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Elevated $p\text{CO}_2$ drives lower growth and yet increased calcification in the early life history of the cuttlefish *Sepia officinalis* (Mollusca: Cephalopoda)

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Ocean acidification is an escalating environmental issue and associated changes in the ocean carbonate system have implications for many calcifying organisms. The present study followed the growth of *Sepia officinalis* from early-stage embryos, through hatching, to 7-week-old juveniles. Responses of cuttlefish to elevated $p\text{CO}_2$ (hypercapnia) were investigated to test the impacts of near-future and extreme ocean acidification conditions on growth, developmental time, oxygen consumption, and yolk utilization as proxies for individual fitness. We further examined gross morphological characteristics of the internal calcareous cuttlebone to determine whether embryonically secreted shell lamellae are impacted by environmental hypercapnia. Embryonic growth was reduced and hatching delayed under elevated $p\text{CO}_2$, both at environmentally relevant levels (0.14 kPa $p\text{CO}_2$ similar to predicted ocean conditions in 2100) and extreme conditions (0.40 kPa $p\text{CO}_2$). Comparing various metrics from control and intermediate treatments generally showed no significant difference in experimental measurements. Yet, results from the high $p\text{CO}_2$ treatment showed significant changes compared with controls and revealed a consistent general trend across the three treatment levels. The proportion of animal mass contributed by the cuttlebone increased in both elevated $p\text{CO}_2$ treatments. Gross cuttlebone morphology was affected under such conditions and cuttlebones of hypercapnic individuals were proportionally shorter. Embryonic shell morphology was maintained consistently in all treatments, despite compounding hypercapnia in the perivitelline fluid; however, post-hatching, hypercapnic animals developed denser cuttlebone laminae in shorter cuttlebones. Juvenile cuttlefish in acidified environments thus experience lower growth and yet increased calcification of their internal shell. The results of this study support recent findings that early cuttlefish life stages are more vulnerable towards hypercapnia than juveniles and adults, which may have negative repercussions on the biological fitness of cuttlefish hatchlings in future oceans.

Keywords: development, growth, metabolism, morphometrics, ocean acidification.

Introduction

An increase in open ocean $p\text{CO}_2$ from present levels of ~ 0.04 kPa to 0.07–0.10 kPa $p\text{CO}_2$ in open ocean habitats is expected to occur by 2100 (IPCC, 2007; Doney *et al.*, 2009). Particularly in many coastal

habitats, much higher $p\text{CO}_2$ values of >0.2 – 0.4 kPa can be expected in the next century. This is due to additional CO_2 production through respiratory processes (Melzner *et al.*, 2013; Wallace *et al.*, 2014). Changes in future ocean chemistry may eventually

cross thresholds in carbonate system speciation that constrain biogenic calcification, especially in species whose carbonate structures are directly exposed to seawater and not covered by protective organic layers (Reis *et al.*, 2009; Tunnicliffe *et al.*, 2009). Elevated seawater $p\text{CO}_2$ causes an acidosis in body fluids of all water-breathing organisms. This acidosis can be countered by some taxa, e.g. fish (Heisler, 1984; Claiborne and Edwards, 2002), crustaceans (Wheatly and Henry, 1992; Spicer *et al.*, 2007), and cephalopods (Gutowska *et al.*, 2010a) via increasing blood bicarbonate concentrations and secretion of protons. These changes in body fluid acid–base chemistry have been shown to induce behavioural pathologies in fish (Munday *et al.*, 2009) and seem to be causally related to hypermineralization in fish and cephalopods (Checkley *et al.*, 2009; Gutowska *et al.*, 2010a).

In some oviparous marine taxa, including fish, crustaceans, and cephalopod molluscs, the final phase of embryogenesis is characterized by challenging abiotic conditions (i.e. naturally high $p\text{CO}_2$) inside the protecting egg capsule. To maintain a sufficient O_2 flux into, and a CO_2 flux output of the perivitelline space, the eggs of many aquatic organisms undergo a swelling process that leads to reduced egg wall thickness and increases gas conductance as well as increased effective surface area available for diffusion (e.g. Cronin and Seymour, 2000; Walther *et al.*, 2010; Tseng *et al.*, 2013). In addition, $p\text{O}_2$ and $p\text{CO}_2$ in the perivitelline fluid (PVF) are modulated to enhance diffusive flux of respiratory gases: this leads to low PVF $p\text{O}_2$ values of <6 kPa and high PVF $p\text{CO}_2$ values of >0.2 – 0.4 kPa and pH values as low as 7.2 (Gutowska and Melzner, 2009; Dorey *et al.*, 2013). Elevated environmental $p\text{CO}_2$ leads to proportional increases in PVF $p\text{CO}_2$, as excretory CO_2 flux depends on a stable $p\text{CO}_2$ gradient between PVF and seawater (Hu *et al.*, 2011b; Dorey *et al.*, 2013). The fact that cephalopod embryos are naturally confronted with strong hypercapnia has led to the evolution of a capable embryonic acid–base regulatory machinery (Hu *et al.*, 2011a).

Members of the cephalopod order Sepiida, the cuttlefish, are direct-developers with a long (ca. 2 months) developmental period *in ovo*, and form an internal shell composed of delicate layers of calcium carbonate (aragonite) and organic matrices. The shell is initially formed in the embryo before hatching. The cuttlebone is formed of overlaid sheets of calcium carbonate, vertically separated to form chambers that function in buoyancy control for the animal (Denton and Gilpin-Brown, 1961). As the animal grows, new lamellae are laid down ventrally and the cuttlebone grows anteriorly in proportion to the rest of the body. Contrary to studies on many calcifying invertebrates (e.g. Wood *et al.*, 2008; Kroeker *et al.*, 2010; Wittmann and Pörtner, 2013), juveniles of the common cuttlefish *Sepia officinalis* actually mineralize more CaCO_3 in their cuttlebones during exposure to elevated seawater $p\text{CO}_2$, while maintaining growth and metabolism (Gutowska *et al.*, 2008, 2010b; Dorey *et al.*, 2013).

Questions remain as to how *S. officinalis* responds to ocean acidification in terms of physiology, development, and growth. To ascertain the effects of realistic near-future ocean acidification, we examined embryonic and juvenile cuttlefish incubated *in ovo*, hatched, and subsequently raised under different $p\text{CO}_2$ treatments. Given that the embryos of this species are naturally capable of secreting a shell under hypercapnic conditions within the PVF, we hypothesized that embryonic calcification is less sensitive to acid–base disturbances compared with post-hatching juveniles. Our experiments were designed to test whether embryonic development of *S. officinalis* under acidified conditions leads to juveniles with

reduced fitness. Growth rates of juvenile *S. officinalis* are reportedly not impacted by elevated $p\text{CO}_2$ (Gutowska *et al.*, 2008), for animals that had not been exposed to elevated seawater $p\text{CO}_2$ as embryos. Recent studies on other marine invertebrate species have found significant negative carry-over effects from one ontogenetic stage to the next in response to simulated ocean acidification, both in molluscs and echinoderms (Dupont *et al.*, 2008; Hettinger *et al.*, 2013). We reared *S. officinalis* embryos under simulated ocean acidification conditions to then assess juvenile growth performance. This study provided an opportunity to examine whether development under OA conditions increases stress at a vulnerable life stage, or alternatively provides an opportunity for early-ontogeny plasticity to cope with altered conditions.

Material and methods

Experimental animals and treatments

Eggs of *S. officinalis* were obtained from captive breeding at the Biological station in Luc-sur-Mer, Université de Caen (Normandy, France) and transported to Germany as very early-stage embryos (Stage 5–7 *sensu* Lemaire, 1970). Experiments were conducted at the facilities on the island Sylt at the Wadden Sea Station of the Alfred Wegener Institute for Polar and Marine Research (AWI), Germany, from May to August 2011.

To avoid bacterial infections, seawater was passed over a 15 W UV sterilizer (HW-Aquaristik, Germany). To avoid alterations of the seawater carbonate system by biological activity of experimental animals and micro-organisms, a flow through system using North Sea water (mean salinity 31.5) was maintained in a 16°C climate chamber with flow rates of seawater adjusted to a minimum twofold water exchange per tank per day (Table 1). All experimental tanks were maintained under a constant 12 h dark:12 h light cycle with water quality parameters monitored weekly to keep concentrations of nitrate below 0.2 mg l^{-1} . Eggs at embryonic stage 10 (after Lemaire, 1970; $n = 525$) were separated and randomly distributed to treatment groups, and each placed individually on the bottom of its assigned tank. Eggs were incubated for ~ 8 weeks before hatching. Post-hatching, temperature was increased to 18°C to improve growth (the range of 16 – 18°C is well within the optimum range of the species), and animals were fed twice-daily *ad libitum*, initially on a diet of mysids (*Neomysis integer*) and progressively transitioned to fresh brown shrimp (*Crangon crangon*).

CO_2 treatments

Three $p\text{CO}_2$ treatments were selected to facilitate comparison with other published studies (Gutowska *et al.*, 2008, 2010b; Hu *et al.*, 2011b; Dorey *et al.*, 2013): control, 0.05 kPa $p\text{CO}_2$; intermediate, 0.14 kPa $p\text{CO}_2$; and high, 0.37 kPa $p\text{CO}_2$ (Table 1). These were controlled by equilibrating experimental aquaria with an air/ CO_2 gas mixture generated by a central automatic gas mixing-facility (Linde Gas, HTK Hamburg, Germany). The gas mixture was introduced into the experimental aquaria using diffuser stones (Dohse, Graftschaft-Gelsdorf, Germany). Fresh and sterilized (hw UV sterilizer 500, Krefeld, Germany) seawater ($S = 28$ – 31.5) was adjusted to a salinity of 32.2 (Instant Ocean, Aquarium Systems, Sarrebourg, France) and pre-equilibrated with the respective air/ CO_2 mixtures in 150 l reservoirs. Daily exchange with pre-equilibrated seawater (40 – 80% of the tank volume) ensured NH_4^+ concentration below 0.2 mg l^{-1} . Temperature, salinity, and pH were checked daily. Seawater samples (0.5 l) for determination of total dissolved inorganic carbon (DIC) were collected weekly and poisoned with

Table 1. Seawater physiochemical parameters in for the control (0.05 kPa CO₂) and hypercapnic treatments (intermediate, ~0.14 kPa CO₂; and high, 0.37 kPa CO₂).

	Control ~0.05 kPa	pCO ₂ ~ 0.14 kPa	pCO ₂ ~ 0.37 kPa
Salinity	32.2 ± 0.2	32.2 ± 0.2	32.2 ± 0.2
Flow rate (ml min ⁻¹)	48.9 ± 7.6	46.4 ± 6.0	46.6 ± 5.0
pH _{NBS}	8.02 ± 0.04	7.76 ± 0.05	7.27 ± 0.03
pH _{NBS} min	7.84	7.45	7.10
pH _{NBS} max	8.07	7.95	7.35
DIC	2333 ± 88	2484 ± 120	2667 ± 140
TA	2567 ± 80	2557 ± 116	2595 ± 132
pCO ₂	0.052 ± 0.008	0.135 ± 0.020	0.368 ± 0.039
Ω _{aragonite}	2.85 ± 0.32	1.33 ± 0.17	0.55 ± 0.05
Day 19— <i>n</i> specimens	19	16	16
Day 34— <i>n</i> specimens	32	32	32
Day 47— <i>n</i> specimens	28	28	28

Measurements presented are the mean among all replicate aquariums over all daily measurements during the full experimental duration. Flow rate and pH_{NBS} (pH NBS scale) were measured daily in each tank, with a mean calculated for each aquarium and each treatment over the experimental period. Flow rate (flow, ml seawater min⁻¹) is the rate with which fresh seawater entered each aquarium. NBS, National Bureau of Standards; DIC, total dissolved inorganic carbon; TA, total alkalinity; Ω_{aragonite}, aragonite saturation state of aragonite; pCO₂, CO₂ partial pressure. pH_{NBS} is the mean of daily measurements. Minimal (min) and maximal (max) values present minimal and maximal values in pH_{NBS} observed in any aquarium and are given as a measure for fluctuation.

100 µl of an HgCl₂ saturated solution. DIC was determined using an AIRICA analyzer (Marianda, Kiel, Germany). DICKSON seawater standard was used as reference (Dickson *et al.*, 2003). Seawater specifications were calculated from pH_{TOTAL} (recalibrated with seawater buffers) and DIC with the open source program CO2SYS (Lewis and Wallace, 1998) using the dissociation constants by Mehrbach *et al.* (1973) as refitted by Dickson and Millero (1987). Seawater pH and temperature (Table 1) were measured daily with a pH meter (WTW 340i pH-analyzer, WTW SenTix 81-measuring chain, precision 0.01 units) that was calibrated with Radiometer IUPAC precision pH buffers 7.00 and 10.00 (S11M44, S11 M007).

Oxygen consumption

Oxygen consumption of stage 29–30 embryos and hatchlings was determined via closed-chamber respirometry. Animals were gently placed into 44 ml (individual eggs, stage 29–30 embryos) or 130 ml (hatchlings, age 1–3 d) glass vessels filled with 0.2 µm-filtered seawater from an incubation tank with the respective pCO₂ level. Respiration chambers containing eggs were closed air-free with glass slides and placed into a temperature controlled water bath at 16.00 ± 0.02°C (Lauda Proline RP855, Lauda-Königshofen, Germany). After an equilibration time of 15 min (for embryos) and 30 min (for hatchlings), chambers were slowly inverted (2–5×) without disturbing the animal to ensure equal oxygen distribution. Chambers were carefully opened, and pO₂ at the beginning and after 2–3 h were determined by inserting a needle-type fibreoptic oxygen sensor (optodes, PreSens GmbH, Regensburg, Germany) connected to a fibreoptic oxygen meter (Oxy-4 Micro, PreSens GmbH). Oxygen never decreased below a critical value of 70% air saturation. Three chambers without animals served as controls. Due to epibiota on the egg capsule surface (Cronin and Seymour, 2000), oxygen consumption by the capsule after removal of the animal was measured separately in the same manner as described above, and embryonic MO₂ was determined as the difference between whole egg and capsule respiration (i.e. subtracting oxygen consumption attributable to epibiota). Measurements were recorded on a personal computer using software provided by the manufacturer (OXY 4 v2.11 Micro) and stable values were obtained after 2–3 min after probe insertion for initial and final values. Calibration of the oxygen sensors

was performed at 16°C using water vapour-saturated air (100% oxygen saturation) and a 1% w/v Na₂SO₃ solution (0% oxygen saturation) at the respective ambient air pressure.

After respiration measurements, eggs were dried on paper tissues and egg wet mass was determined using a precision balance (Sartorius BA110S, Göttingen, Germany). Eggs were dissected, and embryos were checked for vitality. Measurements were omitted when ink was observed in the PVF, indicating a stress response during handling especially in late stage (stage 29–30) embryos. Yolk and animal were carefully wiped on tissue paper and wet masses were determined. Contractions of the mantle removed water from the mantle cavity. Total egg mass and yolk mass could be accurately determined to the nearest 0.01 g, but embryos were recorded to a precision of the nearest 0.05 g due to variation of measured wet masses.

Morphometrics

A subset of animals from each aquarium were retained post-hatching to examine growth of the cuttlebone (3 pCO₂ levels, 4 aquaria per pCO₂ level, 20 cuttlefish in each aquarium, 240 animals in total). After hatching, cuttlefish remained under the same experimental conditions and subsamples of each group were sacrificed at age 19 d (*n* = 16, in each elevated pCO₂ treatment; *n* = 19 in control conditions) and age 34 d (*n* = 32 in each treatment) with all remaining cuttlefish sacrificed at age 47 d (*n* = 28 in each treatment).

Upon termination, the wet mass (g) of each individual was recorded before the cuttlebone being removed by dissection. Care was taken to remove the cuttlebones in their entirety and all further measurements of cuttlebone dry mass (g), length, width, and height (mm) were performed on dried cuttlebones. Cuttlebone dry mass was quantified on a precision balance (AE ADAM, PW 124, Max 120 g, precision 0.0001 g). Length, width, and height of the cuttlebones were measured with digital vernier calipers.

The microstructure of ten cuttlebones each from control and CO₂ treatments was visualized by semi-thin sections of cuttlebones from specimens sacrificed at 47 d. These cuttlebones were selected from the total set based on length to ensure any differences found in internal morphometrics were not confounded by gross size

differences (overall mean length among three groups 26.74 ± 1.53 mm; $F_{2,45} = 0.697$, $p = 0.50$). To rehydrate the dried cuttlebones in preparation for embedding, we adapted protocols used for dry osteological samples. All stages were performed using degassed dH_2O and in a vacuum chamber. Specimens were serially rehydrated in ethanol and then decalcified in 1.5% EDTA. Decalcification was determined by visual inspection, then specimens were dehydrated in ethanol series (30, 50, 70, 90% $\times 2$, 95% $\times 2$, absolute $\times 2$) and cleared in HistoClear (2 h $\times 2$). Specimens were then immediately immersed in a mixture of HistoClear and hot wax (1:3 at 60°C). After 24 h, samples were embedded and allowed to set before sectioning (at $8 \mu\text{m}$) using a rotary microtome (Thermo scientific Finesse E+). Each block was first cut in the sagittal (longitudinal) midline, and semi-thin sections were taken from the central part of the cuttlebone, capturing several sections from each half of each block. These were stained with Coomassie blue (4 min), rinsed with dH_2O ; coverslips were affixed using DPX (Di-*N*-butyle phthalate in xylene) before imaging. Digital images of individual sections were examined for microstructural morphometrics (Figure 1) using the freeware program Image J (Rasband, 2012).

The larval shell layers (i.e. the first eight lamellae deposited before hatching) are visually separate in the shell cross section from the “adult” or post-hatching lamellae (Figure 1). This allowed us to directly measure the size of the larval shell compared with total size at point of death, and the size of the interspace in both the early-stage and post-hatching lamellae. Lamellae are separated by minor, primarily proteinaceous (chitin) laminae which are also visible in stained sections and could therefore be counted.

Analyses

Statistical analyses were conducted using IBM SPSS 13 or SigmaPlot 10 (Systat Software Inc.). The d’Agostino omnibus test for normality was applied to datasets for each treatment group. One-way analysis of covariance (ANCOVA) was conducted to test for differences in embryonic wet mass, egg wet mass, and yolk wet mass; incubation time and embryo wet mass served as covariates. Measured values were transformed to obtain linear regressions between the dependent variables and the covariates. Before testing, linear regressions were tested for homogeneity of slopes. A one-way ANOVA was performed for comparison on oxygen consumption rates in different $p\text{CO}_2$ treatments. MO_2 and specific MO_2 of hatchlings were related to hatchling wet mass of 0.25 g using the mass exponents of the respective power equation regressions.

A two-factor ANOVA was used to test for the interactive effects of $p\text{CO}_2$ treatment and sampling time point on animal size. Because morphometric measurements of cuttlebones were not normally distributed, Kruskal–Wallis tests and Mann–Whitney *U*-tests for *post hoc* comparisons were used to test for (i) further differences in animal wet mass and cuttlebone dry mass at age 19 and 47 d among the three $p\text{CO}_2$ treatments, and (ii) differences among the three treatments at 47 d age for cuttlebone length, width, height, lamellar spacing, and number of minor laminae. A discriminant analysis was applied on the un-standardized measurements for cuttlebone width (mm), cuttlebone length (mm), cuttlebone mass (g), and animal wet mass (g), to investigate separation between the three $p\text{CO}_2$ treatment groups by morphometry. Relationships among these variables were also visualized using *z*-standardized data (using the transform $z = (x - \bar{x})/\sigma$, for each value x for a variable with mean \bar{x} and standard deviation σ), to allow comparisons despite differing units.

Results

Growth, development, and hatching

There were two post-hatching mortalities over the course of the treatments; one animal from the high $p\text{CO}_2$ group at age 25 d, and one animal from the control group at age 26 d. There were no mortalities at age 27 d or later. Daily observation revealed no notable behavioural differences observed either between treatment groups or aquarium groups; however, this was not quantified during the course of this study.

Under control conditions, 50% of animals hatched after 65–69 d of incubation, while hatching was slightly delayed in the intermediate $p\text{CO}_2$ treatment (67–70 d) and further delayed under high $p\text{CO}_2$ (69–72 d). The mean dates were significantly different (Kruskal–Wallis $H_{2,N=531} = 144.46$, $p < 0.001$), with significant separation between both the control and intermediate compared with the high $p\text{CO}_2$ hatching date (Dunn’s multiple comparison: $p < 10^{-6}$).

Overall, yolk wet mass was ~ 0.15 g at 30 d of incubation and exponentially decreased over time with higher values in embryos treated in high $p\text{CO}_2$ (Figure 2a). An ANCOVA revealed a significant effect of treatment on the relationship between yolk wet mass and incubation time ($F_{2,115} = 12.11$, $p < 0.0001$). Yolk mass remained larger in the high $p\text{CO}_2$ group. Pairwise comparisons with Tukey’s HSD showed a significant influence of treatment on yolk mass decrease between the control and intermediate $p\text{CO}_2$ group ($p < 0.0001$), and also the intermediate and high $p\text{CO}_2$ groups ($p < 0.001$). The exponential decrease in yolk wet mass with increasing embryo wet mass was not significantly different between treatments (Figure 2b; ANCOVA, $F_{2,116} = 1.38$, $p > 0.25$). An ANCOVA comparing log-transformed egg wet masses with embryo wet mass as covariate revealed significant effects of treatment ($F_{2,117} = 7.31$, $p < 0.01$; Figure 2c).

Hatchling wet mass was significantly different among treatments, demonstrating a decreased body mass in high $p\text{CO}_2$ -treated animals (Figure 3). An additional one-way ANOVA indicated that exposure to elevated $p\text{CO}_2$ stunted overall animal wet mass at age 19 d post-hatching (Kruskal–Wallis $\chi^2 = 16.43$, d.f. = 2, $p = 0.0003$); *post hoc* analyses showed no significant difference between control and intermediate treatment specimens (*Z*-score = 0.78, $p = 0.44$). At age 19 d, the mean animal wet mass remained depressed in the high $p\text{CO}_2$ group compared with the control treatment (mean mass \pm SD = 0.5507 ± 0.14 g, compared with 0.8054 ± 0.20 g in the control group; $p < 0.001$). This difference becomes more pronounced with age (Figure 3a). To facilitate statistical comparisons, net mass measurements were log-transformed, the resulting slopes of mass over time showed growth rates were significantly lower in high $p\text{CO}_2$ groups ($F_{2,224} = 57.6$, $p < 0.0001$).

The mass of the hatchling cuttlebone was not in equal proportion to animal mass in all three groups and this ratio changed over early post-hatching ontogeny (Figures 3b). At the earliest time point sampled (19 d post-hatching), there was a significant difference between groups for the ratio of the dry (cuttlebone) mass to animal wet mass (Kruskal–Wallis $\chi^2 = 12.20$, d.f. = 2, $p = 0.0022$), but no significant difference separated control and intermediate treatments (*Z*-score = -1.44 , $p = 0.15$). At later time points, the mean dry-shell contribution to animal mass was significantly larger in the intermediate group and significantly larger again in the high $p\text{CO}_2$ group (age 34 d: KW $\chi^2 = 43.05$, $p < 0.001$; day 47: KW $\chi^2 = 48.60$, $p < 0.001$; *post hoc*, $p < 0.01$ for all comparisons; Figure 3b). Animals from the high $p\text{CO}_2$ group were on average 48% lighter than control animals by wet mass but in contrast

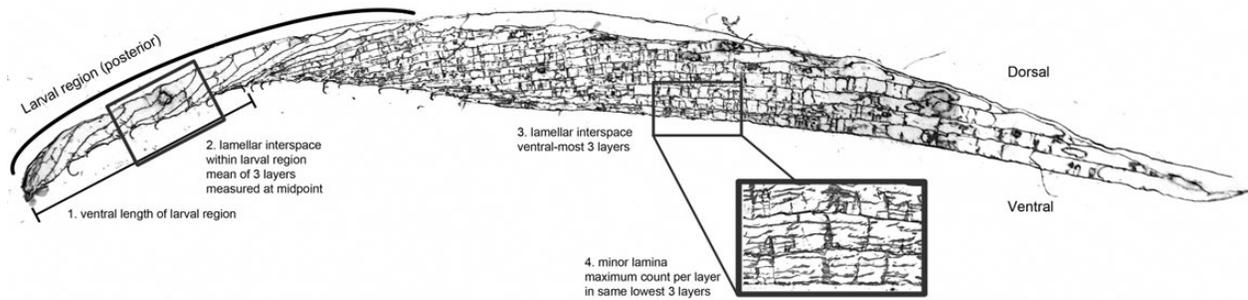


Figure 1. Example semi-thin sagittal section of the cuttlebone, indicating specific areas for microscopic measurements. Inset, higher magnification image of the lamellar structure in the most recent (ventral) part of the cuttlebone, showing minor laminae. The illustrated specimen was reared in an intermediate acidified treatment, 1120 ppm $p\text{CO}_2$ (pH 7.7) and sacrificed at age 47 d.

their cuttlebones were only 18% lighter on average. Shell mass as a percentage of body mass increased with increasing $p\text{CO}_2$ and this became more pronounced over early growth.

Oxygen consumption during development

Embryonic oxygen consumption increased in an exponential fashion over time and approximately tripled during the period of measurement. Compared with control and intermediate $p\text{CO}_2$ embryos, regression curves indicate $\sim 20\%$ lower oxygen consumption in high $p\text{CO}_2$ -treated embryos at late stage incubation (day 62). Metabolic rates normalized to wet mass of hatchlings were around three times higher compared with those of late embryonic stages (Figure 4b). Mean MO_2 did not differ between treatment groups ($\mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$; $F_{2,23} = 0.06$, $p > 0.95$). Corrected rates of MO_2 ($\mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$) per gram fresh wet mass decreased with rising embryo wet mass as expected, but early-stage measurements were highly variable (Figure 4a). There was a greater range of mass-specific oxygen consumption rates in the control group (Figure 4a), but the overall average of all individuals resulted in no significant differences observed for oxygen consumption rates of embryos and hatchlings exposed to the three $p\text{CO}_2$ treatments (Figure 4b).

Cuttlebone morphometrics

The gross biometrics (animal wet mass, cuttlebone dry mass, cuttlebone length, width, and height) generally followed the same pattern, and at each sampled time point, there was a significant difference between treatment groups overall, but no significant difference between control and intermediate- $p\text{CO}_2$ treatment groups. A canonical discriminant analysis (Figure 5a) of the same data (z -transformed normalized data illustrated in Figure 5b) determined that 96.8% of variance among the specimens is explained by the first (of two possible) discriminant function separating treatment groups (Figure 5a). This effectively demonstrates that morphometry is significantly different among the different cohorts. The distribution frequencies of the discriminant scores illustrate that morphometry is largely equivalent at age 19 d, but separates $p\text{CO}_2$ treatments at age 34 d—the high $p\text{CO}_2$ treatment is clearly different from the control but both overlap the intermediate treatment—and by age 47 d, the total shape is distinctly different between all three groups.

The shape and ontogeny of the adult cuttlebone can be gauged by the correlation of the dimensional metrics obtained after a standardization transformation (Figure 5b). The length–width ratio of the total cuttlebone provides a proxy for shape. Cuttlebones in

extreme high $p\text{CO}_2$ were slightly but significantly shorter relative to their width, than those in animals grown in control conditions; overall, the aspect ratio (length/width) was significantly different between treatments (Kruskal–Wallis $\chi^2 = 10.71$, d.f. = 2, $p = 0.0047$), but there was no significant difference between control and intermediate $p\text{CO}_2$ treatment (Z -score = -0.86 , $p = 0.39$).

While specimens in high $p\text{CO}_2$ treatment were smaller and with shorter cuttlebones, the specimens selected for sectioning were deliberately chosen to represent animals of similar size. No significant differences in overall cuttlebone length were found across specimens (age 47 d) selected for sectioning (KW $\chi^2 = 1.31$, d.f. = 2, $p = 0.52$), though the dry mass varied among the three treatment groups (KW $\chi^2 = 6.80$, $p = 0.033$).

Differential influences of the OA treatments were also reflected in the ventral (post-hatching) lamellae at age 47 d; the control group mean interspace was 0.2378 mm, intermediate and high- $p\text{CO}_2$ treatments were increasingly more compact 0.1786 and 0.1748 mm, respectively, which represented a significant difference (KW $\chi^2 = 12.20$, d.f. = 2, $p = 0.0074$). The number of minor (proteinaceous) laminae occurring in the interspace between calcified lamellae on the most recent (ventral) three layers (Figure 1) was also counted. The number of minor laminae ranged from four to six and was not significantly different between treatments ($\chi^2 = 15.31$, d.f. = 18, $p = 0.64$). Cuttlebones became more compact at increased $p\text{CO}_2$.

The embryonic shell is preserved as the posterior-most portion of the cuttlebone and is structurally distinct from the rest of the post-hatching cuttlebone (Figure 1). Embryonic shell size was not significantly different among treatments (KW $\chi^2 = 5.80$, d.f. = 2, $p = 0.055$) and the larval lamellar interspace did not differ between the three treatments (KW $\chi^2 = 1.26$, d.f. = 2, $p = 0.53$). The larval shell did not differ under varying embryonic exposure to elevated exterior $p\text{CO}_2$.

Discussion

In this study, we aimed to take a comprehensive approach to the impact of hypercapnia on *S. officinalis* and to synthesize the body of work on this species (Figure 6). Cuttlefish embryos are exposed to extreme natural hypercapnia *in ovo* which may be increased further under environmental ocean acidification. The developing animals under our near-future and extremely elevated $p\text{CO}_2$ treatments hatched at a slightly delayed tempo and at significantly smaller size. Yet, despite these conditions, the animals maintain an identical embryonic cuttlebone, and proceed to grow a relatively larger cuttlebone per unit wet mass after they have hatched.

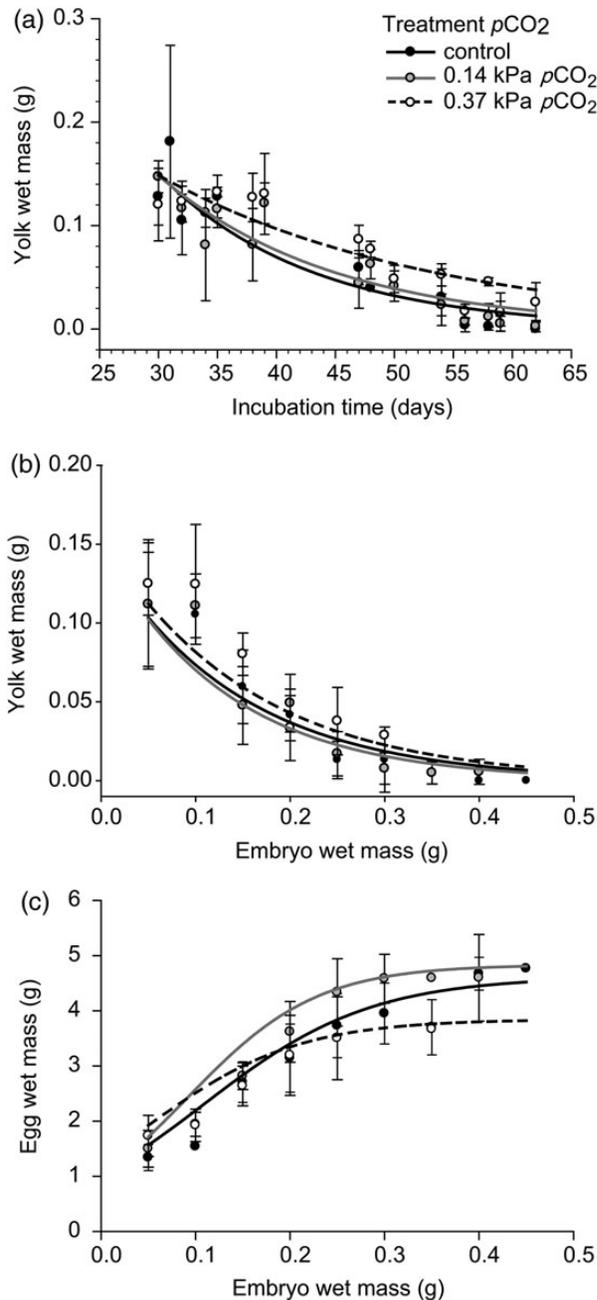


Figure 2. Yolk and egg biometric parameters as a function of time or body mass throughout development of *S. officinalis* in control and elevated CO₂ treatments. Circles and error bars represent mean measurements ± SD. (a) Change in outer yolk wet mass (OYM) over incubation time in days (t). Lines represent exponential regressions of raw data for control [OYM = 1.51 × exp^(0.077×t), R² = 0.77], intermediate pCO₂ [OYM = 1.10 × exp^(0.067×t), R² = 0.74], and the high pCO₂ group [OYM = 0.54 × exp^(0.043×t), R² = 0.67]. (b) Change in outer yolk wet mass with embryo body mass. Lines represent exponential regressions of raw data for the control group [OYM = 0.15 × exp^(6.86×EmM), R² = 0.73], intermediate group [OYM = 0.15 × exp^(7.54×EmM), R² = 0.71], and high pCO₂ [OYM = 0.15 × exp^(6.42×EmM), R² = 0.73]. (c) Total egg wet mass (EggM) as a function of embryo body mass (EmM). Lines represent sigmoid regressions of raw data for the control group [EggM = 4.61/(1 + exp^{-(EmM - 0.11)/0.088})], R² = 0.90, the 0.14 kPa CO₂ group [EggM = 4.83/(1 + exp^{-(EmM - 0.091)/0.069})], R² = 0.92, and the 0.37 kPa CO₂ group [EggM = 3.84/(1 + exp^{-(EmM - 0.050)/0.080})], R² = 0.6924].

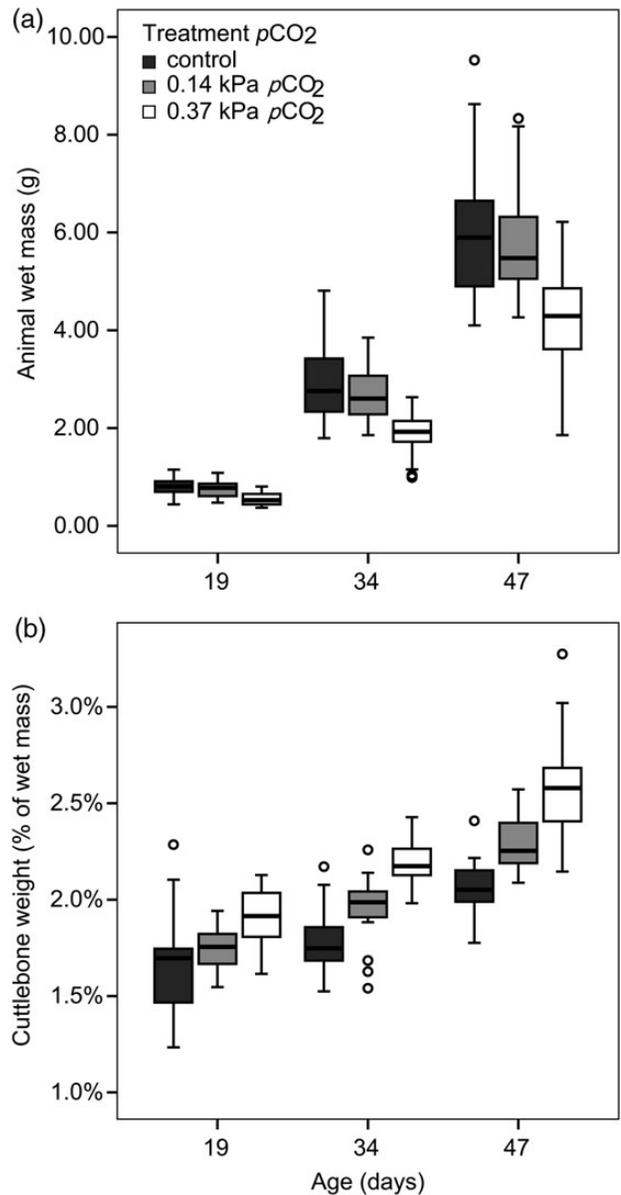


Figure 3. Boxplots illustrating median, upper and lower quartile, and inter-quartile range of size in cuttlefish in three pCO₂ treatments over three time sampling points over juvenile growth (circles indicate individual outliers outside the inter-quartile range). (a) Animal wet mass increases with age but is relatively stunted in elevated pCO₂. (b) The contribution of the cuttlebone as a percentage of total mass increases slightly with growth and is significantly greater in high pCO₂ treatment.

There is evidence that cuttlefish embryonic stages are more sensitive in terms of development and metabolism towards environmental hypercapnia than juveniles and adults (Gutowska *et al.*, 2008). Earlier work on juvenile cuttlefish growth under strongly acidified conditions (ca. 0.4 and 0.6 kPa) showed no change in somatic rates in post-hatching juveniles (Gutowska *et al.*, 2008, 2010b). Similarly, juveniles exposed to moderate hypercapnia (near-future pH) also apparently maintained somatic growth (Dorey *et al.*, 2013). Yet late embryos and hatchlings reared under 0.37 kPa CO₂ (our high pCO₂ treatment) had wet masses reduced

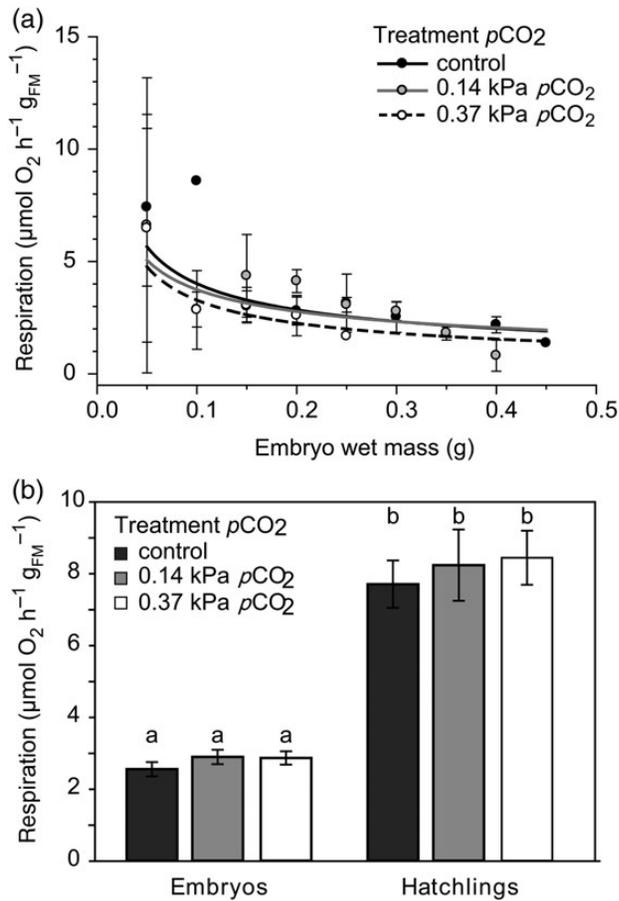


Figure 4. Oxygen consumption rates for embryos of *S. officinalis* (a) as a function of embryo individual wet mass, and (b) mean standard metabolic rates for embryos and hatchlings reared under different $p\text{CO}_2$ concentrations. Oxygen consumption rates were corrected by fresh mass (FM) and presented as mean \pm SD.

by up to 30% in the final phase of embryonic development (Hu *et al.*, 2011b). Contradictory results concerning the greater sensitivity of embryonic growth rates during exposure to near-future acidification, compared with hatchling growth, have been reported (Lacoue-Labarthe *et al.*, 2009; Dorey *et al.*, 2013). The present results clarify this pattern: although there is no statistically significant suppression of growth in our near-future (intermediate) experimental condition, comparison with our extreme (high) pH condition reveal a distinctive pattern of deleterious growth effect (Figure 3a). Indeed in this study, there are limited observable differences between the control group (0.05 kPa $p\text{CO}_2$) and our intermediate elevated $p\text{CO}_2$ treatment (0.14 kPa), yet the significant changes in growth, morphometry, and laminar density at extreme $p\text{CO}_2$ (0.37 kPa) illuminate overall trends in the ways that ocean acidification affects the study organisms.

There is substantial controversy around experimental design and setting threshold $p\text{CO}_2$ levels for studying ocean acidification and this example underlines the empirical value of comparing a range of treatment conditions. To understand OA in the context of natural conditions, special attention should be dedicated to naturally acidified/hypercapnic microhabitats (sediment burrows, shells, egg capsules) in which an organisms lives or develops. For example, within the egg, embryonic cuttlefish are exposed to a

naturally increasingly hypercapnic environment in the PVF (Gutowska and Melzner, 2009). Conditions *in ovo* are further exacerbated in acidified environmental seawater because of the egg capsule acting as a diffusion barrier for metabolic CO_2 generated by the embryo. Increases in external environmental hypercapnia are additive to the already high PVF $p\text{CO}_2$ levels (0.25 kPa), resulting in a PVF and $p\text{CO}_2$ of 0.78 kPa (pH 7.0) in the conditions we used as our high $p\text{CO}_2$ treatment (Hu *et al.*, 2011a). To minimize oxygen limitation, cuttlefish eggs swell in the course of development, which leads to a reduced capsule wall thickness and a higher diffusive area to support the rising oxygen demands and CO_2 excretion rates of the embryo (De Leersnyder and Lemaire, 1972; Cronin and Seymour, 2000; Gutowska and Melzner, 2009). Although the mechanisms of the egg swelling process are not well characterized, it was suggested that osmotically active organic compounds from the environment accumulate inside egg, or that the embryo itself maintains the osmotic gradient (De Leersnyder and Lemaire, 1972; Boyle, 1986). Our results showed that egg mass per volume is potentially slightly increased in embryos exposed to 0.14 kPa CO_2 , but potentially decreased in 0.37 kPa-treated embryos (Figure 2c); this corresponds to observations of *S. officinalis* egg masses exposed to moderate $p\text{CO}_2$ levels of 0.09–0.14 kPa (Lacoue-Labarthe *et al.*, 2009). The increased swelling may be a consequence of physiological responses of embryos to CO_2 within the capsule. Alternatively, cuttlefish embryos may be capable of actively regulating PVF osmolarity, and thus, diffusion properties (i.e. effective surface area and diffusion distance) of the egg capsule to improve CO_2 excretion during environmental hypercapnia. Though the process is unknown, simulated ocean acidification has been demonstrated to additionally challenge the embryonic acid–base regulatory machinery of cephalopods to protect from a CO_2 -induced acidosis (Hu *et al.*, 2013).

In adult cephalopods, the acid–base regulatory machinery of gill epithelia provides competent pH_e control and the resulting protection of the pH-sensitive respiratory pigment haemocyanin (Pörtner, 1990; Hu *et al.*, 2011a, b, 2015). Embryonic stages exhibit a potentially less efficient acid–base regulating machinery; before gill epithelia are functionally developed, cephalopod embryos possess epidermal ionocytes scattered on yolk and skin epithelia, that are capable of secreting proton equivalents (e.g. H^+ or NH_4^+ ; Hu *et al.* 2011a, 2013). The acid–base regulating capacities of the embryos may be limiting in high PVF $p\text{CO}_2$; the consequence would be uncompensated extracellular acidosis that may negatively influence oxygen transport capacities and metabolic processes of the embryo (Decleir *et al.*, 1971; Pörtner *et al.*, 1991). It has been suggested that an uncompensated acidosis as well as limited oxygen supply to the developing embryo directly translates into reduced growth and developmental rates (Reipschläger and Pörtner, 1996; Pörtner *et al.*, 1998).

Hypercapnia-induced reductions in larval and embryonic growth associated with altered metabolic rates have been observed in many marine invertebrates (Kurihara and Shirayama, 2004; Kurihara *et al.*, 2004, 2007; Dupont *et al.*, 2008, 2010; Dupont and Thorndyke, 2009). The oxygen consumption of control and intermediate $p\text{CO}_2$ group embryos at stage 28–29 were similar to those values reported for *S. officinalis* stage 29 embryos (Wolf *et al.*, 1985). During development, cuttlefish embryos reared under high $p\text{CO}_2$ exhibited no overall change in aerobic metabolism when compared with control and intermediate $p\text{CO}_2$ animals. Standard metabolic rate in older *S. officinalis* acclimated to even higher seawater $p\text{CO}_2$

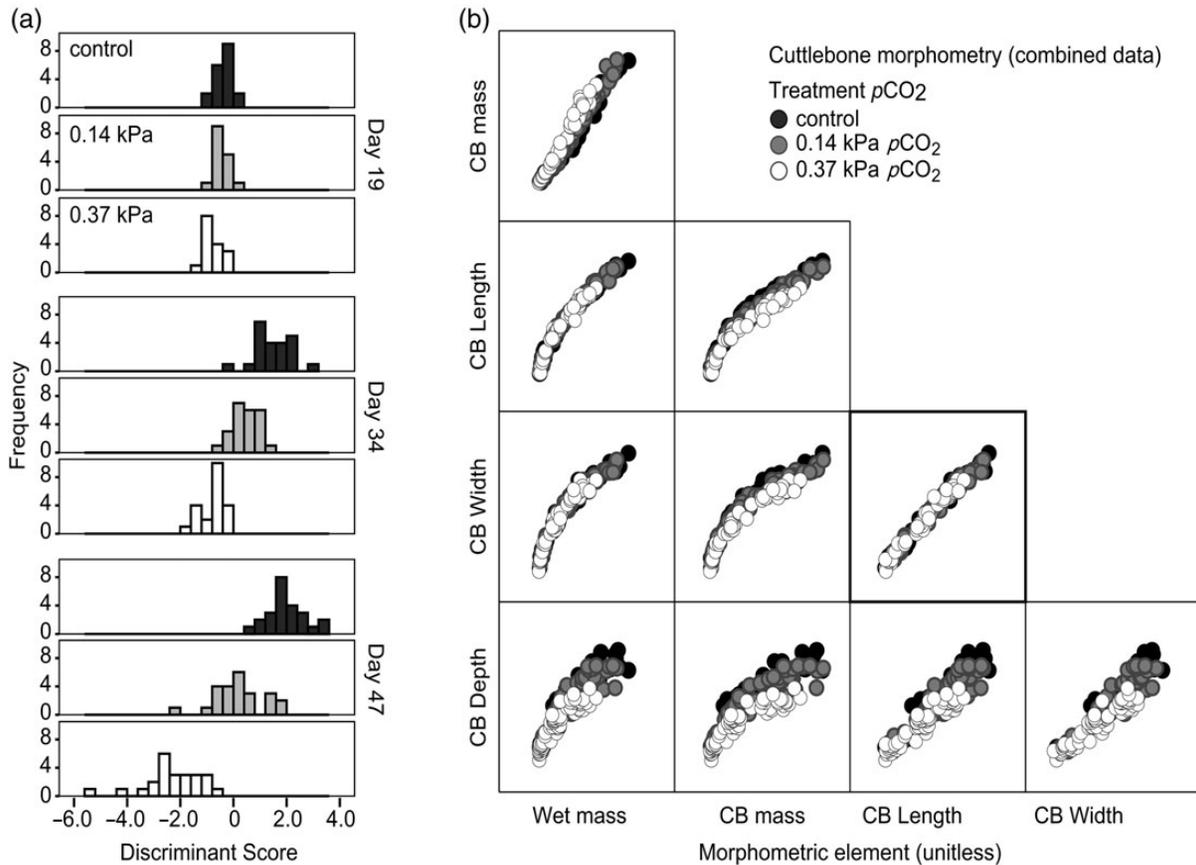


Figure 5. Morphometric discrimination of cuttlebones from animals reared in different ocean chemistry. (a) Stacked histogram of scores from first discriminant function of treatment, i.e. $p\text{CO}_2$, based on morphometry of the total dataset (measurements illustrated in Figure 1), $sep(D) = 1.057$, $D = 6.03(\text{wet mass}) - 5.83(\text{cuttlebone mass}) - 0.75(\text{CB height}) - 0.85(\text{CB width}) + 1.61(\text{CB depth})$. The distribution of individual scores from the first discriminant function illustrates increasing separation of total shape and size between treatment groups during growth over three progressive time points. (b) Matrix scatterplot of z-standardized (i.e. unitless) gross morphometric data for animal wet mass and cuttlebone (CB) dimensions, combining all sampling points (age 19, 34, and 47 d) over early life ontogeny.

(0.6 kPa) also did not differ from that of control animals (Gutowska *et al.*, 2008).

Exposure to low pH during the larval phase is known to adversely affect development in a range of species (reviewed in Byrne, 2011). Pecl *et al.* (2004) hypothesized that smaller cephalopod hatchlings would need more time to escape from the “window of vulnerability” and thus, elevated $p\text{CO}_2$ may negatively influence their survival with respect to predation. There may also be different responses in acclimated adult organisms compared with those that experience a modified environment during embryonic development. Importantly, exposure to high $p\text{CO}_2$ during development here did not prevent stunted growth in early life after hatching and in fact exacerbated size differences between groups in early life.

The present data build on previous work (Gutowska *et al.*, 2008, 2010b; Dorey *et al.*, 2013), by demonstrating that the increased calcification in *S. officinalis* occurs not only at extreme levels of $p\text{CO}_2$ tested in previous studies, but here at a level considered realistic for the oceans within the next 100–200 years as our intermediate treatment (0.14 kPa $p\text{CO}_2$) is similar to predicted ocean conditions in 2100 (IPCC, 2007). The shell of cuttlefish is fully internalized and the organism’s ability to maintain calcification rates are directly dependent on its physiology and not on extrinsic dissolution from the surrounding medium. Other cephalopods are unable to maintain

internal calcified structures under experimental OA (Kaplan *et al.*, 2013). The strong acid–base regulatory abilities, which protect extracellular pH homeostasis by increasing blood HCO_3^- may alter the precipitation kinetics of CaCO_3^- and could explain higher calcification rates induced after hatching. This hypothesis is supported by the fact that the phenomenon of hypercalcification has only been observed in strong-acid–base regulators, including fish (Checkley *et al.*, 2009) crustaceans (Reis *et al.*, 2009), and cephalopods (Gutowska *et al.*, 2010b) that are capable of accumulating significant amounts of HCO_3^- in body fluids to buffer CO_2 -induced acid–base disturbances.

Although animals hatched in the elevated $p\text{CO}_2$ treatments were smaller, including having shorter mantle length and lower wet mass, their calcareous cuttlebones were maintained at a similar size and thus proportionally greater in mass increasing in statistically significant increments between each treatment from 1.8% of wet mass (control) to 2.0% (intermediate) and 2.2% (high $p\text{CO}_2$; Figure 3b). The absolute cuttlebone mass is directly dependent on the animal mass and treatment conditions, including ocean chemistry but perhaps other factors as well. Cuttlebone dry mass is an effective proxy for calcium content (Gutowska *et al.*, 2010b). Increased calcification of the cuttlebone was observed at both a high $p\text{CO}_2$ and to a lesser degree an intermediate treatment

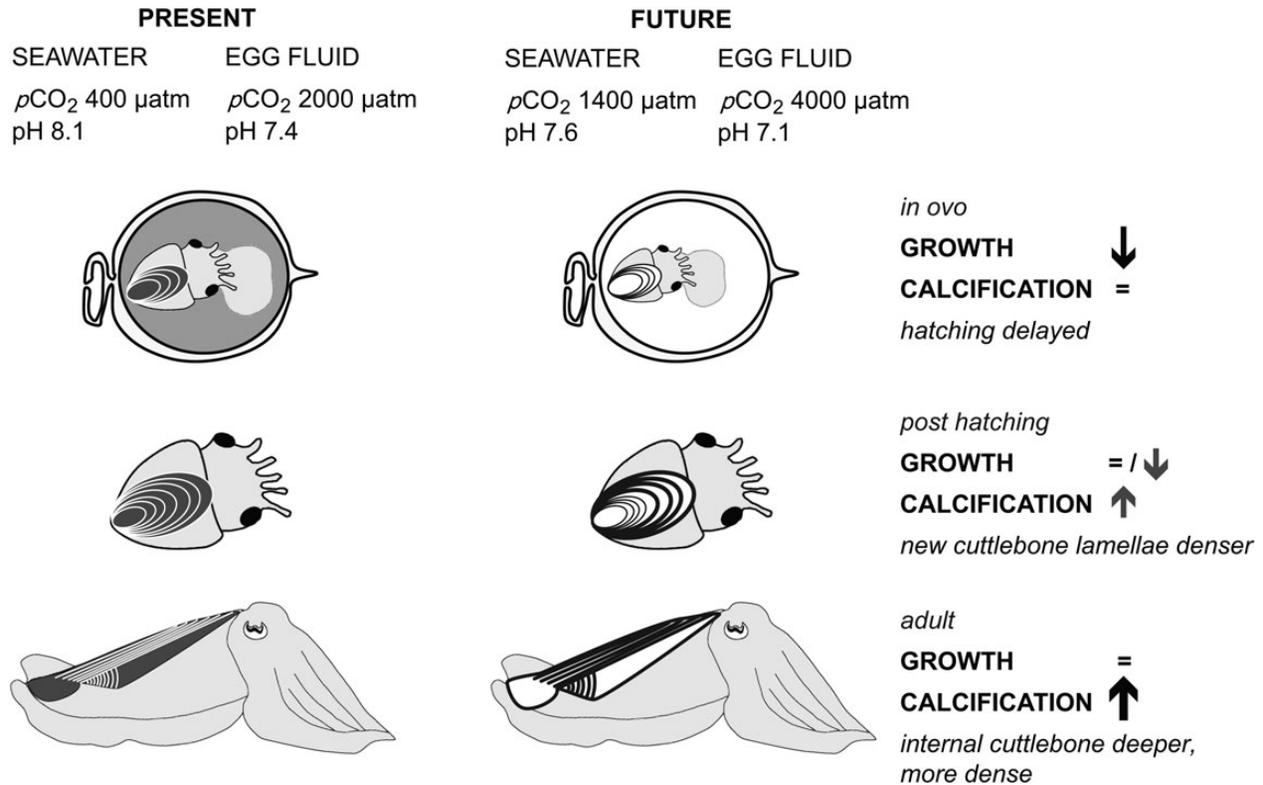


Figure 6. Schematic summary of the influence of ocean acidification on growth rate and cuttlebone calcification in *S. officinalis* embryos and hatchlings. Decreased growth rates observed during embryonic development do not carry over to hatchling growth performance. Cuttlebone hypercalcification does not take place *in ovo* but begins immediately on hatching and can be observed in hatchlings and later growth stages under exposure to elevated seawater $p\text{CO}_2$. An hypothesis is presented that attributes the high growth rates and cuttlebone hypercalcification in hatchlings to their strong acid–base regulatory capacities; the converse is concluded for embryonic life stages.

simulating ecologically relevant, $p\text{CO}_2$. Although there was no evidence of any difference in the embryonic shells (embryonic shells have the same size and same lamellar spacing in all treatments), previous work demonstrated a slight increase in calcium incorporation in embryonic cuttlebones in contrast to post-hatching hypercalcification (Dorey *et al.*, 2013). The cuttlebones in our high $p\text{CO}_2$ were proportionally heavier at age 19 d and with significant differences between all three groups by age 34 d post-hatching. This indicated that the observed influence of pH on ontogeny causes them to become morphologically more separated over time (Figure 5a).

This shift in morphometry was preliminarily described from juveniles, in that individuals exposed to extreme high $p\text{CO}_2$ had slightly shallower cuttlebones with more tightly packed lamellae than control individuals creating a denser cuttlebone (Gutowska *et al.*, 2010b). Such hypercalcification could be detrimental because the cuttlebone acts as a “low cost” or “passive” mechanism for buoyancy regulation, meaning that cuttlefish are able to maintain a position of choice in the water column without expending much energy to do so (Denton and Gilpin-Brown, 1961), by adjusting the gas:fluid ratio within this chambered shell.

Subtle changes in cuttlebone mass can markedly impact cuttlefish buoyancy (Denton and Gilpin-Brown, 1961), making cuttlefish potentially sensitive to over-mineralization through acidification. In the long run, these structural effects may increase and impact upon key currencies related to fitness (i.e. behavioural time budgets and energy costs; Lemon, 1991; Lyons *et al.*, 2012) with consequences for survivorship and reproductive success. Increased

density of the cuttlebone has been found in deeper-water species and may increase strength at higher pressure (Ward and Von Boletzky, 1984; Sherrard, 2000). But the ability of the individual animal to regulate its buoyancy may speculatively be compromised if the total gas volume is reduced, which is correlated with increased cuttlebone density. In this context, malformations of the statoliths were found in the squid *Doryteuthis pealeii* exposed to acidified (0.22 kPa $p\text{CO}_2$; pH 7.3) conditions (Kaplan *et al.*, 2013) and also in *Loligo vulgaris* cultured even at less extreme acidification (pH 7.85 and 7.60: Lacoue-Labarthe *et al.*, 2011). Internal aragonitic statoliths function as balance and acceleration receptors and allow the detection of the particle motion component of a sound pressure wave stimuli in cephalopods (Budelmann, 1988). Quantitative determinations of energy allocations between processes like pH regulation, sensory responses and predation, buoyancy control, and growth will help provide a better understanding regarding the resilience of cuttlefish early life stages in future oceans. That cuttlebones are measurably denser in animals born into a realistic, near-future acidification scenario is a relevant potential threat to cuttlefish as an exploited species.

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