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Medium-term exposure of the North Atlantic copepod *Calanus finmarchicus* (Gunnerus, 1770) to CO_2-acidified seawater: effects on survival and development

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Abstract. The impact of medium-term exposure to CO_2-acidified seawater on survival, growth and development was investigated in the North Atlantic copepod *Calanus finmarchicus*. Using a custom developed experimental system, fertilized eggs and subsequent development stages were exposed to normal seawater (390 ppm CO_2) or one of three different levels of CO_2-induced acidification (3300, 7300, 9700 ppm CO_2). Following the 28-day exposure period, survival was found to be unaffected by exposure to 3300 ppm CO_2, but significantly reduced at 7300 and 9700 ppm CO_2. Also, the proportion of copepodite stages IV to VI observed in the different treatments was significantly affected in a manner that may indicate a CO_2-induced retardation of the rate of ontogenetic development. Morphometric analysis revealed a significant increase in size (prosome length) and lipid storage volume in stage IV copepodites exposed to 3300 ppm CO_2 and reduced size in stage III copepodites exposed to 7300 ppm CO_2. Together, the findings indicate that a pCO_2 level $\leq$ 2000 ppm (the highest CO_2 level expected by the year 2300) will probably not directly affect survival in *C. finmarchicus*. Longer term experiments at more moderate CO_2 levels are, however, necessary before the possibility that growth and development may be affected below 2000 ppm CO_2 can be ruled out.

1 Introduction

Burning of fossil fuels, altered use of land areas and other anthropogenic activities have contributed to a rise in the mean atmospheric concentration of carbon dioxide (CO_2) from a preindustrial level of around 280 ppm to its present level of $\sim$ 390 ppm CO_2 (Solomon et al., 2007). The increasing atmospheric CO_2 level has been identified as an explanation for the global warming phenomenon that has been observed during the last decade (Solomon et al., 2007). Approximately 30 % of the anthropogenic CO_2 has so far been absorbed by the oceans where it lowers the seawater pH through the production of carbonic acid (Sabine et al., 2004) – a process commonly referred to as ocean acidification (OA). As a result of this process, the mean pH of ocean surface water (8.2) has been lowered by 0.1 pH units compared to preindustrial times, corresponding to a 30 % increase in the concentration of H^+-ions. Worst-case scenario estimates based on carbon cycle models predict a CO_2 level of 970 ppm by the end of the century (A1FI, Houghton et al., 2001), and possibly up to 1900 ppm in year 2300 (Caldeira and Wickett 2003), which corresponds to a mean surface-seawater pH of 7.8 and 7.4, respectively. For the year 2300 8000 ppm CO_2 has even been put forward as a "worst-case" scenario (Caldeira and Wickett 2005). The rate of change in the atmospheric CO_2 concentration and ocean pH experienced over the last century is up to a hundred times faster than any change observed during the past 650 000 yr (Siegenthaler et al., 2005). There is a growing concern that the stress due to the rising CO_2 level could be magnified by this rapid change, possibly resulting

in serious consequences for the marine biota (Monaco Declaration, 2009). Some of this concern comes from studies of fossil records that indicate that previous periods of intense ocean acidification, e.g., at the end of the Paleocene, coincided with the mass extinction event of that time (Jackson, 2010).

Carbon capture and storage (CCS) is currently considered to represent one of the most promising alternatives to mitigate CO_2 emissions (Metz et al., 2005). Since the implementation of international legislation concerning sub-seabed CO_2 storage in Europe (EU, 2009; London Protocol, 2006) industrial scale projects are currently undertaken at several locations. Sub-seabed storage is considered to be a relatively safe method to dispose of CO_2, but risk assessments indicate that loss of stored CO_2 to the water column could occur through leakage (Gerlagh and van der Zwaan, 2012). Leakage from such sub-seabed storages will probably affect a relatively limited area, but the CO_2 levels in the affected water column could reach levels that are orders of magnitude higher than the most pessimistic ocean acidification scenarios, with potentially dramatic consequences for the organisms inhabiting the affected area.

The initial concerns regarding OA addressed the possible implications of the reduction in free CO_3^{2-} ions on the formation of calcium-containing structures in calcifying organisms. Indeed, many of the calcareous species investigated have been found to be highly vulnerable to elevated levels of CO_2 (Talmage and Gobler, 2009; Dupont et al., 2008; Comeau et al., 2009; Fabry et al., 2008). However, in addition to affecting the calcification process, elevated levels of CO_2 have also been found to affect various aspects of the normal physiology of marine organisms, such as gene expression (Todgham and Hofmann, 2009), and the energy budgets (Melzner et al., 2011; Stumpp et al., 2011). Negative effects of elevated pCO_2 on reproductive endpoints such as sperm mobility, fertilization and hatching success have been observed in a number of species (Egilsdottir et al., 2009; Ellis et al., 2009; Havenhand et al., 2008; Kurihara et al., 2004a). Also, alterations in growth rate have been observed in many of the investigated species (Clark et al., 2009; Dupont et al., 2008; Findlay et al., 2009; Talmage and Gobler, 2009). Since many of these physiological processes are relevant to non-calcifying organisms, it is important to also investigate the responses of different members of this group to CO_2-induced acidification.

Copepods (Crustacea; Copepoda) are considered to constitute the most numerous multicellular organisms on earth (Mauchline, 1998), and thus play a vital role in marine food webs. However, their exoskeleton is non-calcified (Fitzer et al., 2012), and only a limited number of studies have investigated the vulnerability to elevated CO_2 levels among species from this group. The information available so far indicates considerable stage- and interspecific difference with regard to sensitivity to elevated CO_2 levels. While some species seem to tolerate CO_2 levels that are well above 2000 ppm (the level expected for year 2300), others such as *Acartia tsuensis* (Ito, 1956) (Kurihara and Ishimatsu, 2008), displayed an overall reduced hatching success in the eggs produced at 2300 ppm CO_2 when incubated over multiple generations (although no significant difference was observed within each separate generation). Recently, a study on the harpacticoid copepod *Tisbe battagliai* (Volkmann-Rocco, 1972) revealed a negative effect on hatching success and survival at CO_2 levels well below 1000 ppm (Fitzer et al., 2012), contradicting the perception that copepods are generally resistant to elevated levels of CO_2.

The copepod investigated in the present study, *Calanus finmarchicus* (Gunnerus, 1770), seasonally dominates the zooplankton biomass in the surface waters of the northern North Sea and the North Atlantic (Planque and Batten, 2000; Conover, 1988). The dominance of the northern *Calanus* species has been linked to specific life history traits which involve avoidance of predators and temporary scarcity of food during autumn and winter by descending towards the seafloor, as far as 1500 m, where the late juvenile stages enter into a quiescent state, before re-emerging in the surface water in time for the algal spring bloom (Edvardsen et al., 2006). Through synthesis and accumulation of lipids *Calanus* species are able to concentrate energy, and therefore constitute an important energy link between the phytoplankton and higher trophic level predators, including many fish species (Runge, 1988; Beaugrand and Kirby, 2010) and seabirds (Kwasniewski et al., 2012). Through the production of fecal pellets, *C. finmarchicus*, together with the other calanoid, copepods also constitutes a dominant part of the total vertical carbon flux in the ocean (Bathmann et al., 1987).

The total *Calanus* biomass in the North Sea and North Atlantic has reportedly declined by approximately 70 % between the 1960s and the post 1990s, a reduction that is considered to reflect regional warming (Edwards et al., 2006; Edwards et al., 2012). Due to the importance of *C. finmarchicus* in the marine food webs of northern waters, and its significance for maintenance of commercial fish stocks, negative effects of elevated pCO_2 and climatic changes on the species could have wide-reaching socioeconomic consequences.

Difficulties with successful rearing under laboratory conditions combined with relatively long generation times have so far limited studies examining the potential effects of elevated CO_2 on *Calanus* species to short-term experiments on wild-caught individuals where the focus have been on endpoints such as the hatching success of eggs and early nauplii survival. Using this approach Mayor et al. (2007) found no effects on egg production when wild-caught females were exposed to 8000 ppm CO_2, although the hatching success of the eggs incubated under the same conditions was severely reduced (only 4 % survival). In a similar experiment, where more moderate CO_2 levels were applied, no significant effect on hatching success was observed when eggs from wild-caught *Calanus helgolandicus* (Claus, 1863) females were incubated in seawater with 1000 ppm CO_2 (e.g., a level that

may be reached within the end of the century) (Mayor et al., 2012). Recently, Weydmann et al. (2012) reported that egg production in wild-caught *Calanus glacialis* (Jaschnov, 1955) females were unaffected by a pH level of 7.6 and 6.9 (corresponding to a pCO_2 of ∼ 1000 and ∼ 7000 ppm at the in situ temperature used, respectively), but a reduced hatching success was observed among the eggs that were incubated at pH 6.9. In wild-caught *Calanus sinicus* (Brodsky, 1962), no effect on adult survival and egg production rate was observed below 10 000 ppm CO_2 during an eight day long incubation period (Zhang et al., 2011).

The aim of the present study was to provide data on the long-term effects of elevated pCO_2 in a *Calanus* species. The effects on hatching success, mortality and ontogenetic development were integrated by exposing fertilized *C. finmarchicus* eggs and subsequent developmental stages under controlled laboratory conditions to normal seawater (∼ 390 ppm CO_2), or one of three different levels of CO_2-induced acidification (3300, 7300, 9700 ppm CO_2), over a 28-day period.

2 Methods

2.1 Seawater and exposure facilities

Natural seawater for the experiment was supplied through an inshore sub-sea pipeline in the Trondheimfjorden (Norway), collecting water from about 70 m depth. Prior to use, the seawater was filtered through a sand filter and temperature and gaseous saturation adjusted in the integrated water treatment system available at NTNU SeaLab research facility, which include using a combination of heavy aeration and sprinkling over biofilm carriers (Kaldnes Miljøteknologi, Norway) in a polyethylene holding tank (6 m^3). Before entering the experimental system the water was finally filtered to 1 µ m by inline filters.

All experiments were carried out in a temperature-controlled room maintained at 9–10 °C at the research facility of NTNU Centre of Fisheries and Aquaculture (SeaLab).

2.2 *Calanus* material

Calanus eggs used in the present investigation were produced by females from the culture running at NTNU Centre of Fisheries and Aquaculture (SeaLab) (Hansen et al., 2007). The females (240 individuals) were transferred to a 50 L polyethylene tank where newly laid eggs were collected from the floor after 12 h incubation using a siphon. Prior to use, the collected eggs were gently concentrated using a plankton sieve (mesh size 64 µm) submerged in water.

2.3 Description of the experimental system

A custom flow-through experimental system was developed to include 12 two-liter incubation chambers (borosilicate bottles, Schott) that were maintained in a horizontal position by a rack system (Fig. 1a). Both the inlet and outlet for the continuous addition of seawater to the bottles went through custom developed flask stoppers. A PEEK tube extending from the stopper and further into the bottle created circulation by distributing the inflowing water between three small holes (Ø 0.5 mm), serving as nozzles producing circulation by gentle jet streams. A nylon mesh cloth (mesh size 64 µm, Nitex), mounted in association with the outlet, served as a screen that retained the animals within the bottles (Fig. 1b). The water level in the bottles was determined by a water level controller at the outlet of the system. Two narrow-bored glass tubes (2 mm inner diameter (ID)) mounted through the lids, extending from the headspace in the bottles to the outside, maintained normal ambient air pressure within the bottles. Seawater, pre-equilibrated with different CO_2 enriched air mixtures, was added to each bottle using a 12 channel peristaltic pump (Watson Marlow, model 202) at a constant flow rate of 2.5 mL min^{-1}. All wetted parts of the setup were made from borosilicate glass and high-grade polymers known to be safe to the animals.

2.4 Preparation of the different CO_2 mixtures and equilibration in columns

A gas mixing system was developed for the present study. Briefly, 5000, 10 000 and 15 000 ppm CO_2, in addition to ambient air with 390 ppm (control), was obtained by mixing pressurized air and CO_2 gas (100 %, AGA, Norway). The appropriate gas flow, necessary to produce the different mixtures, was attained by using fine bore polyethylene tubes of different lengths and inner diameter (ID 0.28/0.38 mm) as gas flow restrictors. The equal pressure of the two gases, which is a prerequisite for the mixing principle described above, was obtained using a custom valve developed on the principle described by Parsons et al. (1992). The CO_2 concentration of the different gas mixtures was determined using a NDIR CO_2 gas analyzer (S153 Qubit Systems Inc, Ontario, Canada), calibrated using CO_2-free air and a 1 % CO_2 gas standard (AGA, Norway). The water was equilibrated to the control and the three CO_2-enriched air mixtures using custom developed counter current equilibration columns. The vertical columns consisted of an outer (60 × 16 × 15 cm (L × outer diameter (OD) × ID)) and smaller inner acrylic tube (60 × 6 × 5 cm (L × OD × ID)) mounted within the outer tube (Fig. 1c). The water in the columns was maintained at a constant level using in-house developed floating regulator valves. A submersible aquarium pump (Micro-Jet MC 450, Aquarium systems) was used to lift water from the outer tube to the elevated inlet of the inner tube. The elevated water column of the inner tube that was maintained by the pumping activity caused a gravimetrically determined downward flow of water that drained back to the outer tube through lateral holes near the base of the inner tube, producing a constant circulation. An air stone (lime wood, Aqua Medic) mounted near the base of the inner tube introduced

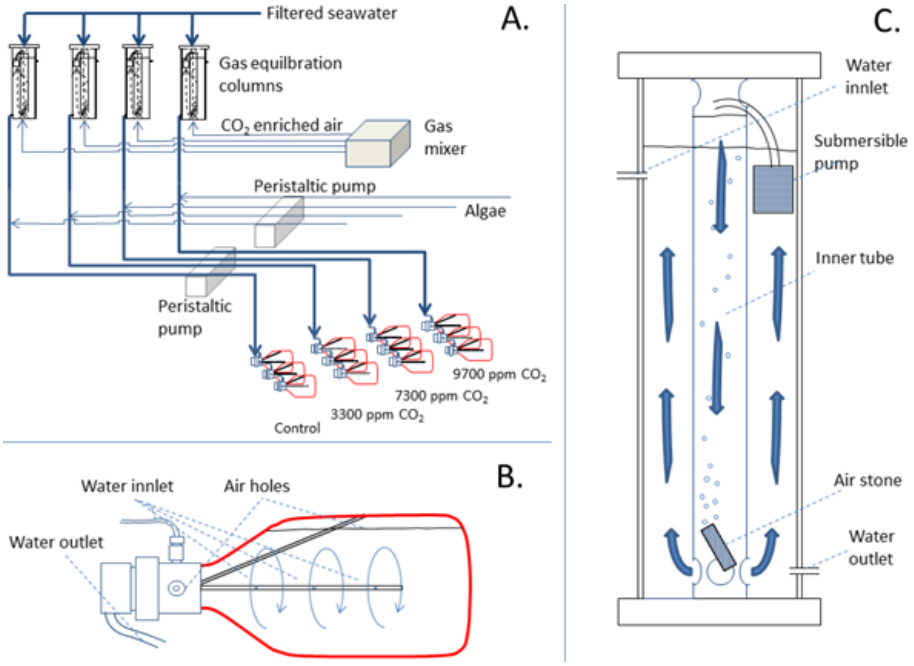

Fig. 1. (A) Schematic outline of the experimental setup, including equilibration columns for preparation of the different CO_2 enriched air mixtures, gas mixer, alga addition and exposure bottles. **(B)** Illustration of the bottles used for the exposure, including water-inlet, water-outlet and air holes. **(C)** Illustration of the columns used for equilibration of the water, including outer and inner tube, submersible pump, and air stone introducing the different CO_2 enriched air mixtures into the water.

small bubbles of the different air mixtures that ascended, counter-current to the descending water current, thus providing a favorable condition for the equilibration process.

2.5 Feeding

The copepods were fed a mixture of three species of micro algae (*Rhodomonas baltica*, Karsten, 1898; *Dunaliella tertiolecta*, Bucher, 1959; and *Isochrysis galbana*, Parke, 1949) during the entire experiment. A carefully prepared algae stock suspension was continuously added to the water stream between the equilibration columns and their respective incubation bottles, at a flow rate of 0.075 mL min^{-1}, using a four-channel peristaltic pump (Watson Marlow model 202) fitted with Marprene tubing. This maintained a stable density of algae in the exposure water corresponding to a total nominal carbon concentration of 600 µg L^{-1}. The three algal species contributed equally in terms of carbon content. To monitor the algae level during the experiment, water samples were collected from the outlet of the exposure bottles and analyzed using a Coulter counter (Multisizer^{TM} 3, Bechman Coulter Inc., USA). The measurements confirmed that the mean concentration was high throughout the experiment (48233 ± 2864 cells mL^{-1}). Even towards the end of the experiment, when the appetite of the copepods peaked, no noticeable change in algae concentration was apparent. Equilibrated seawater with algae was added to the experimental units at a flow rate of 2.5 mL min^{-1}. This flow rate corresponded to a full water exchange two times per day in the bottles.

2.6 Experimental procedure

Batches of 240 newly laid eggs were sorted under a stereo dissection microscope (Leica MZ125, Leica Microsystems, Wetzlar, Germany) and transferred to each incubation chamber (bottle) using a glass Pasteur pipette. New eggs have a more transparent appearance than the older ones and this feature was utilized during the sorting procedure to secure that the eggs used in the experiment were as newly laid, and as synchronized, as possible. The sorted eggs were randomly distributed between the different treatments to avoid potential bias. The fertilization status of the eggs was not checked during the procedure. The eggs and subsequent stages were exposed for a total period of 28 days to four different pCO_2 levels (390, 3300, 7300 and 9700 ppm), and the whole experiment was performed at 10 °C at a 16 : 8 day–night cycle. Three replicate chambers of each CO_2 treatment were included. The experiment was staggered over a three day period, where replicates from the four different treatments were started together in groups on the different days. Based on results from preliminary acute tests, with various CO_2 levels, we suspected that *C. finmarchicus* might be robust with regards to CO_2 levels that are relevant to near-future projections. To reveal the sensitivity range during medium-term exposure, a CO_2 level that was approximately 1000 ppm

above the year 2300 worst-case scenario was selected as the lowest treatment, while the highest CO_2 treatment was chosen to match a whole pH unit drop, which may be relevant to some leakage scenarios from sub-seabed carbon storage sites (Blackford et al., 2008). The incubation bottles were exchanged with clean ones on a weekly basis to reduce the buildup of bacterial microfilm inside the walls. The procedure involved moving the bottles to an upright inverted position, followed by a lowering of the water level by gently tapping the water into a receiving bottle using gravity feed through fine bore tubes (flow restriction). This procedure confined the copepods in a small volume of water above the outlet nylon mesh screen in the bottle stopper. The used bottle could now be detached and replaced by a clean one while reducing the disturbance of the animals to a minimum. The clean bottle was finally gently filled up through the outlet, again using gravity feed. To secure water quality continuity, the "old" water collected during the draining procedure was reused.

2.7 Determination of mortality, stage distribution and morphometry

Following 28 days of exposure the animals were transferred from their respective incubation bottles and inspected using a Leica MZ125 dissecting microscope (Leica Microsystems, Wetzlar, Germany). Pictures of all animals were captured with a digital still-video camera (Sony DWF-sx900, Sony Corporation, Tokyo, Japan), operated by Fire-i software (Unibrain Inc., San Ramon CA, USA). Morphometric characteristics of the animals were measured manually on scaled captured images by the use of the software Image J (National Institutes of Health, Bethesda MD, USA). Length of prosome, urosome, total body length, area of the lipid storage and area of the prosome were measured with the aid of a graphical tablet (Wacom Cintiq 12WX, Wacom Co., Ltd., Saitama, Japan). Volume of lipid storage sac and prosome were calculated according to Miller et al. (1998) from the area and length of the lipid sac and prosome, respectively. Copepodite stages (the 1st copepodite CI to the final, adult stage CVI) and sex of the adults were determined based on the number and shape of the urosome segments (Marshall and Orr, 1972; Mauchline, 1998).

2.8 Carbonate system determination

Initial tests of the stability of the experimental system showed that weekly measurements were sufficient to monitor pH and temperature in the incubation bottles. The pH was determined potentiometrically (PHM240 pH-meter with a pHC2401-electrode and a T201 temperature sensor, Radiometer Analytical) using the NBS scale. The pH meter was calibrated with IUPAC precision pH buffer 4.005 and 7.000 (Radiometer Analytical). Total alkalinity was determined by titration according to the method described by Anderson and Robinson (1946). Seawater carbonate species were calculated using the CO2SYS software (Pierrot et al., 2006) with the dissociation constants for NBS scale of Mehrbach et al. (1973), refitted by Dickson and Millero (1987). Measured values and derived carbon species are presented in Table 1.

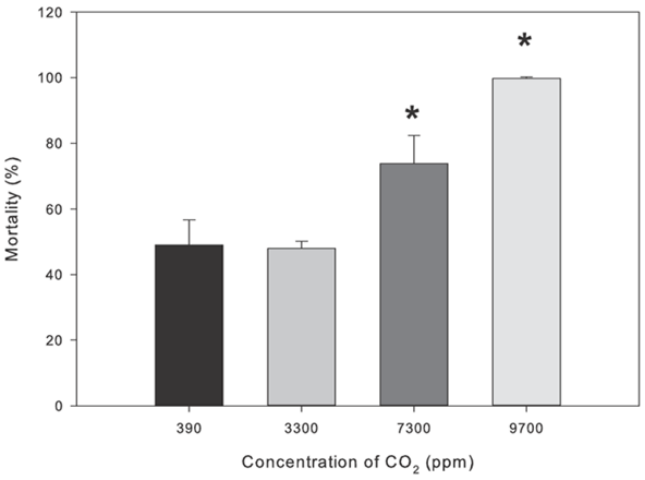

Fig. 2. Mortality recorded after 28-day exposure to different levels of CO_2-acidified seawater. The bars show mean ± 1 std and significant differences between control (390 ppm) and exposed groups are indicated by asterisks (*).

2.9 Statistical treatment

Prior to statistical analysis data on mortality and morphometric parameters were arcsin and log transformed, respectively. Deviation from homogenous variation was examined using Levines test. Statistical comparisons of the different treatments were performed using one way ANOVA followed by Dunnet's *post hoc* test to identify significant differences between control and the elevated CO_2 levels. Due to violation of the assumption of homogeneous variation stage percentages were analyzed using Kruskall–Wallis test. No post hoc test was applied here due to low power. The level of significance was set to 0.05 in all tests. All statistical analyses were performed using the statistical package SPSS.

3 Results

The overall mortality among the animals during the 28-day experiment period was 49 % in the control treatment (Fig. 2). The treatment that received seawater acidified by 3300 ppm CO_2 (47.9 %) showed no significant difference in mortality compared to the control. In the treatment that received seawater acidified by 7300 ppm CO_2 there was a significant increase in the mortality up to 73.8 % ($p < 0.05$) when compared to the control. With the exception of two nauplii found in one of the replicate bottles, no animals developed under

Table 1. Carbonate system speciation in the experimental treatments. Total dissolved inorganic carbon (C_T), pCO_2 and calcium carbonate saturation state for calcite and aragonite (Ω_{Ca}, Ω_{Ar}) were calculated from pH and total alkalinity (A_T). Listed values represent means ±1 std.

pH_{Tot}	A_T (µmol kg^{-1} SW)	S (PSU)	T (°C)	pCO_2 (µatm)	C_T (µmol kg^{-1} SW)	Ω_{Ca}	Ω_{Ar}
8.20 ± 0.01	2353	33	10	365 ± 9	2150 ± 4	3.60 ± 0.07	2.29 ± 0.04
7.31 ± 0.04	2353	33	10	3332 ± 282	2464 ± 15	0.53 ±0.04	0.34 ± 0.03
6.97 ± 0.05	2353	33	10	7281 ± 868	2650 ± 39	0.25 ±0.03	0.16 ± 0.02
6.85 ± 0.03	2353	33	10	9651 ± 597	2755 ± 26	0.19 ±0.01	0.12 ± 0.01

the highest CO_2 treatment (9700 ppm CO_2), resulting in an overall mortality of 99.7 % ($p < 0.05$).

After 28 days of exposure stage V copepodites dominated numerically in controls and the groups exposed to 3300 ppm CO_2 and constituted 82.4 % (±2.6) and 62.7 % (±15.8) of the animals in the two groups, respectively (Fig. 3). While adults (CVI) constituted 5.87 % (±4.19) and 0.53 % (±0.46) of the animals at the end of the experiment in the control and 3300 ppm treatment, respectively, no adults developed in the two highest CO_2 treatments (7300 and 9700 ppm). Increasing CO_2 level caused a significant higher percentage of CIV, while the proportion of the later stages, CV and CVI was reduced ($p < 0.05$), resulting in CIV being the dominant stage in the groups exposed to 7300 ppm. No males were present among all the CVI at day 28.

For the control treatment mean prosome length of stage III, IV and V was 1.25 mm (±0.02), 1.46 mm (±0.03) and 1.91 mm (±0.03), respectively (Fig. 4). The lowest level of CO_2-acidified seawater (3300 ppm) caused a 5.6 % increase of the prosome length of the stage IV copepodites ($p < 0.05$), while exposure to 7300 ppm CO_2 caused a 11.2 % reduction of the prosome length in stage III copepodites compared to the control ($p < 0.05$).

The lipid content of the copepodites in the control treatment increased with increasing developmental stages and constituted 2.1 (±1.5), 2.9 (±1.7), and 7.9 (±1.7) % in stage III, IV and VI, respectively (Fig. 4). The only significant effect of CO_2 exposure observed was a 2.3-fold increase in the lipid content of stage IV copepodites exposed to 3300 ppm.

4 Discussion

The results from the present study on the survival of *C. finmarchicus* egg cohorts raised in seawater with different degree of CO_2 acidification are well in line with the results that have been reported previously in similar, but short-term studies, on *Calanus* species. Exposure to high levels of CO_2-induced acidification has previously been shown to reduce hatching success. The hatching success of *C. finmarchicus* (Mayor et al., 2007) and *C. glacialis* (Weydmann et al., 2012) eggs was severely reduced by incubation in seawater acidified with ∼ 8000 and ∼ 7000 ppm CO_2, respectively. Within this range of CO_2 levels the present study also revealed a strong and significant reduction of the survival in the raised cohorts; survival was reduced by ∼ 50 % in the treatment with 7300 ppm, while no animals developed at 9700 ppm CO_2 (except two individuals which survived arrested as nauplii in one of the three replicates). This suggests that ∼ 10 000 ppm CO_2 may represent the upper limit for successful hatching and continued development of fertilized eggs in *C. finmarchicus*. Such a level of CO_2-induced acidification is within the range that may be relevant to episodes of leakage from sub-seabed storage sites for CO_2 (Blackford et al., 2009). Due to the overwintering strategy of *C. finmarchicus* near the seafloor (Edvardsen et al., 2006), CO_2 leakage from such a storage site may negatively affect the diapausing animals. However, since most of the potential leakage pathways from CO_2 stores are considered most likely to lead to relatively low flux emissions (Holloway, 2007), the affected area is assumed to be relatively limited and any negative impacts on *C. finmarchicus* and other members of the fauna are therefore likely to be only local.

Previous short-term studies on wild-caught animals have reported results indicating that *Calanus* species may be relatively robust to the most pessimistic ocean acidification scenarios expected by the end of this century (∼ 1000 ppm CO_2). Incubation in seawater acidified with ∼ 1000 ppm CO_2 had no significant effect on the hatching success of eggs from wild-caught females of either *C. helgolandicus* (Mayor et al., 2012) or *C. glacialis* (Weydmann et al., 2012). The results from the present medium-term study on cohorts of *C. finmarchicus* eggs revealed no apparent effect on survival after 28 days of exposure to seawater acidified with 3300 ppm CO_2. However, recent studies have shown that some copepods may be negatively affected at pCO_2 levels that are in the range that could occur within the end of this century (∼ 1000 ppm CO_2). A multi-generational study of the harpacticoid copepod *T. battagliai* showed that naupliar production was negatively affected by pH levels as high as 7.82 (≈ 400–470 ppm CO_2) (Fitzer et al., 2012). In light of the results from the present and the short-term studies (Mayor et al., 2007; Mayor et al., 2012; Weydmann et al., 2012) *Calanus* species may rank among the more tolerant copepods with regard to CO_2-induced seawater acidification. Collectively, the results so far available on *Calanus* species suggest that CO_2 levels ≤ 2000 ppm (the worst case CO_2 level predicted for the year 2300 by Caldeira and Wickett 2003)

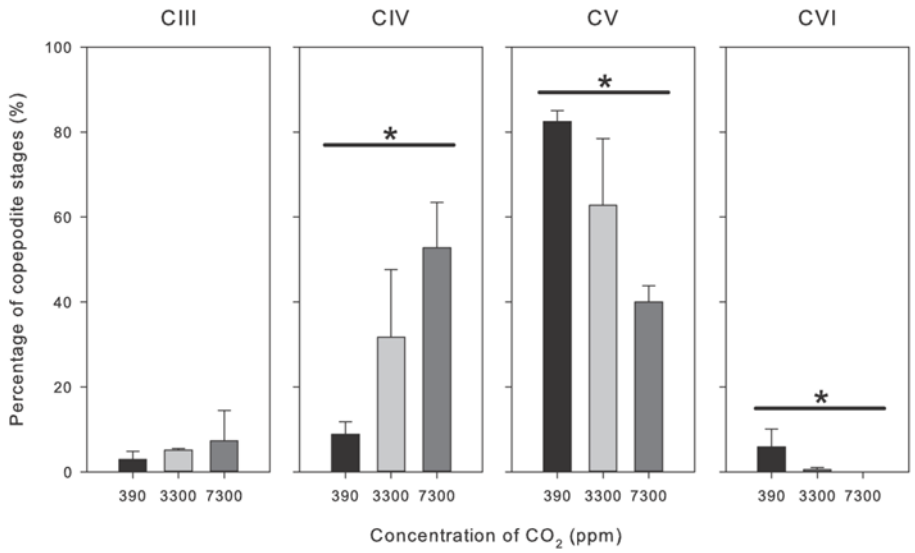

Fig. 3. Stage distribution (percentage) of the copepodites exposed to different levels of CO_2-acidified seawater. Three replicate groups at each exposure level. The bars show mean ± 1 std. Significant differences ($p < 0.05$) between the experimental groups are indicated by asterisks (*).

is not likely to directly affect the survival of individuals from this genus.

Early life stages have been suggested to be the most vulnerable part of the life cycle with regards to elevated pCO_2 in marine organisms in general (Dupont et al., 2008; Kurihara, 2008). Indeed, adult copepods have been found to be much more resistant to elevated levels of pCO_2 than eggs and nauplii (Kurihara et al., 2004b; Mayor et al., 2007). It has been proposed that the negative effect of elevated pCO_2 on the hatching success of eggs may be caused by a reduction of intracellular pH (Kurihara, 2008). Also, the shift in energy source from endogenous yolk to exogenous food represents a critical phase that may explain much of the high mortality rate observed among the early life stages (Takahashi and Ohno, 1996). Additional stress from elevated pCO_2 levels could make this transition an even tighter bottleneck for successful development in these animals. The only information available regarding the relative sensitivity of the early copepod stages (e.g., eggs vs. early nauplii stages) comes from a study on *Acartia erythraea* (Giesbrecht, 1889) where a significant reduction in nauplii survival and hatching success was observed at 5400 and 10 400 ppm, respectively, suggesting that the first nauplii stages may be more sensitive to elevated pCO_2 than the eggs (Kurihara et al., 2004a). If this is the case, results from egg hatching experiments could underestimate the sensitivity to CO_2-acidified seawater, and nauplii survival would perhaps provide a more realistic picture of sensitivity among copepods. The results on survival observed in the present study reflect the sensitivity of the most sensitive developmental stage(s), but the experimental design does not allow the relative contribution of the different life stages to the overall mortality to be identified. More knowledge on the relative sensitivity of eggs and early nauplii stages is required since this information is of vital importance when trying to assess the sensitivity to CO_2-induced acidification in marine species.

Although the present study indicates that survival in *C. finmarchicus* may be relatively robust to pCO_2 levels ≤ 2000 ppm, it should be noted that the use of fertilized eggs in both the present and other studies (e.g., Mayor et al., 2012; Weydmann et al., 2012) could potentially mask any negative effect of CO_2-induced acidification on fertilization processes. Indeed, near-future CO_2 levels have been found to affect fertilization processes in other invertebrate species, including the sea urchin *Heliocidaris erythrogramma* (Valenciennes, 1846) (Havenhand et al., 2008), the oyster *Crassostrea gigas* (Thunberg, 1793) (Barros et al., 2013) and the Antarctic sea star *Odontaster validus* (Koehler, 1906) (Gonzalez-Bernat et al., 2013). Also, the two multigenerational studies on copepods available so far (Kurihara and Isimatsu 2008; Fitzer et al., 2012), which have included in situ fertilization, showed reduced survival at pCO_2 levels ≤ 2300 ppm. In the study by Kurihara and Ishimatsu (2008), an overall reduction in the hatching success was observed when the results from three consecutive generations exposed at 2300 ppm CO_2 were compared to the control, but no significant effect was observed within the separate generations. Similar studies, spanning multiple generations and incorporating in situ fertilization, should also be conducted on *C. finmarchicus* before finally concluding on the sensitivity to pCO_2 levels ≤ 2000 ppm.

The relative contribution of copepodite stages IV, V and VI to the total population in the different treatments were significantly affected in a manner that suggests a retardation

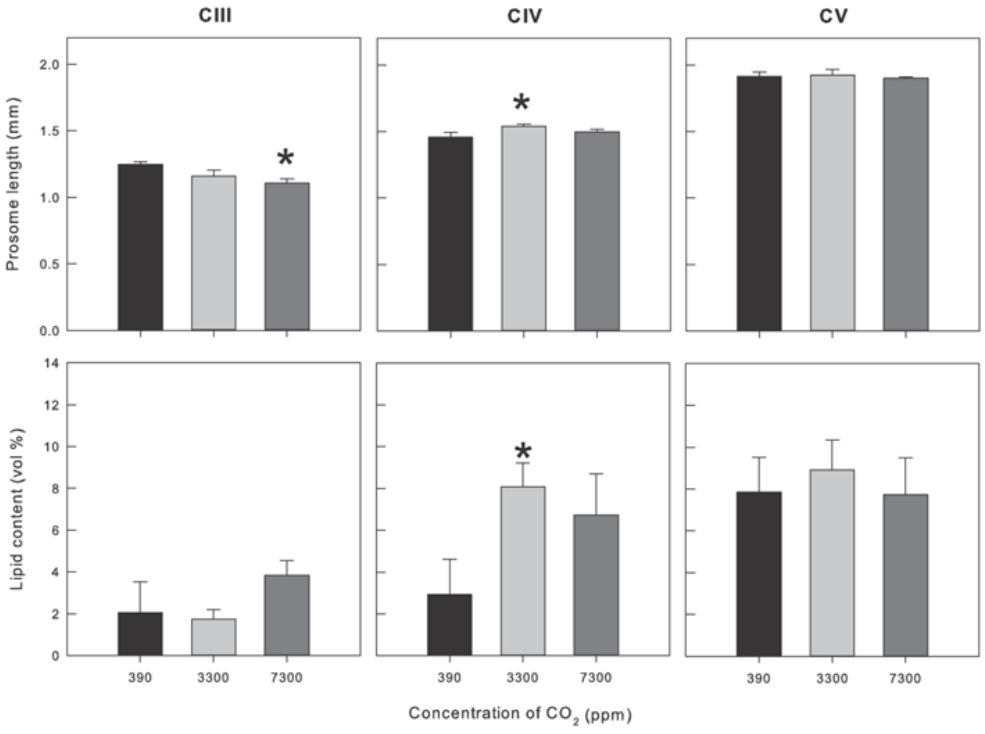

Fig. 4. Stage-specific prosome and lipid content (volume %) of the animals following 28-day exposure to different levels of CO_2-acidified seawater. The bars indicate mean ± 1 std ($n = 3$), and significant differences ($p < 0.05$) between control and exposed groups are indicated by asterisks (*). The total number of individuals measured in the experiment was CIII = 43, CIV = 254, CV = 610.

of the development rate with increasing pCO_2 (Fig. 3). To our knowledge this is the first time effects of elevated pCO_2 on ontogenetic development has been reported in *Calanus* spp. The relationship was relatively weak in the sense that no significant differences could be identified in the post hoc-tests, following the ANOVA. By comparison, stage distribution was not significantly affected by long-term exposure to 2300 ppm CO_2 in a multiple generation study on *A. tsuensis* (Kurihara and Ishimatsu 2008). Retardation in the development may have consequences for the survival since a delayed development can lead to animals staying in more vulnerable stages for longer periods (Lopez, 1996). The reduction in development rate observed for *C. finmarchicus* in the present study could be related to extra energetic costs associated with the induction of compensatory mechanisms in an effort to maintain a normal internal environment. This hypothesis is supported by studies that have shown that extra energy is used for compensatory responses against CO_2-induced stress, leaving less energy to support key biological processes such as growth and development (Wood et al., 2008; Beniash et al., 2010; Stumpp et al., 2011). The fact that only a moderate effect of CO_2 exposure on development rate was observed in the present study may be due to the use of ad libitum feeding conditions. This may potentially have reduced any negative effects related to a reduction of the energy budgets of the animals. Indeed, negative effects of CO_2 exposure on calcification were recently found to be intensified by low algae concentration in the blue mussel *Mytilus edulis* (Linnaeus 1758), and were linked to an overall reduction of the energy budgets in the animals (Melzner et al., 2011).

In addition to development, exposure to CO_2 in the present study was also found to have a profound effect on stage specific morphometric characters. Exposure to 7300 and 3300 ppm CO_2 had opposite effects on stage specific body length (prosome length) and lipid content (volume %). While exposure to 3300 ppm caused a significant increase in both length and lipid content in CIV copepods, a reduced body length was apparent among stage III copepodites at 7300 ppm CO_2 (Fig. 4). The increase in prosome length and lipid content among stage IV copepodites does not necessarily imply a positive effect of 3300 ppm CO_2 on performance of the animals, but is more likely an indirect consequence of a CO_2-induced protraction of the duration of this copepodite stage. Fitzer et al. (2012) observed a marked reduction of the body length ($\sim 25\,\%$) in *T. battagliai*, developing under pH 7.67 (≈ 600 ppm CO_2). Recently, CO_2 exposure was also found to induce developmental delay in the sea urchin *Strongylocentrotus purpuratus* (Stimpson, 1857), and was linked to a reduction in the scope for growth caused by elevated metabolic rate (Stumpp et al., 2011). The authors also observed negative effects on morphological characters,

but attributed these differences to an indirect effect of the delayed development (Stumpp et al., 2011).

Zooplankton like *C. finmarchicus* are highly dependent on a fine-tuned match between their own and phytoplankton blooming events. Thus, even a moderate alteration in full life-cycle developmental time, as observed in the present study, could induce a mismatch between the timing of the phytoplankton bloom and the reproduction cycle, which could ultimately have a large negative impact on the recruitment. This problem could be potentiated by associated increase in seawater temperatures. Svensson et al. (2005) demonstrated that large year to year fluctuations in spring temperatures could lead to the mismatching of larval release with phytoplankton blooming, and thus reduce the recruitment. Combination of ocean acidification and other types of stress (e.g., rising temperature, environmental contaminants) could result in more severe effects at the population and ecosystem level than indicated from the present experiment. Although limited, studies combining ocean acidification scenarios with other stressors are starting to appear. No interaction between exposure to 1000 ppm CO_2 and different temperatures (8, 10 and 12 °C) were observed on egg survival in *C. helgolandicus* (Mayor et al., 2012). In a study on the harpacticoid copepod *Amphiascoides atopus* (Lotufo and Fleeger, 1995) antagonistic effects of ocean acidification on the toxicity of Cu^{2+}-ions were observed, possibly due to the competition between H^{+}-ions and free Cu^{2+}-ions for binding sites at lower pH-levels (Pascal et al., 2010).

The experimental system developed for the present study was capable of maintaining stable exposure conditions during the 28-day-long experiment. However, the moderate volume/surface ratio of the exposure bottles, combined with settling algae, made it necessary to include weekly cleaning procedures for all the experimental units. The fact that no males developed in any of the groups was probably related to the modest volume (2 L) of the bottles used for the exposure of the animals. Limited container size and/or density have previously been reported to negatively affect the proportion of developing males in *C. finmarchicus* (Campbell et al., 2001). Future experiments incorporating multiple generations in a similar system should therefore use larger experimental units to secure development of males, and successful fertilization.

5 Conclusions

By reporting on the effects of CO_2 induced acidification over almost one complete life cycle of *C. finmarchicus*, the results from the present study represent an important complement to the findings reported in previous short-term/acute studies on *Calanus* species, and thereby contribute to improve the understanding of how this genus could be affected by more long-term exposure to elevated CO_2 conditions. Exposure to 7300 and 9700 ppm CO_2, levels that could be reached in case of a leakage from a from sub-seabed storage site, had a strong negative effect in terms of reduced survival, growth and retardation of the ontogenetic development, and clearly shows that long-term exposure to such conditions will have adverse effects on *C. finmarchicus*. Short-term studies have so far shown that exposure to $\sim$1000 ppm CO_2 may not affect the hatching success of *Calanus* eggs, but have not examined if this is also the case for the worst case CO_2 scenario for year 2300 (i.e., $\sim$2000 ppm). Some caution should of course be taken with regards to possible complications on fertilization processes and/or potential carry-over effects, from one generation to the next. However, the absence of any apparent reduction in the overall survival during the present medium-term exposure to 3300 ppm CO_2, indicates that survival of *Calanus* eggs and nauplii may be robust against the direct effects of the worst-case CO_2 scenario predicted for year 2300. Since effects on processes like growth and development were observed in the treatment that received seawater that were acidified by 3300 ppm CO_2, we cannot exclude the possibility that these processes also may be affected by a pCO_2 level $\leq$2000 ppm, especially if the acidification were to be combined with other forms of stress such as rising seawater temperatures or environmental contaminants. If development rate and growth is affected this could have severe impact on the recruitment due to the critical importance of timing of production cycle with alga bloom events. This question would best be addressed through multi-generational studies where several stress factors are combined.

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