temp	**1**	**0,0039816**	**0,0039816**	**11,828**	**0,17032**	**0,013**
Residuals	8	0,0233774	0,0003366		0,1152	
Score after 23,3 days						
	d.f	SS	MS	F	R^2	*p*
CO_2	1	0,0000251	0,0000251	0,0474	0,00235	0,897
Temp	**1**	**0,00566**	**0,00566**	**10,1006**	**0,53051**	**0,003**
CO_2 *Temp	1	0,0007523	0,0007523	1,4	0,07052	0,277
Residuals	8	0,0042315	0,0005289		0,39662	
Proportional duration of stages between N3 to C1						
	d.f	SS	MS	F	R^2	*p*
CO_2	1	0,0006547	0,00065468	1,95	0,12826	0,182
Temp	1	0,0016278	0,00162777	4,85	0,3189	0,074
CO_2 *Temp	1	0,0001353	0,00013535	0,403	0,02652	0,557
Residuals	8	0,0026865	0,00033582		0,52633	
Dry weight						
	d.f	SS	MS	F	R^2	*p*
CO_2	1	0,000716	0,0007164	0,3185	0,01	0,559
Temp	**1**	**0,028414**	**0,0284142**	**12,631**	**0,39674**	**0,011**
CO_2 *Temp	**1**	**0,024492**	**0,0244924**	**10,8876**	**0,34198**	**0,015**
Residuals	8	0,017997	0,0022496		0,25128	
Lipid sac volume						
	d.f	SS	MS	F	R^2	*p*
CO_2	1	0,008713	0,008713	1,846	0,03136	0,196
Temp	**1**	**0,171968**	**0,171968**	**36,44**	**0,61905**	**0,001**
CO_2 *Temp	**1**	**0,059361**	**0,059361**	**12,578**	**0,21369**	**0,01**
Residuals	8	0,037754	0,004719		0,13591	

3.4 Development rate

No significant interaction between temperature and CO_2-concentration (pseudo-F=0.9726, p=0.349) on the MDT for the N3-stage was observed in the different treatments (figure 3.3), although animals exposed to warming and acidification combined had the shortest MDT (4,1 ± 0,31 days) of all treatments. A significant decrease in MDT (pseudo-F=18,7, p= 0,004), as an effect of warming was observed at N3 stage. A post-hoc test revealed a significant reduction of the MDT in the warming alone - (0,44 days, p= 0.042) and in the warming and acidified combined treatment (0,57 days, p= 0.015) when compared to the ambient treatment. No significant effect of acidification on the MDT was observed among the N3-animals (pseudo-F=0.0159, p=0.913).

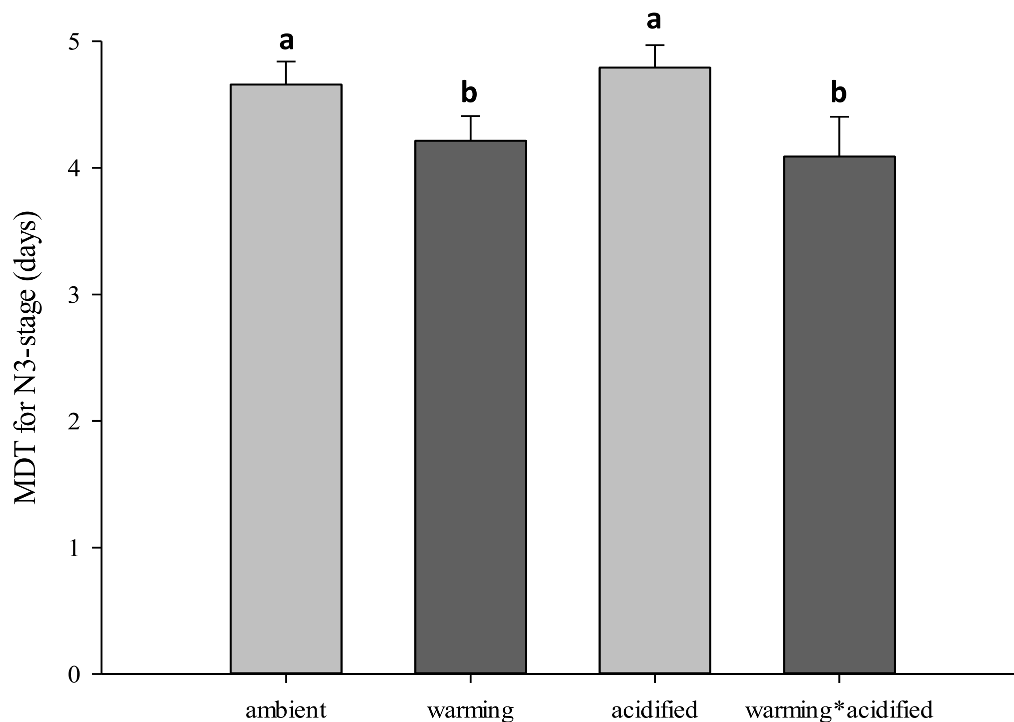

Figure 3.3: The mean values (mean ± SD, n = 3 replicate tanks) of Median Development Time (MDT) for N3-stage of *C. finmarchicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments (p< 0.05).

A significant interaction between temperature and CO_2-concentration (pseudo-F = 11.828, p=0.01) on the MDT for the C1-stage was observed in the different treatments (figure 3.4). A post-hoc test revealed a significant reduction of the MDT in the warming alone - (1,00 days, p<0,001), and in the warming and acidified combined treatment (2,44 days p<0,001) when compared to the ambient treatment. Also, warming and acidification combined caused a significant reduction of the MDT (3,14 days, p<0.001), compared to the warming alone treatment. No significant effect on the acidified treatment alone was observed.

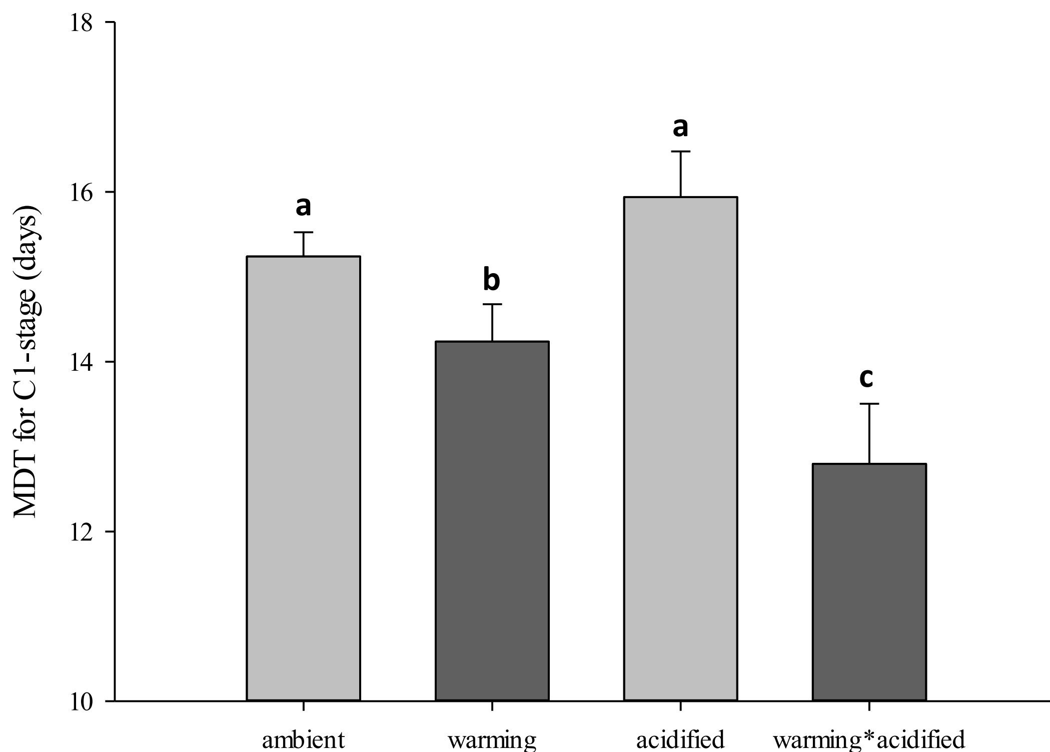

Figure 3.4: The mean values (mean ± SD, n = 3 replicate tanks) of Median Development Time (MDT) for copepodite 1-stage of *C. finmarchicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments (p< 0.05).

An index based on the observed stage distribution was used to estimate the development speed in the different treatments after 26,3 days after mid egg laying point (figure 3.5). No significant interaction between temperature and CO_2-concentration (pseudo-F = 1.422, p = 0.277 was observed, although animals exposed to warming and acidification combined displayed the highest index score (0,19±0,01), indicating that this treatment had the fastest development. Animals exposed to ambient conditions had the lowest score (13,3±0,01) indicating that this group developed slowest. Also, a significant increased score as an effect of warming was observed after 26,3 days (pseudo-F = 10.7, p= 0.003). A post-hoc test revealed a significantly increased score in both the warming - (0,4, p=0,003) and the warming and acidified treatment (0,53, p=0,001). No significant effect of CO_2-concentration on the index score was observed (pseudo-F=0.0159, p=0.913).

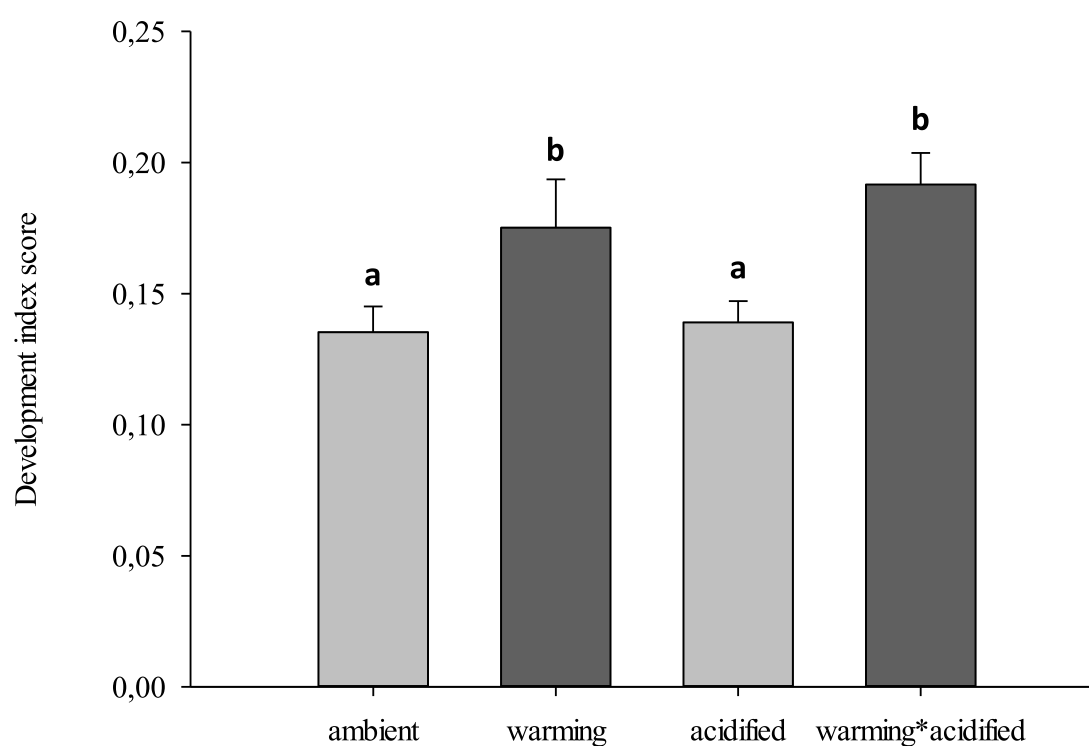

Figure 3.5: The development index score in the different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm), 26,3 days from mid egg-laying point (mean ± SD, n = 3 replicate tanks). A high index-score indicates fast development. Different letters denoted significant differences between treatments (p< 0.05).

3.5 Proportional duration

No significant effect of temperature, CO_2-concentration or interaction between the two factors was observed with regards to proportional duration between the N3 and the C1-stage was observed in the different treatments (figure 3.6)

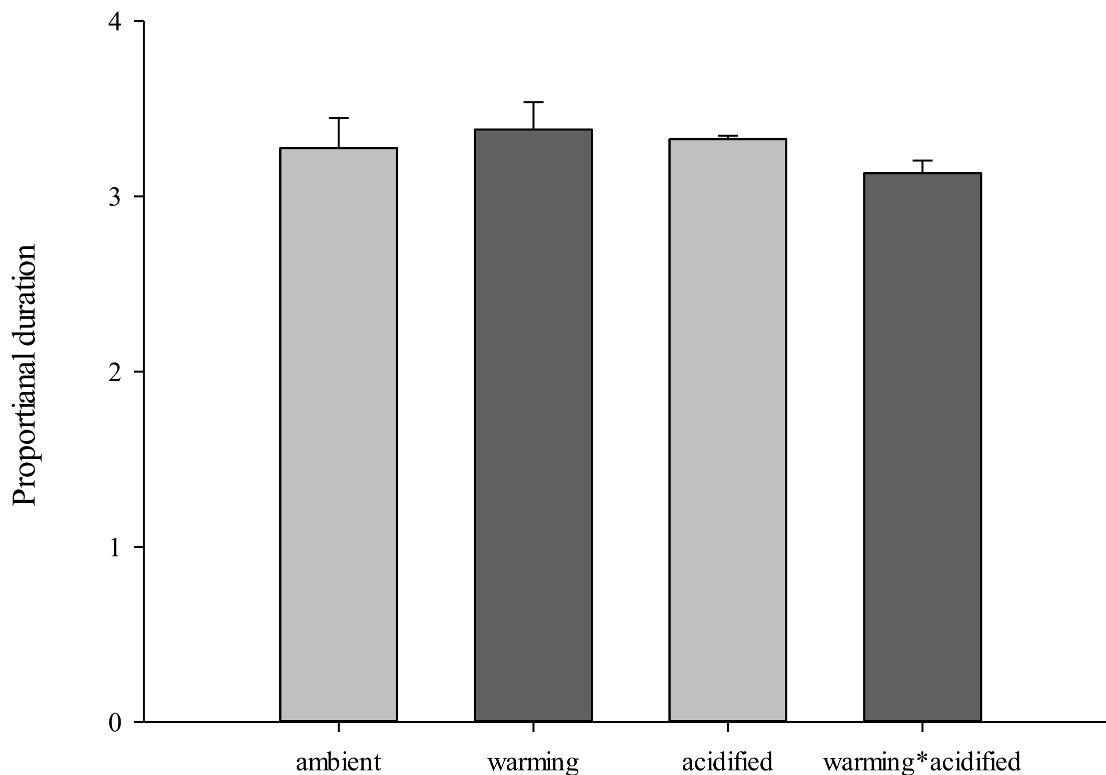

Figure 3.6: Mean values (mean ± SD, n = 3 replicate tanks) of the proportional duration (MDT C1-stage/MDT N3-stage) between N3 and C1 stage *C. finmachicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments ($p < 0.05$).

3.6 Dry weight of *C. finmarchicus*

A significant interaction between temperature and CO_2-concentration on dry weight (pseudo-F = 10.89, p = 0.015) was observed in the different treatment (figure 3.7). The highest dry weight was observed among the animals from the ambient treatment (0,12 ± 0,004mg). The lowest dry weight was observed for animals from the warming treatment (0,08 ± 0,007mg). A post-hoc test revealed a significant reduction in the dry weight of the animals from the warming- (1,46 fold, p=0,001), acidified-(1,22 fold, p=0,019), and warming and acidified combined treatment (1,24 fold, p=0,015) when compared to the ambient treatment. However, warming and acidification combined caused a non-significant increase in dry weight (1,18 fold, p=0.089), compared to the warming alone treatment.

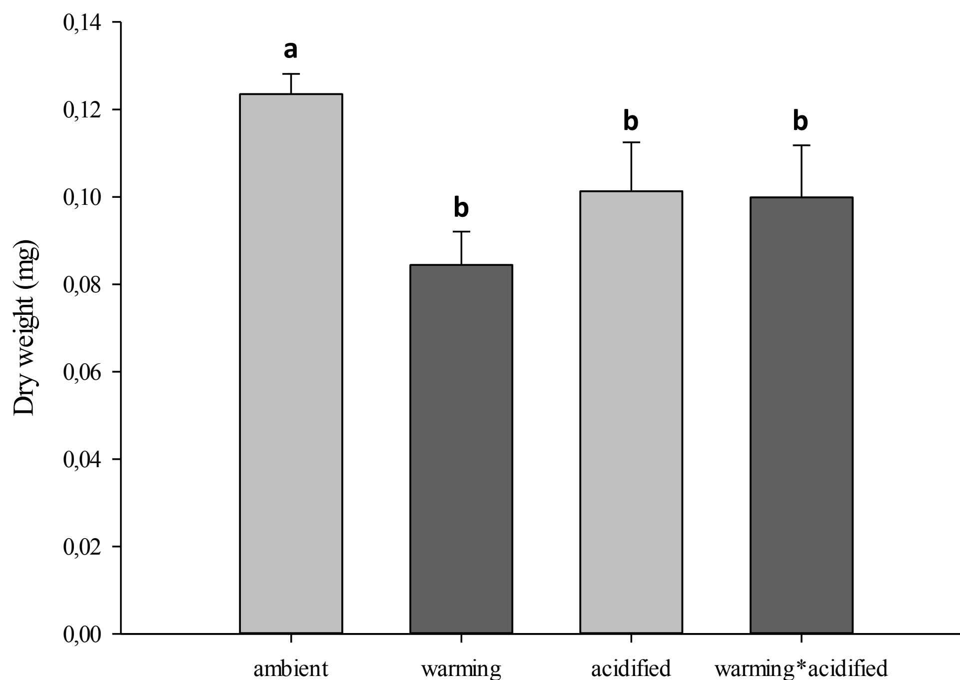

Figure 3.7: Mean values (mean ± SD, n = 3 replicate tanks) of dry weight (mg) of C5-stage *C. finmarchicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Different letters denoted significant differences between treatments (p< 0.05).

3.7 Lipid-sac volume of *C. finmarchicus*

A significant interaction between temperature and CO_2 -concentration (pseudo-F = 12.578, p = 0.01) on the lipid-sac volume was observed among the different treatments (figure 3.8). The highest percentage of lipids was observed in animals from the ambient treatment (10,4 ± 1,1%). A post-hoc test revealed a significant reduction in the lipid content of the animals from the warming- (2,23 fold, p<0,001), acidified-(1,26 fold, p=0,045), and warming and acidified combined treatment (1,56 fold, p=0,003) when compared to the ambient treatment. However, warming and acidification combined caused a nearly-significant increase in lipid content (1,43 fold, p=0.059), compared to the warming alone treatment.

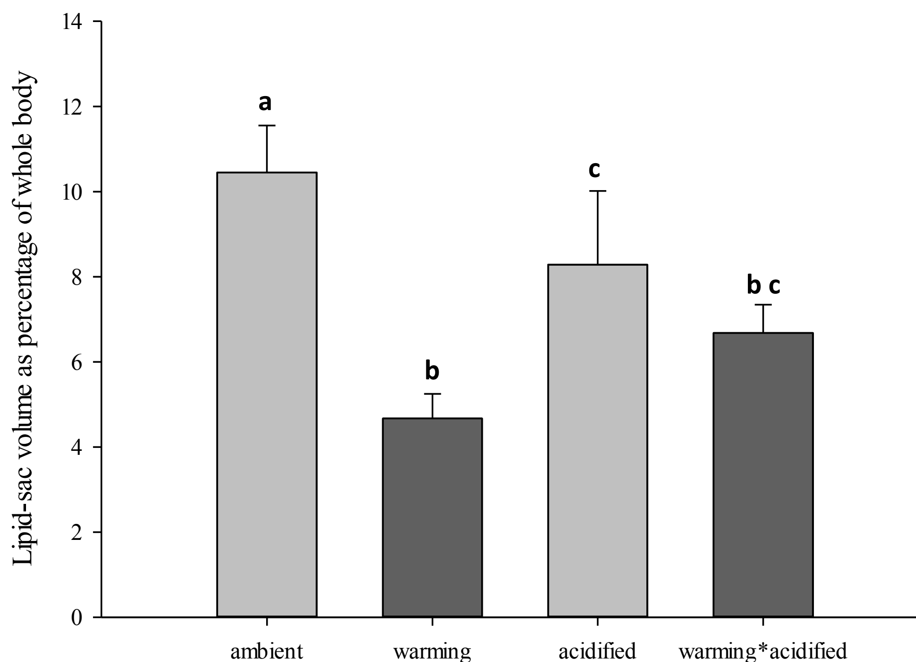

Figure 3.8: Mean values (mean ± SD, n = 3 replicate tanks) of lipid-sac volume as percentage of whole body of C5-stage *C. finmachicus* at four different treatments of temperature and CO_2: ambient (11°C/390ppm), warming (14°C/390ppm) acidified (11°C/2080ppm) and warming*acidified (14°C/2080ppm). Values are calculated from biometric values calculated from 8 x 3 animals from each treatment. Different letters denoted significant differences between treatments (p< 0.05).

4 Discussion

To understand the potential impact of ocean acidification and a concurrent warming, it is critical to cover the natural range of ambient and future levels of the factors experienced by the species in question (Gianguzza et al., 2013). The animals in this experiment were exposed to realistic future regime of both temperature (Solomon, 2007) and CO_2-concentrations (Caldeira and Wickett, 2005) and effects on sub-lethal processes related to development was targeted since these have been considered to be particularly vulnerable (Fitzer et al., 2012, Gianguzza et al., 2013).

The sensitivity of marine animals can increase with exposure time (Yamada and Ikeda, 1999, Kurihara, 2008). However, studies of interaction effects of stressors that exceeds 10 days are rare (Hildebrandt et al., 2014). Also, the understanding of the responses across life cycles to combined stressors associated to oceanic changes is limited (Byrne and Przeslawski, 2013) Thus, the present cross-factorial medium term study from egg to the sub-adult stage (C5-stage) provides an approach to obtain a more realistic estimate of effects caused by climate changes than short-term or single factor studies.

4.1 Evaluating exposure conditions

Water parameters (CO_2-concentration, pH and temperature) measured during the experiment were all relatively close to targeted levels (table 3.1). The mean temperature throughout the exposure period was 14.0°C for the warming- and warming and acidified combined treatment. The temperature in the ambient- and acidified treatment showed small variations, with the latter showing largest divergence with a mean temperature of 0,13°C above the targeted level (11.0°C). The water parameters (CO_2-concentration, pH and temperature) measured during the incubation-period (table 3.2) show small deviation from the targeted levels. This may be because of the animals were incubated in close buckets, where the water was exchanged only once a day.

Animals in the different treatments were provided with approximately the same concentrations of algae during the exposure period, but small variations in algae concentration were detected during the experiment (see figure 3.1). After day 11, treatments with elevated

temperature (i.e. warming and warming*acidified) displayed a reduced algae concentration compared to the treatments with ambient temperatures (i.e. ambient and acidified). As the animals go through the different development stages they become increasingly efficient in filtering out the algae from water (Mauchline, 1998). Thus, low algae concentration observed at 14°C might be due to the faster development of the animals at this temperature (see e.g. figure 3.2). It has also been suggested that increased metabolism may lead to increased food demands to compensate for the energetic cost of having a higher metabolism (Koski and Kuosa, 1999, Gibson et al., 2011). Increased feeding rates to compensate for increased metabolism costs due to CO_2 exposure (Li and Gao, 2012) or increased temperatures (Almeda et al., 2010) has been reported in copepods. Although the observed difference in algae concentration most likely reflects the faster development detected in the treatments that received elevated temperature (+3°C), it is possible that higher stage-specific feeding rates among the animals from these groups, to compensate for metabolic costs, may also have contributed.

Food availability is known to have a strong influence on development rates of copepods (Mullin and Brooks, 1970, Breteler and Gonzalez, 1988, Campbell et al., 2001), and it is therefore possible that the reduced algae concentration observed in the treatments with elevated temperature (warming and warming*acidified) have caused the animals in these treatments to develop somewhat slower than if they had received the targeted concentration of the algae throughout the experiment. This might explain the apparent loss of a significant interaction between temperature and CO_2-concentration on the development rate after 26,3 days (see figure 3.5). In addition, a reduced lipid storage and dry weight might also have resulted from the extra restriction in algae concentration in these treatments and thereby, enhanced the apparent effect of increased temperature on these parameters.

4.2 Method assessment

Estimated development times in this experiment do not account for mortality, as the method intended for assessing mortality (the neutral red staining) did not produce satisfactory results. Bi et al. (2011) found that development of the cuphausiid *Euphausia pacifica* from egg to furcilia V2 was 20% faster when the mortality rates were considered. Thus, if some of the experimental treatment caused higher mortality rates among the animals, this would

potentially lead an underestimation of the development rate in these treatments. In worst case, different mortality rates among treatments might produce different estimates of development rate, where no differences in development rate actually exist.

The method adopted to analyse development speed based on sampling at a single specific time point (26,3 days) accounts for different stage durations in *C. finmarchicus* (Campbell et al., 2001), by weighing the different stages according to their respective relative duration. This was done to prevent underestimation of development rate in treatments with faster development rate, and consequently a higher proportion of late stages with a relatively longer stage-duration. The adopted method is justified by the fact that the relative duration of a given stage have been demonstrated to be constant over a wide range of temperatures (4-12°C) in *C. finmarchicus* (Campbell et al., 2001) and independent of both temperature, CO_2-level and the combination of elevated temperature and acidification demonstrated in the present study (figure 3.6).

The sources of error discussed will tend to reduce and not emphasize observed effects of the different treatments. The observed differences in the development rate between the treatments is therefor likely to reflect the effects caused by increased temperature, CO_2-concentration or in combination.

4.3 Mortality

Mortality is an important endpoint to evaluate the impact of warming and acidified conditions at an ecological level. Unfortunately, assessment of mortality had to be abandoned in this experiment. The variation with regards to coloration intensity among the animals stained with the vital dye was too variable to allow a confident discrimination between live and dead animals. The incubation of animals in the neutral red staining solution was chosen to be 1 hour, witch deviates from the protocol (15min incubation) suggested by Elliott and Tang (2009). Although Tang et al. (2006) found no effect of incubation time on the staining result, they only tested for 5, 15 and 20min incubation. The longitude of the staining incubation used in the present experiment may therefore have contributed to the observed variation in coloration intensity. However, other factor seems more explaining. It has been shown that membrane damage inhibits the accumulation of neutral red and that the amount of dye

released from the cells is proportional to their vitality (Vazzana et al., 2014). Thus, different vitality among animals may cause differences in accumulation of the dye, which may explain the variation with regards to the visual coloration of animals. From this it may be hypothesized that less preferable (sub-lethal) environmental conditions might cause greater variability in the individuals' physical conditions status and thus result in more variable coloration intensity among the animals. Accordingly, it could be interesting to examine the variations in coloration intensity of the animals between treatments. However, this was not examined further as it was not one of the objectives in the present study.

4.4 Development rate

The estimates of development rate in the N3-stage (figure 3.3), C1-stage (figure 3.4) and the development stage reached after 26,3 days into the experiment (figure 3.5) all displayed the same overall trend with regards to the different treatments. Within the scenarios tested in the present study, elevated temperature generally caused a greater effects than decreased pH on the development of *C. finmarchicus* (when they are considered as single factors). Although a significant interaction between temperature and CO_2-concentration was only found for the C1 stage, the groups that received combined exposure to warming and acidification showed the fastest development at the investigated stages (N3, C1) and time points (26,3 days after experiment start), indicating that acidification and warming elicits a positive synergistic effect on the development rate.

4.4.1 Effects of temperature on the development rate

Regardless of CO_2-concentration, a +3°C warming caused a significant increase in the development rate (decreased MDT at N3 and C1-stage, and increased score after 26,3 days). The response to increased temperature on the development of marine invertebrates is generally considered to reflect a balance between facilitation up to a certain level (e.g. enhanced growth at ca. 1-2°C above ambient) and negative effects with grater warming (≥4°C) (Byrne and Przeslawski, 2013). However, negative effects are also widely reported within near future projections (+2-4°C) (Bartolini et al., 2013). Wild stock populations of *C. finmarchicus* display an optimum temperature ranging from 6-10.6°C. Thus, a warming from 11 to 14°C could possibly have a negative effect. However, the temperature-induced increase

in development rate observed in the present study indicates that a 3°C warming is within the thermal window of performance of *C. finmarchicus.* The relatively broad thermal tolerance interval reported in *C. finmarchicus* (Helaouët and Beaugrand, 2007) might lead to the increased development rate found in this study. Hypercapnia is suggested to potentially narrow the thermal window of performance of species (Pörtner, 2008). However, the CO_2-concentration projected to occur within year 2300 (2080ppm) had no negative affect on the development rate of *C. finmarchicus* when the animals were exposed concurrently to a 3°C warming.

4.4.2 Effects of acidification on the development rate

Animals from the acidified treatment displayed the highest MDT among all the treatments at the N3 and C1-stage, indicating that these animals developed slower (although not significantly different compared to the ambient treatment). The process of moulting is energy-demanding (Mauchline, 1998) and as suggested by several studies, hypercapnia constitutes an extra cost due to increased metabolism, which may reduce scope for growth and subsequently the development rate (Stumpp et al., 2011, Whiteley, 2011, Håkedal, 2013). The same trend (not significant) on the development rate as an effect of CO_2-concentration (2080ppm) has previously been reported in *C. finmarchicus* (Gustavson, 2013). However, the absence of significant effects on the development at all investigated stages (N3 and C1) and after 26,6 days, suggests that *C. finmarchicus* may be generally robust to direct effects of ocean acidification alone. This is consistent with the available data regarding sensitivity to elevated CO_2-concentrations in *Calanus sp*. and supports the notion that this genus might be relatively robust towards CO_2-concentrations projected to occur by year 2300 (≤2000ppm) (Pedersen et al., 2014).

4.4.3 Effects of concurrent warming and acidification

A significant interaction observed at the C1-stage indicates that concurrent warming and acidification may cause a positive synergistic effect on the development rate. Although not significantly, a tendency of a positive synergistic effect on the development rate was also observed at N3-stage and after 26,3 days. The animals from the warming and acidified combined treatment displayed the fastest development rate at the N3-stage, C1-stage and after 26,6 days compared to the other treatments, but this was only significant at the C1-stage.

Previous suggestions that ocean acidification may only cause negative responses in animals (Pörtner, 2008), was recently challenged by the introduction of a theoretic framework that integrates the mechanisms involved during both warming and acidified conditions (Gianguzza et al., 2013). It hypothesized that both increased temperature (Peck and Prothero-Thomas, 2002) and decreased pH (Whiteley, 2011) may induce increased growth, since both conditions may cause an up-regulation of metabolism (see figure 4.1). Under non-limiting energy conditions increased metabolism can be translated to an increased scope for growth (Navarro et al., 2006). It can also be hypothesized that the energy state of nauplii and copepodites determine the timing of the moulting to next consecutive stage, as development and the process of moulting is energy demanding (Mauchline, 1998). Thus, an increased scope for growth can translate into an increased development rate. The theoretic framework is therefore consistent with the observed positive synergistic effect of the combined exposure to warming and acidification.

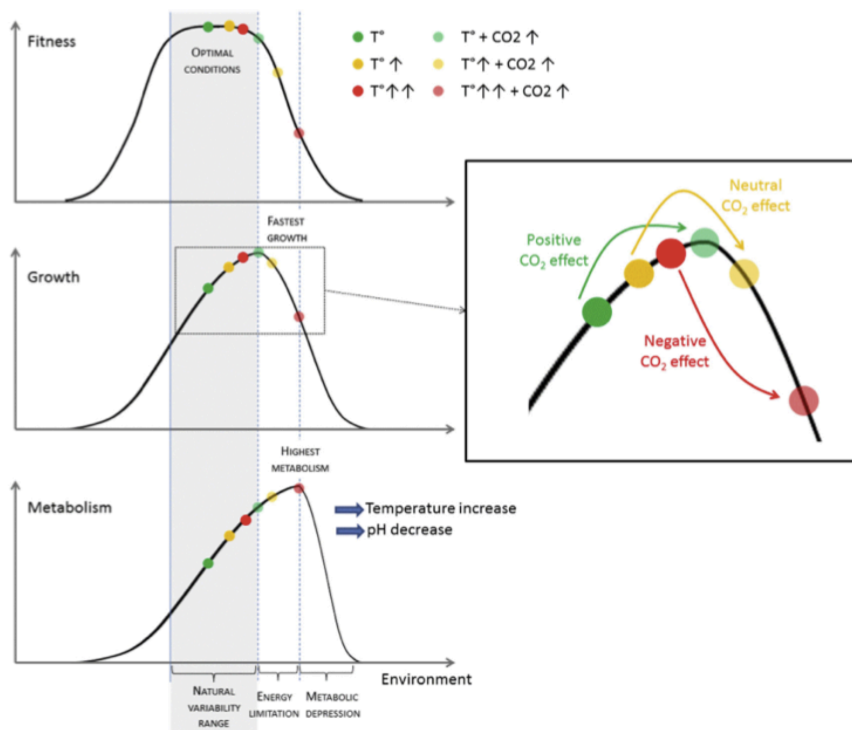

Figure 4.1: Theoretic predictive model showing the relationship between fitness, growth and metabolism under different environmental conditions (temperature and CO_2). An increases scope for growth as an effect of both warming and acidification combined can be achieve because both factors induces an increased metabolism (shift to the right on the metabolic curve) till the point of metabolic depression. Acidification can then lead to a positive effect (under non-limiting energy conditions, green dots) or a negative effect (under limiting energy conditions, red dots) on growth rates depending on the baseline metabolism (Gianguzza et al., 2013)

So far, studies examining interactive effects of warming and elevated CO_2-concentration on development have involved 3 coral, 11 mollusc, 6 echinoderm and 5 crustacean species. Additive negative effects are the most common response to combined exposure (to warming and acidification) in previous studies. While antagonistic effects are also common, synergic effects of combined stress are relatively rare (Byrne and Przeslawski, 2013). The present study is, to the authors` knowledge, the first to detect synergistic positive effects on development of concurrent warming and acidification in a marine invertebrate and supports recent work published on the impact of ocean acidifications, that acidification may not exclusively have a negative impact on marine invertebrates (Gianguzza et al., 2013).

4.4.4 Proportional duration

Relative duration of a given stage has been demonstrated to be constant over a wide range of temperatures (4-12°C) in *C. finmarchicus* (Campbell et al., 2001). By comparing the proportion of time spent between N3 and C1 stage in the different treatments, the present study also provide evidence that proportional duration is independent of both temperature (11-14°C), CO_2-concentration (380-2080ppm) and the combination of elevated temperature and acidification in *C. finmarchicus* (figure 3.6). These results justifies the method adopted to score the development rate after 26,3 days which used the relative duration to account for the different stage duration observed for *C. finmarchicus* (Campbell et al., 2001).

4.5 Lipid content and dry Weight

The C5-animals from the ambient treatment showed a significantly higher dry weight (figure 3.7) and lipid content (figure 3.8) compared to the other treatments. This points to a depletion of C5-animals energy reserves, due to energetic costs of increased metabolism as an effect of both increased temperature (Peck and Prothero-Thomas, 2002) and CO_2-concentration (Whiteley, 2011) separately, but also in combination.

Similar to the indication from the development results, warming alone induces a more pronounced effect on the dry weight and lipid content than acidification alone. This is consistent with available data on the impact of concurrent warming and acidification on marine invertebrates, that indicate that non-calcifying crustaceans may be more sensitive

towards warming that acidification (Byrne and Przeslawski, 2013). However, contrary to the observations on the development rate, acidification alone produced significant negative effects on the dry weight and lipid content, which suggest that *C. finmarchicus* might also be directly negative affected by acidification conditions that are projected to occur by year 2300 (2080 ppm). These suggestion is supported by a significant lower scope for growth and a depletion of the energy reserves of *C. finmarchicus* exposed to elevated CO_2-concentration (2080 ppm) for two consecutive generations (Håkedal, 2013).

The apparent energetic costs of being exposed to concurrent warming and acidification combined, when compared to the ambient treatment (significantly lower dry weight and lipid content), indicate that *C. finmarhicus* might also be sensitive towards a concurrent acidification and warming of the oceans. Similar results have been observed on the artic congeneric species *Calanus hyperboreus* when exposed to combined stress of elevated temperature (0, 5 and 10°C) and increased CO_2-concnetrations (3000 μatm). While increased temperature and CO_2-concentration caused a synergistic negative effect on the carbon content of *C. Hyperboreus* (Hildebrandt et al., 2014), a higher dry weight (not significant) and lipid content (nearly significant) (compared to the warming treatment alone) was observed when *C. finmarchicus* was exposed to warming and acidification combined. The significant interaction between temperature and CO_2-concentration, indicates that acidification may have an antagonistic effect under elevated temperature conditions (+3°C) on both the lipid content and dry weight. Antagonistic effects, where warming reduces the negative effects of acidification, have been commonly reported in multi-stressor studies on marine invertebrates (Byrne and Przeslawski, 2013). The antagonistic effect observed in the present study points to an increased resilience of *C. finmarchicus* towards warming in the presence of a concurrent acidification of the oceans.

The results are also consistent with the theoretic framework developed by Gianguzza et al. (2013) (figure 4.1). The combined effect of both warming and acidification, might under non-limiting energy condition, increase the scope for growth, which could lead to the increased allocation of energy to growth and energy storage (lipids), when compared to warming alone. However, the assumption of non-limiting energy conditions for the theoretic framework developed by (Gianguzza et al., 2013) was not met in this experiment. Compared to development times for *C. finmarchicus* reported by Campbell et al. (2001), under non-limiting

food conditions (500 μg C/L) (calculated from equation 1.5), development times estimated in this study were respectively 28% and 40% slower at ambient (11°C) and warming (14°C), for the N3 stage, and 26% and 40% slower for the C1 stage. From this, it is evident that animals may have been under some constrains with regards to food supply. Also, the animals in the treatment that were exposed to increased temperature and CO_2-concentration combined (together with the warming treatment) showed distinctly lower algae concentration that animals maintained at ambient and acidified conditions, adding further constrain (regarding the energy conditions compared to these treatments).

The apparent contradictory observations of higher development rates (synergistic effect) combined with an increased allocation of energy to growth and energy storage (antagonistic effect) (under limited energy-conditions) in the animals exposed to warming and acidification combined is difficult to explain (without conflicting statements). However, the results highlight the complexity of effects that concurrent stressors (such as warming and acidification) may induce and the importance of study such interactive effects to understand the influence of concurrent stressors on the marine ecosystem. The results also emphasizes the disparity that exists between species as the combined exposure to warming and acidification displayed a synergistic negative effect on the lipid content in *C. hyperboreus* (Hildebrandt et al., 2014) and an antagonistic effects in *C. finmarchius*.

4.6 Ecological implications

The acceleration of development as an effects of warming, or concurring warming and acidification observed in this study could potentially affect the structure and/or function of the marine ecosystem in the areas dominated by *C. finmarchicus*, such as the Norwegian sea (Bagøien et al., 2012). If applicable to natural populations, an acceleration of the development rate might lead to a mismatch between the emergence of development stages and windows of opportunities, timed according to constant cues (e.g. light on algae blooms) (Cushing, 1990, Pörtner, 2008), to which the life cycle of *C. finmarchicus* is finely tuned. The observed increase in development rate might increase the possibility of such a mismatch to occur and should therefor not be interpreted as a positive effect on the fitness of an individual or population.

An effect of a mismatch might cause a reduction in accumulated lipids which are used to support production of reproductive tissue by adult females, but also function as an energy reserve in times of starvation or when food supply is variable (Mauchline, 1998). Also, the significantly reduction in lipid-content as an effect of increased temperature, CO_2-concentration and these conditions combined observed in this study might further reduce the energy state of the animals, implying reduced reproduction and increased vulnerability in adults during periods of low energy supply. Simultaneously, a significantly reduced dry weight observed as an effect of increased temperature, CO_2-concentration and in combination, might lead to reduced clutch size in *C. finmarchicus* (Head et al., 2013). Lower lipid content accompanied with lower body size may be a particularly unfavourable combination with regards to reproductive processes, with potential to affect the population size and ultimately the eco-system level.

5 Conclusion and future work

The results coincide with the initial expectations that concurrent warming and acidification causes interactive effects, but the complexity of these effects was unsuspected. The synergistic positive effect observed on the development rate together with an unfavourable reduction in body size and lipid content suggests that *C. finmarchicus* could be sensitive towards ocean acidification predicted to occur by year 2300 (2080ppm), in combination with increasing temperature (+3°C). However, acidification also displays an antagonistic effect by reducing the negative effect of warming on the body size and lipid storage. The results highlight the importance of future work to consider the combined effect of stressors, as this approach might reveal biological responses which single-factor studies might not be able to detect. From the results it is also evident that warming induce greater effects than acidification on *C. finmarchicus* (when the factors are considered separately). However, *C. finmarchicus* may also be vulnerable towards direct affects from ocean acidification alone.

The results from this study may provide baseline data to elucidate the potential response of *C. finmarchicus* to concurrent warming and acidification of the oceans. The physiological mechanism by which acidification may alter responses of warming conditions should be targeted to improve the understanding of how concurrent warming and acidification may affect the marine biota in the future ocean.

6 References

Almeda, R., Augustin, C. B., Alcaraz, M., Calbet, A. & Saiz, E. 2010. Feeding rates and gross growth efficiencies of larval developmental stages of *Oithona davisae* (Copepoda, Cyclopoida). *Journal of Experimental Marine Biology and Ecology,* **387,** 24-35.

Anderson, M. J. 2001. A new method for non - parametric multivariate analysis of variance. *Austral ecology,* **26,** 32-46.

Anthony, K. R. N., Kline, D. I., Diaz-Pulido, G., Dove, S. & Hoegh-Guldberg, O. 2008. Ocean acidification causes bleaching and productivity loss in coral reef builders. *Proceedings of the National Academy of Sciences,* **105,** 17442-17446.

Bagøien, E., Melle, W. & Kaartvedt, S. 2012. Seasonal development of mixed layer depths, nutrients, chlorophyll and *Calanus finmarchicus* in the Norwegian Sea–A basin-scale habitat comparison. *Progress in Oceanography,* **103,** 58-79.

Bartolini, F., Barausse, A., Pörtner, H. O. & Giomi, F. 2013. Climate change reduces offspring fitness in littoral spawners: a study integrating organismic response and long - term time - series. *Global change biology,* **19,** 373-386.

Beaugrand, G., Reid, P. C., Ibanez, F., Lindley, J. A. & Edwards, M. 2002. Reorganization of North Atlantic marine copepod biodiversity and climate. *Science,* **296,** 1692-1694.

Belehradek, J. 1935. *Temperature and living matter*, Verlag von Gebruder Borntraeger.

Bi, H., Feinberg, L., Shaw, C. T. & Peterson, W. T. 2011. Estimated development times for stage-structured marine organisms are biased if based only on survivors. *Journal of plankton research,* **33,** 751-762.

Breteler, W. C. M. K. & Gonzalez, S. R. 1988. Influence of temperature and food concentration on body size, weight and lipid content of two calanoid copepod species. *Hydrobiologia,* **167,** 201-210.

Byrne, M. & Przeslawski, R. 2013. Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories. *Integrative and comparative biology,* **53,** 582-596.

Båmstedt, U., Nejstgaard, J. C., Solberg, P. T. & Høisæter, T. 1999. Utilisation of small-sized food algae by *Calanus finmarchicus* (Copepoda, Calanoida) and the significance of feeding history. *Sarsia,* **84,** 19-38.

Caldeira, K. & Wickett, M. E. 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *Journal of Geophysical Research-Oceans,* **110**.

Campbell, R. G., Wagner, M. M., Teegarden, G. J., Boudreau, C. A. & Durbin, E. G. 2001. Growth and development rates of the copepod *Calanus finmarchicus* reared in the laboratory. *Marine Ecology Progress Series,* **221,** 161-183.

Conover, R. J. 1988. Comparative life histories in the genera *Calanus* and *Neocalanus* in high latitudes of the northern hemisphere. *Hydrobiologia,* **167,** 127-142.

Corkett, C. J., Mclaren, I. A. & Sevigny, J. M. 1986. The rearing of the marine calanoid copepods *Calanus finmarchicus* (Gunnerus), *C. glacialis* (Jaschnov) and *C. hyperboreus (*Kroyer) with comment on the equiproportional rule. *Syllogeus,* **58,** 539-546.

Cushing, D. H. 1990. Plankton production and year-class strength in fish populations: an update of the match/mismatch hypothesis. *Advances in marine biology,* **26,** 249-293.

De Coen, W. M. I. 1999. *Energy metabolism, DNA damage and population dynamics of the waterflea Daphnia magna Straus under toxic stress.* PhD thesis, University of Ghent

Dell, A. I., Pawar, S. & Savage, V. M. 2011. Systematic variation in the temperature dependence of physiological and ecological traits. *Proceedings of the National Academy of Sciences,* **108,** 10591-10596.
Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. & Martin, P. R. 2008. Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences,* **105,** 6668-6672.
Dickson, A. G., Sabine, C. L. & Christian, J. R. 2007. Guide to best practices for ocean CO_2 measurements. *PICES Special Publication* **3,** 191.
Elliott, D. T. & Tang, K. W. 2009. Simple staining method for differentiating live and dead marine zooplankton in field samples. *Limnology and Oceanography: Methods,* **7,** 585-594.
Fabry, V. J., Seibel, B. A., Feely, R. A. & Orr, J. C. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science: Journal du Conseil,* **65,** 414-432.
Fitzer, S. C., Caldwell, G. S., Close, A. J., Clare, A. S., Upstill-Goddard, R. C. & Bentley, M. G. 2012. Ocean acidification induces multi-generational decline in copepod naupliar production with possible conflict for reproductive resource allocation. *Journal of Experimental Marine Biology and Ecology,* **418,** 30-36.
Frederich, M. & Pörtner, H. O. 2000. Oxygen limitation of thermal tolerance defined by cardiac and ventilatory performance in spider crab, *Maja squinado*. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* **279,** R1531-R1538.
Gianguzza, P., Visconti, G., Gianguzza, F., Vizzini, S., Sarà, G. & Dupont, S. 2013. Temperature modulates the response of the thermophilous sea urchin *Arbacia lixula* early life stages to CO_2-driven acidification. *Marine environmental research,* **93,** 70-77.
Gibson, R., Atkinson, R., Gordon, J., Smith, I. & Hughes, D. 2011. Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. *Oceanography and Marine Biology: An Annual Review,* **49,** 1-42.
Gustavson, L. M. 2013. *Effects of predicted ocean acidification scenarios on the early developmental stages of Calanus finmarchicus.* M.Sc thesis, Norwegian Univeristy of Science and Technology.
Hansen, B. H., Altin, D., Nordtug, T. & Olsen, A. J. 2007. Suppression subtractive hybridization library prepared from the copepod *Calanus finmarchicus* exposed to a sublethal mixture of environmental stressors. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics,* **2,** 250-256.
Harley, C. D. G., Randall Hughes, A., Hultgren, K. M., Miner, B. G., Sorte, C. J. B., Thornber, C. S., Rodriguez, L. F., Tomanek, L. & Williams, S. L. 2006. The impacts of climate change in coastal marine systems. *Ecology letters,* **9,** 228-241.
Head, E. J. H., Harris, L. R., Ringuette, M. & Campbell, R. W. 2013. Characteristics of egg production of the planktonic copepod, *Calanus finmarchicus*, in the Labrador Sea: 1997–2010. *Journal of plankton research,* **35,** 281-298.
Helaouët, P. & Beaugrand, G. 2007. Macroecology of *Calanus finmarchicus* and *C. helgolandicus* in the North Atlantic Ocean and adjacent seas. *Marine Ecology Progress Series,* **345,** 147-165.
Hildebrandt, N., Niehoff, B. & Sartoris, F. J. 2014. Long-term effects of elevated CO_2 and temperature on the Arctic calanoid copepods *Calanus glacialis* and *C. hyperboreus*. *Marine pollution bulletin,* **80,** 59-70.

Hoegh‐Guldberg, O. 2005. Low coral cover in a high‐CO_2 world. *Journal of Geophysical Research: Oceans (1978‐2012),* **110,** 6.
Hofmann, G. E., O'donnell, M. J. & Todgham, A. E. 2008. Using functional genomics to explore the effects of ocean acidification on calcifying marine organisms. *Marine Ecology Progress Series,* **373,** 219-225.
Hönisch, B., Hemming, N. G., Archer, D., Siddall, M. & Mcmanus, J. F. 2009. Atmospheric carbon dioxide concentration across the mid-Pleistocene transition. *Science,* **324,** 1551-1554.
Håkedal, O. J. 2013. *Effects on Scope for Growth due to elevated carbon dioxide in the copepod Calanus finmarchicus.* M.Sc thesis, Norwegian University of Science and Technology.
Kane, J. 1984. The feeding habits of co-occurring cod and haddock larvae from Georges Bank. *Marine ecology progress series. Oldendorf,* **16,** 9-20.
Koski, M. & Kuosa, H. 1999. The effect of temperature, food concentration and female size on the egg production of the planktonic copepod *Acartia bifilosa*. *Journal of plankton research,* **21,** 1779-1790.
Kurihara, H. 2008. Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates. *Marine Ecology Progress Series,* **373,** 275-284.
Kaartvedt, S. 2000. Life history of *Calanus finmarchicus* in the Norwegian Sea in relation to planktivorous fish. *ICES Journal of Marine Science: Journal du Conseil,* **57,** 1819-1824.
Landry, M. R. 1975. Relationship between temperature and the development of life stages of the marine copepod *Acartia clausi* Giesbr. *Limnology and oceanography,* **20**.
Langenbuch, M., Bock, C., Leibfritz, D. & Pörtner, H.-O. 2006. Effects of environmental hypercapnia on animal physiology: A^{13}C NMR study of protein synthesis rates in the marine invertebrate *Sipunculus nudus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **144,** 479-484.
Leong, P. & Manahan, D. 1997. Metabolic importance of Na^+/K^+-ATPase activity during sea urchin development. *Journal of experimental biology,* **200,** 2881-2892.
Li, W. & Gao, K. 2012. A marine secondary producer respires and feeds more in a high CO_2 ocean. *Marine pollution bulletin,* **64,** 699-703.
Marshall, S. M. & Orr, A. P. 1955. *The biology of a marine copepod, Calanus finmarchicus Gunnerus*, Oliver & Boyd Edinburgh 188.
Marshall, S. M., Orr, A. P. 1972. *The biology of a Marine Copepod,* Berlinge, Springer Verlag 1-195.
Mauchline, J. 1998. *The biology of calanoid copepods. In:J.H.S. Blaxter, A.J. southward and P.A. Tylor (eds) Advances in Marine Biology,* San Diego, California, Academica Press 1-710.
Mayor, D. J., Matthews, C., Cook, K. Et Al 2007. CO_2-induced acidification affects hatching success in *Calanus finmarchicus*. *Marine Ecology Progress Series,* **350,** 91-97.
Mayor, D. J., Everett, N. R., Cook, K. B. 2012. End of year century ocean warming and acidification effects on reproductive success in a temerate marine copepod. *J. Plankton Res.,* **34,** 258-262.
Mcardle, B. H. & Anderson, M. J. 2001. Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology,* **82,** 290-297.
Mckenney Jr, C. L. 1998. Physiological dysfunction in estuarine mysids and larval decapods with chronic pesticide exposure. *Microscale Testing in Aquatic Toxicology: Advances, Techniques, and Practice. CRC, Boca Raton, FL, USA,* **45,** 465-476.

Metzger, R., Sartoris, F. J., Langenbuch, M. & Pörtner, H. O. 2007. Influence of elevated CO_2 concentrations on thermal tolerance of the edible crab *Cancer pagurus*. *Journal of Thermal Biology,* **32,** 144-151.
Meyer, B., Irigoien, X., Graeve, M., Head, R. & Harris, R. 2002. Feeding rates and selectivity among nauplii, copepodites and adult females of *Calanus finmarchicus* and *Calanus helgolandicus*. *Helgoland Marine Research,* **56,** 169-176.
Miller, C. B., Crain, J. A. & Morgan, C. A. 2000. Oil storage variability in Calanus finmarchicus. *ICES Journal of Marine Science: Journal du Conseil,* **57,** 1786-1799.
Mullin, M. M. & Brooks, E. R. 1970. The effect of concentration of food on body weight, cumulative ingestion, and rate of growth of the marine copepod *Calanus helgolandicus*. *Limnology and Oceanography,* **15,** 748-755.
Navarro, J. M., Urrutia, G. X. & Carrasco, C. 2006. Scope for growth versus actual growth in the juvenile predatory gastropod *Chorus giganteus*. *Journal of the Marine Biological Association of the United Kingdom,* **86,** 1423-1428.
O'connor, M. I., Bruno, J. F., Gaines, S. D., Halpern, B. S., Lester, S. E., Kinlan, B. P. & Weiss, J. M. 2007. Temperature control of larval dispersal and the implications for marine ecology, evolution, and conservation. *Proceedings of the National Academy of Sciences,* **104,** 1266-1271.
Parmesan, C. & Yohe, G. 2003. A globally coherent fingerprint of climate change impacts across natural systems. *Nature,* **421,** 37-42.
Pechenik, J. A. 1987. Environmental influences on larval survival and development. *Reproduction of marine invertebrates,* **9,** 551-608.
Peck, L. & Prothero-Thomas, E. 2002. Temperature effects on the metabolism of larvae of the Antarctic starfish *Odontaster validus*, using a novel micro-respirometry method. *Marine Biology,* **141,** 271-276.
Pedersen, S. A., Hansen, B. H., Altin, D. & Olsen, A. J. 2013. Medium-term exposure of the North Atlantic copepod *Calanus finmarchicus* (Gunnerus, 1770) to CO_2-acidified seawater: effects on survival and development. *Biogeosciences,* **10,** 7481-7491.
Pedersen, S. A., Våge, V. T., Olsen, A. J., Hammer, K. M. & Altin, D. 2014. Effects of Elevated Carbon Dioxide (CO_2) Concentrations on Early Developmental Stages of the Marine Copepod *Calanus finmarchicus* Gunnerus (Copepoda: Calanoidae). *Journal of Toxicology and Environmental Health, Part A,* **77,** 535-549.
Pelletier, G. J., Lewis, E. & Wallace, D. 2007. CO2SYS. XLS: a calculator for the CO2 system in seawater for Microsoft Excel/VBA. Washington State Department of Ecology, Olympia, Washington.
Przeslawski, R., Ahyong, S., Byrne, M., Woerheide, G. & Hutchings, P. a. T. 2008. Beyond corals and fish: the effects of climate change on noncoral benthic invertebrates of tropical reefs. *Global Change Biology,* **14,** 2773-2795.
Pörtner, H.-O., Reipschläger, A. & Heisler, N. 1998. Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. *The Journal of experimental biology,* **201,** 43-55.
Pörtner, H. O., Bock, C. & Reipschlager, A. 2000. Modulation of the cost of pHi regulation during metabolic depression: a (31) P-NMR study in invertebrate (*Sipunculus nudus*) isolated muscle. *Journal of Experimental Biology,* **203,** 2417-2428.
Pörtner, H. O., Langenbuch, M. & Michaelidis, B. 2005. Synergistic effects of temperature extremes, hypoxia, and increases in CO2 on marine animals: From Earth history to global change. *Journal of Geophysical Research: Oceans (1978–2012),* **110**.
Pörtner, H. O. & Knust, R. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science,* **315,** 95-97.

Pörtner, H.-O. 2008. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Marine Ecology Progress Series* **373,** 203-217.
Pörtner, H. O., Farrell, A.P. 2008. Physiology and climate change *Science,* **322** 690-692.
Repetto, G., Del Peso, A. & Zurita, J. L. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature protocols,* **3,** 1125-1131.
Runge, J. A. 1988. Should we expect a relationship between primary production and fisheries? The role of copepod dynamics as a filter of trophic variability. *Hydrobiologia,* **167,** 61-71.
Sabine, C. L., Feely, R. A., Gruber, N., Key, R. M., Lee, K., Bullister, J. L., Wanninkhof, R., Wong, C., Wallace, D. W. R. & Tilbrook, B. 2004. The oceanic sink for anthropogenic CO_2. *science,* **305,** 367-371.
Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B.,Tignor, M., Miller, H.L 2007. *Climate Change 2007: The physical science basis. Contribution of working group I to the Fourth Assessment Report of theIntergovernmental Panel on Climate Change,* Cambridge, Cambridge University Press,.
Somero, G. N. 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *The Journal of experimental biology,* **213,** 912-920.
Stumpp, M., Wren, J., Melzner, F., Thorndyke, M. C. & Dupont, S. T. 2011. CO_2 induced seawater acidification impacts sea urchin larval development I: Elevated metabolic rates decrease scope for growth and induce developmental delay. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **160,** 331-340.
Tang, K. W., Freund, C. S. & Schweitzer, C. L. 2006. Occurrence of copepod carcasses in the lower Chesapeake Bay and their decomposition by ambient microbes. *Estuarine, Coastal and Shelf Science,* **68,** 499-508.
Taylor, H. H. 1992. Gills and lungs: the exchange of gases and ions. *Microscopic anatomy of invertebrates,* **10,** 203-293.
Vazzana, M., Celi, M., Tramati, C., Ferrantelli, V., Arizza, V. & Parrinello, N. 2014. *In vitro* effect of cadmium and copper on separated blood leukocytes of *Dicentrarchus labrax*. *Ecotoxicology and environmental safety,* **102,** 113-120.
Vehmaa, A., Brutemark, A. & Engström-Öst, J. 2012. Maternal effects may act as an adaptation mechanism for copepods facing pH and temperature changes. *PloS one,* **7,** e48538.
Whiteley, N. M. 2011. Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series,* **430,** 257-271.
Xiaowan Tao, X., Yunyan Ni, Y., 2009. Determination of Total Alkalinity and Calcium Concentration of Seawater Rapidly and Automaticly with Small-Amounts Samples. *The 3rd International Conference on Bioinformatics and Biomedical Engineering (ICBBE 2009).* Beijing, China. *Zoology***,** 1-4.
Yamada, Y. & Ikeda, T. 1999. Acute toxicity of lowered pH to some oceanic zooplankton. *Plankton Biology and Ecology,* **46,** 62-67.
Zeebe, R. E. & Wolf-Gladrow, D. 2001. *CO_2 in Seawater: Equilibrium, Kinetics, Isotopes: Equilibrium, Kinetics, Isotopes,* Amsterdam, Elsevier Science 346.
Zeebe, R. E. 2012. History of seawater carbonate chemistry, atmospheric CO_2, and ocean acidification. *Annual Review of Earth and Planetary Sciences,* **40,** 141-165.

Appendix A – Linear regression data

Table A.1: Linear regressions for the cumulative stage proportions for N3 and C1-stage. The coefficients of the linear equation y=ax+b, and the R^2 –values to describe the linear fit, are included

		Least square linear regressions		
Tank	Stage	a	b	R2
A1	N3	0,009	-0,525	0,930
	C1	0,008	-2,356	0,780
A2	N3	0,026	-2,071	0,990
	C1	0,005	-1,116	0,770
A3	N3	0,010	-0,593	0,840
	C1	0,004	-0,705	0,730
A4	N3	0,009	-0,591	0,780
	C1	0,005	-1,644	0,850
B1	N3	0,010	-0,620	0,820
	C1	0,011	-3,302	0,970
B2	N3	0,068	-0,171	0,730
	C1	0,006	-1,567	0,950
B3	N3	0,009	-0,338	0,800
	C1	0,004	-0,786	0,520
B4	N3	0,010	-0,584	0,750
	C1	0,007	-2,033	0,890
C1	N3	0,006	-0,183	0,670
	C1	0,005	-1,256	0,640
C2	N3	0,094	-0,502	0,600
	C1	0,005	-1,329	0,930
C3	N3	0,033	-2,742	0,820
	C1	0,007	-1,513	0,750
C4	N3	0,026	-2,373	0,970
	C1	0,007	-1,513	0,750

Appendix B – Stage determination data

Table B.1: Raw data of the stage determination. Time is days after mid-egg laying point and volume of the samples is included. Data in bold is excluded from the analysis because of few animals in the samples (<5 animals). The table continues on subsequent pages.

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
A1	3,56	600ml	1		5	13											
A1	3,90	600ml	1	5		6	4	1		1							
A1	**4,23**	**600ml**	**1**			**4**											
A1	4,56	600ml	1	2	2	8	10	1									
A1	4,90	600ml	1			13	14		2								
A1	5,23	600ml	1	1	1		11		2								
A1	5,56	600ml	1	1		1	9										
A1	5,90	600ml	1	5		1	22										
A1	6,23	600ml	1	4		1	19	1	1	1							
A1	6,56	600ml	1	3		2	14	1									
A1	6,90	600ml	1	1		1	12										
A1	7,23	600ml	1				8	3									
A1	7,56	600ml	1	1			15	7									
A1	11,31	600ml	1				6	5	3	1	1						
A1	11,81	1000ml	1,66					1	12	2							
A1	12,31	1000ml	1,66					4	14	9							
A1	12,81	1000ml	1,66						17	9							
A1	13,31	1000ml	1,66						8	15	1	1					
A1	13,81	1000ml	1,66						2	18	2						
A1	14,31	1000ml	1,66				1	1	3	14	5						
A1	14,81	1000ml	1,66						1	12	10	1					
A1	15,31	1000ml	1,66				1		1	2	7	1	1				
A1	15,81	1000ml	1,66					1	2	2	14						
A1	16,31	1000ml	1,66				1		3	4	18						
A1	16,81	1000ml	1,66					1	1		12	1					
A1	17,31	1000ml	1,66					1	1		7	4					
A1	17,81	1000ml	1,66				1		2		9	3					
A1	18,31	1000ml	1,66								1	4	2				
A1	18,81	1000ml	1,66					1	1	1	3	10	3	1			
A1	19,31	1000ml	1,66							1		4					
A1	20,31	1000ml	1,66					1				9	1	1			
A1	21,31	1000ml	1,66							1	3	3	18	1			
A1	26,25	2000ml	3,33										4	16	2		1

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
A2	3,56	600ml	1	4	3	4	2										
A2	3,90	600ml	1	2	2	9											
A2	4,23	600ml	1	3			5										
A2	4,56	600ml	1	2		1	9	1									
A2	4,90	600ml	1				12										
A2	5,23	600ml	1			1	4			1	1						
A2	**5,56**	**600ml**	**1**				**2**	**1**									
A2	5,90	600ml	1			1	12	1	1								
A2	6,23	600ml	1	1		2	7	1									
A2	6,56	600ml	1	1		2	4	5									
A2	6,90	600ml	1			1	2	0				2					
A2	**7,23**	**600ml**	**1**		**1**			**2**									
A2	7,56	600ml	1			2	2	7	1								
A2	11,31	600ml	1					6	4								
A2	11,81	1000ml	1,66				1		2	8	1						
A2	12,31	1000ml	1,66						1	6	2						
A2	12,81	1000ml	1,66						1	7	2						
A2	13,31	1000ml	1,66						1	6	7						
A2	13,81	1000ml	1,66						1	7	3						
A2	14,31	1000ml	1,66						2		6						
A2	14,81	1000ml	1,66							4	7	2					
A2	15,31	1000ml	1,66						2	3	13	3					
A2	15,81	1000ml	1,66					1	2		9	1					
A2	16,31	1000ml	1,66						1	2	8	1			1		
A2	16,81	1000ml	1,66							1	4	6	1				
A2	17,31	1000ml	1,66								2	3	1				
A2	17,81	1000ml	1,66								5	4			1		
A2	18,31	1000ml	1,66							2	10	5	1				
A2	18,81	1000ml	1,66					1		1	2	4	1				
A2	19,31	1000ml	1,66							1	1	1	2				
A2	20,31	1000ml	1,66								2	2	1	3			
A2	21,31	1000ml	1,66									3					
A2	26,25	2000ml	3,33								1		1	13	15		

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
A3	3,56	600ml	1	3	1	7											
A3	3,90	600ml	1	2	3	11	6		1	1							
A3	4,23	600ml	1	3	1	5	8										
A3	4,56	600ml	1	4		3	15	1									
A3	4,90	600ml	1	3	2	1	10	1	1								
A3	5,23	600ml	1	2		2	16										
A3	5,56	600ml	1			1	6										
A3	5,90	600ml	1		1	3	19	1									
A3	6,23	600ml	1			2	9	1									
A3	6,56	600ml	1				14	2									
A3	6,90	600ml	1			1	6	2									
A3	7,23	600ml	1			5	6				2						
A3	7,56	600ml	1				7	10		1	1						
A3	11,31	600ml	1					1	6	3	1						
A3	11,81	1000ml	1,66				1	4	1	11	4						
A3	12,31	1000ml	1,66				1	2	8	10	5						
A3	12,81	1000ml	1,66						2	5	3		1				
A3	13,31	1000ml	1,66				2	1	1		5						
A3	13,81	1000ml	1,66					2		4	16						
A3	14,31	1000ml	1,66				2	2	1	4	6						
A3	14,81	1000ml	1,66					1	1	2	9						
A3	15,31	1000ml	1,66				1		1	1	4	4					
A3	15,81	1000ml	1,66				1	1		1	3	7					
A3	16,31	1000ml	1,66				1	1	1	2	3	3					
A3	16,81	1000ml	1,66					1	1	1	4	7					
A3	17,31	1000ml	1,66					1	3			2		1			
A3	17,81	1000ml	1,66							2	4	6					
A3	18,31	1000ml	1,66							1	3	3					
A3	18,81	1000ml	1,66			1				2	1	2	2				
A3	19,31	1000ml	1,66	1				1			2		2		1		
A3	20,31	1000ml	1,66					1	1	1	1	1		4			
A3	21,31	1000ml	1,66							1		12	3				
A3	26,25	2000ml	3,33									1		3	15		1

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
A4	3,56	600ml	1	2		1	1										
A4	3,90	600ml	1	2	1	16											
A4	4,23	600ml	1	2		18	4										
A4	4,56	600ml	1			13	8										
A4	4,90	600ml	1	6		4	14										
A4	5,23	600ml	1	2		3	13			1							
A4	5,56	600ml	1				9		1								
A4	5,90	600ml	1			3	16	1									
A4	6,23	600ml	1	3		3	16				1						
A4	6,56	600ml	1				12	1	1								
A4	6,90	600ml	1	2		1	16										
A4	7,23	600ml	1	2			17	1			1						
A4	7,56	600ml	1				11	1			1						
A4	11,31	600ml	1				2	3	8								
A4	11,81	1000ml	1,66				2	6	7	3			1				
A4	12,31	1000ml	1,66				2	8	19								
A4	12,81	1000ml	1,66				1	3	9	14			1				
A4	13,31	1000ml	1,66				2	6	10	5	1		2	1			
A4	13,81	1000ml	1,66					2	13	12	2	2	1				
A4	14,31	1000ml	1,66					2	6	15	3						
A4	14,81	1000ml	1,66					1	6	10	1	1					
A4	15,31	1000ml	1,66				1	2	4	9	10	1	2				
A4	15,81	1000ml	1,66					6	4	9							
A4	16,31	1000ml	1,66				1	2	3	6	13	1					
A4	16,81	1000ml	1,66						5	11	13	2	2				
A4	17,31	1000ml	1,66							6	15	1					
A4	17,81	1000ml	1,66				2	1	4	2	14	3					
A4	18,31	1000ml	1,66							4	7	2					
A4	18,81	1000ml	1,66							1	8	4					
A4	19,31	1000ml	1,66					1	2	1	5	6					
A4	20,31	1000ml	1,66					2		1	6	12	2				
A4	21,31	1000ml	1,66						1	1	4	14	4	1	1		
A4	26,25	2000ml	3,33									3	17	22	2		1

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
B1	3,56	600ml	1	5	3	22	5		1								
B1	3,90	600ml	1	1	3	18				1							
B1	4,23	600ml	1	4		11	9										
B1	4,56	600ml	1	1	1	6	4										
B1	4,90	600ml	1			1	25			1	1						
B1	5,23	600ml	1	1		1	12			1							
B1	5,56	600ml	1			3	10										
B1	5,90	600ml	1			3	14			1	1						
B1	6,23	600ml	1			2	5										
B1	6,56	600ml	1	2		1	11										
B1	6,90	600ml	1			1	20	1			1						
B1	7,23	600ml	1			1	10	2			1						
B1	7,56	600ml	1	1		1	4	5	1								
B1	11,31	600ml	1				2		10	1	1						
B1	11,81	1000ml	1,66				1	1	13	1							
B1	12,31	1000ml	1,66				1	2	20	12	3						
B1	12,81	1000ml	1,66					2	5	10	1						
B1	13,31	1000ml	1,66				1	1	2	14	1	1					
B1	13,81	1000ml	1,66				2		5	16	7						
B1	14,31	1000ml	1,66				1	2	2	14	6						
B1	14,81	1000ml	1,66						3	8	9			1			
B1	15,31	1000ml	1,66						1	7	3						
B1	15,81	1000ml	1,66						1	5	12	2					
B1	16,31	1000ml	1,66					1		2	12	2					
B1	16,81	1000ml	1,66					1	1		13	5					
B1	17,31	1000ml	1,66					1		1	11	3					
B1	17,81	1000ml	1,66							1	10	6					
B1	18,31	1000ml	1,66							1	5	5	1				
B1	18,81	1000ml	1,66								5	4					
B1	19,31	1000ml	1,66							2	2	8					
B1	20,31	1000ml	1,66				1			1	2	4	5				
B1	21,31	1000ml	1,66								1	2	5	2			
B1	26,25	2000ml	3,33									2	5	12	2		

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
B2	3,56	600ml	1	3	2	21			1								
B2	3,90	600ml	1			2											
B2	4,23	600ml	1	1		5	7										
B2	4,56	600ml	1		2	6	11										
B2	**4,90**	**600ml**	**1**														
B2	5,23	600ml	1	1	2	3	8	1									
B2	5,56	600ml	1			2	8										
B2	5,90	600ml	1			2	24				1						
B2	6,23	600ml	1				15				1						
B2	6,56	600ml	1			1	14	1									
B2	6,90	600ml	1				8	1	1		1						
B2	7,23	600ml	1	2		1	13	1									
B2	7,56	600ml	1	1			10	2	1								
B2	11,31	600ml	1					2	2	4							
B2	11,81	1000ml	1,66	1				4	9	7							
B2	12,31	1000ml	1,66				1	2	2	11	1	2					
B2	12,81	1000ml	1,66				1		4	7							
B2	13,31	1000ml	1,66				3	3	3	4	7						
B2	13,81	1000ml	1,66				1	2	2	3	4						
B2	14,31	1000ml	1,66							3	3						
B2	14,81	1000ml	1,66					3		4	9		1				
B2	15,31	1000ml	1,66					1	5	1		1					
B2	15,81	1000ml	1,66					2	1	3	5						
B2	16,31	1000ml	1,66				1		1		4	4					
B2	16,81	1000ml	1,66						3	2	9	6					
B2	17,31	1000ml	1,66								5	6	1				
B2	17,81	1000ml	1,66						1		4	7					
B2	18,31	1000ml	1,66							1	5	5	1				
B2	18,81	1000ml	1,66				1	1				4	1				
B2	19,31	1000ml	1,66							2		5	4				
B2	20,31	1000ml	1,66					1		1	1	4	1	1			
B2	21,31	1000ml	1,66							1		2	6	3			
B2	26,25	2000ml	3,33								1	1	4	7	10		

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
B3	3,56	600ml	1	3	2	21											
B3	3,90	600ml	1			11	10				1						
B3	4,23	600ml	1	3		9	18										
B3	4,56	600ml	1			5	13	1									
B3	4,90	600ml	1	5		1	12		1		1						
B3	5,23	600ml	1	2	1		18		1								
B3	5,56	600ml	1				13										
B3	5,90	600ml	1	1		2	21	1									
B3	6,23	600ml	1				15										
B3	6,56	600ml	1			1	12	6			1						
B3	6,90	600ml	1				10	5			2						
B3	7,23	600ml	1			1	6	10									
B3	7,56	600ml	1				3	11	1	1	1						
B3	11,31	600ml	1						5	4							
B3	11,81	1000ml	1,66				1		3	15	5						
B3	12,31	1000ml	1,66				2		2	7	13	1					
B3	12,81	1000ml	1,66					1	1	6	18	2	1				
B3	13,31	1000ml	1,66							5	19						
B3	13,81	1000ml	1,66				1		1		19	2					
B3	14,31	1000ml	1,66						3	3	25	1					
B3	14,81	1000ml	1,66					3	1	4	14	15					
B3	15,31	1000ml	1,66				1	1	1	3	7	9					
B3	15,81	1000ml	1,66					1			6	8					
B3	16,31	1000ml	1,66					1	1	1	8	5	1				
B3	16,81	1000ml	1,66								4	7	1	1			
B3	17,31	1000ml	1,66						2	1	4	7	2				
B3	17,81	1000ml	1,66							2	1	3	5				
B3	18,31	1000ml	1,66								2	6	4				
B3	18,81	1000ml	1,66					1	1	2	2		6	1			
B3	19,31	1000ml	1,66							1		1	4	5			
B3	20,31	1000ml	1,66					1				14	8				
B3	21,31	1000ml	1,66							1	1		5	5			
B3	26,25	2000ml	3,33											11	45	2	1

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
B4	3,56	600ml	1	3	0	17	2	1									
B4	3,90	600ml	1	2	1	12	4	1		1							
B4	4,23	600ml	1	6	2	15	6										
B4	4,56	600ml	1	5		10	17	1	1		1						
B4	4,90	600ml	1	6	1	1	8	1	2								
B4	5,23	600ml	1			2	8										
B4	5,56	600ml	1	2	0	1	13										
B4	5,90	600ml	1	2		3	13	1									
B4	6,23	600ml	1			1	14										
B4	6,56	600ml	1	2		1	15										
B4	6,90	600ml	1	1			11	1			1						
B4	7,23	600ml	1		1	4	15	1									
B4	7,56	600ml	1				9										
B4	11,31	600ml	1					8	2								
B4	11,81	1000ml	1,66				4	5	10	2							
B4	12,31	1000ml	1,66				1	1	11	2	1						
B4	12,81	1000ml	1,66				4	2	8	8		2					
B4	13,31	1000ml	1,66					1	3	10	1						
B4	13,81	1000ml	1,66				5	1	2	9	1						
B4	14,31	1000ml	1,66			1	1	1	2	7	4						
B4	14,81	1000ml	1,66				2	6	2	4	5						
B4	15,31	1000ml	1,66				3	2	4	5	8						
B4	15,81	1000ml	1,66				1	1	1	6	10		1				
B4	16,31	1000ml	1,66				1		1	2	7						
B4	16,81	1000ml	1,66				1		2		8	2		1			
B4	17,31	1000ml	1,66					2			9			1			
B4	17,81	1000ml	1,66						2		8	2		1			
B4	18,31	1000ml	1,66				1			3	8	3	3				
B4	18,81	1000ml	1,66					1		3	3	4	1				
B4	19,31	1000ml	1,66								9	5					
B4	20,31	1000ml	1,66					1			1	13	1				
B4	21,31	1000ml	1,66						1	2	2	1	6				
B4	26,25	2000ml	3,33									1	6	18	4		1

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
C1	3,56	600ml	1		2	16	1										
C1	3,90	600ml	1	4	3	19						1					
C1	4,23	600ml	1	2		6	10	1									
C1	4,56	600ml	1	3		9	7		1								
C1	4,90	600ml	1	3		10	15			2							
C1	5,23	600ml	1	2		2	11		1		1						
C1	5,56	600ml	1	1		2	19	1									
C1	**5,90**	**600ml**	**1**				**1**										
C1	6,23	600ml	1				15										
C1	6,56	600ml	1			2	12	2	1								
C1	6,90	600ml	1	1		2	12				2						
C1	7,23	600ml	1				21				1						
C1	7,56	600ml	1			1	13										
C1	11,31	600ml	1				1	1	9	1							
C1	11,81	1000ml	1,66				1	4	26	3	1						
C1	12,31	1000ml	1,66				1	3	19	5	2	1					
C1	12,81	1000ml	1,66					1	9	12	2						
C1	13,31	1000ml	1,66				2	1	3	16							
C1	13,81	1000ml	1,66				5	1	2	9	1						
C1	14,31	1000ml	1,66						5	15	5	1					
C1	14,81	1000ml	1,66				1	3	5	7	8		1				
C1	15,31	1000ml	1,66					1	1	5	10	1	1	1			
C1	15,81	1000ml	1,66					1	2	5	10		1				
C1	16,31	1000ml	1,66					1	3	9	16	2	1				
C1	16,81	1000ml	1,66					1	2	2	18	2					
C1	17,31	1000ml	1,66						1	6	12	2					
C1	17,81	1000ml	1,66						2		11	3					
C1	18,31	1000ml	1,66					2	2	2	10	5					
C1	18,81	1000ml	1,66						1		4	11	1	1			
C1	19,31	1000ml	1,66					1	1	1	4	10		1			
C1	20,31	1000ml	1,66					1		1	1	14	2	2			
C1	21,31	1000ml	1,66						3			5	4	1			
C1	26,25	2000ml	3,33								1	1	6	26	1		

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
C2	**3,56**	**600ml**	**1**	**2**		**1**											
C2	3,90	600ml	1	7		1	10										
C2	4,23	600ml	1	5		1	6										
C2	4,56	600ml	1	6		2	7	1									
C2	4,90	600ml	1	3		1	4										
C2	5,23	600ml	1	2			6										
C2	5,56	600ml	1				5										
C2	5,90	600ml	1	1			6	2	1								
C2	6,23	600ml	1				9		1								
C2	6,56	600ml	1	3		1	1	4									
C2	6,90	600ml	1				1	6									
C2	**7,23**	**600ml**	**1**				**1**	**3**									
C2	7,56	600ml	1				1	7		1							
C2	11,31	600ml	1					1	5	2	2	1	1				
C2	11,81	1000ml	1,66				1		2	9	1		1	1			
C2	12,31	1000ml	1,66					7	4	2	1	1	1				
C2	12,81	1000ml	1,66						1	4	2						
C2	13,31	1000ml	1,66					1	1	6	5	1		1			
C2	13,81	1000ml	1,66				4		8	9	2						
C2	14,31	1000ml	1,66						1	3	4	10					
C2	14,81	1000ml	1,66							1	17	4		1			
C2	15,31	1000ml	1,66							1	4	4					
C2	15,81	1000ml	1,66							2	5	1					
C2	16,31	1000ml	1,66							3	2	4	1				
C2	16,81	1000ml	1,66						1	1	14	2	1				
C2	17,31	1000ml	1,66							1	2	4	1				
C2	17,81	1000ml	1,66							1	1	5	1				
C2	**18,31**	**1000ml**	**1,66**								**1**		**1**				
C2	18,81	1000ml	1,66						1			2	2				
C2	19,31	1000ml	1,66								1		4				
C2	**20,31**	**1000ml**	**1,66**									**2**	**1**	**1**			
C2	21,31	1000ml	1,66					1			1	1	2				
C2	26,25	2000ml	3,33											3	5		

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
C3	3,56	600ml	1	7		15		1		1							
C3	3,90	600ml	1	2	3	1	10										
C3	4,23	600ml	1			6	21	1									
C3	4,56	600ml	1			3	14				1						
C3	4,90	600ml	1	2			23	1			1						
C3	5,23	600ml	1	1	1	1	18										
C3	**5,56**	**600ml**	**1**				**1**										
C3	5,90	600ml	1	3		2	21										
C3	6,23	600ml	1				16	2	1								
C3	6,56	600ml	1		1		7	6			1						
C3	6,90	600ml	1			1	5	9			2						
C3	7,23	600ml	1					20									
C3	7,56	600ml	1				7	17									
C3	11,31	600ml	1			2	2	2	5	11	2	1	1				
C3	11,81	1000ml	1,66					1	4	26	8						
C3	12,31	1000ml	1,66			1		2	1	10	19						
C3	12,81	1000ml	1,66				2	2		9	20						
C3	13,31	1000ml	1,66				1		4	3	25	1		1			
C3	13,81	1000ml	1,66				1	1	2	4	12	4					
C3	14,31	1000ml	1,66					2	2	2	22	10					
C3	14,81	1000ml	1,66				1			1	8	12	2				
C3	15,31	1000ml	1,66					1	1	1	3	8	2				
C3	15,81	1000ml	1,66					2	1	1	4	13	4				
C3	16,31	1000ml	1,66							2	2	9	1				
C3	16,81	1000ml	1,66							1	3	2	1				
C3	17,31	1000ml	1,66				1		1		1	7	4		1		
C3	17,81	1000ml	1,66						1	1	2	8	4				
C3	18,31	1000ml	1,66						1		1	4	7	2			
C3	18,81	1000ml	1,66					1	1		2	2	2	1			
C3	19,31	1000ml	1,66								2	6	2	1			
C3	20,31	1000ml	1,66						2	1		2	6	4			
C3	21,31	1000ml	1,66									1	2	14	1		
C3	26,25	2000ml	3,33								1		1	10	23		

Sampling		Conversions		Developmental stage													
Tank	Time (days)	Volume	x	Egg	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	M	F
C4	3,56	600ml	1	3	2	11	2										
C4	3,90	600ml	1	2	1	12	1	1									
C4	4,23	600ml	1	3	2	15	5										
C4	4,56	600ml	1	3	2	10	12										
C4	4,90	600ml	1	4	3	4	24	1									
C4	5,23	600ml	1	1	3	2	22										
C4	**5,56**	**600ml**	**1**				**1**										
C4	5,90	600ml	1	1			19	1		1							
C4	6,23	600ml	1	3		4	26		1								
C4	6,56	600ml	1			1	18	1	1								
C4	6,90	600ml	1	2		1	16										
C4	7,23	600ml	1	1		2	24	2			1						
C4	7,56	600ml	1	1			16	1									
C4	11,31	600ml	1	1		2		1	5								
C4	11,81	1000ml	1,66				2	4	24								
C4	12,31	1000ml	1,66					3	13	8	1						
C4	12,81	1000ml	1,66					2	24	14		2					
C4	13,31	1000ml	1,66					3	7	14	2						
C4	13,81	1000ml	1,66				1	2	4	25	4						
C4	14,31	1000ml	1,66				1	1	2	21	7						
C4	14,81	1000ml	1,66						2	14	9		1				
C4	15,31	1000ml	1,66				1	1	5	8	8						
C4	15,81	1000ml	1,66					1	5	5	13	2	1				
C4	16,31	1000ml	1,66				1	1		8	19						
C4	16,81	1000ml	1,66					2	1		2	11	5				
C4	17,31	1000ml	1,66					1		1	15	1					
C4	17,81	1000ml	1,66							4	16	6					
C4	18,31	1000ml	1,66						1	5	9	6	1				
C4	18,81	1000ml	1,66					1		1	8	5					
C4	19,31	1000ml	1,66							2	6	12	1				
C4	20,31	1000ml	1,66								3	11	2				
C4	21,31	1000ml	1,66								2	7	4		1		
C4	26,25	2000ml	3,33								1	1	6	22	8		

Appendix C – Biometry data

Eight C5 *C. finmarchicus* (2days old) were sampled from each of the twelve exposure tanks. Pictures of the animals were taken with a digital still-video camera (Sony DWF-sx900,Sony Corporation, Tokyo, Japan) operated from Fire-i software (Unibrain Inc., San Ramon CA, USA) from which body area, body volume, body length, area of lipid-sac and lengt of lipid-sac were measured. From these values, the fat sac volume (C.1), body, volume (C.2) and fat sac (C.3) (as percentage of whole body) were calculated. All raw data is presented in table C.1

$$Fat\ sac\ volume = \frac{(\pi * Area\ fat\ sac^2)}{(4 * Body\ length)} \tag{C.1}$$

$$Body\ volume = \frac{(\pi * Area\ body^2)}{(4 * Body\ length)} \tag{C.2}$$

$$Lipid\ sac\ \% = \left(\frac{Fat\ sac\ volume}{Body\ volume}\right) * 100 \tag{C.3}$$

Table C.1: Data obtain from biometric analysis and the calculated values of volumes of lipid sac, body volume and lipid sac %. The table continues on subsequent pages.

Animals	Area body (mm$^{2)}$	Length Body (mm^2)	Area lipid-sac (mm$^{2)}$	Length lipid-sac (mm)	Volume lipid-sac (mm^3)	Volume body (mm^3)	Lipid-sac (%)
A1 1	1,082	2,112	0,238	1,673	0,027	0,435	6,108
A1 2	0,955	2,054	0,306	1,691	0,043	0,349	12,471
A1 3	0,772	1,912	0,239	1,181	0,038	0,245	15,519
A1 4	1,018	2,142	0,320	1,890	0,043	0,380	11,199
A1 5	0,946	2,136	0,278	1,807	0,034	0,329	10,208
A1 6	0,931	1,983	0,320	1,709	0,047	0,343	13,708
A1 7	1,018	2,166	0,413	2,054	0,065	0,376	17,357

A1 8	0,964	2,070	0,223	1,541	0,025	0,352	7,188
A2 1	0,758	2,011	0,124	1,100	0,011	0,224	4,892
A2 2	0,902	1,876	0,124	1,377	0,009	0,340	2,575
A2 3	0,696	1,843	0,199	1,671	0,019	0,206	9,016
A2 4	0,888	1,845	0,178	1,638	0,015	0,336	4,526
A2 5	0,869	1,943	0,137	1,364	0,011	0,305	3,540
A2 6	0,758	1,888	0,158	1,379	0,014	0,239	5,949
A2 7	0,898	1,911	0,129	1,262	0,010	0,331	3,125
A2 8	0,733	1,822	0,156	1,267	0,015	0,231	6,513
A3 1	0,926	1,884	0,235	1,467	0,030	0,357	8,271
A3 2	0,790	1,813	0,259	1,672	0,031	0,270	11,655
A3 3	0,778	1,867	0,206	1,530	0,022	0,254	8,555
A3 4	0,917	1,872	0,097	0,508	0,015	0,353	4,123
A3 5	0,571	1,642	0,083	0,987	0,005	0,156	3,515
A3 6	0,872	2,012	0,205	1,442	0,023	0,297	7,711
A3 7	0,827	1,854	0,162	1,398	0,015	0,290	5,089
A3 8	0,873	1,985	0,132	1,164	0,012	0,301	3,899
A4 1	0,905	2,016	0,235	1,405	0,031	0,319	9,675
A4 2	0,979	2,149	0,290	1,766	0,037	0,350	10,678
A4 3	0,935	2,128	0,276	1,661	0,036	0,322	11,163
A4 4	0,899	1,947	0,233	1,517	0,028	0,326	8,621
A4 5	0,914	2,066	0,290	1,620	0,041	0,317	12,839
A4 6	0,977	2,115	0,290	1,770	0,037	0,354	10,528
A4 7	0,953	2,016	0,263	1,774	0,031	0,354	8,655
A4 8	1,050	2,084	0,315	1,858	0,042	0,415	10,095
B1 1	0,928	2,031	0,253	1,541	0,033	0,333	9,796
B1 2	0,840	1,966	0,173	1,278	0,018	0,282	6,525
B1 3	0,870	2,044	0,172	1,434	0,016	0,291	5,571
B1 4	0,875	1,969	0,294	1,825	0,037	0,305	12,180
B1 5	1,145	2,258	0,387	2,085	0,056	0,456	12,372
B1 6	1,003	2,188	0,280	1,650	0,037	0,361	10,334
B1 7	0,888	2,054	0,233	1,257	0,034	0,301	11,250
B1 8	1,098	2,277	0,319	1,979	0,040	0,416	9,712
B2 1	1,063	2,059	0,229	1,587	0,026	0,431	6,021
B2 2	0,714	1,845	0,144	1,323	0,012	0,217	5,672
B2 3	0,804	1,969	0,105	1,780	0,005	0,258	1,887
B2 4	0,722	1,903	0,078	0,849	0,006	0,215	2,616
B2 5	0,735	1,924	0,199	1,532	0,020	0,220	9,206
B2 6	0,739	1,886	0,137	1,378	0,011	0,227	4,704
B2 7	0,865	1,893	0,122	1,019	0,011	0,310	3,695
B2 8	0,850	2,028	0,179	1,467	0,017	0,280	6,131
B3 1	0,846	1,944	0,163	1,396	0,015	0,289	5,169
B3 2	0,884	1,989	0,226	1,537	0,026	0,308	8,458
B3 3	0,832	1,970	0,192	1,425	0,020	0,276	7,362
B3 4	0,780	1,910	0,051	0,945	0,002	0,250	0,864

B3 5	0,986	1,954	0,168	1,377	0,016	0,391	4,120
B3 6	0,849	1,802	0,103	1,173	0,007	0,314	2,261
B3 7	0,988	1,988	0,173	1,274	0,018	0,385	4,784
B3 8	0,823	1,932	0,316	1,839	0,043	0,275	15,488
B4 1	0,741	1,793	0,063	0,746	0,004	0,240	1,737
B4 2	0,857	2,016	0,245	1,636	0,029	0,286	10,071
B4 3	0,803	1,900	0,187	1,256	0,022	0,266	8,204
B4 4	0,885	2,050	0,203	1,421	0,023	0,300	7,590
B4 5	0,871	2,041	0,231	1,469	0,029	0,292	9,773
B4 6	0,807	1,981	0,171	1,262	0,018	0,258	7,048
B4 7	0,842	1,988	0,173	1,265	0,019	0,280	6,634
B4 8	0,878	2,041	0,200	1,526	0,021	0,296	6,940
C1 1	0,908	2,092	0,210	1,524	0,023	0,309	7,342
C1 2	1,028	2,191	0,340	2,034	0,045	0,379	11,783
C1 3	0,949	2,116	0,192	1,390	0,021	0,334	6,231
C1 4	1,064	2,184	0,303	1,786	0,040	0,407	9,917
C1 5	0,967	2,105	0,352	2,010	0,048	0,349	13,877
C1 6	0,779	1,901	0,143	1,170	0,014	0,251	5,475
C1 7	0,888	2,076	0,240	1,537	0,029	0,298	9,866
C1 8	1,108	2,189	0,401	1,937	0,065	0,440	14,802
C2 1	0,732	1,919	0,106	1,164	0,008	0,219	3,457
C2 2	0,782	1,911	0,177	1,681	0,015	0,251	5,824
C2 3	0,818	1,909	0,195	1,444	0,021	0,275	7,513
C2 4	0,699	1,868	0,085	1,039	0,005	0,205	2,659
C2 5	0,821	1,904	0,103	1,151	0,007	0,278	2,604
C2 6	0,709	1,906	0,106	1,238	0,007	0,207	3,441
C2 7	0,968	1,959	0,059	0,825	0,003	0,375	0,882
C2 8	0,839	1,878	0,168	1,333	0,017	0,294	5,649
C3 1	0,901	2,017	0,192	1,475	0,020	0,316	6,210
C3 2	0,806	1,926	0,124	1,070	0,011	0,265	4,260
C3 3	0,802	1,941	0,223	1,566	0,025	0,260	9,583
C3 4	0,686	1,718	0,074	1,008	0,004	0,215	1,983
C3 5	0,756	1,924	0,262	1,175	0,046	0,233	19,673
C3 6	0,794	1,965	0,167	1,352	0,016	0,252	6,430
C3 7	0,736	1,928	0,109	1,308	0,007	0,221	3,233
C3 8	0,992	1,944	0,256	1,692	0,030	0,397	7,652
C4 1	1,046	2,102	0,282	1,709	0,037	0,409	8,940
C4 2	0,806	2,016	0,168	1,375	0,016	0,253	6,370
C4 3	0,747	1,943	0,148	1,129	0,015	0,225	6,756
C4 4	0,804	2,011	0,129	1,233	0,011	0,252	4,199
C4 5	0,755	1,928	0,155	1,413	0,013	0,232	5,751
C4 6	0,737	1,878	0,183	1,459	0,018	0,227	7,936
C4 7	0,817	1,997	0,194	1,534	0,019	0,262	7,340
C4 8	0,950	2,016	0,310	1,909	0,040	0,351	11,245