The influence of elevated seawater pCO_2 on growth, calcification and maintenance of acid-base equilibria in the cephalopod *Sepia officinalis*.

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Summary

The acidification of ocean surface water due to increasing atmospheric CO_2 levels has come into focus as an important global change phenomenon. Research on the potential biological impact of ocean acidification induced changes in seawater chemistry however is still in its infancy. Amongst the invertebrates, cephalopod molluscs have been hypothesized to be extremely vulnerable to elevated seawater pCO_2 due to the pH sensitivity of their blood oxygen binding pigment, hemocyanin. An acidotic shift in blood pH (pHe) could potentially lead to asphyxiation, as the oxygen saturation of the blood pigment would decrease. The experimental work of this thesis was carried out with the cephalopod *S. officinalis* as a model organism. In order to maintain hemocyanin function during exposure to elevated seawater pCO_2, the acid-base regulatory response of the cuttlefish would have to compensate acidosis in pHe. The function of the cuttlebone in *S. officinalis* as a support structure and buoyancy regulation device could also be compromise due to the potential sensitivity of calcification processes to ocean acidification conditions.

The cuttlefish *S. officinalis* exhibited a strong acid-base regulatory response during acute exposure to 0.6 kPa (6,000 ppm) CO_2. Blood HCO_3^- concentrations rose to 10.5 mM through active ion transport mechanisms. The regulatory response partially compensated extracellular acidosis, but the new steady state pHe was still 0.2 units lower than the control value. Despite this shift in blood pH, blood oxygen binding of *S. officinalis* was not significantly compromised and the cuttlefish did not exhibit acute intolerance to hypercapnia. This is due to the combined effects of its lower hemocyanin pH sensitivity compared to pelagic squid, and the strong regulatory response. The acid-base regulatory effort of *S. officinalis* during exposure to 0.6 kPa CO_2 prevented a potential 0.4 unit pH decrease that would have significantly reduced arterial hemocyanin saturation. Intracellular pH was tightly regulated and intracellular phosphagen levels of the mantle muscle remained stable during exposure to acute hypercapnia. It is concluded that the cuttlefish ecotype is not only an efficient acid-base regulator, but is also able to do so without disturbing metabolic equilibria in characteristic tissues.

S. officinalis maintained standard metabolic rates during short-term exposure to 0.6 kPa CO_2 and grew at control rates and gross growth efficiencies during a six-week exposure to 0.4 and 0.6 kPa (4,000 and 6,000 ppm) CO_2. This is in contrast to observations with other, more hypometabolic marine invertebrates, where metabolic depression and growth retardation were observed in both the short- (hours, days) and long-term (weeks), respectively.

In contrast to most invertebrates examined to date *S. officinalis* increased its calcification rate during long-term hypercapnia. Cuttlefish exposed to 0.6 kPa CO_2 accreted 25% more $CaCO_3$ in their cuttlebones due to the thickening of lamellar and pillar walls and a reduction in lamellar spacing. Even though the porosity of the cuttlebones was reduced, the general morphological structure was conserved. The maintenance of calcification under ocean acidification conditions is attributed to the strong ion regulatory abilities of *S. officinalis* and the encasement of the cuttlebone in a transport active epithelium.

Late-stage embryonic development of *S. officinalis* was significantly retarded by a four-week exposure to 0.6 kPa. The arrest of yolk utilization prior to hatching, taken together with reduced embryo growth rates, lead to the conclusion that the embryos experienced metabolic depression when incubated under elevated CO_2. This is not surprising as the perivitelline fluid surrounding the embryo in the egg is already strongly hypercapnic under control conditions. Egg pCO_2 was measured to reach 0.4 kPa and a pH of 7.2 at the end of development. In eggs that were incubated under 0.6 kPa CO_2, CO_2 values rose to over 1.0 kPa and pH decreased to 6.8. High mortality occurred post-hatching, which might be related to compromised, or delayed, differentiation of organs that are essential for metabolic and ion regulatory functions in the more active hatchlings. Our results are in line with existing studies that have found higher sensitivity to elevated seawater pCO_2 of early life stages compared to juveniles and adults.

The experimental results from this thesis indicate a higher tolerance to ocean acidification conditions in juvenile / adult *S. officinalis* compared to more hypometabolic invertebrates. However, the conclusions must be viewed cautiously. Even though *S. officinalis* exhibited a strong acid-base regulatory response and maintained control growth rates under hypercapnia, long-term experiments are necessary to resolve finer changes in the energy budget. Also, the functionality of the cuttlebone in response to changes in ultrastructure must be tested in a complex environment where the cuttlefish are challenged in capturing their prey. In terms of ecologically relevant species sensitivity, experiments that cross the generation boundary are an essential piece that is still missing. Future work on the ontogeny and mechanisms of acid-base regulation in *S. officinalis* will help answer some of the questions that remain open.

Introduction

In the last decade, ocean acidification has emerged as a research focus in the context of global climate change. The present thesis examines the effects of elevated seawater pCO_2 on acid-base regulation, growth and calcification in the cephalopod *Sepia officinalis*. My aim is to contribute to the effort of predicting the sensitivity of marine invertebrates to ocean acidification related changes in seawater carbonate chemistry.

1.1 Ocean acidification induced changes in seawater carbonate chemistry

Due to anthropogenic burning of fossil fuels, atmospheric CO_2 levels have increased from approximately 280 ppm (parts per million) during pre-industrial times to a value of 383 ppm for the globally averaged marine surface annual mean in 2007 (Dr. Pieter Tans, NOAA/ESRL www.esrl.noaa.gov/gmd/ccgg/trends). As increasing CO_2 levels contribute to global warming and acidify the oceans (Caldeira and Wickett 2003 and 2005, Feely et al. 2004, Orr et al. 2005, Cao and Caldeira 2008, Zeebe et al. 2008), the scientific community is aware that the stabilization of CO_2 levels is necessary to prevent dangerous interferences with the climate system (United Nations Framework Convention on Climate Change Article 2). Century-scale SRES (Special Report on Emissions Scenarios) CO_2 marker pathways have been presented by the Intergovernmental Panel on Climate Change (IPCC 2001, 2007) (Fig 1.1).

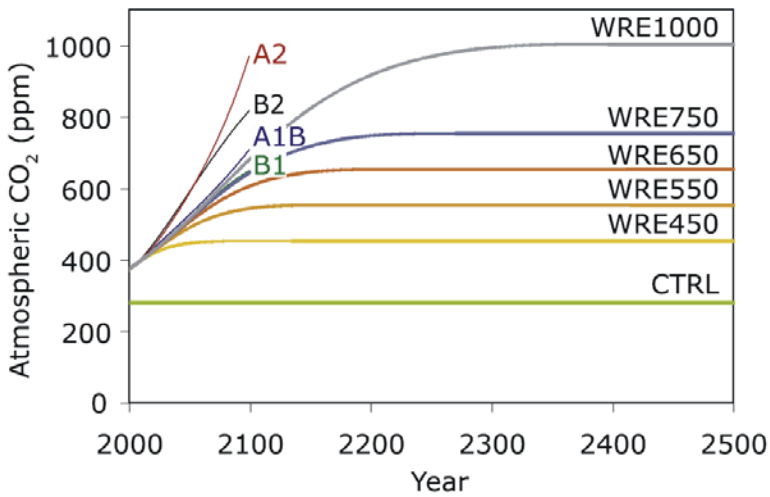


Figure 1.1 Atmospheric CO_2 predictions for the SRES emission pathways and WRE stabilization scenarios using a coupled climate/carbon model (UVic Earth System Climate Model version 2.8, Weaver et al. 2001). The lowest atmospheric CO_2 level predicted for the year 2100 by the SRES emission pathways equals 650 ppm (B1), whereas the highest (A2) is 970 ppm. Fig. 1D from Caldeira and Wickett 2005.

The storylines behind the A1-2 and B1-2 scenarios represent different rates of worldwide technological change and development of energy technologies. A2 and B1 scenarios represent the highest and lowest project emission rates, and reflect a range of 650 – 970 ppm of atmospheric CO_2 levels by the end of the century. The B1 scenario assumes that humanity will develop a globally coherent approach to sustainable development with a high level of environmental and social awareness. Despite even such dramatic changes in global development, the end-of-the-century CO_2 levels predicted by the B1 scenario (650 ppm) would elicit dramatic

changes in ocean chemistry (Caldeira and Wickett 2003 and 2005, Feely et al. 2004). To create a step-wise resolution of more moderate changes in ocean chemistry, stabilization scenarios at lower CO_2 levels (commonly referred to as WRE profiles, developed by Wigley et al. 1996) are also included in many models (Fig 1.1).

The world's oceans have taken up approximately one-third of the anthropogenic carbon dioxide released into the atmosphere in the past 200 years (Sabine et al. 2004). Without the ocean sink, atmospheric CO_2 levels would be 55% higher than the observed 100 ppm increase that has occurred (Sabine et al. 2004). Even though the uptake of anthropogenic CO_2 by the oceans reduces the potential for global warming, marine organisms could be strongly impacted by ocean acidification. The changes that occur in seawater carbonate chemistry when aqueous CO_2 levels increase are describe in the following text (Zeebe and Gladrow 2001). When CO_2 dissolves into seawater, carbonic acid (H_2CO_3) is formed (Eq. 1).

$$CO_2\ (aq) + H_2O \leftrightarrow H_2CO_3 \qquad (1)$$

However, the concentration of H_2CO_3 is so much smaller than that of aqueous CO_2 (<0.3%), the sum of the two chemically non-separable forms is denoted as CO_2 (Eq. 2).

$$CO_2 = CO_2\ (aq) + H_2CO_3 \qquad (2)$$

Using CO_2 from Eq. 2, the carbonate species involved in the dissolution of CO_2 in water can be related by the following equilibria:

$$CO_2 + H_2O \overset{K_1}{\leftrightarrow} HCO_3^- + H^+ \overset{K_2}{\leftrightarrow} CO_3^{2-} + 2H^+ \qquad (3)$$

K_1 and K_2 are the first and second dissociation constants of carbonic acid, they are also referred to as the equilibrium constants (Eq. 3). The equilibrium constants are dependent on temperature, salinity, and pressure and are used for the calculation of carbonate system parameters in seawater. The release of hydrogen ions (H^+) during the dissolution of CO_2 in seawater decreases pH and acidifies the seawater, hence the term ocean acidification. The increased levels of hydrogen ions also interact with carbonate ions (CO_3^{2-}) to form bicarbonate ions (HCO_3^-), thus decreasing seawater CO_3^{2-} concentrations. This phenomenon will be further explained and discussed in section 1.5.

Global average ocean pH has already fallen by approximately 0.1 pH units over the past two centuries as CO_2 levels have increased by 100 ppm, this is equal to a 30% increase in $[H^+]$ (Royal Society 2005). When changes in surface seawater pH are modelled from a WRE stabilization scenario of 550 ppm, pH decreases by more than 0.2 pH units, and over 0.3 units in high latitude areas (Fig. 1.2). Larger increases of atmospheric CO_2 levels, up to ~1000 ppm

following the A2 scenario, will decrease surface ocean pH by nearly 0.5 pH units (Cao and Caldeira 2008). The experimental work of this thesis focuses on contributing to our knowledge about the influences of decreased pH and elevated seawater CO_2 partial pressure (pCO_2) on the physiology of a marine invertebrate.

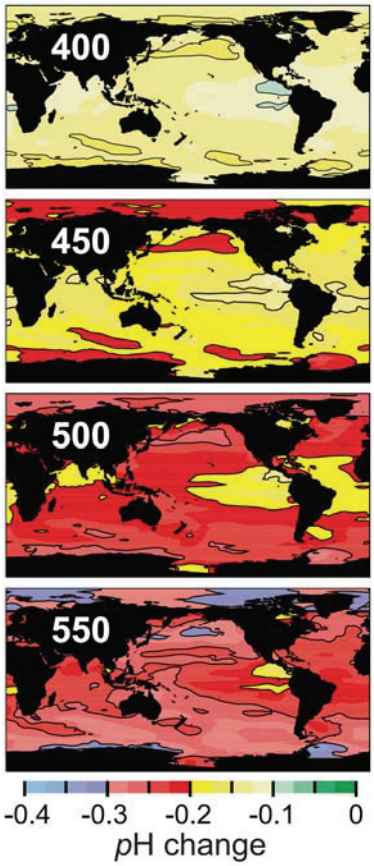


Figure 1.2. Changes in surface ocean pH relative to pre-industrial values for different atmospheric CO_2 stabilization levels. The results were obtained by adding model predicted perturbations in geochemical fields to current observations. Field observations are missing for the Arctic ocean, thus the results represent model simulations. Global average ocean pH has already fallen by approximately 0.1 pH units over the past two centuries, this is equal to a 30% increase in $[H^+]$ (Royal Society 2005). Fig. 3 from Cao and Caldeira 2008.

1.2 Changes in organismal acid-base equilibria during hypercapnia

CO_2 diffuses into both the extra- and intracellular spaces of organisms exposed to elevated seawater pCO_2 (also referred to as hypercapnia), thus creating the potential for acidification and subsequent disturbance of vital biochemical processes. In the extracellular space, a narrow window of pH is necessary for the optimum function of blood pigments, and other membrane proteins such as ion transporters and pumps. Usually, the regulation of pHi is even more tightly controlled than that of pHe, as many cellular processes, such as metabolism, DNA replication and cell division, respond to changes of less than several tenths of a pH unit (Boron, 2004). High non-bicarbonate buffer values in the intracellular space (β_{NB}), about twice as high as those of the extracellular, serve as a buffer reserve and facilitate efficient pH homeostasis (Heisler 1989). Still, buffering is a passive process and can only ameliorate CO_2 induced decreases in pHe. A full restoration of control pHe during acute hypercapnic exposure depends on active ion exchange processes, the export of proton equivalents from the organisms to the seawater (e.g. see Cameron 1986: invertebrates, Heisler 1986: fish). Compensation of pHe during exposure to elevated pCO_2 might be a crucial factor that distinguishes more tolerant marine animal groups from the sensitive ones (Pörtner et al. 1998, Pörtner et al. 2004, Pörtner 2008).

Amongst marine organisms, the mechanisms of transepithelial ion exchange are best understood in teleosts (see Claiborne et al. 2002, Evans et al. 2005, Perry and Gilmour 2006, for reviews), while in most invertebrates, these processes are largely obscure. The gills are the primary sites of acid-base regulation in all marine organisms with higher metabolic rates including fish (Perry and Gilmour 2006), crustaceans (Wheatly and Henry 1992) and probably

also cephalopods (Schipp et al. 1979). In teleost fish, specialized epithelial cells, the mitochondria rich cells (MRCs), contain the ion transport proteins and channels that are important for acid-base regulation. As the transport of acid-base relevant ions is driven to a large degree by the Na^+ -gradient created by Na^+/K^+-ATPase (i.e. low intracellular, high extracellular / seawater Na^+), the activity of this key enzyme is used as an indicator of ion regulatory capacity in different species. Carbonic anhydrase (CA) is an important supporting enzyme involved in the maintenance of acid-base equilbria. During hypercapnia, the compensation of pH through the excretion of acid equivalents potentially follows the following route: CO_2 diffuses into the cell and is hydrated by cytosolic (CA) to form protons and [HCO_3^-]. Subsequently, the protons would be exported via a Na^+/H^+ exchanger (NHE), and [HCO_3^-] could be released into the plasma by means of basolateral Cl^-/ HCO_3^- exchangers or Na^+/ HCO_3^- co-transporters (NBCs), which would also explain the observed decreases in plasma [Cl^-] under hypercapnic conditions (Perry and Gilmour 2006, Larsen et al. 1997). However, the actual mechanisms may be more complicated owing to the large number of transporters and channels present in gill epithelia (see also Deigweiher et al. 2008). The basic mechanisms can be suspected to be similar in decapod crustaceans and cephalopods as well; it is known that similar ion exchange proteins are also expressed in gills of these invertebrates (e.g. Schipp et al. 1979, Donaubauer 1981, Henry and Swenson 2000, Wheatly and Henry 1992) and that high Na^+/K^+ ATPase activities can be measured in gills of all three groups (Siebers et al. 1982, 1983: crustacea, Melzner et al., unpublished: cephalopoda, Gibbs & Somero 1991, Deigweiher et al. 2008: teleost fish).

Generalized groups can be compiled that represent the spectrum of pHe compensation during hypercapnic exposure in marine ectothermic organisms. Fig. 1.3 illustrates the hypothetical acid-base regulatory responses of three groups to a hypercapnic exposure of 0.5 kPa (~5000 ppm). The changes in extracellular pH are represented on the x-axis with HCO_3^- concentration on the y-axis. The CO_2 isopleths in the background represent changes in extracellular pCO_2. Organisms that fully compensate extracellular pH are represented in green in Fig. 1.4. Many teleost fish and some brachyuran crabs (Heisler 1986, Cameron 1986, Larsen et al. 1997, Pane & Barry 2007, Spicer et al. 2007) are capable of accumulating large amounts (>15mM) of HCO_3^- when exposed to hypercapnic conditions and thus fully compensate pHe. Organisms who only partially compensate pHe, like sipunculids and some brachyuran crustaceans (Pörtner et al. 1998, Cameron 1986, Truchot 1975) all exhibit HCO_3^- accumulation that is greater than the passive non-bicarbonate buffer line of their extracellular fluid, but is not sufficiently high enough for full compensation. This group is represented in blue. The red group represents organisms that do not show any active compensatory effort, the pHe of their extracellular fluid just follows the non-bicarbonate buffer slope during hypercapnia. This group

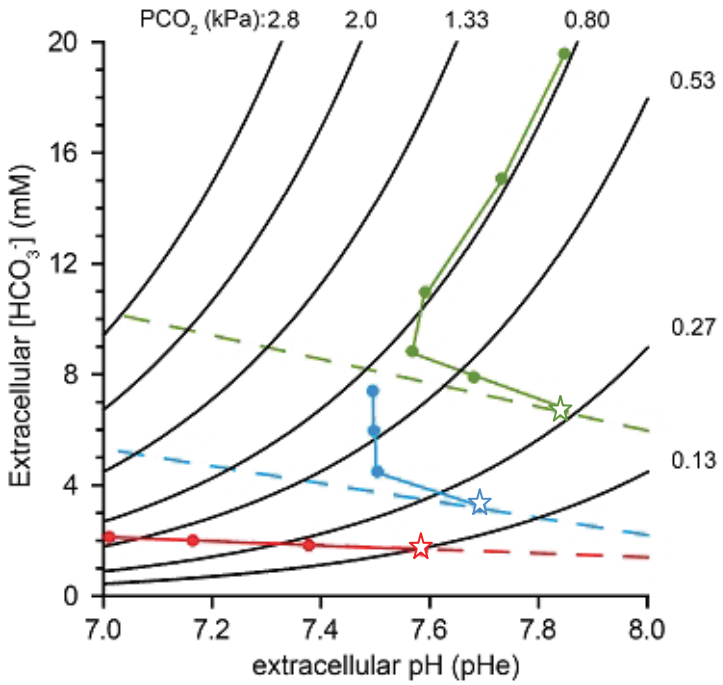


Fig. 1.3. pH-bicarbonate diagram of the acid-base regulatory response in three hypothetical organismal groups during acute exposure to 0.5 kPa (5000 ppm). An experimental time course is represented for each group, the start point, under control conditions, is delineated with a star. Organisms that fully compensate extracellular pH are represented in green (e.g. many teleost fish and some brachyuran crabs). Partial pHe compensation is illustrated in blue (e.g. sipunculids and some brachyuran crabs). Organisms who do not exhibit any compensatory effort are depicted in red (e.g. most bivalves and echinoderms). During hypercapnic exposure, the pH of the extracellular fluid in the red group organisms follows their putative low non-bicarbonate buffer slope and strongly decreases. See text for a detailed discussion.

includes most bivalves and echinoderms (Miles et al. 2007, Thomsen 2008) and most likely many more sessile invertebrates whose acid-base regulatory abilities have not yet been examined.

1.3 Effects of hypercapnia on metabolic rates in marine organisms

Recent studies indicate that some echinoderms and molluscs reduce metabolic rates and somatic growth during hypercapnic exposure. Shirayama and Thornton (2005) documented a 21% and 12 % decrease in growth over a six month period in the sea urchin *Echinometra mathaei* and the gastropod *Strombus luhuanus* when CO_2 was increased to just 560 ppm. The bivalve *Mytilus galloprovincialis* was shown to experience growth and metabolic reductions of 55 and 65 %, respectively, when exposed to ca. 5,000 ppm (0.5 kPa) CO_2 (Michaelidis et al., 2005). The same group of organisms has also been shown to not fully compensate extracellular pH (pHe) during acute exposure to hypercapnia. Both the sea urchin *Psammechinus miliaris* and the mussel *Mytilus galloprovincialis* exhibited a decrease in pHe of at least 0.2 pH units, following 8 days of exposure to hypercapnia at ca. 2,000 ppm (0.2 kPa) and 5,000 ppm (0.5 kPa) CO_2, respectively (Michaelidis et al., 2005; Miles et al., 2007). The onset of metabolic depression in invertebrates during hypercapnic exposure has been demonstrated to be connected to an uncompensated acidotic shift in pHe (Reipschlager and Pörtner 1996, Pörtner et al. 2000, 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). Evidence supporting the hypothesis that metabolic depression is

accompanied by an uncompensated acidotic shift in pHe originates from experimental work with the sipunculid worm *Sipunculus nudus* (Pörtner et al. 1998). Studies with an isolated *S. nudus* muscle preparation showed that decreasing pHe 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 (Pörtner et al. 2000). 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 pCO_2 and HCO_3^-, that 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.

To increase our understanding of the connection between the maintenance of extracellular pH and metabolic depression in invertebrates during hypercapnia, further work with different marine invertebrate species is needed. To date, data on changes in metabolic rate in conjunction with measurement of extracellular acid-base parameters during exposure to elevated seawater pCO_2 is only available for *S. nudus* and the mussel *Mytilus galloprovincialis*. In aquatic vertebrates (marine teleosts), high extracellular acid-base regulatory abilities could be correlated with maintained growth rates under elevated CO_2 conditions. Long-term studies with adult wolffish *Anarichus minor* and *Salmo salar* smolts have found conserved growth rates and condition indices at pCO_2 levels up to 1 kPa (Fivelstad et al., 2003; Foss et al., 2003). In addition, two other studies recently found conserved metabolic rates under elevated pCO_2 conditions (Deigweiher et al. 2008, Melzner et al. 2009). Thus, a link between high acid-base regulatory capacity and growth performance / maintenance of metabolic rate might exist. However, no invertebrates with a high ion- / acid-base regulatory capacity (cephalopoda, decapod crustacean) have been examined so far to test this hypothesis.

1.4 Sensitivity of early life stages to elevated seawater pCO_2.

Several studies indicate that early life stages of marine animals might be most vulnerable to future ocean acidification (summarized in Kurihara 2008). Havenhand et al. (2008) observed reduced sperm motility and fertilization success in a sea urchin species already at pH values of 7.7. Reduced larval growth in two echinoderm species was observed under similar conditions (pH 7.6-7.7, Kurihara & Shirayama 2004, Kurihara et al. 2004). These findings are not entirely surprising, as these early stages, especially the unicellular gametes and zygotes, experience much

larger relative changes in pCO_2 than cells of adult organisms that are surrounded by extracellular fluids with high pCO_2 already under control conditions: The 'extracellular environment' of unicellular stages (the ocean) is characterized by pCO_2 values of about 383 ppm, the extracellular pCO_2 of most adult marine metazoans is located between 1,000 and 4,000 ppm (see publication 5).

Similar results have been obtained for marine vertebrate (teleost) early life stages: Kikkawa et al. (2003) found low 24h LC_{50} values during CO_2 exposure in earliest egg stages (cleavage) of marine teleosts, on average 2-3-fold lower than those of later embryonic, larval and juvenile stages. It has been speculated by Ishimatsu et al. (2005) that the decrease in sensitivity from cleavage to the embryo may reflect the development of ion-regulatory mitochondria rich cells (MRCs) on the yolk sac (Shiraishi et al. 1997). This corresponds to the idea developed above that CO_2 tolerance is supported by a high ion-regulatory capacity.

The early life history of the invertebrate larvae that have been examined for sensitivity to ocean acidification conditions all share a long larval development period outside of the egg (bivalves: Kurihara et al. 2007, ophiuroids: Dupont et al. 2008, sea urchins: Kurihara and Shirayama 2004, copepods: Kurihara and Ishimatsu 2008). The sensitivity of lecithotrophic species whose early-life stage development takes place primarily inside eggs has not been examined and includes its own set of challenges. Egg capsules can provide severe physiological challenges to their inhabitants, as the egg wall represents a barrier to diffusion of gases. Progressively decreasing oxygen levels have been hypothesized to eventually trigger hatching once critical pO_2 values (pO_{2crit}) are reached inside the egg in both vertebrates (reptiles and birds: Vleck and Hoyt 1991, amphibians: Seymour and Bradford 1995) and invertebrates (cephalopods: DeWachter et al. 1988, Cronin and Seymour 2000). Surprisingly, the potential effects of elevated pCO_2, which could correspond to decreasing pO_2 inside eggs, have not been addressed. To our best knowledge, there are currently no published egg fluid pCO_2 values available in the literature for any marine animal, nor has pH been determined in the fluids surrounding the eggs. This is quite surprising, as high pCO_2 values most likely go along with low pH values and potentially constitute another stressor that may significantly affect embryonic physiology. Early life stage invertebrates could be particularly sensitive to hypercapnia induced acidification as ion regulatory epithelia only become fully functional relatively late in most invertebrate and vertebrate larvae, sometimes significantly after hatching (e.g. cephalopods: Schipp et al. 1979, teleost fish: Evans et al. 2005, crustacea: Cieluch et al. 2004).

1.5 Sensitivity of calcification processes in invertebrates to elevated seawater pCO_2

Ocean acidification induced changes in seawater carbonate chemistry reduce the concentration of carbonate ions (CO_3^{2-}) (see section 1.1). A decrease in $[CO_3^{2-}]$ directly influences the calcium carbonate ($CaCO_3$) saturation state of seawater. It is important to distinguish between the two forms of $CaCO_3$, calcite and aragonite, when saturation conditions are discussed. The difference in crystal structure between the two forms makes aragonite more soluble than calcite (Zeebe and Gladrow 2001). The $CaCO_3$ saturation state of seawater (Ω) is expressed as:

$$\Omega = ([Ca^{2+}]_{sw} \times [CO_3^{2-}]_{sw}) K^*_{sp}{}^{-1}$$

K^*_{sp} is the stoichiometric solubility product of either calcite or aragonite. When Ω is calculated > 1, seawater is supersaturated with regards to $CaCO_3$, whereas $\Omega < 1$ corresponds to undersaturation (Zeebe and Gladrow 2001). Predicted ocean acidification conditions will have a dramatic impact on oceanic near-surface Ω (Caldeira and Wickett 2003 and 2005, Cao and Caldeira 2008). The discussion will be focused on the changes in seawater saturation of aragonite. Ω_{arag} in the pre-industrial ocean ranged from 1.4 in polar waters up to 4.7 in tropical areas (Fig. 1.4). Therefore, with increasing atmospheric CO_2 levels, the polar regions will be the first to become undersaturated if CO_2 emissions continue to rise. The predicted changes in Ω_{arag} at the poles are dramatic. Even if atmospheric CO_2 levels are stabilized at 450 ppm, parts of the Southern Ocean will become undersaturated with respect to aragonite (Fig. 1.4). Undersaturation conditions in the polar regions could negatively affect shelled pteropod species that are integral components of the regional food webs Tsurumi et al. 2005, Hunt et al. 2008, McNeil and Matear 2008). Etching and pitting of the external shell in *Clio pyramidata* was found after exposure to seawater with $\Omega_{arag} < 1$ (Orr et al. 2005). ^

Significant changes in Ω_{arag} can also be expected in the tropical regions at atmospheric CO_2 levels of 550 ppm (Fig. 1.4). The potential biological impact of these changes has been discussed in the context of coral reef calcification (Kleypas et al. 1999, Kleypas et al. 2001, Hughes et al. 2003, Orr et al. 2005, Hoegh-Guldberg et al. 2007). In pre-industrial times, when atmospheric CO_2 levels equalled approximately 280 ppm, over 95% of coral reefs were located in areas where Ω_{arag} of near ocean water was greater than 3.5. At CO_2 levels of 550 ppm, no existing coral reefs will be near waters with such high saturation values (Cao and Caldeira 2008). A direct correlation exists between decreasing calcification rates and lower Ω_{arag} values in nearly all coral, and coralline algae species examined to date (Gattuso et al. 1998, Langdon et al. 2000, Marubini et al. 2002). The integrity of coral reef ecosystems has been concluded to be seriously threatened by ocean acidification (Hoegh-Guldberg et al. 2007, Cooper et al. 2008).

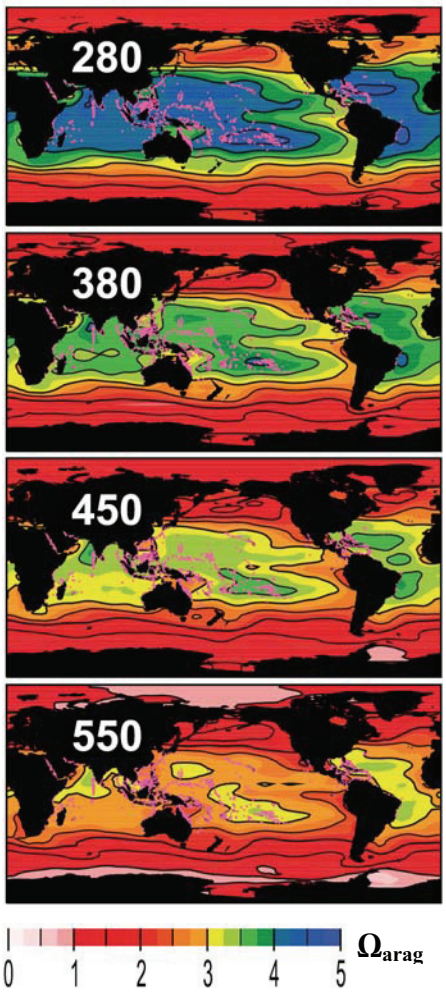


Fig. 1.4. Maps of model-predicted aragonite saturation states at different atmospheric CO_2 stabilization concentrations (ppm) plotted over existing shallow-water coral reef locations (shown as magenta dots). Aragonite saturation values near the reef locations are interpolated from modelled nearby open ocean values. As a strong correlation exists between calcification rate and Ω_{arag} in corals, the integrity of coral reefs has been concluded to be seriously threatened by ocean acidification. Figure 1 from Cao and Caldeira 2008.

From a physiological perspective, the sensitivity of calcification processes to seawater [CO_3^{2-}] is a bit of a conundrum. The carbon source for precipitated $CaCO_3$ is thought to stem from either metabolic CO_2, or active HCO_3^- transport from the seawater (Sikes et al. 1981, Furla et al. 2000, Al-Horani et al. 2005), not directly from the seawater CO_3^{2-} pool. One possible explanation for reductions in calcification rate with decreasing Ω_{arag} could be that sessile, heavily calcified invertebrates are not capable of compensating the internal acidosis they experience when exposed to high pCO_2, low Ω_{arag} seawater. However, as the cellular mechanisms behind calcification are still not fully understood in any invertebrate taxa, we are far from a process based understanding of the observations. The sensitivity of calcification processes to hypercapnia has not been examined in active invertebrates with strong regulatory abilities. Differences in calcification performance between active and inactive taxa could potentially shed light on key processes that are essential for maintaining calcification rates during exposure to hypercapnic seawater.

1.6 *Sepia officinalis* as a model organism

1.6.1 High metabolic rates supported by the oxygen binding pigment hemocyanin

The cephalopod mollusc *Sepia officinalis* is a unique model organism as it calcifies a large aragonitic shell although it leads an active lifestyle. Typically, molluscs which create shells have low metabolic rates and are fairly sessile, When compared to pelagic squid with their 'live fast, die young' life style, nektonic-benthic cuttlefish live in the 'slow lane' (Wells & Wells 1991). Still, their metabolism is, on average, approximately 10-fold higher than that of bivalve molluscs and they can reach masses over 2 kg during their one-two year life cycle (Boletzky 1987, Publication 5).

In order to support such high rates of energy turnover, cephalopods have evolved closed circulatory systems (Fig. 1.5C) and hemocyanin as an extracellular blood oxygen binding protein

to optimise oxygen supply (O'Dor and Webber 1986). However, hemocyanin is less efficient at binding oxygen than hemoglobin by a factor of about 3, 3mM in cephalopods versus 10 mM in some fish (Urich 1994). Blood hemocyanin concentrations cannot be increased to high levels due to the limitation of increasing colloidial osmotic pressure (Mangum 1983 and 1990). Therefore, cephalopods have optimized the function of their blood pigment by increasing the pH sensitivity of oxygen loading and unloading, known as the Bohr effect (Bohr et al., 1904). Cephalopods are one of the invertebrate groups whose hemocyanins have particularly high Bohr coefficients (Redfield and Goodkind, 1929; Bridges, 1994). Maintenance of correct hemocyanin function is dependent on a tight window of extracellular pH as the blood pigment is not protected by an intracellular environment. The acute intolerance of cephalopods to hypercapnic exposure was initially hypothesized by Reipschläger and Pörtner (1996) based on the sensitivity of their hemocyanin to potential changes in pHe. Considerable regulatory ability would be necessary to compensate blood pH and protect blood oxygenation function. The acid-base regulatory ability of cephalopods in response to CO_2 exposure is unknown. Even though cuttlefish are not as "highly tuned" as squid, the oxygen saturation of their blood could still be sensitive to uncompensated changes in blood pH during exposure to elevated pCO_2.

1.6.2 Structure and function of the calcified cuttlebone

The cuttlefish (Sepiida), along with Nautilus (Nautilida) and Spirula (Spirulida), are the only extant cephalopods that utilize a chambered shell for skeletal support and as a buoyancy regulation device (Denton 1974). In the cuttlefish *S. officinalis*, the cuttlebone is dorsally located along the sagittal axis (Fig. 1.6A) and accounts for about 10% of the animal's volume (Denton 1961a). The cuttlebone is surrounded by the cuttlebone epithelium, also referred to as the cuttlebone sac (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 cuttlebone (Appelöf 1893). The combined function of the cuttlebone, both for support and as a light weight buoyancy device, requires an open structure that is uniquely pressure resistant while maintaining a constant volume.

The cuttlebone is composed of two distinct regions, the dorsal shield and the ventrally located aragonitic phragmocone. The phragmocone is made up of parallel lamellae (also refered to as septa in the literature) that are supported by perpendicularly oriented pillars (Fig. 1.6B). Growth of the cuttlebone proceeds through the accretion of subsequent lamellae and extension of the dorsal shield at the anterior end. The cuttlebone epithelium covering the area of active calcification has the characteristics of a secretory epithelium (Appelöf 1893). The various cell types found in epithelium in this region are responsible for the transport of both ions and organic

matrix proteins. The organic matrix is visible in the phragmocone of the cuttlebone as a thin sheet coating the lamellar and pillar surfaces (Fig. 1.6B). It is a critical component of the final structure as it guides calcification by controlling crystal nucleation, polymorph selection and crystal orientation (Weiner and Traub 1984). 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 its cuttlebone as a buoyancy regulation device. The pumping of liquid in and out of the cuttlebone is performed through the creation of an "osmotic pump" (Denton 1961a). Cuttlefish not only adjust their buoyancy according to depth, but also on a diurnal cycle to reduce energy expenditure (Denton 1961a, 1961b). During day time, when *S. officinalis* rests on the ground buried in sand, the posterior chambers of the cuttlebone are filled with fluid, making the cuttlefish negatively buoyant. However, at the onset of night the posterior chambers are emptied, thus decreasing the density of the cuttlefish so it is neutrally buoyant and can maintain its position in the water column hunting with a lower energy expenditure (Webber et al. 2000).

1.6.3 Embryonic development

From the morphology of the surrounding epithelium and function of the cuttlebone, it is obvious that *S. officinalis* has tight control over the ionic environment surrounding its calcified structure. This is in contrast to most other molluscs. In bivalves for example, the extrapallial fluid makes up a large volume and is weakly separated from haemolymph circulation (Ruppert et al. 2004). It stands to reason that bivalve molluscs, with low acid-base regulatory abilities, could potentially not be able to control the ionic composition of the extrapallial fluid when exposed to an abiotic stressor. This could be one explanation for the reductions in calcification that have been measured in Mytilus species during exposure to elevated pCO_2 (Berge et al. 2005, Michaelidis et al. 2005, Gazeau et al. 2007). The influence of hypercapnia on calcification processes in cephalopods has not yet been examined. *S. officinalis* embryos develop inside individual eggs that are clustered together and attached to hard substrate in coastal waters. In the English Channel population (Wolfram et al. 2007) the eggs are laid in the spring and depending on water temperature, 2-4 months after deposition, hatching occurs (Bouchaud and Daguzan 1990). In cuttlefish eggs the embryo is surrounded by perivitelline fluid which is encased by the chorion. The egg capsule coats the surface of the egg and is used as an attachment (Fig. 1.7). Embryonic development of *S. officinalis* is comprised of three main phases: segmentation (stages 1-9), gastrulation (stages 10-17) and organogenesis (stages 18-30, as defined by Lemaire 1970). Within the first half of the developmental period the embryos reach stages 25-26. The second half of development is mainly characterized by significant growth of the embryo, this results in a comparatively, very large hatchling with a high metabolic rate (Wolf et al. 1985, Bouchad 1991,

Cronin and Seymour 2000). At hatching, the mantle length of *S. officinalis* embryos measures approximately 1 cm and the cuttlefish have already calcified the first 8-10 lamellae of their cuttlebones.

The embryonic development of the Sepiodiae is different from most other molluscs in that the cuttlefish hatch as isometric replicates of the adults (Boletzky 2003). The long residence period of a very advanced embryo inside an egg could potentially create unique challenge to the embryo in terms of perivitelline fluid pO_2, pH and pCO_2 (as described in section 1.4). Late-stage embryos of the cephalopod *Sepia officinalis* were used as a model system, to characterize in more detail the abiotic conditions within the perivitelline fluid (PVF), with an emphasis on the pCO_2 / pH gradient between the egg and the environment. If the pCO_2 and $[H^+]$ are indeed elevated in the PVF under control seawater conditions, this could potentially make the developmental stages more susceptible to ocean acidification conditions.

1.7 Experimental Questions and Approaches

Marine organisms with high metabolic rates and acid-base regulatory abilities have been hypothesized to be more tolerant of ocean acidification then their hypometabolic counterparts (Seibel and Walsh 2001, 2003, Knoll et al. 2007). Growth trials under moderate hypercapnic conditions support this hypothesis: metabolic depression and reduced growth rates were observed in bivalves, gastropods and echinoderms, but not in marine teleosts. As to date, the acid-base regulatory ability of an active invertebrate has not been studied in combination with the evaluation of growth performance during long-term exposure to hypercapnia. The relationship between extracellular pH compensation and metabolic rate has also not been examined in active invertebrates. Working with the cephalopod *S. officinalis* as a model organism, the following questions were addressed:

(i) What is the acid-base regulatory ability of the cuttlefish in response to acute hypercapnia?

In vivo blood acid-base parameters were measured in cannulated *S. officinalis* during exposure to acute hypercapnia. The development of respiratory acidosis in the blood was closely monitored as well as compensatory increases in blood $[HCO_3^-]$. Intracellular acid-base regulation was assessed by monitoring pHi in the mantle muscle using non-invasive *in vivo* ^{31}P NMR. It was hypothesized that the cuttlefish would exhibit a strong regulatory response. Additionally, the effect of hypercapnia exposure on the oxygen saturation state of hemocyanin was calculated from the measured changes in pHe and existing oxygen binding curves for *S. officinalis* blood.

(ii) How are metabolism and growth affected by short and long-term exposure to hypercapnia?

Oxygen consumption rates of *S. officinalis* were measured to determine potential changes in metabolism during acute hypercapnic exposure. It was hypothesized that a reduction in metabolic rate would occur if extracellular pH was not fully compensated. High energy phosphates in the mantle muscle of the cuttlefish were monitored using *in vivo* ^{31}P NMR to determine if tissue thermodynamic equilibria were compromised during acute hypercapnia. The influence of exposure to elevated seawater pCO_2 on the energy budget partitioning of *S. officinalis* was examined in a six-week growth trial during which the growth of the cuttlefish as well as their gross growth efficiency were monitored.

(iii) How does elevated seawater pCO_2 affect calcification processes?

The cuttlebones from the cuttlefish raised in the growth trial were analysed for any influence of hypercapnic exposure on calcification processes. Firstly, a subset of the cuttlebones was decalcified to determine the amounts of incorporated $CaCO_3$ and organic matrix. Secondly, the morphometric relationships of the cuttlebones were measured. Finally, scanning electron micrographs were made of the phramocone region to examine if any ultrastructural changes had taken place.

(iv) What is the hypercapnia sensitivity of embryonic development and early life stage growth?

Late-stage *S. officinalis* embryos were incubated through hatching and for two weeks post-hatching (six-weeks total) under hypercapnic conditions. The pO_2, pH and pCO_2 of the egg perivitelline fluid were measured approximately one week prior to hatching. It was hypothesized that pCO_2 would be elevated, and that there would be an acidotic shift in pH towards the end of development inside the egg even under control conditions. The sensitivity of embryonic development in *S. officinalis* to hypercapnic exposure was examined by monitoring the growth rate of the embryos and their yolk utilization. The growth rates and mortality of the hatchlings were recorded for two weeks post-hatching.

Figure 1.5 Abbreviations

A.FI.V.	*Anterior fin vein*
A.M.V.	*Anterior mantle vein*
AU.	*Auricle*
A.V.	*Afferemt branchial vessel*
A.VP.C.	*Aperture of viscero-pericardial coelom*
B.GL.	*Branchial gland*
B.H.	*Branchial heart*
CA.	*Spiral caecum*
C.D.C.	*Cut wall of dorsal chamber of renal sac*
CE.V.	*Cepahlic vein (Anterior vena cava)*
C.I.G.	*Clear centre of ink gland*
D.IS.	*Dorsal surface of inksac*
E.V.	*Efferent branchial vessel*
F.A.M.V.	*Factors from mantle wall of anterior mantle vein*
G.A.V.	*Genital artery and vein*
I.G.	*Ink gland*
IS.A.	*Inksac artery*
IS.A.V.	*Inksac artery and vein*
IS.V.	*Inksac vein*
L.	*Digestive gland (hepatopancreas)*
L.M.V.	*Left mesenteric vein*
L.V.D.	*Lumen between ventral and dorsal chambers of renal sac*
O.D.C.	*Outline of dorsal chamber of renal sac*
O.T.	*Orifice of testis opening into visero-pericardial coelom*
O.VP.	*Outline of viscero-pericardial coelom*
P.AO.	*Posterior aorta*
P.GL.	*Pericardial gland*
P.M.A.	*Posterior mantle artery*
P.M.V.	*Posterior mantle vein*
P.VP.C.	*Posterior limit of viscero-pericardial coelom*
RE.	*Rectum*
RE.A.	*Renal appendages of veins*
RE.P.	*Renal papilla*
R.M.V.	*Right genito-mesenteric vein*
ST.	*Stomach*
TE.	*Testis*
V.B.G.	*Vein of branchial gland*
V.C.	*Vena cava*
V.CH.	*Ventral chamber of renal sac*
V.N.	*Visceral nerve*
VP.	*Viscero-pericardial coelom*

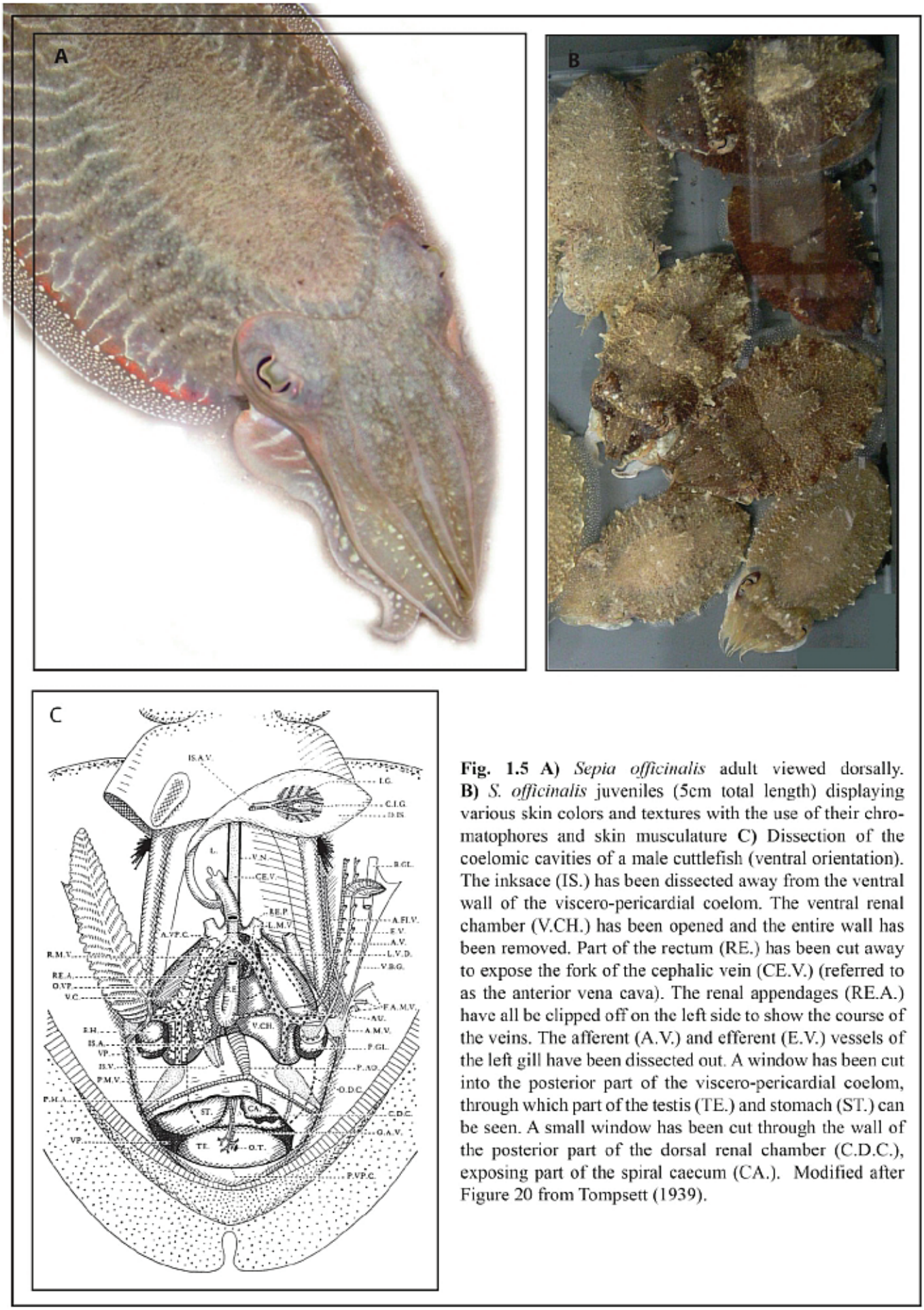


Fig. 1.5 A) *Sepia officinalis* adult viewed dorsally. **B)** *S. officinalis* juveniles (5cm total length) displaying various skin colors and textures with the use of their chromatophores and skin musculature **C)** Dissection of the coelomic cavities of a male cuttlefish (ventral orientation). The inksace (IS.) has been dissected away from the ventral wall of the viscero-pericardial coelom. The ventral renal chamber (V.CH.) has been opened and the entire wall has been removed. Part of the rectum (RE.) has been cut away to expose the fork of the cephalic vein (CE.V.) (referred to as the anterior vena cava). The renal appendages (RE.A.) have all be clipped off on the left side to show the course of the veins. The afferent (A.V.) and efferent (E.V.) vessels of the left gill have been dissected out. A window has been cut into the posterior part of the viscero-pericardial coelom, through which part of the testis (TE.) and stomach (ST.) can be seen. A small window has been cut through the wall of the posterior part of the dorsal renal chamber (C.D.C.), exposing part of the spiral caecum (CA.). Modified after Figure 20 from Tompsett (1939).

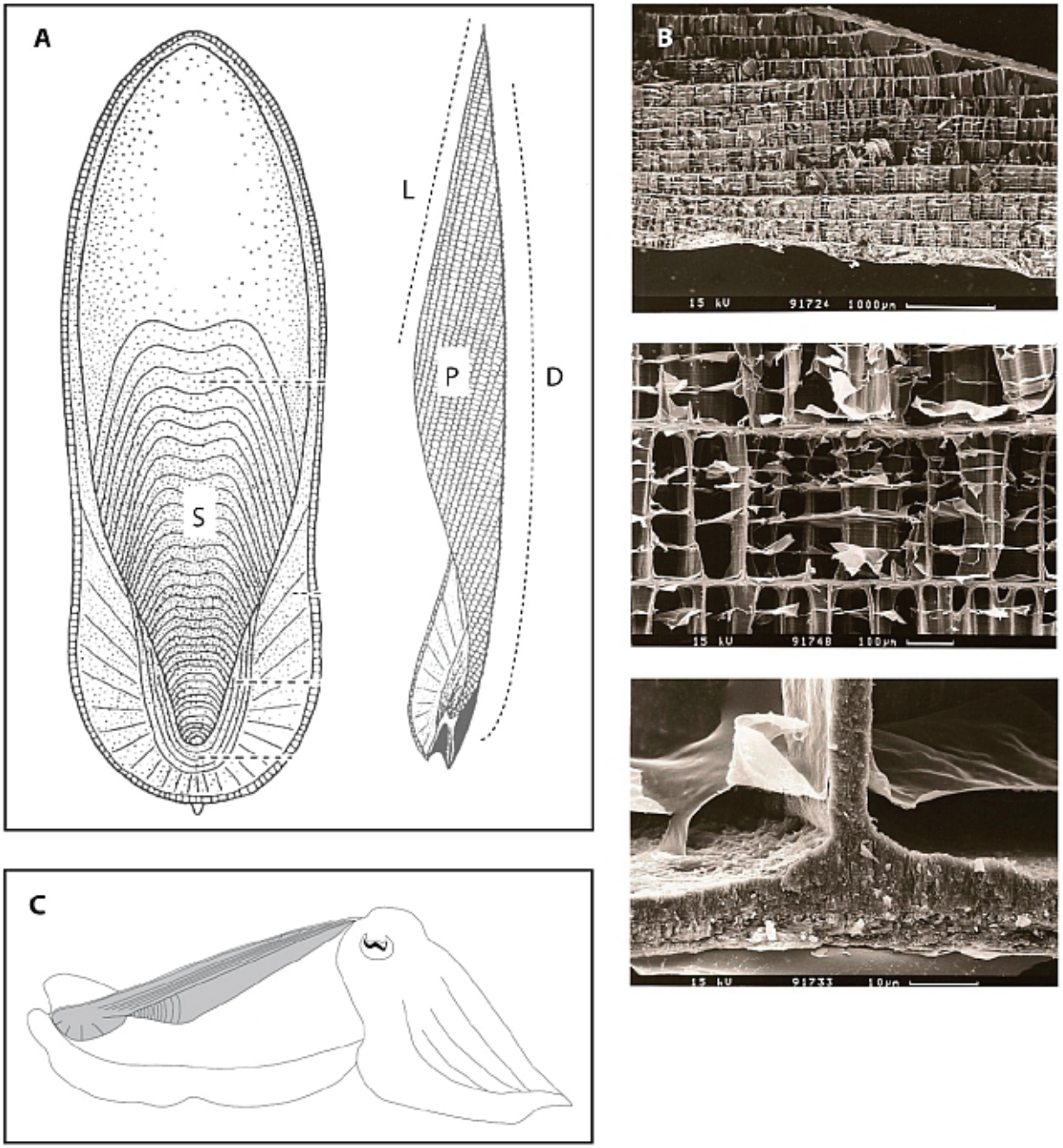
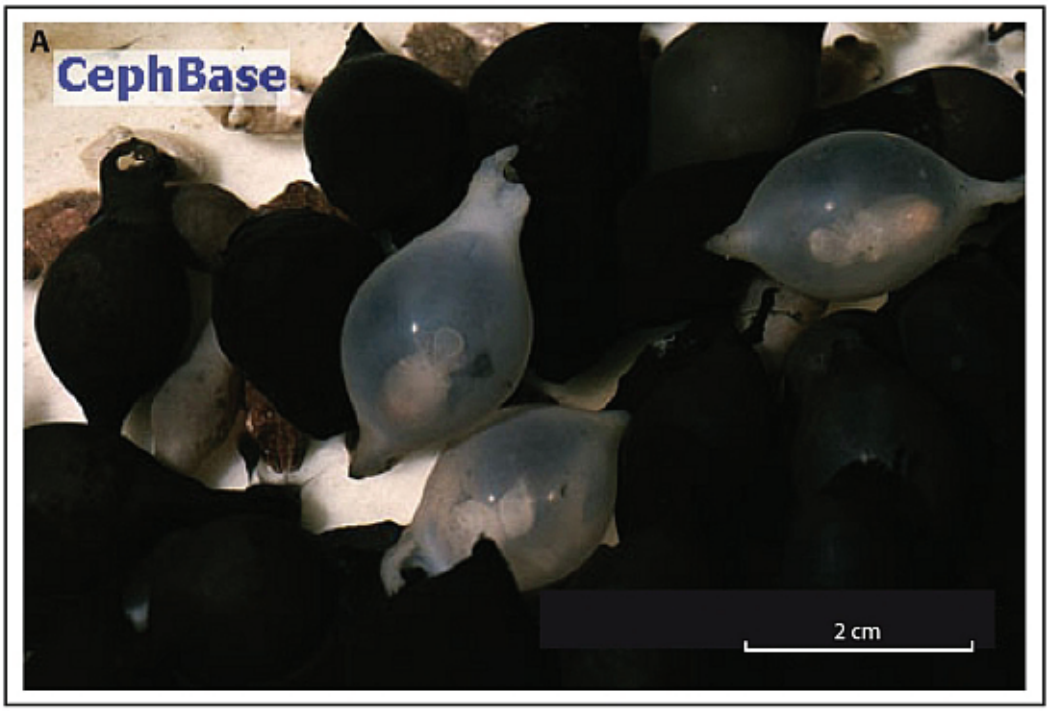


Fig. 1.6 A) Illustrations of an *S. officinalis* cuttlebone, viewed ventrally and in a transverse section. S- Siphuncular surface, D- Dorsal surface, P- phragmacone, L- most recently constructed lamella. Each striation on the siphucular surface represents a lamellar front. Modified from figures 15 and 16 from Tompsett (1939). **B)** Cuttlebone scanning electron micrographs of a transverse section along the midline. The top picture illustrates a large-scale view of eleven lamellae in the phragmacone and the dorsal shield. A closer view of two lamellae and supporting pillars are shown in the middle picture. Note the thin sheets of organic matrix freely suspended perpendicular to the pillars inbetween the lamellae. The bottom picture illustrates a pillar rising off of the lamellar floor in detail. Note the thin sheets of organic matrix that coat the $CaCO_3$ surfaces. **C)** Schematic drawing of *S. officinalis* with the cuttlebone shaded grey.



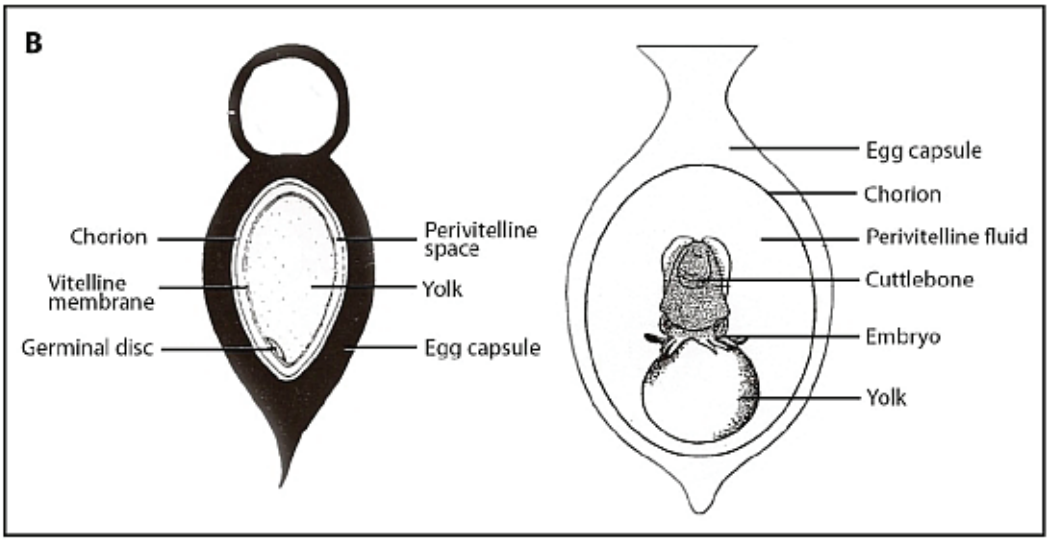


Fig. 1.7 A) *Sepia officinalis* eggs containing late-stage embryos. In rare instances the dark pigment is not incorporated into the egg capsule resulting in a transparent egg. Inside these eggs the embryos are visible along with their large external yolk sacs, tentacles, and cuttelbones. Brown hatchlings are visible on the periphery of the eggs. The portrayed eggs have been individualy separated from the original egg mass. In the field, eggs are attached to hard substrate in groups closely resembling large grape clusters. Photo modified from CephBase archive. **B)** A freshly laid egg is illustrated on the left. Note the large size of the internal yolk sack, and the thin perivitelline space (modified after Wolf et al. 1985). A late-stage embryo is illustrated on the right (modified after Cronin and Seymour 2000). The egg has swollen noticeably and the embryo is surrounded by a large volume (>1 mL) of perivitelline fluid. The thinning of the egg capsule fascilitates gas exchange by reducing the diffussion barrier. Despite the modification of the egg wall, the pO_2 of the perivitelline fluid drops below 5 kPa at the end of development and is hypothesized to induced hatching (DeWachter et al. 1988, Cronin and Seymour 2000).

2. Materials and Methods

2.1 Animals and aquaristics

Laboratory laid *Sepia officinalis* eggs were obtained from the Biological Station in Luc-sur-Mer, Université de Caen (Normandy, France) in the springs of 2005-2008. The cuttlefish were hatched and raised at the Alfred Wegener Institute (AWI, Bremerhaven, Germany) in a closed recirculating system (20m^3 total volume, protein skimmers, nitrification filters, UV-disinfection units) at S=32-34 ppt, T=15 ± 0.1°C, pH=7.9-8.2, constant dark:light cycle (12h: 12h). Water quality parameters were monitored weekly using photometric test kits (Merck, Darmstadt, Germany). Concentrations of ammonia and nitrite were kept below 0.2 mg l^{-1}, and nitrate below 80 mg l^{-1}. The animals were initially fed a daily diet consisting of live mysids (*Neomysis integer*) and live brown shrimp (*Crangon crangon*). After 2 months they were progressively transitioned to frozen brown shrimp.

Table 2.1 gives an overview of the animals used in the experiments conducted in this thesis. In each of the experiments, the animals were exposed to 0.4 or 0.6 kPa CO_2 either sequentially or in parallel to control conditions. All experiments complied with the German animal experimentation laws.

Table 2.1. Experiments and animals discussed in this thesis.

n	Mass(SD) g	Experiment	Publication
5	555 (195)	*In vivo* blood acid-parameters, blood pO_2, ventilation	1
6	110 (16)	*In vivo* ^{31}P NMR spectroscopy studies on mantle muscle	1
6	10.4 (4.3)	Standard metabolic rate	2
80	2.7 (0.3)	Growth rate, gross growth efficiency, cuttlebone analysis	2,3
13	Eggs	Perivitelline fluid acid-base parameters, pO_2	4
120	Eggs	Embryonic growth, yolk utilization, hatchling survival	discussion

2.2 Experimental setups

For the growth trials of juvenile *S. officinalis*, and developmental trials with eggs, each experimental group was maintained in shallow pvc basins (20x40x60 cm) (Fig. 2.1A). Basins drained into reservoir tanks where the seawater was pumped through a nitrifying biofilter (Professional 2, Eheim, Deizisau, Germany) 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. Temperature was closely controlled (16.3±0.1) using aquarium heaters. Water values were maintained at less than 0.2 mg l^{-1} ammonium and 40 mg l^{-1} nitrite.

The cannulated cuttlefish, in which *in vivo* blood-acid base parameters were measured, were held in the following set up during an overnight acclimation period and post-surgery (Fig.

2.1B). The experimental aquarium system consisted of a small animal chamber (20 l volume, with a long lateral opening at the bottom) that was standing inside a thermostatted 17.1±0.2 °C aquarium. The total seawater volume was 200 l. The animal chamber was darkened and perfused at a rate of 5 l per min. Within the chamber, the swimming movements of the cuttlefish were limited with cushioned plastic grids. The blood and pressure catheters were fed through the lateral slit in the chamber wall, and attached to their respective instruments. Water quality was maintained using a 12 W UV-sterilizer and a nitrification filter (Professional 2, Eheim, Deizisau, Germany), ammonia and nitrite values were maintained below 0.1mg l^{-1}. A full water change was done in the system after each experimental animal.

Experimental set ups with a smaller seawater volume (approx. 100 l) were used for the *in vivo* ^{31}P NMR spectroscopy (Fig. 1 Melzner et al. 2006) and respiration rate measurements (Fig. 1.2C). Temperature was maintained at 16°C ± 0.2°C using either a thermostatted water bath or aquarium heaters. A nitrification filter (Professional 2, Eheim, Deizisau, Germany) was used to maintained water quality at ammonia and nitrite values described above. A full water change was done after each experimental run.

2.3 Elevated seawater pCO_2

Two different gas mixing systems were used to produce gas mixtures with elevated pCO_2, a Wösthoff gas mixing pump (Wösthoff model 5KM402/a-F) and MKS gas controller (MKS model GSV-19). Wösthoff pumps were primarily used for applications where only small gas volumes were needed, e.g. for tonometry of blood samples. Two and four channel MKS gas flow controllers were assembled with a variety of valve sizes (3 slm, 500 sccm, 5 scccm) to match gas mixture requirements. In all of the experimental set ups both holding and reservoir tanks were continuously bubbled with the appropriate gas mixture.

2.4 Gas chromatography method to measure C_{CO_2}

The total dissolved inorganic carbon content (C_{CO_2}) of blood and seawater samples was analyzed using a modification of the gas chromatographic method outlined by (Lenfant and Aucutt, 1966), which has been previously modified by Boutilier et al. (1985) and (Pörtner et al., 1990). The underlying concept of this method involves the use of acid to liberate sample C_{CO_2} into the gas phase for subsequent measurement with a gas chromatograph. Blood (30 µl) and seawater (200 µl) samples were injected into 10 ml gas tight vials filled with 3 ml of air equilibrated, 0.1 M HCl. Air equilibrated vials and HCl were used in the modification of this method, instead of N_2 equilibrated, in order to reduce the background variability of blank vials. Vials were processed using an automated headspace sampler (G188 Agilent Technologies, Santa

Clara, Unites States). The gas phase was then injected into a gas chromatograph (6890N Agilent Technologies) equipped with a thermal conductivity detector: split inlet ratio 2.67:1, HP-PLOT Q column (carrier gas helium, flow rate 3.2 mL/min, oven temperature 60°C. Calibration of the system was performed with $NaHCO_3$ standards diluted in distilled water adjusted to pH 7.0 and a salinity of 32 ppt. Data was processed using software provided by the supplier.

2.5 Calculation of HCO_3^- and pCO_2 in seawater and extracellular fluids

Extracellular fluid pCO_2 and $[HCO_3^-]$ were calculated from pH and C_{CO_2} measurements using the following forms of the Henderson-Hasselbalch equation:

$pCO_2 = C_{CO_2} / \alpha \, (10^{\,pH-pK'_1} + 1)$

$[HCO_3^-] = C_{CO_2} - \alpha \, pCO_2.$

where α is the solubility coefficient of CO_2 (0.047 mmol l^{-1} $torr^{-1}$) and pK'_1, the first apparent dissociation constant of carbonic acid (6.020). Both α and pK'_1 were calculated for 17°C and 32 ppt from (Truchot, 1976) values for *Carcinus maenus* hemolymph. The use of constants determined in a decapod for calculations in *S. officinalis* is warranted by the similarity in extracellular hemocyanin concentration, cellular fraction and ionic composition between the two groups.

Seawater pCO_2 and $[HCO_3^-]$ were calculated from pH and C_{CO_2} (also referred to as C_T) measurements with the open-source software CO2SYS (Lewis and Wallace, 1998) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). pH was measured with a WTW 340i meter and SenTix81 electrode calibrated daily with NBS buffers.

2.6 *In vivo* measurements of pH and pO_2

In vivo measurement of pH and pO_2 were made in both blood and egg perivitelline fluid of *S. officinalis*. The cannulation procedure used to obtain blood samples is described in Melzner 2005 section 2.8 and Pub. 1 S1. Perivitelline fluid was withdrawn from the eggs by puncture with the measurement syringe. pH and pO_2 measurements were performed simultaneously using optical sensors (pH HPS-OIW and O_2 PSt1, Presens) that were implanted into 1 ml plastic syringes, such that the tips reached 3 mm inside the syringe. Positioning of the optodes near the tip of the syringe made it possible to measure pH and pO_2 in approximately 100 µl of extracellular fluid (Pub. 1, S2). Stable pH values were obtained after 10 minutes, pO_2 values after 10 seconds. The syringe was temperated by submerging it into a thermostatted bath. Optodes were connected to Microx H and Microx TX2-A units (PreSens, Regensburg, Germany), data were recorded using software supplied by the manufacturer.

O_2 optodes were calibrated following the manufactures instructions. For pH measurements in blood, pH optodes were calibrated in *S. officinalis* serum (blood was centrifuged at 10,000 g for 20 min at 0°C, 5810R Eppendorf, Hamburg, Germany. Three ml of serum were equilibrated in a tonometer (237 Instrumentation Laboratories GmbH, Kirchheim, Germany) with various pCO_2 gas mixtures supplied by a gas-mixing pump (5KM402/a-F, Wösthoff GmbH, Bochum, Germany). pH was measured in the serum with a glass electrode (SenTix 81, WTW GmbH, Weilheim, Germany), and a regression was calculated for the relationship between optode phase angle and measured pH in between pH 7.2 and 8.0. For pH measurements in egg perivitelline fluid, the pH optodes were calibrated using five seawater standards (North Sea seawater, 31 psu, 0.2 µm filtered) adjusted to pH values between 7 and 8 with 1M HCl. A pH electrode (WTW sentix81 and pH340i pH meter, WTW, Weilheim, Germany), calibrated with Radiometer precision buffers, was used to prepare the seawater buffers. Calibration of the pH optodes with sample specific buffers was found necessary as these sensors are sensitive to the ionic strength of the measurement medium.

2.7 Whole animal metabolic rate measurements

Standard metabolic rates (smr) were determined using intermittent closed respirometry (Fig. 2.1C). Oxygen consumption rates (3-4 runs of approximately 20 minutes) were obtained between 8:00 and 20:00 to avoid peak night activity periods of the cuttlefish (Denton & Gilpin-Brown 1961a). Briefly, animals (10.4 ± 4.3 g, n = 6) were starved for 24 hours and then incubated in cylindrical perspex chambers (3x25 cm) for a period of three days during which 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 18-20 kPa in between measurements. Temperature was maintained at 16°C ± 0.2°C by placing the four replicate chambers in a thermostatted water bath. 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 was recorded using software supplied by the manufacturer, and oxygen consumption rates were calculated from linear declines in chamber oxygen partial pressure.

2.8 ^{31}P NMR of pHi and adenylates

Animals were placed in a Perspex perfusion chamber analogous to the one used by (Melzner et al., 2006). Plastic sliders within the chamber were adjusted to restrict the amount of space available to the animal for movement, however, allowing for enough space to guarantee

unrestrained ventilatory movements. The chamber was connected to a closed recirculation seawater system and placed within the magnet as described by Bock et al. (2002). *In vivo* ^{31}P NMR spectroscopy experiments were performed as described by Melzner et al. (2006). Measurements were made in a 47/40 Bruker Biospec DBX system with a 40cm horizontal wide bore and actively shielded gradient coils (50mT m^{-1}). A 5cm triple tunable $^{1}H/^{31}P/^{13}C$ surface coil was used for excitation and signal reception. The coil was placed directly under the animal chamber in such a way to maximize the signal from the posterior mantle muscle section. The position and specific excitation volume of the surface coil were checked by collecting Pilot scans in all three directions using a classical Flash sequence right before the start of the experiments. *In vivo* ^{31}P NMR spectra [sweep width, 5000Hz; flip angle, 45° (pulse shape bp 32; pulse length 200 μs); repetition time (TR), 1s; scans, 256; duration, 3min 40s] were acquired every 21.3 min to measure pH_i. Changes in pHi were represented by the position of the Pi signal relative to the position of the PLA signal. pHi was calculated using the PLA *vs* Pi shift equation obtained by Doumen and Ellington (Doumen and Ellington, 1992), using a pK_a value determined by Pörtner (Pörtner 1990) for an ionic strength of I=0.16. Temperature compensation of the tritration curve was applied according to Kost (Kost, 1990, Bock et al. 2001). ^{31}P NMR spectra were processed automatically using TopSpin V1.0 software (BrukerBioSpin MRI GmbH, Ettlingen, Germany) and a macro (written by R.-M. Witting, AWI) to finally yield integrals of all major peaks within the spectrum (Bock et al. 2001). Concentrations of metabolites, inorganic phosphate (Pi) and phospho-L-arginine (PLA), were expressed as a ratio owing to large changes in overall *in vivo* ^{31}P NMR signal intensities due to animal movement artifacts.

2.9 Measurement of cuttlebone $CaCO_3$ content and morphometrics

Cuttlebones were excised from anesthetized *S. officinalis* individuals at the completion of the growth trial. Extra care was taken to remove the cuttlebones in their entirety, and not to break off the posterior sections of the shell margin. All further measurements were performed on cuttlebones that had been dried for 24 hours at 40° C. Cuttlebone dry mass was measured on a precision balance (ME235S, Sartorius). Length, width and height of the cuttlebones were measured with a caliper to the nearest 0.5 mm. The ultrastructure of six cuttlebones taken randomly from each group, ranging in length from 46 to 52 mm, was further analyzed. Dried cuttlebones were dorsally etched along the posterior-anterior plane and snapped in half. The number of lamellae in a transversal section was counted at the anterior end of the siphuncular region. This transverse section represented the measured height of the cuttlebones.

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 & Thomas (1983).

After 24 hours, the calcified component had entirely dissolved, and the remaining organic matrix was carefully removed, rinsed with distilled water, dried over night in a 40°C oven, and weighed on a precision balance (ME235S, Sartorius).

2.10 Scanning electron microscopy of cuttlebones

Approximately 2 cm sections anterior of the siphuncular region were trimmed and mounted on SEM pedestals stubs with double sided adhesive carbon discs. The sections were sputter-coated with a gold-palladium alloy and investigated using a CamScan-CS-44 SEM. Ultrastructural changes were examined using the freeware program Image J. The 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 $CaCO_3$ deposits, spherical structures, was measured in four 1 mm^2 sections in each cuttlebone on the exposed surface of the 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.

2.11 Abiotic conditions in egg perivitelline fluid

Acid-base parameters and pO_2 were recorded in the perivitelline fluid of S. *officinalis* eggs containing embryos ranging in mass from 134-310 mg. Eggs had been individually cultured in the aquarium system described in section 2.2. All (n=13) eggs of the present study were sampled on the same day (stages 29-30 as defined by Lemaire 1970). Eggs were gently lifted out of the tank and immediately sampled for PVF. All PVF samples were taken within 15 seconds, thus minimizing the chance of artificially increased pCO_2 values caused by disturbed embryos. PVF pH and pO_2 were measured fiber optic sensors implanted in a syringe as describe in section 2.6. Another 350 µl of PVF was sampled with a gas-tight glas syringe for the determination of total dissolved inorganic carbon (C_T). C_T was measured in triplicate (100 µl each) using the gas chromatographic method described in section 2.4. Following PVF sampling, eggs were dissected and embryo and yolk wet mass was determined using a Sartorius precision balance.

2.12 Embryonic development and hatchling viability

The combined effects of accumulating metabolic CO_2 and elevated seawater pCO_2 were examined on embryonic development in the cuttlefish *S. officinalis*. Embryos which were in the

late stages of organogenesis (stage 26 as defined by Lemaire 1970), were incubated under 0.6 kPa CO_2 for approximately 30 days until hatching, and for two weeks post-hatching. Ten eggs were randomly selected from each experimental group on a weekly bases, and hatchling mantle length, dry mass, and yolk dry mass were determined using Leica F6 stereomicroscope and a precision balance (Sartorius ME235S). Hatching success and temporal distribution of hatching were recorded but are not reported due to the small size of the sample group (n=20). Hatchling survival rates were recorded for two weeks post-hatching.

2.13 Statistics

Results were analyzed using GraphPad Prism 4. Analysis of variance (ANOVA), unpaired t-tests, and Dunnett's multiple comparison tests were carried out to assess the significance of differences between control and treatment groups. Regression analyses were performed using GraphPad Prism 4 and Statistica 10. Deviations from nonlinear regression models were tested for significance using a Runs Test. Both linear and nonlinear regression analysis are plotted with 95% confidence intervals. Values are always expressed as means ± SD. For detailed descriptions please refer to the appropriate publication.

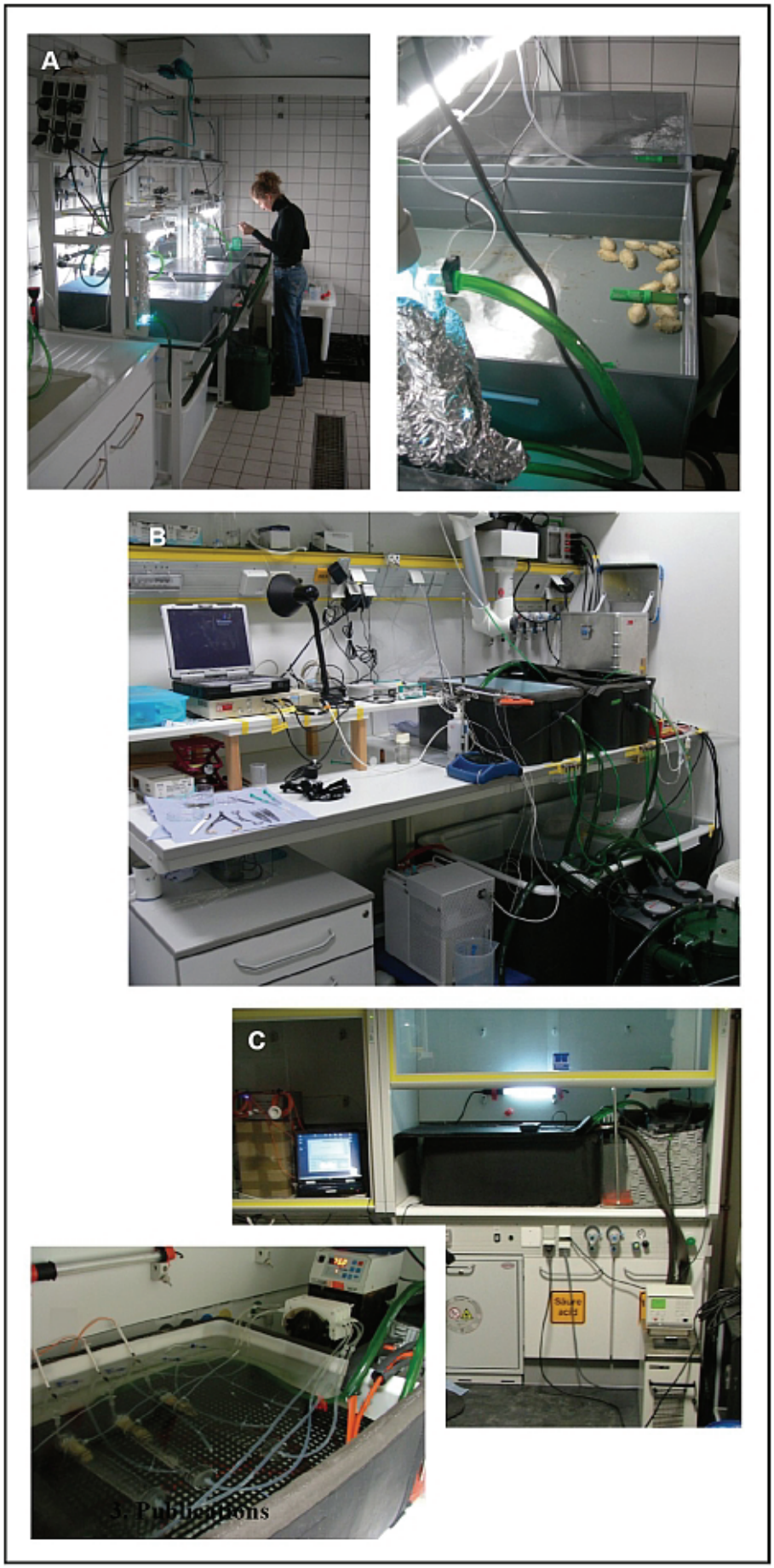


Fig. 2.1 A) Aquaristic system used for the growth trials of juvenile cuttlefish and developmental trials with *S. officinalis* eggs. **B)** Experimental setup and aquaristic system used for incubating cannulated cuttlefish. **C)** Experimental setup and aquaristic system used for oxygen consumption measurements in juvenile *S. officinalis*. For further descriptions please refer to text.

3. Publications

List of publications and declaration of my contribution towards them.

Publication 1

Gutowska, M.A., Melzner, F., Langenbuch, M., Bock, C., Claireaux, G., Pörtner, H.O. Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. (*submitted to the Journal of Experimental Biology*).

Experiments were developed and carried out together with the coauthors. The manuscript was written by myself and revised together with the coauthors.

Publication 2

Gutowska, M. A, Pörtner, H. O., Melzner, F. (2008). Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO_2. Marine Ecology Progress Series. 373, 303-309.

Experiments developed and carried out together with F. Melzner. The manuscript was written by myself and revised together with the coauthors.

Publication 3

Gutowska, M.A., Melzner, F., Pörtner, H.O., Meier, S. Increased cuttlebone calcification during exposure to elevated seawater pCO_2 in the cephalopod *Sepia officinalis*. (*pending submission to Biogeosciences special edition for the 2nd Int. Symposium of The Ocean in a High CO_2 World*).

Experiments developed and carried out by myself. SEM work and analysis together with S. Meier. Manuscript written by myself, revised together with the coauthors.

Publication 4

Gutowska, M.A., Melzner F. (2008). Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2. Marine Biology. 156, 515-519.

Experiments developed and carried out by myself. The manuscript was written and revised together with the second author.

Publication 5

Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M., Bleich, M., Pörtner, H.P. Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifehistory and ontogeny? (*pending submission to Biogeosciences special edition for the 2nd Int. Symposium of The Ocean in a High CO_2 World*).

The original planning, design, and database research for this conceptual manuscript were carried out by FM, MG, and ML. The majority was written by FM and revised together with MG and ML. The remaining coauthors contributed selected passages and final revisions.

Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia.

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Abstract

Acidification of ocean surface waters by anthropogenic carbon dioxide (CO_2) emissions is a currently developing scenario that warrants a broadening of research foci in the study of acid-base physiology. Recent studies working with environmentally relevant CO_2 levels, indicate that some echinoderms and molluscs reduce metabolic rates, soft tissue growth and calcification during hypercapnic exposure. In contrast to all prior invertebrate species studied so far, growth trials with the cuttlefish *Sepia officinalis* found no indication of reduced growth or calcification performance during long-term exposure to 0.6 kPa CO_2. It is hypothesized that the differing sensitivities to elevated seawater pCO_2 could be explained by taxa specific differences in acid-base regulatory capacity. In this study we examined the acid-base regulatory ability of *S. officinalis in vivo*, using a specially modified cannulation technique as well as ^{31}P NMR spectroscopy. During acute exposure to 0.6 kPa CO_2, *S. officinalis* rapidly increased its blood [HCO_3^-] to 10.4 mM through active ion transport processes, and partially compensated the hypercapnia induced respiratory acidosis. A minor decrease in intracellular pH (pHi) and stable intracellular phosphagen levels indicated efficient pHi regulation. We conclude that *S. officinalis* is not only an efficient acid-base regulator, but is also able to do so without disturbing metabolic equilibria in characteristic tissues or compromising aerobic capacities. The cuttlefish did not exhibit acute intolerance to hypercapnia that has been hypothesized for more active cephalopod species (squid). Even though blood pH (pHe) remained 0.18 pH units below control values, arterial O_2 saturation was not compromised in *S. officinalis* because of the comparatively lower pH sensitivity of oxygen binding to its blood pigment. This raises questions concerning the potentially broad range of sensitivity to changes in acid-base status amongst invertebrates, as well as to the underlying mechanistic origins. Further studies are needed to better characterize the connection between acid-base status and animal fitness in various marine species.

Introduction

Physiological studies that examined acid-base regulation in marine organisms have traditionally focused on the maintenance of acid-base homeostasis during exercise or hypoxia induced metabolic depression. A currently developing scenario that warrants a broadening of research foci in the study of acid-base physiology is the acidification of ocean surface waters by anthropogenic carbon dioxide (CO_2) emissions. It is projected that average surface ocean pH could decrease by 0.14 to 0.35 units from pre-industrial values by the year 2100 (Intergovernmental Panel on Climate Change 2007). This would correspond to an increase in seawater pCO_2 from the current value of 0.04 kPa, to values > than 0.1 kPa. In the study of hypercapnia effects on marine organisms, the physiological literature has primarily focused on elaborating mechanistic acid-base regulatory processes in fish and crustaceans using very high CO_2 levels, ranging from 1 to 8 kPa (e.g. Heisler 1986, Cameron 1986). Information on physiological effects resulting from exposure to moderately elevated pCO_2 is very limited (reviewed in Fabry et al. 2008).

However, recent growth trial studies indicate that some echinoderms and molluscs reduce metabolic rates, soft tissue growth and calcification during long-term exposure to hypercapnia. Working with pCO_2 values that were only 0.02 kPa elevated above current levels, Shirayama and Thornton (2005) documented a 21% and 12 % decrease in growth over a six month period in the sea urchin *Echinometra mathaei* and the gastropod *Strombus luhuanus*. Interestingly, in the molluscs examined to date, effects of elevated pCO_2 differ between classes. While the bivalve *Mytilus galloprovincialis* was shown to experience growth and metabolic reductions of 55 and 65 %, respectively, when exposed to ca. 0.5 kPa CO_2 (Michaelidis et al., 2005), cephalopod molluscs appear to be surprisingly tolerant to similar pCO_2 values. In contrast to all prior invertebrate species studied so far, growth trials with the cuttlefish *Sepia officinalis,* conducted under conditions of 0.4 and 0.6 kPa CO_2, found no indication of reduced growth or calcification performance. Growth rates in excess of 3.5 % day^{-1} were conserved over the course of 6-week trials. In addition, food assimilation efficiencies remained at control levels, as did routine metabolic rates (Gutowska et al., 2008). How *S. officinalis* differs from the bivalve physiotype in its ability to conserve growth and calcification under hypercapnic conditions, is unknown at present. However, taxa specific differences in acid-base regulatory capacity may be a crucial factor that could explain differing sensitivities (Pörtner et al. 2004, Pörtner 2008, Melzner et al. 2009).

Incomplete compensation of extracellular pH (pHe) during acute exposure to hypercapnia has been documented in some of the marine invertebrate groups that do not maintain growth and calcification rates during long-term hypercapnic incubations. Both the sea urchin *Psammechinus*

miliaris and the mussel *Mytilus galloprovincialis* exhibited a decrease in pHe of at least 0.2 pH units, following 8 days of exposure to hypercapnia between 0.2 and 0.5 kPa CO_2 (Michaelidis et al., 2005; Miles et al., 2007). The association of uncompensated acidosis in the extracellular space with the onset of metabolic depression during hypercapnia, has been proposed in a recent model (Reipschlager and Pörtner, 1996, Pörtner et al., 2004). In contrast, teleost fish, which are known to have very strong acid-base regulatory abilities (Heisler 1984, Claiborne 1998), are able to maintain their growth rates under comparatively high pCO_2 levels. Long-term studies with adult *Anarichus minor* and *Salmo salar* smolts, have found conserved growth rates and condition indices at pCO_2 levels up to 1 kPa (Fivelstad et al., 2003; Foss et al., 2003). Maintained growth rates in the cuttlefish *S. officinalis* under moderately elevated CO_2 could reflect higher acid-base regulatory abilities as compared to other marine invertebrates.

An uncompensated extracellular acidosis, has the potential to not only depress metabolism, but also to effect the performance of extracellular oxygen binding pigments that are directly exposed to changing physiochemical conditions. A hypercapnia induced decrease in pHe would most dramatically affect the functional capacity of pigments whose oxygen loading and unloading capacity is very sensitive to pH changes (= those with a high Bohr coefficient (Bohr et al., 1904)). Cephalopods are one of the invertebrate groups whose hemocyanins have particularly high Bohr coefficients (Redfield and Goodkind, 1929; Bridges, 1994). The fine tuning of maximal oxygen loading at the gills and unloading in the tissues helps support their extremely high metabolic rates. Oceanic squid species (e.g. *Illex illecebrosus*), who have some of the highest metabolic rates and Bohr coefficients amongst all cephalopods, have been predicted to be one of the most acutely sensitive marine organisms to ocean acidification (Pörtner et al., 2004). In the first study to directly test this hypothesis, the epipelagic squid *Dosidicus gigas* was shown to depress metabolic rates by 31%, and activity levels by 45%, during acute exposure to 0.1 kPa CO_2 (Rosa and Seibel 2008).

Even though cuttlefish are not as highly 'tuned' as oceanic squid, they could also be potentially sensitive to hypercapnic conditions in seawater due to the pH sensitivity of their oxygen binding pigment. We set out to study the acid-base regulatory ability of the cuttlefish *S. officinalis* and to determine to what extent it is capable of compensating its pHe during acute exposure to 0.6 kPa CO_2. We were also interested in examining to what extent hemocyanin oxygen transport would be affected. We successfully developed a cannulation technique to acquire high-quality blood samples for the determination of $pO2$, pH and C_{CO_2}. In addition, we used *in vivo* ^{31}P NMR spectroscopy to measure intracellular pH and high energy phosphates in the mantle muscle of *S. officinalis* during acute hypercapnic exposure.

Material and Methods

2.1 *Animals*

European cuttlefish (*Sepia officinalis*) egg clusters were collected in the Bay of Seine (Normandy, France) in May 2005. The cuttlefish were hatched and raised at the Alfred Wegener Institute (AWI, Bremerhaven, Germany) in a closed recirculating system (20m^3 total volume, protein skimmers, nitrification filters, UV-disinfection units) at S=32-34 ppt, T=15 ± 0.1°C, pH=7.9-8.2, constant dark:light cycle (12h: 12h). 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 animals were initially fed a daily diet consisting of live mysids (*Neomysis integer*) and progressively transitioned to frozen brown shrimp (*Crangon crangon*). Five animals (555±195g wet mass, mean value ± s.d.) were used for the cannulation experiment, and another five (110±16g) for *in vivo* ^{31}P NMR spectroscopy studies on mantle muscle. All experiments complied with the German animal experimentation laws.

2.2 Experiment 1: *In-vivo cannulation measurement of ventilation rate, blood pO_2 and acid-base parameters in response to acute hypercapnia.*

2.2.1 Experimental protocol

S. officinalis individuals were starved for 24 hours prior to surgery within the experimental aquarium system. Surgery was performed in the afternoon of the first day and the animals recovered overnight under control conditions. Control blood parameters were measured the morning of the second day after which the gas supply of the aquarium system was then switched over to a CO_2 – air mixture produced by a gas flow controller (GSV-19, MKS Instruments, Andover, United States). Hypercapnic conditions were observed to stabilize after 4 hours at seawater pH 7.10±0.04 and a pCO_2 of 0.60±0.05 kPa (Table 1). Changes in seawater pCO_2 and [HCO_3^-] were calculated from pH and CO_2 content (C_{CO_2}) (see gas chromatography method below) with the open-source software CO2SYS (Lewis and Wallace, 1998) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). Ventilation rates of the cuttlefish were measured continuously over the course of the experiment. The time course of blood sampling was adjusted for each individual to avoid periods of high activity within the experimental setup (e.g. 'routine jetting', see Melzner el al. 2007) during the 48-hour CO_2 exposure. At the end of the experiment, animals were narcotized, killed, and blood was collected from all individuals for *in vitro* analyses, and pH/oxygen optode calibration.

2.2.2 Anaesthesia and implantation of pressure catheter

Pressure catheters were inserted into the mantle cavity as described in detail in Melzner et al. (2006a). Briefly, individual animals were first anesthetized in oxygenated seawater that was

mixed 1:1 with a 0.4mol l^{-1} $MgCl_2$ solution (Messenger, 1985). Muscle relaxation occurred within 3-5 min, animals were then placed ventral side up on a wet leather cloth, soaked with seawater, to prevent skin damage. During surgery the mantle cavity was perfused with aerated anaesthetic (0.04mol l^{-1} $MgCl_2$) flowing over both gills.

Catheters were constructed from 0.8m long PE tubing (Portex PE tubing, I.D. 0.58 mm; O.D. 0.96 mm), and connected to 23 gauge hypodermic needles on one end and flared, into a disc shape, at the other. For the recording of postbranchial pressure oscillations, the PE catheter was led through the full length of the mantle cavity and finally punctured through the posterior ventro-lateral section of the mantle muscle.

2.2.3 Cannulation of the cephalic vein

After successfully implanting the pressure catheter, a second catheter was inserted into the readily accessible part of the anterior cephalic vein (AVC) between its anterior muscular chamber and the forking into the two vena cava branches (Tompsett, 1939) according to Melzner (2005). The vessel was exposed by “unsnapping” the funnel from the ventral mantle, and was punctured with a modified syringe needle (23 gauge) that was connected to a PE catheter (Portex PE tubing, I.D. 0.58 mm; O.D. 0.96 mm). The catheter was bent 2-4 cm from the front end (depending on the size of the animal) by about 140°, and was completely filled with filtered (0.2 µm) seawater to avoid air bubbles entering the vessel. The needle was tied to the wall of the vessel with a fine surgical suture (Ethicon #N271H, Johnson & Johnson, United States). Three additional sutures were made along the course of the tubing, making use of the larger footing of the available arches, to further stabilize the implanted catheter on the underlying musculature and connective tissue (Fig. S1). All sutures were sealed with cyanoacrylate glue (Hylo Gel, Marston Oelchemie, Germany). The catheter left the mantle cavity at its lateral anterior end, carefully positioned to minimize interference with the activity of the collar flaps, the muscle sheets that create the ventilatory current. “Resnapping” the funnel to the mantle completed surgery. The total surgery time did not exceed 20 min.

2.2.4 Experimental aquarium system and ventilation measurements

Following surgery, the animals were returned to the experimental setup. The experimental aquarium system consisted of a small animal chamber (20 l volume, with a long lateral opening at the bottom) that was standing inside a thermostatted 17.1±0.2 °C aquarium which had a total water volume of 200 l. The animal chamber was darkened and perfused at a rate of 5 l per min. Within the chamber, the swimming movements of the cuttlefish were limited with cushioned plastic grids. The catheters were fed through the lateral slit in the chamber wall,

and attached to their respective instruments. Water quality was maintained using a 12 W UV-sterilizer and a nitrification filter (Pro2, Eheim, Deizisau, Germany), ammonia and nitrite values were maintained below 0.1mg l^{-1}. A full water change was done in the system after each experimental animal.

The implanted pressure catheter was connected to a MLT 0380 reusable pressure transducer; signals were amplified with a ML-110 bridge amplifier and further fed into a PowerLab/8SP data acquisition system (all ADInstruments GmbH, Spechbach, Germany). Pressure transducers were calibrated before every experiment, data acquisition was performed at a rate of 40 Hz. Pressure in the cephalopod mantle cavity is generated to create both a ventilatory water stream past the gills and to enable swimming and escape movements by means of jet propulsion. While ventilatory pressure oscillations are of low amplitude, the latter can reach amplitudes of up to 25kPa (Wells and Wells, 1991). According to the results of Bone et al. (1994) pressure amplitudes > 0.5 kPa were defined as swimming jets. Data evaluation for ventilatory frequency, as well as blood sampling, was accordingly carried out during non jetting periods. Mean ventilatory frequency in beats per minute (bpm) was determined in 20 min sections for every hour.

2.2.5 Blood pO_2 and acid-base parameter measurements

Blood samples were gently pulled with syringes through the AVC catheter. At each sampling point, blood was initially pulled through a prep syringe, 100 µl were then pulled with a gas-tight Hamilton syringe for blood CO_2 content determinations, and 200 µl were pulled with a modified plastic syringe for pH and pO_2 measurements. After completion of the pH and pO_2 measurements the blood was returned to the animal along with 100 µl of sterilized SW to replace the missing blood. pH and pO_2 measurements were performed simultaneously using optical sensors (pH HPS-OIW and O_2 PSt1, PreSens, Regensburg, Germany) that were implanted into 1 ml plastic syringes, such that the tips reached 3 mm inside the syringe. Positioning of the optodes near the tip of the syringe made it possible to measure pH and pO_2 in approximately 100 µl of blood (Fig. S2). The syringe was submerged into the thermostatted water of the outer aquarium. Optodes were connected to Microx_H and Microx TX2-A units (PreSens, Regensburg, Germany), data were recorded using software supplied by the manufacturer. Each measurement lasted no longer than 10 minutes.

The total dissolved inorganic carbon content (C_{CO_2}) of blood and seawater samples was analyzed using a modification of the gas chromatographic method outlined by (Lenfant and Aucutt, 1966), which has been previously modified by Boutilier et al. (1985) and (Pörtner et al., 1990). The underlying concept of this method involves the use of acid to liberate sample C_{CO_2}

into the gas phase, for subsequent measurement with a gas chromatograph. Blood (30 µl) and seawater (200 µl) samples were injected into 10 ml gas tight vials filled with 3 ml of air equilibrated, 0.1 M HCl. Vials were processed using an automated headspace sampler (G188 Agilent Technologies, Santa Clara, Unites States). The gas phase was then injected into a gas chromatograph (6890N Agilent Technologies) equipped with a thermal conductivity detector: split inlet ratio 2.67:1, HP-PLOT Q column (carrier gas helium, flow rate 3.2 mL/min, oven temperature 60°C). Calibration of the system was performed with $NaHCO_3$ standards diluted in distilled water adjusted to pH 7.0 and a salinity of 32 ppt. Data was processed using software provided by the supplier.

Blood pCO_2 and $[HCO_3^-]$ were calculated from pHe and C_{CO_2} measurements for each individual animal, using the following forms of the Henderson-Hasselbach equation:

$pCO_2 = C_{CO_2} / \alpha \, (10^{\,pH-pK'_1} + 1)$

$[HCO_3^-] = C_{CO_2} - \alpha \, pCO_2.$

where α is the solubility coefficient of CO_2 (0.047 mmol l^{-1} $torr^{-1}$) and pK'_1, the first apparent dissociation constant of carbonic acid (6.020). Both α and pK'_1 were calculated for 17°C and 32 ppt from (Truchot, 1976) values for *Carcinus maenus* haemolymph. The use of constants determined in a decapod for calculations in *S. officinialis*, is warranted by the similarity in extracellular hemocyanin concentration, cellular fraction and ionic composition between the two groups.

After completion of the experiment, animals were anaesthesized in the experimental chamber with 2% Ethanol and their blood was collected. The blood was centrifuged at 10,000 g for 20 min at 0°C (5810R Eppendorf, Hamburg, Germany) and the collected serum was divided, with the majority being frozen at -20°C for further *in vitro* studies (see below). The remaining serum was used to calibrate the pH optodes. Three ml of serum were equilibrated in a tonometer (237 Instrumentation Laboratories GmbH, Kirchheim, Germany) with various pCO_2 gas mixtures supplied by a gas-mixing pump (5KM402/a-F, Wösthoff GmbH, Bochum, Germany). pH was measured in the serum with a glass electrode (SenTix 81, WTW GmbH, Weilheim, Germany), and a regression was calculated for the relationship between optode phase angle and measured pH in between pH 7.2 and 8.0. pO_2 optodes were calibrated according to the manufacturer's instructions.

2.2.6 *In-vitro* measurements of blood non-bicarbonate buffer value and cation composition

Serum was thawed on ice and equilibrated with CO_2–O_2 -N_2 mixtures in a tonometer (O_2 = 21%, N_2, CO_2 = variable fractions). At set pH values, 100 µl of blood were removed from the tonometer and analyzed for C_{CO_2}. $[HCO_3^-]$ was calculated from pH and C_{CO_2} values, as described

above. The non-bicarbonate buffer value for *S. officinalis* blood was derived from the linear regression of a pH-bicarbonate graph.

2.3 Experiment 2: *Determination of in-vivo mantle muscle pHi and adenylates using ^{31}P-NMR spectroscopy.*

2.3.1 Experimental Protocol

Experimental animals were starved for 24 h and placed in the experimental set-up at noon of the first day. Control *in vivo* ^{31}P NMR spectra were acquired overnight and until noon of the second day. From the second day onward, the experimental system was equilibrated with air containing 0.60±0.05 kPa CO_2 (Table 1). Hypercapnic conditions stabilized within the same time frame as in the cannulation experiment. The primary focus of the *in vivo* ^{31}P NMR measurements was to monitor the changes in mantle muscle intracellular pH under hypercapnia.

2.3.2 *In vivo* ^{31}P NMR spectroscopy setup and measurements

Animals were placed in a Perspex perfusion chamber analogous to the one used by (Melzner et al., 2006). Plastic sliders within the chamber were adjusted to restrict the amount of space available to the animal for movement, allowing for enough space to guarantee unrestrained ventilatory movements. The chamber was connected to a closed recirculation seawater system and placed within the magnet as described by Bock et al. (2002). Water quality was maintained with a nitrification filter (Eheim Pro2; Eheim, Deizisau, Germany). Water quality parameters were kept within the limits stated above, and all of the system water was replaced after each individual experimental animal.

In vivo ^{31}P NMR spectroscopy experiments were performed as described by Melzner et al. (2006). Measurements were made in a 47/40 Bruker Biospec DBX system with a 40cm horizontal wide bore and actively shielded gradient coils (50mT m^{-1}). A 5cm triple tunable ^{1}H/^{31}P/^{13}C surface coil was used for excitation and signal reception. The coil was placed directly under the animal chamber in such a way to maximize the signal from the posterior mantle muscle section. The position and specific excitation volume of the surface coil were checked by collecting Pilot scans in all three directions using a classical Flash sequence right before the start of the experiments. *In vivo* ^{31}P NMR spectra [sweep width, 5000Hz; flip angle, 45° (pulse shape bp 32; pulse length 200 µs); repetition time (TR), 1s; scans, 256; duration, 3min 40s] were acquired every 21.3 min to measure pH_i. Changes in pHi were represented by the position of the Pi signal relative to the position of the PLA signal. pHi was calculated using the PLA *vs* Pi shift equation obtained by Doumen and Ellington (Doumen and Ellington, 1992), using a pK_a value

determined by Pörtner (Pörtner 1990) for an ionic strength of I=0.16. Temperature compensation of the tritration curve was applied according to Kost (Kost, 1990, Bock et al. 2001). ^{31}P NMR spectra were processed automatically using TopSpin V1.0 software (BrukerBioSpin MRI GmbH, Ettlingen, Germany) and a macro (written by R.-M. Witting, AWI) to finally yield integrals of all major peaks within the spectrum (Bock et al. 2001). Concentrations of metabolites, inorganic phosphate (Pi) and phospho-L-arginine (PLA), were expressed as a ratio owing to large changes in overall *in vivo* ^{31}P NMR signal intensities due to animal movement artifacts.

2.5 Statistics

Results were analyzed using GraphPad Prism 4. Analysis of variance (ANOVA) and Dunnett's multiple comparison tests were carried out to assess the significance of differences between control and treatment groups. Deviations from nonlinear regression models were tested for significance using a Runs Test. Nonlinear regression analysis are plotted with 95% confidence intervals. Values are expressed as means ± SD, n = 4-6.

Results

The time course of changes in extracellular acid-base status was examined in cannulated specimens of *S. officinalis* in response to acute hypercapnia. Cuttlefish were maintained in darkened aquaria under control conditions for 20 hours before being exposed to 0.6kPa (4.5 Torr) CO_2. Fig.1 illustrates the changes in seawater pH, $[HCO_3^-]$ and pCO_2 during the first eight hours of hypercapnic exposure. The following nonlinear regression fits represent the time course of changes in seawater parameters; pCO_2 and $[HCO_3^-]$ third order polynomial, pH exponential decay. Changes in blood acid-base parameters are also represented in Fig 1 for two exemplary animals whose catheters remained open for sampling during the entire period. This allowed for high resolution determination of blood values during the initial stages of acute hypercapnia exposure. The data from these animals are representative of mean values calculated for blood acid-base parameters in all of the animals at selected time points.

pCO_2 increased in the blood of the cuttlefish with nearly no temporal delay in relation to increasing ambient pCO_2 (Fig. 1A). The change in seawater pCO_2, and subsequent diffusion into the extracellular space of the cuttlefish, was complete after 4 hours.The decrease in pH also appeared immediately in the blood with the onset of hypercapnic conditions. However, blood pH only decreased by approximately 0.2 pH units in comparison to the nearly 1 pH unit drop in seawater pH (Fig. 1B). Active and rapid proton equivalent ion exchange by the experimental cuttlefish was clearly evident. After six hours of hypercapnic exposure, blood $[HCO_3^-]$ were nearly four-fold higher than seawater $[HCO_3^-]$.

Control extracellular acid-base parameters, in cannulated *S. officinalis*, were pH = 7.67±0.05, $[HCO_3^-]$ = 3.38±0.12 mM, pCO_2 = 0.22±0.03 kPa (1.65±0.23 Torr) (Fig. 2). A new extracellular steady state value of 0.98±0.03 kPa was calculated for blood pCO_2 during acute exposure to 0.6kPa CO_2. Blood pH decreased to 7.49±0.02 after only 3 hours of hypercapnia, and remained stable at this value for the remainder of the 48 hr exposure. Within 24 hours, blood $[HCO_3^-]$ rose to a new steady state level of 10.37±0.46 mM. However, this large increase in blood $[HCO_3^-]$ was not sufficient to fully compensate the extracellular acidosis, blood pH remained approximately 0.2 pH units lower than control values over the entire 48hr exposure period (Fig. 2, Fig. 3).

The acid-base regulatory response of *S. officinalis* is further depicted in a pH-bicarbonate diagram (Davenport diagram) (Fig. 3). The non-bicarbonate buffer value (β_{NB}) of *S. officinalis* blood was determined to equal 10 mEq l^{-1} pH^{-1}. Within the first hours of hypercapnic exposure, the cuttlefish exhibited partial compensation of the respiratory acidosis through active proton equivalent ion exchange. However, blood $[HCO_3^-]$ and pH remained stable between 24 and 48 hours of exposure time, and no additional compensation of acid-base status occurred.

In vivo ^{31}P NMR revealed a very minor, but significant, decrease in pHi, from a value of 7.534±0.017 to 7.502±0.020. The significance of differences between pHi under control conditions versus hypercapnia were analyzed using a Sigmoidal dose-response fit. Deviations from the model were found not to be significant using a Runs test. The decline in pHi began immediately with the onset of hypercapnia, and remained throughout the 24 hours of exposure Fig. 4A. The decrease in pHi was not accompanied by measureable fluctuations in intracellular phosphagen levels. The Pi/PLA ratio remained stable over the entire experimental period (Fig. 4B). The linear regression fitted through the Pi/PLA ratio did not significantly deviate from zero.

The animals displayed rapid recovery towards control levels of ventilation frequency, 20.62±0.71 bpm, already 4 hours after the cannulation surgery (Fig. 5). Ventilation frequency values significantly increased with the onset of hypercapnia, and stabilized at an elevated value of 22.79±0.90 after 24hours of exposure. A sigmoidal dose-response fit was also used to analyze the time course of changes in ventilation frequency. Deviations from the model were found not to be significant using a Runs test.

Fig. 6A illustrates the changes in blood pO_2 in cuttlefish under control conditions and during the subsequent time course of 48 hr CO_2 exposure. Control values measured in mixed venous blood, returning from the cephalic region through the AVC, were 1.38±0.42 kPa. During acute hypercapnia there was a significant, but slight, increase in pO_2 to 2.66±0.17 kPa. The increase was transient, and returned to control levels after 24hours.

Discussion

This study reports the first *in vivo* measurements of extra-and intracellular acid-base regulation in a decapod cephalopod over a period of two days. Previous studies have measured *in vivo* blood parameters on the time scale of several hours (Johansen et al., 1982 in cuttlefish; Pörtner et al., 1991 in squid). The stability of our catheter preparation (supp. Fig.1) enabled us to collect a greater number of blood samples over a 48 hour period. Since ventilatory frequency was measured simultaneously to our sampling procedure, we were also able to selectively measure control blood values in S*epia officinalis* during periods of rest. The low degree of variability in our measurements reflects this improvement in the sampling method. Ventilation patterns indicate a rapid post-surgery recovery (Fig. 5) and suggest that the experimental animals remained in a stable physiological condition during the entire experiment. The *in vivo* blood parameters we measured match those of the prior study on *S. officinalis* (Johansen et al. 1982). The blood pH values we measured in the AVC (7.67±0.04) are very similar to those of Johansen et al. (7.63±0.08). However, Johansen et al. (1982) measured 1.5 mM higher [HCO_3^-], which leads to the calculation of slightly higher blood pCO_2 (0.33 kPa versus 0.22 kPa in the present study). Whether the slightly higher pCO_2 in the study of Johansen et al. resulted from the larger body size of their cuttlefish (1.5kg) and correspondingly higher diffusion gradients across the gills, remains to be investigated. Overall, our optimization of the cannulation preparation allowed us to accurately measure cuttlefish blood acid-base parameters *in vivo* and quantify acid-base regulation in response to elevated seawater pCO_2 over an extended period of time.

The compensation of acid-base disturbances elicited by hypercapnia is accomplished by proton equivalent ion exchange in most organisms (Cameron 1986 reviews invertebrates, Heisler 1986 reviews fish). According to the reactions between H^+, HCO_3^- ions and water, the removal of H^+ ions results in the same effect as the addition of OH^- or HCO_3^-, during a compensatory reaction in body fluid compartments. However, it is still unknown which species of the acid-base relevant ions is actually transferred across the regulatory epithelia in most marine organisms, including cephalopods. In the subsequent text we refer to the general compensatory effort as HCO_3^- accumulation. The following discussion compares the acid-base regulatory ability of the cuttlefish to that of more inactive invertebrates, decapod crustaceans, and to that of teleost fish. We focus on discussing experimental work performed under comparable hypercapnic regimes and time intervals (see Table 3).

S. officinalis exhibited a pH compensation pattern which typifies organisms with a considerable acid-base regulatory ability. In response to acute 0.6 kPa CO_2 exposure, the cuttlefish partially compensated the respiratory acidosis present in its blood through a rapid increase in extracellular [HCO_3^-] to 9.8 mM within 8 hours and a stable value of 10.4 mM after

24 hours. The HCO_3^- accumulatory response we measured in *S. officinalis* is considerably higher than that of inactive invertebrates examined in recent hypercapnia studies (Table 3). Work done with the sea urchin *Psammechinus miliaris*, at pCO_2's of 0.24 and 2.31 kPa, found that [HCO_3^-] in the coelomic fluid passively followed the non-bicarbonate buffer line with increasing pCO_2. This resulted in 0.5 and 0.9 unit decreases, respectively, in pHe during 8 days of exposure (Miles et al., 2007). In contrast, the mussel *Mytilus galloprovincialis* partially compensated the respiratory acidosis induced by exposure to 0.5 kPa CO_2, and thus limited the decrease in its haemolymph pH to 0.23 units. However, the compensatory effort only involved a 2.4 mM increase in [HCO_3^-]e over a period of 8 days (Michaelidis et al.2005). Additionaly, the source of acid-base relevant increases in bivalve haemolymph [HCO_3^-] has been primarily attributed to dissolution of shell $CaCO_3$, and not to active ion-transport by regulatory epithelia (Lindinger et al. 1984, Dwyer and Burnett 1996).

Calcifying marine invertebrates that are weak acid-base regulators may experience shell dissolution during exposure to pCO_2 levels high enough to lower the calcium carbonate saturation state of seawater (Ω) to <1. Elevated haemolymph Ca^{2+} and or Mg^{2+} concentrations in both *M. galloprovincialis,* and *P. miliaris* during hypercapnic exposure have been interpreted to result from the dissolution of calcified structures (Michaelidis et al. 2005, Miles et al. 2007). The contribution of elevated [HCO_3^-] from dissolved structures, to the compensation of acid-base equilibria, was found to be negligible in *P. miliaris* (Miles et al. 2007) but played a significant role in *M. galloprovincialis* (Michaelidis et al. 2005). More then equimolar increases in haemolymph [Ca^{2+}] compared to [HCO_3^-] were found in *M. galloprovincialis* during 8 days of exposure to 0.5 kPa CO_2. Lindinger et al. showed that similar increases found in [HCO_3^-]e of *Mytilus edulis* during hypercapnia, could be reproduced in hypercapnic seawater containing cleaned mussel shells due to the dissolution of shell $CaCO_3$ (Lindinger et al. 1984). In *S. officinalis*, the rate and magnitude of HCO_3^- accumulation during acute exposure, along with elevated calcification rates under long-term hypercapnic conditions (Gutowska et al, 2008), led us to conclude that cuttlebone dissolution did not contribute to elevated blood [HCO_3^-]. As in fish, active ion-transport processes must be responsible for this response (e.g. Deigweiher et al, 2008). The identification and localization of the relevant transport molecules involved in the compensatory HCO_3^- accumulatory response remains open to investigation. As to date, almost nothing is known about the gill ion transport machinery in cephalopods.

Despite the rapid acid-base regulatory response, *S. officinalis* did not fully compensate its extracellular pH (pHe). Blood pH remained 0.18 units below control values over the course of the 48 hour exposure period. If pHe was to be fully compensated at a blood pCO_2 around 1.0 kPa, *S. officinalis* would need to increase [HCO_3^-]e to approximately 17 mM (see Fig. 3).

However, the time course of HCO_3^-e accumulation does not suggest that such values would be reached: [HCO_3^-]e followed the typical hyperbolic regulation pattern found in many other powerful ion-regulators (e.g. Claiborne and Evans 1992, Toews et al. 1983), with 90% of the accumulatory response already being accomplished after 8h. Marine organisms that have been shown to fully compensate pHe typically do so within one continuous HCO_3^-e accumulation regulatory reaction, often within 24 hours. However, due to time limited periods of viable cannulation it is unknown whether a slow phase of bicarbonate accumulation complements compensation during long term exposures.

The high acid-base regulatory abilities of crustaceans and fish have been widely studied and their compensatory capacities have been nicely summarized (Whiteley et al., 2001; Claiborne 1998). A recent study of the effect of hypercapnia on *Cancer magister* has shown that the species increased its blood [HCO_3^-] by 12 mM within 24 hours in response to a pCO_2 of 1 kPa to fully compensate pHe (Pane and Barry, 2007). Studies on *Carcinus maenus* and *Callinectes sapidus* also indicate similarly high acid-base regulatory abilities (Cameron and Iwama, 1987; Truchot, 1984), as do studies on teleost fish, where rapid and complete compensation of pHe during moderate hypercapnia is accompanied by [HCO_3^-]e increases in excess of 20 mM (Larsen et al., 1997) (Table 3). Thus, some decapod crustaceans and teleost fish appear to be even more powerful ion regulators than the cuttlefish *S. officinalis*. However, our results could also be interpreted in a different way: Cuttlefish display rapid HCO_3^-e accumulation that is sufficient to maintain proper functioning of their respiratory pigment, hemocyanin, but avoid the increased energetic costs that go along with full pHe compensation and the maintenance of very high blood [HCO_3^-] and [H^+] gradients. We explain this possibility in more detail in the following paragraphs.

The acute intolerance of more active cephalopod ecotypes (squid) to ocean acidification was initially hypothesized by Pörtner et al. (2004). This idea was conceptually based on the high pH sensitivity of oxygen binding to the blood pigment, hemocyanin, in oceanic squid *Illex illecebrosus* ((Pörtner, 1990a). A 0.2 unit decrease in blood pH was calculated from the blood non-bicarbonate buffer line ($ß_{NB}$) in response to a hypothetical exposure of 0.6 kPa CO_2. Taking into consideration the steep slopes of the oxygen saturation curves along the *in vivo* pH range, a 0.2 pH unit decrease would reduce hemocyanin saturation by about 50% and lead to lethal asphyxiation.

However, the present study demonstrates that blood pHe does not follow $ß_{NB}$ when *S. officinalis* is exposed to hypercapnic conditions. Rather, the bicarbonate accumulation response sets in immediately and prevents the drop of pHe below 7.5. At a blood pH of 7.5, hemocyanin function does not appear to be significantly compromised in the cuttlefish: Fig. 6B illustrates the

comparative insensitivity of *S. officinalis* blood to a 0.2 pH unit reduction starting from the *in vivo* pH value of 7.67. Oxygen saturation curves for *S. officinalis* blood (measured at 20°C) are replotted from (Zielinski et al., 2001) in Fig. 6B, along with the in vivo blood pH value we measured adjusted according to alpha-stat pH regulation pattern (Reeves 1976). Assuming an arterial pO_2 around 13 kPa (Johansen et al. 1982), only a very slight decrease in arterial hemocyanin oxygenation (<5%) is evident in response to a 0.2 unit shift in blood pH. Slightly greater changes in hemocyanin saturation are evident in the venous blood. Tracing a 1.7 kPa O_2 isobar, which is relatively close to the 1.38 kPa venous pO_2 measured in this study, hemocyanin saturation is reduced by about 10% in response to a 0.2 unit pH shift. The relative insensitivity of hemocyanin saturation to limited pH changes in *S. officinalis* protects the cuttlefish from oxygen limitation during acute hypercapnia exposure. It is important to keep in mind that if no active HCO_3^- accumulatory response had taken place, blood pH would have fallen below 7.3, and consequently reduced arterial saturation by at least 20%. The acid-base regulatory response in *S. officinalis* during hypercapnic exposure significantly reduced the decrease in pHe thus allowing for the maintenance of full oxygen transport pigment saturation.

Acid-base regulation in response to hypercapnia has not been directly measured in the most active cephalopods, oceanic squid. However, acid-base regulatory changes in *I. illecebrosus* and *Loligo pealei* were studied during exhaustive exercise (Pörtner et al., 1991). When blood pCO_2 rose to 0.37 kPa during jet locomotion, a 1.7 mM increase in $[HCO_3^-]$, likely released from the musculature, protected the blood from acidification and thus maintained hemocyanin saturation in arterial blood (see Fig. 5 in Pörtner et al. 1991, Pörtner 1994). The tight regulation of blood parameters by *I. illecebrosus* in order to optimize hemocyanin function, most likely goes hand in hand with a high capacity for acid-base regulation. It is probable, that like the cuttlefish, *I. illecebrosus* is capable of accumulating significant amounts of compensatory HCO_3^- in response to hypercapnia. In order to fully compensate its pHe during exposure to 0.6 kPa of CO_2, it would need to accumulate approximately 5 mM of HCO_3^-. This is based on the assumption of a blood pCO_2 of approximately 0.8 kPa during exposure, as control ΔpCO_2 between blood and seawater is approximately 0.2 kPa (calculated from Fig. 5, Pörtner et al. 1991). The HCO_3^- levels that would be present in the blood of *I. illecebrosus* if pHe was fully compensated during hypercapnic exposure, are much lower than those in *S. officinalis*. This is because the acid-base equilibrium in squid blood is shifted in a more acidic direction compared to that of the cuttlefish (see Fig. 5 in Pörtner et al. 1991). As the acute sensitivity of both squid and cuttlefish to hypercapnia is highly dependent on the magnitude and rate of exposure to elevated CO_2, further work is needed to define their tolerance limits.

During hypercapnic exposure, CO_2 diffuses into both the extra- and intracellular spaces,

creating the potential for intracellular acidification and subsequent disturbance of vital biochemical processes. High non-bicarbonate buffer values in the intracellular space, about twice as high as that of blood, serve as a buffer reserve and facilitate efficient pHi homeostasis (Boron, 2004). In several studies of invertebrates, a preferential compensation of pHi has been shown when pHe values remained uncompensated during hypercapnia exposure (Michaelidis et al. 2005, Pörtner et al. 1998). It is worth noting however, that the compensation of pHi depends on the time course of pHe regulation and is supported by extracellular bicarbonate accumulation (Pörtner et al., 1998). In our study, *S. officinalis* did not fully compensate for acidotic shifts in either pHe or pHi during the first 48 hours of 0.6 kPa CO_2 exposure. The decrease we measured in pHi, was very minor at 0.03 pH units, but still significantly different from control values over the entire 48 hour experimental period (Fig.4A). The 0.03 pH unit decrease we measured in pHi during acute hypercapnia, versus 0.2 pH units in pHe, reflects that intracellular pH is regulated at a lower level than pHe, thus it requires less bicarbonate accumulation for the compensation of intracellular acidosis. It is questionable if the very minor 0.03 pH unit decrease we measured in intracellular pH during acute hypercapnic exposure to 0.6 kPa CO_2 is a physiologically relevant stressor for the cuttlefish *S. officinalis*.

The close regulation of pH during acid-base disturbances is energetically costly as various ATP dependent transporters are responsible for the maintenance of organismic acid-base homeostasis (Dubyak, 2004). One way that the potential increases in metabolism can be monitored, is by examining fluctuations in intracellular phosphagen levels e.g. (Storey and Storey, 1979). Working with *in vivo* ^{31}P NMR, Melzner et al. (2006) showed that changes in intracellular phosphagen levels during the upregulation of metabolism in response to exercise were quantifiable in the mantle muscle of *S. officinalis*. During spontaneous activity, concentrations of phospho-L- arginine (PLA), a rapidly available energy reserve, decrease in the mantle muscle of *S. officinalis*. Transphosphorylation of PLA helps buffer cellular [ATP] when muscle fibres require rapid ATP provision, but also leads to the accumulation of inorganic phosphate. Using the same method, we monitored the Pi /PLA ratio in mantle muscle of *S. officinalis* during hypercapnic exposure, and found it to remain stable over the entire experimental period (Fig. 4B). This demonstrates efficient pHi regulation and that anaerobic metabolic pathways were not challenged by providing the extra energy demand for acid-base regulation during hypercapnia. Stable ATP, PLA and Pi concentrations during minor decrements in pHi are indicative of the balanced thermodynamic environment (i.e. a high Gibb's free energy of ATP hydrolysis, Kammermeier 1984) that is necessary for the proper function of cellular ATPases. This is in contrast to previous work on less active invertebrates that are weak acid-base regulators.

In the worm *Sipunculus nudus* the transient intracellular acidosis, caused a significant increase in the Pi/PLA ratio and in concentrations of free ADP and AMP (Pörtner et al. 1998) parallel to metabolic depression. The stable Pi/PLA ratio in *S. officinalis*, and its implications for metabolism, are in line with results from a prior study, where whole animal standard metabolic rates were shown to remain stable during acute hypercapnic exposure (Gutowska et al. 2008). This stability indicates an immediate regulatory response to the hypercapnic stimulus. The very slight increase in ventilation frequency we measured in this study, also suggests that oxygen demand was not significantly elevated during hypercapnia. Using the correlation between ventilation frequency and oxygen consumption rate from (Melzner et al., 2006a), the 2 bpm increase we measured during acute hypercapnia corresponds to a less than 5 % increase in whole animal oxygen consumption. It is quite apparent that the cuttlefish species is not only an efficient acid-base regulator, but is also able to do so without disturbing metabolic equilibria in characteristic tissues or compromising aerobic capacities.

Conclusiom

This study, together with a companion paper (Gutowska et al. 2008), provides evidence that maintenance of whole animal growth rates by invertebrates during long-term hypercapnic exposure could be supported by significant acid-base regulatory capacities. Previous work has shown that the cuttlefish *S. officinalis* is capable of maintaining control growth rates and food assimilation efficiencies during long-term exposure to 0.6 kPa CO_2. Here we show that the acid-base regulatory response of *S. officinalis* is considerably greater than that of less active invertebrates which have also been shown to be much more sensitive to hypercapnia. During acute exposure to 0.6 kPa CO_2 cuttlefish rapidly increase their blood [HCO_3^-] to 10.4 mM and partially compensate the hypercapnia induced respiratory acidosis. However, blood pH still remains 0.18 units below control values. The observed time course of [HCO_3^-]e accumulation also does not suggest any major increases beyond the initial 48 hour exposure period, thus it is likely that blood pH remains depressed over longer periods of time. As we measured control growth, and elevated calcification rates in *S. officinalis* during a six-week exposure at the same pCO_2 (Gutowska et al., 2008), the possibility exists that some molluscs may be able to maintain performance levels under hypercapnic conditions despite a 0.2 unit acidotic shift in pHe. This is an interesting consideration, as a similar decrease of approximately 0.2 pHe units during acute hypercapnic exposure in the bivalve *Mytilus. galloprovincialis* was correlated with acute metabolic depression to 40% of normocapnic values and a 55% reduction in long-term growth (Michaelidis et al., 2005).

The response of *S. officinalis* to hypercapnic exposure raises questions concerning the

potentially broad range of sensitivity to changes in acid-base status amongst invertebrates, as well as to the underlying mechanistic origins. The association of uncompensated acidosis in the extracellular space with the onset of metabolic depression during hypercapnia has been proposed to occur through a reduction in ATP cost of intracellular pH regulation, in a recent model based on experimental work with the infaunal worm *Sipunculus nudus* (Pörtner et al., 2004; Reipschlager and Pörtner, 1996, Pörtner et al., 2000) However, further studies are needed to better characterize the connection between acid-base status and animal fitness in various marine species, especially during long-term hypercapnic exposures and at lower seawater pCO_2's. More work in this direction is particularly critical if we aim to predict the sensitivity of marine invertebrates to ocean acidification based on their acid-base regulatory abilities. An intriguing possibility exists that extracellular sensing and regulation in the cuttlefish are predominantly focused on adequate oxygen supply, rather than on strict control of pCO_2 / pH, as cephalopods rely on highly efficient blood oxygen extraction to support their high metabolic rates and low blood oxygen carrying capacities (e.g. Melzner et al,, 2007a, O'Dor & Webber 1986). This hypothesis remains open for future investigations.

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Figure Legends

Figure 1. Time course of changes in seawater and *Sepia officinalis* blood (A) pCO_2, (B) pH, and (C) HCO_3^- during acute hypercapnic (0.6 kPa CO_2) exposure. Seawater conditions are delineated with a solid line. *S. officinalis in vivo* blood parameters are represented by two model animals whose catheters remained open during the entire period, and thus allowed for high resolution determination of blood values during the initial stages of acute hypercapnic exposure. The data are representative of calculated mean values.

Figure 2. Time course of *in vivo* changes in *Sepia officinalis* blood (▲) pCO_2, (●) pH, and (□) HCO_3^- during 48 hours of hypercapnic (0.6 kPa CO_2) exposure. n = 5, means ± SD.
Acid-base parameters significantly different from control values are delineated with a horizontal line.

Figure 3. pH-bicarbonate (Davenport) diagram showing the time course of acid-base compensation during 48 hours of hypercapnic (0.6 kPa CO_2) exposure in *Sepia officinalis* blood. The non-bicarbonate buffer line ($ß_{NB}$) is delineated with a dashed line. The solid curved lines represent CO_2 isopleths. The point labeled U illustrates the theoretical uncompensated acid-base status of *S. officinalis* blood if no regulatory response occurred, and blood [HCO_3^-] passively followed $ß_{NB}$. Point C illustrates the theoretical fully compensated blood acid-base status. n = 5, means ± SD.

Figure 4. *In vivo* changes in (A) pHi and the (B) ratio P_i / PLA (phospho-L-arginine) in *Sepia officinalis* mantle muscle during control conditions and subsequent 24 hours of hypercapnic (0.6 kPa CO_2) exposure. n = 5, means ± SD.

Figure 5. Changes in ventilation frequency in *Sepia officinalis* during recovery from surgery, 12 hours of control conditions and subsequent 48 hours of hypercapnic (0.6 kPa CO_2) exposure. n = 5, means ± SD

Figure 6. (A) Changes in *Sepia officinalis* blood (●) pO_2 and (○) pH during 48 hours of hypercapnic (0.6 kPa CO_2) exposure. n = 5, means ± SD. Time points marked with an asterix significantly differ from control values. (B) Potential changes in *S. officinalis* arterial and venous blood saturation in response to a 0.18 pH unit decrease in pH using the pH saturation analysis of Zielinski et al., 2001.

Table 1. Seawater physiochemical parameters in all of the experimental setups for both normoxic control conditions and normoxic hypercapnia, S=32.2 ± 0.4 ppt, T=16.1 ± 0.2 °C.

	Control	Hypercapnia
pH	8.12 ± 0.05	7.10 ± 0.04
HCO_3^- (mM)	2.09 ± 0.1	2.42 ± 0.2
pCO_2 (kPa)	0.05 ± 0.01	0.6 ± 0.05

Table 2. Changes in extracellular acid-base parameters of selected marine organisms in response to acute hypercapnia. Values are expressed as means (SE). Sea urchin *P. miliaris*, 3-5cm test diameter 10°C (Miles et al. 2007); Mussel *M. galloprovincialis*, size n.d. 18°C (Michaeilidis et al. 2005); Cephalopod *S. officinalis*, 300-700g 17°C (this study); Crab *C. magister*, 500-1000g 10°C (Payne and Barry 2007); Teleost *S. aurata*, 50g 18°C (Michaelidis et al. 2007); Teleost *G. morhua*, 230-525g 12°C (Larsen et al. 1997).

	Control extracellular values			Hypercapnic exposure		Hypercapnic extracellular values		
Species	pH	$[HCO_3^-]$ (mM)	pCO_2 (kPa)	Seawater pCO_2 (kPa)	Exposure (days)	Maximum Δ pH	Final Δ pH	Δ $[HCO_3^-]$ (mM)
Psammechinus miliaris	7.40 (0.05)*	1.8 (0.2)	0.13 (x)*	0.25	8	-0.55	-0.55	+1.5*
Mytilus galloprovincialis	7.55 (0.02)	1.62 (0.12)	0.15 (0.03)	0.51	8	-0.19	-0.19	+2.4
Sepia officinalis	7.67 (0.05)	3.38 (0.12)	0.22 (0.03)	0.60	2	-0.18	-0.18	+6.7
Cancer magister	7.82 (0.05)*	6.5 (0.5)*	0.28 (x)*	1.10	1	-0.41*	-0.07*	+12.0
Sparus aurata	7.65 (0.03)	7.34 (0.54)	0.34 (0.04)	0.51	10	-0.24	-0.06*	+19.2*
Gadus morhua	7.90 (x)*	10.5 (x)*	0.43 (x)*	1.10	1	-0.18	+0.02	+21.0*

* = values read from figures
x = SD < minimum readable from figure
Maximum ΔpHe = maximum pH decrease measured in the respective study during acute hypercapnic exposure.
Final ΔpHe and Δ$[HCO_3^-]$ (mM) = values reported for the endpoint measurement in each study.

Figure 1

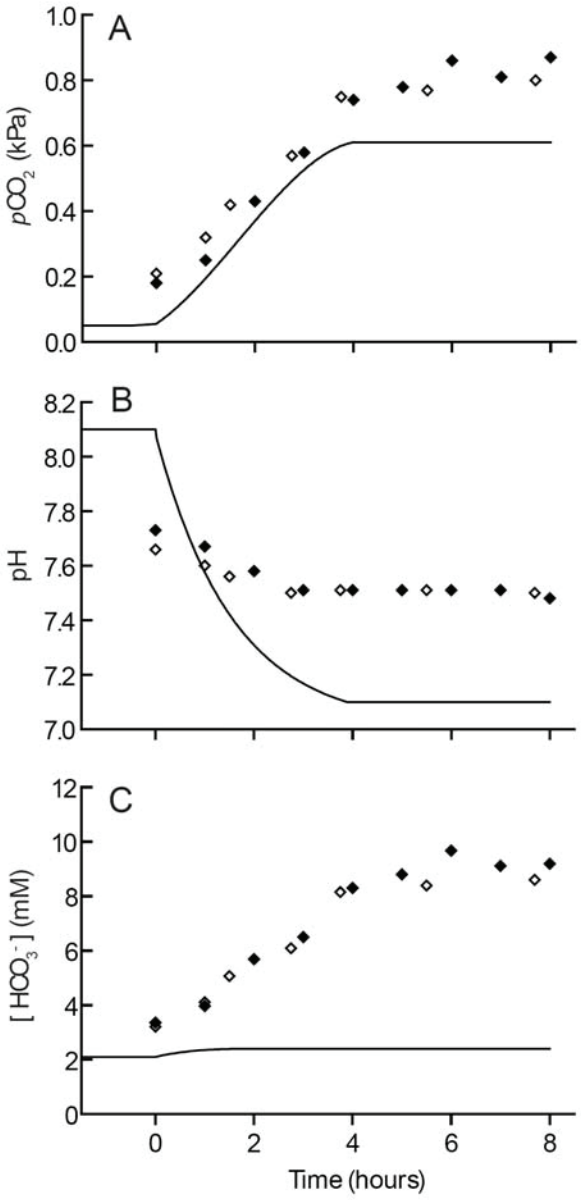

A
B
C
pCO_2 (kPa)
pH
[HCO_3^-] (mM)
Time (hours)
1.0
0.8
0.6
0.4
0.2
0.0
8.2
8.0
7.8
7.6
7.4
7.2
7.0
12
10
8
6
4
2
0
0
2
4
6
8

Figure 2

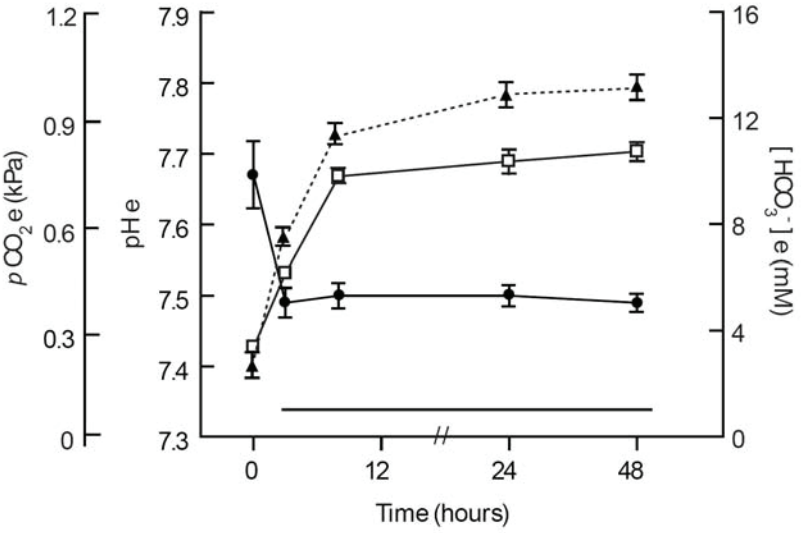


Figure 3

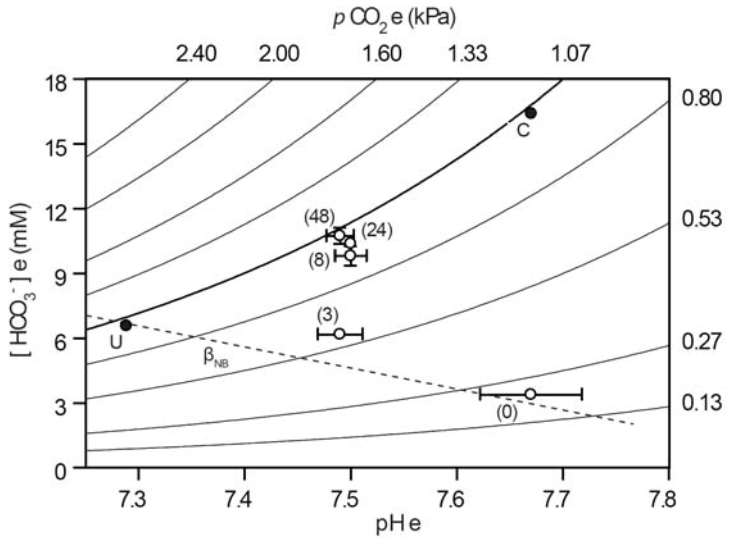


Figure 4

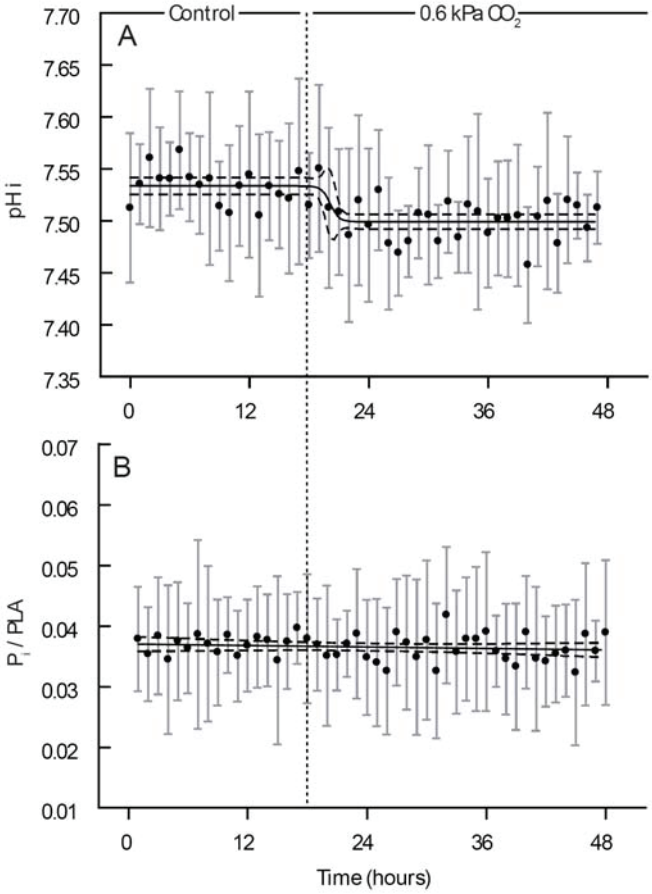

Control
0.6 kPa CO_2
A
B
pHi
P_i / PLA
7.70
7.65
7.60
7.55
7.50
7.45
7.40
7.35
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
12
24
36
48
Time (hours)

Figure 5

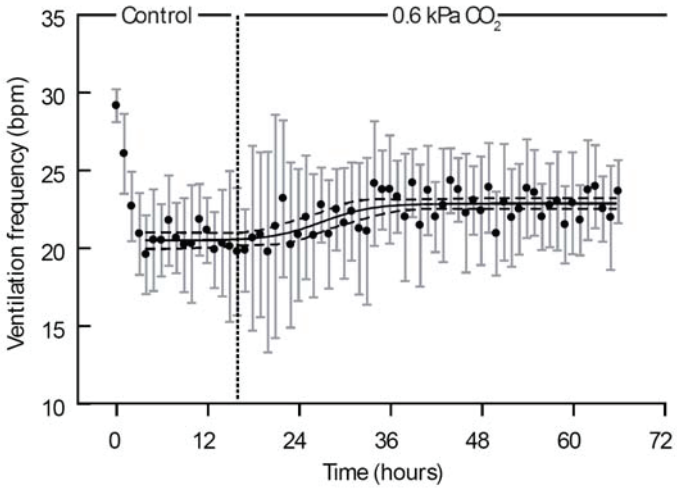
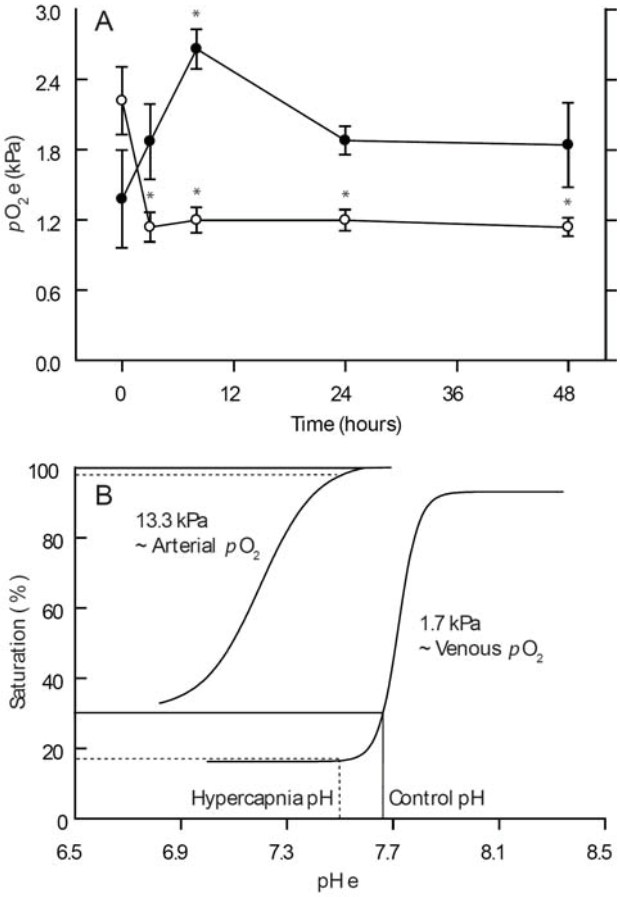

Control
0.6 kPa CO_2
Ventilation frequency (bpm)
35
30
25
20
15
10
0
12
24
36
48
60
72
Time (hours)

Figure 6


A
3.0
2.4
1.8
1.2
0.6
0.0
pO_2 e (kPa)
0
12
24
36
48
Time (hours)
B
100
80
60
40
20
0
Saturation (%)
13.3 kPa
~ Arterial pO_2
1.7 kPa
~ Venous pO_2
Hypercapnia pH
Control pH
6.5
6.9
7.3
7.7
8.1
8.5
pH e

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Contribution to the Theme Section 'Effects of ocean acidification on marine ecosystems'

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Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO_2

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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 (pCO_2). During a 6 wk period, juvenile *S. officinalis* maintained calcification under ~4000 and ~6000 ppm CO_2, 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*

INTRODUCTION

Anthropogenic carbon dioxide (CO_2) 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 CO_2 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 (CO_3^{2-}) 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 saturation 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 CO_2 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 pCO_2, and ceases when pCO_2 is above 1800 ppm (data replotted from their Table 1 in

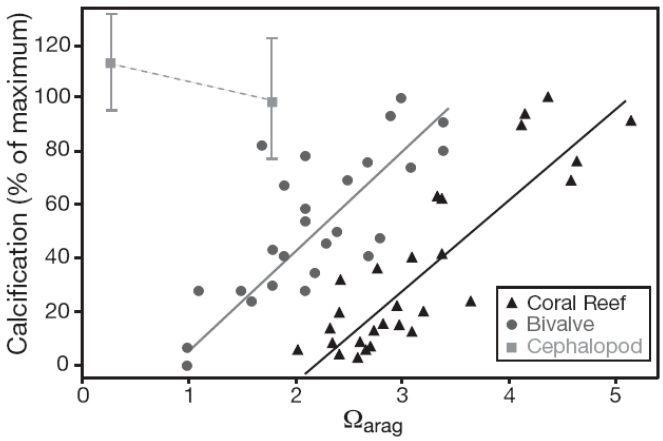

Fig. 1. The dependence of calcification on CO_2-dependent seawater calcium carbonate saturation (Ω_{arag}) in marine invertebrates. Long-term coral reef data set recorded in the Biosphere 2 mesocosm (Langdon et al. 2000, data replotted from their Table 4), acute changes in *Mytilus edulis* (bivalve) 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 ± 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 $\Omega_{arag} < 1$, decreasing calcification at $\Omega_{arag} > 1$ 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 m^3 total volume, protein skimmer, nitrification filter, UV disinfection unit (Sander), salinity 32 to 34, temperature (mean ± SD) 15 ± 0.1°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 pCO_2 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 ± SD

Incubation group	Temperature (C°)	Salinity	pH_{NBS}	C_T (µmol kg^{-1})	pCO_2 (ppm)	Ω_{arag}
Control	16.32 ± 0.12	32.8 ± 0.5	7.94 ± 0.06	2047 ± 68	705 ± 101	1.47
CO_2 ~4000 ppm	16.37 ± 0.12	32.9 ± 0.4	7.23 ± 0.04	2451 ± 54	4271 ± 373	0.34
Control	17.45 ± 0.16	31.4 ± 0.4	8.01 ± 0.04	2104 ± 56	628 ± 60	1.78
CO_2 ~6000 ppm	17.43 ± 0.15	32.3 ± 0.6	7.10 ± 0.03	2583 ± 43	6068 ± 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 ($CaCO_3$) 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 $CaCO_3$ (aragonite). We determined $CaCO_3$ content by back-calculating from the dry mass of the remaining organic matrix after dissolution of the cuttlebone $CaCO_3$ 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 ± SD; 10.4 ± 4.3 g, n = 6) were fasted for 24 h and then incubated in cylindrical perspex chambers (3 × 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 ± SD) 16 ± 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 ± SD.

RESULTS

No differences in soft-tissue growth performance were measured between cuttlefish incubated at ~4000 and ~6000 ppm CO_2 and controls (Table 2). Final average body mass for the cuttlefish incubated at ~4000 ppm CO_2 equaled 11.16 ± 1.40 g compared with 11.63 ± 1.39 g for the control group. In those incubated at ~6000 ppm CO_2 the corresponding mass was 23.06 ± 4.15 g compared with 24.15 ± 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 ± 6.2% and 39.5 ± 4.5%, and there were no significant differences (Table 2).

Standard metabolic rates of cuttlefish exposed acutely to ~6000 ppm CO_2 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 ± 0.004 µmol O_2 g^{-1} min^{-1}, and after 24 h of CO_2 exposure were 0.088 ± 0.003 µmol O_2 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 $CaCO_3$. At the end of the trial period, there

Table 2. *Sepia officinalis*. Growth and calcification during each of 2 separate trials under elevated CO_2 conditions. Values are mean ± 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 ± 0.30	20.53 ± 0.14	11.63 ± 1.39	37.16 ± 1.88	4.0	36.6 ± 6.2
CO_2 ~4000 ppm	2.70 ± 0.33	20.71 ± 0.17	11.16 ± 1.40	36.33 ± 2.29	3.8	38.9 ± 3.6
Control	4.61 ± 1.01	27.83 ± 2.47	24.15 ± 5.25	52.84 ± 4.03	3.9	39.5 ± 4.5
CO_2 ~6000 ppm	4.50 ± 1.08	27.90 ± 2.39	23.06 ± 4.15	52.01 ± 4.76	3.7	39.4 ± 3.7

were no significant differences between the mantle lengths of control cuttlefish and those incubated at ~6000 ppm CO_2 (52.01 ± 4.76 mm versus 52.84 ± 4.03 mm, respectively), nor between the control and ~4000 ppm CO_2 incubated cuttlefish (37.16 ± 1.88 mm 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 CO_2 growth trial, the CO_2 incubated animals incorporated significantly more $CaCO_3$ 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.

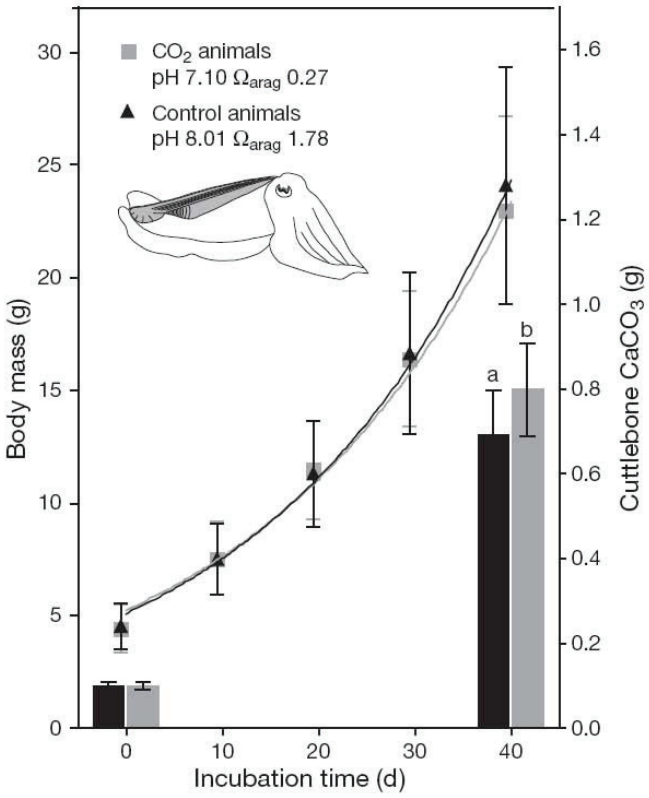

Fig. 2. *Sepia officinalis*. Growth (■▲, left *y*-axis) and calcification (bars, right *y*-axis) in the cuttlefish incubated under ~6000 ppm CO_2 (grey) and control conditions (black). For $CaCO_3$ accretion, means not sharing the same letter above bars are significantly different. Data are mean ± SD (n = 20). The calcified cuttlebone is shaded grey in the schematic drawing of *S. officinalis*

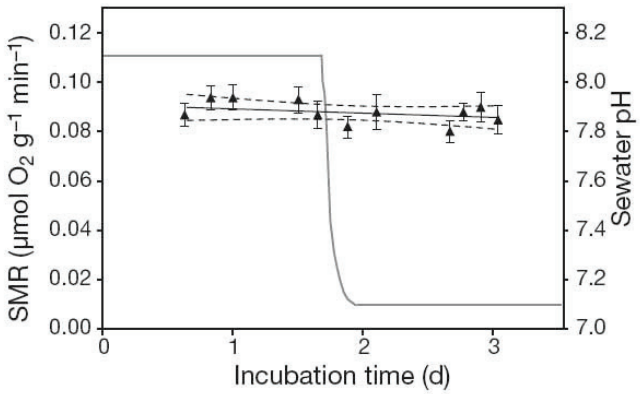

Fig. 3. *Sepia officinalis*. Standard metabolic rate (SMR) of cuttlefish during acute exposure to ~6000 ppm CO_2 (▲). Cuttlefish were placed in the chambers at Time = 0 and CO_2 exposure was started after 40 h of control measurements; the change in seawater pH (grey curve) reflects the time course of CO_2 exposure. Data are mean ± SD, n = 6

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 CO_2 concentrations.

Growth

Sepia officinalis juveniles cultured at ~4000 and ~6000 ppm CO_2 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 CO_2 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 CO_2. Michaelidis et al. (2005) found that under comparable CO_2 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 CO_2 for half a year. Clearly, *S. officinalis* does not exhibit sensitivity to elevated CO_2 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 CO_2 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 pCO_2 and HCO_3^-, 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 CO_2 exposure, which matches the conserved growth rates observed in our study. Standard metabolic rates of around 0.09 µmol O_2 g^{-1} min^{-1} were maintained at a constant level during acute exposure to ~6000 ppm CO_2 (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 CO_2 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 CO_2 (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 CO_2 concentrations up to 17 000 ppm CO_2. Fish are capable of maintaining growth rates under elevated CO_2 conditions because of their high ion transport and acid–base regulatory abilities. During acute hypercapnic exposure they rapidly increase HCO_3^- levels in their blood, and are able to fully compensate their extracellular pH (Toews et al. 1983, Claiborne & 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 HCO_3^- 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 CO_2 conditions, but it also maintains high calcification rates of its cuttlebone. *S. officinalis* is capable of calcifying under ~6000 ppm CO_2 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 CO_2 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 CO_2 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 CO_2 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 (Ca^{2+}) 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 $\Omega_{arag} < 1$ 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 CO_2 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 CO_2 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, CO_2 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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Increased cuttlebone calcification 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 increases in the cephalopod *Sepia officinalis* during long-term exposure to elevated seawater pCO_2. The present study investigated structural changes in the cuttlebones of *S. officinalis* calcified during six-weeks of exposure to 615 Pa CO_2. Cuttlebones of CO_2 incubated animals accreted 22-55% more $CaCO_3$, depending on animal size (mantle lengths 44-56 mm). No major morphological differences were distinguishable between the cuttlebones from the two treatments. However, the average height of the CO_2 exposed cuttlebones was reduced. A decrease in spacing of the cuttlebone lamellae accounted for the height reduction, 195 ± 38 µm versus 384 ± 26 µm in the control group. An increase in thickness of the lamellar and pillar walls contributed to the greater dry mass of the CO_2 incubated cuttlebones. Particularly, pillar thickness increased from 2.6 ± 0.6 µm to 4.9 ± 2.2 µm. The apparent robustness of calcification processes in *S. officinalis* to elevated seawater pCO_2 is discussed to result from the high ion- and acid-base regulatory abilities of this active invertebrate. Also, the fully internalized cuttlebone is disconnected from changes in seawater chemistry as it is enclosed by an epithelium that contains highly specialized transport cells that control the extracellular environment. We discuss the potential negative impact of increased calcification in the cuttlebone of *S. officinalis* during exposure to elevated CO_2 on its function as a lightweight and highly porous buoyancy regulation device.

1. Introduction

Ocean acidification related changes in seawater carbonate chemistry have been hypothesized to reduce calcification rates in marine molluscs (Orr et al. 2005, Fabry et al. 2008). Shell length growth has been shown to significantly decrease in *Mytilus edulis* (Berge et al. 2006), *Mytilus galloprovincialis* (Michaelidis et al. 2005) and *Strombus luhuanus* (Shirayama and Thornton 2005) during long term exposure to elevated seawater pCO_2. In contrast, we have shown that metabolism and growth rates remain at control levels during long-term exposure to 615 Pa CO_2 in a cephalopod mollusc, the cuttlefish *Sepia officinalis*, and calcification actually increased (Gutowska et al., 2008). In this study we present data on ultrastructural changes that occurred in the cuttlebones of *S. officinalis* during six-weeks of exposure to 615 Pa CO_2. We discuss the observed morphological findings with regard to potential limitations on buoyancy control.

Cuttlefish (family Sepiidae), along with *Nautilus spp.* (Nautilidae) and *Spirula* (Spirulidae), are the only extant cephalopods that utilize a chambered shell for skeletal support and as a buoyancy regulation device (Denton 1974). In the cuttlefish *S. officinalis*, the cuttlebone is dorsally located along the anterior-posterior axis (Fig. 1) and accounts for about 10% of the animal's volume (Denton 1961a). The cuttlebone is surrounded 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 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 1961d). Ion transporters in the cuttlebone epithelium reduce the ionic concentration of the fluid in the cuttlebone chambers by selectively exporting Na^+ and Cl^- ions. When enough osmotic pressure is created, liquid begins to leave the cuttlebone chambers, making use of an extracellular system that connects to veins below the basement connective tissue via ampullae and ducts (Denton 1961a,d). In *Spirula spirula*, the osmolarity of shell fluid is reduced to one-fifth of that of seawater when a chamber is emptied (Denton 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 1961a, 1961b). During day time, when *S. officinalis*

rests on the ground buried in sand, the posterior chambers of the cuttlebone are filled with fluid, making the cuttlefish negatively buoyant. However, at the onset of night these chambers are emptied, thus decreasing the density of the cuttlefish. The animal 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 ionic environment surrounding its calcified structure.

The combined function of the cuttlebone, both for support, and as a lightweight buoyancy device, requires an open structure that is uniquely pressure resistant while maintaining a constant volume. The porosity of the cuttlebone is high at 93% (Birchall and Thomas 1983) and yet it has been tested to withstand pressures of 20 atm (Denton 1961c). This corresponds to approximately 200 m depth, and suffices to cover the 150 m depth distribution of *S. officinalis*. 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. 2). Growth of the cuttlebone proceeds through 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 3 to 5 % organic matrix, whereas the dorsal shield contains 30 to 40 % (Birchall and Thomas, 1983). In the phragmocone, the 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 a protein-chitin complex made up of β-type chitin (Birchall and Thomas 1983). While many proteins of the soluble 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 been identified so far in cuttlebones (Dauphin 1996).

We have previously shown that the mantle length (which is representative of cuttlebone length) of animals grown under 615 Pa CO_2 for six weeks did not significantly differ from those of control animals, but that the total $CaCO_3$ content of the cuttlebones was greater in the group exposed to elevated pCO_2 (Gutowska et al. 2008). In this study, we first focus on characterizing the gross morphometric characteristics of the cuttlebones. Secondly, using scanning electron microscopy, we examine ultrastructural 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.

2. *Material* and Methods

2.1 Animals

European cuttlefish (*Sepia officinalis*) egg clusters were collected in the Bay of Seine (Normandy, France) in May 2006. The cuttlefish were hatched and raised at the Alfred Wegener Institute (AWI, Bremerhaven, Germany) in a closed recirculating system (20m^3 total volume, protein skimmers, nitrification filters, UV-disinfection units) at S=32-34 ppt, T=15 ± 0.1°C, pH=7.9-8.2, constant dark:light cycle (12h: 12h). 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 animals were initially fed a daily diet consisting of live mysids (*Neomysis integer*) and progressively transitioned to frozen brown shrimp (*Crangon crangon*). Forty animals (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 and elevated pCO_2 conditions for a period of six weeks. Upon termination of the experiment, the animals were sacrificed and their cuttlebones removed for further analyses. An additional control group of 50 animals, ranging in size from 5 to 36 g, was sampled from the maintenance aquaria, to compare the relationships between animal wet mass and cuttlebone morphometrics over a broader range of animal sizes.

2.2 Growth trial under elevated pCO_2 conditions.

Each group of n = 20 *S. officinalis* was maintained in shallow pvc basins (20x40x60 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. 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 & Wallace 1998) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

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

2.3 Cuttlebone morphometrics and organic matrix content

Cuttlebones were excised from anesthetized *S. officinalis* individuals at the completion of the growth trial. Extra care was taken to remove the cuttlebones in their entirety, and not to break off the posterior sections of the shell margin. All further measurements were performed on cuttlebones that had been dried for 24 hours 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 hours, the calcified component had entirely dissolved, and the remaining organic matrix was carefully removed, rinsed with distilled water, dried over night in a 40°C oven, and weighed on a precision balance (ME235S, Sartorius).

2.4 Cuttlebone ultrastructure examined with light and scanning electron microscopy

The ultrastructure 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 posterior-anterior 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 pedestals stubs with double sided adhesive carbon discs. The sections were sputter-coated with a gold-palladium alloy and investigated using a CamScan-CS-44 SEM.

Ultrastructural changes were examined in the approximately 2 cm long cuttlebone sections that were mineralized during the six week growth trial period using the freeware program Image J. The 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 $CaCO_3$ deposits, spherical structures, was measured in four random 1 mm^2 sections in each cuttlebone on the exposed surface of the 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.

3. 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 the 95% confidence intervals.

4. Results

In *S. officinalis* raised under control conditions at 17° C, animal wet mass (g) related to cuttlebone dry mass (g) following the equation $y = 0.034x – 0.055$ ($R^2 = 0.99$), over an animal size range of 5-35 g (Fig. 3A). Cuttlebone length (mm) and cuttlebone dry mass (g) followed the equation $y = 0.209 - 0.02x + (6.03x10^{-4})x^2$ ($R^2 = 0.99$), over the same animal size range (Fig.3B). Cuttlebone dry mass made up 3% of animal wet mass.

Changes in cuttlebone morphology were compared in *S. officinalis* incubated under both control and elevated CO_2 conditions, 615 Pa CO_2, for six 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 animals raised under elevated CO_2 conditions weighed 0.2 g more on average than control cuttlebones of the same length (Fig. 4A). 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. 4B, 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, the slope of the regression did not significantly differ from zero $F_{(1,18)}= 4.28$, $p = 0.05$. (Table 2, Fig. 4C).

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 ultrastructural changes described below. Ten lamellae were accreted on average by *S. officinalis* incubated under 615 Pa CO_2 over a period of six-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 six-week experimental period.

Changes in both lamellar and pillar spacing, as well as thickness (Fig. 5) were evident in SEM micrographs between the two experimental groups. However, the general ultrastructure 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). Changes in pillar spacing were visible, but were not quantified due to their more irregular structure.

A higher occurrence of irregular $CaCO_3$ deposition, specific to the CO_2 treatment group, was identified in the form of spherical structures (Fig. 6). The spherical structures were covered with a sheet of organic matrix and primarily attached to the ventral side of the lamellae (Supplementary Fig. 1). 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 spherical 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 animals incubated under elevated pCO_2 contained significantly less NASOM than control cuttlebones, $p<0.005$ (Fig. 7A). 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 $CaCO_3$ (Fig. 7B). 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).

5. Discussion

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

5.1 Changes in cuttlebone ultrastructure induced by exposure to elevated seawater pCO_2

S. officinalis individuals grew to a range of lengths spanning 40-55 mm, nearly doubling their cuttlebone length, during a six-week growth trial. 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 growth trial animals due to the greater inaccuracy of the measurement in representing cuttlebone length. Interestingly, despite their slightly shorter length, the hypercapnic cuttlebones accreted 0.2 g more dry mass on average, which corresponds to a 20-55% relative increase in cuttlebone mass (depending on animal size, Fig. 4A). Despite the different mass to length relationship, no major morphological differences were clearly visible. The relationship between cuttlebone length and width was preserved (Fig. 4B). However, the height of the hypercapnic incubated cuttlebones in relation to length was reduced (Fig. 4C).

To further understand the structural changes underlying the morphometric shifts in *S. officinalis* cuttlebones during long-term hypercapnic exposure, the ultrastructure of a subset of cuttlebones that were no more than 15% different in final attained length, 46 to 52 mm, was examined. Cuttlefish from both groups accreted 10 lamellae during the six-week experimental period, which gives a lamellar accretion rate of 4.2 days. A lamellar accretion rate of ca. four 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. Tightly spaced sections of lamellae are found in cuttlebones of adult wild animals that correspond to slow growth periods during exposure to cold winter conditions (Goff et al. 1998, Hall et al 2007). Severely reduced 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 is visible in both groups, however the CO_2 treatment cuttlebones have a significantly reduced lamellar spacing despite maintained growth rates (Table 2). Both the length and height of cuttlebones are affected by changes in lamellar spacing. 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 ultrastructure between the two groups clearly indicates the formation of a less porous structure by the cuttlefish exposed to elevated seawater pCO_2 (Fig. 5). Not only did the lamellar spacing decrease, but the thickness of the lamellar and pillar walls increased significantly (Table 2). Particularly the pillar walls doubled their average thickness from 2.6±0.5µm to 4.9±2.1µm. This increase in deposited $CaCO_3$ substantially contributed to the greater mass of the CO_2 treatment cuttlebones. The general ultrastructure of the cuttlebones was conserved; however a greater degree of irregularity was present in the linearity and thickness of the lamellar walls and pillars. This is evident from 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 of exposure 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). In *S. officinalis* cuttlebones exposed to six-weeks of 615 Pa CO_2, the mass of non-acid soluble organic matrix (NASOM) was reduced by 30% on average (Fig.7A). Interestingly, the freely suspended non-calcified organic matrix sheets in between adjoining lamellae were present in both experimental groups (Figs. 5 and 6). This is in contrast to observations of cuttlebones calcified by *S. officinalis* on very low feeding rations, where the inter-lamellar organic sheets are entirely missing in young animals (Boletzky and Wiedmann, 1978), and significantly reduced in older animals (Wiedmann and Boletzky, 1982). As the cellular pathways of organic matrix synthesis and secretion by the cuttlebone epithelium are unknown, interpretation of the reduction in NASOM is difficult. To summarize the general pattern however, we see a distinct increase in $CaCO_3$ deposition accompanied by a reduction the non-acid soluble organic matrix, which in *S. officinalis* is a protein-chitin complex made up of β-type chitin.

The increase in $CaCO_3$ content in the cuttlebone sections calcified during long-term exposure to elevated pCO_2 should be viewed as potentially pathological. The density of the cuttlebone structure must remain low if it is to preserve its function as a buoyancy regulation device. In wild caught adult animals, a change of only ±16.5% in cuttlebone mass, from the state of neutral buoyancy (cuttlebone density 0.6), 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. Unfortunately as far as we are aware, there is no supporting literature that discusses changes in the ultrastructure of cephalopod chambered shells and the potential impacts on animal energetics and physiology.

We were not able to resolve the behavioral and energetic changes necessary to access potential changes in functional control of the cuttlebones. We hypothesize that the significant increases in cuttlebone mass, 22-55% depending on cuttlefish size, in *S. officinalis* incubated under 615 Pa CO_2, will affect function of the cuttlebone as buoyancy regulation device. Considering the extent of changes, long-term incubation under environmentally relevant seawater pCO_2 (<100-200 Pa) could also potentially affect the density of the cuttlebone in *S. officinalis* and the partitioning of their energy budget. It is important to note that in our growth trial the majority of the region of the cuttlebone responsible for buoyancy control was mineralized by the experimental animals under control conditions before the onset of CO_2 exposure. Future growth studies, where animals are raised under elevated pCO_2 conditions from

the beginning of cuttlebone formation are necessary to fully assess how ultrastructural changes may influence functional control. Quantification of animal 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 elevated CO_2 conditions.

5.2 Sensitivity of calcification process in *S. officinalis* to elevated seawater pCO_2

It has been hypothesized that organisms with high metabolic rates and ion-regulatory abilities are less sensitive to elevated seawater CO_2 levels (hypercapnic exposure) as they possess highly efficient mechanisms to maintain physiological homeostasis (Seibel and Walsh 2003, Pörtner et al. 2004, Pörtner 2008, Melzner et al. 2009). During hypercapnic exposure, one of the most important compensatory actions is the regulation of acid-base equilibria, as unbuffered increases in pCO_2 elicit acidosis. The sensitivity of calcification processes to elevated CO_2 conditions can also be included in the same conceptual framework. 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 biomineralization during exposure to elevated CO_2.

The question that arises is, if *S. officinalis* is capable of maintaining soft tissue growth rates and metabolism at control levels during hypercapnia (Gutowska et al. 2008), why does calcification increase? An important point could be that active organisms, like the cuttlefish, significantly increase HCO_3^- levels and partially compensate pH in their extracellular fluids during exposure to high seawater pCO_2's (Gutowska et al. 2009b, Table 2). In *S. officinalis* exposed to 615 Pa CO_2, blood [HCO_3^-] is elevated to 10.4 mM (Gutowska et al. 2009b). This could change ion transport kinetics across the cuttlebone epithelium, and the $CaCO_3$ saturation state of the extracellular fluid surrounding the cuttlebone could increase. Measurement of the extracellular acid-base parameters in the fluid around the cuttlebone during long-term exposure to elevated seawater pCO_2 would help answer some of these questions. It would be interesting to consider the calcification response of other invertebrates with high acid-base regulatory abilities to long-term elevated CO_2 exposure, such as decapod crustaceans (Truchot, 1984; Cameron and Iwama, 1987; Spicer et al., 2006; Pane and Barry, 2007), however, to date no such studies are available.

An interesting example of increased calcification during long-term exposure to high CO_2 levels in vertebrates can be found in aquaculture studies on teleosts. The frequency of pathological calcareous precipitates observed in the kidneys, i.e. nephrocalcinosis, increases during long-term exposure to elevated CO_2 concentrations (*Anarhichas minor*, Foss et al., 2003; *Salmo salar*, Fivelstad et al., 1999; Fivelstad et al., 2003; Hosfeld et al., 2008; *Dicentrarchus*

labrax, Vandeputte et al., 2009). Like the cuttlefish, teleosts elevate their blood HCO_3^- levels during exposure to high seawater pCO_2 conditions. The compensatory response is significantly stronger than that of *S. officinalis*, during exposure to hypercapnia levels >1000 Pa blood [HCO_3^-] in teleosts increase to values > 20mM (Toews et al., 1983; Larsen et al 1997, Hayashi et al., 2004; Michaelidis et al., 2007, Melzner et al. 2009). Perhaps there is a common physiological basis behind the deposition of greater amounts of $CaCO_3$ during exposure to elevated CO_2 conditions in organisms that significantly elevate their extracellular HCO_3^- levels for pHe compensation. In *S. officinalis* both the $CaCO_3$ content of the regular cuttlebone structure, as well the number of irregular deposits within the cuttlebone, increased during exposure to 615 Pa CO_2 (Table 2, Figs. 5 and 6). Deposition of pathological calcareous deposits as a result of disturbance in acid-base and electrolyte status has also been described in *Homarus americanus* (Dove et al. 2004, Dove 2005).

Finally, it is interesting to consider the embryonic development of *S. officinalis* in the context of calcification sensitivity to elevated pCO_2 conditions. Unlike most molluscs, the early life stage development of the cuttlefish takes place entirely inside individual eggs. It has been recently shown, that the perivitelline fluid inside the egg is strongly acidified over the course of development through the accumulation of metabolic CO_2 (300 Pa) (Gutowska and Melzner 2009a). It is fascinating that embryonic cuttlefish mineralize the first approximately eight lamellae of their cuttlebone, as well as their statoliths, inside the egg (Lemaire 1970, Fioroni 1990, Ré and Narciso 1994) under progressively more hypercapnic conditions. In contrast, calcification has been shown to be particularly sensitive to hypercapnia in the early developmental stages of bivalve molluscs (Kurihara 2008). Larval shells of *M. galloprovincialis* and *C. gigas* mineralized during exposure to approx. 200 Pa CO_2 exhibited morphological abnormalities such as convex hinges, protrusions of the mantle and malformations. However, embroyogenesis was unaffected until the trochophore stage when shell formation began. As development progressed, CO_2 exposed veliger larvae remained 20% smaller compared to the control group (Kurihara et al. 2007, Kurihara et al. 2009).

The data available to date is not sufficient to draw a causal relationship between long-term exposure to elevated seawater pCO_2 and reduced calcification rates in adult molluscs, as reductions in calcification have only been reported as shell length, coupled to reductions in somatic growth (Michaelidis et al. 2005, Shirayama and Thornton 2005, Berge et al. 2006). It remains open to investigation, whether decreased shell length 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, which then requires lower rates of shell growth (Pörtner et al., 2004; Pörtner, 2008, Melzner et al. 2009). In the cephalopod mollusc

S. officinalis, we have shown that metabolism and growth rates remain at control levels during exposure to elevated seawater pCO_2 while mineralization of $CaCO_3$ significantly increases. Ultimately, studies that bring us closer to understanding the mechanistic processes underlying calcification are necessary to increase our ability of predicting molluscan sensitivity to ocean acidification.

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Figure Legends

Figure 1. A) Illustration of a *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 a neutrally buoyant *S. officinalis* (modified after Denton 1974). The cuttlebone is illustrated in a sagittal section, liquid filled sections are shaded. The represented cuttlebone has a density of about 0.6, and provides a net lift of approximately 4% of the animal's weight in air. The oldest and most posterior lamellae are almost full of liquid. In very light cuttlebones (density 0.5) even the oldest chambers are pumped dry, whereas in dense cuttlebones (0.7) almost all of the lamellae contain liquid. S- Siphuncular surface, L- most recently constructed lamella when it is still filled with liquid.

Figure 2. Cuttlebone scanning electron micrographs of a transverse section along the midline. **A)** Large-scale view of eleven lamellae in the phragmocone and the dorsal shield **B)** Closer view of two lamellae and supporting pillars. Note the thin sheets of organic matrix freely suspended perpendicular to the pillars in between the lamellae. **C)** Detail of pillar rising off of a lamellar floor. Note the thin sheets of organic matrix that coat the $CaCO_3$ surfaces.

Figure 3. Morphometric relationships between animal mass, cuttlebone mass and length in *S. officinalis* raised under control conditions at 17° C. **A)** Animal wet mass(g) relates to cuttlebone dry mass (g) following the equation $y = 0.034x - 0.055$ ($R^2 = 0.99$), over an animal size range of 5-35 g. **B)** Cuttlebone length (mm) and cuttlebone dry mass (g) follow the equation $y = 0.209 - 0.02x + (6.03x10^{-4})x^2$ ($R^2 = 0.99$).

Figure 4. Comparison of morphometric relationships between cuttlebone length, mass, height and width in the control (black) and CO_2 incubated (grey) groups. **A)** The average mass of the CO_2 incubated cuttlebones was significantly greater than that of the control group, 0.80±0.15 versus 0.70±0.16 g, $p<0.03$. **B)** The average width of the CO_2 incubated cuttlebones was significantly narrower than in the control group, 16.2±0.9 versus 17.1±0.9 mm, $p<0.005$.
C) The average height of the CO_2 incubated cuttlebones was significantly reduced compared to the control group, 4.6±0.4 versus 5.4±0.5 mm, $p<0.0001$ (n =20).

Figure 5. SE micrographs comparing ultrastructural changes in cuttlebones mineralized under control **(A)** and 615 Pa CO_2 **(B)** seawater conditions. 1- Pillar thickness, 2- Lamellar thickness, 3- Lamellar spacing. Note the reduced lamellar and pillar spacing in the cuttlebone section mineralized during CO_2 exposure.

Figure 6. SE micrographs illustrating the differences in irregular spherical $CaCO_3$ deposition between control **(A)** and CO_2 exposed **(B)** cuttlebones. Control cuttlebones contained 0.3 ± 0.3 irregular spheres per 1 mm^2 on average, along the midline transverse fracture. The spheres ranged in height from 20 to 65 μm. Cuttlebone sections mineralized during exposure to 615 Pa CO_2 contained significantly more irregular spheres, 1.6 ± 0.5 per 1 mm^2, $p<0.005$ (n = 3). The spheres ranged in height from 12 to 127 μm.

Figure 7. A) Significantly less non-acid soluble organic matrix (NASOM) was incorporated into the cuttlebones mineralized during exposure to 615 Pa CO_2 (grey), $p<0.005$ (n = 14). **B)** Cuttlebones mineralized during exposure to 615 Pa CO_2 (grey) contained significantly more $CaCO_3$ than the control group, (n = 14).

Supplementary Figure 1. SE micrographs **(A-B)** illustrating the detailed ultrastructure of irregular spherical $CaCO_3$ deposits that were primarily associated with the ventral surfaces of the lamellae. **C)** The surfaces of the irregular spheres were partially coated with organic matrix and hexagonal $CaCO_3$ crystals that had an approximately 1μm diameter.

Table 1. Seawater physicochemical parameters during six-week long growth trial. Mean value during six-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

Table 2. Comparison of ultrastructural changes and irregular spherical $CaCO_3$ deposition in *S. officinalis* cuttlebones exposed to elevated seawater pCO_2.

Experimental Group	Lamellar Spacing (µm)	Lamellar Thickness (µm)	Pillar Thickness (µm)	Sphere # $1mm^{2\ -1}$	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

Figure 1

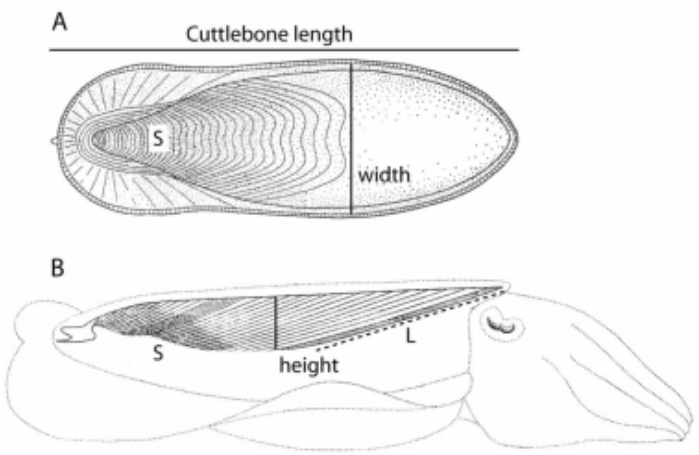


Figure 2

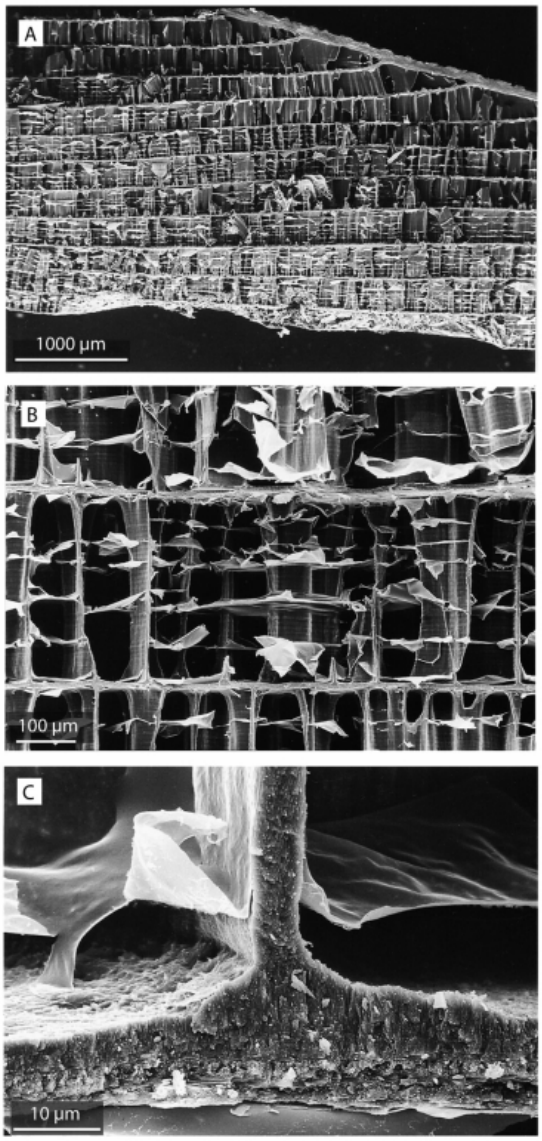


Figure 3

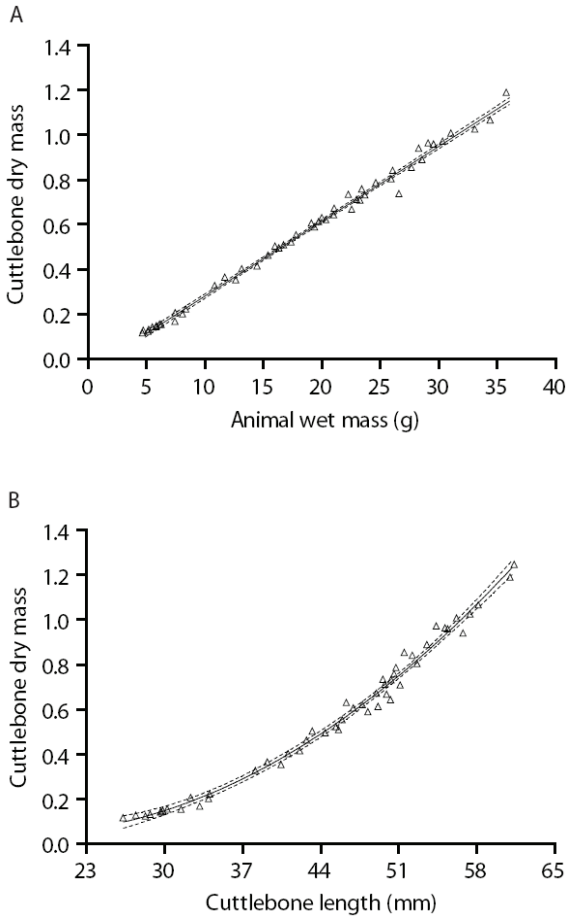

A
Cuttlebone dry mass
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
Animal wet mass (g)
B
Cuttlebone dry mass
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
23
30
37
44
51
58
65
Cuttlebone length (mm)

Figure 4

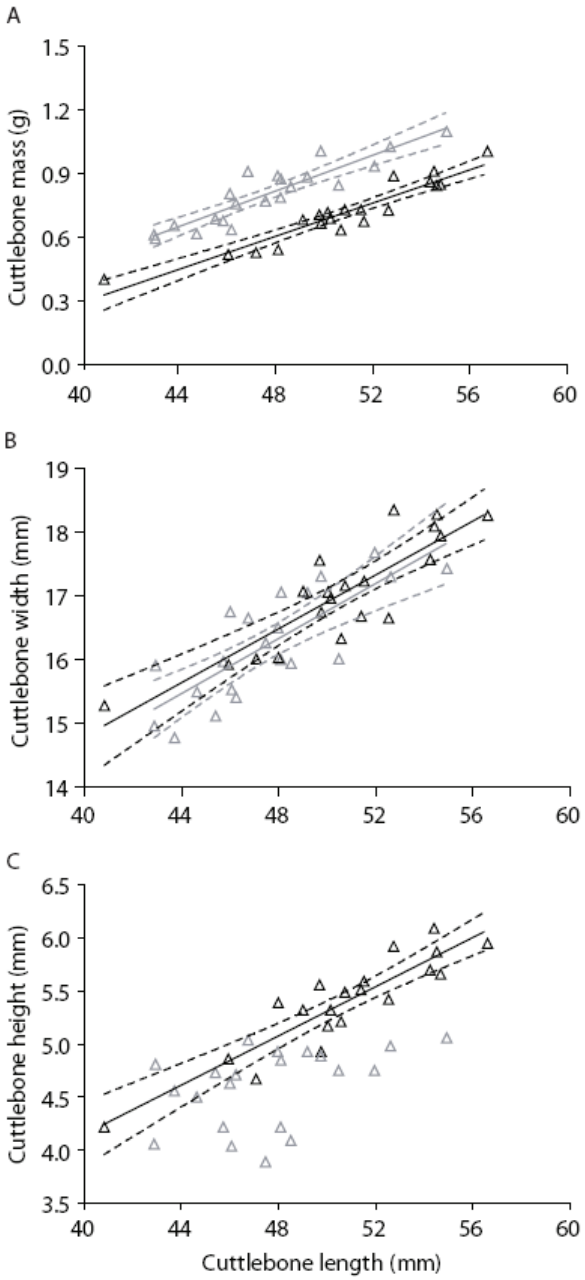

A
Cuttlebone mass (g)
1.5
1.2
0.9
0.6
0.3
0.0
40
44
48
52
56
60
B
Cuttlebone width (mm)
19
18
17
16
15
14
40
44
48
52
56
60
C
Cuttlebone height (mm)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
40
44
48
52
56
60
Cuttlebone length (mm)

Figure 5

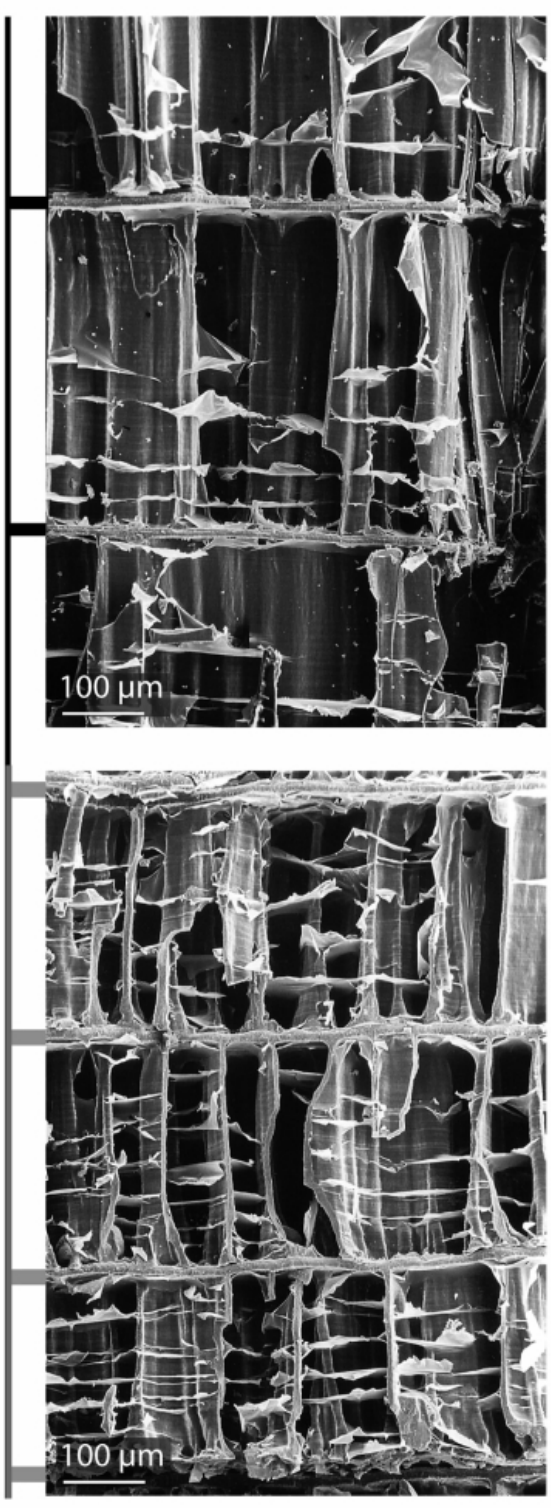


Figure 6

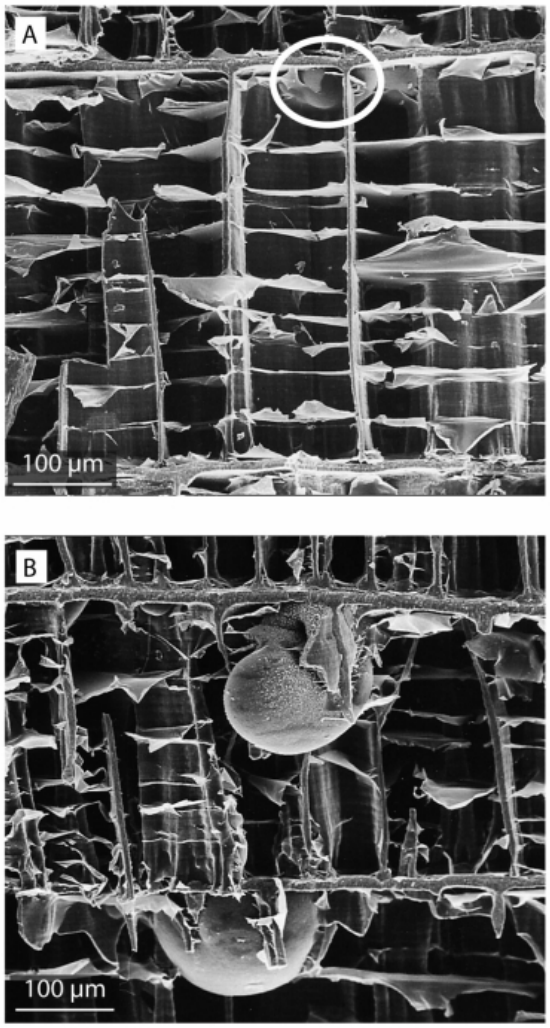
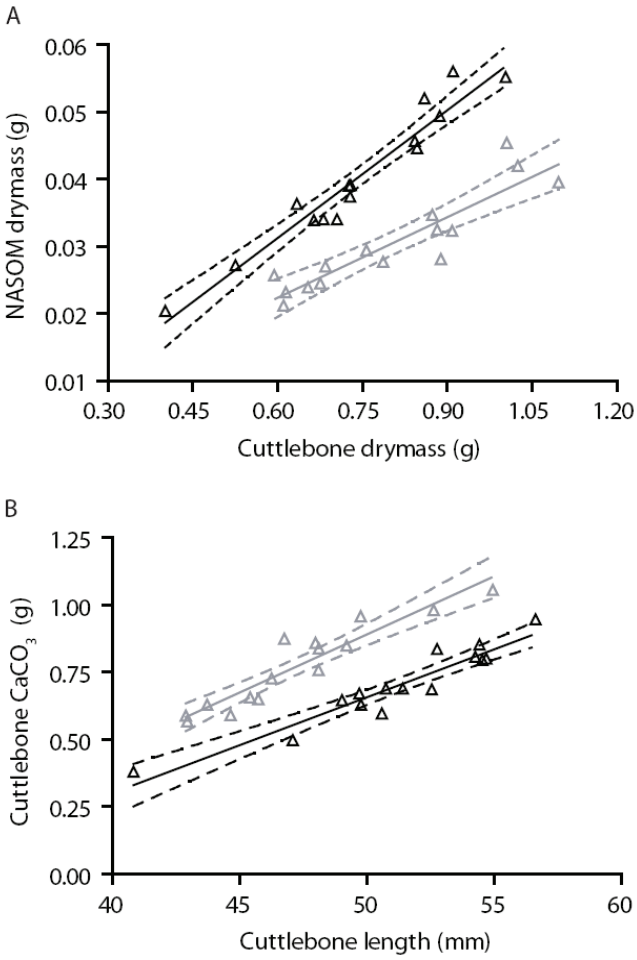
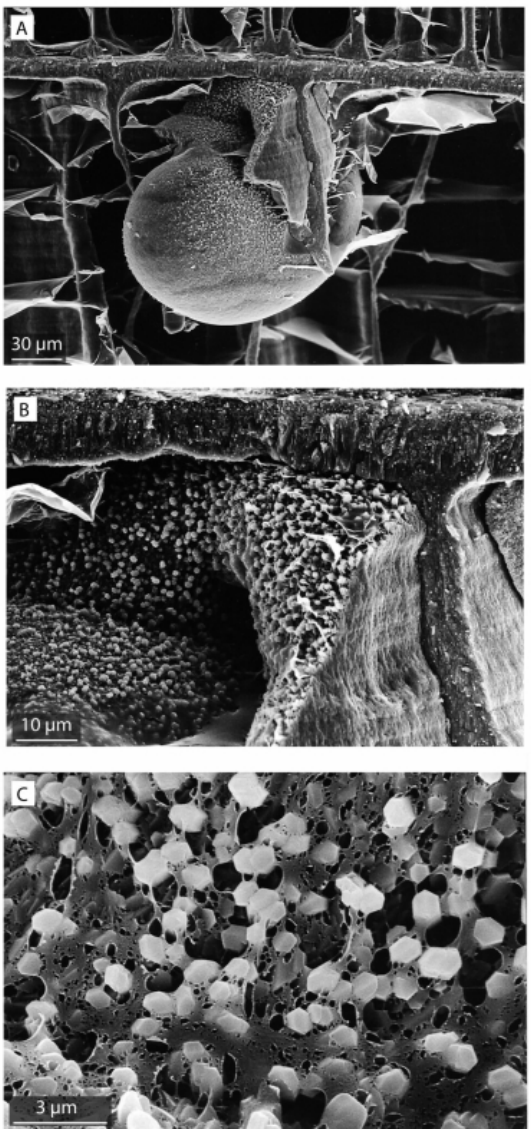


Figure 7


A
NASOM drymass (g)
0.06
0.05
0.04
0.03
0.02
0.01
0.30
0.45
0.60
0.75
0.90
1.05
1.20
Cuttlebone drymass (g)
B
Cuttlebone CaCO3 (g)
1.25
1.00
0.75
0.50
0.25
0.00
40
45
50
55
60
Cuttlebone length (mm)

Supplementary Figure 1



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SHORT COMMUNICATION

Abiotic conditions in cephalopod (*Sepia officinalis*) eggs: embryonic development at low pH and high pCO_2

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Abstract Low pO_2 values have been measured in the perivitelline fluids (PVF) of marine animal eggs on several occasions, especially towards the end of development, when embryonic oxygen consumption is at its peak and the egg case acts as a massive barrier to diffusion. Several authors have therefore suggested that oxygen availability is the key factor leading to hatching. However, there have been no measurements of PVF pCO_2 so far. This is surprising, as elevated pCO_2 could also constitute a major abiotic stressor for the developing embryo. As a first attempt to fill this gap in knowledge, we measured pO_2, pCO_2 and pH in the PVF of late cephalopod (*Sepia officinalis*) eggs. We found linear relationships between embryo wet mass and pO_2, pCO_2 and pH. pO_2 declined from >12 kPa to less than 5 kPa, while pCO_2 increased from 0.13 to 0.41 kPa. In the absence of active accumulation of bicarbonate in the PVF, pH decreased from 7.7 to 7.2. Our study supports the idea that oxygen becomes limiting in cephalopod eggs towards the end of development; however, pCO_2 and pH shift to levels that have caused significant physiological disturbances in other marine ectothermic animals. Future research needs to address the physiological adaptations that enable the embryo to cope with the adverse abiotic conditions in their egg environment.

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Introduction

Designed to protect embryonic stages from predation, egg capsules also can provide severe physiological challenges to their inhabitants, as the egg wall represents a barrier to diffusion of gases. Previous work has demonstrated that oxygen diffusion coefficients (K_{O_2}) of marine animal egg capsules are typically 10–20% that of pure water (e.g., Brante 2006). In molluscs (as in all other developing embryos), oxygen consumption rates rise dramatically during development (e.g., Cronin and Seymour 2000; Brante 2006). Thus, in order to enable rising oxygen fluxes by means of diffusion, many molluscan eggs swell during development, leading to enhanced surface areas and reduced egg wall thicknesses (e.g., Kress 1972; Cronin and Seymour 2000), and consequently increased oxygen conductances (Seymour 1994). In addition, embryos inhabiting fluid filled capsules often produce convective currents that prevent the formation of pO_2 gradients within the egg fluid (amphibians: Burggren 1985, fish: Rombough 1988, molluscs: Cronin and Seymour 2000). While thinning of egg capsule walls has been shown to enable consistently high pO_2 values in eggs of the marine gastropod *Fusitriton oregonensis* (ca. 12 kPa; Brante 2006), the combination of egg swelling and convection does not prevent pO_2 from consistently falling during embryonic development of the cephalopod *Sepia apama*, from initial values of 14 kPa down to 5–6 kPa close to hatching (Cronin and Seymour

2000). It has been hypothesized that this might eventually trigger hatching once critical pO_2 values (pO_{2crit}) are reached within the egg (pO_{2crit} = 5–8 kPa for *S. apama* and *S. officinalis* late embryonic stages (DeWachter et al. 1988; Cronin and Seymour 2000). While low pO_2 values in eggs also have been proposed to stimulate hatching in vertebrates (reptiles and birds: Vleck and Hoyt 1991, amphibians: Seymour and Bradford 1995), little attention has been devoted to the flip side of the coin: egg pCO_2 and pH, especially in marine animals. In order to excrete metabolically produced CO_2 at a rate that almost equals that of oxygen consumed (of course, depending on the respective respiratory quotient), a pCO_2 gradient has to be maintained in the opposite direction, with high values inside the egg. To our best knowledge, there are currently no published egg fluid pCO_2 values available in the literature for any marine animal. This is quite surprising, as high pCO_2 values most likely go along with low pH values and potentially constitute another stressor that may significantly affect embryonic physiology. Using late embryos of the cephalopod *Sepia officinalis* as a model system, we aimed at characterizing in more detail the abiotic conditions within the perivitelline fluid (PVF), with an emphasis on the pCO_2/pH gradient between the egg and the environment.

Materials and methods

Laboratory laid *Sepia officinalis* eggs were obtained from the Biological Station in Luc-sur-Mer, Université de Caen (Normandy, France) and transported to Germany at the age of 7–8 days. They were then incubated in a recirculating aquarium system (200 l volume) at the AWI Bremerhaven at 17°C (±0.2°C) for approximately four weeks. A nitrification filter (Eheim Professional 2, Eheim, Deizisau, Germany), a protein skimmer (AquaCare 2000, AquaCare, Germany) and a 36 Watt UV sterilizer (Rebie, Bielefeld, Germany), in combination with frequent water changes, aided in maintaining a high water quality within the system (pH > 8.1, pCO_2 < 0.042 kPa, pO_2 > 20 kPa, $[NH_4^+]$ < 0.1 mg/l, $[NO_2^{2-}]$ < 0.1 mg/l, $[NO_3^-]$ < 5 mg/l, S > 32 ppt). pH, pO_2 and salinity were checked daily using a WTW multimeter (WTW, Weilheim, Germany), nitrogenous waste products were assessed bi-weekly using photometric test kits (Merck, Darmstadt, Germany). Carbonate system parameters were calculated from pH_{NBS} and weekly determinations of total dissolved inorganic carbon (C_T) (see below).

Eggs were placed individually on the bottom of the incubation tank until close to hatching (stages 29–30). All (n = 13) eggs of the present study were sampled on the same day. Eggs were gently lifted out of the tank and immediately sampled for PVF. All PVF samples were taken within 15 s, thus minimizing the chance of artificially increased pCO_2 values caused by disturbed embryos. To measure pH and pO_2, a 1 ml plastic syringe was equipped with miniature fiber optic sensors (optodes, tip diameter 140 µm, Presens GmbH, Regensburg, Germany) and filled with 200–300 µl PVF, (previously described in Melzner 2005). Stable pH values were obtained after 10 min, pO_2 values after 10 s. During the measurement period, the syringe and sensors were placed in a thermostatted water bath at 17°C. The oxygen optodes were calibrated according to the manufacturer's instructions with water vapor saturated air and a Na_2SO_3 solution. The pH optodes were calibrated using five seawater standards (North Sea seawater, 31 psu, 0.2 µm filtered) adjusted to pH values between 7 and 8 with 1 M HCl. A pH electrode (WTW sentix81 and pH340i pH meter, WTW, Weilheim, Germany), calibrated with Radiometer precision buffers, was used to prepare the seawater buffers. Calibration of the pH optodes with seawater buffers was found necessary as these sensors are sensitive to the ionic strength of the measurement medium.

Another 350 µl of PVF was sampled with a gas-tight glas syringe for the determination of total dissolved inorganic carbon (C_T). C_T was measured in triplicate (100 µl each) using a gas chromatographic method (Lenfant and Aucutt 1966, modified after Pörtner et al. 1990) on an Agilent 6890 N gas chromatograph. Carbonate system speciation (i.e., pCO_2, $[HCO_3^-]$) was calculated from C_T and pH_{NBS} using CO2SYS software (Lewis and Wallace 1998), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Following PVF sampling, eggs were dissected and embryo and yolk wet mass was determined using a Sartorius precision balance (Sartorius, Göttingen, Germany). Embryonic stages were determined according to Lemaire (1970).

PVF pO_2, pCO_2, $[HCO_3^-]$ and pH were graphed against embryo wet mass. Subsequent regression analyses were performed using GraphPad InStat 3.0 software (GraphPad Software, San Diego, USA). A Runs test was used to test for deviations from linearity, an ANOVA was used to assess whether slopes differed from zero.

Results and discussion

The eggs of *S. officinalis* are particularly suited for the investigations of abiotic parameters in the PVF due to their large size. At hatching, the eggs contain >1 ml of PVF and have a diameter between 1.5 and 2 cm (see supplementary movie, S1). Schematic drawings of a freshly laid egg, as well as of late stage embryos, are nicely illustrated in

Wolf et al. (1985) (Figs. 1 and 2). All of the cuttlefish eggs we investigated contained living embryos, ranging in wet mass between 134 and 310 mg (mean 238 mg, SD 44 mg). Organogenesis was completed in all embryos (stages 29–30, Lemaire 1970), and embryos had mostly absorbed their external yolk (mean wet mass of external yolk 18 mg, SD 20 mg). The larger embryos (>250 mg) were probably <1 week away from hatching, especially the two largest embryos, who had consumed all external yolk reserves (Lemaire 1970). Measured PVF pO_2, pH and pCO_2 values were tightly linked to embryo wet mass, increasing (pCO_2) or decreasing (pO_2, pH) in a linear fashion (Fig. 1a, b, c). The results of all regression analyses are depicted in Table 1.

PVF Oxygen partial pressures

Sepia officinalis PVF oxygen partial pressures varied between 12.8 kPa (ca. 61% air saturation) and 4.6 kPa (ca. 22% air saturation). Wolf et al. (1985) found *S. officinalis* PVF pO_2 values to rise with embryonic development, increasing from 12 kPa (stage 24) to 15.5 kPa (stage 29). They concluded that improved diffusion properties of the swelling and thinning egg case would aid in the maintenance of high PVF pO_2. Unfortunately, Wolf et al. did not provide embryo masses, making it difficult to draw meaningful conclusions between our studies. However, the smallest of our embryos was also classified as stage 29 and its PVF pO_2 value was high (>12 kPa, see Fig. 1a). It is thus not unlikely that Wolf et al. missed the final growth phase in their study, as this is when PVF pO_2 rapidly declines. Support comes from the study by Cronin and Seymour (2000), who also demonstrated an inverse correlation between embryo mass and pO_2 in Australian giant cuttlefish eggs (*Sepia apama*, 12°C). In *S. apama*, pO_2 declined to values of about 6 kPa during the last 50 days of development. As Cronin and Seymour determined a pO_{2crit} of 8 kPa for hatchling animals, they suggested that late embryos probably experience diffusion limitation close to hatching. pO_{2crit} for 200–300 mg *S. officinalis* incubated at 18–19°C has been shown to be in the 4.5–5 kPa range (DeWachter et al. 1988), thus in the range of our lowest PVF values (4.6 kPa). These findings support the idea of oxygen diffusion limitation being one critical factor in *S. officinalis* late embryonic development.

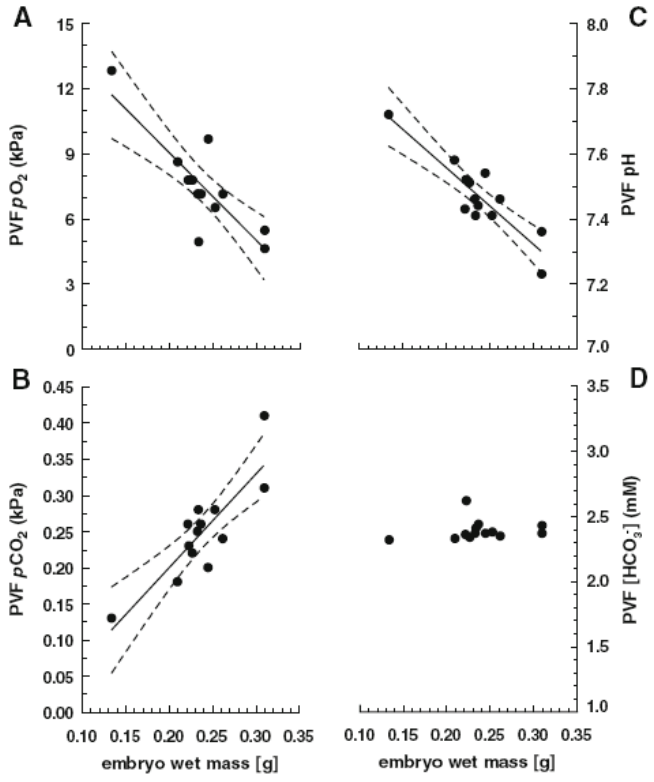

Fig. 1 pO_2 (**a**), pCO_2 (**b**), pH (**c**) and $[HCO_3^-]$ in perivitelline fluid (*PVF*) of *Sepia officinalis* eggs (stages 29–30), displayed against embryo wet mass (excluding the external yolk sac). pCO_2 and $[HCO_3^-]$ were calculated from PVF C_T and pH. See Table 1 for regression analyses and equations

PVF pCO_2 and pH

As expected, PVF pCO_2 values rose with increasing embryo mass, from 0.13 kPa in the smallest up to 0.41 kPa in the largest embryos (Fig 1b). Thus, cuttlefish embryos are surrounded by tenfold higher pCO_2 values than those of ambient sea water (ca. 0.04 kPa) at the end of their embryonic development. High pCO_2 values in the PVF also imply that blood pCO_2 values must be even higher in order to maintain CO_2 excretion rates across the gill/skin epithelia by means of diffusion. Typically, pCO_2 values in extracellular fluids of high-power animals such as fish or cephalopods are 0.2–0.4 kPa above those of the ambient seawater (e.g., Heisler 1986; Johansen et al. 1982; Pörtner et al. 1991). Therefore, cuttlefish embryos are probably exposed to blood pCO_2 values of 0.6–0.8 kPa at the end of their development. It would be quite rewarding to study blood pH regulation in embryos under such conditions, as cephalopods are known for the high pH sensitivity of their extracellular respiratory pigment hemocyanin (Melzner et al. 2007). The occurrence of special embryonic hemocyanins (Decleir et al. 1971) may be one adaptation to the high pCO_2 values encountered during late embryogenesis.

The CO_2 gradient between the PVF and seawater was much shallower than that of oxygen, almost by a factor of 70 ($\Delta pO_2/\Delta pCO_2 = 66.6$, SD 11.3). This ratio is higher than expected from Henry's and Graham's laws, which suggest an approximately 26-fold higher partial pressure gradient of O_2 versus CO_2 across the egg envelope (for seawater at 17°C, Dejours 1975). These results can only be

Table 1 Linear regression analyses. $N = 13$ eggs were analyzed, pCO_2 and $[HCO_3^-]$ are derived parameters (calculated from pH and C_T, see text), pCO_2 and pO_2 in kPa

Regression	ANOVA	Runs test	R^2	Equation
pO_2 vs. pCO_2	$F_{(1,11)} = 36.5$, $P < 0.001$	0.88, NS	0.77	$y = -27.9x + 14.5$
pO_2 vs. mass	$F_{(1,11)} = 25.1$, $P < 0.001$	0.15, NS	0.70	$y = -40.3x + 17$
pCO_2 vs. mass	$F_{(1,11)} = 31.9$, $P < 0.001$	0.98, NS	0.74	$y = 1.31x - 0.06$
pH vs. mass	$F_{(1,11)} = 42.6$, $P < 0.001$	0.88, NS	0.80	$y = -2.32x + 8.02$
$[HCO_3^-]$ vs. mass	$F_{(1,11)} = 0.3$, $P > 0.59$	0.47, NS	0.03	NS

See also Fig. 1

explained if either a significant portion of the excretory CO_2 is retained within the embryo to aid in the formation of the internal $CaCO_3$ shell, or if Krogh's diffusion coefficient for CO_2 (K_{CO_2}) is much higher than K_{O_2} for *S. officinalis* egg capsules. It has been demonstrated for sea urchin larvae and corals that significant portions of inorganic carbon for calcification are derived from metabolic CO_2 (>50%, Sikes et al. 1981; Furla et al. 2000). However, a more extensive follow-up study that will focus on the determination of oxygen consumption and CO_2 excretion, as well as egg capsule surface area and thickness, will enable us to give estimates for both, K_{O_2} and K_{CO_2}.

As pCO_2 increased, pH strongly decreased, from 7.72 to 7.23 (Fig. 1c). Again, there are no pH measurements for marine animal eggs at present that our data could be compared with. In freshwater fish (salmonids), 0.3–1.0 unit lower pH values have been recorded in egg PVF at ambient pH between 7 and 8 (Kugel and Peterson 1989). No accumulation of HCO_3^- was visible in *S. officinalis* PVF in order to actively buffer pH (Fig. 1d), a mechanism that is used by many marine organisms to compensate for extra- and intracellular acidification (e.g., Heisler 1986; Cameron 1986). $[HCO_3^-]$ fluctuated around 2.39 mM (SD 0.08 mM) in all eggs.

It is noteworthy that cuttlefish embryos are able to form an internal $CaCO_3$ (aragonite) shell under the low pH and high pCO_2 prevailing in their egg environment. We recently demonstrated, that calcification rate in juvenile cuttlefish is not impaired at external pCO_2 values of 0.4 and 0.6 kPa (Gutowska et al. 2008). This sets *S. officinalis* apart from other marine invertebrates studied so far, as the majority show a decrease in calcification under comparable conditions (see Fabry et al. 2008 for a review). It is tempting to propose that this capacity is causally linked to an embryo that is already adapted to cope with relatively high pCO_2/low pH values.

Perspectives

As mentioned, no data on egg fluid pCO_2 and pH is available for other marine organisms; however, judging from the pO_2 versus pCO_2 ratios obtained in our study, in comparison to pO_2 values in- and around eggs or egg masses of other marine animal taxa, it seems likely that many embryos will also be surrounded by fluids of high pCO_2 and low pH: For example, Diez and Davenport (1987) showed that pO_2 values in shark eggs decrease from ca. 18 kPa in early embryos to ca. 10 kPa in late embryos, Fernandez et al. (2000, 2002) measured water pO_2 values <5 kPa in decapod crustacean egg masses, Cohen and Strathmann (1996) found pO_2 values of less than 6 kPa in egg masses of opisthobranch gastropods and those of a polychaete worm.

Delayed development of embryos in central positions of egg masses has usually been causally linked to reduced metabolic rates due to low ambient pO_2 (e.g., Chaffee and Strathmann 1984). However, high pCO_2/low pH may be important factors as well, as they could also elicit reductions in respiration rates: Metabolic depression, in combination with reduced growth and calcification performance, has recently been observed in mussels (*Mytilus galloprovincialis*) exposed to sea water of similar pCO_2 (ca. 0.5 kPa, Michaelidis et al. 2005).

Quite clearly, PVF pCO_2 and pH represent important abiotic factors that might influence the physiological performance of marine animal embryos to a large degree. To date, these factors have been thoroughly neglected. We hope that this study sparks some interest in studying CO_2 excretion and pH homeostasis in eggs of marine animals.

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Biogeosciences

Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny?

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Abstract. Future ocean acidification has the potential to adversely affect many marine organisms. A growing body of evidence suggests that many species could suffer from reduced fertilization success, decreases in larval- and adult growth rates, reduced calcification rates, and even mortality when being exposed to near-future levels (year 2100 scenarios) of ocean acidification. Little research focus is currently placed on those organisms/taxa that might be less vulnerable to the anticipated changes in ocean chemistry; this is unfortunate, as the comparison of more vulnerable to more tolerant physiotypes could provide us with those physiological traits that are crucial for ecological success in a future ocean. Here, we attempt to summarize some ontogenetic and lifestyle traits that lead to an increased tolerance towards high environmental pCO_2. In general, marine ectothermic metazoans with an extensive extracellular fluid volume may be less vulnerable to future acidification as their cells are already exposed to much higher pCO_2 values (0.1 to 0.4 kPa, ca. 1000 to 3900 μatm) than those of unicellular organisms and gametes, for which the ocean (0.04 kPa, ca. 400 μatm) is the extracellular space. A doubling in environmental pCO_2 therefore only represents a 10% change in extracellular pCO_2 in some marine teleosts. High extracellular pCO_2 values are to some degree related to high metabolic rates, as diffusion gradients need to be high in order to excrete an amount of CO_2 that is directly proportional to the amount of O_2 consumed. In active metazoans, such as teleost fish, cephalopods and many brachyuran crustaceans, exercise induced increases in metabolic rate require an efficient ion-regulatory machinery for CO_2 excretion and acid-base regulation, especially when anaerobic metabolism is involved and metabolic protons leak into the extracellular space. These ion-transport systems, which are located in highly developed gill epithelia, form the basis for efficient compensation of pH disturbances during exposure to elevated environmental pCO_2. Compensation of extracellular acid-base status in turn may be important in avoiding metabolic depression. So far, maintained "performance" at higher seawater pCO_2 (>0.3 to 0.6 kPa) has only been observed in adults/juveniles of active, high metabolic species with a powerful ion regulatory apparatus. However, while some of these taxa are adapted to cope with elevated pCO_2 during their regular embryonic development, gametes, zygotes and early embryonic stages, which lack specialized ion-regulatory epithelia, may be the true bottleneck for ecological success – even of the more tolerant taxa.

Our current understanding of which marine animal taxa will be affected adversely in their physiological and ecological fitness by projected scenarios of anthropogenic ocean acidification is quite incomplete. While a growing amount of empirical evidence from CO_2 perturbation experiments suggests that several taxa might react quite sensitively to ocean acidification, others seem to be surprisingly tolerant. However, there is little mechanistic understanding on what physiological traits are responsible for the observed differential sensitivities (see reviews of Seibel and Walsh, 2003; Pörtner et al., 2004; Fabry et al., 2008; Pörtner, 2008). This leads us to the first very basic question of how to define general CO_2 tolerance on the species level.

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1 Defining tolerance towards elevated seawater pCO_2

When trying to classify marine organisms into CO_2 sensitive and CO_2 tolerant groups, we encounter a major complication: Projected ocean acidification progresses at a rate much too slow to be simulated in the laboratory, and differences in genetic adaptation potential vary at orders of magnitude between taxa. Organisms with a high generation turnover time (e.g. bacteria, unicellular auto- and heterotrophs) will have time for thousands of generations to select for genotypes that can cope with an ocean characterized by pCO_2 values of up to ca. 0.2 kPa (ca. 2000 μatm) by the year 2300 (Caldeira and Wickett, 2003, 2005), while in long-lived species, such as the ocean quahog (*Arctica islandica; mollusca: bivalvia*), with a maximum life expectancy of ca. 400 years (Abele et al., 2008), today's genotypes may be exposed to the high pCO_2 values of the year 2300. Thus, species longevity/generation time is a crucial factor that could possibly determine future success in an – on evolutionary timescales – rapidly changing habitat.

Multi-generation experiments will be very important to understand the adaptation potential of a given species, however, such approaches are only beginning to emerge (Kurihara and Ishimatsu, 2008; Dupont and Thorndyke, 2009), especially in metazoans with long generation cycles (months, years). Thus, by reading the ideas we propose in this concept paper we should keep in mind the problems of rate of change in ocean carbonate chemistry and the genetic adaptation potential of a given species. In addition, the largely unexplored problems of species interaction and food-web feedbacks will be major factors shaping ecological performance of marine species in a future high pCO_2 ocean. For example, delayed larval development, as observed in echinoderm early life stages subjected to elevated pCO_2 (Dupont and Thorndyke, 2009), probably will increase predation related mortality in the field, even if there is no mortality difference in experimental cultures (Elkin and Marshall, 2007).

Considering the limited availability of multi-generation experiments, the best we can do at the moment to define tolerance versus sensitivity to ocean acidification, is to look at indicators for animal performance during long-term (weeks to months) CO_2 perturbation experiments. We use the term "animal performance" as the sum of the major relevant traits that ensure ecological success of a species (on a species level), i.e. among others, aerobic scope, locomotory scope, reproductive output, calcification and somatic growth, which, together, influence animal fitness. Aerobic metabolic scope (the difference between active and standard metabolic rates, see Fry, 1948, for a definition) is a parameter that can be (more or less) easily assessed in mobile animals, e.g. crustaceans, cephalopods or fish (e.g. Booth et al., 1984a; Wells and Wells, 1985; Pörtner et al., 1991; Melzner et al., 2009), whereas it can only be approximated in sessile animals, sometimes via the specific dynamic action of food (e.g. Vahl, 1984; Widdows, 1973). Aerobic metabolic scope of a species is also often directly related to locomotory scope and growth performance. While the measurement of standard metabolic rates can potentially indicate how the costs for homeostatic regulation are altered under an acute abiotic stress regime, somatic and reproductive growth performance can integrate cost re-allocation over a longer time interval. Thus, "footprints" in the energy budget in response to an abiotic stressor regime can be detected more easily in long-term growth trials. While somatic/reproductive growth may be one of the best performance indicators, it has already become clear that in order to consider possible trade-offs between single parameters all relevant indicators have to be included simultaneously to generate a meaningful assessment of a given species' vulnerability to future ocean acidification (e.g. see below, Wood et al., 2008; Kurihara et al., 2008). Unfortunately, to date there are few comprehensive performance assessments for marine metazoan species subjected to long-term elevated pCO_2. Thus, in the following text, we will place emphasis on those taxa where most information is available, hoping that future studies will focus on the simultaneous assessment of multiple performance indicators in long-term CO_2 perturbation experiments. The aim of the present review paper is thus not to compare single parameters between different species but to pool data on higher taxonomic levels to improve our understanding of major physiological characteristics that provide the basis for a high degree of CO_2 tolerance. While it is clear already now that due to the synergistic effects of a complex set of parameters CO_2 tolerance at near-future levels of ocean acidification is difficult to predict, even for closely related species (e.g. echinoderm larval stages: Dupont and Thorndyke, 2009; Widdicombe and Spicer, 2008), we will make use of those studies that have used higher pCO_2 values (>0.3 to 0.5 kPa) to elucidate some fundamental tolerance mechanisms that are closely related to lifestyle and metabolic rates of more active taxa.

2 Sensitive vs. tolerant physiotypes: which taxa perform best?

If we combine evidence from the few long-term CO_2 perturbation experiments (weeks to months) until now, it appears that (adult) marine ectothermic vertebrates are the most CO_2-tolerant group – various performance parameters seem not to be compromised by chronic hypercapnia at levels >0.3 to 0.6 kPa. Teleost species studied in long-term growth trials (wolffish, *Anarhichas minor*; salmon, *Salmo salar*) did not display reductions in somatic growth performance when exposed to pCO_2 values of up to 0.6 kPa and higher (5900 μatm; Foss et al., 2003; Fivelstad et al., 1998, 2003). In addition, recent findings indicate that long-term acclimation of Atlantic cod (*Gadus morhua*) to pCO_2 values of 0.3 and 0.6 kPa (ca. 3000 to 5900 μatm) does not seem to impact swimming performance (critical swimming speed, U_{crit}), standard and active metabolism, as well as aerobic scope

Table 1. Impact of CO_2 exposure on various physiological performance indicators like metabolic rate, acid-base regulation, growth and calcification at high seawater pCO_2 values >0.5 kPa (ca. 4900 μatm). The table gives an overview on effects assessed in marine taxa of different hypercapnia tolerance; references are noted in parenthesis. Note the scarce knowledge in specific areas (active metabolic rate under hypercapnia) and organism groups (brachyuran crabs). Active bicarbonate accumulation excludes cases where ions most probably stem from passive shell dissolution and subsequent enrichment in a closed system (e.g. in bivalves). Cited references are: (1) Fivelstad et al., 2003, (2) Foss et al., 2003, (3) Melzner et al., 2009, (4) Larsen et al., 1997, (5) Michaelidis et al., 2007, (6) Truchot, 1979, (7) Pane and Barry, 2007, (8) Spicer et al., 2007, (9) Gutowska et al., 2008, (10) Gutowska et al., submitted, 2009, (11) Siikavuopio et al., 2007, (12) Kurihara and Shirayama, 2004, (13) Dupont et al., 2008, (14) Miles et al., 2007, (15) Michaelidis et al., 2005, (16) Gazeau et al., 2007, (17) Booth et al., 1984/Lindinger et al., 1984.

	Somatic Growth	Rate of calcification	Standard/routine metabolic rate (SMR/RMR)	Active metabolic rate (AMR)	active extracellular pH compensation/ (HCO_3^-) accumulation
teleost fish	o (1,2)	?	o (3)	o (3)	+ (4,5)
brachyuran crustacea	?	?	?	?	+ (6,7,8)
cephalopoda	o (9)	o/+ (9)	o (9)	?	+ (10)
echinodermata	– (11)	– (12, 13)	?	?	– (14)
bivalvia	– (15)	– (15,16)	– (15)	?	– (15,17)

o/+/–= measured values or rates remain constant /increase /decrease;
?= no data available.

(Melzner et al., 2009). In contrast, marine invertebrates generally seem less tolerant at high levels of hypercapnia. Several studies have documented decreased growth and/or calcification rates in long-term exposure studies, e.g. in mussels (Michaelidis et al., 2005), echinoderms (Siikavuopio et al., 2007), coral reef communities and individual coral species (Langdon et al., 2000; see review by Hoegh-Guldberg et al., 2007), at levels that teleost fish are not affected by. In contrast to these invertebrates, the cephalopod *Sepia officinalis* is characterized by maintained somatic growth and slightly elevated calcification rates at pCO_2 values of 0.4 and 0.6 kPa (ca. 3900 to 5900 μatm; Gutowska et al., 2008), making it the only marine invertebrate species so far that to some degree approaches adult teleost performance standards (see Table 1). We suspect that shallow water brachyuran crustaceans could be another marine invertebrate taxon likely to approach teleost CO_2 tolerance, mainly due to their high ion-regulatory capacity (Wheatly and Henry, 1992). Unfortunately, no long-term growth and calcification experiments have been conducted using this group as a model to date.

Table 1 summarizes the effects of CO_2 exposure studied so far in representatives of the different marine taxa. Interestingly, a common feature of all more CO_2 tolerant species studied so far (again, at high pCO_2 values of >0.3 to 0.6 kPa) is their ability to perform a pH compensatory reaction to protect their extracellular fluids (blood, hemolymph) from excessive acidification. This might be a crucial trait, as it has been suggested that uncompensated extracellular pH is causally linked to metabolic depression in some of the more sensitive marine invertebrates (e.g. see Reipschläger and Pörtner, 1996; Pörtner et al., 2004; Michaelidis et al., 2005; Fabry et al., 2008). Metabolic depression, while beneficial during short-term abiotic stress (e.g. Guppy and Withers, 1999), would lead to long-term reductions in growth performance, aerobic and locomotory capacity, and thus, decreased ecological fitness in general (cf. Langenbuch and Pörtner, 2004). For our line of reasoning, it is thus quite important to fully understand the mechanisms leading to extracellular pH stabilization in these more tolerant organisms. If we speak of pH, to simplify matters, all data mentioned throughout the text, tables and figures refer to the NBS scale.

3 Mechanisms of extracellular pH regulation in tolerant vs. sensitive physiotypes

Buffering of free protons builds the first line of defence against CO_2 induced acidification of body fluids: The two buffering systems that are functional in all organisms studied so far are (I) the CO_2-bicarbonate system itself and (II) the so called non-bicarbonate buffering system. Unfortunately, the CO_2-bicarbonate system is of only small efficiency for buffering in marine animals. In response to high proton concentration the chemical equilibrium between the weak carbonic acid and bicarbonate leads to a rise in aqueous CO_2. In air breathers, the resulting higher pCO_2 is typically

eliminated by means of increased ventilation. However, this process is seriously impaired by the (comparatively) low pCO_2 values in body fluids of water breathers and the resulting very small diffusion gradients between organism and the surrounding water (see Heisler, 1986, for an extended discussion). Consequently, binding of respiratory protons (originating from CO_2 hydration) by so called non-bicarbonate buffers is the first step to minimize pH changes under acidified conditions. Non-bicarbonate buffering is mainly provided by partially protonated amino acid side chains (mostly from histidine or cysteine at physiological pH values), N-terminal α-amino groups of proteins or organic/inorganic phosphate groups. As buffering can only mask protons during an acidotic pH shift and thus reduce pH changes compared to a non-buffered system, surplus protons have to be eliminated to restore the original fluid pH. This can only be achieved by means of active ion transport across specialized epithelia, such as gills, renal or digestive tissue. Although the involved ion exchange mechanisms as a whole are poorly understood and may vary between different marine taxa, the processes contributing to pH compensation are summarized as proton equivalent ion exchange. Concerning the reduction of proton activity in body fluids, it is not important if a pH change is realized by higher proton excretion rates, rising bicarbonate import from the seawater or increased retention of metabolic bicarbonate.

A useful tool to visualize the correlation of the three acid-base parameters pH, pCO_2 and bicarbonate concentration for a specific physiological environment is the so called Davenport diagram (Fig. 1a, see figure caption and Davenport, 1974). All pCO_2 isopleths in such diagrams can be calculated with the help of the Henderson-Hasselbalch Eq. (1) from fixed pH and $[HCO_3^-]$ values, if the apparent dissociation constant of carbonic acid (pK'_1) and CO_2 solubility coefficient (α_{CO2}) for the particular fluid of interest (e.g. blood, hemolymph, coelomic fluid) are known (e.g. see Truchot, 1976, Heisler, 1986, Boutilier et al., 1984). When extracellular pCO_2 rises in vivo, extracellular pH decreases, while the increment in $[HCO_3^-]$ follows the non-bicarbonate buffer line (termed "respiratory acidosis", see Fig. 1a). This is due to the production of both, protons and $[HCO_3^-]$ during the CO_2 hydration reaction in the extracellular fluid when dissociating protons are largely bound to non-bicarbonate buffers, while bicarbonate remains. Thus, a slight increase in extracellular $[HCO_3^-]$ is caused by an increase in pCO_2. The magnitude of this buffering reaction is reflected in the slope of the non-bicarbonate buffer lines. These can be constructed from in vitro measurements by equilibrating samples of extracellular fluid with known pCO_2 to subsequently measure pH and $[HCO_3^-]$ (see Fig. 1). The negative slope of the non-bicarbonate buffer line, $\Delta[HCO_3^-]/-\Delta pH$, is typically called the non-bicarbonate buffer value (β_{NB}), expressed in $m\,Eq\,l^{-1}pH^{-1}$, or slykes. In molluscs, for example, extracellular β_{NB} values range from 0.4 to 0.6 slykes in bivalves (*Mytilus edulis*; Booth et al., 1984, Lindinger et al., 1984) to values of 3 to 10 slykes in cephalopods (Pörtner et al. 1991; Gutowska et al., 2009). Thus, an acute increase in hemolymph pCO_2 would lead to a much more pronounced decrease in extracellular pH in the bivalve vs. the cephalopod. Typically, β_{NB} is directly proportional to the protein concentration in the extracellular fluid (e.g. Truchot, 1976). The red and blue non-bicarbonate buffer lines in figure 1B approximate the conditions in bivalves and cephalopods (β_{NB} blue line = ca. 3 slykes, red line = ca. 0.4 slykes).

Whether an increase in extracellular fluid $[HCO_3^-]$ is due to buffering, or whether active proton equivalent transport processes are occurring, can be easily depicted from Davenport diagrams: If, under elevated pCO_2, pH and $[HCO_3^-]$ follow the course of the non-bicarbonate buffer line in vivo, then passive buffering prevails and no active bicarbonate accumulation is contributing to the observed increase in $[HCO_3^-]$. The red symbols in Fig. 1b illustrate such a case, which may be typical for certain echinoderms, bivalves or deep-sea crustaceans under hypercapnic conditions (Miles et al., 2007; Pane and Barry, 2007; Thomsen, 2008). In this hypothetic example, environmental hypercapnia of 0.5 kPa (ca. 4900 μatm) would lead to a hemolymph pCO_2 of 0.65 kPa (ca. 6500 μatm) and extracellular pH would drop dramatically, from 7.6 to 7.0 (note: extracellular pCO_2 is always higher than seawater pCO_2, see below). Figure 1b also illustrates cases, in which active transepithelial ion-exchange processes contribute to the increase in $[HCO_3^-]$. Upon acute exposure to a pCO_2 of 0.5 kPa (ca. 4900 μatm), organisms initially follow the course of the non-bicarbonate buffer line, until ion-transport processes kick in (typically after minutes to hours) to actively elevate $[HCO_3^-]$ above the slope of non-bicarbonate buffer line (often termed "metabolic or non-respiratory alkalosis", see also Fig. 1a). Partial compensation of extracellular pH (blue dots) has been observed in sipunculids, cephalopods, some brachyuran crustaceans and some teleost fish (Heisler, 1986; Pörtner et al., 1998; Cameron, 1986; Truchot, 1975; Gutowska et al., 2009). Full compensation, i.e. restoration of the original control extracellular pH (Fig. 1b, green dots), has been demonstrated for a range of teleost fish and some brachyuran crabs tested (Heisler, 1986; Cameron, 1986; Pane and Barry, 2007; Spicer et al., 2007). The amount of bicarbonate necessary for full compensation during hypercapnic stress can easily be assessed using the Henderson-Hasselbalch equation:

$$pH = pK'_1 + log([HCO_3^-]\alpha_{CO2}^{-1} pCO_2^{-1}) \quad (1)$$

with pK'_1= apparent first dissociation constant of carbonic acid, α_{CO2}=CO_2 solubility coefficient of the respective fluid (e.g. blood, hemolymph, coelomic fluid; $[mmol\,l^{-1}\,Pa^{-1}]$)

In order to maintain extracellular pH constant, any factorial change in extracellular pCO_2 has to be balanced by an equivalent change in $[HCO_3^-]$ such that the ratio between the two remains constant, e.g. a 1.5-fold change in blood pCO_2 from 0.2 to 0.3 kPa (ca. 2000 to 3000 μatm) would need to

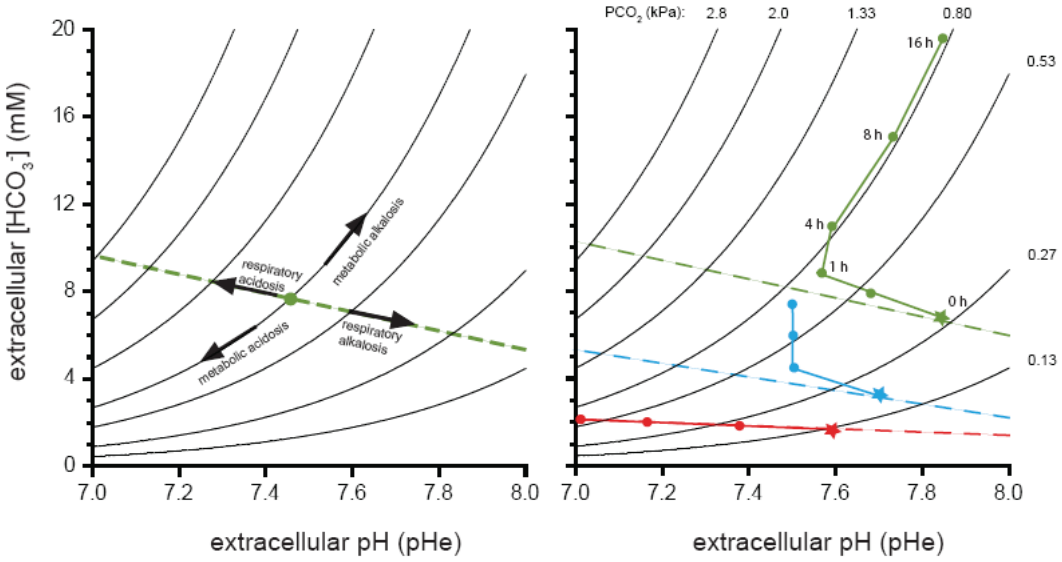

Fig. 1. Davenport diagrams. **(A)**: Schematic illustration of non-bicarbonate buffer line, dashed green line. Arrows indicate changes in pCO_2 and $[HCO_3^-]$ during respiratory acidosis/alkalosis and metabolic acidosis/alkalosis. See text for explanations. **(B)**: Three different hypothetical organisms subjected to 0.5 kPa (ca. 4900 μatm) environmental hypercapnia. Red symbols: No active accumulation of bicarbonate in the extracellular space to compensate pH, pH follows the non-bicarbonate buffer line. Blue symbols, green symbols: partial/full pH compensation through active bicarbonate accumulation. Stars indicate control parameters, numbers indicate time (h) exposed to elevated pCO_2 (hypothetical time course!). See text for a detailed discussion.

result in a 1.5-fold increase in $[HCO_3^-]$ to maintain extracellular pH at the control level.

The main prerequisite for such a rapid and efficient bicarbonate accretion are high net proton equivalent fluxes between ectothermic organisms and the surrounding seawater. Such data are currently only available for decapod crustaceans and for teleost/elasmobranch fish as well as an invertebrate (sipunculid) worm. Values of about 100 μEq kg^{-1} h^{-1} net acid efflux have been recorded for the crustacean *Carcinus maenas* exposed to a pCO_2 value of about 0.7 kPa (ca. 6900 μatm; Truchot, 1979), even higher values have been recorded in the marine teleost *Conger conger*, where exposure to 1.3 kPa CO_2 (ca. 12 800 μatm) produced a net acid efflux of 920 μEq kg^{-1} h^{-1} (Holeton et al., 1983). Rates were much lower in the sipunculid and mirrored transiently enhanced net proton release during transition to a new steady state in acid-base status under hypercapnia (Pörtner et al., 1998).

In summary, it appears that a relative degree of tolerance towards hypercapnic exposure can be found mainly in such marine ectothermic organisms that possess the ability to actively accumulate large amounts of bicarbonate ions to stabilize extracellular pH. In addition, these organisms are typically equipped with relatively high non-bicarbonate buffering capacities, which protect extracellular pH during acute CO_2 exposure. While hypercapnia typically is not a relevant stressor in the natural habitat of many marine organisms (however, see Sects. 8 and 9), high capacities for net acid extrusion directly result from an active mode of life, high metabolic rates and frequent as well as rapid metabolic rate fluctuations. We will follow this line of argument in the following paragraphs.

4 A common denominator: metabolic rate and metabolic rate fluctuations

Allowing for considerable intra-taxon variability, there are strong common ties between teleost fish, brachyuran crustaceans and cephalopod molluscs when compared with e.g. echinoderms and bivalve molluscs: All more tolerant taxa are characterized by high (specific) metabolic rates and high levels of mobility/activity. Figure 2a gives an overview of the range of metabolic rates that can be encountered in the aforementioned taxa, with standard/routine metabolic rates displayed in black, and those obtained during (exhaustive) exercise in white. For clarity sake, only subtidal and intertidal species from temperate regions were considered for this comparison. It is quite obvious that all active taxa are characterized by considerably higher metabolic rates, and, maybe even more important, higher metabolic rate fluctuations, than members from less active taxa (for references see Fig. 2). Maximum differences in oxygen consumption can be 100 to 200-fold between certain sessile echinoderms and exercising cephalopods. Even more revealing is a closer look at the flipside of the coin: Depending on the composition of their diet, marine animals have to excrete close to equimolar quantities, i.e. between 0.7 (fatty acids) and 1.0 (carbohydrates) moles

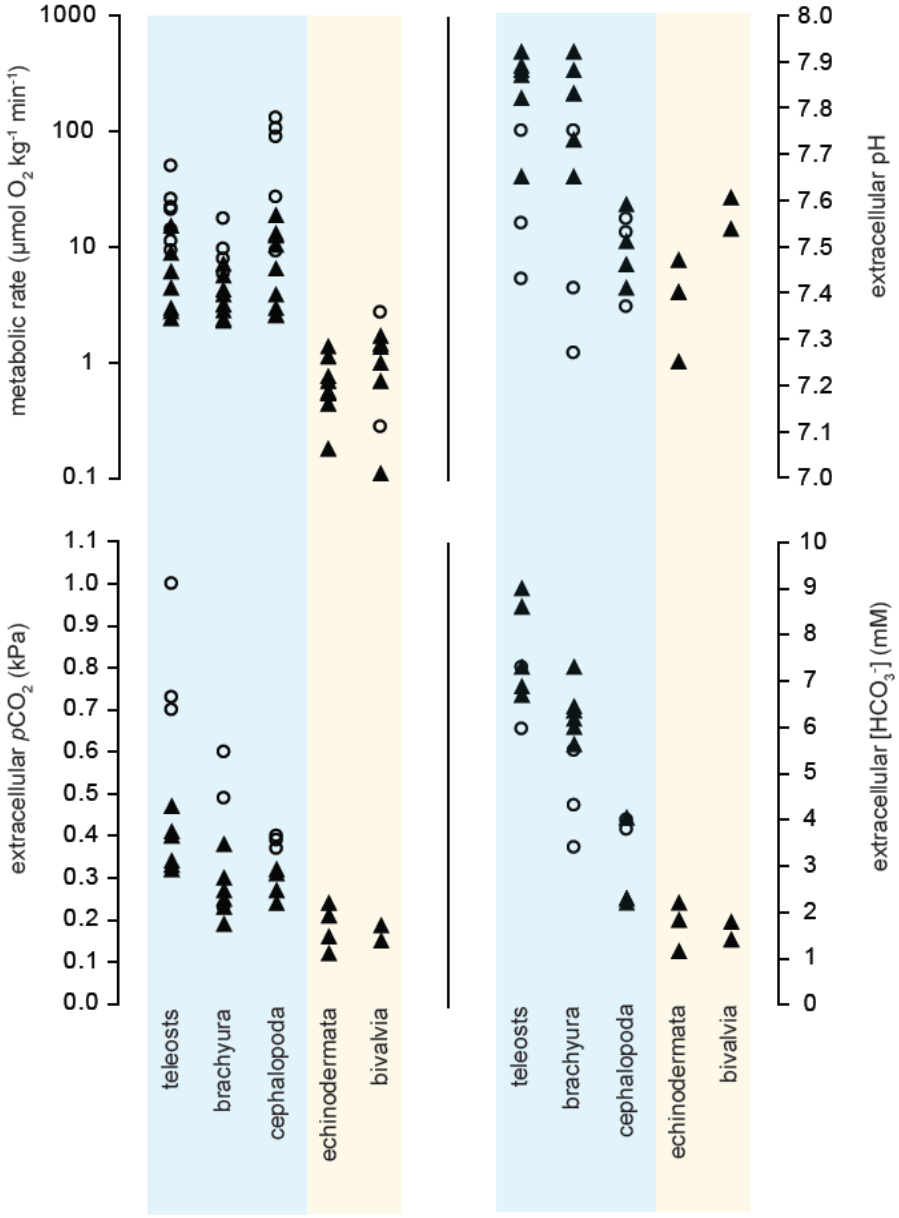

Fig. 2. (A): Routine (black symbols) and active (white symbols) metabolic rates for groups of randomly chosen marine subtidal ectothermic animals from temperate ocean regions. To ensure comparability, all metabolic rates have been scaled to an animal weight of 20 g (total body weight) at 15°C, using a Q_{10} value of 2.5 and a mass exponent of b=0.75 (see supplementary file: http://www.biogeosciences.net/6/2313/2009/bg-6-2313-2009-supplement.pdf). **(B), (C), (D)**: Acid-base parameters for groups of randomly chosen marine subtidal ectothermic animals under control (black symbols) conditions or after exercise (white symbols). (B) depicts pCO_2 values, 2C pH_{NBS} values and 2-D bicarbonate concentrations determined in extracellular fluids (blood or hemolymph) of various marine taxa. In most cases pCO_2 and bicarbonate values have been calculated from measurements of pH_{NBS} and dissolved inorganic carbon using the Henderson-Hasselbalch equation and appropriate constants (pK'_1, αCO_2). See supplementary for more detailed information and a table of references: http://www.biogeosciences.net/6/2313/2009/bg-6-2313-2009-supplement.pdf.

of CO_2 per mole of O_2 consumed. Thus, the flux of CO_2 that active vs. more inactive marine ectotherms have to channel from their mitochondria across the cell membranes into the blood space (or coelomic fluid/hemolymph) and, finally, across respiratory epithelia, also varies at the same order of magnitude. Exercise induced alterations in oxygen consumption thus are always coupled to almost equimolar changes in CO_2 flux. Such 3 to 5-fold fluctuations in O_2/CO_2 exchange in active species can occur within minutes, elicited both, by exercise and food consumption. Thus, taxa with

high metabolic rates must possess an advanced machinery for the elimination of CO_2 and associated acid-base disturbances. As a consequence, this machinery might also be helpful in coping with high pCO_2 values originating from seawater hypercapnia.

5 High extracellular pCO_2 in marine ectothermic metazoans

All marine ectothermic metazoans have one feature in common: their cells are surrounded by an extracellular fluid compartment (blood, coelomic fluid or hemolymph) that is used as a convective transport system for various substances, including dissolved gases. As with O_2, CO_2 exchange between this fluid and the external medium (seawater) is mainly realized by means of diffusion according to the following equation (Dejours, 1975):

$$MCO_2 = K_{CO2}(AE^{-1})(pCO_{2e} - pCO_{2sw}) \quad (2)$$

with MCO_2=CO_2 flux in moles, K_{CO2}= species (and organ) specific diffusion constant, pCO_{2e}= extracellular pCO_2, pCO_{2sw}= seawater pCO_2, A= functional diffusion area, E= thickness of the diffusion barrier.

Thus, CO_2 excretion is directly proportional to the CO_2 partial pressure gradient from the inside (extracellular fluid) to the outside (seawater). Consequently, higher marine metazoan animals are characterized by extracellular fluids with several-fold higher pCO_2 values than the surrounding seawater in order to produce a substantial net outward flow of CO_2 (see Fig. 2B), although diffusion areas also scale with metabolic rate. Minimum extracellular pCO_2 values in some marine metazoans (some echinoderms, bivalves) are little higher than 0.1 kPa (ca. 1000 μatm), most animals, however, live with extracellular pCO_2 values of 0.2 kPa (ca. 2000 μatm) and greater. Highest extracellular pCO_2 values in those water breathers are found in teleost fish (0.3 to 0.5 kPa; ca. 3000–4900 μatm). Most ectothermic marine animals maintain relatively constant extracellular pCO_2 values that go along with taxon specific extracellular $[HCO_3^-]$ and pH (under comparable abiotic conditions). Common patterns can be observed in both, brachyuran crustaceans and teleost fish: Relatively high $[HCO_3^-]$ values of 5 to 10 mM usually help support high extracellular pH values of 7.6 to 7.95 (Fig. 2c, d). On the other end of the scale, echinoderms are typically characterized by low extracellular pH (7.0 to 7.5) and low bicarbonate concentrations that are barely higher than those of seawater. Coleoid cephalopods, despite their fish like performance display relatively low extracellular pH and bicarbonate values.

Extracellular pCO_2 values may be first line indicators of an animals' susceptibility towards future ocean acidification. A simple example can illustrate this idea: any unicellular marine organism (e.g. a coccolithophorid, sperm and oocytes of broadcast spawners) today is surrounded by "extracellular" fluid (= seawater) with a pCO_2 of about 0.04 kPa (ca. 400 μatm). An increase in seawater pCO_2 by another 0.04 kPa therefore leads to a 100% increase in "extracellular" pCO_2 for that organism. A similar increase in seawater pCO_2 would probably only lead to a 40% increase in coelomic fluid pCO_2 of an echinoderm with a control coelomic fluid pCO_2 of 0.1 kPa (ca. 1000 μatm), and to a 10% increase in blood pCO_2 of a teleost fish with a control extracellular pCO_2 of 0.4 kPa (ca. 3900 μatm). In both cases, extracellular pCO_2 would need to be increased by 0.04 kPa in order to maintain a constant CO_2 diffusion gradient. Thus, the higher the pCO_2 values that cells are exposed to now, the lower the relative change that will come with future ocean acidification. Thus, fish/cephalopod/brachyuran cells will be exposed to a lower relative change in pCO_2 than cells of typical bivalves/echinoderms, while unicellular organisms (and life stages) will experience the greatest relative changes in their respective extracellular environment.

Figure 2b indicates, that following exhaustive exercise, even higher extracellular pCO_2 values can be encountered: Respiratory and metabolic acidosis result in maximum pCO_2 values between 0.4 kPa (ca. 3900 μatm, cephalopods) and >1.0 kPa (>9900 μatm, teleost fish). Thus, these taxa are adapted to cope (at least occasionally) with extracellular pCO_2 values that are up to five times higher than maximum values we might expect through ocean acidification in surface waters within the next few hundred years, i.e. 0.2 kPa (ca. 2000 μatm: Caldeira and Wickett, 2003).

Interestingly, little information is available on extracellular pCO_2 values during sub-maximal (exclusively aerobic) exercise. While one would expect that animals simply increase their extracellular pCO_2 in order to enhance CO_2 diffusion rates across gill epithelia, the few examples available for teleost fish suggest that pCO_2 is not dramatically elevated under such conditions (van den Thillart et al., 1983; Brauner et al., 2000). For other taxa (brachyuran crustaceans, cephalopods) such measurements have not been performed. It is thus quite rewarding to take a closer look at the physiological basis that enables elevated O_2/CO_2 exchange rates in teleost fish during aerobic exercise and to look at some physiological consequences of exhaustive exercise in active taxa in general. These mechanisms probably form the basis of efficient pH compensation as exploited during hypercapnia.

6 High CO_2 fluxes during (exhaustive) exercise

The capacity to live with elevated pCO_2 values in the extracellular fluid and to cope with extreme and rapid fluctuations in pCO_2 during muscular exercise is a challenge for active taxa. In order to support high metabolic rates, active groups discussed above rely on efficient circulatory systems. These do not only operate at high pressure and volume flow, but also contain intra- (fish) or extracellular (decapod crustaceans,

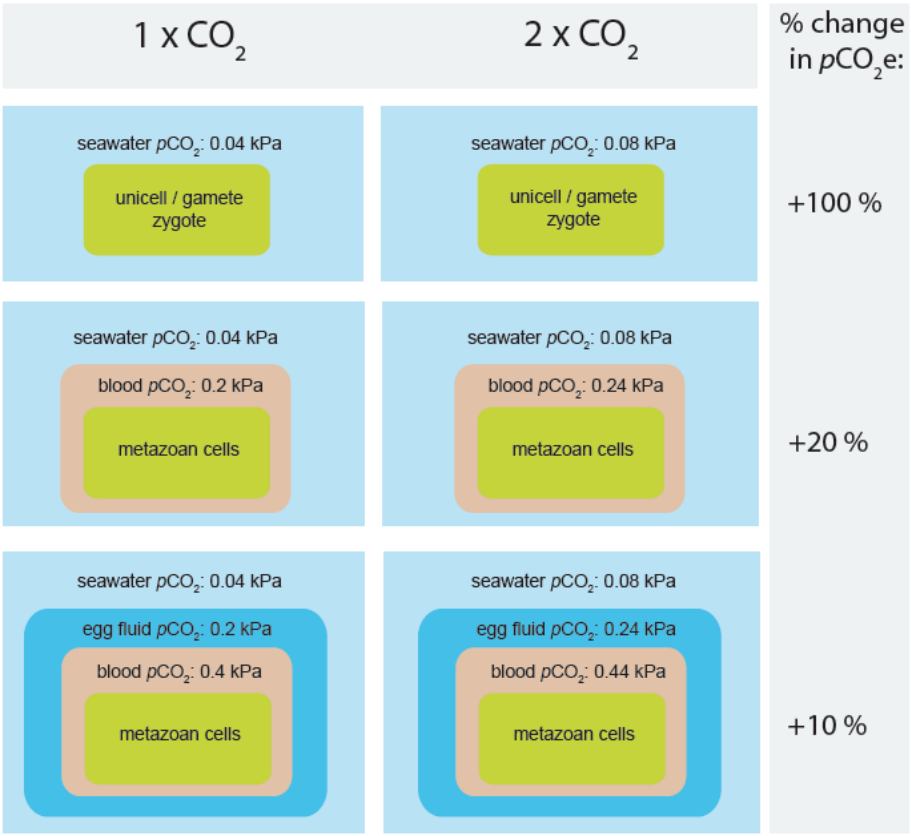

Fig. 3. Schematic illustration of relative changes in pCO_2 that a cell experiences upon doubling of ocean pCO_2 from 0.04 to 0.08 kPa (ca. 400 to 800 μatm). Unicellular organisms experience the greatest relative change in pCO_2, as their extracellular environment is the ocean. Metazoan cells are surrounded by extracellular fluid, which typically is characterized by pCO_2 values between 0.1 and 0.4 kPa (ca. 1000 to 3900 μatm). An elevation of ocean pCO_2 to 0.08 kPa (ca. 800 μatm) would probably only lead to a 20% increase in a metazoan with a control extracellular pCO_2 of 0.2 kPa. Cells of metazoan embryos, like those of cuttlefish, have to overcome yet another diffusion barrier, thus probably are exposed to even higher extracellular pCO_2 values. An equivalent change in pCO_2 by 0.04 kPa would possibly lead to an only 10% change in extracellular pCO_2. The lower relative degree of change in extracellular pCO_2 might render juvenile/adult metazoans less susceptible to future ocean acidification; however, their gametes might be the most sensitive stages.

cephalopods) respiratory pigments that greatly increase the oxygen carrying capacity of the blood. Typical active crustacean and cephalopod hemolymph can contain 70 to 200 g of respiratory protein per litre of blood, providing oxygen carrying capacities of 0.7 to 3 mM (e.g. Brix et al., 1989; Truchot, 1976; Johansen et al., 1982; Zielinski et al., 2001). Thus, in comparison to a mussel without a respiratory pigment, 3 to 8 times less blood has to be circulated per unit oxygen consumed. However, some respiratory pigments evolved to react quite sensitively to disturbances in blood homeostasis, especially in pH, to allow for fine controlled oxygen and CO_2 transport (e.g. Mangum, 1990; Melzner et al., 2007). It is thus not surprising that there have been high evolutionary pressures on the selection for physiotypes that on the one hand are able to cope with highly variable CO_2 fluxes, but on the other hand simultaneously "protect" extracellular pH within acceptable limits.

Excretory CO_2 is mainly transported in the form of bicarbonate in the extracellular fluid, as the capacity for transport of physically dissolved CO_2 is quite limited. In all active animal taxa investigated so far, this process is greatly dependent on the ubiquitous enzyme carbonic anhydrase. Currently, most information on CO_2 excretion in aquatic ectothermic animals is available for teleost fish: CO_2 diffuses from the metabolically active tissues into capillaries and into red blood cells, where bicarbonate ions are formed via carbonic anhydrase catalyzed hydration (as teleost fish lack plasma carbonic anhydrase). Protons generated during this reaction are bound to the respiratory pigment and thereby aid in the release of oxygen (Bohr shift). Bicarbonate is then transported into the plasma in exchange for Cl^- via electroneutral anion exchangers. In the gill vasculature the reverse process takes place: Transport of bicarbonate into the red blood cells and carbonic anhydrase catalyzed dehydration enable rapid diffusion of molecular CO_2 across the thin

gill epithelium and release into the surrounding water (see Tufts and Perry, 1998, for a review). During the short transit time through the gill vasculature (0.5 to 2.5 s; Cameron and Polhemus, 1974) approximately 12 to 35% of blood [HCO_3^-] is transformed and excreted (Perry, 1986). While sufficient capacities of carbonic anhydrase are necessary within the red blood cells to enable a rapid dehydration of bicarbonate during the gill passage (Henry and Swenson, 2000), the rate limiting step in CO_2 excretion in teleosts is thought to be the transfer of plasma bicarbonate into the red blood cell via the band 3 anion exchanger (e.g. Perry and Gilmour, 1993; Wood and Munger, 1994). Recent experimental evidence could convincingly establish that the rate of CO_2 excretion across gill epithelia is diffusion limited (e.g. Perry and Gilmour, 2006). Each anaemia (i.e. a low content of red blood cells in the blood) and elevated blood flow were observed to lead to elevated blood pCO_2, an effect, that could be reversed by experimentally making carbonic anhydrase available in fish plasma (Desforges et al., 2002; Gilmour and MacNeill, 2003).

During aerobic exercise, provision of oxygen to the working muscles becomes paramount and increases in metabolic rate are compensated for by elevated rates of blood convection (cardiac output). Other changes in the gill vasculature enable more efficient gas exchange, helping to maintain pCO_2, extracellular pH and [HCO_3^-] at control levels. Most important are increases in the perfused gill area (A in Eq. 2) and decreases in the gill epithelial thickness (E in Eq. 2), which are caused by increases in ventral aortic blood pressure (e.g. Kiceniuk and Jones, 1977; Randall and Daxboeck, 1984). However, elevated cardiac output can reduce gill transit time by a factor of three (Randall, 1982). As the CO_2 excretion system is already limited by the capacity of the red blood cell HCO_3^-/Cl^- exchange system, higher swimming velocities can result in slightly elevated blood pCO_2, a respiratory acidosis may develop (e.g. Brauner et al., 2000). Brauner et al. (2000) could also demonstrate that when their experimental fish (sea water acclimated rainbow trout, *Oncorhynchus mykiss*) were approaching their critical swimming speed (shortly before exhaustion), arterial pH was protected from acidification by rapid active accumulation of HCO_3^-. Extremely high blood pCO_2 values (>0.6 kPa; ca. 5900 μatm) and low extracellular pH values <7.5 are only encountered during and following exhaustive exercise (Fig. 2b, white symbols) in brachyuran crustaceans and teleost fish. These are mainly caused by anaerobic metabolism ("metabolic acidosis"): Force production by aerobic swimming muscles is complemented by the recruitment of anaerobic ("white") fibers; lactate and protons originate as metabolic end products. Both are eventually released into the extracellular fluid, where the protons can titrate plasma [HCO_3^-], thus decreasing extracellular pH (see Figs. 1a, 2c). However, rapid compensation processes are occurring during exhaustive exercise and particularly during the recovery phase. Gill ion-regulatory epithelia produce enormous net proton equivalent fluxes from the organism into the surrounding seawater, ranging in magnitude between 1200 μEq kg^{-1} h^{-1} (rainbow trout, *O. mykiss*; Holeton et al., 1983) and 4800 μEq kg^{-1} h^{-1} (blue crab, *Callinectes sapidus*; Booth et al., 1984) to restore the original acid-base status. Clearly, a powerful ion regulatory machinery can be made visible under conditions of extreme physical stress, the very same machinery that will then enable active organisms to compensate extracellular pH during hypercapnic disturbances (see above). It thus makes sense to take a closer look at ion-regulatory epithelia in the more active taxa.

7 The acid-base regulatory machinery and its main motor

Species specific mechanisms of transepithelial ion exchange have been reviewed, e.g. in Boron (2004), Claiborne et al. (2002), Perry and Gilmour (2006), and Wheatly and Henry (1992). However, we are far from exactly understanding the whole system of ion exchange mechanisms, especially in the invertebrate taxa. Interestingly, similar molecular components have been conserved in different marine animal groups. Gills are the primary sites of acid-base regulatory processes in all high metabolic rate marine taxa discussed in this text, in fish (Perry and Gilmour, 2006), crustaceans (Wheatly and Henry, 1992) and probably also in cephalopods (Schipp et al., 1979). In fish, specialized epithelial cells, the mitochondria rich cells, contain a set of ion transporting proteins and channels that are important for acid-base regulation. Cells that are active in acid secretion contain electroneutral Na^+/H^+ exchangers or V-type H^+ ATPases, coupled energetically to apical Na^+ channels. While the latter system is thought to be more important for freshwater organisms which have to absorb Na^+ (e.g. Wilson et al., 2000), the former can operate on the favourable Na^+ gradient between seawater and cytosol, shuttling one H^+ out of the cell for each Na^+ imported. While Na^+/H^+ exchangers do not directly consume energy (there is no ATPase directly linked to these proteins), they essentially operate on the energy spent by the ATP consuming sodium pump (Na^+/K^+ ATPase). Basolateral Na^+/K^+ ATPase is thus commonly considered the motor of the ion-regulatory machinery in marine animal gills. Pumping two K^+ into the cell while simultaneously removing three Na^+, it creates the low intracellular [Na^+] typical for all animal cells and thus is partly responsible for the cell's membrane potential. One potential mechanism for the removal of acid during a respiratory acidosis could be the following (established from results of studies in teleost fish; see Fig. 4): Excess CO_2 diffuses into the mitochondria rich cells and is instantly hydrated by cytosolic carbonic anhydrase into protons and bicarbonate ions. While the protons are exported via the Na^+/H^+ exchanger, bicarbonate could be released into the plasma by means of basolateral Cl^-/HCO_3^- exchangers or Na^+/HCO_3^- co-transporters.

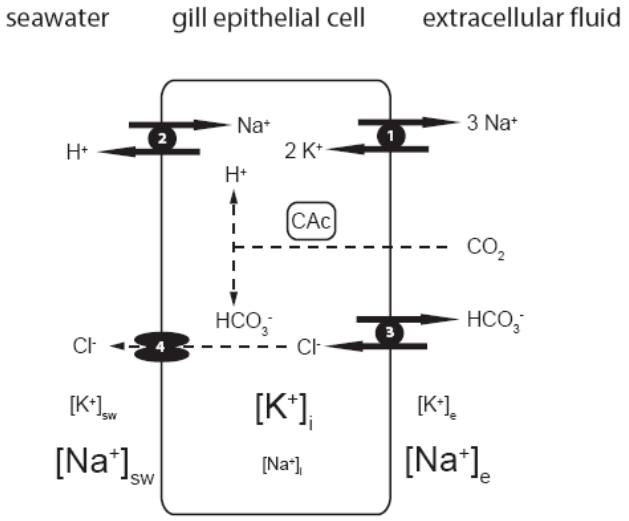

Fig. 4. Simplified schematic depiction of an epithelial gill cell (ionocyte) of a teleost fish (adapted from Perry and Gilmour, 2006). Decapod crustacean and cephalopod gill epithelia are equipped with similar proteins. **(1)**=Na^+/K^+ ATPase, **(2)**=Na^+/H^+ exchanger, **(3)**=Cl^-/HCO_3^- exchanger, **(4)**=Cl^- channel (e.g. CFTR), CAc = cytoplasmatic carbonic anhydrase. Na^+/K^+ ATPase is responsible for the low intracellular Na^+ and high K^+ concentration. Secondary active transporters, such as Na^+/H^+ exchanger can utilize the sodium gradient to export H^+. H^+ are produced when CO_2 is hydrated by CAc. The resulting HCO_3^- can be transferred into the extracellular fluid (blood, hemolymph), while Cl^- is exported to the seawater through chloride channels to maintain electroneutrality.

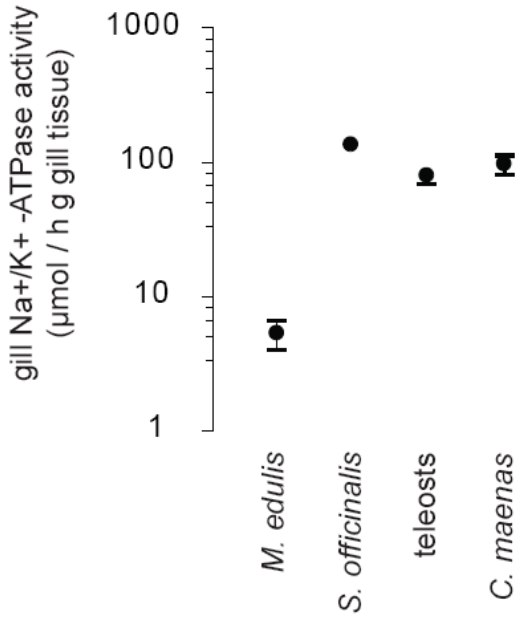

Fig. 5. Gill Na^+/K^+-ATPase activity measure in crude gill homogenates in two molluscs, the cephalopod *S. officinalis*, the bivalve *M. edulis* and the crustacean *Carcinus maenas*, acclimated and measured at 14 to 15°C vs. similar measurements on fish gill homogenates measured at 10°C. The teleost value represented in the figure is the mean of six species of shallow water teleosts from Gibbs and Somero (1990, their Table 1) and the eelpout *Z. viviparus* from Deigweiher et al. (2008). The mussel, cephalopod and crustacean measurements (Melzner and Lucassen, unpublished) were performed according to the protocol outlined in Melzner et al. (2009; see supplementary file for details: http://www.biogeosciences.net/6/2313/2009/bg-6-2313-2009-supplement.pdf).

This plasma bicarbonate may then undergo further protonation/dehydration/hydration cycles leading to a net proton extrusion via the gills. In order to maintain electroneutrality in the plasma, Cl^- is typically excreted, possibly via apical Cl^- channels (e.g. CFTR; see Perry and Gilmour, 2006; Deigweiher et al., 2008, for an extended discussion). However, the true mechanisms may be more complicated owing to the large number of transporters and channels present in gill epithelia (see also Deigweiher et al., 2008). However, basic processes can be suspected similar for decapod crustaceans and cephalopods as well; it is known by now that similar ion exchange proteins are also expressed in gills of these invertebrates (e.g. Schipp et al., 1979; Piermarini et al., 2007; Virkki et al., 2003; Henry and Swenson, 2000; Wheatly and Henry, 1992; Hu, Lucassen and Melzner, unpublished).

As Na^+/K^+ ATPase activity is the main energy sink and driving force for gill ion exchange processes in marine ectothermic animals, it can serve as a useful indicator for the overall capacity in ion and acid-base regulation. Consequently, gill Na^+/K^+ ATPase activity has been shown to correlate with metabolic rate in marine teleost species: Gibbs and Somero (1990) found highest Na^+/K^+ ATPase activities in shallow water, active species, while more inactive, deep-sea species activities were an order of magnitude lower. These relationships correspond with lower metabolic rates (e.g. Torres et al., 1979), lower gill surface areas (Hughes and Iwai, 1978) and lower muscle glycolytic enzyme capacities (Somero and Childress, 1980) in deep-sea vs. shallow water teleost species. The latter feature suggests that deep-sea fish rely less on aerobic as well as high-intensity, anaerobic "burst" swimming, thus likely would experience metabolic acidosis less often than shallow water species. Based on similar considerations, it has already been suggested that deep-sea marine animals might be significantly more vulnerable with respect to ocean acidification than shallow living species (Seibel and Walsh, 2001, 2003).

The gills of hypercapnia tolerant, shallow water marine taxa are characterized by surprisingly similar activities of Na^+/K^+ ATPase, an order of magnitude higher than those of sessile, hypometabolic species such as the blue mussel (see Fig. 5). While the comparison between high-power taxa and bivalves is confounded by the fact that the mussel gill primarily serves as a feeding organ, the lack of a true ion-regulatory organ in bivalves itself illustrates a key point: The evolution

of high metabolic rate physiotypes is closely connected to the development of extremely specialized organ structures to promote respiration and ion regulation that are very similar in their ultrastructural design (e.g. Evans et al., 2005: fish; Budelmann et al., 1999: cephalopoda; Taylor and Taylor, 1999: decapod crustacea).

Na^+/K^+ ATPase activities are modulated in vivo during metabolic rate transitions (e.g. exercise, specific dynamic action) on a short term basis by several second messenger pathways finally leading to a change in protein phoshorylation (e.g. Ramnanan and Storey, 2006). The most impressive example is the beta adrenergic stimulation of the enzyme in skeletal muscle which compensates the large K^+ efflux during exercise. Also changes in cytosolic ion composition, namely Na^+ and H^+ concentrations are involved in the regulation of Na^+/K^+-ATPase activity. In addition interaction with the cytoskeleton and membrane trafficking of the pump are regulatory mechanisms acutely controlling its function and availability (Bertorello and Katz, 1993). Long term regulation of Na^+/K^+-ATPase is under control of nuclear hormones. They trigger transcription of the subunits by binding to nuclear hormone responsive elements on the respective genes (Féraille and Doucet, 2001).

However, as has been shown that phosphorylation/dephosphorylation can activate or deactivate the enzyme, high-power animals may operate with a functional reserve that can be activated upon demand. Whether such a reserve is important for the rapid extracellular HCO_3^- accumulatory reaction observed upon acute hypercapnic exposure (see above, Fig. 1b) remains to be investigated. It has been recently shown in two marine teleost fish species, that gill Na^+/K^+ ATPase activity increases during acclimation to higher levels of hypercapnia (Deigweiher et al., 2008; Melzner et al., 2009). Rapid increases in activity in the eelpout *Zoarces viviparus* upon exposure to 1 kPa of CO_2 (ca. 9900 μatm) within two days have been observed to be related to elevated Na^+/K^+ ATPase mRNA and protein levels, suggesting that the enzyme is under tight transcriptional control. Longer acclimation (6 weeks) led to a ~80% increase in Na^+/K^+ ATPase activity. In cod (*Gadus morhua*) long term acclimation (4–12 months) led to increases in Na^+/K^+ ATPase activity and protein concentration at a pCO_2 of 0.6 kPa (ca. 5900 μatm), whereas no significant changes were observed at 0.3 kPa (ca. 3000 μatm; Melzner et al., 2009). Although this occurred in specimens from two distinct populations, it could nevertheless indicate that the control fitting of the gill ion regulatory machinery in many teleosts has high enough of an excess capacity to cope with the additional ion-regulatory challenge due to hypercapnia under more realistic scenarios of ocean acidification (i.e. 0.1 to 0.2 kPa; Caldeira and Wickett, 2003). Clearly, further studies need to address this exciting possibility.

8 Environmental hypercapnia

Further above it was stated that typical marine ectothermic animals are seldom exposed to environmental hypercapnia. This applies for large areas of the pelagic open ocean, however there are some special habitats that do provide elevated pCO_2 values to its inhabitants: intertidal regions, estuaries, oxygen-minimum zones, upwelling coastal regions or deep-sea vent systems (see e.g. Frankignoulle et al., 1996, 1998; Weigelt and Rumohr, 1986; Dwyer and Burnett, 1996; Feely et al., 2008; Wotton et al., 2008). While present mean surface ocean pCO_2 values average around 0.04 kPa (ca. 400 μatm) much higher values are reached in the above mentioned habitats. For example, pCO_2 values in Kiel Fjord, home to numerous calcifying organisms, can rise above 0.1 to 0.2 kPa (ca. 1000 to 2000 μatm) for prolonged times during summer and autumn (Thomsen, 2008). Similarly, upwelling processes lead to elevated near-shore pCO_2 values of up to 0.1 kPa (about 1000 μatm) in continental shelf areas off the Californian coast (Feely et al., 2008). Animals living in intertidal rockpools experience even stronger short-term fluctuations (Truchot and Duhamel-Jouve, 1980): depending on respective light conditions pCO_2 values during low tide emersion periods can range between about 0.35 kPa (3500 μatm, due to extensive nighttime respiration) and almost zero (due to high photosynthetic activity). High pCO_2 values (around 1200 μatm) have also been observed in oceanic oxygen minimum layers of intermediate depths (200–1000 m) where high community respiration rates cause hypoxia and associated hypercapnia (Brewer and Peltzer, 2009). However, special physiological and biochemical adaptations enable various animal groups to populate even the most extreme habitats with respect to hypercapnic, temperature and other chemical conditions – the deep-sea hydrothermal vent ecosystems. Amongst other things, this inhospitable environment challenges its inhabitants with pCO_2 conditions as high as 7 kPa (about 69 000 μatm). Nevertheless, the vent mussel *Bathymodiolus brevior* has been found able to precipitate shells under such high pCO_2/low-pH conditions (Tunnicliffe et al., 2009). Again, following our rationale from above, organisms already living under elevated pCO_2 in their particular habitats may encounter less of a relative change in pCO_2 than e.g. oceanic species, thus may be better adapted to future acidification (however, pCO_2 in CO_2 enriched habitats may not necessarily increase at the same rate as projected for the open ocean; see calculations in Brewer and Peltzer, 2009).

9 Ontogenetic hypercapnia: the hostile environment within egg capsules

Of large evolutionary relevance might be a special "ontogenetic habitat": the egg case and egg masses of many marine ectothermic animals. Recent determinations of pH, pO_2 and pCO_2 in the fluid surrounding the cephalopod

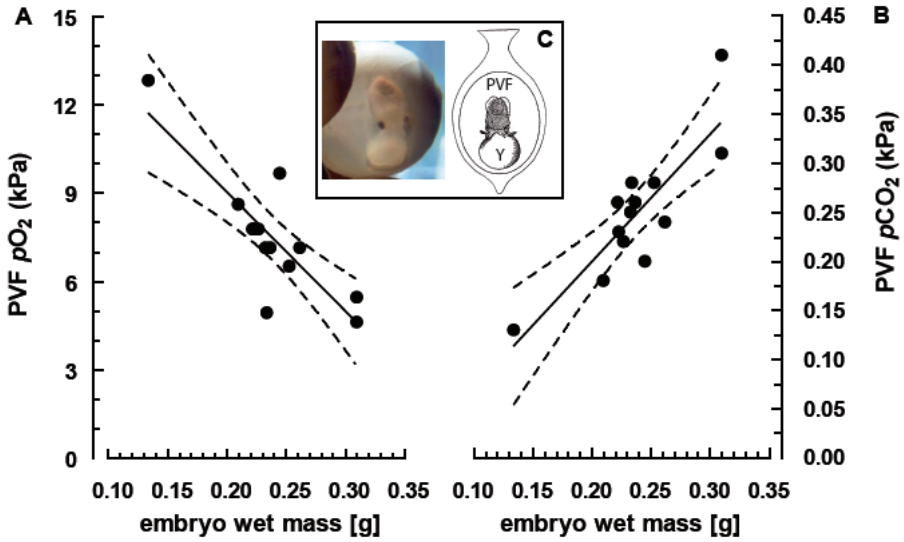

Fig. 6. Abiotic conditions in cuttlefish (cephalopod) eggs (modified, from Gutowska and Melzner, 2009). **(A)**: pO_2 in the fluid surrounding the embryo (perivitelline fluid) graphed against embryo wet mass. **(B)**: Perivitelline fluid pCO_2. Cuttlefish experience hypoxic and hypercapnic conditions towards the end of their embryonic development as the egg case serves as a diffusion barrier. **(C)**: Schematic illustration and photo of a late embryonic stage cuttlefish in its egg. These eggs can reach a diameter of almost 2 cm (see also supplementary video supplied by Gutowska and Melzner (2009) on Marine Biology homepage).

Sepia officinalis in its egg casing (perivitelline fluid) suggest that the egg case serves as a significant barrier to diffusion, of both CO_2 and O_2 (Gutowska and Melzner, 2009). Thus, embryos become progressively exposed to hypercapnic and hypoxic conditions the larger they grow within their capsules. Figure 4a shows linear relationships between wet mass and perivitelline fluid pO_2, pCO_2 and pH in late stage embryos shortly before hatching (stage 29, 30; Lemaire, 1970). Oxygen partial pressure declined to values around 4.6 kPa (from >12 kPa), while pCO_2 increased from 0.13 to 0.41 kPa (ca. 1300 to 4000 μatm); embryos thus were surrounded by about tenfold higher CO_2 values than those of ambient seawater (0.04 kPa; ca. 400 μatm) and pH values as low as 7.2 for 1 to 2 weeks at the end of their embryonic development. As extracellular pCO_2 values are always significantly elevated above ambient (see above), we can expect blood pCO_2 values of at least 0.6 to 0.7 kPa (ca. 5900 to 6900 μatm) in late *S. officinalis* hatchlings. These tolerated values have been shown to cause significant physiological disturbances in other, even adult, but more sensitive organisms such as echinoderms or bivalves. During their development within the egg case, cuttlefish even start to form their calcium carbonate (aragonite) shell, as well as their statoliths (Nixon and Mangold, 1998). Thus, obviously, hypercapnic stress is an integral part of the life cycle of *S. officinalis*. Coupled with special physiological adaptations (e.g. embryonic hemocyanins; Decleir et al., 1971), powerful net proton excretion mechanisms can be expected to be present already in these early life stages to cope with high perivitelline fluid pCO_2. Whether these high perivitelline fluid pCO_2 values render late embryonic stages of *S. officinalis* more vulnerable to additional hypercapnic stress in a progressively acidic ocean, needs to be determined. In order to maintain diffusion rates of CO_2 excretion elevated pCO_2 values would be additive to the already high perivitelline fluid pCO_2 values (see Fig. 3).

Unfortunately, no comparable data are available for egg fluid pCO_2 or pH of other marine ectothermic animals. However, assuming similar perivitelline fluid pO_2 to pCO_2 ratios, it seems likely that embryos from other marine taxa will also be surrounded by fluids of high pCO_2 and low pH. Decreased oxygen partial pressures have been measured for instance in shark eggs (Dietz and Davenport, 1987) and decapod crustacean egg masses (Fernandez et al., 2000, 2002). In this context, it is a striking analogy that embryos from most of the more CO_2 tolerant marine taxa like fish, cephalopods or brachyuran crustaceans all share a common ontogenetic characteristic, namely their relatively long developmental period and their growth to comparatively large size within the protecting egg shell. In contrast, development of many other marine invertebrate taxa (cnidarians, echinoderms, bivalves) is characterized by external fertilization, early hatching and the succession of various small, free larval stages within the water column: 55 to 85% of all benthic invertebrate species produce long-lived planktotrophic larvae spending weeks to months in the plankton, 5% produce short-lived planktotrophic larvae (spending hours to days in plankton), and about 10% produce lecithotrophic larvae (Thorson, 1950, 1966).

It is thus tempting to speculate, that the demand to cope with high pCO_2/low pH conditions during embryogenesis in *S. officinalis* and potentially, other species with lecithotrophic larval stages, selects for hypoxia- and hypercapnia-tolerant physiotypes with a highly developed ion regulatory apparatus that can efficiently export proton equivalents. The flip side of the coin may be that species like *S. officinalis* only can afford a direct mode of development and growth to comparatively large sizes (200 to 300 mg wet mass) within their egg capsules because the species is equipped with a capable gill ion-regulatory machinery to begin with. Clearly, this question of whether the life-history strategy selects for a certain physiological machinery, or vice versa, must remain unanswered at the moment.

Following our argumentation from above (see Sect. 5) we would expect embryonic stages of *S. officinalis* to be more tolerant towards future ocean acidification than larval stages of species that develop in open seawater from early on, as any changes in ocean pCO_2 result in relatively smaller changes in perivitelline fluid pCO_2 (Fig. 3). Unfortunately, at present we lack more data for fish, decapod crustaceans and cephalopods to follow this hypothesis further. However, several studies have shown that echinoderm and bivalve larval stages sometimes can react extremely sensitive towards hypercapnia (Kurihara, 2008; Dupont et al., 2008). A slight decrease in pH can have dramatic effects, inducing 100% mortality in only 8 days post fertilization in calcifying pelagic larvae of the brittlestar *Ophiothrix fragilis* due to larval and skeletal malformations (Dupont et al., 2008). An increased mortality is also observed in many other calcifying species such as some crustaceans, molluscs and echinoderms (see Dupont and Thorndyke, 2009). Nevertheless, available data on early developmental stages reveal contradictory results and apparent paradox. In the same phyla, different species are not, or sometimes positively, affected by near-future levels of ocean acidification, e.g. by decreases in mortality. For example, at low pH a significantly higher proportion of larvae successfully reached metamorphosis in the sea urchin *Strongylocentrotus droebachiensis* (see Dupont and Thorndyke, 2009).

Little information is available on the differential sensitivities towards hypercapnia in adult vs. embryonic/larval stages. Data presented by Kikkawa et al. (2003) on CO_2 tolerance of early life stages of marine teleosts indicate that the earliest stages (cleavage) were characterized by 2 to 3-fold lower values of lethal CO_2 concentration (LC_{50}=50% of test animals die within 24 h) than later stage embryos, larval stages and juveniles. The increase of this lethal concentration from the cleavage to the embryo stages may reflect the development of ion-regulatory chloride cells on the yolk sac membrane (cf. Ishimatsu et al., 2004, 2005). These results fit our concept of ion-regulatory ability defining hypercapnia tolerance in marine animals and indicates, that even in those organisms that display a high tolerance as juveniles/adults, the true bottleneck might be the earliest stages: gametes, zygotes and cleavage stages. Essentially, at the level of gametes and zygotes, broadcast-spawning teleosts (e.g. herring) do not differ much from the echinoderm situation, i.e. both groups release cells that are directly exposed to seawater and would experience large relative changes in pCO_2 during future ocean acidification. Thus, we would expect these stages to be similarly impacted by hypercapnia. However, while Havenhand et al. (2008) could demonstrate reductions in sperm mobility and fertilization success in an echinoderm species at relatively low seawater pCO_2 values (0.1 kPa, ca. 1000 μatm), no reductions in fertilization success, embryonic growth, mortality and hatching rate could be observed in herring (*Clupea harengus*) exposed to pCO_2 values between 0.05 and 0.4 kPa (ca. 500 to 3900 μatm; Franke, 2008). The latter results go along with an alternative hypothesis: Hamdoun and Epel (2007) have argued that embryos and larvae have an inherent set of cellular defense mechanisms that provide robustness to "buffer" environmental variability. However, these authors also agree that there may be some level of external (anthropogenic) stress that "depletes" the buffering reserve given to early life stages. Clearly, many more studies are needed that titrate sensitivities of these early life stages to elevated pCO_2 to create meaningful comparisons.

In summary, recent measurements of pCO_2 in marine animal eggs showed that significant levels of hypercapnia up to 3900 μatm can be part of the normal life cycle of cephalopods (Gutowska and Melzner, 2009) and, judging from oxygen partial pressures in eggs of other taxa, probably also of decapod crustaceans and teleost/elasmobranch fish. Thus, the defence machinery against hypercapnia might already be developed (and challenged) at the beginning of the life cycle, probably in many of the more hypercapnia tolerant species.

10 Synthesis and conclusions

Hypometabolism has previously been suggested to render animals more sensitive to ocean acidification. Seibel and Walsh (2001, 2003) convincingly argued that hypometabolic deep-sea animals might be significantly more vulnerable to future ocean acidification. Knoll et al. (2007) suggested that mass extinction of primarily hypometabolic marine genera at the Permian-Triassic boundary was mainly triggered by high levels of seawater hypercapnia.

In this paper, we tried to summarize some of those physiological traits that distinguish temperate, subtidal high-power marine ectothermic taxa (brachyuran crustaceans, teleost fish, cephalopods) from more hypometabolic ones (bivalves, echinodermata) and outlined their possible relevance for an increased tolerance towards environmental hypercapnia. We explained why the evolution of high metabolic physiotypes simultaneously led to the co-evolution of a physiotype that might be pre-adapted to cope with future ocean acidification, as it has acquired an ion-regulatory machinery that can

protect body fluids from excessive acidification and, potentially, metabolic depression. Here we will briefly summarize these adaptations and point at the crucial role of further studying early life stages, as they might be most vulnerable.

High-power physiotypes, such as teleosts, cephalopods and many brachyuran crustaceans, need advanced blood oxygen and CO_2 transport mechanisms to support their active lifestyles. The invention of blood oxygen binding proteins facilitated oxygen (and CO_2) transport, but also constrained the taxa, as blood pigment pH sensitivity rendered the animals more vulnerable to extracellular pH disturbances. While the high amount of respiratory protein provided them with a high pH buffering capacity, an efficient acid-base and ion-regulatory machinery was nonetheless needed to balance shifts in extracellular pH. For apparent reasons (high rates of sea water perfusion), this apparatus was incorporated into the primary gas exchange organs, the gills. High metabolic rates called for almost equimolar rates of CO_2 excretion, which were causally linked to an efficient carbonate system manipulation machinery (carbonic anhydrase, anion exchangers in fish) in order to transport the necessary amounts of CO_2/dissolved inorganic carbon. High extracellular pCO_2 values of 0.1 to 0.4 kPa (ca. 1000 to 3900 μatm) under control conditions provided the diffusion gradient for CO_2 excretion across gill epithelia. These high extracellular pCO_2 values might be another key correlate and contribute to why marine metazoans with an extracellular convection system should be more tolerant towards future ocean acidification than unicellular organisms, as any future change in seawater pCO_2 is less of a relative change for organisms, whose cells are already surrounded by a high pCO_2 fluid (see Fig. 3). Numerous studies have shown that during extensive exercise many of the high-power taxa experience high pCO_2 oscillations due to combined respiratory and metabolic acidosis, illustrating that cells are occasionally exposed to very high pCO_2 values. In addition, the proton excretion machinery of the gills needs surplus capacity to eliminate the metabolic protons generated during aerobic and, especially, anaerobic exercise. This machinery scales with metabolic rate and the magnitude of metabolic rate fluctuations and may be extremely important under future conditions, as ocean acidification can be seen as a long-lasting respiratory acidosis. Comparable to rebalancing acid-base status after bursts of exercise, important extracellular pH compensation processes during hypercapnia rely on efficient (net) proton excretion. Thus, the more CO_2 tolerant physiotype that can more or less manipulate its extracellular environment is a direct consequence of an active, high metabolic life style.

It is quite likely that these physiological traits already play a very important role during embryonic development, when high pCO_2 values develop in and around egg capsules owing to the egg case serving as a diffusion barrier (cephalopods, fish) or due to perfusion problems (crustacean egg masses). Thus hypercapnia may be an important natural stressor during the course of ontogeny in many marine animal taxa.

An active high-metabolic lifestyle and natural hypercapnia during the course of embryonic development, as well as within the natural habitat constitute factors that pre-adapt animals to cope better with hypercapnia/future ocean acidification. Of course it needs to be emphasized that we are discussing probabilities rather than certainties; biological diversity is too great to make universal statements on the potential vulnerability on the species/genus level. An analogy from the past exemplifies this point: Knoll et al. (2007) state that selectively, more hypo-metabolic, heavily calcified taxa suffered higher species losses during the Permian-Triassic mass extinction than high-metabolic taxa with more advanced circulatory systems and respiratory surfaces. While 81% of hypometabolic genera (calcareous sponges, corals, brachiopods, bryozoans and most echinodermata) were lost during this time period, only 38% of the more hypermetabolic genera (molluscs, arthropods, chordates) suffered this fate. Multiple transition stages will most likely be observed between "tolerant" and "sensitive" groups, however, the general physiological principles outlined in this paper will, on average, define the trends.

The true bottleneck, even for the seemingly hypercapnia tolerant organisms might, however, be located in very early ontogeny. Gametes of broadcast spawners, the fertilization reaction in open seawater, the zygote and early cleavage stages might be especially vulnerable in all taxa, as for these stages, any change in ocean pCO_2 constitutes a much higher relative change in external pCO_2 than for cells of the later ontogenetic stages (see Fig. 3; however, the alternative views of Hamdoun and Epel, 2007, also should be kept in mind). In addition, specialized ion-regulatory cells develop well after cleavage. However, these stages are the most difficult to study and only little information is available at present. The generation boundary thus clearly should be a major area of research activity in the near future.

We see this paper as an attempt to highlight some common features of certain animal groups that have a high capacity to perform well in physiological terms on the short to medium scale despite high environmental pCO_2. As was stated in the beginning, multi generation experiments are largely missing at the moment and crucial life stages have not been considered. In addition, a large research effort is currently focusing on those taxa that are seemingly most sensitive to ocean acidification, while for a mechanistic understanding it is quite important to study the (apparently) more tolerant ones as well. We therefore are only beginning to see the "patterns" that define tolerance vs. sensitivity to future ocean acidification. However, past extinction events give us a good idea where to look for potential survivors in a more acidified ocean.

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4. Discussion

Acidification of seawater has been shown to negatively affect growth and calcification in many marine invertebrates (Hoegh-Guldberg et al. 2007, Fabry et al. 2008). In order to be able to better predict the biological impacts of future ocean acidification, common physiological mechanisms that underlie hypercapnia sensitivity need to be identified and understood in various invertebrate groups (Pörtner 2008). The present thesis examined the physiological responses of the cephalopod *S. officinalis* to acidified seawater conditions due to elevated pCO_2.

Many inactive marine invertebrates are weak acid-base regulators and are unable to compensate the acidotic shift in extracellular pH induced by elevated pCO_2. It is hypothesized that more active invertebrates with high metabolic rates and higher ion regulatory capacities, will therefore be able to more tightly regulate their acid-base equilibria (Seibel and Walsh 2001, 2003, Pörtner 2008). The cuttlefish *Sepia officinalis* is a very active invertebrate with a high metabolic rate. The strong acid-base regulatory response of *S. officinalis* to elevated seawater pCO_2 is discussed in section 4.1 (Publication 1).

The acute intolerance of cephalopods to hypercapnia was hypothesized to be based on the high pH sensitivity of hemocyanin oxygen binding (Reipschläger and Pörtner 1996). It was suggested that cephalopods could potentially be unable to compensate the acidotic shift in their blood pH during hypercapnic exposure, thus risking suffocation due to the incomplete oxygenation of arterial blood. The relative insensitivity of cuttlefish blood oxygenation to pH changes, compared to that of squid, is discussed in section 4.2, along with the stabilizing effect of partial blood pH compensation during hypercapnia in *S. officinalis* (Publication 1).

The association of uncompensated acidosis in the extracellular space with the onset of metabolic depression during hypercapnia has been proposed in a recent model (Pörtner et al., 2004). Amongst the molluscs, a 0.2 unit shift in haemolymph pH was found in parallel to reduced metabolism and growth rates in the mussel *Mytilus galloprovincialis* during exposure to a seawater pCO_2 of 0.5 kPa. (Michaelidis et al. 2005). In contrast, *S. officinalis* maintains control growth rates and gross growth efficiencies during long-term exposure to a pCO_2 of 0.6 kPa. Long-term growth performance of *S. officinalis* under elevated seawater pCO_2 is discussed in section 4.3 (Publication 2).

The physiological mechanisms behind reduced calcification rates during exposure to elevated pCO_2 in marine molluscs, echinoderms, and cnidarians (Michaelidis et al. 2005, Shirayama and Thornton 2005, Langdon and Atkinson 2005) are still unkown. A closer examination of calcification sensitivities in invertebrates with a greater degree of control over the extracellular environment at the mineralization site could provide insights to the mechanistic processes that underlie reduced calcification rates during hypercapnic exposure. In contrast to the majority of previous studies, calcification rates actually increased in *S. officinalis* during long-

term exposure to 0.6 kPa CO_2. Changes in cuttlebone calcification are discussed in section 4.4 (Publication 3).

Early life stages of marine invertebrates have been shown to be more sensitive to elevated seawater pCO_2 and lower pH (Kurihara 2008). The effects of long-term incubation at a pCO_2 of 0.6 kPa, on embryonic and hatchling *S. officinalis* stages was compared to that of adults. The delayed growth and increased mortality of early stage *S. officinalis* during exposure to elevated seawater pCO_2, is discussed in section 4.5 (Publication 4).

4.1 The acute regulatory response of *S. officinalis* to elevated seawater pCO_2.

During acute exposure to a pCO_2 of 0.6 kPa *S. officinalis* exhibited a extracellular pH (pHe) compensatory pattern which typifies organisms with a considerable acid-base regulatory ability. The cuttlefish partially compensated the respiratory acidosis present in its blood through a rapid increase in extracellular [HCO_3^-] (Fig. 4.1). However, *S. officinalis* did not fully compensate its pHe, blood pH remained 0.18 units below control values following 48 hours of hypercapnic exposure. If pHe was to be fully compensated at a blood pCO_2 around 1.0 kPa, *S. officinalis* would have needed to increase [HCO_3^-]e to approximately 17 mM (Fig. 4.1). However, the time course of observed HCO_3^-e accumulation does not suggest that such values would eventually be reached: [HCO_3^-]e followed the typical hyperbolic regulation pattern found in many other powerful ion-regulators such as teleosts and brachyuran crabs (e.g. Claiborne and Evans 1992, Toews et al. 1983), with 90% of the accumulatory response already being accomplished after 8h. Marine organisms that have been shown to fully compensate pHe typically do so within one continuous regulatory reaction, often within 24 hours. However, it is not known whether long-term changes in the ion transport machinery of the gills can further alleviate acid-base disturbances. A remodelling of the gill ion-regulatory machinery has been observed in a marine fish after 6 weeks of acclimation to hypercapnia (Deigweiher et al. 2008).

The HCO_3^- accumulatory response that was measured in *S. officinalis* is considerably higher than that of inactive invertebrates examined in recent hypercapnia studies (Table 4.1). In the sea urchin *Psammechinus miliaris* HCO_3^-accumulation only followed the slope of the non-bicarbonate buffer line (β_{NB}), indicating no active buffering effort during acute exposure to 0.25 kPa CO_2 (Miles et al. 2007). In the mussel *Mytilus galloprovincialis*, the minor increase in haemolymph [HCO_3^-] during exposure to 0.5 kPa CO_2, was attributed to shell dissolution and not active transport (Michaelidis et al. 2005), a conclusion also reached by Lindinger et al. (1984) for the mussel *M. edulis*. In all of the comparatively inactive invertebrates examined to date, the weak regulatory effort during hypercapnia resulted in an uncomplete compensation of pHe. The acid-base regulatory abilities of crustaceans and teleost fish have been found to be significantly greater than those of the inactive invertebrates. During exposure to moderate and high CO_2

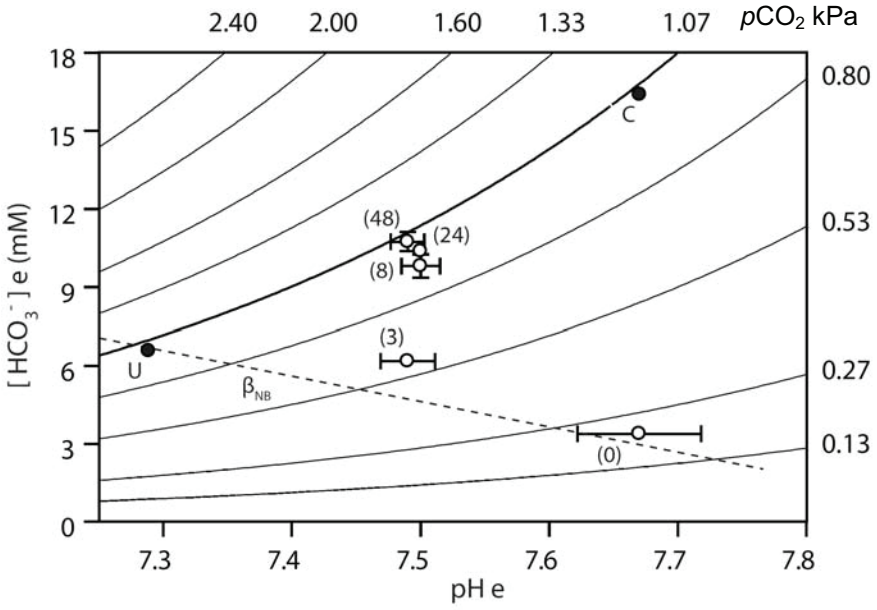

Figure 4.1. pH-bicarbonate diagram showing the time course of acid-base compensation during 48 hours of hypercapnic (0.6 kPa CO_2) exposure in *S. officinalis* blood. The non-bicarbonate buffer line (NBB) is delineated with a dashed line. The solid curved lines represent CO_2 isopleths. The point labeled U illustrates the theoretical uncompensated acid-base status in the absence of active HCO_3^- accumulation. Point C illustrates the theoretical full compensation of blood pH. n = 5, means ± SD (Pub. 1).

levels, 0.5-1.0 kPa, fish increase the [HCO_3^-] in their blood by about 20mM and fully compensate an initial acidotic shift in pHe (Table 4.1) (Michaelidis et al. 2007, Larsen et al. 1997, Iwama et al. 1993, Toews et al. 1983).

The only invertebrates know to date that are capable of fully compensating pHe through a strong regulatory response during hypercapnia, are some decapod crustaceans. *Cancer magister* has been shown to nearly compensate pHe (-0.07 units) by increasing its HCO_3^-e by 12 mM during 24 hours of acute exposure to 1.1 kPa CO_2 (Pane and Barry 2007). An even stronger regulatory response was measured in *Necora puber* during exposure to 1.1 kPa CO_2, [HCO_3^-] rose by over 20mM and pHe was actually 0.07 units more alkaline than control values (Spicer et al. 2006). Strong HCO_3^- accumulatory responses to hypercapnia in decapod crustaceans have also been shown in several older studies (Truchot 1975, 1979 (*Carcinus maenas*); Cameron 1978, Cameron and Iwama 1987, Booth et al. 1984 (*Callinectes sapidus*)). The acid-base regulatory ability of *S. officinalis* appears to fall between that of the weakly regulating inactive invertebrates and the strong regulators such as crustaceans. It is concluded that the regulation of acid-base equilibria in *S. officinalis* takes place through active proton-equivalent ion transport, as in crustaceans and fish, and not through dissolution of the calcified cuttlebone. This conclusion is based on the rate and magnitude of HCO_3^- accumulation during acute exposure, and elevated calcification rates under long-term hypercapnic conditions (see section 4.4).

The primary sites of acid-base regulation in *S. officinalis* are most likely the gill epithelia (Schipp et al. 1979). However some of the excretory organs, such as the renal and pancreatic appendages (Schipp et al. 1975, Schipp and Boletzky 1976), could also play an important role, as in teleosts (Grosell 2006). The epithelium of the gill (branchial epithelium) is divided into transport and respiratory regions (Figure 4.2). The morphology of the inner transport region,

with high mitochondrial and vesicle density, a basal labyrinth and a blurred brush border, is typical of ion transport active epithelia. Enzymes involved in energy metabolism (malate, succinate, and glucose-6-phospate dehydrogenase), as well as $Na^{+/}K^{+}$-ATPase, are located in this region of the gill (Schipp et al. 1979, Donaubauer 1981). The functions of the renal and pancreatic appendages are associated with osmotic regulation, active ion transport and excretion, also making them potentially important for acid-base regulation. Preliminary studies on the renal fluid of *S. officinalis*, have found an acidic pH<6, low [HCO_3^-] and high [NH_4^+] (20% of the total cations, Gutowska unpublished, Robertson 1953). Thus, the excretion of renal fluid could also be a path for H^+, and NH_4^+ excretion (Schipp and Boletzky 1975, Schipp et al. 1975).

Table 4.1. Changes in extracellular acid-base parameters of selected marine organisms in response to acute hypercapnia. Values are expressed as means (SE). Sea urchin *P. miliaris*, 3-5cm test diameter 10°C (Miles et al. 2007); Mussel *M. galloprovincialis*, size n.d. 18°C (Michaeilidis et al. 2005); Cephalopod *S. officinalis*, 300-700g 17°C (this study); Crab *C. magister*, 500-1000g 10°C (Payne and Barry 2007); Teleost *S. aurata*, 50g 18°C (Michaelidis et al. 2007); Teleost *G. morhua*, 230-525g 12°C (Larsen et al. 1997). (Pub.1).

	Control extracellular values			Hypercapnic exposure		Hypercapnic extracellular values		
Species	pH	[HCO_3^-] (mM)	pCO_2 (kPa)	Seawater pCO_2 (kPa)	Exposure (days)	Maximum Δ pH	Final Δ pH	Δ [HCO_3^-] (mM)
Psammechinus miliaris	7.40 (0.05)*	1.8 (0.2)	0.13 (x)*	0.25	8	-0.55	-0.55	+1.5*
Mytilus galloprovincialis	7.55 (0.02)	1.62 (0.12)	0.15 (0.03)	0.51	8	-0.19	-0.19	+2.4
Sepia officinalis	7.67 (0.05)	3.38 (0.12)	0.22 (0.03)	0.60	2	-0.18	-0.18	+6.7
Cancer magister	7.82 (0.05)*	6.5 (0.5)*	0.28 (x)*	1.10	1	-0.41*	-0.07*	+12.0
Sparus aurata	7.65 (0.03)	7.34 (0.54)	0.34 (0.04)	0.51	10	-0.24	-0.06*	+19.2*
Gadus morhua	7.90 (x)*	10.5 (x)*	0.43 (x)*	1.10	1	-0.18	+0.02	+21.0*

* = values read from figures
x = SD < minimum readable from figure
Maximum ΔpHe = maximum pH decrease measured in the respective study during acute hypercapnic exposure.
Final ΔpHe and Δ[HCO_3^-] (mM) = values reported for the endpoint measurement in each study.

Future experimental work will focus on the identification and localization of the relevant transport molecules responsible for acid-base regulation in *S. officinalis*. As to date, almost nothing is known about the ion transport machinery in cephalopods. The first studies will focus on identifying potential anion exchangers Cl^-/ HCO_3^-, Na^+/ HCO_3^-cotransporters, and Na^+/H^+ exchangers, as these are some of the primary ion transport proteins that have been identified to be responsible for the maintenance of acid-base equilibria in marine teleosts (Evans et al. 2005, Perry and Gilmour 2006). It will be interesting to follow the changes in expression patterns of key ion transporters during both acute and long-term exposure to hypercapnic conditions in *S. officinalis*. A recent study working with the teleost *Zoarces viviparous* found that Na^+/K^- ATPase capacities steadily increased during six weeks of exposure to hypercapnic conditions (Deigweiher et al. 2008). The measurement of expression levels, as well as activities, of key enzymes used for acid-base regulation, such as Na^+/K^- ATPase and carbonic anhydrase, will also be important in increasing our mechanistic understanding of acid-base regulation in the cuttlefish.

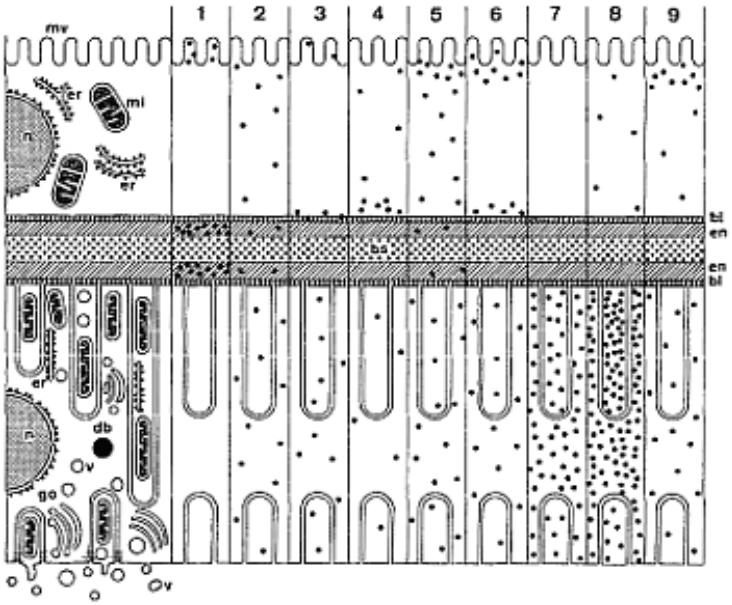

Figure 4.2. Schematic illustration of the inner transporting (below) and the outer respiratory (above) parts of the branchial epithelium (from Schipp et al. 1979). Basal lamina (bl), dense bodies (db), endothelium (en), endoplasmic reticulum (er), Golgi system (go), microvilli (mv), nucleus (n), vacuoles (v). The histological localization of enzymes is summarized: alkaline phosphatase (1), acid phosphatase (2), carbonic anhydrase (3), Mg^{2+}-adenosine triphosphatase (4), β-glucuronidase (5), glucose-6-phosphate dehydrogenase (6), malate dehydrogenase (7), succino-dehydrogenase (8), monoamine oxidase (9). The dehydrogenases which are involved in energy metabolism occur mostly in the inner epithelium which is rich in mitochondria. The enzyme pattern and morphology of the inner epithelium are typical of transport active epithelia.

The close regulation of pH during acid-base disturbances is energetically costly as many of the ion transport proteins responsible for the maintenance of organismic acid-base homeostasis are ATP dependent (Dubyak, 2004). Working with *in vivo* ^{31}P NMR, the inorganic phosphate (Pi) / phospho-L- arginine (PLA) ratio in the mantle muscle of *S. officinalis* was monitored during hypercapnic exposure 0.6 kPa CO_2, and found it to remain stable over the entire experimental period (Pub.1 Fig.4). This demonstrates efficient pHi regulation and that anaerobic metabolic pathways were not challenged to provide the extra energy demand for acid-base regulation during hypercapnia. It is questionable if the very minor, 0.03 unit, decrease measured in steady state intracellular pH during acute hypercapnia was a physiologically relevant stressor for the cuttlefish *S. officinalis*. In contrast to studies on inactive invertebrates that are weak acid-base regulators (Pörtner et al. 1998), our findings of stable ATP, PLA and Pi concentrations during minor decrements in pHi are indicative of a balanced thermodynamic environment that is necessary for the proper function of cellular ATPases (Kammermeier 1984).

The implications of a stable Pi/PLA ratio in *S. officinalis*, for the maintenance of routine metabolism during acute hypercapnia, are in line with our measurements of conserved standard metabolic rates during acute exposure to 0.6 kPa CO_2 (Pub. 2 Fig. 3). The absence of a either a depression or elevation of metabolism indicates an immediate regulatory response to the hypercapnic stimulus. The very slight increase measured in ventilation frequency also suggests that oxygen demand was not significantly elevated during hypercapnia (Pub.2 Fig.5). Using the correlation between ventilation frequency and oxygen consumption rate from Melzner et al. (2006a), the 2 bpm increase that was measured during acute hypercapnia corresponds to a less than 5 % increase in whole animal oxygen consumption. Such an increase would, however, probably be concealed by the inherent variability of the metabolic rate determinations. Again, this matches the conserved standard metabolic rates measured during acute hypercapnic

exposure. It is quite apparent that the cuttlefish ecotype is not only an efficient acid-base regulator, but is also able to do so without disturbing metabolic equilibria in characteristic tissues or compromising aerobic capacities.

4.2 Sensitivity of blood oxygenation in the cuttlefish during acute hypercapnic exposure.

Oceanic squid species (e.g. *Illex illecebrosus*), who have some of the highest metabolic rates and Bohr coefficients amongst all cephalopods (Pörtner, 1990), have been predicted to be one of the most acutely sensitive marine organisms to acute CO_2 exposures as in CO_2 disposal scenarios (Pörtner et al., 2004). In the oceanic squid *Illex illecebrosus*, a hypothetical exposure to 0.6 kPa CO_2 was calculated to elicit a 0.2 unit decrease in blood pH if no compensatory HCO_3^- accumulation took place and blood pH followed the non-bicarbonate buffer line (NBB). Taking into consideration the steep slopes of the oxygen saturation curves along the *in vivo* pH range, a 0.2 pH unit decrease would reduce hemocyanin saturation by about 50% and lead to lethal asphyxiation. In fact, a recent study by Rosa and Seibel (2008) demonstrated slightly decreased rates of metabolism in the jumbo squid (*Dosidicus gigas*) exposed to acute hypercapnia (0.1 kPa CO_2). Even though cuttlefish are not as highly 'tuned' as oceanic squid, they could also be potentially sensitive to hypercapnic conditions in seawater due to the pH sensitivity of their oxygen binding pigment.

The present study demonstrates that blood pHe does not follow NBB when *S. officinalis* is exposed to hypercapnic conditions (Fig. 4.3). Rather, the bicarbonate accumulation response sets in immediately and prevents the drop of pHe below 7.5. At a blood pH of 7.5, hemocyanin function does not appear to be significantly compromised in the cuttlefish: Figure 4.3 illustrates the comparative insensitivity of *S. officinalis* blood to a 0.2 pH unit reduction starting from the *in vivo* pH value of 7.67 in venous blood from the anterior vena cava. In *S. officinalis* the pH value of venous and arterial blood have been reported to be nearly equal (< 0.04 unit difference) under control conditions (Johansen et al. 1982). It is important to keep in mind that if no active HCO_3^- accumulatory response had taken place, blood pH would have fallen below 7.3, and consequently reduced arterial saturation by at least 20%. The acid-base regulatory response in *S. officinalis* during hypercapnic exposure significantly reduced the decrease in pHe thus allowing for the maintenance of full oxygen pigment saturation. It is hypothesize that cuttlefish accumulate enough HCO_3^-e to maintain proper functioning of their respiratory pigment, but avoid the increased energetic costs that go along with full pHe compensation and the maintenance of very high blood [HCO_3^-] and [H^+] gradients. I further explain this hypothesis in section 4.3.

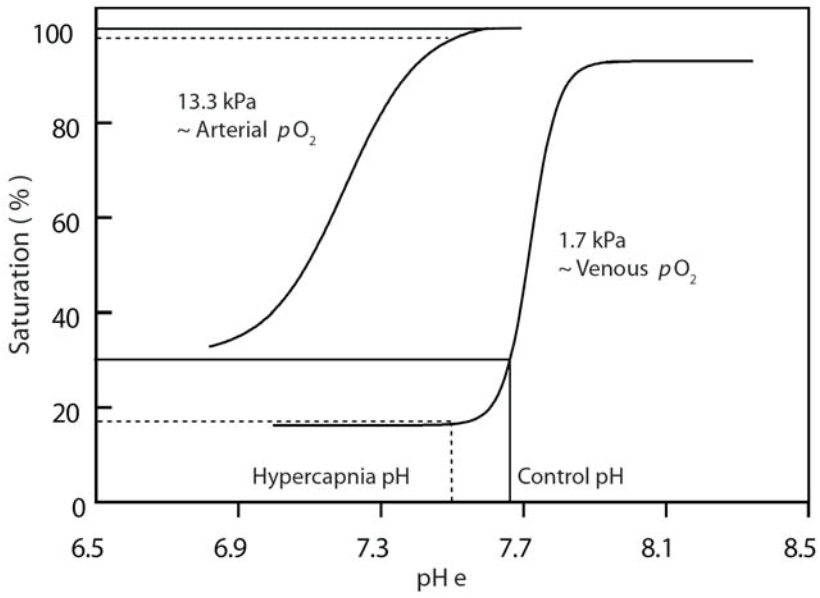

Figure 4.3. Potential changes in *S. officinalis* arterial and venous blood oxygen saturation in response to a 0.18 pH unit decrease in pH induced by acute exposure to 0.6 kPa CO_2. Oxygen saturation curves (measured at 20°C) are replotted from Zielinski et al., 2001, along with the *in vivo* blood pH value measured in venous blood from the anterior vena cava, adjusted according to the alpha-stat pH regulation pattern (Reeves 1976). The relative insensitivity of hemocyanin saturation to limited pH changes in *S. officinalis* protects the cuttlefish from oxygen limitation during acute hypercapnic exposure. (Pub.1).

Acid-base regulation in response to hypercapnia has not been directly measured in the most active cephalopods, oceanic squid. However, acid-base regulatory changes in *I. illecebrosus* and *Loligo pealei* were studied during exhaustive exercise (Pörtner et al., 1991). When blood pCO_2 rose to 0.37 kPa during jet locomotion, a 1.7 mM increase in [HCO_3^-], likely released from the musculature, protected the blood from acidification and thus maintained hemocyanin saturation in arterial blood (see Fig. 5 in Pörtner et al. 1991, Pörtner 1994). The tight regulation of blood parameters by *I. illecebrosus* in order to optimize hemocyanin function, most likely goes hand in hand with a high capacity for acid-base regulation. It is probable, that like the cuttlefish, *I. illecebrosus* is capable of accumulating significant amounts of compensatory HCO_3^- in response to hypercapnia. As the acute sensitivity of both squid and cuttlefish to hypercapnia is highly dependent on the magnitude and rate of exposure to elevated CO_2, further work is needed to define their tolerance limits.

4.3 Long-term growth performance of *S. officinalis* under elevated seawater pCO_2.

The results of our growth trial with juvenile *S. officinalis* show for the first time, that at least one marine invertebrate species is capable of maintaining somatic growth performance and gross growth efficiency levels at control values during long-term exposure to significantly elevated seawater CO_2 concentrations. *Sepia officinalis* juveniles cultured at ~4,000 and ~6,000ppm (0.4 and 0.6 kPa) CO_2 grew at the same rate as control animals, gaining body mass at a rate of approximately 4% body mass day^{-1} (Fig. 4.4). They also maintained their gross growth efficiency at control levels (Table 4.2). 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. These results are in stark contrast to existing invertebrate growth studies under elevated CO_2. Michaelidis et al. (2005) found that under comparable CO_2 levels to our

study, and over a growth period of 3 months, shell length and soft body mass in *Mytilus galloprovincialis* were reduced by 55% and 70% respectively (as calculated from their Fig. 3). Even more striking is the study reported by Shirayama and 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 CO_2 for half a year. Clearly, *S. officinalis* does not exhibit sensitivity to elevated CO_2 levels within the range of concentrations that elicits a negative response in most other invertebrates studied to date.

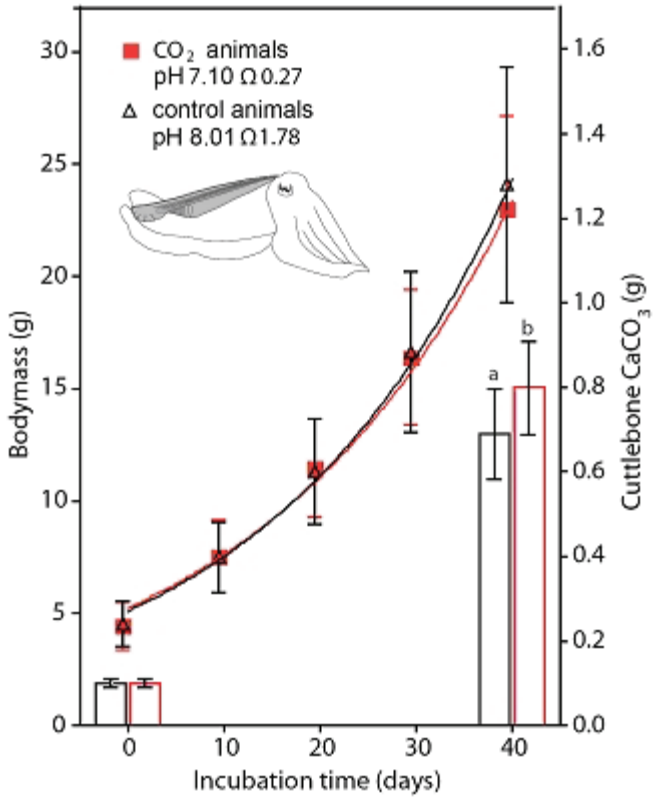

Figure 4.4. Growth and calcification in *S. officinalis* incubated under 0.6 kPa CO_2 (■) and control conditions (Δ). $CaCO_3$ accretion is shown (bars), means not sharing the same letter are significantly different. Data are mean values ± SD (n = 20). The calcified cuttlebone is shaded grey in the schematic drawing of *S. officinalis* Cuttlefish juveniles in both experimental groups gained body mass at a rate of approximately 4% body mass day $^{-1}$.During the six-week growth period all of the cuttlefish increased the mass of their cuttlebones by over 500%. The CO_2 incubated animals incorporated significantly more $CaCO_3$ in their cuttlebones than the control group, 0.80 ± 0.15 g versus 0.71 ± 0.15 g. Functional control of the cuttlebones (i.e. buoyancy regulation) did not appear to be negatively affected by low pH conditions. (Pub.2).

Taxa specific differences in acid-base regulatory capacity may be a crucial factor that could partially explain differing sensitivities to hypercapnia amongst marine organisms. The association of uncompensated acidosis in the extracellular space with the onset of metabolic depression during hypercapnia, has been proposed in a recent model (Pörtner et al., 2004; Reipschlager and Pörtner, 1996). It is hypothesized that this depression of metabolism is responsible for reduced growth rates during long-term hypercapnic exposure (Michaelidis et al. 2005). Inactive invertebrates with low metabolic rates and weak acid-base regulatory abilities are not able to fully compensate the acidotic shift in pHe during hypercapnia and have been found to be the least tolerant to elevated seawater pCO_2. At the other end of the spectrum, teleost fish who mostly have high metabolic rates and strong acid-base regulatory abilities, and fully compensate their pHe during hypercapnia are the most tolerant to elevated pCO_2. Even though the upper limits of long-term growth tolerance to elevated pCO_2 have not been examined in the cuttlefish, it will most likely not be as high as that of the most tolerant physiotypes like fish. This

conclusion is based on the fact that the acid-base regulatory ability of *S. officinalis* fits between those of the sensitive and tolerant types.

Table 4.2. Growth and calcification during each of two separate trials under elevated CO_2 conditions. Values are mean ± SD, n=20 in each of the incubation groups. (Pub. 2).

Incubation group	Initial wet mass (g)	Initial mantle length (mm)	Final wet mass (g)	Final mantle length (mm)	Daily mass gain%	GGE (%)
Control	2.69 ± 0.30	20.53 ± 0.14	11.63 ± 1.39	37.16 ± 1.88	4.0%	36.6 ± 6.2
CO_2 ~4000	2.70 ± 0.33	20.71 ± 0.17	11.16 ± 1.40	36.33 ± 2.29	3.8%	38.9 ± 3.6
Cnt	4.61 ± 1.01	27.83 ± 2.47	24.15 ± 5.25	52.84 ± 4.03	3.9%	39.5 ± 4.5
CO_2 ~6000	4.50 ± 1.08	27.90 ± 2.39	23.06 ± 4.15	52.01 ± 4.76	3.7%	39.4 ± 3.7

The long-term tolerance of *S. officinalis* to 0.6 kPa CO_2 is particularly interesting because there is a potential that the cuttlefish supported growth performance at control levels despite an uncompensated acidotic shift in pHe. pHe after long-term exposure has not been measured, but the time-course of HCO_3^- accumulation suggests that no major compensatory increases occurred beyond the initial 48 hour exposure. It is worth noting that during the first 48 hours of exposure to 0.6 kPa CO_2, when pHe had decrease by 0.18 units, the standard metabolic rates of *S. officianlis* remained at controlled levels and no indication of metabolic depression was evident. In order to support long-term growth rates of 4% body mass per day, the metabolic rates of *S. officinalis* must have remained high and supported a conserved energy budget. As far as I am aware only one other invertebrate has been reported to not experience metabolic depression during exposure to elevated pCO_2. The brittle star *Amphiura filiformis* actually increased its metabolic rate during exposure to a seawater pH of 7.3 (Wood et al. 2008). However, unlike in the cuttlefish, a restructuring of the energy budget is evident in the brittle stars due to the significant wastage of arm muscle. The reasons behind elevated metabolic rates during hypercapnia in the brittle star remain unknown, both elevated activity levels as well as increased costs of acid-base regulation could have contributed to an elevation in metabolism.

The response of *S. officinalis* to hypercapnic exposure raises questions concerning the potentially broad range of sensitivity to changes in acid-base status amongst invertebrates, as well as to the underlying mechanistic origins. An intriguing possibility exists that extracellular sensing and regulation in the cuttlefish are predominantly focused on adequate oxygen supply, rather than on strict control of pCO_2 / pH, as cephalopods rely on highly efficient blood oxygen extraction to support their high metabolic rates and low blood oxygen carrying capacities (e.g. Melzner et al,, 2007a, O'Dor & Webber 1986). Further studies are needed to better characterize the connection between acid-base status and animal fitness in various marine species, especially during long-term hypercapnic exposures and at lower seawater pCO_2's. More work in this

direction is particularly critical when testing the hypothesis that the sensitivity of marine invertebrates to ocean acidification is based on their acid-base regulatory abilities.

4.4 Increased calcification of the cuttlebone during exposure to elevated seawater pCO_2.

Unlike most invertebrates examined to date, *S. officinalis* is capable of maintaining high calcification rates during exposure to elevated seawater pCO_2. In fact, cuttlefish increased their calcification rate during a six-week growth trial where they nearly doubled the length of their cuttlebones during exposure to seawater CO_2 levels of ~6,000ppm and Ω_{arag} values of 0.27. The average cuttlebone length attained by the experimental group incubated under 0.6 kPa CO_2 was slightly, but still significantly, shorter than the control group. The small 3 mm difference in average length was not detected in the mantle length measurements of the growth trial animals due to the greater inaccuracy of the measurement. Interestingly, despite their slightly shorter length, the hypercapnic cuttlebones accreted 0.2 g more dry mass on average (Fig. 4.5A). The relationship between cuttlebone length and width was preserved. However, the height of the hypercapnic incubated cuttlebones in relation to length was significantly reduced (Fig. 4.5B). This is an interesting point as this means that the cuttlebones incubated under elevated pCO_2 became heavier, and less porous.

Comparison of the cuttlebone ultrastructure between the two groups clearly indicated the formation of a denser $CaCO_3$ structure by the cuttlefish exposed to elevated seawater pCO_2. The CO_2 treatment cuttlebones have a dramatically reduced lamellar spacing (Fig. 4.6). The reduction in the height of the CO_2 incubated cuttlebones can fully be accounted for by the 50% reduction in lamellar spacing. Not only did the lamellar spacing decrease, but the thickness of the lamellar and pillar walls significantly increased (Table 2, Pub.3). This increase in deposited $CaCO_3$ substantially contributed to the greater mass of the CO_2 treatment cuttlebones. The general ultrastructure of the cuttlebones was conserved, however a greater degree of irregularity was present in the linearity and thickness of the lamellar walls and pillars. Irregularity of $CaCO_3$ deposition can be attributed to reduction in organic matrix incorporation (Pub.3, Fig.10 and 11). The role of the organic matrix is crucial in the formation of calcified structures as it controls crystal nucleation, polymorph selection and crystal orientation of the mineralized $CaCO_3$ (Weiner and Traub 1984, Weiner and Dove 2003, Wilt et al. 2003). As the cellular pathways of organic matrix synthesis and secretion by the cuttlebone membrane are not known, no further interpretation of the reduction in non soluble matrix can be provided. The sensitivity of the structure and function of the organic matrix to long-term CO_2 exposure will be an important component determining the impact of ocean acidification on invertebrate calcification processes.

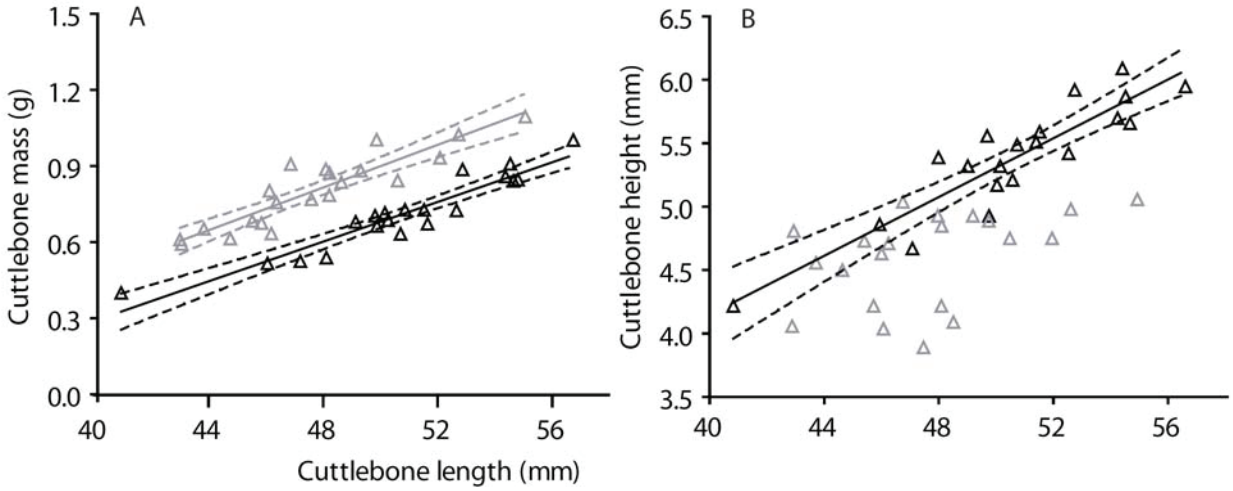

Figure 4.5. Comparison of morphometric relationships between cuttlebone length, mass, and height in control (black) and CO_2 incubated (grey) groups. A) The average mass of the CO_2 incubated cuttlebones was significantly greater than that of the control group, 0.80±0.15 versus 0.70±0.16 g, $p<0.03$ (n =20). B) The average height of the CO_2 incubated cuttlebones was significantly reduced compared to the control group, 4.6±0.4 versus 5.4±0.5 mm, $p<0.0001$ (n =20). Cuttlebones from animals incubated under 0.6 kPa CO_2 had a reduced volume compared to control cuttlebones, and yet a greater mass. (Pub. 3).

Despite significant ultrastructural changes, the functional role of the cuttlebone did not appear to be affected by long-term exposure to elevated seawater pCO_2. No changes in animal buoyancy or body posture that would have been indicative of functional failure were observed. However, the increase in $CaCO_3$ content of the cuttlebone should be regarded as a pathology and not a potentially beneficial modification. The density of the cuttlebone structure must remain low if it is to preserve its function as a buoyancy regulation device. A large enough increase in the weight of the cuttlebone could decrease the buoyancy of the cuttlefish, thus requiring it to invest more energy into maintaining its swimming posture while hunting. It is possible that such fine scale energetic changes were not resolved in the long-term growth trials, as the animals were fed *ad libitum* and did not need to be very active to succeed in prey capture. Unfortunately, as far as I am aware, there is no supporting literature that correlates changes in the microstructure of cephalopod chambered shells with animal energetics and physiology. It is also important to note that the majority of the cuttlebone region responsible for buoyancy control was mineralized under control conditions before the start of CO_2 exposure by the experimental animals. Future growth studies, where animals are raised under elevated pCO_2 conditions from the beginning of cuttlebone formation are necessary to fully assess how ultrastructural changes influence functional control.

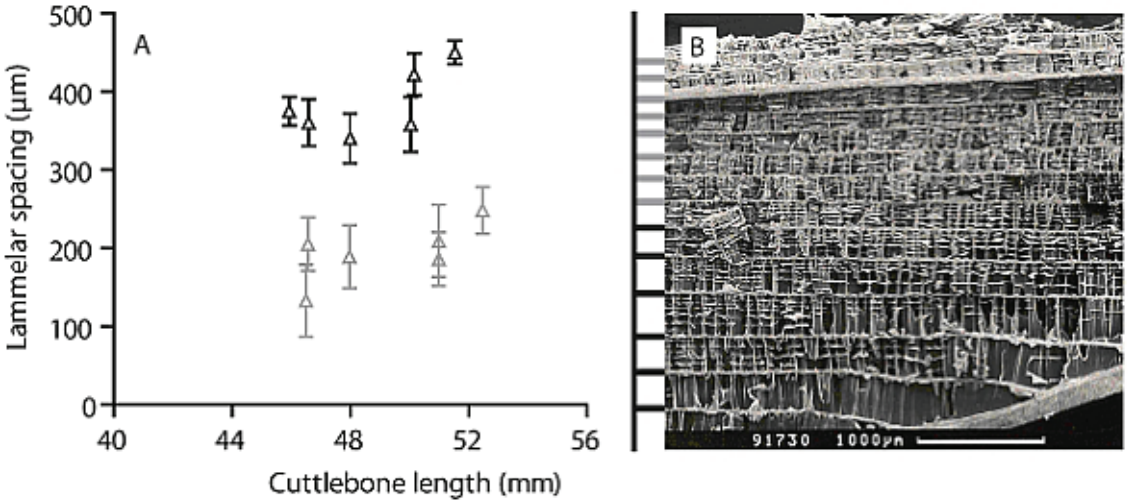

Figure 4.6. Comparison of ultrastructural changes in cuttlebone mineralization during exposure to elevated seawater pCO_2. Control data is represented in black, hypercapnic in grey. A) Lamellar spacing was significantly reduced in the cuttlebone sections mineralized during exposure to 0.6 kPa CO_2, 195 ± 38 versus 384 ± 26 µm, $p<0.0001$ ($n = 6$). B) Phragmocone section containing lamellae mineralized under both control and elevated CO_2 condition. Note the reduced lamellar spacing in the cuttlebone section mineralized during CO_2 exposure (top section). (Pub. 3).

The increase measured in calcification rate during hypercapnia puts *S. officinalis* in a unique position in relation to existing studies, since most invertebrates examined to date, cnidarians, molluscs, and echinoderms, exhibit a negative influence of elevated CO_2 concentrations on calcification. Interestingly, some of these organisms display strong linear relationships of calcification rate with Ω_{arag} (Fig. 4.7). The changes in calcification recorded over a two-year period in the Biosphere 2 mesocosm (Langdon et al. 2000: data replotted from their Table 4 in 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 *Mytilus edulis* decreases linearly with increasing pCO_2, and ceases at pCO_2's above 1,800ppm (data replotted from their Table 1 in Fig. 1). While the latter might be explained by external shell dissolution when $\Omega_{arag} < 1$, decreasing calcification at $\Omega_{arag} > 1$ may indicate that there exists significant physico – chemical control over calcification in mussels. In contrast, it is evident that in the cuttlefish, calcification of the fully internalized cuttlebone is independent of decreasing seawater Ω_{arag} values. As far as I am aware, only one other study working with long-term hypercapnic exposure in invertebrates, has shown increased calcification rates under elevated seawater CO_2 levels. In the brittle star *Amphiura filiformis* increased calcification was reported as a ratio of arm mass and total calcium measure in dissolved arms after 40 days of exposure to seawater pH's off 7.7-6.8 (Wood et al. 2008). To further understand the calcification response of *A. filiformis* to long-term hypercapnic exposure it will be important to measure its acid-base regulatory capacity and the amount, and ultrastructure, of $CaCO_3$ mineralized in its skeleton.

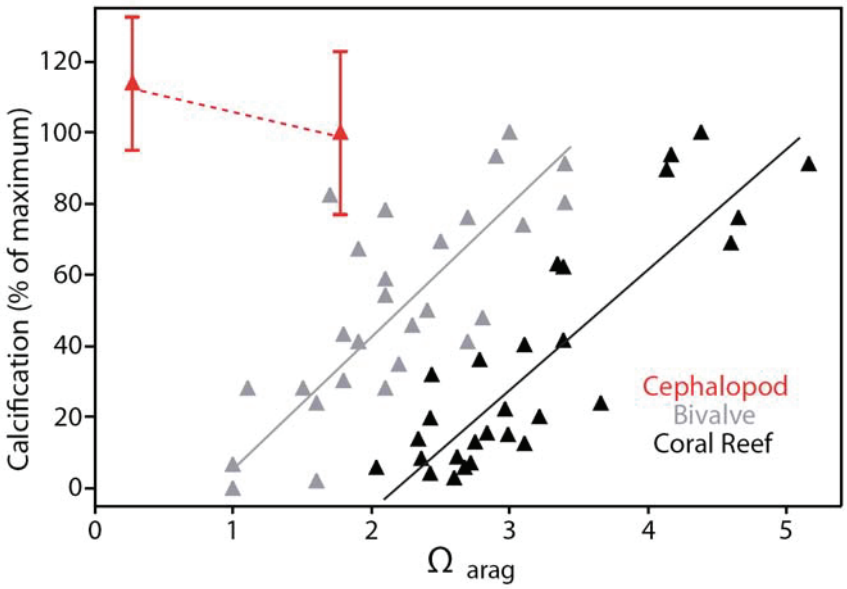

Figure 4.7. The dependence of calcification on CO_2 dependent seawater calcium carbonate saturation (Ω_{arag}) in marine invertebrates: Long-term coral reef data set recorded in the Biosphere 2 mesocosm (Langdon et al. 2000, data replotted from their Table 4), acute changes in *Mytilus edulis* calcification (Gazeau et al. 2007, data replotted from their Table 1), *Sepia officinalis* calcification measured over six weeks in this study (data are mean ± SD, n = 20). The highest calcification rates in the respective data sets were set at a value of 100%. (Pub. 3).

An interesting question that remains is, if *S. officinalis* is capable of maintaining soft tissue growth rates and metabolism at control levels during hypercapnic exposure, why does calcification increase? An important point could be that active organisms, like cuttlefish, significantly increase HCO_3^- levels and partially compensated pH in their extracellular fluids during exposure to high seawater pCO_2's (Table 4.1). If similar compensatory changes took place in the extracellular space around the cuttlebone, the $CaCO_3$ saturation state (Ω) of the extracellular space would increase. Even if [HCO_3^-] did not increase due to compensatory activity in the extracellular space around the cuttlebone, elevated blood [HCO_3^-] could change ion transport kinetics across the cuttlebone epithelium. Either way, it is possible that Ω could be increased around the cuttlebone, thus favoring the precipitation of $CaCO_3$ crystals. Measurement of the extracellular acid-base parameters in the fluid around the cuttlebone during long-term exposure to elevated seawater pCO_2 would help answer some of these questions. It would be interesting to consider the calcification response of other invertebrates with high acid-base regulatory abilities to long-term elevated CO_2 exposure, such as brachyuran crustaceans. However, to date no long-term growth studies have been performed with brachyuran crustaceans under hypercapnic conditions.

Considering that calcification requires tight control of ionic composition and pH in the micro-environment at the deposition site (Weiner & Dove 2003), it could be that *Mytilus spp.*, and other invertebrates with low metabolic rates / low ion exchange capacities, are not capable of maintaining conditions favorable to mineral deposition under the acidification stress of hypercapnia. Findings of elevated [Ca^{2+}] in *Mytilus galloprovincialis* hemolymph, in combination with the already mentioned uncompensated pHe reduction (Michaelidis et al. 2005), have been interpreted to support such a hypothesis. Our lack of understanding of the mechanistic processes behind calcification in molluscs, limits our interpretation of the apparent dependency of calcification on relatively high seawater [CO_3^{2-}] and Ω's > 1. The question that remains is to

what extent reductions in calcification rate are due to a specific decrease in the ability of the organism to accrete $CaCO_3$ during exposure to elevated pCO_2, or due to general reductions of metabolism that reduce growth (Pörtner et al. 2004). The currently available data about long-term reductions in invertebrate calcification is not sufficient to draw a causal relationship between long-term exposure to elevated seawater pCO_2 and reduced calcification rates. The measured long-term reductions in calcification have only been reported as gross shell length and animal mass (Michaelidis et al. 2005, Shirayama and Thornton 2005, Berge et al. 2006). To advance our understanding of the sensitivity of calcification processes in molluscs and echinoderms to elevated seawater pCO_2, further work is needed that disentangles the parallel effects of metabolic regulatory processes and the potential dependency of calcifying organisms on high seawater [CO_3^{2-}]. Future studies should take into consideration the morphological localization of the calcification site as well as the ultrastructure of the mineralized $CaCO_3$ and organic matrix.

4.5 The sensitivity of embryonic development in *S. officinalis* to hypercapnia.

The egg capsule of *S. officinalis* eggs creates a diffusion barrier for metabolic wastes produced by the embryo. Even under control seawater conditions, the gas tensions and pH of the perivitelline fluid (PVF) inside the egg differ strongly from those of the surrounding seawater over the course of development (Pub. 4 Fig. 1). In order to maintain rates of diffusive gas flux due to rising metabolic rates of growing embryos, oxygen partial pressures of the PVF decrease from 12.8 kPa (ca. 61% air saturation) to <5 kPa (ca. 22% air saturation). These measurements support the idea of an oxygen diffusion limitation being one critical factor in *S. officinalis* late embryonic development and correspond to similar values measured in the past (DeWachter et al. 1988, Cronin and Seymour 2000). However, as pO_2 declined, PVF pCO_2 values rose from 0.13 kPa in the smallest embryos up to 0.41 kPa in the largest (Fig. 8A). Thus, cuttlefish embryos are surrounded by 10-fold higher pCO_2 values than those of ambient sea water (ca. 0.04 kPa) at the end of their embryonic development. As pCO_2 increases, pH strongly decreases, from 7.72 to 7.23 (Fig. 8B). There was no measureable accumulation of HCO_3^- in the PVF of *S. officinalis* in order to actively buffer pH (Pub. 1 Fig. 1). There are no pH measurements for marine animal eggs available at present that these measurements could be compared with. In salmonids, 0.3-1.0 unit lower pH values have been recorded in egg PVF at ambient pH between 7 and 8 (Kügel and Peterson, 1989).

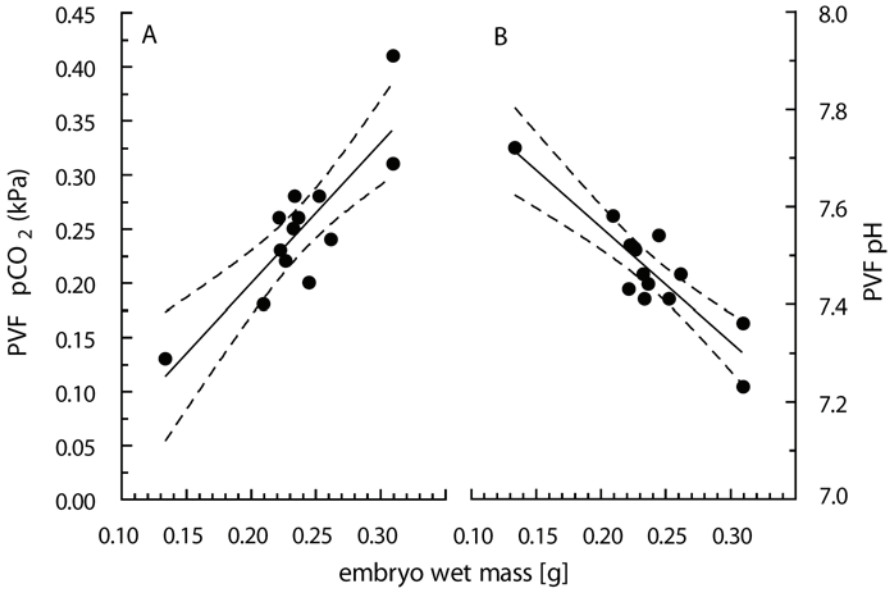

Figure 4.8. A) pCO_2 and B) pH in perivitelline fluid (PVF) of *S. officinalis* eggs (stages 29-30), displayed against embryo wet mass (excluding external yolk sac). pCO_2 was calculated from PVF C_T and pH. (Pub. 4).

High pCO_2 values in the PVF also imply that blood pCO_2 values must be even higher in order to maintain CO_2 excretion rates across the gill / skin epithelia by means of diffusion. Typically, pCO_2 values in extracellular fluids of high-power animals such as fish or cephalopods are 0.2-0.4 kPa above those of the ambient seawater (e.g. Heisler 1986, Johansen et al. 1982). Therefore, cuttlefish embryos are probably exposed to blood pCO_2 values of 0.6-0.8 kPa at the end of their development. It would be quite rewarding to study blood pH regulation in embryos under such conditions, as cephalopods are known for the high pH sensitivity of their extracellular respiratory pigment hemocyanin (Brix et al. 1981, Pörtner 1994, Melzner et al. 2007). The occurrence of special embryonic hemocyanins (Decleir et al., 1971) may be one adaptation to the high pCO_2 values encountered during late embryogenesis.

As mentioned before, no data on egg fluid pCO_2 and pH is available for other marine organisms; however, judging from the pO_2 vs. pCO_2 ratios obtained in our study, in comparison to pO_2 values in- and around eggs or egg masses of other marine animal taxa, it seems likely that many embryos will also be surrounded by fluids of high pCO_2 and low pH: For example, Dietz and Davenport (1987) showed that pO_2 values in shark eggs decrease from ca. 18 kPa in early embryos to ca. 10 kPa in late embryos, Fernandez et al. (2000) and Fernandez et al. (2003) measured water pO_2 values <5 kPa in decapod crustacean egg masses, Cohen and Strathmann (1996) found pO_2 values of less than 6 kPa in egg masses of opisthobranch gastropods and those of a polychaete worm. Delayed development of embryos in central positions of egg masses has usually been causally linked to reduced metabolic rates due to low ambient pO_2 (e.g. Chaffee and Strathmann, 1984). However, high pCO_2 / low pH may be important factors as well, as they could also elicit reductions in metabolic rates. Clearly, PVF pCO_2 and pH represent important abiotic factors that might influence the physiological performance of marine animal embryos to a large degree.

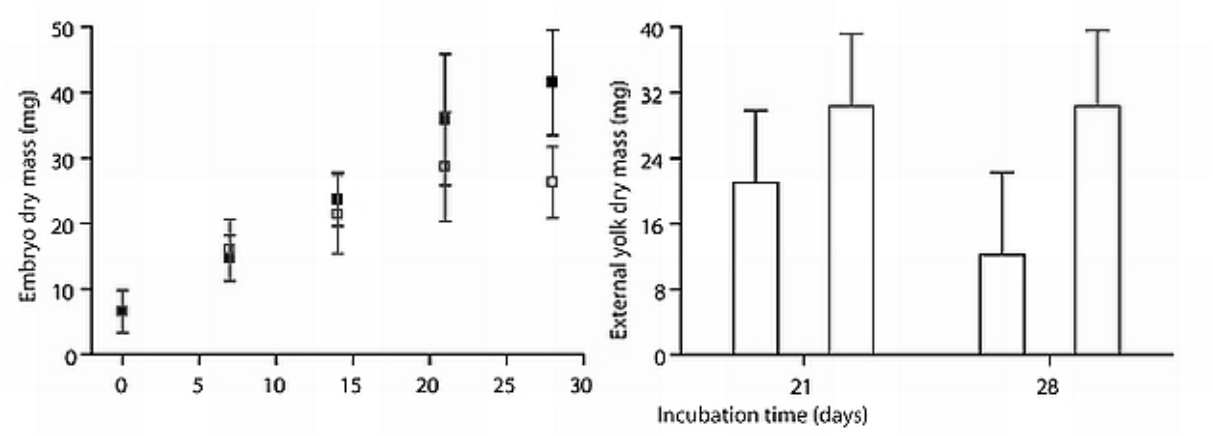

Figure 4.9. Embryonic development of *S. officinalis* under control conditions (black) and 0.6 kPa CO_2 (red). Embryos hatched after approximately 30 days of incubation. A) Growth of *S. officinalis* embryos. The increment of dry weight is significantly reduced in CO_2 treated embryos in the last weeks of development. B) External yolk utilization was slowed in CO_2 incubated embryos (grey). In the last week of development yolk utilization was fully inhibited. Lower yolk utilization and reduced growth rates point towards a reduction of metabolism in late-stage embryos exposed to 0.6 kPa CO_2. (Gutowska et al. unpub.)

The fact that *S. officinalis* embryos are exposed to hypercapnic conditions during development inside the egg capsule under control conditions, could make them potentially more sensitive to elevated seawater pCO_2. Preliminary studies have shown that during exposure to seawater CO_2 levels of 0.6 kPa, the pCO_2 of the perivitelline fluid also increased by 0.6 kPa. This resulted in PVF pCO_2's > 1 kPa before hatching (Gutowska et al. unpub.). It is not surprising, that the developmental rates of embryos incubated under 0.6 kPa CO_2, for four weeks prior to hatching were delayed. After three weeks of incubation under elevated CO_2 conditions, embryonic dry mass of the hypercapnic eggs was observed to be lower than that of control eggs (Fig. 4.9A). The masses of the external yolks sacs also differed between the experimental groups towards the end of the developmental period (Fig. 4.9B). The CO_2 incubated embryos had significantly larger yolks sacs than the control group at the last two sampling points. At the last sampling point prior to hatching, the masses of the yolk sacs from CO_2 incubated embryos had not significantly decreased from the 21 days sampling point. Thus, it appears that yolk utilization was dramatically reduced towards the very end of embryonic development when pCO_2's of the PVF were the highest. Considering the reduced embryonic growth rates, as well as decreased yolk utilization, it was hypothesized that *S. officinalis* embryos undergo metabolic depression during long-term incubation under 0.6 kPa CO_2 and that their final growth inside the egg is retarded.

The most dramatic impact of elevated CO_2 exposure on younger developmental stages of *S. officinalis* occurred within the first few weeks post-hatching. During the two weeks after the peak hatching date, the experimental group that had been incubated and maintained under 0.6 kPa CO_2 experienced 100% mortality. In contrast, there was 0% mortality among the control group hatchlings (Gutowska et al. unpub.). Even though hatchling *S. officinalis* look like

isometric replicates of adults, they still undergo further differentiation of important organs post-hatching. The work of Schipp et al. has illustrated that the initially uniform gill (branchial) epithelium (Fig. 10A) of the cuttlefish differentiates into transport and respiratory regions within the first weeks post- hatching (Fig. 10B, see section 4.1, Fig. 4.2, for further description). It is hypothesized the high mortality of *S. officinalis* post-hatching, instead of during embryonic development, is connected to compromised or delayed differentiation of organs that are essential for metabolic and ion regulatory functions in the more active hatchlings. Future studies should examine the morphogenetic changes of the branchial epithelium in *S. officinalis* embryos and the development of acid-base regulatory capacity.

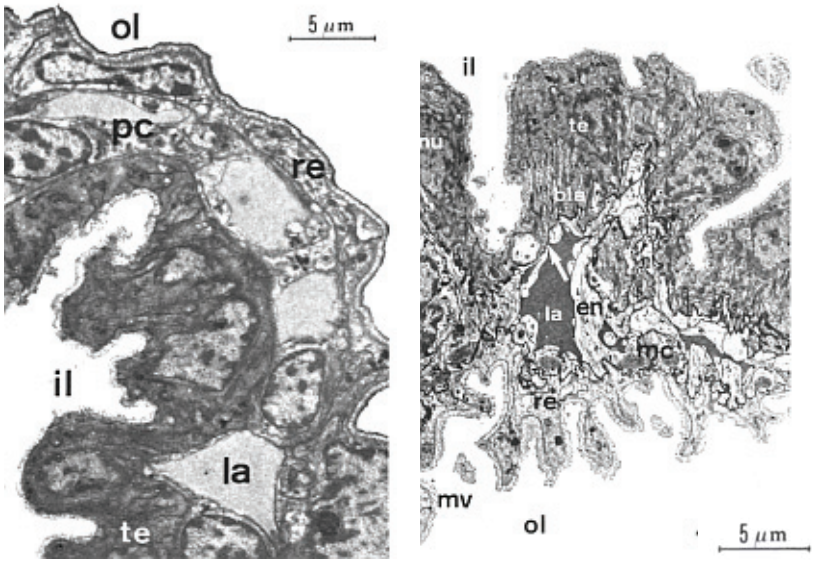

Figure 4.10. A) Cross section of a secondary branchial fold in a late-stage *S. officinalis* embryo. The outer respiratory epithelium (re) is oriented towards the outer lumen (ol) represented by the open space of the mantel cavity. The inner transporting epithelium (te) is oriented towards the inner lumen (il) in the recess of the secondary branchial fold. **B)** Further differentiation of the branchial epithelia is evident in a 5 day old hatchling. Basal labyrinth (bla), basal lamina (white arrow), blood lacunae (la), endothelium (en), microvilli (mv), nucleus (nu), obliquely striated muscle cells (mc), pilaster cells (pc). (Modified from Schipp et al. 1979).

The preliminary data on CO_2 sensitivity of *S. officinalis* embryos and hatchlings indicates that the early developmental stages of the cuttlefish are less tolerant to elevated seawater pCO_2 than juveniles and adults. In contrast to juvenile *S. officinalis*, the embryos had reduced growth rates and high hatchling mortality when exposed to 0.6 kPa CO_2. High sensitivity of early life stages to elevated seawater pCO_2 has also been found amongst bivalves and echinoderms (reviewed in Kurihara 2008). Calcification processes appear to be particularly sensitive to hypercapnia in larval invertebrates (Kurihara and Shirayama 2004, Kurihara et al 2007, Dupont et al. 2008). It is interesting to consider the embryonic development of *S. officinalis* in the context of calcification sensitivity to elevated pCO_2 conditions. Cuttlefish embryos form the first eight lamellae of their internal aragonitic shell and statoliths under the low pH and high pCO_2 conditions that prevail in their egg environment (Lemaire 1970, Fioroni 1990, Ré and Narciso 1994). This sets *S. officinalis* embryos apart from other marine invertebrate larvae studied so far, as the majority show shell dissolution under seawater conditions comparable to those found in the PVF (reviewed in Fabry et al. 2008). It is tempting to propose that the capacity to maintain

calcification rates in hypercapnic condition is causally linked to an embryo that is already adapted to cope with relatively high pCO2 / low pH values.

Conclusion

The experimental work in this thesis supports the hypothesis that marine organisms with high metabolic rates and strong acid-base regulatory abilities will be more tolerant of changes in seawater carbonate chemistry induced by ocean acidification (Seibel and Walsh 2001, 2003, Pörtner 2008, Pub. 5). The cephalopod *S. officinalis* has a higher acid-base regulatory ability than invertebrates who have been shown to be sensitive to elevated seawater pCO_2 (Pub.1). During acute exposure to hypercapnic seawater, bicarbonate was rapidly accumulated in the extracellular space to partially compensate blood pH. Despite a new steady state blood pH value approximately 0.2 units below control, metabolic rate and long-term growth performance were not found to be depressed. This is a surprising finding, as it has been hypothesized that invertebrates who are unable to fully compensate their pHe during exposure to hypercapnic seawater undergo metabolic depression (Pörtner and Reipschläger 1996, Pörtner et al. 1998, Pörtner et al. 2004).

However, the invertebrates that have been observed to suffer from hypercapnia induced metabolic depression are typically characterized by much lower metabolic rates than those of cephalopods and other more hypercapnia tolerant taxa, such as brachyuran crustaceans and teleost fish (Fig.2,. Pub.5). Comparing these groups, it becomes immediately evident, that exercise induced elevation of metabolic rates not only leads to metabolic rates that are occasionally 100-fold higher than those of hypometabolic organisms (e.g. echinoderms, bivalves), but also to high CO_2 excretion rates (Pub. 5). As metabolic rates are proportional to CO_2 excretion rates, high-power taxa typically maintain relatively high extracellular pCO_2 values to enhance diffusional excretion of CO_2 across the gill epithelia (0.2-0.4 kPa during control conditions, 0.3-1.0 during exercise). The cells of high power metazoans are thus already exposed to elevated pCO_2 values on a regular basis, and could be less sensitive to the comparatively subtle changes in ocean pCO_2 expected during the next decades. Multiple studies have also shown that species from active groups are characterized by powerful ion regulatory machinery in their gill epithelia that aid in the removal of protons produced during anaerobic exercise. This means that the very same ion regulatory machinery that is needed for the compensation of ocean acidification induced acidosis is already expressed in active marine organisms. The ultrastructural basis (highly folded transport epithelia with high mitochondrial densities) and machinery (ion regulatory proteins, ion channels) for effective regulation of extracellular pH and pCO_2 does not to be 'invented' in response to rising abiotic stress in the future. Phenotypic plasticity, i.e. modulation of existing inventory might be sufficient to keep active organisms

'afloat' in a future more acidic ocean.

Such considerations are supported by the fact that hypercapnia is an inherent feature of the cephalopod life cycle, and potentially, in that of other active organisms such as fish and crustaceans (Pub.4). Maximum pCO_2 values of 0.4 kPa (4,000 ppm) were observed in the perivitelline fluid surrounding the embryo of *S. officinalis* at the end of development. Although no comparable data is available at present, low pO_2 values recorded in and around fish and crustacean eggs / egg masses (e.g. Dietz and Davenport 1987, Fernandez et al. 2000, 2003) indicate that these organisms also might encounter high pCO_2 values as embryos. This suggests that powerful ion regulatory machinery is already present in early life stages. It is especially noteworthy, that *S. officinalis* embryos calcify the first lamellae of their aragonitic cuttlebone and statoliths in the egg. Considering the high ion regulatory capacity of the adults and the ability of embryos to calcify in a hypercapnic egg environment, it is not entirely surprising to find conserved rates of calcification (Pub.3) and growth (Pub.2) in juvenile cuttlefish exposed to long-term elevated seawater pCO_2.

In summary, cephalopods like *S. officinalis* and other highly active species, i.e. teleost fish and some decapod crustaceans, benefit from highly developed ion regulatory machinery when exposed to ocean acidification conditions characterized by more acidic seawater pH and elevated pCO_2. High fluxes of CO_2, and high extracellular pCO_2 values, encountered by these organisms day to day may provide the basis for effective hypercapnia compensation. Interestingly, the costs of compensation are not so high as to leave a measurable footprint in the species' energy budget during exposure to moderately high CO_2 levels. It is proposed that *S. officinalis* will be much less vulnerable to future ocean acidification than its hypometabolic relatives, such as bivalves. This corresponds to observations made by Knoll et al. (2007) that during the Permo-Triassic mass extinction many more hypometabolic genera were lost, possibly due to hypercapnia stress. However, there are some aspects of the present thesis that require further research efforts in the future. These will be briefly outlined in the following section.

Some notes of caution need to be added to the statements made above. Animals in the present study were not acclimated for time periods longer than 6 weeks, thus more subtle long-term effects could not be resolved. In addition, the generation boundary was not crossed in this study. Several recent publications suggest that gametes, the fertilization reaction and zygotes could be the true bottleneck stages in terms of sensitivity towards ocean acidification (e.g. Kikkawa et al. 2003, Havenhand et al. 2008, Kurihara 2008). Further study of these processes deserves attention.

Significant ultrastructural modifications were observed in the cuttlebones of *S. officinalis* acclimated to high seawater pCO_2. It is not clear, whether these changes have any relevance for functionality and ecological fitness. Subjecting long-term hypercapnia acclimated cuttlefish to a

more realistic three-dimensional habitat, in which prey capture is dependent on full-buoyancy control mediated by the cuttlebone and adjacent ion regulatory epithelia, could provide the missing information. Such experiments should be conducted with cuttlefish whose cuttlebones were calcified solely under hypercapnic conditions. In addition, the cost of buoyancy control could be assessed using an experimental approach equivalent to that of Webber et al. (2000).

Exposure of cuttlefish eggs to pCO_2 values equivalent to those used for juvenile growth and calcification trials, led to retarded embryonic growth and high post-hatching mortality. This is not surprising as pCO_2 values in the perivitelline fluid around the embryos increases additionally by the pCO_2 added to the seawater. It appears that the hypercapnia levels already tolerated by the embryos inside the eggs, renders them more sensitive to additional stress. The mechanisms that lead to mortality are unclear at present, as are the development of ion-regulatory epithelia in the embryo. To further differentiate the effects of hypercapnia exposure on the ontogeny of acid-base regulation in *S. officinalis*, much younger embryos will need to be incubated, than the late-stage (post-organogenesis) embryos used in this study.

An exciting topic for further study is the ion regulatory machinery of *S. officinalis* in general, as so little is known about its mechanistic basis at present (e.g. Schipp et al. 1979). The comparison with similarly powerful ion-regulators such as teleost fish and decapod crustaceans will be very important to gain an understanding of how hypercapnia tolerant physiotypes have evolved.

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