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ORIGINAL PAPER

Cuttlebone calcification increases during exposure to elevated seawater pCO_2 in the cephalopod *Sepia officinalis*

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Abstract Changes in seawater carbonate chemistry that accompany ongoing ocean acidification have been found to affect calcification processes in many marine invertebrates. In contrast to the response of most invertebrates, calcification rates increase in the cephalopod *Sepia officinalis* during long-term exposure to elevated seawater pCO_2. The present trial investigated structural changes in the cuttlebones of *S. officinalis* calcified during 6 weeks of exposure to 615 Pa CO_2. Cuttlebone mass increased sevenfold over the course of the growth trail, reaching a mean value of 0.71 ± 0.15 g. Depending on cuttlefish size (mantle lengths 44–56 mm), cuttlebones of CO_2-incubated individuals accreted 22–55% more $CaCO_3$ compared to controls at 64 Pa CO_2. However, the height of the CO_2-exposed cuttlebones was reduced. A decrease in spacing of the cuttlebone lamellae, from 384 ± 26 to 195 ± 38 μm, accounted for the height reduction The greater $CaCO_3$ content of the CO_2-incubated cuttlebones can be attributed to an increase in thickness of the lamellar and pillar walls. Particularly, pillar thickness increased from 2.6 ± 0.6 to 4.9 ± 2.2 μm. Interestingly, the incorporation of non-acid-soluble organic matrix (chitin) in the cuttlebones of CO_2-exposed individuals was reduced by 30% on average. The apparent robustness of calcification processes in *S. officinalis*, and other powerful ion regulators such as decapod cructaceans, during exposure to elevated pCO_2 is predicated to be closely connected to the increased extracellular [HCO_3^-] maintained by these organisms to compensate extracellular pH. The potential negative impact of increased calcification in the cuttlebone of *S. officinalis* is discussed with regard to its function as a lightweight and highly porous buoyancy regulation device. Further studies working with lower seawater pCO_2 values are necessary to evaluate if the observed phenomenon is of ecological relevance.

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Introduction

Ocean acidification–related changes in seawater carbonate chemistry have been hypothesized to reduce calcification rates in marine mollusks (Orr et al. 2005; Fabry et al. 2008). Shell growth has been shown to significantly decrease in several species of bivalves and gastropods (Michaelidis et al. 2005; Shirayama and Thornton 2005; Berge et al. 2006; Ries et al. 2009) during long-term exposure to elevated seawater pCO_2. However, the available data are not sufficient to draw a causal relationship

between long-term exposure to elevated seawater pCO_2 and reduced calcification rates in adult mollusks. Reductions in calcification have been reported as shell length decrements, coupled to reductions in somatic growth (Michaelidis et al. 2005; Shirayama and Thornton 2005; Berge et al. 2006) or reductions in net calcification calculated from buoyant weight (Ries et al. 2009). It remains open to investigation whether decreased shell growth and body mass under high pCO_2 conditions are a consequence of a reduced ability to calcify, or whether metabolic depression is the primary mechanism leading to reduced rates of growth (Pörtner et al. 2004; Pörtner 2008; Melzner et al. 2009b). In contrast to previous studies on mollusks, we have shown that metabolism and growth rates remain at control levels during exposure to highly elevated seawater pCO_2 in the cephalopod *Sepia officinalis*, while mineralization of $CaCO_3$ significantly increases (Gutowska et al. 2008).

Cuttlefish (family Sepiidae), along with *Nautilus* spp. (Nautilidae) and *Spirula spirula* (Spirulidae), are the only extant cephalopods with a chambered shell that provides skeletal support and acts as a buoyancy regulation device (Denton 1974). In the cuttlefish *S. officinalis*, the aragonitic cuttlebone is dorsally located along the anterior–posterior axis (Fig. 1a, b) and accounts for about 10% of the cuttlefish's volume (Denton and Gilpin-Brown 1961a). The cuttlebone is encased by the cuttlebone epithelium, also referred to as the cuttlebone sac. Dorsally, it is covered by a skin layer, and ventrally, connective tissue separates it from the visceral mass (Tompsett 1939). The cuttlebone epithelium transports the constituents of the cuttlebone to the calcification site and maintains the ionic and protein composition of the extracellular environment around the

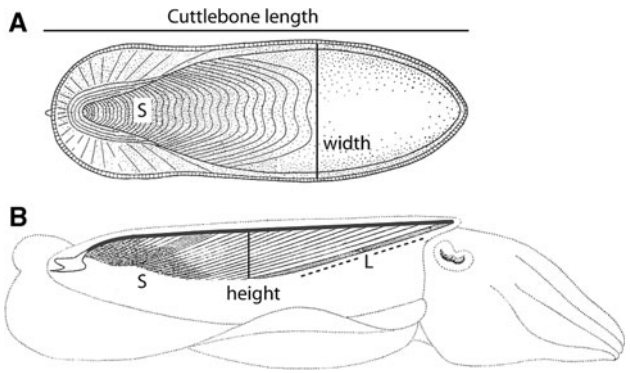

Fig. 1 **a** Illustration of *S. officinalis* cuttlebone viewed ventrally (modified after Tompsett 1939). Cuttlebone length, width and height are delineated as they were measured for morphometric analysis. **b** Schematic drawing of *S. officinalis* (modified after Denton 1974). Cuttlebone is illustrated in a sagittal section, liquid-filled parts are *shaded*. The posterior lamellae are pumped dry over the siphuncular surface on a daily cycle. *S* siphuncular surface, *L* most recently mineralized lamella

cuttlebone (Appellöf 1893; Wendling 1987). In cephalopods, the functional morphology of the epithelium responsible for calcification processes has been best described in *Nautilus pompilius* (Westermann et al. 2005).

In the posterior ventral region of the cuttlebone, the cuttlebone epithelium transports ions and water over the siphuncular surface, thus enabling *S. officinalis* to use this structure as a buoyancy regulation device. The flow of liquid in and out of the cuttlebone is enabled by the creation of an osmotic pump (Denton and Gilpin-Brown 1961a; Denton et al. 1961d). In *Spirula spirula*, the osmolarity of shell fluid is reduced to one-fifth of that of seawater when a chamber is emptied (Denton and Gilpin-Brown 1971). Similarly, strong ion-transport processes most likely occur over the siphuncular surface in *S. officinalis*. Cuttlefish not only adjust their buoyancy according to depth, but also on a diurnal cycle to reduce energy expenditure (Denton and Gilpin-Brown 1961a; Denton and Gilpin-Brown 1961b). During the day, when *S. officinalis* rests buried in sand, the posterior chambers of the cuttlebone are filled with fluid, making the cuttlefish negatively buoyant. At the onset of night, these chambers are emptied, thus decreasing the density of the cuttlefish. The individual is then neutrally buoyant and can maintain its position in the water column during hunting with lower energy expenditure. From the morphology of the surrounding epithelium, and function of the cuttlebone, it is obvious that *S. officinalis* has tight control over the extracellular environment surrounding its calcified structure.

The dual functions of the cuttlebone as support and a lightweight buoyancy device requires an open structure that is pressure resistant while maintaining a constant volume. The porosity of the cuttlebone is high at 93% (Birchall and Thomas 1983); however, it has been tested to withstand pressures of 20 atm (Denton and Gilpin-Brown 1961c). This corresponds to approximately 200 m depth, and suffices to cover the 150-m depth distribution of adult *S. officinalis* (Ward and Boletzky 1984; Neige and Boletzky 1997). The cuttlebone is composed of two distinct regions. The dorsal shield, with a high fraction of organic matrix, plays an important mechanical role by increasing the flexural strength of the cuttlebone (Birchall and Thomas 1983). The ventrally located aragonitic phragmocone consists of parallel lamellae (also referred to as septae in the literature) that are supported by perpendicularly oriented pillars (Fig. 2a, b). Growth of the cuttlebone proceeds by the accretion of subsequent lamellae and extension of the dorsal shield at the anterior end.

The cuttlebone of *S. officinalis* contains more organic material than other molluscan shells (Hare and Abelson 1965). The phragmacone consists of 10% organic matrix, whereas the dorsal shield contains 30–40% (Birchall and Thomas 1983; Florek et al. 2009). In the phragmocone, the

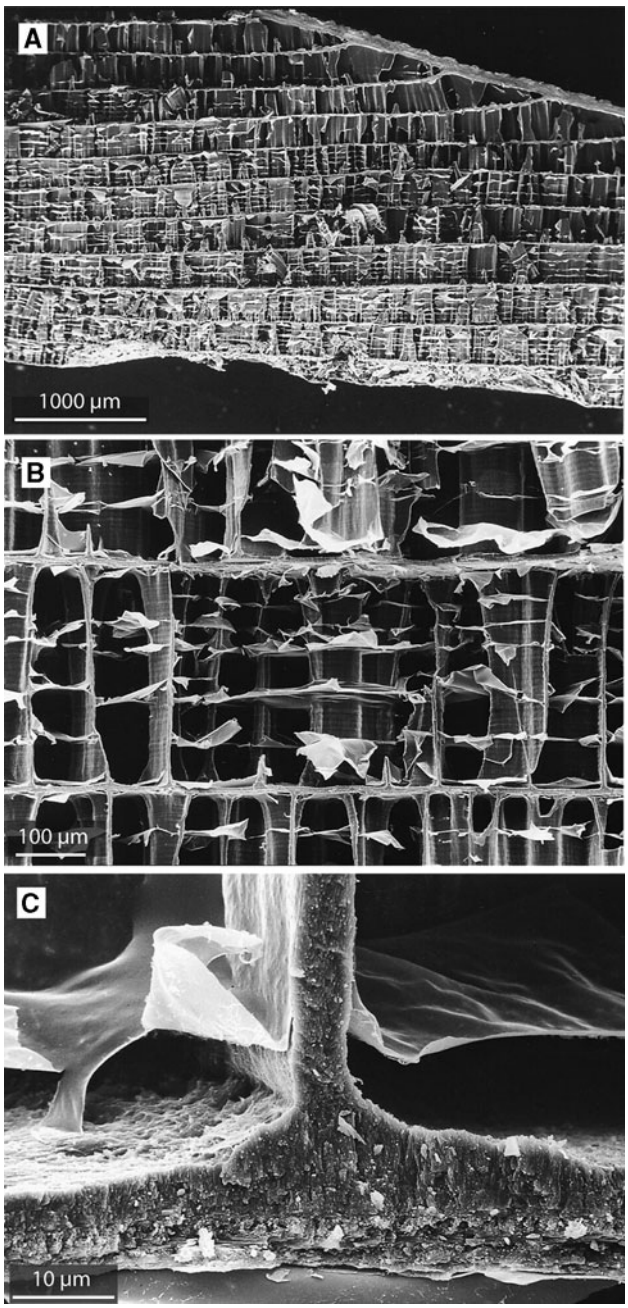

Fig. 2 SE micrographs along the sagittal midline of a control *S. officinalis* cuttlebone. **a** Large-scale view of eleven lamellae in the phragmocone and dorsal shield **b** Closer view of a lamella and supporting pillars. Note thin sheets of organic matrix freely suspended perpendicular to pillars. **c** Detail of pillar rising off of lamellar floor. Note organic matrix coating of $CaCO_3$ surfaces

organic matrix is visible as a thin sheet coating the lamellar and pillar surfaces (Fig. 2c), and as freely suspended non-calcified sheets parallel to the lamellae. The primary, non-acid-soluble constituent of the organic matrix in the cuttlebone is β-type chitin (Dauphin and Marin 1995; Florek et al. 2009). While many of the acid-soluble components from the organic matrix are known in bivalve shells (Weiner 1979; Weiner and Traub 1984; Marin et al. 2000; Wilt et al. 2003; Marin and Luquet 2004), none have yet been specifically identified in cuttlebones (Dauphin 1996).

In complementary studies, we have previously shown that the cuttlefish *S. officinalis* has a significant acid–base regulatory capacity and maintains controlled somatic growth rates during 6 weeks of exposure to 615 Pa CO_2 (Gutowska et al. 2008; Gutowska et al. 2010). We chose to work with a very high pCO_2 as it presented the cuttlefish with a strong regulatory challenge and facilitated our examination of acid–base regulation. The conclusions that can be drawn from our results are clearly mechanistic in nature and cannot be further extrapolated to future species fitness in an ecologically relevant context of ocean acidification. In this study, we first focus on characterizing the gross morphometric characteristics of *S. officinalis* cuttlebones that showed increased calcification during 6 weeks of exposure to 615 Pa CO_2. Secondly, using scanning electron microscopy, we examine microstructural changes of the lamellae and pillars and identify irregular $CaCO_3$ deposits. Finally, we compare organic matrix incorporation in the cuttlebones of the CO_2-incubated and control cuttlefish.

Materials and methods

Cuttlefish

European cuttlefish (*Sepia officinalis*) egg clusters were collected in the Bay of Seine (Normandy, France) in May 2006 from multiple clutches. The cuttlefish were hatched and raised at the Alfred Wegener Institute (AWI, Bremerhaven, Germany) in a closed recirculating system (20-m^3 total volume, protein skimmers, nitrification filters, UV-disinfection units) at $S = 32$–34, $T = 15 \pm 0.1$°C, pH = 7.9–8.2, 12 h *L*: 12-h D photoperiod. Water quality parameters were monitored weekly and concentrations of ammonia and nitrite were kept below 0.2 mg l^{-1}, and nitrate below 80 mg l^{-1}. The cuttlefish were initially fed a daily diet consisting of live mysids (*Neomysis integer*) and progressively transitioned to frozen brown shrimp (*Crangon crangon*). Forty individuals (4.56 ± 1.04 g), with an initial mantle length of 27.86 ± 2.13 mm, were raised in a growth experiment under both control (64 Pa CO_2) and elevated pCO_2 (615 Pa) conditions for 6 weeks. Upon termination of the experiment, the cuttlefish were killed and their cuttlebones removed for further analyses. An additional control group of 50 individuals, ranging in size from 5 to 36 g (mantle lengths 29–66 mm), was sampled from the maintenance aquaria to compare the relationships between

cuttlefish wet mass and cuttlebone morphometrics over a broad range of sizes.

Growth trial under elevated pCO_2 conditions

Each group of $n = 20$ *S. officinalis* 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 less than 0.2 mg l^{-1} ammonium and 40 mg l^{-1} nitrate. 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 incubations are given in Table 1, control seawater pCO_2 equaled 64 Pa and the treatment 615 Pa. pH was measured with a WTW 340i meter and SenTix81 electrode calibrated daily with NBS buffers. Total dissolved inorganic carbon (C_T) was measured using a gas chromatographic method modified after Lenfant and Aucutt (1966) and Pörtner et al. (1990). Seawater carbonate chemistry parameters were calculated from C_T and pH_{NBS} using the program CO2SYS (Lewis and Wallace 1998) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Throughout the duration of the 6-week growth trial, cuttlefish were fed ad libitum with live shrimp (*Crangon crangon)*. For further information on the weekly recordings of individual growth as well as food assimilation efficiencies, see Gutowska et al. (2008). The average wet mass of the cuttlefish sampled at the end of the growth trial was 23.61 ± 4.7 (g) and their mantle lengths had nearly doubled.

Cuttlebone morphometrics and organic matrix content

Cuttlebones were excised from anesthetized *S. officinalis* individuals at the completion of the growth trial. Particular care was taken to remove the cuttlebones in their entirety and not to break off the margins of the posterior section. All further measurements were performed on cuttlebones that had been dried for 24 h at 40° C. Cuttlebone dry mass was measured on a precision balance (ME235S, Sartorius). Length, width and height of the cuttlebones (Fig. 1a) were measured with a caliper to the nearest 0.5 mm. To determine the relative contributions of non-acid-soluble organic matrix and $CaCO_3$ to cuttlebone mass, cuttlebones were placed in 4 M HCl according to Birchall and Thomas (1983). After 24 h, the calcified component had entirely dissolved, and the remaining organic matrix was carefully removed, rinsed with distilled water, dried overnight in a 40°C oven and weighed on a precision balance (ME235S, Sartorius).

Cuttlebone microstructure examined with light and scanning electron microscopy

The microstructure of six cuttlebones each from control and CO_2 treatments, ranging in length from 46 to 52 mm, was further analyzed. Dried cuttlebones were dorsally etched along the sagittal plane and snapped in half. The number of lamellae in a transversal section was counted at the anterior end of the siphuncular region using a light microscope (MZ8, Leica). This transverse section represented the measured height of the cuttlebones.

Approximately, 2-cm sections anterior of the siphuncular region were trimmed and mounted on SEM pedestal stubs with double-sided adhesive carbon disks. The sections were sputter coated with a gold–palladium alloy and examined using a CamScan-CS-44 SEM.

Microstructural changes were examined in the approximately 2-cm long cuttlebone sections mineralized during the 6-week growth trial period using the freeware program Image J. Spacing between adjoining lamellae was measured in each cuttlebone. Lamellar width was calculated from the average of seven measurements of three lamellae in each cuttlebone. Changes in pillar spacing between the two groups were not quantified due to the complex sigmoidal orientation of the pillars. However, pillar thickness was measured for seven pillars in between three lamellae in each cuttlebone. The number and height of irregular, approximately spherical, $CaCO_3$ deposits were measured in four random 1-mm^2 sections in each cuttlebone on the exposed surface of the sagittal midline fracture. The number of non-calcified organic matrix sheets in between the lamellae was not quantified due to the variable separation of the sheets from the pillar walls during the initial fracture.

Table 1 Seawater physicochemical parameters mean value during 6-week growth trial ±SD

Incubation group	Temp °C	Salinity	pH_{NBS}	DIC μmol kg^{-1}	CO_2 Pa	Ω_{arag}
Control	17.45 ± 0.16	31.4 ± 0.4	8.01 ± 0.04	2,104 ± 56	63.6 ± 6.1	1.78
CO_2	17.43 ± 0.15	32.3 ± 0.6	7.10 ± 0.03	2,583 ± 43	614.8 ± 39.4	0.27

Statistical analyses

Results were analyzed using GraphPad Prism 4. Unpaired *t* tests were carried out to assess the significance of differences between experimental groups for cuttlebone morphometric measurements at $P < 0.05$. Linear regression analysis was used to determine whether the morphometric relationships of CO_2-incubated cuttlebones differed from the control group. All results are presented as means ± SD. Linear regression analyses are plotted with 95% confidence intervals.

Results

In *Sepia officinalis* raised under control conditions at 15°C, individual wet mass (g) related to cuttlebone dry mass (g) following the equation $y = 0.034x - 0.055$ ($R^2 = 0.99$), over a cuttlefish size range of 5–35 g (S1a). Cuttlebone length (mm) and cuttlebone dry mass (g) followed the equation $y = 0.209 - 0.02x + (6.03 \times 10^{-4})x^2$ ($R^2 = 0.99$), over the same size range (S1b). Cuttlebone dry mass made up 3% of cuttlefish wet mass.

Effect of elevated seawater pCO_2 on cuttlebone morphology

Changes in cuttlebone morphology were compared in *S. officinalis* incubated under both control, 64 Pa CO_2, and elevated CO_2 conditions, 615 Pa CO_2, for 6 weeks. The final length attained by the cuttlebones was minimally, but still significantly, shorter in the CO_2 treatment, 47.6 ± 3.2 versus 50.7 ± 3.6 mm ($P < 0.005$). Interestingly, despite being slightly shorter, cuttlebones from individuals raised under elevated CO_2 conditions weighed 0.2 g more on average than control cuttlebones of the same length (Fig. 3a). This corresponds to a relative mass increase of 22–55% depending on the length of the cuttlebone. Thus, while the slopes of the cuttlebone length to mass relationships were not significantly different between the two experimental groups, $F_{(1,37)} = 0.31$, $P = 0.58$, the y-intercepts differed significantly, $F_{(1,38)} = 122$, $P < 0.0001$. Still, no gross morphological differences stood out when comparing the two experimental groups.

Due to their smaller size, the average width of the CO_2-treatment cuttlebones was also slightly narrower compared to the control group, 16.2 ± 0.9 versus 17.1 ± 0.9 mm ($P < 0.005$). The relationship between cuttlebone length and width did not differ between experimental conditions (Fig. 3b, slopes, $F_{(1,37)} = 0.01$, p = 0.92, *y*-intercepts, $F_{(1,38)} = 0.76$, $P = 0.39$). However, the average height of the CO_2-treatment cuttlebones was significantly reduced, 4.6 ± 0.4 versus 5.4 ± 0.5 mm ($P < 0.0001$). Cuttlebone length and height were also no longer linearly related in the CO_2 treatment, and the slope of the regression did not significantly differ from zero $F_{(1,18)} = 4.28$, $P = 0.05$. (Table 2, Fig. 3c).

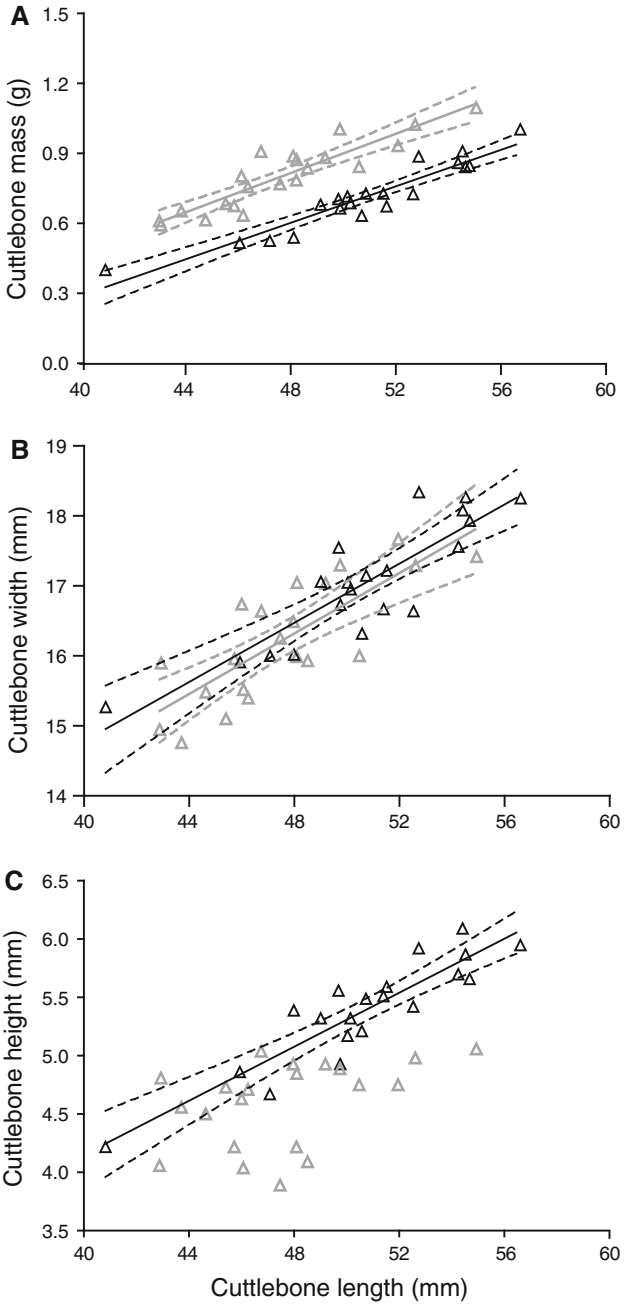

Fig. 3 Morphometric relationships between cuttlebone mass, width and height in control (*black*) and CO_2-incubated (*gray*) groups. **a** Average mass of CO_2 cuttlebones was significantly greater, 0.80 ± 0.15 versus 0.70 ± 0.16 g, ; $P < 0.03$. **b** Average width of CO_2 cuttlebones was significantly narrower, 16.2 ± 0.9 versus 17.1 ± 0.9 mm, $P < 0.005$. **c** Average height of CO_2 cuttlebones was significantly reduced, 4.6 ± 0.4 versus 5.4 ± 0.5 mm, $P < 0.0001$ ($n = 20$)

Because the growth of each particular individual was not traceable in our study, we were not able to measure the number of lamellae individuals accreted per mm increase in cuttlebone length. However, the lamellae accreted during the CO_2 treatment are distinguishable due to microstructural changes described below. Ten lamellae were accreted on average by *S. officinalis* incubated under 615 Pa CO_2 over a period of 6 weeks. Since the total number of lamellae counted in a transverse section at the anterior end of the siphuncular region equaled 17 in both experimental groups, we conclude that cuttlefish from the control group also accreted ten lamellae during the 6-week experimental period.

Effect of elevated seawater pCO_2 on cuttlebone microstructure and organic matrix incorporation

Changes in both lamellar and pillar spacing, as well as thickness (Fig. 4) were evident in SEMs between the two experimental groups. However, the general microstructure of the cuttlebones was conserved. The distance between lamellae significantly decreased in cuttlebones from the CO_2 treatment ($P < 0.0001$), while both lamellar and pillar thickness increased ($P < 0.01$; Table 2).

A higher occurrence of irregular $CaCO_3$ deposition, specific to the CO_2 treatment group, was identified in the form of spherical structures (Fig. 5). The structures were covered with a sheet of organic matrix and primarily attached to the ventral side of the lamellae (S2). The average number of spherical structures visible along the midline fracture was significantly greater in the cuttlebones exposed to elevated CO_2 than in the control group, $P < 0.005$. The height of the structures in the CO_2 cuttlebones spanned a greater range of values (Table 2); however, the average heights were not significantly different due to the large variability of sizes.

Cuttlebone composition, in terms of the ratio between $CaCO_3$ and non-acid-soluble organic matrix (NASOM), also changed during hypercapnic exposure. The cuttlebones from individuals incubated under elevated pCO_2 contained significantly less NASOM than control cuttlebones, $P < 0.005$ (Fig. 6a). NASOM mass in CO_2-exposed cuttlebones was reduced by 30% on average compared to control cuttlebones. Thus, the greater mass of the CO_2 treatment cuttlebones can clearly be attributed to an increase in mineralized $CaCO_3$. Despite the decrease in NASOM in the CO_2-incubated cuttlebones, non-calcified organic matrix sheets were still present. Cuttlebone sections with eight suspended organic matrix sheets in between lamellae were found in both groups (data not shown).

Discussion

In this study, we show that changes in cuttlebone microstructure underlie increased calcification rates measured in the cephalopod *Sepia officinalis* during long-term exposure to elevated seawater pCO_2 (Gutowska et al. 2008).

During a 6-week growth trial, *Sepia officinalis* individuals nearly doubled their cuttlebone length. The average cuttlebone length attained by the experimental group incubated under 615 Pa CO_2 was slightly, but significantly shorter than the control group. The small 3 mm difference in average length was not detected in the mantle length measurements of the cuttlefish in the study by Gutowska et al. (2008) due to the greater inaccuracy of dorsal mantle length measurements in live specimens. Interestingly, despite their slightly reduced length, high CO_2 treatment, cuttlebones accreted 0.2 g more dry mass on average, which corresponds to a 20–55% relative increase in cuttlebone mass. Despite the different mass to length relationship, no gross morphological differences were readily visible. The relationship between cuttlebone length and width was preserved, but the height of the hypercapnic-incubated cuttlebones was reduced in relation to their length.

To further understand the structural changes underlying the morphometric shift in *S. officinalis* cuttlebones during long-term hypercapnic exposure, the microstructure of a subset of cuttlebones that was no more than 15% different in final attained length, 46–52 mm, was examined. Cuttlefish from both groups accreted 10 lamellae during the 6-week experimental period, which gives a lamellar accretion rate of 4.2 days. A lamellar accretion rate of ca. 4 days fits into the published range of values at 17°C (Goff et al. 1998; Bettencourt and Guerra 2001). Lamellar spacing is known to strongly correlate with growth rate.

Table 2 Comparison of microstructural changes and irregular, spherical $CaCO_3$ structures in *S. officinalis* cuttlebone sections calcified under control conditions 64 Pa CO_2, and during exposure to 615 Pa CO_2

Experimental group	Lamellar spacing (μm)	Lamellar thickness (μm)	Pillar thickness (μm)	Sphere # 1 mm^{2-1}	Sphere height (μm)	Sphere height max/min (μm)
Control	384 ± 26	5.4 ± 1.8	2.6 ± 0.6	0.3 ± 0.3	39.9 ± 13.8	65.0/20.2
CO_2	195 ± 38*	8.2 ± 3.1*	4.9 ± 2.2*	1.6 ± 0.5*	47.5 ± 28.3	127.2/11.6

* $P < 0.01$

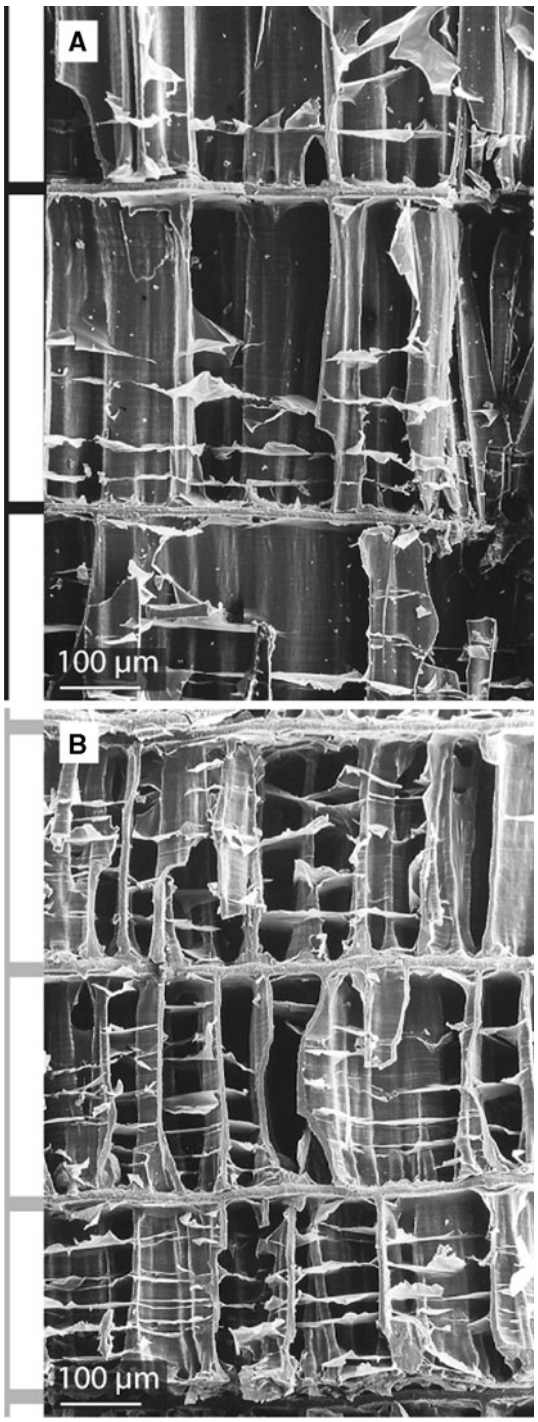

Fig. 4 SE micrographs along the sagittal midline comparing microstructural changes in cuttlebone sections mineralized under control 64 Pa CO_2 **a** and 615 Pa CO_2 **b** seawater conditions. Note the reduced lamellar and pillar spacing in CO_2 cuttlebones

Tightly spaced sections of lamellae are found in cuttlebones of adult wild cuttlefish that correspond to slow growth periods during exposure to cold winter conditions (Goff et al. 1998; Hall et al. 2007). Severely reduced

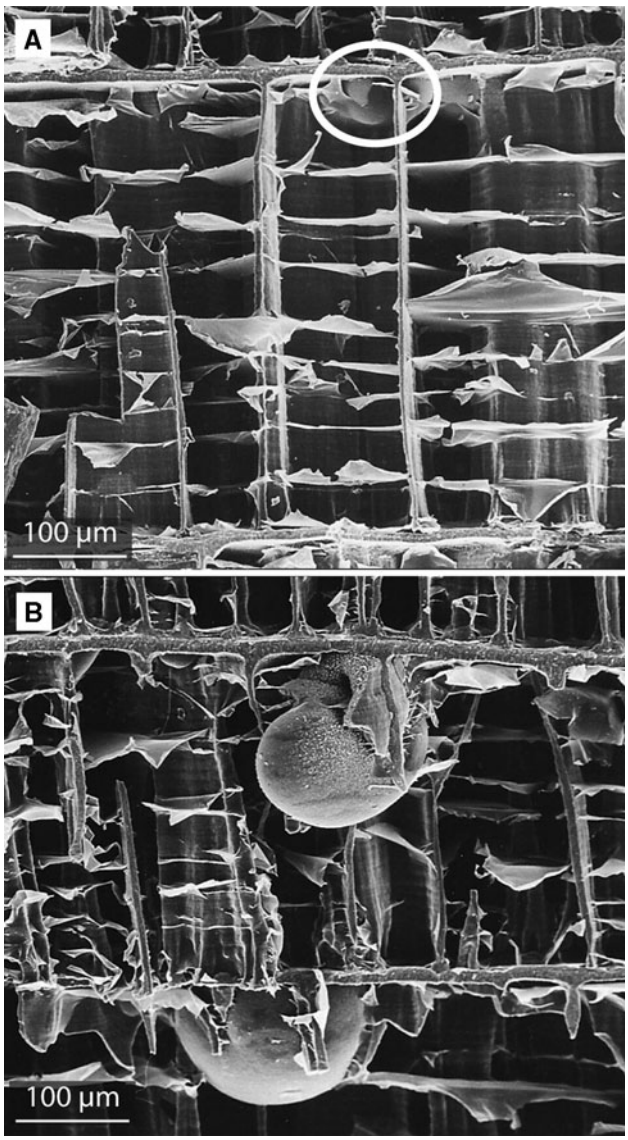

Fig. 5 SE micrographs along the sagittal midline illustrating differences in irregular $CaCO_3$ deposition. Control cuttlebones **a** contained 0.3 ± 0.3 spherical structures per 1 mm^2, ranging in height from 20 to 65 µm. Cuttlebone sections mineralized during exposure to 615 Pa CO_2 **b** contained significantly more spherical structures, 1.6 ± 0.5 per 1 mm^2, $P < 0.005$ ($n = 3$), ranging in height from 12 to 127 µm

lamellar spacing and body size have also been documented in cuttlefish cultured under very low feeding rations (Boletzky and Wiedmann 1978; Wiedmann and Boletzky 1982). In our study, the correlation of increasing lamellar spacing with faster growth rates was visible in both groups; however, the CO_2 treatment cuttlebones had a significantly reduced lamellar spacing despite maintained growth rates. The reduction in the height of the CO_2-incubated cuttlebones can be fully accounted for by the 50% reduction in lamellar spacing.

A comparison of the cuttlebone microstructure between the two groups clearly indicates the formation of a less

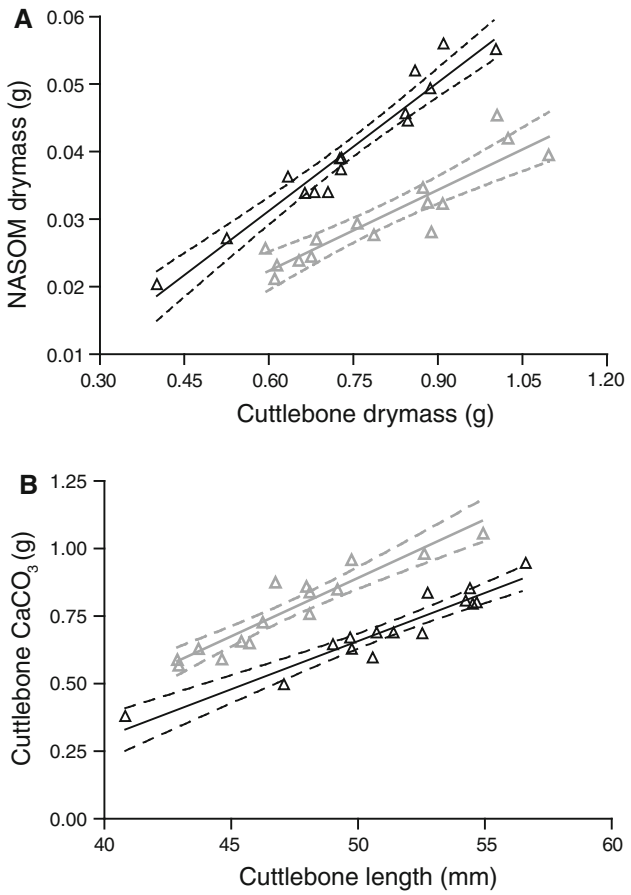

Fig. 6 Significantly less non-acid-soluble organic matrix (NASOM) was incorporated into cuttlebones mineralized during exposure to 615 Pa CO_2 (*gray*), $P < 0.005$ ($n = 14$)

porous structure by the cuttlefish exposed to elevated seawater pCO_2. Not only did the lamellar spacing decrease, but the thickness of the lamellar and pillar walls increased significantly. Notably, the pillar walls nearly doubled their average thickness from 2.6 ± 0.5 to 4.9 ± 2.1 µm. This increase in deposited $CaCO_3$ substantially contributed to the greater mass of the CO_2-treatment cuttlebones. Our finding of increased $CaCO_3$ deposition specifically along the pillar walls complements micro-Raman analysis of *S. officinalis* cuttlebones. It has been shown that the richest aragonite areas are along the pillar walls and the poorest in the lamellae, as these are comprised of significantly more organic matrix (Florek et al. 2009). Even though the general microstructure of the cuttlebone sections calcified during exposure to 615 Pa CO_2 was conserved, a greater degree of irregularity was present in the linearity and thickness of the lamellar walls and pillars, as evidenced by the high standard deviations of the measurements.

When examining the influence of elevated seawater pCO_2 on calcification processes, it is also important to consider the potential effects on the constituents of the organic matrix. The role of the organic matrix is crucial as it controls crystal nucleation, polymorph selection and crystal orientation of the mineralized $CaCO_3$ (Marin and Luquet 2004; Addadi et al. 2006; Marie et al. 2009). The mass of incorporated non-acid-soluble organic matrix (NASOM) in cuttlebones calcified during 6 weeks of exposure to 615 Pa CO_2 was reduced by 30% on average compared to controls. Interestingly, the freely suspended non-calcified organic matrix sheets in between adjoining lamellae were present in both experimental groups. This is in contrast to observations of cuttlebones calcified by *S. officinalis* on very low feeding rations, where the interlamellar sheets were entirely missing in young cuttlefish (Boletzky and Wiedmann 1978), and significantly reduced in older individuals (Wiedmann and Boletzky 1982). As the cellular pathways of organic matrix synthesis and secretion by the cuttlebone epithelium are still unknown, further interpretation of the reduction in NASOM is limited. To summarize the general pattern, however, we see a distinct increase in $CaCO_3$ deposition and a decrease in the incorporation of chitin, the primary structural component of the organic matrix.

Increased $CaCO_3$ deposition in cuttlebones during long-term exposure to elevated pCO_2 could be potentially detrimental to cuttlefish. If the cuttlebone is to preserve its function as a buoyancy regulation device, the structural density must remain low. In wild caught adult cuttlefish, a change of only ±16.5% in cuttlebone mass from the state of neutral buoyancy is sufficient to make *S. officinalis* either negatively or positively buoyant (calculated from Denton and Gilpin-Brown 1961a). A large enough increase in the mass of cuttlebone dry matter, accompanied by a decrease in volume due to the reduction in lamellar spacing, could significantly increase the density of the cuttlebone. This, in turn would decrease the buoyancy of the cuttlefish, requiring it to invest more energy into maintaining its swimming posture while hunting. The experimental design of this study did not include the resolution of behavioral modifications that could have indicated potential changes in functional control of the cuttlebones. Supporting literature that discusses changes in the microstructure of cephalopod chambered shells and the potential impacts on energetics and physiology is limited. However, Sherrard (2000) concludes that cuttlefish species living at greater depths have thicker lamellae and more densely spaced pillars compared to shallow living species. The greater structural strength of cuttlebones from deep-water species is suggested to come at the cost of increased cuttlebone density and thus reduced buoyancy function.

The extent of changes found in cuttlebone mass during 6 weeks of exposure to 615 Pa CO_2 suggests longer exposure periods under environmentally relevant seawater pCO_2 (<100–200 Pa) could also potentially affect the density of the cuttlebone in *S. officinalis*. It is important to note that in

this study, the majority of the region of the cuttlebone responsible for buoyancy control was mineralized by the experimental individuals under control conditions before the onset of CO_2 exposure. Future growth studies, where cuttlefish are raised under elevated pCO_2 conditions from the beginning of cuttlebone formation are necessary to fully assess how microstructural changes may influence functional control of the cuttlebone. Quantification of cuttlefish behavior and metabolic rates, taken together with measurements of cuttlebone density, could be used to calculate a new cost of buoyancy for *S. officinalis* (Webber et al. 2000) raised under future ocean acidification conditions.

It has been hypothesized that organisms with high metabolic rates and ion-regulatory abilities are less sensitive to elevated seawater CO_2 levels (hypercapnia) as they possess highly efficient mechanisms to maintain physiological homeostasis (Seibel and Walsh 2003; Pörtner et al. 2004; Pörtner 2008; Melzner et al. 2009b). During hypercapnic exposure, the maintenance of acid–base equilibria is challenged as a significant increase in pCO_2 elicits extracellular acidosis when not compensated. Considering the sensitivity of calcification processes to elevated pCO_2 and $[H^+]$, organisms that exhibit a greater degree of control over extra- and intracellular homeostasis will be more capable of conserving protein function and ion concentrations necessary for calcification.

The question that arises is, if *S. officinalis* is capable of maintaining somatic growth and metabolism at control levels during hypercapnia (Gutowska et al. 2008), why does calcification increase? It is important to consider that active marine organisms, like the cuttlefish, significantly increase extracellular HCO_3^- levels and partially, or fully, compensate the acidosis in their extracellular fluids during exposure to high seawater pCO_2 (Gutowska et al. 2010, Table 2). In *S. officinalis* exposed to 615 Pa CO_2, blood HCO_3^- is elevated to 10.4 mM (Gutowska et al. 2010). A significant increase in extracellular $[HCO_3^-]$, combined with partially compensated extracellular pH, could change ion-transport kinetics across the cuttlebone epithelium, and increase the $CaCO_3$ saturation state in the extracellular space surrounding the cuttlebone. In contrast, the sensitivity of shell growth in bivalves to ocean acidification conditions could potentially be attributed to the weak acid–base regulatory abilities of these comparatively inactive mollusks (Lindinger et al. 1984; Michaelidis et al. 2005).

Increased calcification during exposure to elevated seawater pCO_2 has also been described in other active marine organisms with high metabolic rates and ion-regulatory abilities. Brachyuran crabs are known to be proficient regulators of acid–base status. During exposure to hypercapnia of 500–1000 Pa, crabs nearly compensate extracellular pH and elevate blood $[HCO_3^-]$ to values between 10 and 30 mM (Truchot 1984; Cameron and Iwama 1987; Spicer et al. 2007; Pane and Barry 2007). Recent work has shown that the net calcification rate of carapaces in several decapod crustaceans (*Callinectes sapidus*, *Homarus americanus*, *Penaeus plebejus*) significantly increased during 60 days of exposure to ~300 Pa (Ries et al. 2009). It has also been noted that during periods of disturbance in acid–base and electrolyte status, pathological calcareous deposits occur in the antennal glands and gills of *Homarus americanus* (Dove et al. 2004; Dove 2005).

Studies on marine teleosts also provide examples for both hypercalcification of $CaCO_3$ structures, as well as calcinosis (mineralization of tissues), during exposure to elevated seawater pCO_2. As in cuttlefish and crabs, blood HCO_3^- levels in teleosts are elevated during exposure to high seawater pCO_2. However, the pH compensatory response of most teleosts is even stronger than that of active invertebrates. During exposure to hypercapnia >1,000 Pa, teleosts increase blood $[HCO_3^-]$ to values >30 mM, and often fully compensate the acidosis in blood pH (Toews et al. 1983; Larsen et al. 1997; Hayashi et al. 2004; Michaelidis et al. 2007; Melzner et al. 2009a). During long-term exposure to high levels of hypercapnia (~1,000 Pa), teleosts maintain control growth rates; however, the frequency of calcareous precipitates observed in the kidneys, i.e. nephrocalcinosis, has been found to increase (*Anarhichas minor*, Foss et al. 2003; *Salmo salar*, Fivelstad et al. 1999, 2003; Hosfeld et al. 2008; *Dicentrarchus labrax*, Vandeputte et al. 2009). Similarly, elevated calcification has also been observed in otoliths, where otolith mass increased by 25% in 7-day-old larvae of *Atractoscion nobilis*i raised at 260 Pa CO_2 (Checkley et al. 2009).

We hypothesize that increased deposition of $CaCO_3$ in organisms that actively compensate extracellular acidosis during exposure to elevated CO_2 conditions can be attributed to increased extracellular $[HCO_3^-]$ and resulting changes in $CaCO_3$ saturation at the calcification site. Ongoing work examining the transport physiology of the cuttlebone epithelium in the cephalopod *Sepia officinalis* will help explain the phenomenon of increased calcification during exposure to elevated seawater pCO_2 in marine invertebrates. Ultimately, studies that bring us closer to understanding the cellular mechanisms of calcification are necessary for predicting molluscan sensitivity to ocean acidification.

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