

# Pre-hatching seawater $p\text{CO}_2$ affects development and survival of zoea stages of Arctic spider crab *Hyas araneus*

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**ABSTRACT:** The sensitivity of marine crustaceans to anthropogenic  $\text{CO}_2$  emissions and the associated acidification of the oceans may be less than that of other, especially lower, invertebrates. However, effects on critical transition phases or carry-over effects between life stages have not been comprehensively explored. Here we report the impact of elevated partial pressure of  $\text{CO}_2$  ( $p\text{CO}_2$ ) values (3100  $\mu\text{atm}$ ) in seawater on *Hyas araneus* during the last 2 wk of their embryonic development (pre-hatching phase) and during development while in the consecutive zoea I and zoea II larval stages (post-hatching phase). We measured oxygen consumption, dry weight, developmental time and mortality in zoea I to assess changes in performance. Feeding rates and survival under starvation were investigated at different temperatures to detect differences in thermal sensitivities of zoea I and zoea II larvae depending on pre-hatch history. When embryos were pre-exposed to elevated  $p\text{CO}_2$  during maternal care, mortality increased about 60% under continued  $\text{CO}_2$  exposure during the zoea I phase. The larvae that moulted into zoea II displayed a developmental delay by about 20 d compared to larvae exposed to control  $p\text{CO}_2$  during embryonic and zoeal phases. Elevated  $p\text{CO}_2$  caused a reduction in zoea I dry weight and feeding rates, while survival of the starved larvae was not affected by seawater  $\text{CO}_2$  concentration. In conclusion,  $\text{CO}_2$  effects on egg masses under maternal care carried over to the first larval stages of crustaceans and reduced their survival and development to levels below those previously reported in studies exclusively focussing on acute  $p\text{CO}_2$  effects on the larval stages.

**KEY WORDS:** *Hyas araneus* · Zoea · Larvae · Embryos · Ocean acidification

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## INTRODUCTION

Ongoing acidification of the world's oceans due to absorption of anthropogenic  $\text{CO}_2$  is receiving increasing public interest. Since the early Miocene, about 24 million yr ago, atmospheric  $\text{CO}_2$  concentrations have remained below 500 ppm and were thus relatively stable (Pearson & Palmer 2000). Today's changes have already caused ocean pH to decline by more than 0.1 unit below values characterising pre-industrial times (Caldeira & Wickett 2003). Atmospheric  $\text{CO}_2$  might reach values of 3000 ppm by the

year 2300 (Caldeira & Wickett 2005) and cause pH to fall to 0.8 unit below pre-industrial values.

Studies of the relative vulnerability of marine ectotherms to ocean acidification scenarios revealed a high inter-taxa variation in  $\text{CO}_2$  sensitivity (Melzner et al. 2009a, Kroeker et al. 2010). Marine fish and cephalopods, and to some extent, crustaceans, seem to be more tolerant to high  $\text{CO}_2$  levels, while e.g. echinoderms and bivalves, which at the same time are more heavily calcified, appear to be more sensitive (Siikavuopio et al. 2007, Gutowska et al. 2008, Melzner et al. 2009b, Thomsen & Melzner 2010). In

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crustaceans, as in other taxa, tolerance to elevated CO<sub>2</sub> levels was found to be species-dependent and linked to different ion-regulation capacities (Truchot 1979, Pane & Barry 2007, Spicer et al. 2007).

An increasing number of studies in the field of ocean acidification research focus on early developmental and reproductive stages of calcifiers, which are believed to be the most vulnerable (Kurihara 2008). In crustaceans, most studies dealt with effects on post-embryonic (post-hatching) larval stages, but disregarded effects of partial pressure of CO<sub>2</sub> (*p*CO<sub>2</sub>) levels in seawater on embryos pre-hatching as well as any carry-over effects on the later larval or adult stages, which had been seen in molluscs or echinoderms (Parker et al. 2012, Dupont et al. 2013).

Elevated seawater *p*CO<sub>2</sub> of 1200 µatm CO<sub>2</sub> did not influence development, mortality or growth in the first 2 zoea stages of the European lobster *Homarus gammarus* (Arnold et al. 2009) nor zoea mortality or growth in the subtidal spider crab *Hyas araneus*. Yet, it affected the transition to the megalopa stage (Walther et al. 2010, 2011). In line with these findings, effects of elevated seawater *p*CO<sub>2</sub> on oxygen consumption, weight and elemental composition in developing zoea I larvae of *Hyas araneus* from an Arctic population were small, and developmental duration and survival remained unaffected (Schiffer et al. 2013). However, perturbation experiments on individual larval stages are mostly limited by the stage duration, and long-term experiments are hence more difficult to carry out. The transition between larval stages may be even more critical than progression within any individual stage per se. Short-term experiments have been criticised because they may over- or underestimate the real impacts of chronic exposure to high *p*CO<sub>2</sub>, as there might not be enough time for acclimation or not enough time to induce effects (Dupont et al. 2013). Studies should start with the earliest and pre-hatching embryonic stages. Such efforts would take more stages into account and would extend incubation time considerably. Putative bottlenecks during development, such as the transition to the megalopa stage (Walther et al. 2010, 2011), should also be identified for a comprehensive picture.

Hatching might be another critical bottleneck. The eggs of almost all decapod crustaceans are attached to the female's pleopods and subject to maternal care. Thus, embryos are not only exposed to the same environmental conditions as the ovigerous females, but their well-being and, ultimately, recruitment also depend on the performance capacity of the female to facilitate gas exchange around and within the egg

mass (Fernández et al. 2000). Abiotic factors such as salinity (Giménez 2002) and temperature (Petersen 1995) experienced during the pre-hatching phase influence zoea I hatching rate, survival and development.

The first aim of the present study was to investigate possible carry-over effects from CO<sub>2</sub>-exposed embryos to the successive life history stages (zoea I and zoea II larvae) in the spider crab *Hyas araneus* collected from an Arctic population. We exposed females with late-stage eggs to 2 different levels of seawater *p*CO<sub>2</sub> and performed a time series study on zoea I larvae hatched from these egg masses. We determined mortality and stage duration to examine larval fitness. We measured embryonic oxygen consumption (eggs) and followed the course of oxygen consumption and weight increment in developing zoea I larvae to estimate energy demands during continued CO<sub>2</sub> exposure. Heart rate and maxilliped beat rate of zoea I larvae were measured before moulting to the second stage (zoea II). We compared our present results to those of an earlier study on zoea I of Arctic *H. araneus* where larvae had been CO<sub>2</sub>-exposed just after hatching but not during the preceding embryonic phase (Schiffer et al. 2013).

The second aim was to assess whether temperature sensitivities differ between zoea I or zoea II developed from control and pre-exposed egg masses. Since food availability influences the survival and development of crustacean larvae and interacts or masks effects of abiotic factors (Giménez 2002), we measured feeding rates and survival under starvation at 4 different temperatures in zoea I and zoea II larvae developed from control and pre-exposed eggs. Larval feeding behaviour was then compared to observed growth rates.

## MATERIALS AND METHODS

### Arctic *Hyas araneus*

*Hyas araneus* inhabits rocky, sandy and muddy bottoms on continental shelves. This eurythermal species has a wide distribution from the temperate southern North Sea to sub-Arctic waters (Christiansen 1969), with temperatures ranging from 0 to 21°C. It is an excellent organism to study the effects of elevated seawater *p*CO<sub>2</sub> on successive life-history stages (Walther et al. 2010, 2011, Schiffer et al. 2013). Females carry egg masses for approximately 2 yr when seawater temperatures remain below 12°C (Petersen 1995). Zoea I larvae hatch from the eggs

and are released into the water column. After going through 2 zoeal and 1 megalopa stage, juveniles settle in the adult habitat. Successful development of *H. araneus* larvae from the North Sea has been reported for temperatures between 3 and 18°C (Anger 1983). Developmental duration of larval stages varies highly with temperature and population (Anger 1983, Walther et al. 2010) and ranges from 13 d at 15°C to 60 d at 3°C in zoea I and zoea II larvae of the Arctic *H. araneus* population (Walther et al. 2010) used in the present study.

### Egg and larval collection and maintenance

Ovigerous females of *Hyas araneus* were collected in Kongsfjorden (Ny Alesund, Svalbard; 78° 55' N, 11° 56' E; Arctic population) by scientific divers in spring 2010 at 8 m depth and a water temperature of 0°C. They were transferred to the Alfred Wegener Institute in Bremerhaven and maintained in flow-through aquaria at 4°C during summer and at 0°C during early winter to avoid early hatching. Salinity was maintained at 31. In January 2011, females with late stage III eggs (Petersen 1995) were placed individually in 2 l flow-through aquaria within recirculating CO<sub>2</sub> incubation systems (volume: 1 m<sup>3</sup> seawater each) at 4.5 ± 0.1°C to induce hatching. Seawater CO<sub>2</sub> manipulation was achieved by injecting the feeder tank and the header tank with a defined air/CO<sub>2</sub> mixture using a mass flow controller (HTK 6 channel). Aquaria were directly provided with seawater from the header tank. Egg-carrying females were held at 2 different CO<sub>2</sub> concentrations of 350 µatm (controls) and 3100 µatm CO<sub>2</sub> (high CO<sub>2</sub> treatment), respectively (Fig. 1). Embryos were sampled for respiration measurements after 1 wk of incubation (approximately 7 d prior to hatch). All

embryos that were selected for experimentation exhibited a small yolk-filled area, which was divided into 2 separate parts indicating the pre-hatching phase. Hatching started approximately 2 wk after the females had been transferred to the incubation systems.

A scheme of the experimental design is depicted in Fig. 1. Experiments were conducted with zoea I larvae that had hatched within 24 h. Hatched larvae from 3 females were pooled and groups of 30 individuals were transferred into closed 0.5 l culture vessels, with seawater CO<sub>2</sub> concentrations maintained. Larvae were kept at a constant temperature of 4.4 ± 0.4°C. Seawater was provided from reservoir tanks (60 l) continuously injected with a defined air/CO<sub>2</sub> mixture using a mass flow controller (HTK 6 channel). Water in culture vessels and food (freshly hatched *Artemia* sp., Sanders Brine Shrimp Company) were changed daily and dead larvae and moults were removed. Zoea II that had moulted on the same day were pooled and transferred to a new culture vessel. Water physicochemistry was monitored weekly over the entire experimental duration (approximately 10 wk) by determining pH with a pH electrode (WTW ProfiLine pH 3310) that was calibrated with NIST buffers and analysis of dissolved inorganic carbon (DIC). The pH was converted to total scale (pH<sub>T</sub>) via measurements of Dickson standards. Water pCO<sub>2</sub> was calculated from DIC and pH<sub>T</sub> using the program CO<sub>2</sub> SYS (Lewis & Wallace 1998) (Table 1).

### Mortality and developmental time

Twenty-two culture vessels containing about 30 larvae each were used for investigating the effect of control (11 vessels) or elevated CO<sub>2</sub> (11 vessels) on

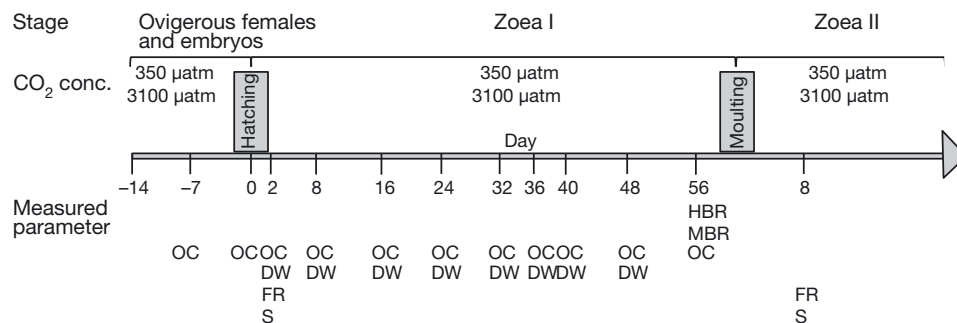


Fig. 1. *Hyas araneus*. Measurements conducted on specific days of development in control (350 µatm) and elevated (3100 µatm) partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) of seawater. DW: dry weight; FR: feeding rate; HBR: heartbeat rate; MBR: maxilliped beat rate; OC: oxygen consumption; S: survival under starvation

Table 1. Seawater parameters measured during incubation (N = 9). Values are mean  $\pm$  SD. pH<sub>T</sub>: pH total scale; DIC: dissolved inorganic carbon; pCO<sub>2</sub>: partial pressure of CO<sub>2</sub>

Incubation	Temperature (C°)	pH <sub>T</sub>	DIC (μmol kg <sup>-1</sup> )	pCO <sub>2</sub> (μatm)	Salinity
Control	4.5 $\pm$ 0.5	8.13 $\pm$ 0.05	2308 $\pm$ 83	349 $\pm$ 57	31.2 $\pm$ 0.9
CO <sub>2</sub> 3100	4.4 $\pm$ 0.2	7.20 $\pm$ 0.02	2492 $\pm$ 73	3103 $\pm$ 146	31.2 $\pm$ 0.9

Table 2. *Hyas araneus*. Zoea I stage duration and effect size used for calculating age (virtual age = real age  $\times$  effect size). Effect size is defined as the ratio of zoea I stage duration at 350 μatm CO<sub>2</sub> to zoea I stage duration at 3100 μatm CO<sub>2</sub>

pCO <sub>2</sub> (μatm)	Zoea I stage duration (d)	Effect size
350	51.95	1
3100	71.30	0.72

mortality and duration of the larval stage. Mortality and moulting (number of zoea II) were recorded on a daily basis until all larvae were either dead or moulted into zoea II. Survival curves for larvae were generated and median survival was determined using GraphPad Prism 4 software.

As high CO<sub>2</sub> concentrations frequently affect larval developmental time, the virtual age of zoea I larvae was calculated following Pörtner et al. (2010) to detect day-specific differences in larval dry weight (DW). Virtual age was calculated as: real age (days post hatching)  $\times$  effect size (Table 2), where effect size is defined as the ratio of zoea I stage duration at 350 μatm CO<sub>2</sub> to zoea I stage duration at 3100 μatm CO<sub>2</sub>.

### Oxygen consumption and DW

Oxygen consumption rates of eggs and individual zoea I larvae were measured in closed, water-jacketed respiration chambers (OXY041 A, Collotec Meßtechnik) perfused by thermostatted water to maintain control temperature at 4°C. Oxygen saturation was recorded by oxygen micro-optodes (needle type: NTH-PSt1-L5-TF-NS\*46/0,80-YOP, fibre-optic microsensor, flat broken tip, diameter: 140 μm; PreSens), connected to an oxygen meter (Microx TX3, PreSens).

Oxygen consumption rates were measured in egg batches from 8 individual females after 1 wk of exposure to corresponding seawater CO<sub>2</sub> concentrations.

Approximately 20 eggs were carefully removed from each brooding female and placed on a fine grid in the respiration chamber. A magnetic stirrer was placed beneath the grid and the water in the respiration chamber was gently mixed to prevent oxygen stratification. The plunger of the chamber lid was inserted and the volume of the chamber was reduced

to 250 μl. Respiration measurements were stopped when the oxygen saturation of the chamber water had decreased to about 80% air saturation. Before and after each measurement, blanks were run to consider bacterial oxygen consumption.

Larvae were sampled for respiration and DW measurements 2 d post hatching and subsequently every 8 d. Hatched larvae were carefully transferred from the culture vessel into the respiration chamber, which contained seawater of the corresponding CO<sub>2</sub> condition. Afterwards the plunger of the chamber lid was inserted and the volume of the chamber was reduced to 150 μl. The needle of the micro-sensor was inserted into the chamber through a hole in the lid and the sensitive tip of the optode was placed in the middle of the chamber. The almost constant swimming of the larvae caused mixing of the water in the chamber. Respiration measurements were stopped after 30 min or when the oxygen saturation of the chamber water had decreased to about 80%. Before or after each measurement, blanks were run to account for bacterial oxygen consumption. Larval oxygen consumption was expressed as μgO<sub>2</sub> mgDW<sup>-1</sup> h<sup>-1</sup>  $\pm$  SE to allow for treatment-specific differences in larval DW.

After oxygen consumption measurements, eggs/larvae were removed from the chamber and briefly rinsed with deionised water. Excessive water was removed using a paper towel. Larvae/eggs were killed by snap-freezing in liquid nitrogen and stored at -20°C in pre-weighed tin cartridges for DW determination by freeze-drying to constant weight determined on a high-precision balance (Mettler Toledo). Larval DW is given as μg  $\pm$  SE per individual. On all sampling days, 8 zoea I larvae and 8 egg masses from each CO<sub>2</sub> treatment were used to measure oxygen consumption. The same 8 individuals were used for DW analyses. Oxygen consumption rates and weight were plotted against real age (days post hatching) and virtual age (days post hatching  $\times$  effect size) to compare their change over time courses in regard to actual developmental day as well as compensated for developmental delay in high-CO<sub>2</sub> larvae.

### Heart rate and maxilliped activity

In addition to oxygen consumption, heart rate and maxilliped activity were determined in 5 zoea I larvae on Day 50 post-hatching by using a digital camera (AxioCam MRm, Carl Zeiss Mikroimaging) mounted onto a microscope (Axio Observer A1, Carl Zeiss). Larvae were measured under the microscope in a custom-built temperature-controlled flow-through micro-chamber (AWI workshop) with a flow rate of 5 ml min<sup>-1</sup> to avoid a decrease in oxygen concentration due to larval respiration in the closed chamber. Temperature-controlled seawater (temperature: 4°C, salinity: 32) was provided from a reservoir vessel placed in the thermostat water bath and was pumped through the chamber. Before closing the chamber, individual larvae were positioned in the centre of the micro-chamber by gluing the carapace to a thin glass spine, which itself was attached to a glass table. Then the chamber was closed and water-flow through the chamber was started. Larvae were left for 1 h to recover from handling stress and were videotaped for 1 min periods. The video sequence was analysed for heartbeat and maxilliped activity, respectively, by counting the beats min<sup>-1</sup>. The beating heart can easily be seen through the transparent carapace. Heart rate and maxilliped beat rate were calculated for each larva as the mean number of beats min<sup>-1</sup> ± SE from three 10 s intervals.

### Feeding rate

Feeding rates were measured on Day 8 post-hatching in zoea I and on Day 8 post-moulting in zoea II larvae. To determine feeding rates, larvae were held individually in closed vials containing 10 ml of seawater at constant salinity of 32. One day prior to experiments, 6 vials per CO<sub>2</sub> treatment were placed at 4 different temperatures (3, 9, 15 and 21°C; 48 vials in total for zoea I and 48 vials for zoea II) in a temperature-controlled table providing a stable temperature gradient (custom-made by AWI workshop). These temperatures were chosen to cover the upper thermal limit where the CO<sub>2</sub> effect is supposed to be highest. Larvae of the Arctic population successfully developed at 3, 9 and 15°C (Walther et al. 2010). The highest temperature of 21°C is the highest temperature this species frequently experiences at their southernmost distribution limit during summer. Larvae were starved for 2 d at rearing temperature and then transferred to experimental temperatures for 1 d prior to feeding experiments. On Day 4, *Artemia* sp.

(Sanders Brine Shrimp Company), at a density of 10 shrimps per ml, were added to each vial containing one *H. araneus* larvae. After 24 h, larvae were carefully removed from the incubation vial, leaving *Artemia* sp. specimens behind. The remaining *Artemia* sp. individuals were counted under a microscope, and *H. araneus* larvae were killed by snap-freezing and stored at -20°C. Feeding rate is given as number of *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> ± SE (where 'ind.' refers to individual *H. araneus* larvae).

### Survival under starvation

Larvae were fed until Day 8 post-hatching in zoea I or until Day 8 post-moulting in zoea II as described above. Subsequently, larvae were transferred individually into closed vials containing 10 ml seawater of a constant salinity of 32. Individual rearing was necessary to avoid cannibalism during the starvation experiment. One day prior to experiments, 6 vials per CO<sub>2</sub> treatment were exposed to 4 different temperatures (3, 9, 15 and 21°C) in a temperature-controlled water bath. Seawater was provided from reservoir tanks (4.5 ± 0.1°C) that were injected with a defined air/CO<sub>2</sub> mixture. Water in the experimental vials was changed daily and checked for dead larvae. Larvae were considered dead when no heartbeat could be detected under a microscope.

### Statistical analyses

Results were analysed using GraphPad Prism 4 software. All data were checked for outliers by use of Nalimov's test (Noack 1980). The normality of the data set was tested according the Kolmogorov-Smirnov normality test and the data were tested for homogeneity of variances by Bartlett's test. A 2-way ANOVA was used to investigate the effects of CO<sub>2</sub> concentration and day of development on larval oxygen consumption and DW, as well as the effects of CO<sub>2</sub> concentration and temperature on survival under starvation and feeding rates. Bonferroni tests were used for *a posteriori* analyses. When a disordinal interaction between factors was detected, a 1-way ANOVA was run additionally for each CO<sub>2</sub> concentration to detect differences among days of development or temperatures. Tukey's multiple comparison tests were used for *a posteriori* analyses. An unpaired *t*-test was conducted to analyse the effect of CO<sub>2</sub> on egg respiration, zoea I development time and heartbeat rate and maxilliped beat rate in 50 d old



zoea I larvae. A multiple linear regression was calculated with DW and respiration as dependent variables using SigmaPlot (version 12, Systat Software). Differences in survival curves between control and high- $\text{CO}_2$  exposures were tested by a log-rank test using GraphPad Prism 4. Mean developmental times for larvae under control and elevated  $\text{CO}_2$  levels were determined from means of the 11 replicates using GraphPad Prism 4.

## RESULTS

### Mortality and developmental time (zoea I larvae)

Survival curves of larvae reared at control versus elevated  $p\text{CO}_2$  values were significantly different (log-rank test,  $p < 0.0001$ ) (Fig. 2). Larvae from females pre-exposed to high  $\text{CO}_2$  had a median survival ( $\text{LD}_{50}$ ) of 74 d, while no median survival could be determined for control larvae, as percent survival at the end of the study exceeded 50%. 90.1% of the larvae reared at control  $\text{CO}_2$  levels and 26.2% kept at high  $\text{CO}_2$  levels, survived the first zoea stage and moulted into zoea II (Fig. 2). Seawater  $\text{CO}_2$  concentration had a significant effect on the duration of the

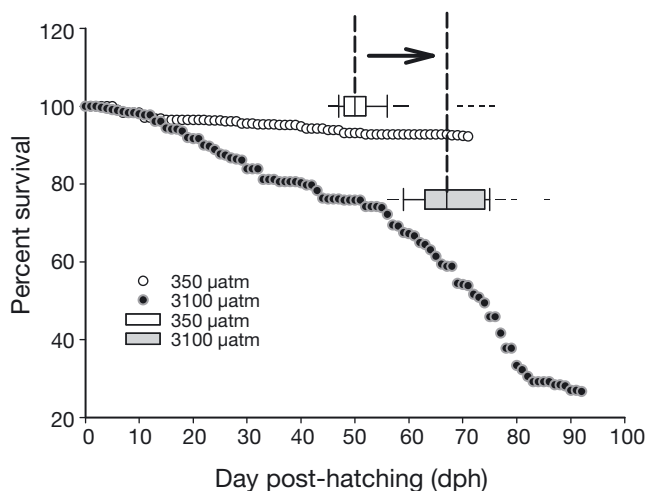


Fig. 2. *Hyas araneus*. Survival of zoea I larvae reared under 350  $\mu\text{atm}$   $\text{CO}_2$  (control) and 3100  $\mu\text{atm}$   $\text{CO}_2$  after 2 wk of pre-exposure of ovigerous females and eggs. Data were collected from hatching onward until larvae were either dead or moulted to the second stage. Box whisker plots show developmental time of zoea I larvae from hatching until moulting to the second stage. Box limits represent 25th and 75th percentiles, the line within the box marks the median and whiskers indicate 90th and 10th percentiles. Dots outside the whiskers represent outliers. Arrow indicates shift of developmental time of larvae reared at high seawater  $p\text{CO}_2$  in comparison to control larvae

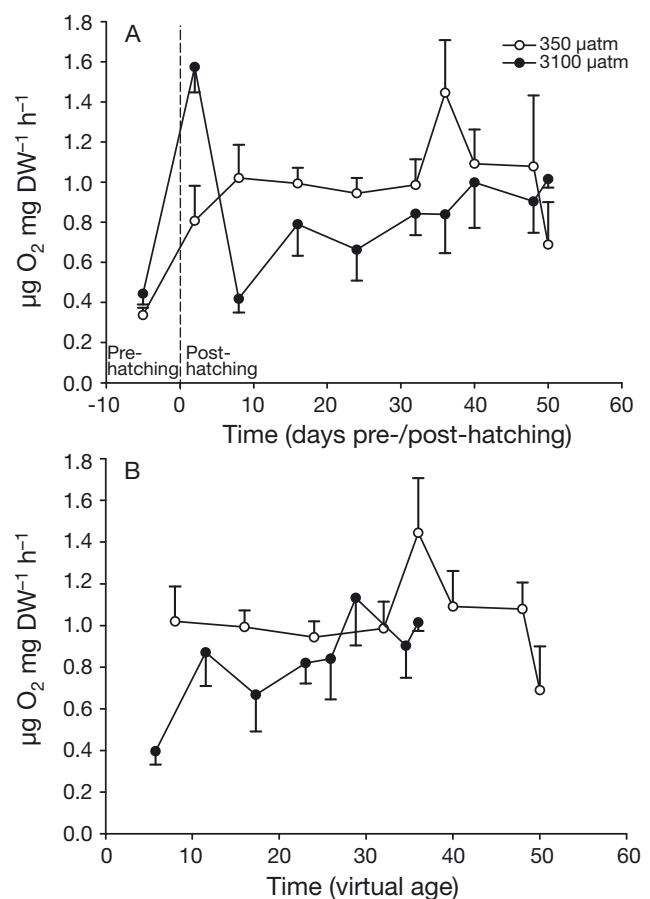


Fig. 3. *Hyas araneus*. (A) Weight-specific oxygen consumption of embryos (approximately 7 d pre-hatching) and zoea I larvae (post-hatching) under 350 and 3100  $\mu\text{atm}$   $\text{CO}_2$ . (B) Relationship between virtual age (days) and weight-specific oxygen consumption of zoea I larvae reared under 350 and 3100  $\mu\text{atm}$ . Data for Day 2 were excluded to improve the curve fit. Data are mean  $\pm$  SE ( $N = 8$ ). DW: dry weight

first zoea stage (unpaired  $t$ -test,  $p < 0.0001$ ). Stage duration until moulting into zoea II increased from  $47.7 \pm 0.8$  d under control conditions to  $68.1 \pm 6.2$  d at high  $\text{CO}_2$  levels (Fig. 2).

### Oxygen consumption (eggs and zoea I larvae)

There was no effect of seawater  $\text{CO}_2$  concentration on mean egg respiration rates (unpaired  $t$ -test,  $p = 0.1309$ ), which was  $0.33 \pm 0.03$  SE  $\mu\text{gO}_2$   $\text{mgDW}^{-1}$   $\text{h}^{-1}$  in eggs from females under control  $p\text{CO}_2$  and  $0.44 \pm 0.05$   $\mu\text{gO}_2$   $\text{mgDW}^{-1}$   $\text{h}^{-1}$  in eggs from the high- $\text{CO}_2$  treatment (Fig. 3A).

There was a significant but disordinal interaction in the 2-way ANOVA between day of development and  $\text{CO}_2$  concentration in zoea I larvae (Table 3),

Table 3. *Hyas araneus*. 2-way ANOVAs investigating effects of CO<sub>2</sub> concentration and larval age on oxygen consumption and dry weight of zoea I larvae. **Bold** values indicate statistical significance

Response variable	CO <sub>2</sub> effect			Day of development			Interaction		
	F	df	p	F	df	p	F	df	p
Oxygen consumption	2.662	1	0.1060	1.720	7	0.1130	2.583	7	<b>0.0174</b>
Oxygen consumption (Day 2 excluded)	8.096	1	<b>0.0055</b>	1.469	6	0.981	0.8920	6	0.5044
Dry weight	91.02	1	<b>&lt;0.0001</b>	53.74	7	<b>&lt;0.0001</b>	2.717	7	<b>0.0132</b>

which renders an interpretation of both main factors (CO<sub>2</sub> and day of development) impossible. The interaction was significant because larvae reared at high CO<sub>2</sub> showed higher respiration rates on Day 2 after hatching and lower rates during further development in comparison with control larvae. When data from Day 2 were excluded from the 2-way ANOVA, seawater CO<sub>2</sub> concentration had a significant effect on larval respiration rates, while day of development showed no effect (Table 3). There was a significant but disordinal interaction in the 2-way ANOVA between day of development and CO<sub>2</sub> concentration when egg respiration was included in the 2-way ANOVA, which renders a global interpretation of both main factors (CO<sub>2</sub> and day of development) impossible.

Subsequent 1-way ANOVAs revealed no significant change in respiration rates of zoea I larvae reared under control conditions (1-way ANOVA,  $p < 0.05$ ,  $F_{7,46} = 0.9124$ ) (Fig. 3A). Respiration rates ranged from 0.80  $\mu\text{gO}_2 \text{ mgDW}^{-1} \text{ h}^{-1}$  on Day 2 to a maximum of 1.44  $\mu\text{gO}_2 \text{ mgDW}^{-1} \text{ h}^{-1}$  on Day 36. Respiration rates changed slightly with development when egg respiration was included in the 1-way ANOVA ( $p = 0.0088$ ,  $F_{8,53} = 2.924$ ) and increased significantly from Day 5 pre-hatching to Day 36 post-hatching ( $p < 0.001$ ). There was a significant effect of age on overall respiration rates in zoea I larvae incubated at high CO<sub>2</sub> levels (1-way ANOVA,  $p < 0.05$ ,  $F_{7,52} = 3.506$ ). Application of an *a posteriori* Tukey's test showed a significant decrease in respiration rates from 1.57  $\mu\text{gO}_2 \text{ mgDW}^{-1} \text{ h}^{-1}$  on Day 2 to 0.41  $\mu\text{gO}_2 \text{ mg DW}^{-1} \text{ h}^{-1}$  on Day 8 ( $p < 0.001$ ), and constant rates between Day 8 and Day 48 ( $p > 0.05$ ). When egg respiration was included in the 1-way ANOVA, an *a posteriori* Tukey's test showed a significant increase between Day 5 pre-hatching and Day 2 post-hatching ( $p < 0.001$ ).

The zoea I stage duration was 1.37 times longer in the CO<sub>2</sub> treatment compared to the control treatment (Table 2). When related to virtual age, a multiple regression analysis indicated a dependence of oxygen consumption rates on seawater CO<sub>2</sub> concentra-

tion ( $p < 0.001$ ) (Fig. 3B) but not on time (virtual age) ( $p < 0.884$ ). Data for Day 2 were excluded from this analysis to improve the curve fit.

#### DW (zoea I larvae)

There was a significant ordinal interaction in the 2-way ANOVA between day of development and CO<sub>2</sub> concentration (Table 3). Both main factors (CO<sub>2</sub> and day of development) affected larval DW. Larval DW increased over time in both treatments (Fig. 4A). In zoea I larvae reared at control CO<sub>2</sub> levels, DW increased between Day 2 and Day 32 ( $p < 0.001$ ) and remained constant between Day 32 and Day 48 ( $p > 0.05$ ) (Fig. 4A). DW of larvae from the incubation at 3100  $\mu\text{atm}$  CO<sub>2</sub> increased significantly between Days 16 and 48 ( $p < 0.001$ ). Starting with the same DW as control larvae on Day 2, larvae reared in high CO<sub>2</sub> conditions showed a reduced DW during their development ( $p < 0.05$ ), but finally DW equalled that of control larvae on Day 48 (Fig. 4A). When related to virtual age, the linear regression in DW in larvae from both CO<sub>2</sub> concentrations was dependent on both factors, day ( $p < 0.001$ ) and seawater CO<sub>2</sub> concentration ( $p < 0.001$ ), with lower DW in larvae from the high-CO<sub>2</sub> treatment (Fig. 4B).

#### Oxygen consumption, heart rate and maxilliped activity (zoea I larvae)

Oxygen consumption of zoea I larvae at 50 d post-hatch did not differ between CO<sub>2</sub> treatments ( $p > 0.05$ ), while heartbeat rate was significantly reduced at elevated pCO<sub>2</sub> ( $p = 0.0061$ ) (Fig. 5). Heartbeat rate was  $97 \pm 5 \text{ beats min}^{-1}$  in larvae reared at control CO<sub>2</sub> and  $69 \pm 3 \text{ beats min}^{-1}$  in larvae reared at high CO<sub>2</sub> levels (Fig. 5). Maxilliped activity decreased significantly with elevated seawater pCO<sub>2</sub>, from  $85 \pm 15 \text{ beats min}^{-1}$  in control larvae to  $33 \pm 7 \text{ beats min}^{-1}$  in larvae under high CO<sub>2</sub> levels ( $p = 0.0394$ ) (Fig. 5).

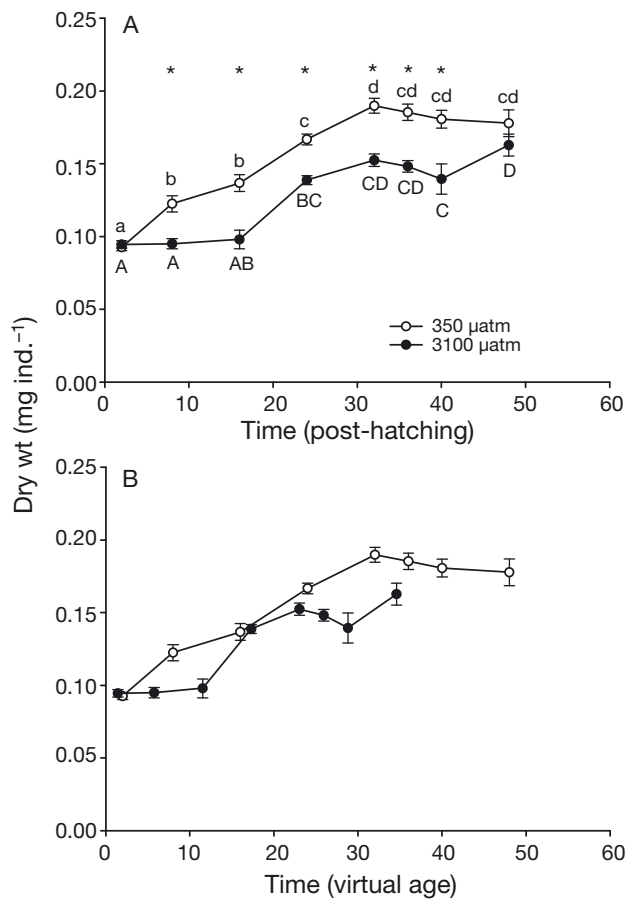


Fig. 4. *Hyas araneus*. (A) Dry weight of zoea I larvae reared under 350 and 3100  $\mu\text{atm}$   $\text{CO}_2$  during development. \*Significant differences ( $p < 0.05$ ) between treatments on the same developmental day. Different letters indicate significant differences ( $p < 0.05$ ) between days of development within one treatment (lowercase letters: 350  $\mu\text{atm}$   $\text{CO}_2$ ; uppercase letters: 3100  $\mu\text{atm}$   $\text{CO}_2$ ). (B) Relationship between virtual age (days) and dry weight of zoea I larvae reared under 350 and 3100  $\mu\text{atm}$   $\text{CO}_2$ . Data are mean  $\pm$  SE ( $N = 8$ )

#### Feeding rates (zoea I and II larvae)

Feeding rates of zoea I larvae (8 d post-hatching) were significantly affected by both  $\text{CO}_2$  concentration and temperature (Table 4). Under control conditions, feeding rates of zoea I larvae increased significantly from  $17 \pm 2$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> at 3°C to  $33 \pm 4$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> at 9°C ( $p < 0.01$ ), remained constant between 9 and 15°C ( $p > 0.05$ ), and decreased significantly at 21°C to  $17 \pm 2$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> ( $p < 0.01$ ) (Fig. 6A). Zoea I larvae reared under elevated  $\text{CO}_2$  levels displayed no significant changes in feeding rate with temperature, ranging from  $8 \pm 2$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> at 3°C to  $17 \pm 2$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> at 15°C ( $p > 0.05$ ) and were always below those of the

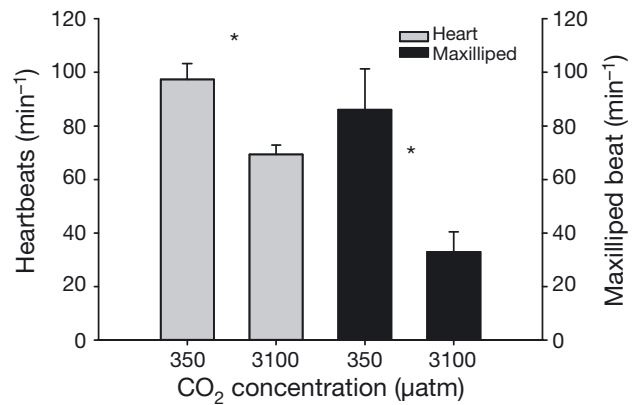


Fig. 5. *Hyas araneus*. Heartbeat rate and maxilliped beat rate of 50 d old zoea I larvae reared under 350 and 3100  $\mu\text{atm}$   $\text{CO}_2$ . \*Significant differences ( $p < 0.05$ ) between treatments. Data are mean  $\pm$  SE ( $N = 5$ )

control larvae. While seawater  $p\text{CO}_2$  had no effect on feeding rate at 3 and 21°C, feeding rates at 9 and 15°C were significantly reduced in zoea I larvae from the high- $\text{CO}_2$  treatment in comparison to control zoea I larvae (9°C:  $p < 0.001$ ; 15°C:  $p < 0.05$ ).

A similar trend could be found in zoea II larvae (8 d post-moulting). There was a significant ordinal interaction in the 2-way ANOVA between temperature and  $\text{CO}_2$  concentration (Table 4). Feeding rates of control larvae at 21°C were excluded from the ANOVA as no data were available from larvae raised at high  $\text{CO}_2$  levels at 21°C due to 100% mortality. Both  $\text{CO}_2$  concentration and temperature had a significant effect on zoea II feeding rates (Table 4). Feeding rates increased significantly with increasing temperature, from  $19 \pm 1$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> at 3°C to  $70 \pm 3$  *Artemia* ind.<sup>-1</sup> d<sup>-1</sup> at 15°C in larvae reared under control conditions ( $p < 0.01$ ) (Fig. 6B). In zoea II larvae raised at elevated  $p\text{CO}_2$ , feeding rates increased significantly between 3 and 15°C ( $p < 0.01$ ). There were no differences in feeding rates between 3 and 9°C or between 9 and 15°C ( $p > 0.05$ ), respectively. At 9 and 15°C, feeding rates of larvae reared under high  $p\text{CO}_2$  were significantly reduced in comparison to control zoea II larvae (9°C:  $p < 0.001$ ; 15°C:  $p < 0.001$ ).

#### Survival under starvation (zoea I and II larvae)

Temperature had a significant effect on the survival time of starved zoea I larvae (experiment started 8 d post-hatching, Table 4) with decreasing survival at increasing temperature (Fig. 7A). The



mean survival time of starved larvae incubated under control conditions decreased significantly from  $26 \pm 5$  d at 3°C to  $19 \pm 2$  d at 9°C and  $7 \pm 1$  d at 15°C ( $p < 0.01$ ). There was no difference in larval survival between 15 and 21°C ( $p > 0.05$ ). Survival of starved

zoea I larvae reared at high CO<sub>2</sub> levels decreased significantly between 3 and 9°C ( $p < 0.001$ ) and remained constant between 9 and 21°C ( $p > 0.05$ ). Seawater CO<sub>2</sub> concentration had no effect on the survival of starving zoea I larvae (Table 4).

Table 4. *Hyas araneus*. 2-way ANOVAs investigating effects of CO<sub>2</sub> and temperature on feeding rate and survival under starvation of zoea I and II larvae. **Bold** values indicate statistical significance

Response variable	CO <sub>2</sub> effect			Temperature			Interaction		
	F	df	p	F	df	p	F	df	p
Feeding rate of zoea I	29.612	1	<b>&lt;0.0001</b>	6.611	3	<b>0.0012</b>	1.583	3	0.2114
Feeding rate of zoea II	116.32	1	<b>&lt;0.0001</b>	55.39	2	<b>&lt;0.0001</b>	16.21	2	<b>&lt;0.0001</b>
Survival of starved zoea I	0.6109	1	0.4396	21.54	3	<b>&lt;0.0001</b>	2.227	3	0.1018
Survival of starved zoea II	3.423	1	0.0723	8.896	3	<b>0.0001</b>	0.568	3	0.6393

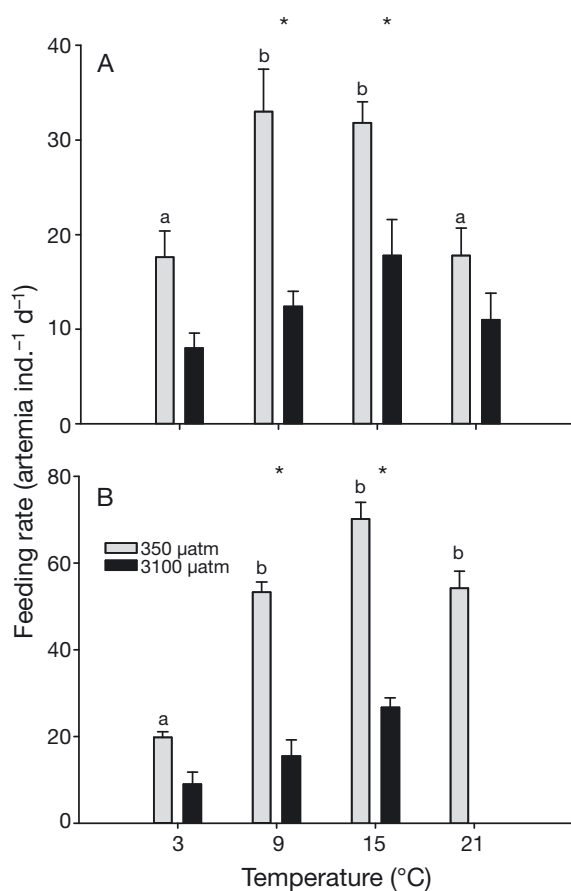


Fig. 6. *Hyas araneus* feeding on *Artemia* sp. Feeding rate (no. of *Artemia* ind.<sup>-1</sup> d<sup>-1</sup>, where 'ind.' refers to individual *H. araneus* larvae) of (A) zoea I and (B) zoea II larvae reared under 350 and 3100 µatm CO<sub>2</sub> at different temperatures. \*Significant differences ( $p < 0.05$ ) between treatments at the same experimental temperature. Different letters indicate significant differences ( $p < 0.05$ ) between temperatures within one treatment. Data are mean  $\pm$  SE (N = 6)

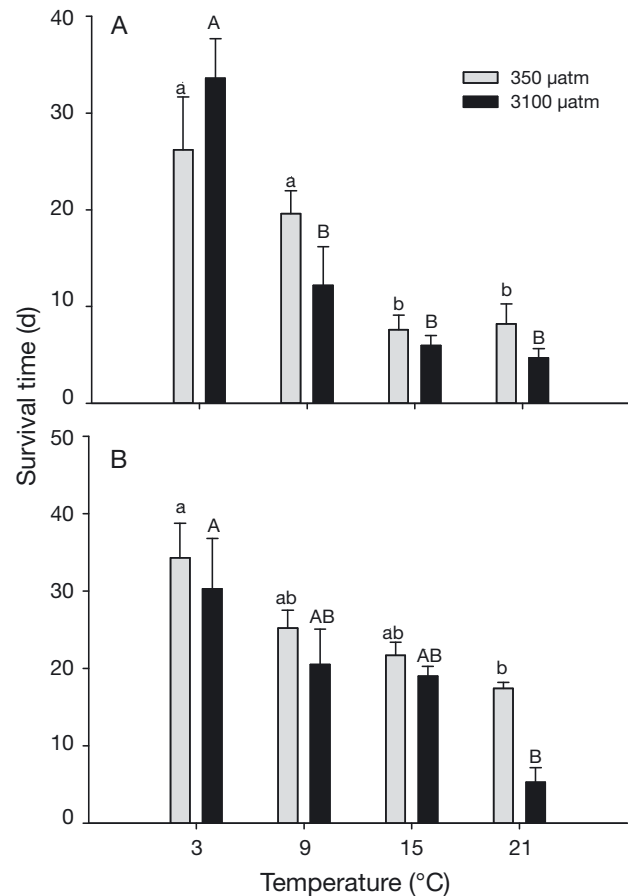


Fig. 7. *Hyas araneus*. Survival time of starved (A) zoea I and (B) zoea II larvae reared under 350 and 3100 µatm CO<sub>2</sub> at different temperatures. Different letters indicate significant differences ( $p < 0.05$ ) between treatments (lowercase letters: 350 µatm CO<sub>2</sub>; uppercase letters: 3100 µatm CO<sub>2</sub>). Note that there was no significant difference between treatments at the same experimental temperature. Data are mean  $\pm$  SE (N = 6)

Survival of zoea II *Hyas araneus* larvae (experiment started 8 d post-moulting) was also significantly affected by temperature (Table 4, Fig. 7B). Larvae from both CO<sub>2</sub> treatments showed a significantly decreased survival at 21°C compared to survival at 3°C (350 µatm CO<sub>2</sub>;  $p < 0.05$ ; 3100 µatm CO<sub>2</sub>;  $p < 0.001$ ) (Fig. 7B). Survival time under starvation decreased from 34 ± 4 and 30 ± 6 d at 3°C to 17 ± 0.5 and 5 ± 1 d at 21°C under control and high CO<sub>2</sub> conditions, respectively. Seawater CO<sub>2</sub> concentration had no effect on survival of starved zoea II larvae (Table 4).

## DISCUSSION

The early embryonic development of Arctic *Hyas araneus* is unusual, as the total time span of approximately 2 yr between the spawning of eggs and the hatching of larvae is exceptionally long (Petersen 1995). Therefore, it was surprising that exposure of females with late-stage eggs to high seawater *p*CO<sub>2</sub> values, during a comparatively short period of 2 wk, had fundamental consequences for larval performance, indicated by high mortality, prolonged developmental time and lower DW increment in the first larval stage (zoea I).

These findings indicate carry-over effects between embryonic and larval stages, as has been found in other studies (Kurihara et al. 2004). Comparing high mortality (over 70%) and prolonged developmental duration after pre-hatch exposure of *Hyas araneus* zoea I larvae to ocean acidification studies on other species and taxa is difficult because of varying experimental conditions such as seawater CO<sub>2</sub> concentrations, temperature and incubation time. However, the sea urchin *Strongylocentrotus droebachiensis* revealed extremely high mortality (95%) of juveniles produced from gametes of adults acclimated to elevated seawater *p*CO<sub>2</sub> of 1200 µatm at 12°C for 4 mo when larvae and juveniles were reared at the same high seawater CO<sub>2</sub> (Dupont et al. 2013). In contrast, Parker et al. (2012) showed a positive effect on larval developmental time in oysters after 5 wk of exposure, when adults were pre-exposed to an elevated level of seawater *p*CO<sub>2</sub> of 850 µatm at 24°C. The authors suggested that an increase in the maternal energy investment due to environmental stress might help to compensate or reduce the negative effects of elevated seawater *p*CO<sub>2</sub> on the larvae (Parker et al. 2012). Studies regarding effects of elevated seawater CO<sub>2</sub> concentrations on more than one life history stage of crustaceans have mainly focused on cope-

pods (Kurihara et al. 2004, Kurihara & Ishimatsu 2008). Neither the egg production nor hatching rate and egg survival of the copepod *Acartia tsuensis* was affected by elevated seawater *p*CO<sub>2</sub> of 2000 µatm CO<sub>2</sub> (Kurihara & Ishimatsu 2008). A negative effect on hatching rate and nauplius mortality could only be found at extremely high seawater *p*CO<sub>2</sub> values of 5000 and 10000 µatm CO<sub>2</sub> in the copepod *A. erythraea* raised at 27°C for 8 d (Kurihara et al. 2004). A relatively high seawater CO<sub>2</sub> concentration was used in the present study, similar to previous studies on the effect of elevated seawater *p*CO<sub>2</sub> on *H. araneus* zoea (Walther et al. 2010, 2011, Schiffer et al. 2013). The comparatively high mortality of zoea I larvae of *H. araneus* pre-exposed to elevated seawater *p*CO<sub>2</sub> indicates that the embryonic stage is a potential physiological bottleneck within the life cycle of *H. araneus*, and negative effects can be attributed to carry-over effects from embryos to larvae. Further experiments are needed to verify these results at lower seawater CO<sub>2</sub> concentrations. Embryos of the marine porcelain crab *Petrolisthes cinctipes* showed higher sensitivity towards an elevated seawater *p*CO<sub>2</sub> of 1300 µatm in comparison to zoea larvae, indicated by lower metabolism and DW of embryos exposed to elevated seawater *p*CO<sub>2</sub> (Carter et al. 2013).

A previous study on post-hatch effects of elevated seawater *p*CO<sub>2</sub> on the first larval stage of *Hyas araneus* strengthens the assumed hypothesis of adverse carry-over effects between the embryonic and the first larval stage (Schiffer et al. 2013). In contrast to the present study, there was no effect of post-hatching exposure to elevated seawater *p*CO<sub>2</sub> on developmental duration and survival of zoea I larvae, while oxygen consumption and growth were only slightly affected (Schiffer et al. 2013). Experimental conditions were, however, slightly different between the present and the previous study. The rearing temperature in the present study was approximately 4.5°C, while larvae were reared at approximately 6.1°C in the previous study (Schiffer et al. 2013). Furthermore, zoea larvae of *H. araneus* were exposed to a seawater CO<sub>2</sub> concentration of 2400 µatm post-hatching (Schiffer et al. 2013), while the pre- and post-hatching seawater *p*CO<sub>2</sub> was 3100 µatm in the present study. While 73% of zoea I larvae died when pre-exposed to elevated seawater *p*CO<sub>2</sub> of 3100 µatm during their embryonic phase, only 13% of larvae died during the first zoea stage after post-hatch exposure to 2400 µatm (Schiffer et al. 2013). Thus, the lower survival of pre-exposed zoea I larvae could be due to the higher seawater *p*CO<sub>2</sub> of 3100 µatm

used for the present experiments. However, post-hatching exposure to even higher seawater CO<sub>2</sub> levels of 3300 µatm at 10°C did not considerably increase zoea I mortality in a temperate population of *H. araneus* (M. Schiffer et al.). Total mortality was 15% in larvae exposed to a control pCO<sub>2</sub> of 420 µatm and 17% in zoea I larvae exposed to 3300 µatm (M. Schiffer et al. unpubl.). This is further supported by studies by Walther et al. (2010, 2011), who found no effect of elevated seawater pCO<sub>2</sub> (2400 µatm) on mortality or growth of the first zoea stages in *H. araneus*. However, zoea I larvae of *H. araneus* exhibited a prolonged developmental time when reared at a high seawater pCO<sub>2</sub> of 2400 µatm at 3°C post-hatching, while CO<sub>2</sub> effects on developmental time vanished at 6, 9 and 15°C (Walther et al. 2010, Schiffer et al. 2013). Zoea I development was prolonged from 61.4 to 66.8 d at 2400 µatm CO<sub>2</sub> and 3°C (Walther et al. 2010). In the present study, developmental duration of zoea I larvae was extended by approximately 20 d, from 47.7 to 68.1 d, at a rearing temperature of 4.5°C, when larvae were exposed to elevated seawater pCO<sub>2</sub> during maternal care. The results indicate a CO<sub>2</sub> effect on the development of zoea I larvae of *H. araneus* at low temperatures when the zoeal development between zoea I and zoea II is comparatively long. This effect might be enhanced by pre-hatch exposure of zoea larvae to elevated seawater pCO<sub>2</sub>.

To our knowledge, this is the first study showing lower DW increment under elevated seawater pCO<sub>2</sub> levels in the first larval stage of decapod crustaceans. Differences in DW increment between control and CO<sub>2</sub>-treated crustacean larvae have so far only been found in late developmental stages (Arnold et al. 2009, Walther et al. 2010). Larval DW is a suitable indicator for growth and feeding in crustacean larvae and reflects the level of food supply. Especially during the first 2 wk of development, the DW increment was less in *Hyas araneus* zoea I exposed to elevated pCO<sub>2</sub> than in control larvae (3% compared to 50% between Day 2 and Day 16), indicating decreased growth and low feeding rate. Energy depletion and energy accumulation and the associated growth and weight increment are highly relevant factors, substantially shaping crustacean larval survival and developmental duration (Anger & Dawirs 1981, Anger 1987). Arctic *H. araneus* larvae are planktonic and depend on immediate food supply for successful development. Earlier studies indicated the crucial importance of sufficient food supply for development and survival of crustacean larvae (Anger & Dawirs 1981, Dawirs 1983). The influence of starvation on larval survival and development became par-

ticularly evident when larvae were starved at the beginning of their development (Anger & Dawirs 1981). Zoea I mortality in *H. araneus* from the North Sea was over 90% when feeding was prevented on the second day post-hatching. However, after Day 4 post-hatching, starvation did not influence survival to the second stage. Feeding conditions also affected the duration of larval development in zoea I. Development was almost twice as long in larvae starved during the first 6 d post-hatching than under normal feeding conditions (Anger & Dawirs 1981).

Following this reasoning, prolonged developmental time, high mortality and decreased growth may relate to low feeding rates. Indeed, our experiments showed that *Hyas araneus* zoea I and zoea II larvae experiencing elevated seawater pCO<sub>2</sub> during their embryonic phase, displayed reduced feeding rates in early zoea I and zoea II larvae and reduced activity (maxilliped beat rates) in older zoea I larvae. At elevated pCO<sub>2</sub>, lower feeding rates were accompanied by lower oxygen consumption rates, indicating lower energy demands, possibly due to pH-induced metabolic depression, which then involves lower maxilliped activity for oxygen supply. Less energy might also be used for swimming and feeding. Lowered energy demand is also indicated by lower heart rates in 50 d old, pCO<sub>2</sub>-treated larvae. This is consistent with results from Ellis et al. (2009) and Chan et al. (2011) showing that exposure to elevated pCO<sub>2</sub> affects the activity levels and feeding of embryos and hatched larvae of marine ectotherms. At a seawater pCO<sub>2</sub> of 1100 µatm CO<sub>2</sub> during embryogenesis, embryos of the intertidal snail *Littorina obtusata* spend more time stationary instead of crawling and displayed slower rotation rates (Ellis et al. 2009). In *H. araneus* larvae, the differences in feeding rates between controls and CO<sub>2</sub> treatment were more pronounced at 3, 9 and 15°C than at 21°C. At 21°C, feeding rates of control larvae decreased and no differences were found between larvae from the 2 CO<sub>2</sub> treatments. Within the thermal window, increasing oxygen consumption rates with increasing seawater temperature indicates higher metabolic costs for maintenance in *H. araneus* zoea larvae (Jacobi & Anger 1985). Higher metabolic costs were met up to a certain temperature by increased feeding rates in control larvae, but not in larvae kept at high pCO<sub>2</sub> levels. Zoea of *H. araneus* are carnivorous as well as herbivorous (Meyer-Harms & Harms 1993). Zoea larvae of brachyuran crabs catch live prey by using the telson to scoop up the prey and hold it from below (Gonor & Gonor 1973). Good swimming abilities are a crucial prerequisite for successful foraging and to

actively swim in the upper water column. However, elevated temperatures as used in feeding experiments can influence animal performance, which depends on aerobic scope (Pörtner & Farrell 2008). Elevated seawater  $p\text{CO}_2$  might limit the scope for aerobic performance at elevated temperature in larvae reared at high seawater  $\text{CO}_2$  and might prohibit an increase in activity levels and thereby, appropriate feeding rates. A reduction of aerobic scope at thermal extremes has already been shown by Walther et al. (2009) for *H. araneus* adults exposed to elevated seawater  $p\text{CO}_2$ .

To sum up, we assume that the high mortality and prolonged development found in *Hyas araneus* zoea I larvae reared at elevated  $p\text{CO}_2$  can partly be attributed to reduced feeding rates. This assumption is further supported by the fact that the seawater  $p\text{CO}_2$  did not affect survival time of *H. araneus* zoea I and zoea II once starved. Then, temperature but not  $\text{CO}_2$  concentration determines survival time. Hence, sustained feeding would support larval survival under elevated seawater  $\text{CO}_2$  concentrations. Reduced feeding and developmental delay at elevated seawater  $p\text{CO}_2$  have also been found in sea urchin larvae (Stumpp et al. 2011). Larvae of similar size had a comparable feeding efficiency, indicating that reduced feeding paralleled the developmental delay. In the present study, feeding rates in *H. araneus* zoea I larvae were solely investigated on Day 8 post-hatching, when larvae reared at high  $p\text{CO}_2$  had reached a virtual age of 6 d. Hence, reduced feeding rates and a lower increase in DW might cause the developmental delay in larvae reared at elevated  $p\text{CO}_2$ . We hypothesise that elevated  $p\text{CO}_2$  causes developmental delay through reduced feeding or vice versa, mirroring a coordinated reduction of both.

## CONCLUSIONS

We investigated how the exposure of ovigerous females and their embryos to a late ocean-acidification scenario affects the subsequent larval stages of the spider crab *Hyas araneus*. We demonstrated that after such pre-exposure, the survival and development of the first zoea stage is highly affected by elevated seawater  $p\text{CO}_2$ . In contrast, there was no effect of seawater  $p\text{CO}_2$  on the survival and development of zoea I acutely exposed to different seawater  $p\text{CO}_2$  values (Schiffer et al. 2013). Our data collected under elevated  $\text{CO}_2$  levels demonstrate the need to focus on early ontogenetic development across various life stages rather than on selected life stages.

Sensitivities of different life stages to  $\text{CO}_2$  are co-defined by their preceding life stages or the transition from one stage to the next. Carry-over effects between life stages and/or  $\text{CO}_2$ -induced disturbances of the transition phases from one stage to the next have the potential to severely impact species survival in a high- $\text{CO}_2$  world.

**Acknowledgements.** Financial support was provided by the Federal Ministry of Education and Research (BMBF), Germany, within phase I of the BIOACID research programme (FKZ 03F0608B, subproject 2.2.1 PI D. Storch). We thank the scientific divers of the Alfred Wegener Institute for animal collection, and L. Preis, P. Augustin, M. Zhu and P. Schulze for technical assistance.

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Submitted: January 28, 2013; Accepted: December 9, 2013  
Proofs received from author(s): March 7, 2014