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Impacts of temperature and acidification on larval calcium incorporation of the spider crab *Hyas araneus* from different latitudes (54° vs. 79°N)

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Abstract The combined effects of ocean warming and acidification were compared in larvae from two populations of the cold-eurythermal spider crab *Hyas araneus*, from one of its southernmost populations (around Helgoland, southern North Sea, 54°N, habitat temperature 3–18°C; collection: January 2008, hatch: January–February 2008) and from one of its northernmost populations (Svalbard, North Atlantic, 79°N, habitat temperature 0–6°C; collection: July 2008, hatch: February–April 2009). Larvae were exposed to temperatures of 3, 9 and 15°C combined with present-day normocapnic (380 ppm CO_2) and projected future CO_2 concentrations (710 and 3,000 ppm CO_2). Calcium content of whole larvae was measured in freshly hatched Zoea I and after 3, 7 and 14 days during the Megalopa stage. Significant differences between Helgoland and Svalbard Megalopae were observed at all investigated temperatures and CO_2 conditions. Under 380 ppm CO_2, the calcium content increased with rising temperature and age of the larvae. At 3 and 9°C, Helgoland Megalopae accumulated more calcium than Svalbard Megalopae. Elevated CO_2 levels, especially 3,000 ppm, caused a reduction in larval calcium contents at 3 and 9°C in both populations. This effect set in early, at 710 ppm CO_2 only in Svalbard Megalopae at 9°C. Furthermore, at 3 and 9°C Megalopae from Helgoland replenished their calcium content to normocapnic levels and more rapidly than Svalbard Megalopae. However, Svalbard Megalopae displayed higher calcium contents under 3,000 ppm CO_2 at 15°C. The findings of a lower capacity for calcium incorporation in crab larvae living at the cold end of their distribution range suggests that they might be more sensitive to ocean acidification than those in temperate regions.

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Introduction

The anthropogenic emission of carbon dioxide (CO_2) causes an accumulation of CO_2 in the oceans, which co-occurs with ocean warming (IPCC 2001; 2007). CO_2 concentrations of about 710 ppm are predicted for the year 2100, and values of about 3,000 ppm might be reached by the year 2300, depending on fossil fuel utilization (IPCC 2001; Caldeira and Wickett 2005). Atmospheric CO_2 equilibrates with the ocean surface water and will be distributed by ocean circulation (Orr et al. 2001). The ongoing trends of ocean warming and acidification emphasize the importance of identifying their specific and combined impacts on marine organisms and ecosystems (Pörtner et al. 2005; Pörtner 2008).

The impact of ocean acidification on the calcification of marine animals has been a focus in recent studies (Fabry et al. 2008; Ries et al. 2009). The rising CO_2 concentrations in the oceans have negative effects on calcified structures of organisms like corals (Gattuso et al. 1998; Kleypas et al. 2006), echinoderms (Kurihara et al. 2004; Wood et al. 2008) and crustaceans (Findlay et al. 2009), due to the lowering of calcification rates which may even lead to abnormal skeletogenesis. Early developmental stages are an important part of the successful life cycle of an animal

and may be the most sensitive. Limited information exists on the effects of ocean acidification on developmental stages of invertebrates. Some studies report negative effects of CO_2 on the development, morphology, growth, hatching success or survival of larval stages of, for example, echinoderms or crustaceans (Mayer et al. 2007; Dupont et al. 2008; Kurihara 2008). Reduced calcification rates were found in larval stages of crustaceans like the lobster *Homarus gammarus* (Arnold et al. 2009) or the barnacles *Amphibalanus amphitrite* and *Semibalanus balanoides* (McDonald et al. 2009; Findlay et al. 2010a, b). In contrast, positive effects of elevated CO_2 concentrations on the growth of larval echinoderms were reported by Gooding et al. (2009) and Dupont et al. (2010).

Since aquatic CO_2 enrichment depends on latitude and associated temperatures, investigations of ocean acidification effects on the first calcifying developmental stages of marine invertebrates at various latitudes are considered very important (Fabry et al. 2008). However, studies of crustacean larvae living at different latitudes and various temperatures are scarce. Those available focus on older stages, the so-called post-larvae of barnacles (Findlay et al. 2010a, b).

Investigations of CO_2 effects on larvae from the same species across a wide latitudinal cline will exclude the influence of species-specific effects and will offer a clear view of how combined acidification and temperature trends affect early development stages and their physiological capacities. For this purpose, the spider crab *Hyas araneus* was chosen as a model organism for the present study. This species is distributed from the temperate southern North Sea (near Helgoland, Germany) to the sub-Arctic waters of Svalbard (Norway) (Christiansen 1969). In the southern North Sea, the species is exposed to a temperature range from 3 to 18°C (Wiltshire and Manly 2004), whereas the region around Svalbard is characterized by temperatures from 0 to 6°C (Svendsen et al. 2002). Adult spider crabs live on stony, sandy and soft bottoms from <1 m down to 360 m, most commonly at depths less than 50 m (Christiansen 1969). Ovigerous females release their larvae depending on ambient temperature and associated plankton blooms (Starr et al. 1994; Anger 2001; Hop et al. 2002), around February in the southern North Sea and around March in the sub-Arctic region (Walther et al. 2010). The larvae hatch into the first zoeal stage (Zoea I) and moult to Zoea II in a temperature-dependent way. Moreover, the duration of larval development is temperature dependent. The developmental time of both zoeal stages ranged from about 120 days at 3°C to 37–42 days at 9°C and 22–25 days at 15°C under normocapnic conditions. At those temperatures, larvae of the Svalbard population displayed a prolonged development (Walther et al. 2010). The zoeal stages use thoracopods (maxilliped I and II) to move in the water column (Christiansen 1971) and have long spines attached to their body as a protection against predators (Anger 2001). The carapace of zoeal larvae is soft and has unmineralized chitin layers, which supports a reduction in body weight and thereby enhanced mobility (Anger 2001). After moulting to the Megalopa stage, larvae start to incorporate calcium carbonate into their exoskeleton (Höcker 1988; Anger 2001). Megalopae display a semi-benthic life style and select a suitable habitat for the benthic juvenile and adult life-history stages, before they metamorphose to the first crab instar (Anger 2001). Development of Megalopae under normocapnic conditions took longer in the Svalbard population (40 days at both 9 and 15°C, respectively; 3°C not tested) than in the Helgoland population (30 days at 9°C; 27 days at 15; 3°C not tested). Additionally, only few Megalopae from Svalbard were able to metamorphose into the first juvenile stage at 9 and 15°C (~1%), whereas ~25% of those from Helgoland moulted to juveniles (Walther et al. 2010). This delay indicates the expression of polar characteristics (cf. Pörtner 2006), but may also reflect a high sensitivity of larval development during warming to 9 and 15°C. Altogether, larval stages of *H. araneus* are found around Helgoland for about 6 months. Thus, in their natural environment, larvae of the Zoea I stage exist from February to April (at 3–6°C), Zoea II larvae from April to May (6–9°C) and Megalopae from May to July (9–15°C) (Anger and Nair 1979; Anger 1983). Data on the duration of larval development in the natural environments around Svalbard are not yet available. The small temperature range around Svalbard (0–6°C) and the temperature dependence of development support the assumption that larval development around Svalbard occurs for a minimum of 6 months (March–August) but likely even longer. Because of their semi-benthic lifestyle, Megalopa larvae might be more exposed to the larger coastal pH fluctuations (Wootton et al. 2008; Waldbusser et al. 2010), than the pelagic Zoea larvae.

In crustaceans, calcification occurs as the incorporation of calcium carbonate ($CaCO_3$) into the chitin layer. Crustacean exoskeletons mainly consist of amorphous calcium phosphate and crystalline magnesium calcite in the form of nanocrystals as well as alpha-chitin (Boßelmann et al. 2007). Calcium is taken up from the ambient water via the gills (Cameron and Wood 1985; Neufeld and Cameron 1993; Flik et al. 1994) and transported into the cells via calcium channels in the basolateral membrane of gill tissue. For mineralizing the matrix of the cuticle, calcium is transported to the extracellular space through the apical membrane by Ca^{2+}-ATPase (Wheatly 1999). The calcium is forwarded to the space between haemolymph and carapace through the basolateral membrane of the carapace by use of ion transporters like Ca^{2+}-ATPase, Na^{+}/Ca^{2+} exchanger and Ca^{2+}/H^{+} exchanger (Neufeld and Cameron

1993; Wheatly 1999). Here, calcium and carbonate combine to form $CaCO_3$ (Cameron 1985a, b), which is precipitated to the chitin-protein fibres of the exo- and epicuticle (Travis 1955). Carbonate is provided by the reaction of CO_2 with water catalysed by carbonic anhydrase and the pH-dependent dissociation of bicarbonate (Giraud 1981; Henry and Cameron 1983). Upon acidification, the large $CaCO_3$ reservoir of the shell can be re-mobilized yielding Ca^{2+} and HCO_3^- for buffering purposes (Henry et al. 1981; Cameron 1985a, b; Pratoomchat et al. 2002). This may lead to structural changes in the carapace such that it can no longer fully protect the organisms from predators and pathogens (Pratoomchat et al. 2003). Saturation and dissolution of calcite structures in the carapace may depend on the temperature-dependent saturation state of calcite (Ωca) in the ambient water (Feely et al. 2009). Dissolution of calcite directly exposed to sea water occurs at Ωca below 1. In Arctic oceans, the saturation state of calcite is about 3.8, and reaches 5.3 in the North Atlantic (Feely et al. 2009). The saturation state of aragonite, which is lower than that of calcite, does not play a role in crustacean carapace calcification. The present study investigates to what extent the exposure to combined ocean warming and acidification affects crustacean larvae from different latitudes and their ability to calcify during their development. The specific responses of larval development to temperature in both populations (Helgoland and Svalbard) (Walther et al. 2010) led to the hypothesis that different rates of calcium incorporation occur in both populations. In addition, reduced pH values may cause lower rates of calcification, hence, contributing to lower calcium contents in larvae from both populations.

Materials and methods

Collection of crabs

Ovigerous females (15 ind.) of *Hyas araneus* were dredged in January 2008 at 30–50 m depths near Helgoland (German Bight, North Sea, 54° 11′N, 7° 53′E). Each female was kept in a flow-through aquarium at ambient water temperatures (4–6°C) and salinity (32) at the Biologische Anstalt on Helgoland (Alfred-Wegener-Institute, Germany). Experiments were performed with larvae hatched from the end of January to the end of February 2008.

In July 2008, 60 ovigerous females of *Hyas araneus* were caught by divers in the Kongsfjorden (Svalbard, Norway, 78° 55′N, 11° 57′E). Females were transported to the Alfred-Wegener-Institute, Bremerhaven (Germany), and kept for 8 months at 5°C in flow-through seawater aquaria. Before hatching, the females were transported to the Biologische Anstalt on Helgoland (Alfred-Wegener-Institute, Germany) where the larvae hatched from the end of February to the beginning of April 2009.

Due to extended and variable periods of reproduction, the number of females contributing to sufficient numbers of offspring during the experimental period was small, including the first three females with hatchlings from Helgoland and the first four females with hatchlings from Svalbard. From each population (Helgoland; Svalbard), a total of 9,450 freshly hatched larvae were collected from three females from Helgoland and a total of 8,100 freshly hatched larvae from four females from Svalbard. Larvae were reared at three different temperatures (3, 9 and 15°C) in combination with three different CO_2 concentrations at each temperature (normocapnia (=380 ppm CO_2), 710 and 3,000 ppm). Per combination of treatments (3 temperatures, 3 CO_2 conditions), 1,050 larvae from Helgoland population and 900 larvae from Svalbard population, provided by three females of Helgoland population and four females of Svalbard population, respectively, were kept separate, in aliquots of 50 larvae, each in 0.5–l Kautex flasks. On each day, the seawater was changed, dead larvae were removed and larvae were fed with freshly hatched nauplii of *Artemia* sp. (50–100 food ind. ml^{-1}) (San Francisco, Bay Brand).

Zoea I-larvae from flasks of a single female that moulted to the next instar Zoea II on the same day were pooled together into a new flask with the same treatment. When up to 12 animals had moulted into Zoea II, they were transferred into 0.2-l Kautex flasks. When more than 12 and up to 30 individuals moulted, we used 0.5-l Kautex flasks. After moulting from Zoea II to the Megalopa instar, we pooled the Megalopa per female and treatment in 0.2-l Kautex flasks (1–5 ind.) and 0.5-l Kautex flasks (6–15 ind.).

Treatments

Under all CO_2 conditions, seawater filtered at 0.2 μm was used. For CO_2 concentrations of 710 or 3,000 ppm, 60-l Kautex bottles were filled with filtered seawater and equilibrated with commercial gas mixtures (0.071% carbon dioxide, 21% oxygen in nitrogen or 0.3% carbon dioxide, 21% oxygen in nitrogen, provided by Air liquide). Flasks were closed with a lid to avoid contamination with air and to ensure stable water conditions over 24 h. On each day seawater removed with the sampling of larvae from the Kautex bottles was replenished, and water pH was recorded (WTW 340i, WTW SenTix HWS). Water samples removed repeatedly (10 times) during experimentation for alkalinity assays were stabilized by addition of 0.02% $HgCl_2$ in water and stored at 3°C in 250-ml borosilicate flasks. Total alkalinity was measured by potentiometric titration (Brewer et al. 1986) and calculated from linear

Table 1 Parameters of the seawater carbonate system calculated from temperature, pH (NIST-scale), total alkalinity (TA) and salinity (32) using the CO2Sys program (Lewis and Wallace 1998), mean values ± SD, $N = 10$

Temp. (°C)	CO_2 treatment (ppm)	pH	TA (μmol kg^{-1})	PCO_2 (μatm)	Ωca	Ωar
3	380	8.11 ± 0.05	2,405 ± 7	354 ± 65	3.12 ± 0.44	1.96 ± 0.28
	710	7.81 ± 0.03	2,405 ± 4	754 ± 37	1.65 ± 0.07	1.03 ± 0.05
	3,000	7.33 ± 0.03	2,405 ± 6	2,378 ± 164	0.57 ± 0.04	0.36 ± 0.02
9	380	8.12 ± 0.07	2,404 ± 8	346 ± 80	3.72 ± 0.68	2.35 ± 0.43
	710	7.81 ± 0.03	2,403 ± 6	786 ± 54	1.88 ± 0.11	1.18 ± 0.07
	3,000	7.35 ± 0.04	2,406 ± 8	2,443 ± 238	0.68 ± 0.06	0.43 ± 0.04
15	380	8.05 ± 0.04	2,409 ± 2	401 ± 30	4.02 ± 0.21	2.56 ± 0.14
	710	7.79 ± 0.04	2,409 ± 4	846 ± 42	2.25 ± 0.09	1.43 ± 0.06
	3,000	7.34 ± 0.04	2,409 ± 2	2,637 ± 160	0.82 ± 0.04	0.52 ± 0.03

Gran plots. The carbonate system was calculated from temperature, pH, alkalinity and salinity by the CO2Sys program (Lewis and Wallace 1998) using equilibrium constants provided by Mehrbach et al. (1973) and refitted by Dickson and Millero (1987). The parameters of the carbonate system characterizing the respective treatments are compiled in Table 1.

Ion chromatography

Analyses of calcium contents were carried out in six to 13 replicate samples of freshly hatched Zoea I (ZI) and in samples of Megalopa on days 3 (M3), 7 (M7) and 14 (M14). Randomized sampling for each temperature and CO_2 treatment ensured independence from the mother. Larvae were briefly rinsed in Millipore water, blotted on filter paper and transferred in pre-weighed 500-μl cups. After determination of the fresh weight, 100-μl Millipore water was added and samples stored were frozen at −20°C. For calcium content analyses, larvae were defrosted at room temperature and treated with 200 μl 1 M HCl, mixed on a vortex and incubated for 1 h (modified after Price and Dendigner 1983). Preliminary trials with different acid concentrations confirmed maximized extraction of whole body calcium by 1 M HCl. Subsequently, samples were centrifuged for 5 min at 20,800 rcf (relative centrifugal force). The supernatant was removed and diluted 100-fold with Millipore water. After sample preparation, calcium content of the larvae was determined by ion chromatography (ICS-2000, Dionex, Idstein, Germany). Cations (Na^+, K^+, Mg^{2+}, Ca^{2+}) were separated on an IonPac CS15 column with methane sulphonic acid (30 mmol l^{-1}) as an eluent at a flow rate of 0.36 ml min^{-1} and 40°C. Calcium content was calculated in mg l^{-1} relative to the Dionex Six Cation-II Standard and is given in mg l^{-1} per mg fresh weight (fwt).

Statistical analysis

Statistical analyses were performed with STATISTICA software (Version 7.1, StatSoft Inc.) and GraphPad Prism (Version 4, GraphPad Software Inc.). In order to test for the effect of developmental stage (levels: ZI, M3, M7, M14), original population (levels: Helgoland, Svalbard) and temperature (levels: 3, 9, 15°C) on the calcium content of the larvae, individual three-way ANOVAs were applied to the three experimental CO_2 conditions (380, 710, 3,000 ppm). In all three cases, data were square-root transformed to meet the assumptions of normal distribution (Kolmogorov–Smirnov-test, $P > 0.05$) and homogeneity of variances (Bartlett's test, $P > 0.05$). Square-root transformation prevented the slightly skewed distributions in the tested data sets after log transformation. For the 3,000 ppm CO_2 treatment, transformation did not lead to the required agreement. However, according to Underwood (1997 cited in Coleman et al. 2006), large designs incorporating three factors are robust against the consequences of deviation from this assumption. Significant differences between individual groups were identified through pairwise comparisons (Tukey's post hoc test).

The different maintenance periods for the ovigerous females required until larval hatching (8 months for Svalbard and 2 weeks for Helgoland) may have affected the observed differences between populations. However, the results and the reasoning presented here indicate significant functional differences between the two populations under the same temperature and CO_2 conditions and agree with previous research on evolutionary adaptation to different climate regimes.

Independent of age and therefore, pooled over all developmental stages (M3, M7 and M14), the calcium content data from the 380 ppm CO_2 (normocapnia) treatment were analysed for effects of temperature (3, 9, 15°C)

in each population with separate one-way ANOVAs. Assumptions of the normal distribution of data and homogeneity of variances were confirmed by the Kolmogorov–Smirnov-test ($P > 0.05$) and by Bartlett's test ($P > 0.05$), respectively. In addition, the Q_{10} values of calcium incorporation in each developmental stage (M3, M7 and M14) were calculated from the calcium content data of Megalopae reared at 380 ppm CO_2 for the temperature ranges 3–9 and 9–15°C in both populations. With $N = 3$ replicates per temperature range and population, differences between Q_{10} values were tested by pairwise comparisons with student's *t* test for unpaired data sets. Because of the small sample sizes ($N = 3$), normal distribution of data was checked by visual inspection. All data sets were considered normally distributed, the more so as the data points (i.e. the mean values) originated from normally distributed data sets as previously tested. The homogeneity of variances was confirmed by application of the *F* test ($P > 0.05$).

In order to test for the effect of developmental stage (levels: ZI, M3, M7, M14), temperature (levels: 3, 9, 15°C) and experimental CO_2 conditions (levels: 380, 710, 3,000 ppm) on the calcium content of the larvae, individual three-way ANOVAs were applied to each the Helgoland and Svalbard population. In both cases, data were square-root transformed to meet the assumption of normal distribution (Kolmogorov–Smirnov-test, $P > 0.05$) and of the homogeneity of variances (Bartlett's test, $P > 0.05$). Significant differences between individual groups were identified through pairwise comparisons (Tukey's post hoc test).

Graphs were prepared using GraphPad Prism (Version 4, GraphPad Software Inc.). Values are given as means ± 1 SE. All analyses were tested at the 0.95% confidence level.

Results

The analysis of larval calcium contents in the two populations (Helgoland, Svalbard) of Zoea I, of Megalopa after 3 (M3), 7 (M7) and 14 (M14) days of development under various temperatures (3, 9 and 15°C) and normocapnic conditions revealed that all factors (developmental stage, population and temperature) significantly affected the content (main factors ($P < 0.01$; Fig. 1a, suppl. Table 1)). The calcium content in larvae from both *Hyas araneus* populations reared under normocapnic conditions were compared in Fig. 1a. The calcium content of freshly hatched Zoea I (ZI) of both Helgoland and Svalbard larvae was about 2 mg l^{-1} mg(fwt)$^{-1}$. At 3°C, the calcium content of Zoea I and M3 larvae from Helgoland were similar and remained constant at about 4 mg l^{-1} mg(fwt)$^{-1}$ after 7 and 14 days of development during the Megalopa stage. The calcium content of Svalbard larvae at 3°C was the same in all developmental stages and significantly lower in M7 and M14 stages from Svalbard than from Helgoland. The same principle difference between the two populations could be seen at 9°C, while the calcium content of the Svalbard larvae increased significantly with developmental stage. No significant differences between the two populations could be found at 15°C. Within each population at 15°C, the calcium content of all measured Megalopa stages was significantly higher than in Zoea I.

Analyses of the calcium contents of pooled Megalopae at three temperatures (3, 9, 15°C) and under normocapnic conditions revealed no significant effect of temperature within the population from Helgoland (Fig. 2, suppl. Table 2). Consequently, the calculated Q_{10} values of Helgoland Megalopae were low between 3 and 9°C ($Q_{10} = 1.5 \pm 0.4$) or 9 and 15°C ($Q_{10} = 1.0 \pm 0.3$) ($N = 3$ each, Fig. 2). In contrast, temperature affected the calcium content of the Megalopae from Svalbard ($P < 0.01$; suppl. Table 2, Fig. 2), where it increased significantly from 3 to 9°C and then 15°C, with Q_{10} values of 1.5 ± 0.1 between 3 and 9°C and $Q_{10} = 2.1 \pm 0.3$ between 9 and 15°C (Fig. 2) (*t* test, $P < 0.05$). Furthermore, both populations differed significantly in the temperature-dependent increment of the calcium contents, reflected in the higher Q_{10} value of Svalbard larvae at 9–15°C compared to Helgoland larvae at 3–9 and 9–15°C (*t* test, $P < 0.05$).

The analyses of the calcium content of Megalopa from each population (Helgoland, Svalbard) after 3 (M3), 7 (M7) and 14 (M14) days at various temperatures (3, 9 and 15°C) and different CO_2 conditions (380, 710, 3,000 ppm) revealed that all factors (developmental stage, temperature and CO_2) affected the content (for all main factors $P < 0.01$; suppl. Table 3). At 3°C, all Megalopa stages (M3, M7, M14) of the Helgoland population showed a significant decrease of the calcium content from 380 to 3,000 ppm CO_2 (Fig. 3a). In contrast, differences remained insignificant between Megalopae reared under 380 ppm CO_2 compared to those reared under 710 ppm CO_2. At 9°C, Helgoland Megalopae of the 3rd and 7th day displayed a significant decrease of the calcium content under 3,000 ppm CO_2 compared to larvae reared under 380 ppm CO_2 (Fig. 3a). However, the calcium content of M14 Megalopae at 9°C had increased and was again similar under control (380 ppm) and all CO_2 conditions. At 15°C, all Megalopae maintained the same calcium level under all CO_2 levels (Fig. 3a).

Like Megalopae from Helgoland at 3°C, Megalopae from Svalbard reared at 3,000 ppm CO_2 and 3°C displayed a significantly lower calcium content compared to Megalopae from the same population reared under 380 or

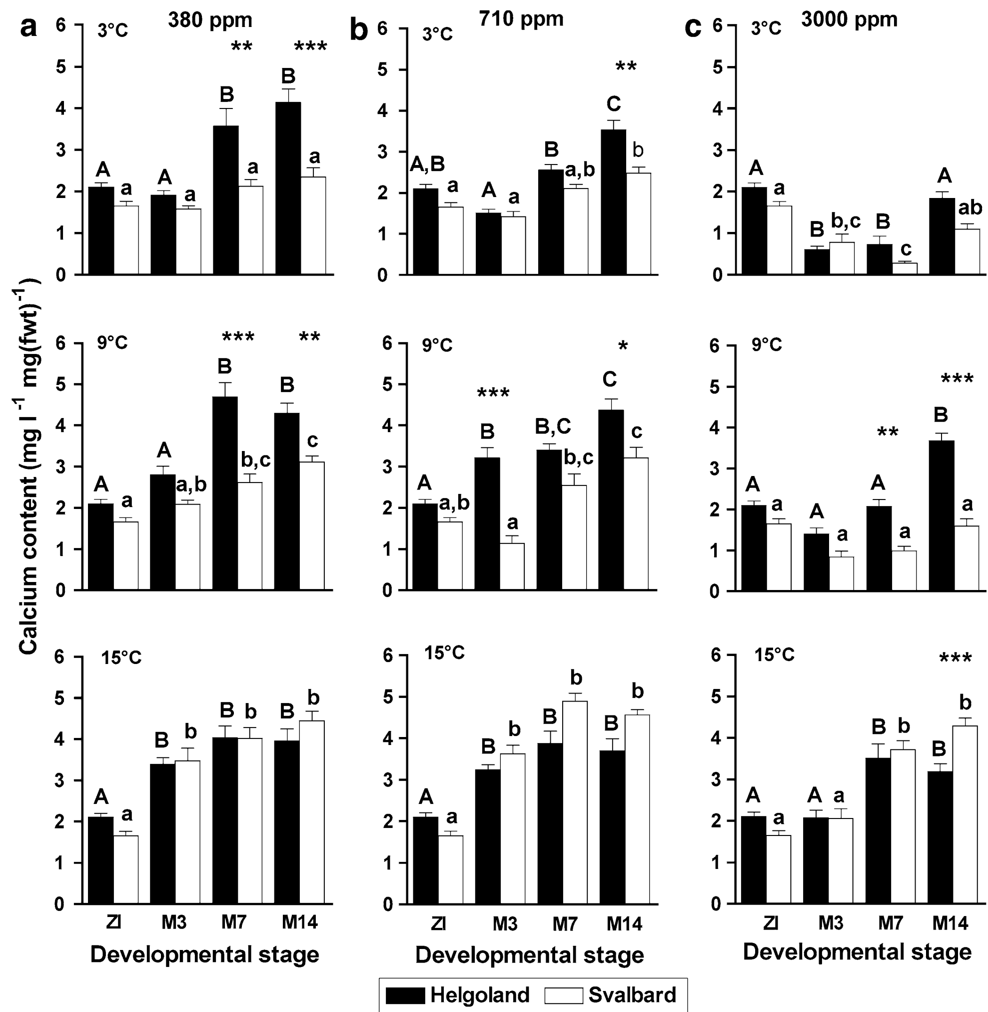

Fig. 1 Comparison of two populations of *Hyas araneus*, (*Helgoland*, *black*; *Svalbard*, *white*) with respect to calcium content of Zoea I (*ZI*) and Megalopa after 3 (*M3*), 7 (*M7*) and 14 days (*M14*) reared at 3, 9 and 15°C under 380 ppm (**a**), 710 ppm (**b**) and 3,000 ppm CO_2 (**c**). Significant differences between populations are indicated by *asterisks*, within populations by capital (*Helgoland*) or small letters (*Svalbard*): three-way ANOVAs of each CO_2 condition, $N = 6–13$

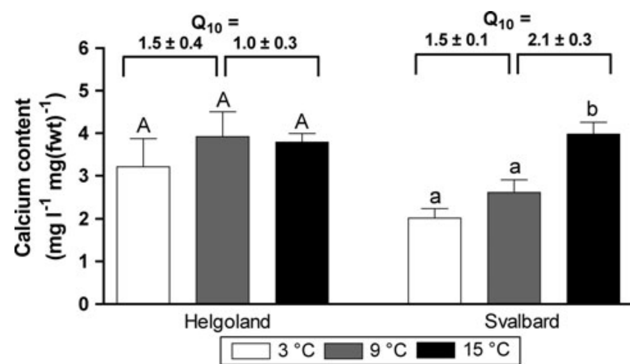

Fig. 2 Comparison of pooled Megalopae of *Hyas araneus* from Helgoland and Svalbard reared at 3°C (*white*), 9°C (*grey*) and 15°C (*black*) under normocapnic conditions and associated Q_{10} values between 3–9°C and 9–15°C of each population. Significant differences within each population are indicated by capital (*Helgoland*) or small letters (*Svalbard*): one-way ANOVA, $N = 3$

710 ppm CO_2 (Fig. 3b). At 9°C, the calcium contents of Megalopae sampled after 7–14 days were significantly decreased under 3,000 ppm CO_2 when compared to 380 and 710 ppm CO_2. The calcium content of M3 Megalopae reared under 710 ppm fell earlier at 9°C than at the other temperatures and reached similarly low calcium levels as seen in Megalopae under 3,000 ppm CO_2 (Fig. 3b). At 15°C (Fig. 3b), the significant drop in calcium contents of M3 under 3,000 ppm CO_2 was reversed leading to levels similar to those in M7 and M14 under 380 ppm.

The analyses of the calcium contents of Zoea I and Megalopae from the two populations (Helgoland, Svalbard) after 3 (M3), 7 (M7) and 14 (M14) days under various temperatures (3, 9 and 15°C) and at 710 or 3,000 ppm CO_2, respectively, revealed that all factors (developmental stage, population and temperature) significantly affected the content (for all main factors $P < 0.001$; Fig. 1b, c, suppl. Table 1). Although not all interactions between two factors were significant, the results indicate that the calcium content of individuals originating from Helgoland and Svalbard were affected differently by temperature and that this was different between the developmental stages under study (P for interactions of all three factors <0.001; suppl. Table 1).

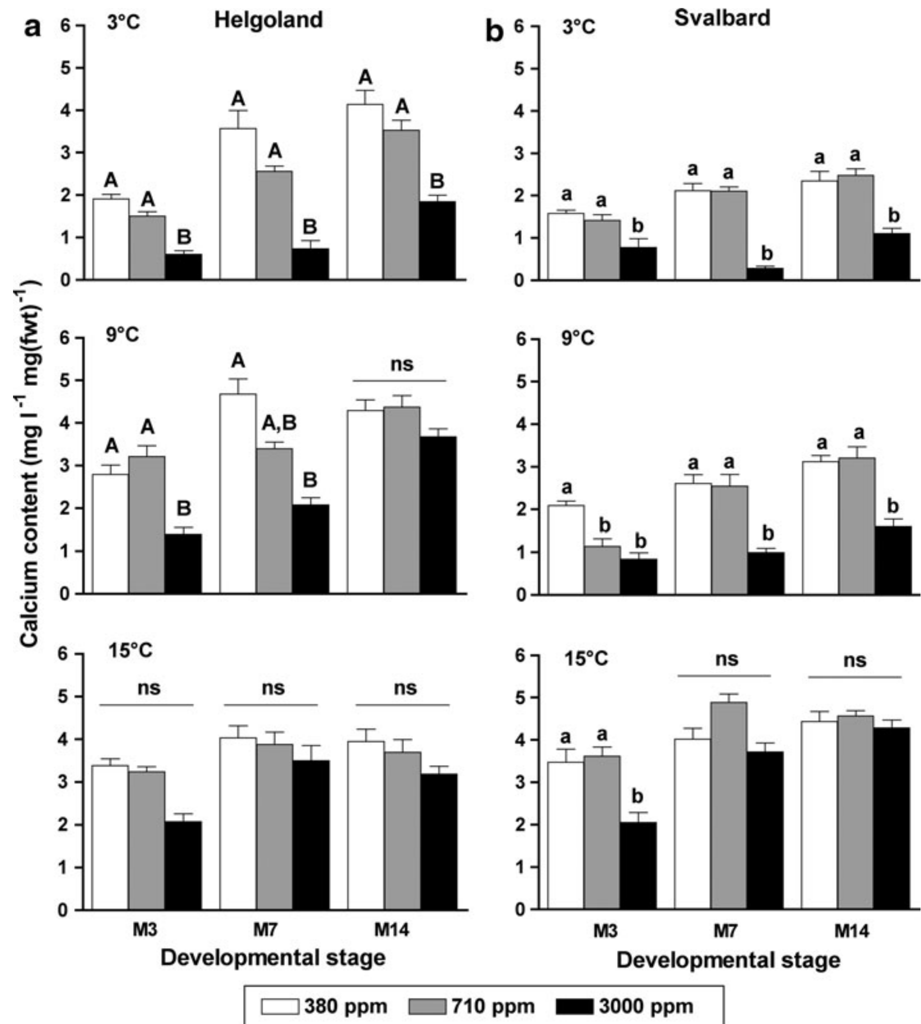

Fig. 3 Calcium content of Helgoland (**a**) and Svalbard (**b**) Megalopa of *Hyas araneus* after 3 (*M3*), 7 (*M7*) and 14 days (*M14*) reared at 3, 9 and 15°C under 380 ppm CO_2 (*white*), 710 ppm CO_2 (*grey*), 3,000 ppm CO_2 (*black*). Significant differences within populations are indicated by capital (*Helgoland*) or small letters (*Svalbard*): three-way ANOVA, $N = 6–13$

The calcium content of larvae from both populations reared at 710 ppm CO_2 displayed an increment with progressive development at 3 and 9°C, which differed significantly between both populations (Fig. 1b). At 3°C, the calcium content of 14-day Megalopa (M14) from Helgoland was significantly higher than in M14 from Svalbard. At 9°C, the calcium content of larvae from Helgoland was also significantly higher than in those from Svalbard, now in M3 and also in M14. At 15°C, however, the calcium content increased similarly in both populations from Zoea I to M3/M7/M14.

Figure 1c shows the calcium content of larvae from Helgoland and Svalbard reared under 3,000 ppm CO_2. At 3°C, the calcium content of Helgoland larvae decreased significantly from ZI to M3 and increased within 14 days to the same level as in ZI. The calcium content in larvae from Svalbard decreased from ZI to M7, and then rose to similar levels in M14 as in Zoea I and M3. At 9°C, calcium contents in Megalopae from Svalbard remained low and revealed no rise during development under elevated CO_2 levels. In contrast, the calcium content of larvae from Helgoland first decreased significantly from ZI to M3. This decrease was compensated for after 14 days at the Megalopa stage. Moreover, the calcium content in M7 and M14 was significantly higher in specimens from Helgoland than in those from Svalbard. At 15°C, a significant increase in calcium content from ZI/M3 and M7/M14 could be detected in both populations. Interestingly, the main difference between populations was seen in M14, with a higher calcium content in larvae from Svalbard than in those from Helgoland.

Discussion

The calcification of decapod crustacean larvae starts with the moult to the Megalopa stage (Anger 2001). Immediately after hatching, the first Zoea stage inflates its body

with ambient water. Ions like calcium are able to permeate the whole Zoea body surface through the thin unmineralized cuticula of the Zoea larvae by diffusion (Pütz and Buchholz 1991; Anger 2001) and are in equilibrium with the ambient water. In Megalopae, a mineralized cuticula forms and water and ions can only enter the body across the gill surface, either passively or by active transport mechanisms (Cameron and Wood 1985; Neufeld and Cameron 1993; Flik et al. 1994).

The calcification process proceeds during a defined moulting cycle with several phases (postmoult, intermoult, premoult, ecdysis) (Roer and Dillaman 1984; Pratoomchat et al. 2002). In crustacean larvae, moulting is important for growth and for the formation of various larval stages with morphological differences and functional specialization (Anger 2001). The Megalopa stage starts with an early postmoult phase, which starts the mineral nucleation of bound calcium salts (Pratoomchat et al. 2002). The following intermoult of the Megalopa stage is characterized by the incorporation of calcite and intense calcareous thickening (Roer and Dillaman 1984). The accumulation of calcium in the body of Megalopae over time thus mostly reflects the rate of calcification during assembly of calcified structures, which are dissolved by exposure to HCl (Price and Dendigner 1983).

The progressive embedding of calcium into the carapace occurs with progressing Megalopa age (Fig. 1a) and growth (Walther et al. 2010). The regular progress in cuticular mineralization under normocapnic conditions becomes visible through the continuous increase in the weight-specific calcium content of Megalopae from day 3 to day 14 (Fig. 1a). The highest calcium contents are thus found in older larval stages of *H. araneus*, similar to observations in lobster larvae (Arnold et al. 2009).

In line with the hypothesis that different rates of calcium incorporation occur in both populations (see Introduction), larval calcium contents differed between both populations (Fig. 1a). Overall, the comparison of both populations under 380 ppm CO_2 revealed that Svalbard Megalopae during the first 14 days at 3°C could not set their calcification rate to the same level as Megalopae from Helgoland at the same temperature. Possibly, enzymes and transporters supporting the active influx of calcium ions and their assembly into the chitin layer (Giraud 1981; Neufeld and Cameron 1993; Wheatly 1999) differ in their expression or activity between both populations. Alternatively, energy allocation to calcification may be less in the population from Svalbard and thereby delay the accumulation of carbonates.

However, while showing lower calcium contents at low temperature than their conspecifics from Helgoland, only the Svalbard larvae enhanced their calcium to control levels during warming to temperatures higher than reached in the natural environment (see Q_{10}; Fig. 2). The observed temperature-dependent calcium accumulation under normocapnia is an indicator of the coupling of the calcification process to temperature-dependent enzymatic (including transport) mechanisms.

Similar to *Hyas araneus* from Svalbard, the barnacle *Chtalamus montagui* (Clavier et al. 2009), and the crayfish *Asthacus asthacus* (Lahti 1988) displayed a temperature-dependent level of calcification. Ostracods (*Herpetocypris intermedia*), living between 13 and 20°C, also demonstrated a temperature-dependent calcification rate, with decreased moulting and calcification rates associated with reduced survival at the low end of the temperature range (Mezquita et al. 1999). It is surprising though that Svalbard Megalopae adapted to live permanently at temperatures between 0 and 6°C display lower calcification rates in the cold at 3 and 9°C than Helgoland larvae which experience 3°C as their lowest habitat temperature in winter (Fig. 1b, c). This pattern suggests that cold compensation of calcification occurs in the temperate eurythermal North Sea population more than in the cold adapted Svalbard population. Furthermore, these findings together with the one of a stronger response to temperature may suggest a reduction in energy turnover in the Arctic population, which may in fact have developed cold stenothermy characteristics (cf. Pörtner 2006).

Further explanations might lie in the different saturation states of calcite in both regions and in the associated adaptive strategies. Lower Ωca in Arctic waters than in the North Atlantic are caused by lower temperatures (Feely et al. 2009). The low Ωca values at the crab's natural temperature of 3°C may limit calcium contents of Arctic *H. araneus* Megalopae. These crabs may then be prepared to sacrifice calcification even more and to successfully put energy into growth, as indicated by stable dry weights under elevated CO_2 conditions at 3°C (Walther et al. 2010). Under elevated CO_2 tensions, the observation of low calcium contents at body dry weights unchanged from controls in larvae from Svalbard at 3 and 9°C suggests that they compensated for the reduced amount of calcium carbonate by increased chitin contents in the carapace. Similarly, Pratoomchat et al. (2003) observed an increased content of polymerized chitin in the epidermis of the exoskeleton of the mud crab (*Scylla serrata*) after exposure to pH 7.5. Reduced calcification at habitat temperatures may cause reduced strength of the exoskeleton as an efficient protection mechanism against predators or pathogens. Such explanation may suggest reduced reliance of *Hyas araneus* on calcification in its cold-water habitat at Svalbard, possibly, because predator pressure may be less in the cold water habitat. For a detailed explanation of the different calcium levels, future studies of the enzymes and transporters involved in the calcification process are necessary.

Supported by higher Ωca at 9 and 15°C, Helgoland larvae may put an emphasis on calcification and may

allocate energy to calcification during elevated CO_2 concentrations at the expense of soft tissue growth, indicated by a reduction in dry weight (Walther et al. 2010). However, larvae from Svalbard are also able to increase calcification rate and do so at elevated non-natural temperature when they reach even higher calcium contents than their conspecifics from Helgoland (Fig. 1a). This indicates a stronger thermal response of the calcification process in the Svalbard population, which again is similar to the thermal response of other processes in polar ectotherms (Pörtner 2006).

In larvae of each population, lower calcium contents caused by higher CO_2 concentrations were possibly due to the reduced availability of carbonate for calcium precipitation at the deposition site and associated with the low Ωca values in ambient water (Table 1). At $\Omega ca < 1$ in the water, which is the case at 3,000 ppm CO_2, dissolution of calcium carbonates to their components prevails (Feely et al. 2009). At 3,000 ppm CO_2, the accumulation of calcium in all larvae (of Helgoland and Svalbard population) was initially disturbed, especially at 3 and 9°C (Fig. 1c), but larvae were able to restore these levels over time when reaching M14. Active transport by ion exchange likely caused the accumulation of calcium levels to support the calcification of the carapace (Cameron and Wood 1985; Neufeld and Cameron 1993; Flik et al. 1994). The capacity of this compensation process may depend on the parallel compensation of a CO_2 induced disturbance in acid–base status (cf. Pörtner 2008). Larvae at 9°C were able to undergo compensation faster than at 3°C (Fig. 1c). Hence, the effect of ocean acidification is more prominent at 3°C than at warmer temperatures.

Under control CO_2 levels (380 ppm CO_2) at 15°C, similar calcium contents in Megalopae from both populations (Fig. 1a) combined with progressively lower growth rates and fitness levels in Megalopae from Svalbard than in those from Helgoland (Walther et al. 2010) support the hypothesis that high non-natural temperatures (15°C) may cause energy to be reallocated to calcification, but this occurs at or beyond the limits of thermal tolerance or even at the expense of somatic fitness and development during the first 14 days of the Megalopa stage at Svalbard. Loss of fitness at beyond habitat temperatures under normocapnia is reflected in the high mortality of *Hyas araneus* Megalopae from Svalbard at 9 and 15°C, associated with reduced success rates in the metamorphosis of Megalopae to the first juvenile stage (Walther et al. 2010). For comparison, the barnacle *Semibalanus balanoides* displayed reduced calcification together with reduced survival and growth rates at 19°C (Findlay et al. 2010a). Overall, an optimized balance between calcification and developmental rates may be crucial for recruitment success and is lost in Svalbard larvae in the warmth.

Interestingly and in contrast to findings at 3 and 9°C, calcium content in M14 from Svalbard at 15°C, a non-natural temperature, and exposed to 3,000 ppm increased significantly over time compared to those from Helgoland (Fig. 1c) and reached levels seen under normocapnia (Fig. 3b). This observation resembles those in the temperate barnacle *Amphibalanus amphitrite*, exposed to enhanced CO_2 conditions (pH 7.4) and can be explained by compensatory calcification in active growth zones of the shells. However, the elevated calcium content was insufficient to prevent the weakening of shell walls at reduced pH in the barnacles (McDonald et al. 2009). The reduced survival and disturbed metamorphosis of *Hyas araneus* Megalopae also suggests that the compensatory increase in calcification rate cannot alleviate all effects of elevated CO_2 levels and the associated unfavourable shift in energy budget. It remains to be explored whether the compensation process is due to the kinetic stimulation of calcification at warmer temperatures or whether it involves a change in the gene expression of associated mechanisms.

Elevated CO_2 concentrations caused a decrease in larval calcium content at all temperatures (3, 9 and 15°C) (Fig. 3b), however, did not affect fitness and dry mass in Svalbard Megalopae more than under control conditions (Walther et al. 2010). In contrast, Helgoland Megalopae displayed reduced fitness and lowered dry weight after exposure to 3,000 ppm CO_2 at 9°C (Walther et al. 2010). Arnold et al. (2009) showed reduced dry weight and calcification in the last zoeal stage of lobster (*Homarus gammarus*) at 1,200 ppm CO_2. A similar reduction in calcification could be seen in adults of the mud crab *Scylla serrata*, at low water pH (pH 7.5, pH reduction caused by fixed acid titration) (Pratoomchat et al. 2003).

Developmental time may influence the rate of calcification in both populations (cf. Pörtner et al. 2010). A reduction in developmental rates at elevated CO_2 levels, paralleled by reduced calcification rates, may thus explain the lower calcium contents found when comparing larvae from different CO_2 treatments after the same time periods. A reduction in developmental rates also means that Zoea larvae exposed to different temperature and CO_2 conditions spend different periods under the respective conditions before they moult to Megalopa stage (Anger and Nair 1979; Walther et al. 2010). Larvae in the Megalopa stage also show those effects (Anger and Nair 1979; Walther et al. 2010). It can, therefore, be assumed that the development of zoeal stages and Megalopa stage slowed by ocean acidification (Walther et al. 2010) translates to lower fitness and possibly, reduced calcification status of the Megalopa stages.

In Megalopae at 15°C, the maintenance of calcium at control levels in larvae from Svalbard contrast findings in barnacle post-larvae, where the calcium content remained

reduced even after long term exposure (20 days) under elevated CO_2 conditions (Findlay et al. 2010a). It remains unclear, however, whether such reduction occurred at high temperatures beyond the borders of their window of thermal tolerance. In case of *H. araneus* from Svalbard at 15°C, the slower development than in Helgoland larvae may reflect the surpassing of thermal limits (Walther et al. 2010), but this may not necessarily imply an early loss of calcium levels in the exoskeleton.

Overall, ocean acidification has the potential to cause an imbalance between somatic and carapace growth and composition as well as associated disturbances in energy budget. These disturbances have species-specific implications and consequences depending on the climate regime where such influences are observed. For a species at its lower temperature limits such as *Hyas araneus* from Svalbard, the patterns observed indicate an imbalance in energy budget, at the expense of growth and development and thus a higher sensitivity to ocean acidification than seen in the same species at more Southern, warmer latitudes.

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