

Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled pteropod *Limacina helicina*: mortality, shell degradation, and shell growth

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Received: 27 October 2010 – Published in Biogeosciences Discuss.: 5 November 2010

Revised: 11 April 2011 – Accepted: 12 April 2011 – Published: 15 April 2011

Abstract. Due to their aragonitic shell, thecosome pteropods may be particularly vulnerable to ocean acidification driven by anthropogenic CO₂ emissions. This applies specifically to species inhabiting Arctic surface waters that are projected to become temporarily and locally undersaturated with respect to aragonite as early as 2016. This study investigated the effects of rising partial pressure of CO₂ ($p\text{CO}_2$) and elevated temperature on pre-winter juveniles of the polar pteropod *Limacina helicina*. After a 29 day experiment in September/October 2009 at three different temperatures and under $p\text{CO}_2$ scenarios projected for this century, mortality, shell degradation, shell diameter and shell increment were investigated. Temperature and $p\text{CO}_2$ had a significant effect on mortality, but temperature was the overriding factor. Shell diameter, shell increment and shell degradation were significantly impacted by $p\text{CO}_2$ but not by temperature. Mortality was 46% higher at 8 °C than at in situ temperature (3 °C), and 14% higher at 1100 μatm than at 230 μatm . Shell diameter and increment were reduced by 10 and 12% at 1100 μatm and 230 μatm , respectively, and shell degradation was 41% higher at elevated compared to ambient $p\text{CO}_2$. We conclude that pre-winter juveniles will be negatively affected by both rising temperature and $p\text{CO}_2$ which may result in a possible decline in abundance of the overwintering population, the basis for next year's reproduction.

thereby also diminishing the saturation state of seawater with respect to calcite and aragonite. This effect is strongest in high-latitude surface waters, which are also experiencing the steepest increase in surface ocean temperature (Orr et al., 2005; Steinacher et al., 2009). Due to increasing CO₂ emissions since the industrial revolution, global mean surface temperatures have risen by 0.76 °C and global mean seawater pH has decreased by 0.1 unit (IPCC, 2007).

Calcite and aragonite are two common types of calcium carbonate secreted by marine organisms. Surface waters of the Arctic are specifically affected by carbonate undersaturation due to the high solubility of CO₂ in cold waters, for example in the Canada Basin, undersaturation with respect to aragonite was already detected in 2008 (Steinacher et al., 2009; Yamamoto-Kawai et al., 2009).

Changes in the carbonate chemistry can have severe consequences for marine organisms, particularly to those that build skeletons, shells, and tests of biogenic calcium carbonate. For instance, reduced calcification rates in corals, coralline macroalgae, coccolithophorids, bivalves, and echinoderms have been reported during the last years as a consequence of rising CO₂ partial pressures (e.g. Gattuso et al., 1999; Riebesell et al., 2000; Gazeau et al., 2007; Fabry et al., 2008). On contrast, a recent study by Gutowska et al. (2010) revealed increased calcification in the cuttlebone of the cephalopod *Sepia officinalis* during exposure to elevated CO₂ partial pressures. Hence, organisms' response of calcification to carbonate system variations is diverse.

A widely held view, as articulated by Wilson (1973), has been that “*living things during early developmental stages are more sensitive than at any other time in their life cycle to adverse influences in the environment*”. Also with respect to ocean acidification, several studies corroborate this view in that early developmental and reproductive stages of calcifiers are the most vulnerable stages within a life cycle (e.g. Kurihara et al., 2007; Kurihara, 2008; Clark et al., 2009;

1 Introduction

Anthropogenic CO₂ emissions affect the seawater carbonate chemistry and cause a decrease of seawater pH (termed ocean acidification) and carbonate ions in the worlds' oceans,



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Comeau et al., 2010a). These authors reported, for example, on retarded larval development, reduced shell mineralization/calcification, increased mortality and degradation of larval skeleton in sea urchin and pteropod larvae, respectively. Acting on early developmental and reproductive stages, ocean acidification can have a direct impact on the size of the population, reduce fitness and increase mortality of the offspring (Kurihara, 2008). Thus it can lead to changes in species distribution and abundances that could propagate through multiple trophic levels of the marine food web (Guinotte and Fabry, 2008).

Thecosome pteropods are widely distributed small-sized holoplanktonic marine mollusks. While their species diversity is high in tropical regions, only two epipelagic species, both of the genus *Limacina*, occur in temperate areas of the North Atlantic and in Arctic regions (van der Spoel, 1967). In northern and polar regions, pteropods can dominate zooplankton communities at times and are key species in epipelagic food webs both as consumers of and prey for various marine organisms, including commercially important fish, seabirds and baleen whales (Gilmer and Harbison, 1991; Falk-Petersen et al., 2001; Karnovsky et al., 2008).

Shelled pteropods of the genus *Limacina* (*L. helicina*, *L. retroversa*) also contribute significantly to vertical fluxes of both organic matter and biogenic calcium carbonate (Berner and Honjo, 1981; Bathmann et al., 1991; Hunt et al., 2008). Furthermore, thecosomes have a very thin and fragile shell made of aragonite, a particularly soluble form of calcium carbonate, and are the main planktonic producers of aragonite in the world's oceans (Lalli and Gilmer, 1989; Seibel et al., 2007). Due to the chemical structure of their shell, pteropods are expected to be among the first major group of calcifying organisms to be adversely affected by undersaturation in CaCO_3 (Orr et al., 2005; Seibel et al., 2007). Hence, due to its polar distribution, this will specifically apply to *Limacina helicina*.

In Svalbard waters *Limacina helicina* has a one-year life cycle. It develops to adults in early summer, and after reproduction in July/August their veligers grow to juveniles that overwinter until the next spring before development is completed (Gannefors et al., 2005; Lischka unpublished data). To cope with the high seasonality of food supply, many polar organisms reduce their metabolism to save energy and/or live on their energy reserves during winter (e.g. Hirche, 1996; Hagen and Auel, 2001; Lee et al., 2006). As for *L. helicina* it is not clear whether or not it lowers its metabolism during overwintering and, if it does, to what extent. Juveniles accumulate lipids and probably utilize them during the dark period (Gannefors et al., 2005). Hence with respect to ocean acidification, *L. helicina* will have to withstand the most threatened period in a presumably vulnerable life stage and time.

Recently, the response to elevated partial pressure of CO_2 ($p\text{CO}_2$) of adult *L. helicina* was investigated by Comeau et al. (2009, 2010b). In the later study, calcification was re-

duced as a function of $p\text{CO}_2$ at control and elevated temperature, whereas respiration was unaffected at control temperature but increased significantly as a function of $p\text{CO}_2$ at higher temperature. Hence, temperature can be a contributing factor to a species' response to changing $p\text{CO}_2$. In another investigation, Comeau and co-workers studied larvae of the mediterranean species *Cavolinia inflexa* and reported on malformations, reduced shell growth and even shell-less, though still alive, larvae grown under low pH, respectively (Comeau et al., 2010a).

Furthermore, studies of pteropod shells collected in deep-sea sediment and traps provide evidence that shell integrity is lost with increasing degree of dissolution (Almogi-Labin et al., 1986; Acker and Byrne, 1989; Haddad and Droxler, 1996; Gerhardt et al., 2000; Manno et al., 2007). However, it is not possible from these findings to conclude on the effect of undersaturated waters on the shell of living pteropods. In fact, shell dissolution in live pteropods (*Clio pyramidata*) has so far only been described by Orr et al. (2005) by a coincidental observation and in larvae of *Cavolinia inflexa* by Comeau et al. (2010a).

The present paper reports on the first experimental study that focuses on the combined effects of ocean acidification and elevated temperature on juveniles of the pteropod *L. helicina* from the Arctic Kongsfjord (Svalbard) prior to the overwintering period (mid September to end of October 2009). To study the synergistic effects of elevated $p\text{CO}_2$ and temperature, incubation experiments were carried out at three different temperatures and four $p\text{CO}_2$ partial pressures to qualitatively assess the impact on species mortality, shell degradation, and shell growth. Levels of $p\text{CO}_2$ chosen cover a range from pre-industrial values to levels projected to occur within this century (according to the A2 scenario in the report of the Intergovernmental Panel on Climate Change, IPCC, 2007). The combination of three temperatures and four $p\text{CO}_2$ levels permitted conclusions regarding the separate, as well as combined, effects of temperature and CO_2 .

2 Material and methods

2.1 Field sampling of pteropods

Juvenile *Limacina helicina* (Pteropoda, Thecosomata) were collected in Kongsfjord (northwest Spitsbergen) between 21 and 23 September 2009 using a plankton net (70 μm mesh size, 0.2 m^2 mouth opening, 1 l plastic cod end) onboard R/V *Teisten*. Integrated vertical hauls were taken in the deepest accessible part of the fjord from 300 or 200 m depth to the surface to allow collection of the deeper living overwintering individuals (Fig. 1). Onboard, freshly collected pteropods were stored in 20 l plastic containers in ambient seawater, and immediately transported to the Kings Bay Marine Laboratory where they were transferred into filtered seawater. About 200 individuals were caught per haul, less than 5% were damaged

due to collection. In the laboratory, specimens were kept for 1 to 2 days at in situ temperature (approximately 3 °C) in 1 µm filtered seawater until the start of experiments. Seawater supplied in the Marine Laboratory was pumped at 80 m depth from Kongsfjord and filtered through 20 µm filters. For experimental purpose, seawater was additionally filtered through Whatman GF/B filters (approximately retaining particles larger than 1 µm). Furthermore, to characterize abiotic parameters, depth, temperature, and salinity were measured with a STD/CTD model SD-204 (SAIV A/S, Bergen, Norway) prior to each sampling event.

2.2 Calcein staining

For qualitative assessment of shell growth under different temperature and $p\text{CO}_2$ conditions, pteropods were stained in a calcein bath (50 mg l⁻¹) for 1 h prior to incubation at experimental conditions. After staining, animals were rinsed with filtered seawater four times to remove excessive dye. Only actively moving individuals were taken for the experiments. Under UV-light, calcein has its fluorescence maximum at 515 nm (green) and marks the aperture margin at the time of staining (Comeau et al., 2009).

2.3 Experimental setup

In order to simulate past, present and future carbonate chemistries (atmospheric $p\text{CO}_2$ levels), GF/B filtered seawater was bubbled with air enriched with CO₂ using gas mixing pumps (Wösthoff, Germany) to CO₂ levels of 180, 380, 750 and 1150 ppm $p\text{CO}_2$ (target values) in storage containers at three temperatures (3, 5.5, and 8 °C). Temperatures were chosen according to a projected 1–2 °C temperature increase for the upper 100–200 m of the Arctic ocean (Steinacher et al., 2009). The natural temperature range of *Limacina helicina* goes from -0.4 °C to +4 °C, infrequently up to 7 °C (van der Spoel, 1967). Hence, experimental temperatures are within its natural range (3 and 5.5 °C) and according to a projected 2 °C increase slightly above the upper temperature limit (8 °C). Six replicates were set up for each of the twelve treatment combinations. Prior to pipetting 10 optically clean and actively moving juvenile *L. helicina* into each of the 440 ml jars (= one replicate), 5 ml filtered seawater were put in the jars to accommodate pteropods before filling the jars with the manipulated seawater. The whole procedure was done on crushed ice. After completion of all replicates of one temperature treatment (6 replicates × 4 $p\text{CO}_2$ levels = 24 jars), jars were filled with the manipulated seawater at treatment temperature, closed with an air-tight lid and stored in a water bath in a climate room at the experimental temperature. Incubations were performed in darkness in order to mimic the conditions at the depth of collection. Directly before filling experimental jars with manipulated seawater, water samples of each of the CO₂ manipulated seawater storage containers were taken for total alkalinity (A_T) and nutrients

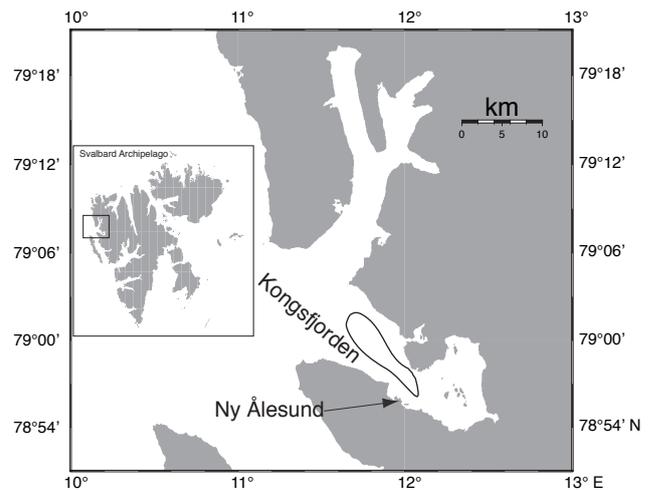


Fig. 1. Map of Kongsfjord highlighting the sampling area.

and pH on the total scale (pH_T) was measured (see below). Nutrient samples were immediately deep-frozen (-20 °C) whereas A_T samples were poisoned with HgCl₂ and stored at 4 °C (Dickson et al., 2007). After 29 days the experiment was terminated. Before harvesting juvenile *Limacina helicina*, pH_T was measured in each jar and water samples for A_T and nutrient analyses were taken. Subsequently, juveniles were collected from the jars and inspected for survival/mortality under a stereomicroscope and after rinsing in Milli-Q water, live (actively moving) individuals were preserved in 70% EtOH until inspection for shell degradation state and shell increment. Additionally, fjord water samples were taken for in situ A_T , dissolved inorganic carbon (C_T) and nutrient measurements.

2.4 Analyses of the carbonate chemistry

pH on the total scale was calculated from voltage readings according to SOP6a in Dickson et al. (2007). Instead of using TRIS buffers we used certified reference material (CRM) with a known pH calculated from known A_T and C_T (Prof. A. Dickson, Scripps Institution of Oceanography, La Jolla, California) as standard. Measurements were performed using a pH Mobile 826 pH meter (Metrohm, Switzerland), precision was within 0.2 mV units.

Total alkalinity (A_T) was determined using a potentiometric titration device (Titrand 808, Metrohm; Bradshaw et al., 1981). A_T was calculated from the Gran function as described by Dickson et al. (2003). The accuracy was determined by measuring CRM's as described for the pH measurements and precision was within 2 µmol kg⁻¹ (max. difference between two replicate measurements). For fjord water, dissolved inorganic carbon (C_T) was quantified with continuous-flow analysis (Bran & Luebbe QUAAIRO photometer) according to Stoll et al. (2001). A_T accuracy was between 1 to 15 µmol kg⁻¹ (differences between measured

Category	Scale					max. value
	clear (for category I + II) no (for category III –IV)	slight	medium	strong	very strong	
	0	1	2	3	4	
I: shell transparency (oblique transmitted light): milky						4
II: shell transparency (transmitted light): brownish						4
III: scarred structures						4
IV: corrosion						4
	0 perforations	1–2 perforations	3–4 perforations	≥5 perforations		3
	0	1	2	3		
V: number of perforations						max. possible sum across all categories
						19

Fig. 2. Categories of shell degradation (I to V) and their levels of conspicuities on a scale from 0 to 4 (for category I to IV) and 0 to 3 (for category V). Examples for each level and its associated score in each category are shown. See also Figure 3 for category IV and V.

and target A_T in the CRM's from A. Dickson et al. (2003) depending on prepared solutions and temperatures etc.) and C_T accuracy was between 40 to 50 $\mu\text{mol kg}^{-1}$. Remaining carbonate system parameters for the experiments and fjord were calculated from A_T and pH, and A_T and C_T , respectively with the free software CO2SYS (Pierrot and Wallace, 2006) using the constants from Mehrbach et al. (1973) refitted by Dickson and Millero (1987). Nutrients were analyzed according to Koroleff and Grasshof (1983).

CO₂ partial pressures at the start of the experiment differed slightly between replicates of the same CO₂ level, with consistently lower values at lower temperatures. This difference is attributed to temperature dependent equilibration rates, which increase with increasing temperature. Apparently three days of bubbling with CO₂/air gas mixtures were insufficient to achieve full equilibration of the medium.

2.5 Mortality

After termination of the experiment, all juvenile *Limacina helicina* were inspected for survival under a stereomicroscope. For this, individuals were categorized optically into five different stages of activity (I=highest activity, V=no activity, identified as dead)

- Stage I: animal expanded, actively moving, soft tissue appears clear and in good condition

- Stage II: animal retracted, actively moving inside shell, soft tissue appears clear and in good condition
- Stage III: animal retracted, no discernable active movements, but soft tissue appears clear and in good condition, individual most likely alive
- Stage IV: animal retracted, no discernable movements, soft tissue appears decomposed, individual most likely dead
- Stage V: animal retracted, soft tissue appears strongly decomposed, individual clearly dead.

For statistical evaluation stages I–III (alive) and IV–V (dead) were pooled.

2.6 Shell degradation

The soft tissue was removed with chlorine bleach (24 h at room temperature) prior to analyzing shell degradation and growth (see below).

Shell integrity of all surviving individuals of all replicates was examined for surface degradation under a stereomicroscope (Leica MZ 16F). Pteropod shells lose their integrity with increasing dissolution (e.g. Almogi-Labin et al., 1986; Gerhardt et al., 2000). Similar to Gerhardt et al. (2000), shell degradation was qualitatively described according to five categories, which are briefly described in the following (Fig. 2):

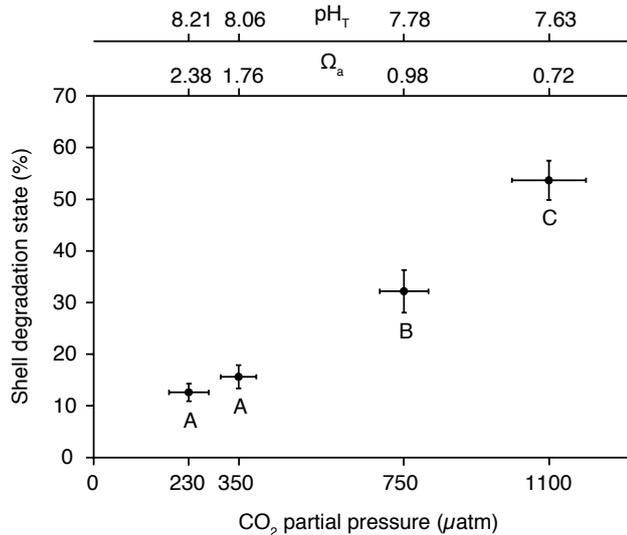


Fig. 3. Percent shell degradation of *Limacina helicina* after 29 days of incubation. Mean for the effect of $p\text{CO}_2$. Vertical bars denote 0.95 confidence intervals for shell degradation while horizontal bars denote 0.95 confidence intervals for $p\text{CO}_2$. Results from PERMANOVA pair-wise tests are depicted by letters with levels not connected by same letter being significantly different.

- Category I: Shell transparency (applying oblique transmitted light with mirror in an acute angle to the source of light). Shells were evaluated according to their transparency under oblique transmitted light. The scale extends from total transparency/clearness to different states of milky and cloudy shells, respectively.
- Category II: Shell transparency (applying transmitted light, with mirror in an obtuse angle to the source of light). Using transmitted light, shells appear brownish to various extents.
- Category III: Scarred structures. Category III describes the frequency of scarred structures of any kind.
- Category IV: Corrosion. Category IV describes the deepness of scarred structures in the shell that can be estimated by focusing the stereomicroscope, hence category IV describes the severity of corrosion.
- Category V: Perforation. In this category the number of holes in the shell is considered.

The condition of a single shell according to the different categories was described on a scale from 0 to 4 for categories I to IV and 0 to 3 for category V. The maximum possible sum of the five categories was 19 for a single shell. The maximum value was calculated for all inspected shells and graphically depicted as percentage of maximum possible surface degradation (see Fig. 3). For statistical evaluation, raw scores for each category were used (see below). In

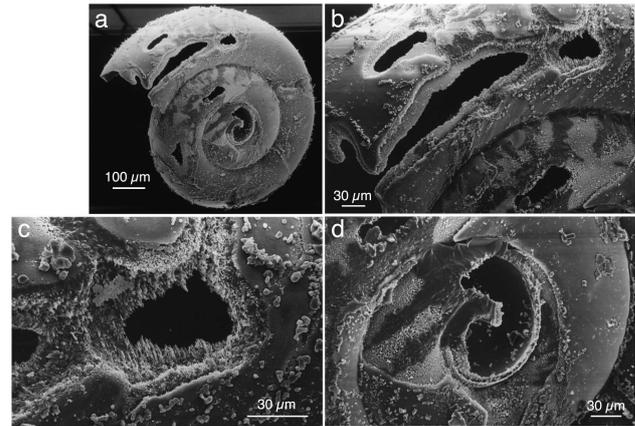


Fig. 4. Scanning electron micrographs (SEM) of representative *Limacina helicina* reared at 3 °C and 1100 µatm to illustrate shell degradation state specifically with respect to category IV and V. Magnification and scale bars are shown. The individual shown is the same as the one shown in Table 1 for category V with ≥ 5 perforations.

some cases, shells were mechanically damaged during processing. However, perforations due to corrosion can usually be distinguished from those resulting from mechanical damage. Therefore, only those perforations were counted in category V that could be identified as non-mechanical. Hence, shell surface degradation with respect to category V is underestimated.

Additionally, selected shells were examined by Scanning Electron Microscope (CamScan-CS-44, Institute of Geosciences, Kiel University) to illustrate shell degradation state in categories IV and V (Fig. 4).

2.7 Analysis of shell growth

Shell growth estimated from calcein staining was measured on a Leica MZ 16 F stereomicroscope equipped with a UV-epifluorescence lamp and the Leica Application Suite 3.5.0. To standardize for individual size differences, not only the shell increment was measured but also the shell diameter and the ratio of shell increment to shell diameter was used for comparison between temperature treatments and CO_2 levels. One individual of each of the 6 replicates was analyzed (Fig. 5).

2.8 Statistics

Multifactorial analysis was performed to test for significant effects of the factors temperature and $p\text{CO}_2$ on mortality, shell diameter and the ratio of shell increment to shell diameter using a general linear model Type IV (GLM) for equal sample size in case of mortality and a GLM Type III model for unequal sample size in case of shell diameter and shell increment/diameter. Percentage data of mortality

Table 1. Mean carbonate system parameters calculated from samples taken from the prepared manipulated seawater at start and from all experimental jars at the end of the experimental period. The treatment column refers to target temperature and $p\text{CO}_2$, of which the $p\text{CO}_2$ levels refer to glacial partial pressure CO_2 (180 μatm , 18 kyr BC), present day $p\text{CO}_2$ (380 μatm , year 1990), high $p\text{CO}_2$ I (750 μatm , year 2080), and high $p\text{CO}_2$ II (1150 μatm , > year 2100). The concentration of total CO_2 (C_T), partial pressure of CO_2 ($p\text{CO}_2$), and the saturation state of aragonite (Ω_a) were derived from pH_T , total alkalinity (A_T), salinity (S) and temperature (T). Si is silicate.

Treatment		S	T (°C)		A_T ($\mu\text{mol kg}^{-1}$)		pH_T		C_T ($\mu\text{mol kg}^{-1}$)		$p\text{CO}_2$ (μatm)		Ω_a		Si ($\mu\text{mol g}^{-1}$)	
T (°C)	$p\text{CO}_2$ (μatm)		start	end	start	end	start	end	start	end	start	end	start	end	start	end
3	180	34.7	3.5	3.6	2280	2288	8.27	8.28	2045	2047	216	211	2.45	2.51	2.63	6.49
	380	34.7	3.3	3.5	2280	2287	8.12	8	2115	2164	323	435	1.81	1.45	2.63	7.27
	750	34.7	3.1	3.6	2280	2291	7.81	7.78	2223	2241	703	764	0.95	0.91	2.63	11.82
	1150	34.7	3	3.5	2280	2296	7.69	7.62	2260	2291	948	1110	0.72	0.65	2.63	11.25
5.5	180	34.7	5.4	5.4	2280	2288	8.24	8.09	2041	2118	231	347	2.5	1.87	2.63	3.47
	380	34.7	5.5	5.4	2280	2295	8.09	8.03	2111	2150	348	411	1.86	1.65	2.63	8.91
	750	34.7	5.6	5.4	2280	2293	7.8	7.74	2215	2246	732	851	1.02	0.9	2.63	4.38
	1150	34.7	5.6	5.4	2280	2299	7.66	7.63	2256	2283	1026	1104	0.76	0.71	2.63	12.28
8	180	34.7	8	7.6	2280	2290	8.26	8.12	2012	2092	223	322	2.8	2.15	2.63	7.3
	380	34.7	7.9	7.6	2280	2293	8.11	8	2086	2146	333	445	2.11	1.68	2.63	15.22
	750	34.7	7.9	7.5	2280	2302	7.81	7.73	2199	2247	711	876	1.15	0.96	2.63	16.18
	1150	34.7	8.1	7.5	2280	2305	7.62	7.58	2256	2297	1138	1279	0.77	0.69	2.63	16.46
Fjord (200 m)		35	3.9	4.3	2296	2287	7.98	8.03	2174	2145	448	398	1.45	1.61	3.27	3.13

were arcsin transformed, and values for the ratio shell increment/diameter were Box-Cox transformed ($\lambda = 2.467508$). Data were tested for normality (Shapiro-Wilks test). In case of significant effects of temperature and/or $p\text{CO}_2$, homogeneity of variances was tested with Levene's test. To determine significant differences between temperature and $p\text{CO}_2$ treatments, respectively, a post-hoc Tukey's honest significant difference test (HSD or HSD for unequal n, respectively) with α -level 0.05 was used.

Raw scores for each shell degradation category as well as the compound score for shell degradation were analyzed using non-metric multidimensional scaling (NMDS). PERMANOVA was applied using all five shell degradation categories as response variables to test for significant effects of the factors temperature and $p\text{CO}_2$ and possible interactions between factors. For NMDS and PERMANOVA Euclidean Distance was used to create a resemblance matrix. PERMANOVA was conducted according to a two-way crossed design (factors being temperature and $p\text{CO}_2$) with type III partitioning of the sums of squares. Permutation of residuals under a reduced model was applied based on 9999 permutations to obtain the P-value, α -level was 0.05.

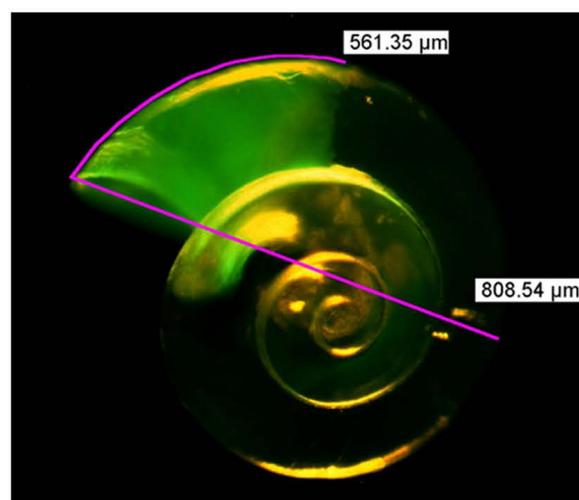


Fig. 5. Calcein stained shell of *Limacina helicina* showing shell increment during the experiment and measurement of increment length and diameter.

Statistics were carried out using Statistica version 8 (Statsoft) and PRIMER 6 (including PERMANOVA+, PRIMER-e Ltd), respectively.

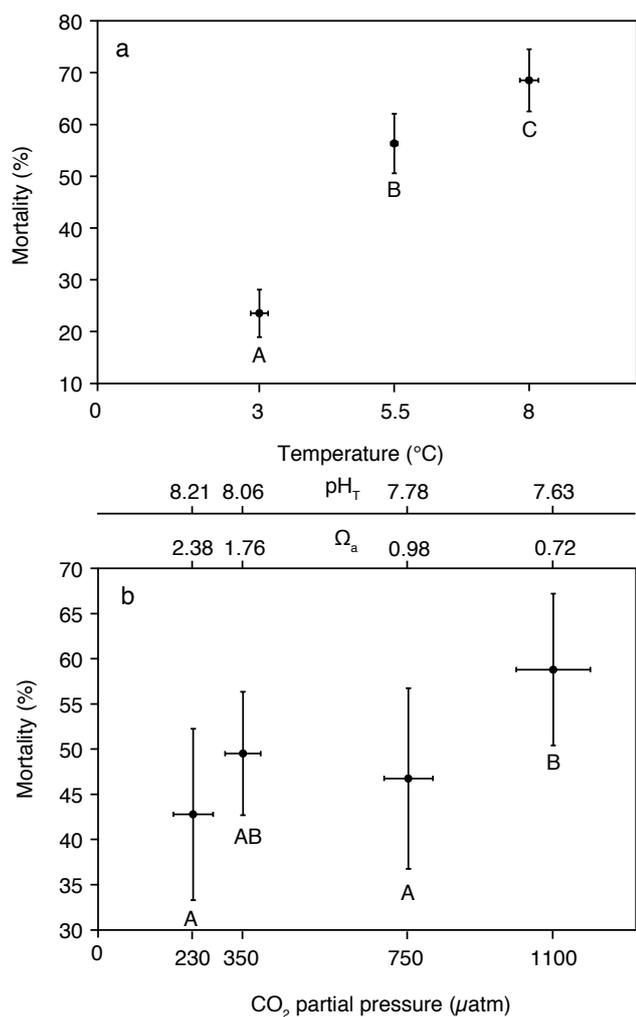


Fig. 6. Percent mortality after 29 days of incubation of *Limacina helicina*. Means for the effects of temperature (a) and $p\text{CO}_2$ (b). Vertical bars denote 0.95 confidence intervals for mortality, horizontal bars denote 0.95 confidence intervals for temperature and $p\text{CO}_2$, respectively. Results from the Tukey HSD post-hoc test are depicted by letters with levels not connected by same letter being significantly different.

3 Results

3.1 Carbonate system

Carbonate chemistry conditions of the fjord ($p\text{CO}_2$ of $448 \pm 15 \mu\text{atm}$) were roughly simulated in the $380 \mu\text{atm}$ treatment as revealed by A_T , C_T and temperature (Table 1). Compared to in situ conditions the $180 \mu\text{atm}$ treatment had an increased pH level and Ω_a whereas the CO_2 enriched treatments had diminished pH levels and subsaturated Ω_a conditions. At the end of the experiment, CO_2 partial pressures were higher compared to initial partial pressures. Accordingly pH and Ω_a levels were lower and $p\text{CO}_2$ was higher. Interestingly, also A_T values were slightly higher.

Table 2. Univariate tests of significance indicating the effect of temperature and $p\text{CO}_2$ on mortality. Significant results are represented in bold.

Effect	SS	df	MS	F	<i>p</i>
Intercept	84.734	1	84.734	1362.562	0.000
Temp	7.469	2	3.735	60.055	0.000
$p\text{CO}_2$	1.088	3	0.363	5.831	0.0009
Temp \times $p\text{CO}_2$	0.442	6	0.074	1.184	0.32
Error	8.209	132	0.062		

In the following, $p\text{CO}_2$ values (μatm) shown in figures are the means of values measured at experimental start.

3.2 Mortality

Temperature and $p\text{CO}_2$ both had a significant effect on mortality of *Limacina helicina* ($F = 60.055$, $p < 0.0001$ and $F = 5.831$, $p = 0.0009$, respectively) (Table 2). However, the temperature effect was stronger than the $p\text{CO}_2$ effect (Fig. 6a and b). There were no interactions between both factors ($F = 1.184$, $p = 0.32$). Tukey HSD post-hoc test showed mortality to be significantly higher at 5.5°C and 8°C ($p < 0.0001$ and $p < 0.0001$, respectively) than at in situ temperature (3°C) (Fig. 6a). Moreover, mortality was significantly higher at 8°C than at 5.5°C ($p = 0.03$). $p\text{CO}_2$ caused a significantly higher mortality at $1100 \mu\text{atm}$ as compared to 230 and $750 \mu\text{atm}$ ($p < 0.0001$ and $p = 0.03$, respectively) (Fig. 6b), however, the mortality at $1100 \mu\text{atm}$ did not significantly differ from $350 \mu\text{atm}$ ($p = 0.10$).

3.3 Shell degradation

Ordination of the raw scores for each degradation category and the compound score by non-metric multidimensional scaling (NMDS) revealed differences in the effect size and direction of the factors temperature and $p\text{CO}_2$. Furthermore, sizes and/or directions of factor effects were different within different levels of the other factor, thus implying interactions between both factors. Distances between data clouds showed that the effect size of the factor $p\text{CO}_2$ was generally clearer and larger than the effect size of the factor temperature (Fig. 7). However, a temperature effect was specifically distinct for category V (perforation), where temperature has a clear and large effect within level four ($1100 \mu\text{atm}$) of factor $p\text{CO}_2$ (Fig. 7e). Moreover, temperature effects within all levels of factor $p\text{CO}_2$ were larger in categories III, IV and V (scarred structures, corrosion, perforation) as compared to categories I, II (milky, brownish) and the compound value for all categories.

Statistical significance of factor effects was determined applying PERMANOVA. PERMANOVA revealed a significant

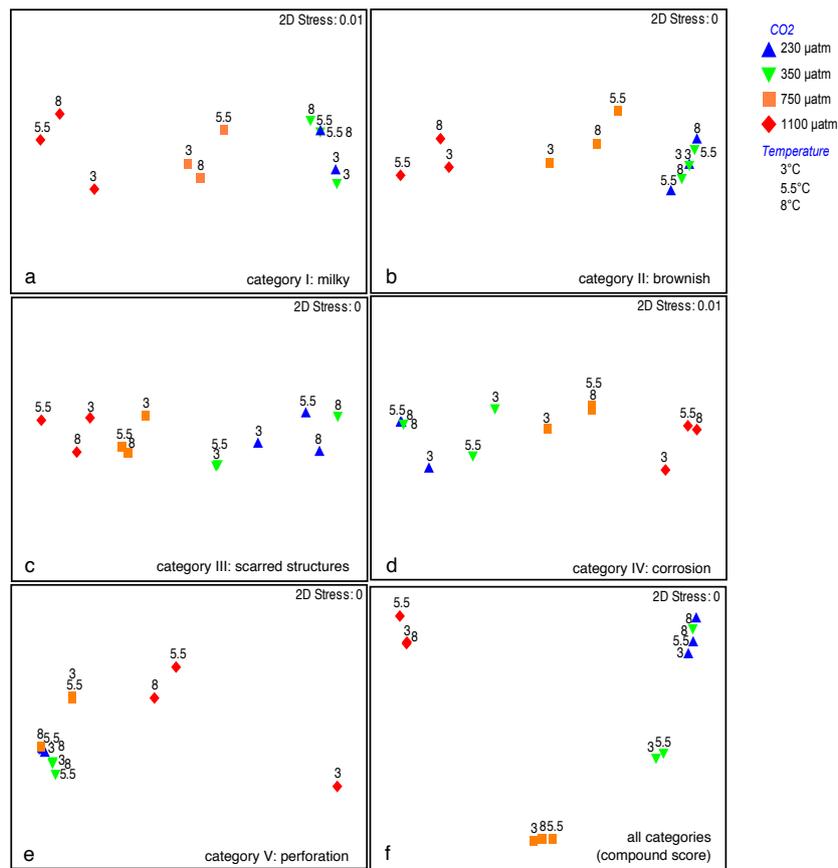


Fig. 7. MDS ordination of shell degradation categories with labels for the two-way crossed design: analyses of raw scores for each category (a to e) and of the compound value of all categories (f). 2-D stress is the goodness of the representation, i.e. a stress of < 0.05 gives an excellent representation with no prospect of misinterpretation.

Table 3. PERMANOVA using all five shell degradation categories as response variables indicating the effect of temperature and $p\text{CO}_2$ on shell degradation. Significant results are represented in bold.

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms
Temperature	2	2.306	1.153	1.150	0.320	9938
$p\text{CO}_2$	3	146.03	48.678	48.565	0.0001	9947
Temp \times $p\text{CO}_2$	6	9.560	1.593	1.590	0.095	9936
Res	60	60.139	1.002			
Total	71	220.36				

effect of $p\text{CO}_2$ on shell degradation (Pseudo-F=48.565, P (perm)=0.0001; Table 3). The effect of temperature was not statistically significant (Pseudo-F=1.1501, P (perm)=0.32), and no significant interaction between both factors was found (Pseudo-F=1.59, P (perm)=0.095). Pair-wise tests for the factor $p\text{CO}_2$ showed significant differences in shell degradation between all level combinations of $p\text{CO}_2$ (P (perm)=0.0001) except for the pair 230/350 μatm (P (perm)=0.361) (Table 4, Fig. 3).

3.4 Shell growth

Univariate test of significance revealed a significant effect of $p\text{CO}_2$ on shell diameter ($F=4.955$, $df=3$, $p=0.003$) but not of temperature ($F=1.767$, $df=2$, $p=0.17$; Table not shown). Also, there was no significant interaction between the two factors ($F=0.548$, $df=6$, $p=0.76$). Tukey HSD post-hoc test revealed shell diameter of the high $p\text{CO}_2$ treatment (1100 μatm) to be significantly lower than at 230 μatm and 750 μatm , however, it was not significantly different from the 350 μatm treatment (Fig. 8).

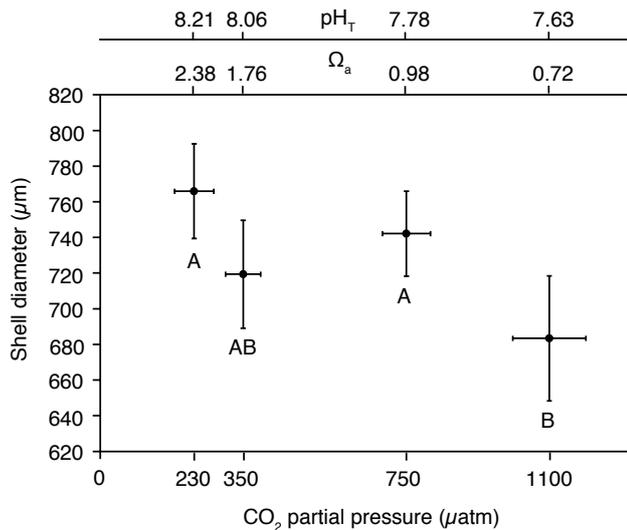


Fig. 8. Mean diameter of *Limacina helicina* after 29 days of incubation for the effect of $p\text{CO}_2$. Vertical bars denote 0.95 confidence intervals for diameter, horizontal bars denote 0.95 confidence intervals for $p\text{CO}_2$. Results from the Tukey HSD post-hoc test are depicted by letters with levels not connected by same letter being significantly different.

The ratio of shell increment versus diameter was significantly affected by $p\text{CO}_2$ ($F=4.344$, $p=0.008$), but there was no significant effect of temperature ($F=1.353$, $p=0.27$) and no significant interaction between both factors ($F=2.044$, $p=0.07$; Table 5). Tukey HSD post-hoc test revealed the shell increment/diameter ratio at the high $p\text{CO}_2$ treatment (1100 μatm) to be significantly lower than at 230 and 350 μatm , but not significantly different from the 750 μatm treatment (Fig. 9).

4 Discussion

The present experiments were carried out with unfed juvenile *Limacina helicina*; this species feeds on particles entangled in its mucus web, mainly on phytoplankton and small protozoa (Lalli and Gilmer, 1989). Phytoplankton biomass is low in Kongsfjorden already in late summer (Hop et al., 2002), and phytoplankton abundance was extremely low during the whole period of investigation (own observations from a 20 μm net haul in the upper 20 m). Moreover, *L. helicina*'s downward migration to overwintering depth (>100 m) begins in the first half of September in Kongsfjorden (Lischka, unpublished data) and the larger part of the population already dwelled between 100 to 200 m in mid September (as determined by stratified vertical net hauls). Thus, feeding on phytoplankton can most likely be excluded. Rather lipid consumption during overwintering is likely (Gannefors et al., 2005) presumably at lower level metabolism (e.g. Lee et al., 2006). Although it is possible, that *L. helicina* still fed on

Table 4. PERMANOVA, Pair-wise tests for factor $p\text{CO}_2$. Significant results are represented in bold.

Groups	t	P (perm)	Unique perms
230, 350	1.027	0.3609	9946
230, 750	4.5986	0.0001	9944
230, 1100	8.7309	0.0001	9927
350, 750	6.1461	0.0001	9941
350, 1100	11.556	0.0001	9926
750, 1100	5.3049	0.0001	9941

Table 5. Univariate tests of significance indicating the effect of temperature and $p\text{CO}_2$ on the ratio of shell increment to shell diameter. Significant results are represented in bold.

	SS	df	MS	F	p
Intercept	34.772	1	34.772	4249.554	0.000
Temp	0.022	2	0.011	1.353	0.27
$p\text{CO}_2$	0.107	3	0.036	4.344	0.008
Temp \times $p\text{CO}_2$	0.100	6	0.017	2.044	0.07
Error	0.507	62	0.008		

organic particles during this time of the year, which could affect the effect size of temperature and $p\text{CO}_2$ on the response of *L. helicina*, a change in effect direction is unlikely since individuals in all treatments were kept under the same conditions with respect to food.

4.1 Carbonate system

A_T at the end of the experiment showed a slightly increased trend with increasing temperature and $p\text{CO}_2$ that correlates with silicate concentrations. Most likely the A_T increase resulted from silicate and other components dissolved from the experimental jars.

Increased C_T concentrations and therefore decreased pH and Ω values as well as increased CO_2 partial pressures at the end are most likely explained by respiration of pteropods (and bacteria) in the closed experimental set up.

4.2 Effects of elevated temperature

Concerning mortality, temperature was the dominant factor as revealed by the higher F and lower p value compared to those associated with $p\text{CO}_2$ (Table 2). *Limacina helicina* at the beginning of the overwintering phase are very sensitive to changes of 3 to 5 $^\circ\text{C}$. *L. helicina* is a north Atlantic/polar species that is adapted to a relatively narrow and low temperature range between -0.4°C and $+4^\circ\text{C}$ and infrequently up to 7°C (van der Spoel, 1967; Bé and Gilmer, 1977). As

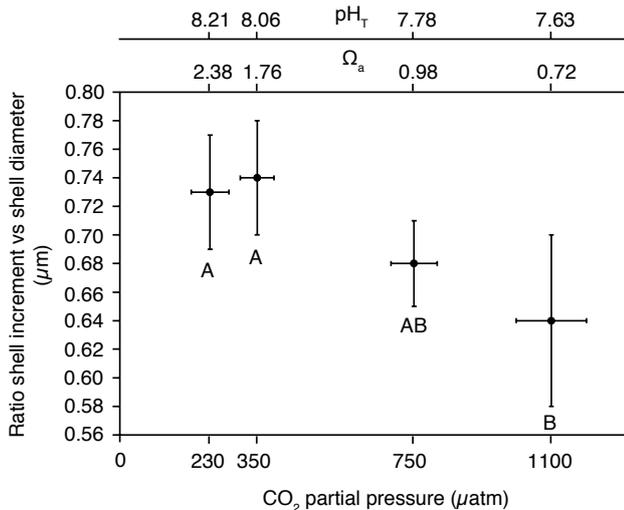


Fig. 9. Mean ratio of shell increment to diameter of *Limacina helicina* after 29 days of incubation for the effect of $p\text{CO}_2$. Vertical bars denote 0.95 confidence intervals for mean ratio while horizontal bars denote 0.95 confidence intervals for $p\text{CO}_2$. Results from the Tukey HSD post-hoc test are depicted by letters with levels not connected by same letter being significantly different.

mentioned earlier, peak occurrence of *L. helicina* during the present investigation was between 100 and 200 m depth. In September/October 2009, a relatively warm water lens of about 5 to 5.5 °C occurred in Kongsfjorden between 40 and 110 m depth. Below 110 m, temperature decreased quickly to values below 4 °C down to a minimum of about 2 °C at 300 m. Furthermore, at the time of this investigation, *L. helicina* was at the onset of the overwintering period presumably not feeding anymore, likely living on lipid reserves. Temperature adaptation is a complex and costly metabolic response and costs of existence (the sum of all processes that are necessary to maintain viability, excluding reproduction, growth, and activity) vary significantly with temperature (Clarke, 2003). Hence, temperature stress during this period of *L. helicina*'s life cycle can be a severe threat for its ability not only to survive the food-scarce winter period, but to survive with still enough energy reserves available to proceed development to adults and fuel reproduction in spring/summer.

4.3 Effects of elevated $p\text{CO}_2$

4.3.1 Shell increment and shell diameter

Similar to Reusch (1998), calcein was used here as a qualitative measure of shell growth. In juvenile *L. helicina*, staining was not only depicted as a distinct green line at the growing margin, but usually two main regions of coloration indicating calcification activity were observed. One region usually started with a distinct line on the outer whorl extending to

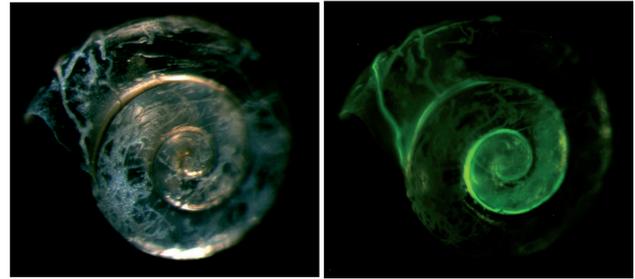


Fig. 10. Example for “repair” calcification: shell degradation of *Limacina helicina* (left, oblique transmitted light) and the respective regions of “repair” calcification indicated by enhanced fluorescence due to calcein incorporation (right).

the aperture (the actual shell increment – in terms of lengthening), the other was found around the protoconch. This is in contrast to Comeau et al. (2009) who reported a distinct green line marking the shell edge (apertural margin) at the time of staining. The findings presented here are proposed as indication of “repair” calcification, since regions of coloration usually showed up where shell integrity was affected (Fig. 10). Indeed, thecosomes are able to repair shell parts (van der Spoel, 1967).

The question arising from this is why the region defined as shell increment was completely green instead of a single line marking the shell edge at time of staining and whether or not this region is indeed incremental or rather a region of enhanced shell thickening (in terms of increase of shell thickness) at the inner surface (presumably the inner prismatic layer, Sato-Okoshi et al., 2010)? Concomitant shell diameter measurements on juveniles freshly caught in the field during the whole period of the experiment (data not shown) as well as the increasing shell diameters of the incubated individuals indicate growth of juvenile *L. helicina* during September/October 2009. To further address this question, shell cross sections were made and revealed a fluorescence band at the inner shell surface, implying inward shell thickening (Fig. 11a and b). However, close to the aperture, the whole shell cross section (sensu Sato-Okoshi et al., 2010 the outer prismatic, middle crossed-lamellar, and inner prismatic layer) was fluorescent, thus implying shell increment (Fig. 11c). This region is suggested to exhibit shell increment, where the proximal (“older”) part of new increment is still thickened inwardly (inner prismatic layer), and close to the apertural margin true shell increment proceeds. A similar process was described for the pteropod *Cuvierina columnella* (Cavoliniidae), which after attaining maximum shell length continues to thicken the entire shell inwardly (Lalli and Gilmer, 1989). Moreover, if our hypothesis is true for *L. helicina*, the fact that the entire region of increment was green points to storage of calcein during the entire incubation time.

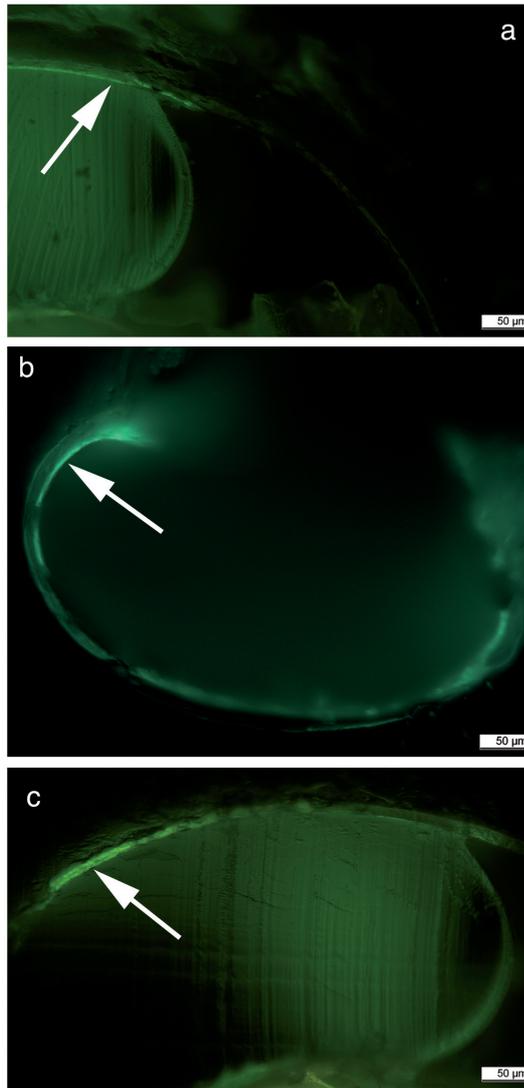


Fig. 11. Cross sections of *Limacina helicina* shells in the region of shell increment. (a) Dorso-lateral section (higher magnification of the right part of (c) showing a fluorescence band at the inner shell surface, arrow pointing at the thin inner fluorescence line). (b) Cross section of the aperture region distal from the area of increment, the arrow pointing at the line of constructive processes at the inner prismatic shell layer. (c) Dorso-lateral section showing calcein incorporation in all shell layers (prismatic, middle crossed-lamellar, inner prismatic) close to the aperture (arrow).

Shell growth of *Limacina helicina* kept at 1100 μatm was significantly reduced as compared to individuals kept at 350 and 230 μatm (Fig. 9). However, *L. helicina* was able to grow at $\Omega_a < 1$. Consequently, the present results principally agree with the conclusion by Comeau et al. (2009) deduced from adult *L. helicina* that seemed to be relatively well adapted to aragonite undersaturation. However, in addition to shell growth, shell degradation state of juvenile *L. helicina* revealed pronounced dissolution processes at the same

time and as mentioned earlier, “repair” calcification is suggested. Therefore, three crucial questions arise: (1) How well can juvenile *L. helicina* adapt to aragonite undersaturation?, (2) What are the costs for different life stages of *L. helicina* to facilitate calcification and repair dissolution damage under aragonite undersaturation?, and (3) Will it be possible for *L. helicina* to maintain the integrity of its shell and, at the same time, sustain development, reproduction and accumulation of energy reserves to overwinter? The intensity of shell degradation under the two highest $p\text{CO}_2$ levels leads to the conclusion that juvenile pre-winter *L. helicina* may be seriously threatened under aragonite undersaturation.

The present study shows that $p\text{CO}_2$ is the driving factor influencing shell growth and calcification of juvenile *L. helicina*, whereas for temperature no significant influence was detected. This is in agreement with recent findings for adult *L. helicina* that exhibited declined precipitation rates of CaCO_3 as a function of $p\text{CO}_2$ (Comeau et al., 2010b). However, inconclusive results with respect to a temperature effect in the present study might be due to i.e. experimental constraints (i.e. experiment/test could not detect an effect) and does not necessarily mean that temperature had no effect.

4.3.2 Shell degradation

Both elevated $p\text{CO}_2$ levels caused a significant effect on shell degradation, especially at 1100 μatm (Fig. 3). Hence, shell degradation state gives clear evidence that the shell of juvenile *L. helicina* is negatively affected by aragonite saturation levels below 1. To the authors knowledge dissolution in live pteropods has so far only been reported by Orr et al. (2005) and Comeau et al. (2010a) from a Subarctic Pacific and a Mediterranean species, respectively (*Clio pyramidata* and *Cavolinia inflexa*). Similar to the present results, shell surface alterations due to CaCO_3 undersaturation are reported by e.g. Green et al. (2009) on live juvenile benthic bivalves, and from *Limacina* spp. shells collected in sediment traps by Gerhardt et al. (2000) and Manno et al. (2007).

PERMANOVA neither revealed a significant effect for temperature nor significant interactions between the two factors but P-value for the interaction term was quite small and thus rather inconclusive (Table 3). Although not statistically significant, NMDS ordination (Fig. 7), however, suggests that temperature may have an influence on shell degradation as well as the occurrence of interactive effects between temperature and $p\text{CO}_2$ are suggested. The ordination of the 3 °C and 1100 μatm CO_2 treatment in Fig. 7e shows a clear and large temperature effect. Highest incidence of perforated shells was at ambient temperature and high $p\text{CO}_2$, whereas at higher temperatures shells were considerably corroded but less perforated. Hence, at higher temperature and perhaps due to a higher metabolism, *L. helicina* might be better able to counteract dissolution processes by ‘repair’ calcification and therefore avoid perforation at least for a limited time. Similarly, McDonald et al. (2009)

describe compensatory calcification in the intertidal barnacle *Amphibalanus amphitrite* that was exposed to acidified conditions and showed compensatory calcification of their basal shell plates but central shell wall plates were significantly weakened.

5 Conclusion

The findings shown here give insight to the response (with respect to mortality, shell degradation and shell increment) of juvenile *Limacina helicina* just before overwintering as a function of temperature and $p\text{CO}_2$. Increased temperature and $p\text{CO}_2$ act differently on *L. helicina*, both significantly increasing species mortality but only the latter significantly affecting shell integrity and shell growth. $p\text{CO}_2$ change projected for the near future may severely affect pre-winter juveniles, and rising temperatures could contribute to a decline in abundance of the overwintering population, which represents the basis for next year's reproduction. Hence, population dynamics are likely to be changed and consequences for the Arctic epipelagic ecosystem may arise.

Various questions arise from this study with respect to *L. helicina*'s fate in a high CO_2 ocean. This study suggests higher metabolic activity and thus higher metabolic costs to compensate for dissolution and as possible reason for higher mortality of *L. helicina* at higher temperature (Wood et al., 2008; Comeau et al., 2010b; Thomsen and Melzner, 2010). This raises the question as to energetic costs/trade offs and to what extent compensatory metabolic costs can be tolerated without seriously threatening winter survival and ultimately reproductive success? To address these questions, detailed physiological studies of the effects of rising CO_2 and temperature on different life stages of *Limacina helicina* are needed (e.g. respiration, lipid utilization).

Supplementary material related to this article is available online at:
<http://www.biogeosciences.net/8/919/2011/bg-8-919-2011-supplement.pdf>.

Acknowledgements. We thank the staffs of the AWIPEV Station of the Alfred-Wegener-Institute for Polar and Marine Research and Kings Bay AS in Ny-Ålesund for excellent logistical support during our field campaign. The Institute for Polar Ecology in Kiel is acknowledged for making logistics and optical facilities available to us. The scanning electron microscopy work was performed in the Institute of Geosciences in Kiel, particularly we are indebted to Ute Schuldt for her supervision in the SEM Lab. Thanks are also due to Armin Form for helpful discussions. Dieter Piepenburg (Institute for Polar Ecology) provided access to PERMANOVA + for PRIMER. We also thank Jon Havenhand and an anonymous referee for their valuable suggestions and critics on the manuscript. This work is a contribution to the German BMBF (Federal Ministry of Education and Research) funded project "Biological Impacts of Ocean Acidification" (BIOACID, Grant number 03F0608A) as

well as to the "European Project on Ocean Acidification" (EPOCA) which received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 211384. This work also received funding from the European Centre for Arctic Environmental Research (ARCFAC, Grant number 026129-2008-54).

Edited by: J.-P. Gattuso

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