Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO₂

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ABSTRACT: Ocean acidification and associated changes in seawater carbonate chemistry negatively influence calcification processes and depress metabolism in many calcifying marine invertebrates. We present data on the cephalopod mollusc *Sepia officinalis*, an invertebrate that is capable of not only maintaining calcification, but also growth rates and metabolism when exposed to elevated partial pressures of carbon dioxide (pCO₂). During a 6 wk period, juvenile *S. officinalis* maintained calcification under ~4000 and ~6000 ppm CO₂, and grew at the same rate with the same gross growth efficiency as did control animals. They gained approximately 4 % body mass daily and increased the mass of their calcified cuttlebone by over 500 %. We conclude that active cephalopods possess a certain level of pre-adaptation to long-term increments in carbon dioxide levels. Our general understanding of the mechanistic processes that limit calcification must improve before we can begin to predict what effects future ocean acidification will have on calcifying marine invertebrates.

KEY WORDS: Ocean acidification · Calcification · Metabolism · Growth · Marine invertebrate · Cephalopod · *Sepia officinalis*

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INTRODUCTION

Anthropogenic carbon dioxide (CO₂) emissions are acidifying the world's oceans. While current ocean pH values are already more than 0.1 units below those of pre-industrial times, further increases in atmospheric CO₂ concentrations to values of 1500 to 2000 ppm could result in a drop of ocean pH of up to 0.8 units within the next 300 yr (Caldeira & Wickett 2003). Together with declining pH values, ocean carbonate ion (CO₃²⁻) concentrations will decrease, which in turn will lead to a reduction of calcium carbonate saturation (Ω) in seawater (Zeebe & Wolf-Gladrow 2001). As many marine organisms form shells or skeletons from calcium carbonate minerals (primarily aragonite or calcite), considerable attention has been devoted to studying calcification processes in response to seawater acidification. Surface ocean waters are currently supersaturated with respect to both calcite and aragonite. However, recent measurements and models predict that surface seawater calcium carbonate satura-

tion states are decreasing globally (Feely et al. 2004). By the year 2050 it is predicted that high latitude regions will become undersaturated ($\Omega < 1$) with respect to aragonite (Ω_{arag}) as a consequence of ocean acidification (Orr et al. 2005).

Most marine invertebrates respond negatively to elevated CO₂ concentrations. Many cnidarians, molluscs and echinoderms display reduced rates of calcification (Fabry et al. 2008). Interestingly, some of these organisms display strong linear relationships of calcification rate with the saturation of calcium carbonate (Ω) (Fig. 1). The changes in calcification recorded over a 2 yr period in the Biosphere 2 mesocosm (Langdon et al. 2000; data replotted from their Table 4 in our Fig. 1) illustrate the high sensitivity of reef building communities to calcium carbonate undersaturation. Bivalve molluscs also react sensitively to decreasing pH and Ω_{arag} . The work of Gazeau et al. (2007) shows that net calcification in the mussel *Mytilus edulis* decreases linearly with increasing pCO₂, and ceases when pCO₂ is above 1800 ppm (data replotted from their Table 1 in

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Fig. 1. The dependence of calcification on CO_2 -dependent seawater calcium carbonate saturation (Ω_{arag}) in marine invertebrates. Long-term coral reef data set recorded in the Biosphere 2 mesocosm (Langdon et al. 2000, data replotted from their Table 4), acute changes in *Mytilus edulis* (bivalve) calcification (Gazeau et al. 2007, data replotted from their Table 1), *Sepia officinalis* (cephalopod) calcification measured over 6 wk in this study (data are mean $\pm$ SD, $n = 20$). The highest calcification rates in the respective data sets were set at a value of 100 %

our Fig. 1). While the latter might be explained by external shell dissolution when $\Omega_{\text{arag}} < 1$, decreasing calcification at $\Omega_{\text{arag}} > 1$ may indicate that significant physicochemical control exists over calcification in mussels.

Marine invertebrates whose calcification processes are disturbed by elevated CO_2 are also characterised by comparatively low metabolic rates and activity levels. These factors may increase a marine organisms' sensitivity to ocean acidification, as suggested by Seibel & Walsh (2003). In response to this possibility, the present study explores the calcification and growth capacity of an active mollusc (cephalopod) with a high metabolic rate, the European cuttlefish *Sepia officinalis*, under acidified conditions. Cuttlefish possess an internal aragonite shell ('cuttlebone', see Fig. 3) that serves as a structural support and, with the help of ion transport mechanisms, as a buoyancy control device (Denton & Gilpin-Brown 1961a). Interestingly, we find that *S. officinalis* does not reduce its growth or calcification rate when exposed to ~6000 ppm CO_2 for a period of 6 wk.

MATERIALS AND METHODS

Experimental animals. *Sepia officinalis* egg clusters were collected in the Bay of Seine, Normandy, France, in May 2006 and 2007. Cuttlefish were hatched and raised at the Alfred-Wegener-Institute, Bremerhaven, Germany, in a closed recirculating system (20 m³ total volume, protein skimmer, nitrification filter, UV disinfection unit (Sander), salinity 32 to 34, temperature (mean $\pm$ SD) $15 \pm 0.1^\circ\text{C}$, pH 7.9 to 8.2, constant 12 h dark:12 h light cycle). Water quality parameters were monitored weekly and concentrations of ammonia and nitrite were kept below 0.2 and 50 mg l⁻¹, respectively. The cuttlefish were initially fed a daily diet consisting of live mysids *Neomysis integer* and progressively transitioned to feed exclusively on frozen brown shrimp *Crangon crangon*.

Growth trials of *Sepia officinalis* under elevated pCO₂ conditions. For the 2 growth trials, each group of 20 *Sepia officinalis* ind. was maintained in shallow PVC basins (20 × 40 × 60 cm). Basins drained into reservoir tanks where the seawater was pumped through a nitrifying biofilter (Eheim Pro 2) and past a 12 W UV sterilizer before being recirculated into the holding tanks. The total seawater volume of each system was approximately 300 l. Water values were maintained at <0.2 mg l⁻¹ ammonium and <40 mg l⁻¹ nitrite. Holding and reservoir tanks were continuously bubbled with the appropriate gas mixture supplied by an MKS gas controller (MKS, model GSV-19). Specific seawater conditions for the various incubations are given in Table 1. The pH was measured with a WTW 340i meter and SenTix81 electrode calibrated daily with National Bureau of Standards (NBS) buffers. Total dissolved inorganic carbon (C_T) was measured using a gas chromatographic method modified from Lenfant & Aucutt (1966) and Pörtner et al. (1990). Seawater carbonate chemistry parameters were calculated from C_T and pH_{NBS} with the software CO2SYS (Lewis & Wallace 1998) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson & Millero (1987).

Throughout the duration of the growth trials, cuttlefish were fed ad libitum with live brown shrimp. The wet mass of shrimp consumed daily by each group was

Table 1. Seawater physiochemical conditions during 6 wk growth trials. NBS: National Bureau of Standards; C_T : total dissolved inorganic carbon; pCO_2 : partial pressures of CO_2 . Values (except aragonite saturation state, Ω_{arag}) are mean $\pm$ SD

Incubation group	Temperature (°C)	Salinity	pH_{NBS}	C_T (μmol kg ⁻¹)	pCO_2 (ppm)	Ω_{arag}
Control	16.32 $\pm$ 0.12	32.8 $\pm$ 0.5	7.94 $\pm$ 0.06	2047 $\pm$ 68	705 $\pm$ 101	1.47
CO_2 ~4000 ppm	16.37 $\pm$ 0.12	32.9 $\pm$ 0.4	7.23 $\pm$ 0.04	2451 $\pm$ 54	4271 $\pm$ 373	0.34
Control	17.45 $\pm$ 0.16	31.4 $\pm$ 0.4	8.01 $\pm$ 0.04	2104 $\pm$ 56	628 $\pm$ 60	1.78
CO_2 ~6000 ppm	17.43 $\pm$ 0.15	32.3 $\pm$ 0.6	7.10 $\pm$ 0.03	2583 $\pm$ 43	6068 $\pm$ 389	0.27

recorded. Cuttlefish wet masses and mantle lengths were determined weekly over a period of 6 wk. Slopes of the exponential growth curves were used to determine the daily increase in percent body mass. Gross growth efficiency (percent conversion of ingested shrimp into biomass) was calculated for each group on a weekly basis by dividing the weekly increase in animal wet mass (g) by the mass of the food consumed by that group over the same time interval (Forsythe et al. 2002).

Cuttlebone dry mass and calcium carbonate (CaCO₃) content were determined upon termination of the experiment. The organic matrix contributed only 5 to 8% of total cuttlebone dry mass in the size range of sampled individuals (data not shown), the remainder of the mass being CaCO₃ (aragonite). We determined CaCO₃ content by back-calculating from the dry mass of the remaining organic matrix after dissolution of the cuttlebone CaCO₃ fraction with 4 M HCl following Birchall & Thomas (1983). All samples were weighed on a precision balance (ME235S, Sartorius).

Determination of standard metabolic rate under hypercapnia. Standard metabolic rates (SMR) were determined using intermittent closed respirometry. Oxygen consumption rates (3 to 4 runs of approximately 20 min each) were obtained between 08:00 and 20:00 h to avoid peak night activity periods of the cuttlefish (Denton & Gilpin-Brown 1961b). Briefly, cuttlefish (mean $\pm$ SD; 10.4 $\pm$ 4.3 g, n = 6) were fasted for 24 h and then incubated in cylindrical perspex chambers (3 $\times$ 25 cm) for a period of 3 d during which time they were acutely exposed to hypercapnic conditions. The chambers were perfused with seawater using an Ismatec peristaltic pump (ISM 404B) and gas-tight Tygon tubing (T4406-23). Applied flow rates (100 ml min⁻¹) ensured chamber oxygen partial pressures of approximately 18 to 20 kPa between measurements. Seawater from the growth trial reservoirs was pumped through a UV sterilization unit and then used to perfuse the respiration chambers (see Table 1 for seawater values under control and hypercapnic conditions). Temperature was maintained at (mean $\pm$ SD) 16 $\pm$ 0.2°C by placing the 4 replicate chambers in a water bath fitted with a thermostat. Oxygen partial pressures were measured using a fiber optic oxygen sensing system (Oxy-4

Micro, PreSens) and needle-type optodes, incorporated into the closed loop. Data were recorded using software supplied by the manufacturer, and oxygen consumption rates were calculated from linear declines in chamber oxygen partial pressure.

Statistical analyses. Results were analyzed using GraphPad Prism 4. Unpaired t -tests were carried out to assess the significance of differences between incubation groups at p < 0.05. A linear regression analysis was used to determine whether oxygen consumption rates varied with exposure time. All values are expressed as means $\pm$ SD.

RESULTS

No differences in soft-tissue growth performance were measured between cuttlefish incubated at ~4000 and ~6000 ppm CO₂ and controls (Table 2). Final average body mass for the cuttlefish incubated at ~4000 ppm CO₂ equaled 11.16 $\pm$ 1.40 g compared with 11.63 $\pm$ 1.39 g for the control group. In those incubated at ~6000 ppm CO₂ the corresponding mass was 23.06 $\pm$ 4.15 g compared with 24.15 $\pm$ 5.25 g in the controls. All 4 of the experimental groups grew at high rates typical of juvenile cephalopods (Forsythe et al. 1994, Melzner et al. 2005), increasing body mass exponentially at a rate of approximately 4% d⁻¹. There were no significant differences between the exponential curves used to calculate daily growth (Fig. 2). Gross growth efficiencies (GGE), calculated from weekly means, were also similar between the 4 incubation groups; the values ranged between 36.6 $\pm$ 6.2% and 39.5 $\pm$ 4.5%, and there were no significant differences (Table 2).

Standard metabolic rates of cuttlefish exposed acutely to ~6000 ppm CO₂ showed no significant increase or decrease over time ($F_{1,9}$ = 2.9, p > 0.1; Fig. 3). Mean oxygen consumption values during the control period were 0.092 $\pm$ 0.004 μ mol O₂ g⁻¹ min⁻¹, and after 24 h of CO₂ exposure were 0.088 $\pm$ 0.003 μ mol O₂ g⁻¹ min⁻¹.

Growth of the calcified cuttlebone was determined both indirectly, from the mantle length of the cuttlefish, and directly, by measuring the amount of deposited CaCO₃. At the end of the trial period, there

Table 2. *Sepia officinalis*. Growth and calcification during each of 2 separate trials under elevated CO₂ conditions. Values are mean $\pm$ SD, n = 20 in each of the incubation groups

Incubation group	Initial wet mass (g)	Initial mantle length (mm)	Final wet mass (g)	Final mantle length (mm)	Daily mass gain (%)	Gross growth efficiency (%)
Control	2.69 $\pm$ 0.30	20.53 $\pm$ 0.14	11.63 $\pm$ 1.39	37.16 $\pm$ 1.88	4.0	36.6 $\pm$ 6.2
CO ₂ ~4000 ppm	2.70 $\pm$ 0.33	20.71 $\pm$ 0.17	11.16 $\pm$ 1.40	36.33 $\pm$ 2.29	3.8	38.9 $\pm$ 3.6
Control	4.61 $\pm$ 1.01	27.83 $\pm$ 2.47	24.15 $\pm$ 5.25	52.84 $\pm$ 4.03	3.9	39.5 $\pm$ 4.5
CO ₂ ~6000 ppm	4.50 $\pm$ 1.08	27.90 $\pm$ 2.39	23.06 $\pm$ 4.15	52.01 $\pm$ 4.76	3.7	39.4 $\pm$ 3.7

were no significant differences between the mantle lengths of control cuttlefish and those incubated at ~6000 ppm CO₂ (52.01 ± 4.76 mm versus 52.84 ± 4.03 mm, respectively), nor between the control and ~4000 ppm CO₂ incubated cuttlefish (37.16 ± 1.88 mm

Fig. 2. *Sepia officinalis*. Growth (■▲, left y-axis) and calcification (bars, right y-axis) in the cuttlefish incubated under ~6000 ppm CO₂ (grey) and control conditions (black). For CaCO₃ accretion, means not sharing the same letter above bars are significantly different. Data are mean ± SD (n = 20). The calcified cuttlebone is shaded grey in the schematic drawing of *S. officinalis*

Fig. 3. *Sepia officinalis*. Standard metabolic rate (SMR) of cuttlefish during acute exposure to ~6000 ppm CO₂ (▲). Cuttlefish were placed in the chambers at Time = 0 and CO₂ exposure was started after 40 h of control measurements; the change in seawater pH (grey curve) reflects the time course of CO₂ exposure. Data are mean ± SD, n = 6

versus 36.33 ± 2.29 mm, respectively) (Table 2). During the 6 wk growth period all of the cuttlefish increased the mass of their cuttlebones by over 500 % (Fig. 3). Interestingly, in the ~6000 ppm CO₂ growth trial, the CO₂ incubated animals incorporated significantly more CaCO₃ into their cuttlebones than did the control group, 0.80 ± 0.15 g versus 0.71 ± 0.15 g, respectively. Functional control of the cuttlebones (i.e. buoyancy regulation) did not appear to be negatively affected by low pH conditions.

DISCUSSION

The results of our growth trial show that at least 1 marine invertebrate species is capable of maintaining both metabolic rates and somatic growth performance at control levels during long-term exposure to significantly elevated seawater CO₂ concentrations.

Growth

Sepia officinalis juveniles cultured at ~4000 and ~6000 ppm CO₂ grew at the same rate as did control individuals, gaining body mass at a rate of approximately 4 % body mass d⁻¹ (Table 2). These growth rates closely correspond with results from previous work, where *S. officinalis* of similar size gained 3.5 % body mass d⁻¹ at 17°C (Forsythe et al. 2002). Under both CO₂ conditions, there was no significant difference between control and treatment final wet mass gained during the 6 wk growth intervals. All cuttlefish more than quadrupled their body mass (Table 2). These results are in stark contrast to existing invertebrate growth studies under elevated CO₂. Michaelidis et al. (2005) found that under comparable CO₂ levels to our study, and over a growth period of 3 mo, shell length and soft body mass in the mussel *Mytilus galloprovincialis* were reduced by 55 and 70 %, respectively (as calculated from their Fig. 3). Even more striking is the study reported by Shirayama & Thornton (2005) where significant differences in total body mass were measured in the sea urchin *Echinometra mathaei* and the gastropod *Strombus luhuanus* incubated under just 560 ppm CO₂ for half a year. Clearly, *S. officinalis* does not exhibit sensitivity to elevated CO₂ levels within the range of concentrations that elicits a negative response in most other invertebrates studied to date.

Metabolism

Reduced growth performance in marine invertebrates under elevated CO₂ conditions has been suggested to

be a result of the organisms entering a state of metabolic depression (Pörtner et al. 2004). The cellular processes mediating metabolic depression have been extensively reviewed (Hand & Hardewig 1996, Guppy & Withers 1999, Storey & Storey 2007), and hypercapnia alone as an environmental stressor has been found to induce metabolic depression (Barnhart 1989, Rees & Hand 1990). Recent case studies on marine invertebrates support this conclusion; in *Sipunculus nudus* (Pörtner et al. 1998) and *Mytilus galloprovincialis* (Michaelidis et al. 2005) a decrease in metabolic rate in response to both acute and long-term hypercapnia exposure was accompanied by an uncompensated decrease in extracellular pH (pH_e). Working with an isolated muscle model, Pörtner et al. (2000) suggested that decreasing pH_e slows down the rate of H⁺ equivalent ion exchange between the extra- and intracellular space, and this in turn reduces the work load of Na⁺/K⁺-ATPase in maintaining the transepithelial electrochemical gradient. With this arrangement, organisms could effectively lower the energy requirements of acid–base regulation in their cells. However, they would still face new steady-state levels of decreased extracellular pH, elevated pCO₂ and HCO₃[−], which might have long-term effects on metabolic function (Reipschläger & Pörtner 1996). These could include changes in amino acid catabolism, with a preference towards net formation of metabolic bicarbonate for buffering (Langenbuch & Pörtner 2002). In combination with reduced rates of protein biosynthesis under low pH conditions (Smith et al. 1996, Reid et al. 1997, Langenbuch & Pörtner 2003), such processes would eventually limit somatic growth.

Metabolic depression is not evident in *Sepia officinalis* in response to acute CO₂ exposure, which matches the conserved growth rates observed in our study. Standard metabolic rates of around 0.09 μmol O₂ g^{−1} min^{−1} were maintained at a constant level during acute exposure to ~6000 ppm CO₂ (Fig. 2). The control metabolic rates we measured in *S. officinalis* match previously published values for similarly sized animals (Melzner et al. 2007a). A recent study working with the brittle star *Amphiura filiformis* also found no evidence of metabolic depression during long-term hypercapnic exposure under similar CO₂ levels (Wood et al. 2008). In fact, a significant increase in metabolic rate was found along with dramatic arm muscle wastage at an incubation pH of 7.3 (Wood et al. 2008). The catabolism of arm muscle to support elevated metabolic costs during hypercapnia, however, is indicative of a restructuring of the energy budget that significantly compromises long-term animal fitness.

In contrast, the cuttlefish in this study were not only capable of conserving growth and metabolic rates, but they also maintained their GGE at control levels under

both ~4000 and ~6000 ppm CO₂ (Table 1). This suggests that the partitioning of their energy budget was conserved under hypercapnia, and that they did not simply ingest more food to maintain growth performance. Our GGE values, ranging from 36 to 39%, correspond with published values of 30 to 50% (Forsythe et al. 2002) for *Sepia officinalis* cultured at 17°C. A similar response is also known in fish, where metabolic rates and growth are not influenced even by high degrees of hypercapnia. Working with juvenile spotted wolffish *Anarhichas minor*, Foss et al. (2003) reported conserved growth rates, as well as food conversion efficiencies, at CO₂ concentrations up to 17 000 ppm CO₂. Fish are capable of maintaining growth rates under elevated CO₂ conditions because of their high ion transport and acid–base regulatory abilities. During acute hypercapnic exposure they rapidly increase HCO₃[−] levels in their blood, and are able to fully compensate their extracellular pH (Toews et al. 1983, Clai-borne & Evans 1992, Larsen et al. 1997, Hayashi et al. 2004, Michaelidis et al. 2007). Thus, in contrast to most invertebrates, pH_e is not depressed in fish during moderate, long-term hypercapnic exposure and, thus, does not influence potential reductions in metabolism and growth. The elevation of HCO₃[−] levels in response to hypercapnia-induced acidification is a response common to most organisms (Heisler 1989); however, the degree to which pH is compensated is dependent on ion-regulatory capacity and is species specific.

Calcification

Not only does *Sepia officinalis* successfully acquire soft tissue mass under elevated CO₂ conditions, but it also maintains high calcification rates of its cuttlebone. *S. officinalis* is capable of calcifying under ~6000 ppm CO₂ and Ω_{arag} values of 0.27. Cuttlebone formation rate, as determined from mantle length measurements, was equal between all of the growth trial groups (Table 2). The cuttlebone is a fully internalized shell that is encased in a cuttlebone sac (Appellöf 1893), dorsally positioned along the anterior–posterior plane (see Fig. 3). When directly measured, total calcium carbonate accumulation in the cuttlebones of the ~6000 ppm CO₂ incubated individuals was actually found to be significantly higher than in the control group (Fig. 3). This puts *S. officinalis* in a unique position in relation to existing studies, since most invertebrates examined to date exhibit a negative influence of elevated CO₂ concentrations on calcification, and in some organisms there is a linear decrease of calcification rate with decreasing Ω_{arag} (Fig. 1). As far as we are aware, only one other study working with long-term hypercapnic exposure in invertebrates has shown in-

creased calcification rates under elevated seawater CO₂ levels (Wood et al. 2008).

Considering that calcification requires tight control of ionic composition and pH in the micro-environment at the deposition site (Weiner & Dove 2003), it seems likely that *Mytilus galloprovincialis*, and other invertebrates with low metabolic rates or low ion exchange capacities, are not capable of maintaining conditions favorable to mineral deposition under the acidification stress of hypercapnia. Findings of elevated calcium ions (Ca²⁺) in *M. galloprovincialis* hemolymph, in combination with the previously mentioned uncompensated pH_e reduction (Michaelidis et al. 2005), support such a hypothesis. In contrast, calcification at $\Omega_{\text{arag}} < 1$ in *Sepia officinalis* could be directly related to high, 'fish-like', ion regulatory capacities in this active invertebrate.

SUMMARY

We conclude that marine ectothermic organisms with high metabolic rates (teleost fish, cephalopods) might be characterised by a certain level of pre-adaptation to acidification enabling them to grow and calcify under long-term elevated CO₂ conditions. By means of competition for similar resources, both fish and cephalopods have been forced into an active, high-power style of living (e.g. O'Dor & Webber 1986, 1991). During exercise, cephalopods are known to encounter CO₂ partial pressures >3000 ppm in their blood (Pörtner et al. 1991), which are values that are twice as high as those predicted for the world's oceans for the year 2300 (Caldeira & Wickett 2003). However, they are known to protect their blood from exercise-induced acidification by recycling octopine and associated protons in their mantle tissue (Pörtner et al. 1993). Since a stable blood pH is necessary for the proper function of their extracellular oxygen pigment hemocyanin (e.g. Melzner et al. 2007b), active cephalopods must possess a sophisticated ion transport machinery (and appropriate buffering systems) to cope with high, exercise-induced, CO₂ concentrations on a daily basis. Ongoing work on the blood acid–base parameters and the general ion regulatory ability of *Sepia officinalis* in response to hypercapnia will provide further insights.

Our work underlines the importance of improving our understanding of the processes responsible for biocalcification, growth and physiological homeostasis, when aiming towards predicting sensitivities of marine invertebrates to future climate change. The cuttlefish *Sepia officinalis* might, therein, serve as an important invertebrate model organism to identify specific biological mechanisms that promote tolerance to long-term reductions in seawater pH and calcium carbonate saturation.

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