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Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.)

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Abstract

Oceanic uptake of anthropogenic CO_2 results in a reduction in pH termed "Ocean Acidification" (OA). Comparatively little attention has been given to the effect of OA on the early life history stages of marine animals. Consequently, we investigated the effect of culture in CO_2-acidified sea water (approx. 1200 ppm, i.e. average values predicted using IPCC 2007 A1F1 emissions scenarios for year 2100) on early larval stages of an economically important crustacean, the European lobster *Homarus gammarus*. Culture in CO_2-acidified sea water did not significantly affect carapace length or development of *H. gammarus*. However, there was a reduction in carapace mass during the final stage of larval development in CO_2-acidified sea water. This co-occurred with a reduction in exoskeletal mineral (calcium and magnesium) content of the carapace. As the control and high CO_2 treatments were not undersaturated with respect to any of the calcium carbonate polymorphs measured, the physiological alterations we record are most likely the result of acidosis or hypercapnia interfering with normal homeostatic function, and not a direct impact on the carbonate supply-side of calcification per se. Thus despite there being no observed effect on survival, carapace length, or zoeal progression, OA related (indirect) disruption of calcification and carapace mass might still adversely affect the competitive fitness and recruitment success of larval lobsters with serious consequences for population dynamics and marine ecosystem function.

1 Introduction

The ocean is a substantial reservoir of CO_2 (Feely et al., 2004; Sabine et al., 2004; Morse et al., 2006). Addition of CO_2 to sea water alters the carbonate chemistry and reduces pH, in an effect recently termed "Ocean Acidification" (OA). Over the past 200 years increasing pCO_2 has resulted in a decrease in surface sea water pH of 0.1 units (Caldeira and Wickett, 2003; Orr et al., 2005). It is predicted that atmospheric CO_2 concentrations could reach 1200 ppm by the year 2100 resulting in a decrease

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in average surface ocean pH of 0.3 to 0.4 units (Caldeira and Wickett, 2003; Raven et al., 2005). Our understanding of the biological and ecological consequences of OA is, however, still in its infancy (Raven et al., 2005). Reductions in seawater pH have been demonstrated to affect the physiological and developmental processes of a number of marine organisms (e.g. Pörtner et al., 2004, 2005; Raven et al., 2005) through reduced internal pH (acidosis) and increased CO_2 (hypercapnia), raising the possibility that not only species, but also ecosystems, will be affected (Widdicombe and Spicer, 2008).

As CO_2 increases and pH decreases there is a concomitant reduction in carbonate ion (CO_3^{2-}) availability, which can lead to increased dissolution of calcium carbonate ($CaCO_3$) structures. This may have a significant impact on species, which have $CaCO_3$ skeletons, such as reef building organisms, some phytoplankton, molluscs, crustaceans and echinoderms. Research into the effects of OA has thus far primarily investigated impacts on these calcareous marine organisms, particularly focusing on corals (e.g. Reynaud et al., 2003; Langdon and Atkinson, 2005), molluscs (e.g. Michaelidis et al., 2005; Gazeau et al., 2007) and coccolithophores (e.g. Riebesell, 2000; Zondervan et al., 2002; with reviews by Paasche, 2001; Hinga, 2002; and Riebesell, 2004). Studies to date have demonstrated a number of potentially important effects including reduced growth rates (Gazeau et al., 2007), decreased reproductive success (Kurihara et al., 2004b), shell dissolution (Bamber, 1990) as well as acidification of internal body fluids (Spicer et al., 2007), compromise of induced defences (Bibby et al., 2007), increased susceptibility to infection (Holman et al., 2004) and impairment of immune function (Bibby et al., 2008). Unfortunately the majority of these studies have investigated short-term responses with most attention being paid to effects on adult organisms alone. Thus there is an urgent need, both for more long-term data, especially for invertebrates (Langenbuch and Pörtner, 2002; Pörtner et al., 2004; Michaelidis et al., 2005) and studies of species at different stages of their larval development as well as on adults (Widdicombe and Spicer, 2008). Research into the effects during larval stages has mainly focused on echinoderms (Kurihara and Shirayama, 2004, Havenhand et al., 2008; Dupont et al., 2008), fish (Ishimatsu et al.,

2004), copepods (Kurihara et al., 2004b; Kurihara et al., 2007), amphipods (Egilsdottir et al., 2009) and gastropods (Ellis et al., 2009) However, it is important to investigate potential effects of CO_2-induced acidification during early life stages, as they are likely to be more sensitive to such environmental stressors than adults (e.g. Kikkawa et al., 2003; Ishimatsu et al., 2004), especially as many benthic species possess pelagic larval stages which occur in the surface waters where increasing pCO_2 is occurring first (Calderia and Wickett, 2003). Determining how OA might affect larval development of benthic organisms, particularly those that initiate calcification processes while still in their planktonic phase, is critical to predicting how these impacts might propagate through the ecosystem. Early investigations suggest that early life stage development may be slowed (Egilsdottir et al., 2009; Findlay et al., 2009) or even completely disrupted (Dupont et al., 2008; Havenhand et al., 2008) at CO_2 levels predicted for the end of this century.

The European lobster *Homarus gammarus* is an economically important species contributing substantially to the continued survival of small coastal communities. Calcification in the Norway lobster *Nephrops norvegicus*, closely related to *H. gammarus*, occurs during very early development, with a major change in the pattern of calcification observed as the individual makes the transition from a planktonic zoea to a benthic postlarva (Spicer and Eriksson, 2003). While crustacean exoskeletons contain $CaCO_3$, this most likely predominates in the more soluble polymorph, magnesium calcite ($MgCaCO_3$) (Boßelmann et al., 2007). It is believed that crustaceans utilise either CO_2 or bicarbonate (HCO_3^-), not carbonate (CO_3^{2-}) as the primary source of carbon for the formation of their $CaCO_3$ structures (Cameron, 1989). Therefore reduction in CO_3^{2-} as a result of OA may not always be expected to directly impact their ability to produce $CaCO_3$. *H. gammarus* is cultured within hatcheries to aid commercial catch and hence is an ideal model to use to understand the impacts that OA might have on the early development stages of a commercially important crustacean. Consequently, this study investigates the effect of CO_2-induced acidification on the early life stages of *H. gammarus*. We have followed aspects of growth and development through early ontogeny

and investigated changes in calcium and magnesium concentrations in the carapace of developing lobsters. As Mg-$CaCO_3$ is more soluble than other polymorphs of calcium carbonate, aragonite and calcite, (Feely et al., 2004) it may be less advantageous to form Mg-$CaCO_3$ under decreasing pH as it will be the first to dissolve (Kleypas et al., 2006), hence these lobsters may be at greater risk from dissolution. Exposure to long-term hypercapnia may be energetically costly to marine organisms and therefore may be detrimental to developmental processes, such as growth, reproduction, natural recruitment and survival (Barry et al., 2005; Wood et al., 2008).

2 Materials and methods

2.1 Animal material

Ovigerous females were supplied by local fishermen and held in aquaria (160×100×35 cm) at the National Lobster Hatchery (NLH) in Padstow, Cornwall, UK. Each aquarium was constantly supplied with aerated, filtered re-circulating sea water (T=19±1°C, S=35) pumped directly from waters adjacent to the NLH. Water was pre-treated in a pressurized sand filter, passed through activated carbon, and finally UV-irradiated. Adult lobsters were fed ad libitium with blue mussels, *Mytilus edulis*. When required, newly-hatched, free-living larvae were removed from aquaria and used as described below. Experiments were carried out between June and July 2007, to coincide with the natural hatching season (between April and September).

Newly-hatched Zoea I larvae, from different mothers, were (carefully) distributed haphazardly between a number of aquaria (flasks vol.=1 l; N=50 zoea per flask; T=19±1°C), which contained one of the following aerated media: sea water ("untreated control") or sea water with elevated CO_2 (1200 ppm) (N=5 for each treatment). Media changes were performed every 24 h, with flasks left to acclimate for 2 h, or until the required CO_2 concentration had been reached, before larvae were added. Both moulted exoskeletons and mortalities were removed before changing media. Larvae were fed

(*Artemia nauplii*, density = 5 indiv.ml^{-1} sea water) after media changes (Carlberg and Van Olst, 1976). Sea water was placed in ten flasks (vol.=1 l) and the CO_2 concentration was modified by equilibrating the water with air containing different CO_2 concentrations exactly as described by Findlay et al. (2008). Air/CO_2 mixtures were produced using a bulk flow technique where known amounts of scrubbed air (CO_2 removed using KOH) and CO_2 gas were supplied, via flow meters (Jencons, UK, Roxspur, France), and mixed before equilibrating with sea water. Control flasks were aspirated (10 l min^{-1}) with air containing 365 ppm of CO_2. To produce the reduced pH treatment, sea water was aspirated (1 ml min^{-1} CO_2 mixed with 10 l min^{-1} scrubbed air) with the high-CO_2 air containing 1200 ppm of CO_2. The CO_2 concentration chosen for the elevated exposure is close to increases expected in surface ocean waters under "business as usual scenarios" by the year 2100 (Caldeira and Wickett, 2003; Raven et al., 2005). The pCO_2 was monitored regularly using a pCO_2 micro-electrode (LazarLabs) and pH using a pH probe (Denver). Any fluctuations in pH were noted, and adjusted, via the flow meters, accordingly.

2.2 Larval growth and survival

Larvae were sampled haphazardly from each flask (N=9), at four different stages in their development (i.e. Zoea I, II, III, and IV). Carapace length (CL) and carapace area (CA) were measured under lower power magnification (×10) using ImageJ software. Larval survival was recorded daily and any morphological differences observed were recorded. Moult stage was determined using the schemes of Aiken (1973) and Chang et al. (2001), which involve detailed examination of the exoskeleton and pleopods.

2.3 Measurements of mineral content

Measurements of the calcium and magnesium content of the carapace from individuals in each treatment (N=9), for each of the four developmental stages (Zoea I, II, III, and IV), were made using Inductively Coupled Plasma Spectrometry (ICP). Indi-

viduals were washed briefly in distilled water, carefully blotted dry with paper tissue, and stored frozen ($T = -20^{\circ}$C). They were subsequently freeze-dried (LYOVAC GT2, Leybold-Heraeus, Germany) to a constant mass, before the carapace was carefully removed and weighed using a microbalance (AT200, Mettler-Toledo, Switzerland). The carapace was dissolved in concentrated nitric acid (75% pro analysis) to extract the mineral portion. The resultant solution was diluted with Milli-Q water before ICP analysis. These concentrations are expressed as percentage of total mass of animal carapace and also per unit of total carapace area, exactly as presented by Spicer and Eriksson (2003).

2.4 Statistical analysis

Data were expressed as mean ±1 S.E.; the data were tested for normality using the Kolmogorov-Smirnov test and homogeneity of variances and applying Levene's test prior to analysis. Two-way repeated-measures ANOVA was used to investigate significant differences in physiological parameters as a result of CO_2 and of exposure time, and least significant difference post hoc tests were carried out to assess the significance of differences between treatment groups.

3 Results

There was no significant effect of hypercapnia on carapace length or development ($p>0.05$ in each case) with both remaining coupled throughout their respective experimental exposures (circa 28 days) (Fig. 1). There was however, an effect of CO_2 on carapace mass over time, causing a significant reduction in mass at Zoea IV ($p<0.05$, Fig. 2).

Changes in calcium concentration of the carapace during larval development are presented in Fig. 3. The calcium content in the carapace was significantly effected over time, with respect to treatment, this was detectable both when expressed as con-

centration per surface area ($p<0.05$; Fig. 3a) and when presented as a percentage of the total carapace content ($p<0.05$; Fig. 3b). In the high CO_2 treatment, the calcium concentration was almost half of the control at Zoea IV ($0.13\,\mu g\,l^{-1}\,mm^{-2}$ S.E. 0.01 vs. $0.23\,\mu g\,mm^{-2}$ S.E. 0.01). This indicates that after metamorphosis into Zoea IV the increased CO_2 significantly impacted the carapace calcium content.

The effect of increased CO_2 on magnesium in the carapace, when expressed as a concentration per surface area (Fig. 4a), produced a reduction in concentration over time when compared to the control, with significant differences occurring at Zoea IV ($p<0.05$). Reduction in magnesium concentration, due to culture in CO_2-acidified sea water, were also apparent when expressed as a percentage of total carapace content ($p<0.05$), with significant differences evident at Zoea III (Fig. 4b); 0.81% (S.E 0.07) compared to 1.16% (S.E 0.13) in the control larvae.

4 Discussion

This is the first study, to the authors' knowledge, to follow the pattern of calcification during larval development of any lobster species. Calcium and magnesium form the principal mineral portion of the crustacean exoskeleton (Neufeld and Cameron, 1992); the concentrations of which are most likely to be determined by environmental conditions (Wickins, 1984). The pattern of calcification displayed by *H. gammarus* generally follows an increase in calcium as the larvae moult through each progressive zoea, with metamorphosis into the Zoea IV containing the largest concentration of calcium. However when expressed as a percentage of mass, the proportion of calcium in relation to other exoskeletal components stays fairly constant throughout larval development. The only other study to investigate calcification in larval lobsters was completed by Spicer and Eriksson (2003), on the Norway lobster *Nephrops norvegicus*. However, only Zoea III was examined and while *N. norvegicus* Zoea III had $1.7\,Ca\,\mu g\,mm^2$ (4.3% of mass), *H. gammarus* had $0.2\,Ca\,\mu g\,mm^2$ (9.7% of mass). *H. gammarus* Zoea III values, in term of calcium concentration per surface area, were much lower than those found in

N. norvegicus. However, the percentage of calcium in relation to mass was double the value in *H. gammarus* compared to *N. norvegicus*, indicating that the processes involved in calcification may be reasonably different between the two species. Magnesium concentrations were also measured and similarly displayed an increase with development; although the ratios of calcium to magnesium can be variable in many marine calcifying species (Boßelmann et al., 2007). It is hypothesised that in crustaceans, magnesium is often used as a substitute for calcium in the mineral matrix of the exoskeleton (Richards, 1951). However, when the percentage of calcium increased at Zoea IV the percentage of magnesium decreased, possibly showing that calcium plays a more important role during the final stages of development in *H. gammarus*.

This is also the first study to examine the effects of CO_2-induced acidification on development and calcification of any lobster species. Increased moult frequency and high rates of mortality are associated with larval development; therefore these early stages may be particularly vulnerable to ocean acidification due to an increased energy requirement for calcification of the exoskeleton (Haugan et al., 2006). Certain morphological parameters were measured in the present study (carapace length and mass) along with determining calcium and magnesium concentrations, as they all provide proxies of an organism's ability to grow and calcify (Findlay et al., 2009). Survival, zoeal progression, and carapace length in *H. gammarus* were not significantly affected by culture in CO_2-acidified sea water (1200 ppm CO_2), with all remaining coupled throughout. However, both carapace mass and calcification were considerably reduced during metamorphosis into Zoea IV due to increased CO_2. The fact that growth (length) was not affected by culture in CO_2-acidified sea water even though both mass and mineral content did appear to decrease, is feasible because Spicer and Eriksson (2003) indicates that the majority of growth occurs through laying down organic material and chitin and not $CaCO_3$. The reduced carapace mass observed was therefore most likely due to CO_2-induced acidification resulting in a decline in the net production of $CaCO_3$, which would potentially result in a lighter carapace.

It is thought that lobsters utilize HCO_3^- or CO_2 for the precipitation of $CaCO_3$

(Cameron, 1989) not CO_3^{2-} therefore if the experimental conditions had resulted in undersaturation with respect to carbonate minerals, we still might not expect a direct impact on calcification in larval lobsters. However, the sea water in both the control and the elevated CO_2 treatment had relatively high levels of CO_3^{2-}, and neither became low enough to cause undersaturation with respect to any of the calcium carbonate polymorphs relevant here ($\Omega_{aragonite}$>2.5; Table 1). This seems to occur as a result of naturally high alkalinity levels in the sea water (>2450 μEq kg^{-1}), and indicates that neither CO_3^{2-} or HCO_3^- were limiting calcification, nor was there any indication of enhanced dissolution. One caution to these results is that pH and pCO_2 were measured during the experiment but total alkalinity and DIC were calculated using CO2sys (Pierrot et al. 2006), with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 using Dickson (1990), and therefore these may not represent exact values, particularly as the sea water comes from a coastal environment. The increased CO_2, added through gas bubbling, increased the total dissolved inorganic carbon, and hence lowered the pH, although not below levels perceived to be "normal". Therefore the observed impacts on mass and reduced calcium concentration in these larval *H. gammarus* may primarily be the result of hypercapnia interfering with normal homeostatic function (perhaps via a trade-off), and not a direct impact on calcification per se, e.g. general physiological stress can result in a reduction in the energy allocated to shell thickening (Henry et al., 1981). A similar study by Gazeau et al. (2007), using comparable CO_2 values, also displayed a net decline in the calcium carbonate structure in the mussel, *Mytilus edulis*, and the Pacific oyster, *Crassostrea gigas*. As in this study, they too found that calcium carbonate polymorphs did not become undersaturated, but did suggest that saturation states were correlated with net calcification rates. Here we suggest that saturation states may not always be the main limiting factor for calcification under increasing CO_2, as the processes and mechanisms for calcification in multi-cellular organisms are complex and are closely linked with many other physiological processes (Portner, 2008). As the solubility of a calcite structure increases with the incorporation of magnesium, under increasing CO_2 concentrations

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(Raven et al., 2005), several species have the ability to secrete a lower concentration of magnesium in response to environmental changes (Stanley et al., 2002). However, as the decrease in magnesium ions was fairly consistent with the decrease in calcium ions it seems unlikely that this mechanism for dealing with an altered environment was occurring in the present study.

CO_2-induced acidification affected the calcified exoskeleton in late zoea larval stages, which is arguably the most critical period for production of viable post-larvae. There has been no examination of the effects of OA on post-larval lobsters completed to date. As OA is predicted to occur over ocean surface waters, with a great degree of certainty, and corrosive waters are already seasonally observed in shelf sea upwelling areas (Feely et al., 2008) and around major rivers (Salisbury et al., 2008), marine organisms inhabiting the pelagic zone will be unable to avoid these unfavourable changes in ocean chemistry (Haugan, 1997).

From most studies on marine organisms to date there appears to be a substantial cost involved with increased CO_2 on developmental processes, whether it be decreased calcification or shell dissolution in order to maintain internal chemistry (Gazeau et al., 2007; Michaelidis et al., 2005), or increase muscle wastage in order to maintain skeletal integrity (Wood et al., 2008). A net decline in calcification, along with a reduced shell mass of developing larval lobsters may affect their competitive fitness and recruitment success; this could trigger cascading trophic effects on population dynamics and potentially on the functioning of marine ecosystems. It is not certain whether the adverse effects associated with OA may be counteracted by physiological acclimatization and/or genetic adaptation of marine organisms (Riebesell, 2004). However initial findings do not appear promising and assessments of potential impacts are hampered by the scarcity of relevant research (Orr et al., 2005).

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Table 1. System data (mean ± standard error) for the control and the high CO_2 treatment. Salinity, temperature, pH and pCO_2 were measured, all other data (DIC = dissolved inorganic carbon, A_T = total alkalinity; CO_3^{2-} = carbonate ion concentration; $\Omega_{calcite}$ = calcite saturation state; $\Omega_{aragonite}$ = aragonite saturation state) were calculated from pH and pCO_2 using CO2sys (Pierrot et al., 2006), with dissociation constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and KSO_4 using Dickson (1990).

	Control	High CO_2 Treatment
CO_2 (ppm)	315±18.83	1202±29.83
pH	8.39±0.006	8.10±0.009
Temp (°C)	17.0	17.0
Sal (psu)	35.0	35.0
A_T (μEq kg^{-1})	2544±148.9	4290±145.9
DIC (μmol kg^{-1})	2152±131.1	3967±132.8
$\Omega_{calcite}$	6.71±0.43	6.79±0.32
$\Omega_{argonite}$	4.33±0.28	4.38±0.20
HCO_3^- (μmol kg^{-1})	1863±112.9	3651±119.5
CO_3^{2-} (μmol kg^{-1})	281±18.1	285±13.3

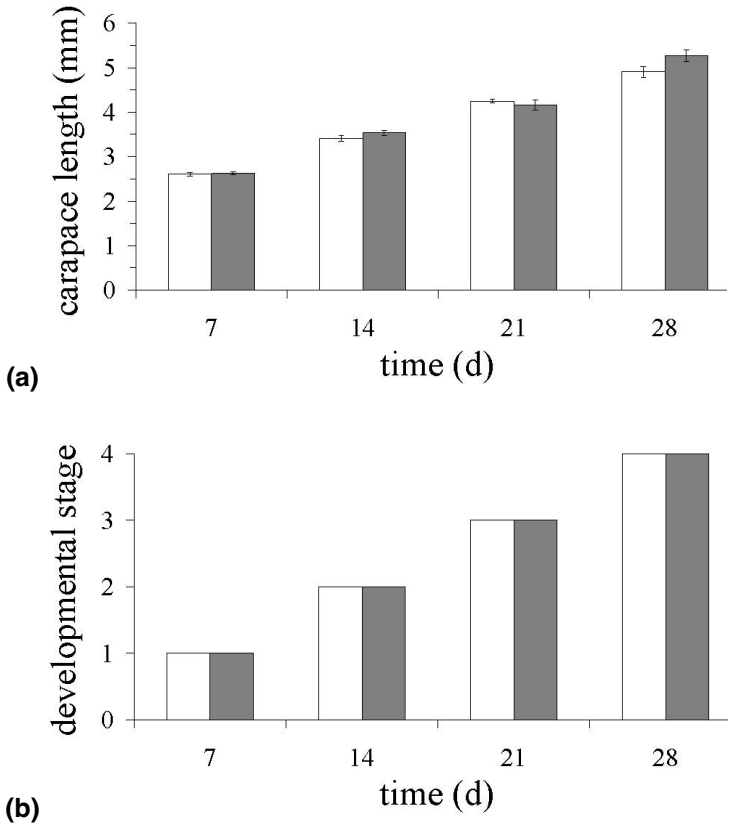

Fig. 1. Larval growth measured by length of the carapace (mm) **(a)** and developmental stage **(b)** of various aged *H. gammarus* larvae during culture in 1000 ppm of CO_2. Values represent mean ±1 standard error; white, control; grey, 1200 ppm of CO_2.

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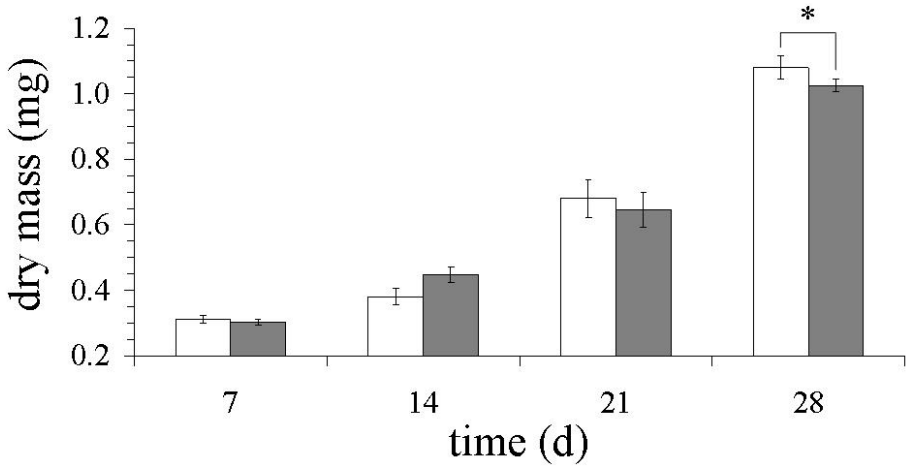

Fig. 2. Dry mass of carapace (mg) of different ages of *H. gammarus* larvae during culture in 1200 ppm of CO_2. Values are means ±1 standard error; white, control; grey, 1200 ppm of CO_2. Asterisk denotes significant differences from control ($p<0.05$).

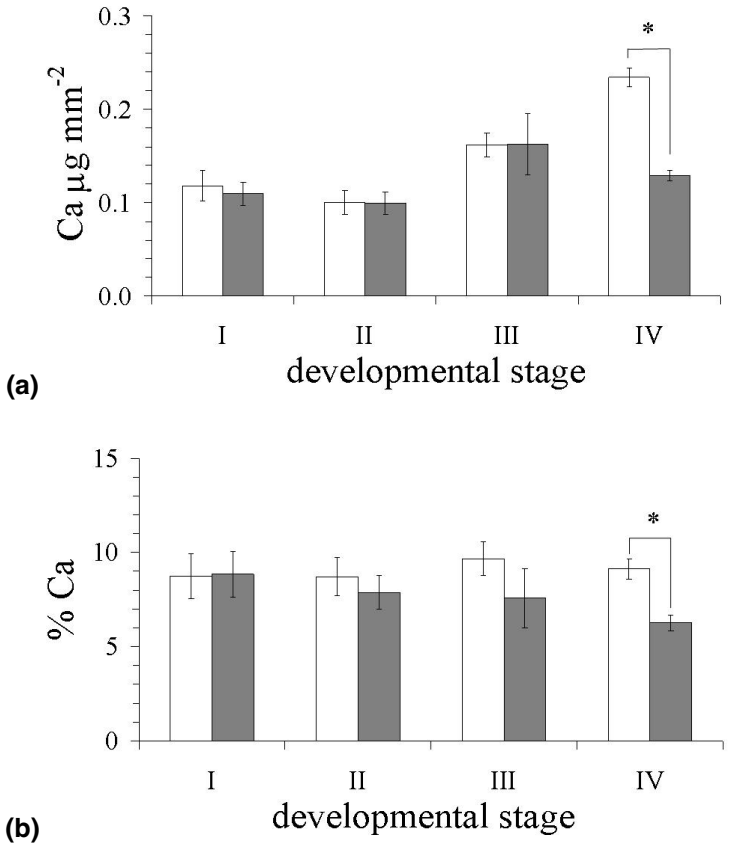

Fig. 3. Changes in the calcium concentration of the carapace during development and exposure to CO_2-acidification (1200 ppm), expressed as concentration per surface area **(a)** and % carapace dry mass **(b)**. Values are means ±1 standard error; white bar, control; grey bar, 1200 ppm of CO_2. Asterisk denotes significant differences from control ($p<0.05$).

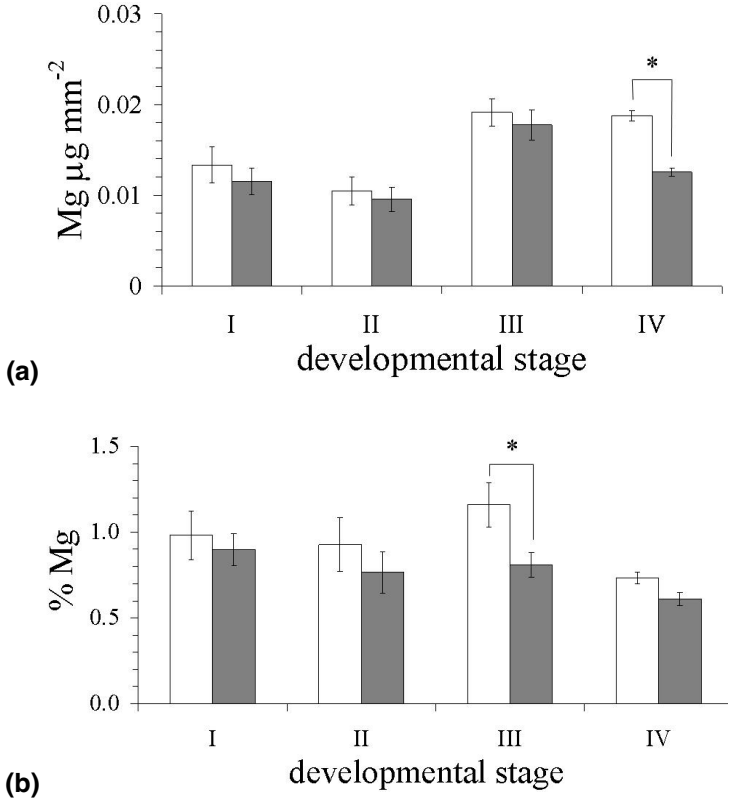

Fig. 4. Changes in the magnesium concentration of the carapace during development and exposure to CO_2-acidification (1200 ppm), expressed as concentration per surface area **(a)** and % carapace dry mass **(b)**. Values are means ±1 standard error; white bar, control; grey bar, 1200 ppm of CO_2. Asterisk denotes significant differences from control ($p<0.05$).