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Faculty 2: Biology / Chemistry

Master-Thesis

MSc Marine Biology

Effects of hypoxia and hypercapnia on blood and tissue physiology of the common cuttlefish *Sepia officinalis*

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List of abbreviations

Incubations:

H+H	Hypoxia & hypercapnia (~60% O_2 air saturation, ~0.1 kPa CO_2)
C_H+H	Control for H+H incubation (~100% O_2 air saturation, ~0.04 kPa CO_2)
HOx	Hypoxia (~50% O_2 air saturation, ~0.04 kPa CO_2)
C_HOx	Control for HOx incubation (~100% O_2 air saturation, ~0.04 kPa CO_2)
HCa	Hypercapnia (~100% O_2 air saturation, ~0.12 kPa CO_2)
C_HCa	Control for HCa incubation (~100% O_2 air saturation, ~0.04 kPa CO_2)

Tissues:

M	Mantle	F	Funnel
SH	Systemic heart	BH	Branchial hearts

Physiological parameters:

ATP	Adenosine-5'-triphosphate	ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate	Arg	Arginine
PCO_2	CO_2 partial pressure	P_i	Inorganic phosphate
PLA	Phospho-L-arginine	PO_2	O_2 partial pressure
ΔG/Δξ	Gibbs free energy change of ATP hydrolysis		

Chemicals:

EDTA	Ethylenediaminetetraacetic acid	NTA	Nitrilotriacetic acid
PCA	Perchloric acid	TRA	Triethanolamine

Others:

CE	Capillary electrophoresis	ETS	Electron transport system
SD	Standard deviation		

Abstract

Two major factors that will change due to climate change are ocean oxygen and CO_2 content. To assess possible consequences for cephalopods, which are considered especially vulnerable to these changes, the effects of hypoxia and hypercapnia on the common cuttlefish *Sepia officinalis* were studied in a laboratory experiment.

Muscle and heart samples of cuttlefish incubated under simultaneous hypoxia and hypercapnia were compared to samples of earlier experiments where *S. officinalis* was exposed either to hypoxia or hypercapnia. The incubations lasted at least five weeks and for each treatment a corresponding control incubation with the same number of replicates was run in parallel. Concentrations of different metabolites of aerobic and anaerobic metabolism (arginine, phospho-L-arginine, octopine, ATP, ADP, AMP, inorganic phosphate) were measured in muscular mantle, funnel, systemic heart and branchial hearts. If possible, succinate concentration, intracellular pH, PCO_2 and bicarbonate concentration were determined and free ADP and AMP concentrations and Gibbs free energy change of ATP hydrolysis were calculated. Blood pH, PCO_2 and bicarbonate were measured during exposure to hypoxia and hypercapnia as well as in the respective control.

In the funnel, hypoxia caused a decrease of octopine and inorganic phosphate, while phospho-L-arginine, ATP and ADP increased. Hypercapnia caused a decrease in arginine, phospho-L-arginine and ATP of funnel tissue. During simultaneous hypoxia and hypercapnia, intracellular PCO_2, AMP and ADP were elevated in the mantle tissue. Intracellular pH was reduced, but free AMP, free ADP and Gibbs free energy were not affected. Blood PCO_2 and bicarbonate increased during simultaneous hypoxia and hypercapnia, while blood pH was reduced. Parameters of systemic heart and branchial hearts were not affected by any of the incubations and the metabolite concentrations were generally lower in hearts than in mantle or funnel.

Results indicate that *S. officinalis* is able to acclimate to long-term exposure to moderate levels of hypoxia and hypercapnia. Blood oxygen supply was secured. Survival could have been supported by a hypoxia-induced metabolic depression, but the exact triggering mechanism is still unknown. The differences between the tissues reflect the different activity patterns of the tissues. The absence of effects in systemic heart and branchial heart was maybe caused by low test power, but could also reflect the essential role of these tissues in the distribution of oxygen inside the body.

Zusammenfassung

Zwei wichtige Faktoren, die sich im Zuge des Klimawandels verändern werden, sind Sauerstoff- und CO_2-Gehalt der Ozeane. Cephalopoden werden als besonders empfindlich gegenüber solchen Veränderungen erachtet. Um die möglichen Konsequenzen für diese Tiergruppe abzuschätzen, wurden die Effekte von Hypoxie und Hyperkapnie auf den gemeinen Tintenfisch *Sepia officinalis* untersucht.

Muskel- und Herzproben von Tintenfischen, die simultan Hypoxie und Hyperkapnie ausgesetzt wurden, wurden mit Proben von Tieren verglichen, die in früheren Experimenten entweder Hypoxie oder Hyperkapnie ausgesetzt wurden. Jede Inkubation dauerte mindestens 5 Wochen und für jede Behandlung gab es eine parallele Kontrolle mit der gleichen Anzahl von Replikaten. Die Konzentrationen verschiedener aerober und anaerober Metabolite (Arginin, Phospho-L-Arginin, Octopin, ATP, ADP, AMP, anorganisches Phosphat) wurden in Mantel, Trichter, systemischen Herz und Kiemenherzen gemessen. Wenn möglich wurden die Succinatkonzentration, intrazellulärer pH, PCO_2 und die Bikarbonatkonzentration. Zudem wurden wenn möglich die Konzentration von freiem ADP und freiem AMP sowie der Energiestatus (Gibbs free energy of ATP Hydrolysis) berechnet. Blut pH, PCO_2 und Bikarbonatgehalt wurden in der unter Hypoxie und Hyperkapnie inkubierten Tieren und der entsprechenden Kontrolle gemessen.

Hypoxie verursachte eine Reduktion der Konzentrationen von Octopin und anorganischem Phosphat im Trichter, während Phospho-L-Arginin, ATP und ADP erhöht waren. Hyperkapnie löste eine Verringerung der Konzentrationen von Arginin, Phospho-L-Arginin und ATP im Trichter aus. Unter simultaner Hypoxie und Hyperkapnie stiegen intrazellulärer PCO_2, AMP und ADP im Mantelgewebe an. PCO_2 und Bikarbonat im Blut stiegen an, während der Blut pH konstant blieb. Keine der Inkubationen hatte einen Einfluss auf die in den Herzen gemessenen Parameter, jedoch waren die Metabolitkonzentration in den Herzen niedriger als im Mantel oder im Trichter.

Die Ergebnisse lassen vermuten, dass *S. officinalis* sich an eine langanhaltende Hypoxie und Hyperkapnie anpassen kann. Die Versorgung mit Blutsauerstoff war sichergestellt. Das Überleben wurde möglicherweise durch eine Reduktion der Stoffwechselaktivität unterstützt, jedoch ist der genaue Auslösemechanismus noch unbekannt. Die Unterschiede zwischen den Geweben spiegeln die verschiedenen Aktivitätsmuster der Gewebe wieder. Das Fehlen von Effekten auf das systemische Herz und die Kiemenherzen könnte aus einer geringen statistischen Teststärke resultieren oder die entscheidende Rolle der Herzen für die Sauerstoffverteilung im Körper wiederspiegeln.

1 Introduction

1.1 A changing ocean

Within the last decades, the world's oceans have faced rising water temperatures as well as decreasing O_2 and increasing CO_2 content (Caldeira & Wickett 2003, Meehl et al. 2007, Diaz & Rosenberg 2008). Average sea surface temperature (SST) is expected to rise by 1.5-4.0℃ until the year 2100 (Meehl et al. 2007). Rising temperatures do and will cause changes in the latitudinal distribution and migration of marine species (Sims et al. 2001, Beaugrand et al. 2002, Perry et al. 2005, Zeidberg & Robison 2007). The distribution changes reflect the thermal window of the species, which defines the temperature limits for survival (critical temperature, T_{crit}) and optimal performance (pejus temperature, T_p). Frederich & Pörtner (2000) showed that these limits are often set by a species' ability to maintain a sufficient O_2 supply. This can either be impaired by the increasing metabolic rates and oxygen demands caused by increasing temperature (Q_{10}) or by reduced oxygen transport capacity of the circulatory system due to temperature changes (Pörtner 2001). This concept of "oxygen & capacity-limited thermal tolerance (OCLTT)" points out possible severe influence of climate change on marine species for increasing water temperatures can not only impair oxygen supply but additionally reduces ambient water oxygen content (Pörtner 2010).

1.1.1 Oxygen

The solubility of O_2 in water is temperature dependent and rising SSTs will reduce the amount of oxygen in the upper water layers. At 15℃ the oxygen concentration of saturated seawater at sea level (35 psu, 101.325 kPa) is 248 µmol*L^{-1}. At 20℃ the concentrations is only 225 µmol*L^{-1} (Boutilier et al. 1984). Decreasing ocean oxygen concentrations (hypoxia) have already been reported and they are usually confined to well-defined areas or timescales (Whitney et al. 2007, Diaz & Rosenberg 2008). The sizes of these areas can vary over several magnitudes and their occurrence can be infrequent, periodic or permanent (Diaz & Rosenberg 2008, Stramma et al. 2008). Generally, water bodies are termed hypoxic, if their oxygen content lies below 10 µmol*L^{-1} (Gray et al 2002). Hypoxic events are most often found in neritic bottom waters or in oceanic waters between 200 m and 800 m depth (Fig. 1.1) (Helly & Levin 2004, Diaz & Rosenberg 2008, Stramma et al. 2008). Their occurrence is typically

connected to a previous plankton bloom in the overlying surface waters (Diaz 2001). Increasing SSTs (see above) furthermore support the development of hypoxic zones, as they reinforce stratification and thus prohibit the convection of fresh (O_2-rich) water to deeper layers.

Hypoxic events have become more frequent and severe in the last decades and this trend is expected to continue (Diaz 2001). One, if not the main reason for this, is the increasing nutrient influx from land caused by human activities (Cloern 2001, Dethlefsen 1983, Diaz 2001). A clear correlation between the density of human centers along the coast and the accumulation of hypoxia zones can be seen (Fig. 1.1). In the future, hypoxic events are expected to become more frequent, more severe and to last longer (Diaz & Rosenberg 2008). This development is expected to have strong effects on individual marine organisms as well as on complete ecosystems (Diaz 2001).

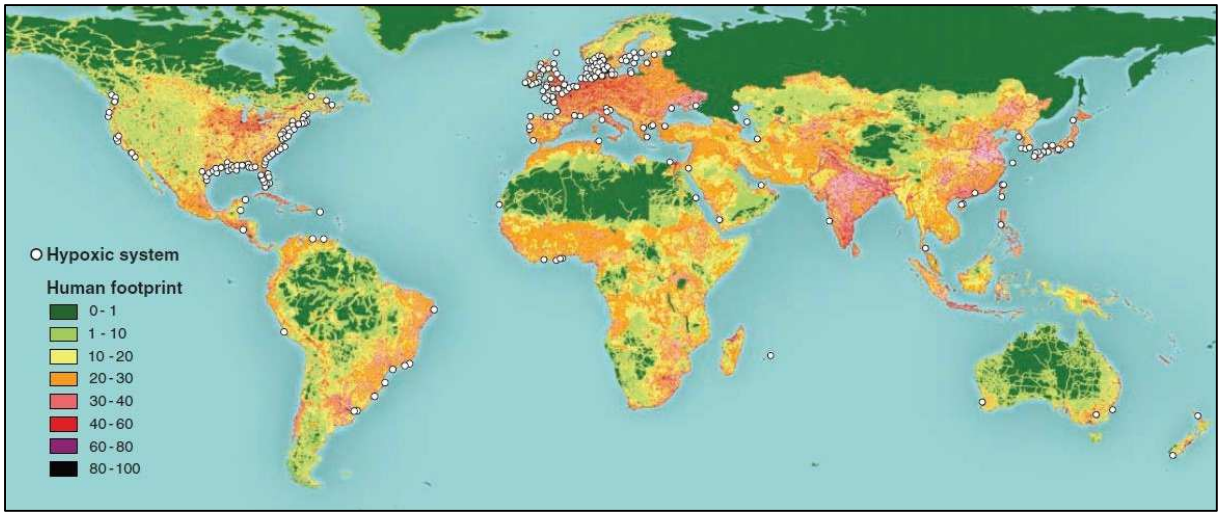

Fig. 1.1: Global distribution of eutrophication associated hypoxic systems compared to the human influence on the terrestrial environment. White cycles represent hypoxic systems. The color of the land areas indicates the human influence on the respective area. The influence is shown as "human footprint" (given as %), which was defined by Sanderson et al. (2002). A clear correlation becomes evident between the strength of the human footprint and the occurrence of hypoxic systems. Figure adopted from Diaz & Rosenberg (2008).

1.1.2 CO_2 & pH

Anthropogenic CO_2 emissions have caused an increase in the partial pressure of carbon dioxide (PCO_2) at the ocean surface from the preindustrial (year ~1750) value of 0.028 kPa to the present value of 0.04 kPa (Orr et al. 2005). By the year 2100 a PCO_2 of 0.07-0.113 kPa will be reached depending on the applied scenario (Meehl et al. 2007). A situation of elevated PCO_2 is generally termed hypercapnia. In earth history, organism usually had enough time to evolve adaptations to the changing

conditions, as similar changes in atmospheric PCO_2 took at least several millennia (often millions of years), but the rapid CO_2 rise observed at present will probably have severe consequences for various marine species (Pearson & Palmer 2000).

The major factor of ocean chemistry, which is affected by the dissolution of atmospheric carbon dioxide, is the seawater pH. Sea surface pH has already decreased from a pH of 8.2 in 1750 (preindustrial) to the present value of ~8.05-8.00. In 2100, a sea surface pH of 7.8-7.75 is expected (Caldeira & Wickett 2003). The change in pH will be most pronounced and fastest in the surface layers, because of the direct interaction with the atmosphere (Caldeira & wicket 2003). Besides the accumulation of atmospheric carbon dioxide, an increase in water PCO_2 can also be caused by the aforementioned process of eutrophication (see 1.1.1). The O_2 dependent degradation of organic matter in deeper water layers or on the seafloor sets free large amounts of CO_2 and thus causes hypercapnia. This can result in CO_2 partial pressures exceeding 0.10 kPa in the respective area (Rosa & Seibel 2008, Melzner et al. 2012).

Hypercapnia affects various biological processes ranging from molecular transport mechanisms over metabolic regulation to complex behavior patterns (Rees & Hand 1990, Pörtner et al. 2004, Munday et al. 2009). In interaction with other factors like hypoxia or increasing temperatures, the effects might be even stronger. In this thesis the focus lies on the effects of hypoxia and hypercapnia on the physiology of the cephalopod *Sepia officinalis*. To understand and assess these effects, however, it will be necessary to first understand the biological processes that could be affected.

1.2 Cephalopod physiology

Cephalopods are often considered the most highly evolved group of invertebrates and they are the only group that shows activity and performance levels, which are similar to those of fish or even higher (O'Dor & Webber 1991, Wells 1994, Rosa & Seibel 2008). Even less active species still display comparably high metabolic rates. In the mostly bottom-dwelling cuttlefish *Sepia officinalis*, O_2 consumption at rest lies between 0.077 and 0.094 µmol $O_2 * g^{-1} * min^{-1}$ (body mass: 105 g, 17℃) (Johansen et al. 1982b, Wells & Wells 1991, Melzner et al. 2006b). However, the rising activity levels, as well as their highly developed nervous system (Packard 1972) also strongly increased the energy demands (Wells 1994).

Cephalopods have evolved several morphological adaptations to fulfill their high oxygen requirements. Gill surface area per gram increased, while thickness of the blood-water barrier decreased (Wells 1994). In cuttlefish and many coastal squid species, locomotion and ventilation are uncoupled by the use of lateral fins for movement. This means that contractions of the muscle mantle, which maintain locomotion by jet-propulsion in squids, can be greatly reduced (Wells 1994). This reduces energy costs and also increases O_2 extraction from the ventilatory water current. In *S. officinalis*, oxygen extraction from ambient water at 17°C is ~ 70% (Melzner et al. 2006b), whereas in squids the extraction does rarely exceed 10% (Wells et al. 1988, Shadwick et al. 1990). Unlike other molluscs, cephalopods have a closed high-pressure vascular system to distribute the O_2 taken up by the gills (Fig. 1.2). In addition to the systemic heart (SH), which distributes the O_2-rich blood in the body, it includes two branchial hearts (BH), which receive the O_2-poor venous blood and create the pressure to pump it through the gills (Schipp 1987). Venous blood transport can be supported by ventilatory mantle contractions (Melzner et al. 2007a).

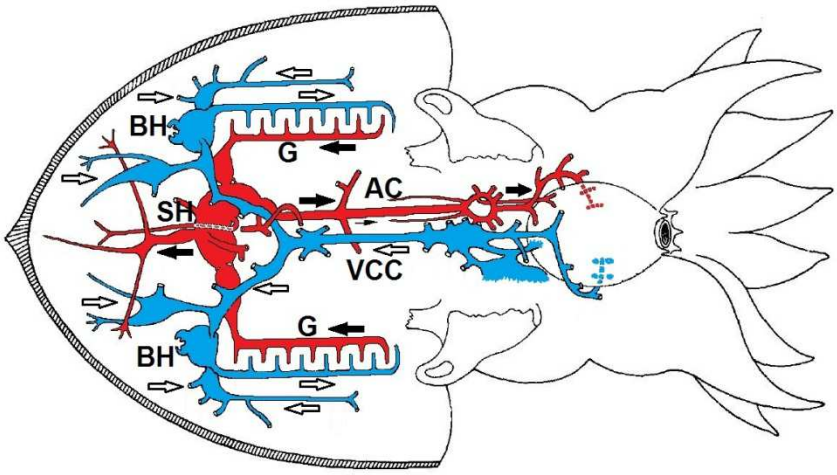

Fig. 1.2: Vascular system of the common cuttlefish *Sepia officinalis*. View from the ventral side. Major blood vessels are shown. O_2-rich blood is shown red; O_2-poor blood is shown blue. Black and white arrows indicate the flow direction of O_2-rich and O_2-poor blood, respectively. SH = systemic heart, BH = branchial heart, G = gill, AC = *Aorta cephalica*, VCC = *Vena cava cephalica*. Figure adopted from Schipp (1987) and changed.

Generally, cephalopods need higher metabolic rates than fish of similar activity to reach the same performance level (reviewed by O'Dor & Webber 1991). This is mainly attributed to their mollusc heritage, which sets constraints to their circulatory system and thus oxygen supply (Wells 1994).

1.2.1 Blood physiology

The oxygen carrying capacity of cephalopod blood (1-2 mmol*L^{-1}) is clearly below that of fish blood (4-5 mmol*L^{-1}) (Pörtner 1994). The reason for this is the use of

freely dissolved hemocyanin as an O_2-carrier in molluscs (Mangum 1990). In vertebrates, the blood pigment hemoglobin is highly concentrated inside the erythrocytes. Cephalopods lack such blood cells and the concentration of the free pigment in the plasma directly affects blood viscosity (Mangum 1990, Pörtner 1994). Therefore, the blood concentration of hemocyanin is limited to 150 mg protein per mL, which is still the highest known blood concentration of hemocyanin known so far (Pörtner 1994, Strobel et al. 2012). To counteract the problem of viscosity, cephalopods have evolved a hemocyanin protein with eight domains, each consisting of ten subunits. Each subunit is able to bind one O_2-molecule (Miller 1994). Therefore 80 O_2-molecules can be carried by only one hemocyanin protein that affects viscosity. Additional O_2 supply (up to 50% of total O_2) by skin respiration has been reported for squids (Pörtner 1994, 2002). Data on cutaneous respiration are lacking for other cephalopods but were estimated to contribute up to 25% of total O_2 in *S. officinalis* (Melzner et al. 2006b).

Most of oxygen bound in the blood is extracted from the blood on its way through the body leaving only a small venous reserve (Johansen et al. 1982a, Wells 1994). The size of the reserve seems to depend on the activity level of the species. In *S. officinalis* venous blood is still 19% saturated with O_2 at 20℃ (Zielinski et al. 2001), whereas the venous hemocyanin of the highly active northern shortfin squid *Illex illecebrosus* is < 5% saturated at 15℃ (Pörtner 1990). This is enabled by the strong Bohr-effect of the cephalopod hemocyanin (Bohr-coefficient < -1) (Pörtner 1994). The Bohr-effect describes the pH dependent oxygen affinity of the blood pigment. The affinity of the hemocyanin subunits is closely correlated to the surrounding pH. At a low pH, the concentration of protons (H+) is high and the protons bind to histidine groups of the hemocyanin. This causes a reduction of the hemocyanin O_2-affinity. Protons are taken up during the release of oxygen by hemocyanin. Because the protons used here derive from the bicarbonate system in the blood, their uptake and release also affects the dissociation equilibrium of CO_2/HCO_3^-. A release of O_2 thereby decreases the H^+-concentration and shifts the equilibrium from $CO_2 + H_2O$ (H_2CO_3) towards $HCO_3^- + H^+$ (Pörtner 1994). As a consequence, more CO_2 e.g. from the tissue can be taken up into the blood. A Bohr-coefficient < -1 means that per mol O_2 released by hemocyanin, more than 1 mol H^+ is taken up. Thus, already small drops of the blood pH (pH_e) can strongly reduce oxygen affinity of the hemocyanin. Generally, the maximum oxygen carrying capacity of the hemocyanin decreases with

decreasing pH_e according to the Root-effect (Bridges 1994). The increase of the bicarbonate concentration and CO_2-flow into the blood due to the O_2-dependent proton exchange at the blood pigment is termed the Haldane-effect and its strength is proportional to the Bohr-effect (Wyman 1964, Pörtner 1994). The cooperativity of the hemocyanin subunits ensures that the released oxygen is not taken up again.

pH_e in cephalopods is around 7.4 and the affinity curve of the hemocyanin has its highest slope in this range (Pörtner 1990, Zielinski et al. 2001). This means that the cooperativity is highest in this range and that already small changes in pH_e will strongly affect the O_2-binding at the pigment. To ensure the O_2-transport to the tissue, the arterial pH_e in cephalopods therefore increases from the gills to the tissue due to the H^+-uptake during deoxygenation caused by the strong Bohr-effect (Pörtner 1994). In squids, an additional release of HCO_3^- from the tissue into the blood further supports blood buffering and increases pH_e (Pörtner et al. 1991). This is a contrast to the classical functioning of the Bohr-effect, which shall support the unloading of the hemocyanin at the tissue. Therefore, there has to be a decrease in pH_e at the tissue to support oxygen-release. However, at Bohr coefficients < -1 the amount of CO_2 produced from blood oxygen respiration (1 mol O_2 → 1 mol CO_2) is insufficient to cause such a drop in pH_e. Additional CO_2 may be provided by cutaneous O_2 uptake (Pörtner 1994, 2002) or (in *S. officinalis*) by O_2-linked CO_2 binding in the hemocyanin, which carries CO_2 from the gills to the tissue (Lykkeboe et al. 1980).

1.2.2 Aerobic metabolism

The crucial role of O_2 for cephalopod performance is also reflected in the metabolic pathways. Aside cytosolic glycolysis, energy (ATP) is mainly produced by the electron transport system (ETS or respiratory chain) of the mitochondria (Fig. 1.3) (Hoeger et al. 1987, Pörtner 1987). The Krebs-cycle produces most of the reduction equivalents ($NADH+H^+$ / $FADH_2$) for the ETS and is fueled by two major sources. The first source is the carbohydrate glycogen that is also considered being the major energy storage compound (Hochachka et al. 1975). Glycogen entering the glycolysis is metabolized to pyruvate and enters the Krebs-cycle as acetyl-CoA (Fig. 1.3).

The second energy source is the large pool of free amino acids, especially proline, in the cytosol (Hochachka 1994, Lee 1994). Proline is found in very high concentrations and has also been proposed as the major energy source (Mommsen et al. 1982, Lee 1994). Other major amino acids are aspartate, arginine and glutamate and there is

interconversion between them (Hochachka et al. 1975, Mommsen et al. 1982, Hochachka & Fields 1982). Before proline enters the Krebs-cycle, it gets oxidized to glutamate (Fig. 1.3). This step consumes ½ mol O_2 per mol proline, which reduces the energy yield (Sacktor 1970, Hochachka & Fields 1982). Therefore, glycogen is preferred energy substrate, especially under oxygen limited conditions like hypoxia or high ambient temperatures (Mommsen et al. 1982, Hochachka 1994, Oellermann et al. 2012). When temperature decreases, the energy output of both pathways is reduced and succinate may act as an additional substrate, which is less temperature sensitive but also less efficient (Oellermann et al. 2012). The coupled catabolism of glycogen and amino acids increase the ATP production per mol O_2 by 50% and thus strongly raises energy efficiency (Lee 1994).

Rapid uptake of blood glucose has been proposed as another possible energy source, but low concentrations of the enzyme hexokinase, which is essential for fast glucose uptake, argue against this assumption (Hochachka et al. 1975). The cellular glycogen storages are supposed to derive from the conversion of the major amino acids to pyruvate followed by gluconeogenesis (Hochachka & Fields 1982). Fatty acids concentrations are very low and they are hardly used for energy production (Culkin & Morris 1970). A reason for this could be the higher O_2 consumption during fatty acid metabolization. For cephalopods, oxygen is considered the limiting factor of performance (see above) and thus, it makes sense that they rely in energy sources that consume less O_2 per produced ATP like carbohydrates or amino acids (Hochachka 1994, Oellermann et al. 2012).

Different cephalopod tissues receive different amounts of oxygen. For example, the cephalopod mantle consists of three regions of muscles fibers for mantle contraction. The thin inner and the outer layers of the mantle (adjacent to the ambient or mantle cavity water) receive oxygen via cutaneous respiration and are strongly perfused by blood vessels. The muscle cells of these layers are densely filled with mitochondria, thus indicating large aerobic capacity (Bone et al. 1981). The muscle fibers of the central mantle are poorly perfused and display low mitochondria density. The fibers in the periphery are used for constant ventilator mantle contraction, which is maintained fully aerobic. The central fibers are used to create strong water jets during escaping or attack on prey. This burst activity is mainly fueled by anaerobic metabolism (see 1.2.3). Another difference occurs between the systemic heart, which receives O_2-rich blood from the gills, and the branchial hearts, which receive the O_2-poor blood

returning from the body. The systemic heart has a more active energy metabolism, which is also reflected by higher enzyme activities (Driedzic et al. 1990, Oellermann et al. 2012). The branchial hearts consume less ATP and produce lower blood pressures, but have a higher hypoxia tolerance, while the systemic heart is considered obligatory aerobic (Wells & Wells 1983, Driedzic 1985).

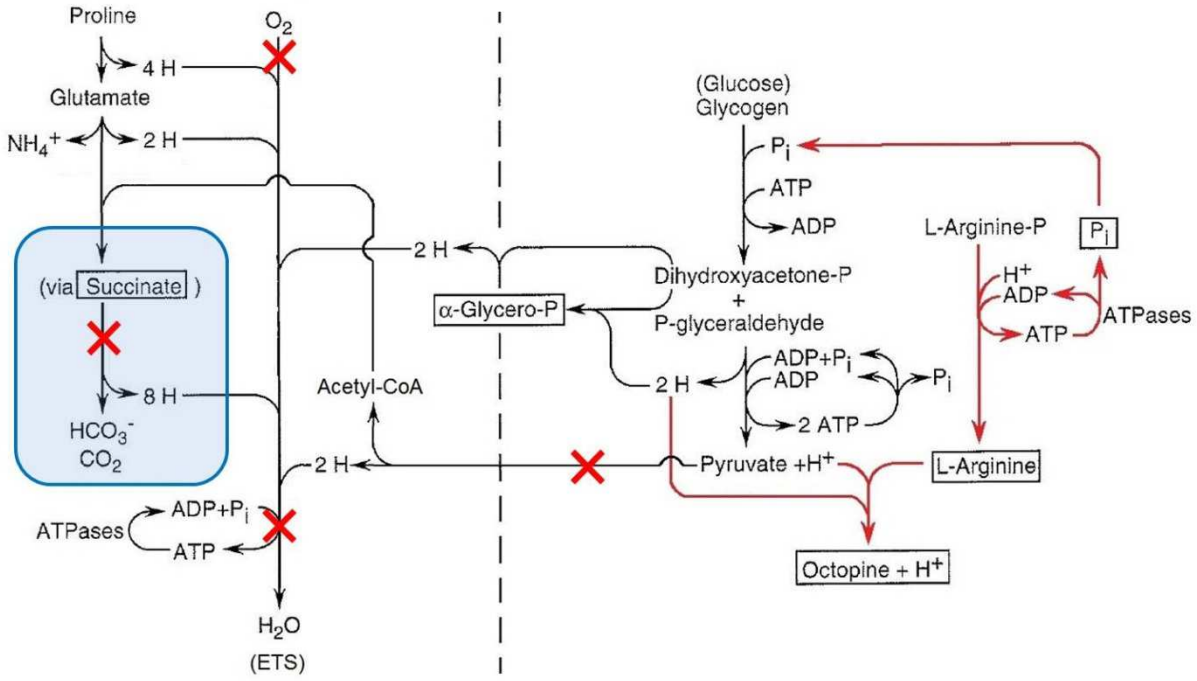

Fig. 1.3: Major metabolic pathways during aerobic and anaerobic metabolism in cephalopods (simplified, stoichiometry not correct). Red arrows represent reactions, which only take part during anaerobic metabolism. Red crosses indicate reactions, which do not take place during anaerobic metabolism. Metabolites in boxes accumulate during anaerobic metabolism. The blue box represents the Krebs-cycle. The vertical dashed line represents the border between mitochondrion (left) and cytosol (right). The transport of proline from the cytosol to the mitochondrion and the O_2 consumption during proline conversion are not shown. Figure adopted from Pörtner & Zielinski (1998) and changed.

Generally, aerobic metabolism is designed to create a constant supply of ATP for cellular functioning. This implies a steady-state situation with stable concentrations of ATP, ADP and AMP. The steady-state equilibrium may shift depending on the ambient conditions, but there is no accumulation of end products (Hochachka 1994). As a consequence, the energy status of the tissue (expressed as Gibbs free energy change of ATP hydrolysis, $\Delta G/\Delta\xi$) is kept constantly high (Pörtner 1987). Gibbs free energy values under control conditions usually lie at -56 kJ*mol^{-1} in cephalopods (Pörtner et al. 1996, Melzner et al. 2006a). A value of -44.7 kJ*mol^{-1} is considered the critical value for cellular ATPase functioning and indicated the onset of muscle fiber contractile failure (Pörtner et al. 1996, Pörtner 2002).

1.2.3 Anaerobic metabolism

Despite their sophisticated adaptations for the protection of aerobic metabolism, cephalopods regularly have to face situations when the O_2 supply is insufficient (e.g. environmental hypoxia). Under these conditions cephalopods can resort to a well-developed anaerobic metabolism to overcome such oxygen-limited situations (Storey & Storey 1979, Pörtner 1987, Grieshaber et al. 1994). Energy demands during fast jet-propelled swimming usually exceed the energy provision by aerobic metabolism, as this is limited by the O_2-supply (Hoeger et al. 1987, Pörtner 1994, Finke et al. 1996, see 1.2 & 1.2.1). A similar situation occurs during environmental hypoxia, when the energy demands are stable, but the O_2 supply is reduced and less ATP can be produced.

Anaerobic metabolic pathways help to resolve this impairment of energy demands, albeit creating an oxygen debt due to the accumulation of end products (Lewis et al. 2007, Rosa & Seibel 2008). Anaerobic metabolism in cephalopods is based on the use of glycogen and phospho-L-arginine (PLA) and creates the anaerobic end product octopine + H^+ (Fig. 1.3) (Grieshaber & Gäde 1976, Pörtner 1987). The proton actually derives from pyruvate formed during anaerobic glycolysis. The pyruvate is condensed with L-arginine from phosphagen mobilization yielding octopine, which means that per mol octopine (or pyruvate), 1 mol H^+ is produced (Grieshaber & Gäde 1976, Pörtner 1987). The production of protons supports the mobilization of the phosphagen and the transfer of the phosphate group yielding ATP. Although the phosphagen mobilization consumes protons (0.24 mol H^+ per 1 mol PLA at pH 7.3, Pörtner 1987), the anaerobic metabolism causes a drop in intracellular pH (pH_i), which is clearly correlated to the production of octopine + H^+ (Pörtner et al. 1991, Pörtner et al. 1993). Other accumulating end products are α-glycerophosphate and NADH+H^+ from the anaerobic glycolysis (Fig. 1.3). Due to the lack of oxygen, the mitochondrial ETS can no longer consume reduction equivalents and NADH+H^+ is no longer shuttled into the mitochondria via α-glycerophosphate (Fig. 1.3) (Grieshaber & Gäde 1976, Pörtner 1987). Generally, anaerobic pathways provide less ATP than aerobic ATP production by the ETS. The aerobic metabolization of 1 mol glucose to CO_2 + H_2O provides ~36 mol ATP + ~18 additional ATP, if proline is metabolized in parallel (Hochachka et al. 1975, Storey & Storey 1983). The anaerobic degradation to intermediates like succinate does not create more than 4 mol ATP / mol glucose (Hochachka et al. 1975, Storey & Storey 2005). Therefore, glycolytic enzyme activity

is elevated during anaerobic metabolism to increase the ATP output (Finke et al. 1996). Anaerobic metabolism is time-limited due to the depletion of energy storages (glycogen, PLA) and the accumulation of end products (Pörtner 1987).

The net degradation of ATP during anaerobiosis results in a higher total ADP-concentration, as well as a higher percentage of unbound ADP in the cytosol (Pörtner et al. 1993). AMP-concentrations increase too, with a higher fraction of AMP remaining unbound (Pörtner et al. 1993). The increase in free ADP and AMP affects enzymatic functions. It has been shown that free ADP enhances glycolytic enzyme activities (Storey & Storey 1978). The preferred way of PLA mobilization differs between cephalopod species. In the hypoxia-tolerant brief squid *Lolliguncula brevis*, total and free ADP stay almost constant during anaerobic metabolism and the use of PLA is mostly triggered by a drop in pH_i (Pörtner et al. 1996, Pörtner 2002). In contrast, anaerobic metabolism causes only small intracellular acidosis in the longfin inshore squid *Loligo pealei* and PLA mobilization is mainly caused by a strong rise in free ADP while total ADP stays more or less constant (Pörtner et al. 1993, Pörtner 2002). The accumulating inorganic phosphate (P_i) released during ATP-hydrolysis additionally enhances glycogen mobilization (Fig. 1.3) and thus provides fuel for the glycolysis (Pörtner & Zielinski 1998). The accumulation of $NADH+H^+$ and octopine would usually inhibit glycolytic enzyme activities (Storey 1981, Pörtner et al. 1993), but the increase in free AMP overrides this inhibition and activates phosphofructokinase, which is a key enzyme of glycolysis (Storey & Storey 1983, Pörtner et al. 1993). The maintenance of proper glycolytic function during anaerobiosis by high free AMP levels is an exclusive feature of cephalopods (Storey & Storey 1983).

As indicated by the accumulation of α-glycerophosphate (see above), hypoxia is not confined to the cytosol, but does also affect mitochondria (Pörtner 1987, Finke et al. 1996). During hypoxia, the O_2 supply is insufficient to ensure metabolization of Krebs-cycle and ETS intermediates. Thus, the intermediates accumulated and stop both pathways by product inhibition. The onset of anaerobic metabolism in the cytosol also seems to be triggered by mitochondrial hypoxia as shown in *L. brevis* (Pörtner 1995, Finke et al. 1996). One intermediate of mitochondrial anaerobic metabolism is succinate (Fig. 1.3) and acetate and propionate may accumulate during long-term anaerobic metabolism (Pörtner 1987, Grieshaber et al. 1994). All three metabolites derive from reactions of the Krebs-cycle, which utilize malate and

may also be fueled by proline degradation (Pörtner 1987, Storey & Storey 1978, Mommsen et al. 1982) However, the concentration of succinate during hypoxia has been found to be much lower than that of octopine indicating a minor role of mitochondrial anaerobic ATP production during hypoxia (Zielinski et al. 2000, Rosa & Seibel 2010). If the ETS-induced membrane potential is maintained, there is no drop in mitochondrial pH during anaerobiosis, as protons are consumed in the Krebs-cycle (Pörtner 1987).

Despite the buffering of ATP levels by the use of PLA storages, anaerobic metabolism ultimately leads to a decrease and ATP and Gibbs free energy. There are several mechanisms to delay the drop of the Gibbs free energy, which were nicely summarized by Pörtner (2002). The accumulation of free ADP supports the buffering of ATP, as it activates glycolysis and thus ATP production. The production of octopine removes arginine, which would antagonize PLA mobilization and thus ATP buffering. The intracellular acidosis and the accumulation of P_i from ATP degradation both decrease muscle performance and thereby also reduce ATP consumption (Pörtner 2002).

The fate of the anaerobic end products is still under discussion. Storey & Storey (1979) found a rapid uptake of octopine injected into the blood by aerobic tissues (brain, ventricle) and proposed a blood transport of octopine from sites of production (anaerobic tissues) to sites of O_2-consuming degradation (aerobic tissues). This assumption is supported by the presence of different isoforms of the octopine creating/degrading enzyme octopine-dehydrogenase (ODH) in the different tissues of *S. officinalis*. Whereas an octopine forming isoform is dominant in anaerobic tissues, an octopine degrading isoform predominates in aerobic tissues (Storey 1977). However, findings of constantly low blood octopine levels in squids during rest, exercise and recovery (Pörtner et al. 1991) contradict this hypothesis. Pörtner et al. (1991) found octopine and metabolic protons almost completely retained in the cells of squid mantle tissue during exercise and postulated that anaerobic ends products are recycled within the tissue, when the O_2 is again sufficient (Pörtner et al. 1993). As protons do not leave the cellular space, a drop in pH_i should not affect pH*e*. Only if stressful conditions (e.g. exercise, hypoxia) are too severe or last too long, a drop in pH_e can be observed indicating an H^+ leakage from the tissue (Pörtner et al. 1991).

1.3 Physiological effects of hypoxia and hypercapnia

Oxygen is an essential energy source for all animals. As mentioned above, the availability of O_2 is considered one of the most important factors affecting an animal's performance (Pörtner 2001, Pörtner et al. 2005). Hypoxia causes reduced growth and performance in various taxa (Jones 1971, Walsh et al. 1984, Driedzic 1985, Houlihan et al. 1987, Taylor & Miller 2001). Reduced oxygen availability can also have pronounced effects on a species' vulnerability to other factors like changing temperatures or pathogens (Pörtner 2001, Cheng et al. 2002, Pörtner et al. 2005). Physiological response includes the use of amino acids with a low O/N-ratio, which means that less oxygen is needed for energy production by amino acids catabolism (Langenbuch & Pörtner 2002). This mechanism was identified in the peanut worm *Sipunculus nudus* and could also be present in other taxa relying on amino acids as energy source (e.g. cephalopods).

During hypoxia, many cephalopods show a rise in venous pH_e, which then can even exceed arterial pH_e (Johansen et al. 1982a, Houlihan et al. 1982, Häfker & Seibel unpubl.). This is partially caused by the acting of the strong Bohr-effect, which results in an uptake of protons by the hemocyanin, when the pigment is not loaded completely with O_2 (see 1.2.1). Additional blood alkalosis can be caused by an increase of blood bicarbonate concentrations during hypoxia (Johansen et al. 1982a). The HCO_3^- is supposed to derive from anaerobic metabolism in the tissue, where the degradation of PLA causes a rise in bicarbonate levels (Burton 1978). The increase of HCO_3^- concentrations has been reported for squids and cuttlefish during hypoxia (Johansen et al. 1982a, Zielinski et al. 2000). In squids, this alkalosis can contribute to a higher oxygen extraction rate from the water as standard extraction rates are naturally low (Wells 1994). A similar hypoxia-induced rise in arterial pH_e was measured in *Octopus vulgaris* and was attributed to the acting of the Bohr-effect and an increased blood HCO_3^- concentration (Houlihan et al. 1982). Whereas a high pH_e supports the O_2 loading of the hemocyanin at the gills, it impedes the unloading at the tissue (Johansen et al. 1982a). However, the oxygen affinity of hemocyanin is reduced during hypoxia and the complete saturation curve is shifted towards higher pH values (Zielinski et al. 2001, Melzner et al. 2007b). Thus, the rise in pH_e could aid proper functioning of the Bohr-effect. CO_2 supply by cutaneous O_2 respiration (mainly squids) or transport by the blood pigment (*S. officinalis*) can support blood

acidification and oxygen unloading (Lykkeboe et al. 1980, Pörtner 1994, 2002), but total tissue O_2 supply is clearly reduced during hypoxia (Houlihan et al. 1982).

In the tissue, the most prominent change during hypoxia is the onset of anaerobic metabolism with the associated changes in metabolite concentrations and energy status (see 1.2.3). Anaerobic metabolism does not replace aerobic metabolism, but provides additional ATP, if the energy requirements can no longer be met by the aerobic ATP production alone. A decrease in total energy consumption and in some cases heart rate (metabolic depression) has also been reported for several cephalopod species during hypoxia (Houlihan et al. 1982, Johansen et al. 1982b, Rosa & Seibel 2008, 2010). This down regulation of ATP consuming processes enables animals to stay aerobic, if the ATP production by the ETS is reduced, thus delaying the onset of anaerobic metabolism. The metabolic depression also extends the time for anaerobic metabolism, because the energy storages are depleted slower at low metabolic rates. The oxygen concentration at which the first hypoxia response can be detected usually lies between 60 µmol*L^{-1} and 120 µmol*L^{-1}, but can differ strongly among taxa and species (Gray et al. 2002). If hypoxia becomes too severe to allow adaption, anaerobic metabolism begins.

Hypercapnia can have quite paradoxical effects on animal physiology. On the one hand, it can cause hyperventilation in several marine taxa including cephalopods like the cuttlefish *S. officinalis* (Pörtner et al. 2005, Gutowska et al. 2010). The accumulation of CO_2 in the blood decreases the pH_e and reduces hemocyanin O_2 binding due to the acting of the Bohr-effect (see 1.2.1). Animals try to reduce the blood CO_2 content by hyperventilation, but this is rather inefficient because of the small gradient between blood *P*CO2 and ambient water *P*CO2 (Rahn 1966, Heisler 1986). Additionally, respiration does only reduce blood CO_2, which is the minor component of the bicarbonate system (< 1%), while HCO_3^- (~90%) and CO_3^{2-} (~10%) stay unaffected (Dickson 2010, Melzner et al. 2012). On the other hand, hypercapnia is also a very common trigger for metabolic depression, which means that while ventilation increases, O_2 consumption decreases (Rees & Hand 1990, Pörtner et al. 1998, Michaelidis et al. 2005a). The effects of hypercapnia on metabolic rate in cephalopods are diverging. Whereas moderate hypercapnia (0.1 kPa) together with hypoxia caused significantly reduced activity and metabolic rates in a squid (Rosa & Seibel 2008), exposure to severe hypercapnia (0.6 kPa) caused hyperventilation in

S. officinalis, but had no effect on metabolic rate, growth and calcification (Gutowska et al. 2008).

The triggering mechanism of metabolic depression is not fully understood yet. In the bivalves *Mytilus galloprovincialis* and *Crassostrea gigas*, a metabolic reduction was clearly correlated to a decrease in pH_e (Michaelidis et al. 2005a,b), but in *Octopus vulgaris* and the jumbo squid *Dosidicus gigas* a decrease in O_2 consumption occurred during hypoxia, despite a parallel rise in pH_e (Houlihan et al. 1982, Häfker & Seibel, unpubl.). Intracellular acidosis is also considered as a possible factor causing metabolic depression. As discussed before (see 1.2.3), H^+ accumulation affects muscular activity and thus reduces energy consumption. Another assumed mechanism is a central nervous control regulated by the accumulation of adenosine (Reipschläger et al. 1997, Schwartz et al. 2003). The accumulation of adenosine is in turn supposed to be correlated to intracellular acidosis or the accumulation of HCO_3^- (Pörtner et al. 2005).

1.4 Study objectives

There are several studies that investigated the effects of hypoxia or hypercapnia on marine animal physiology in general and cephalopod physiology in particular (Grieshaber et al. 1994, Hochachka et al. 1996, Wu 2002, Gutowska et al. 2008, 2010), but studies assessing the effects of simultaneous exposure to both stressors are scarce (Pörtner et al. 2005, Rosa & Seibel 2008, Melzner et al. 2012). However, hypoxia and hypercapnia often co-occur in the ocean (see 1.1.1 & 1.1.2) and the combination of both factors could therefore represent a situation, which is closer to the natural environment (Pörtner et al. 2005, Melzner et al. 2012). In cephalopods, this interaction of factors might be of special relevance, as hypercapnia should counteract the rise in pH_e during hypoxia and could thus support oxygen unloading at the tissue. The fact that hypercapnia is a common trigger for metabolic depression (see above) could cause reduced O_2 consumption during simultaneous hypoxia and would thus support long-term survival under low oxygen conditions.

Although the changes induced by hypercapnia, hypoxia or both, support resistance to stressful ambient conditions, they do also reduce performance, growth and reproductive output and increase the vulnerability to predators and changing temperatures (Pörtner 2001, Pörtner et al. 2005). Despite being advantageous for the individual, the said factors could thus have negative effects on stock sizes and

community composition on the long-term (Pörtner et al. 2005). However, studies often focused on the determination of critical oxygen and CO_2 tensions during acute exposure while long-term effects on physiology and development are poorly investigated (Wells et al. 1988, Gutowska et al. 2008, 2010, Rosa & Seibel 2008, 2010). Climate change and human activities are expected to have profound permanent effects on ocean O_2 and CO_2 content, as well as other factors (see 1.1), which might have severe consequences for marine life. Cephalopods may be especially affected by this trend to their high activity levels and the limited physiological capacities (see 1.2).

This study assesses the effects of hypoxia and hypercapnia on the physiology of a well-investigated cephalopod model organism, the common cuttlefish *Sepia officinalis*. The species is considered a good acid-base regulator (Gutowska et al. 2010), thus indicating a certain potential for resistance to both stressors. The species lives in the bottom waters of the continental margins (see 2.1). These areas are expected to be strongly affected by future changes in ocean O_2 and CO_2 content (Melzner et al. 2012). It is the aim to determine the separate effects of hypoxia and hypercapnia as well as to identify possible synergistic effects of both factors. These effects are studied in muscular tissues, which are naturally exposed to different oxygen concentrations within the body (mantle, systemic heart, branchial hearts). The effects on blood acid-base parameters, which are important for oxygen supply, are also investigated. The results of this study could help to formulate unifying principles of cephalopod physiology and to predict population development in a future ocean. Additionally, it could help to get a better insight into the physiology of *S. officinalis*, an important cephalopod model and target species for fisheries, which also has the potential for large-scale food production in aquaculture (see 2.1).

2 Materials & Methods

2.1 *Sepia officinalis* (Linnaeus, 1758), Sepiidae, Cephalopoda

The common cuttlefish, *Sepia officinalis* is widespread in neritic waters around Europe. Its distribution ranges from the North Sea over the western shelf areas of the British Isles, France and the Iberian Peninsula down to the coast of Mauretania and the Senegal (Fig. 2.1B). The species is also found in nearly all shelf areas of the Mediterranean (von Boletzky 1983, Jereb & Roper 2005). A number of other subspecies is described, but their status is still partially unclear (Jereb & Roper 2005). Genetic analysis revealed several distinct populations. For example, the *S. officinalis* populations from the English Channel can be distinguished from the Mediterranean population (Pérez-Losada et al. 2002, Wang et al. 2003, Wolfram et al. 2006).

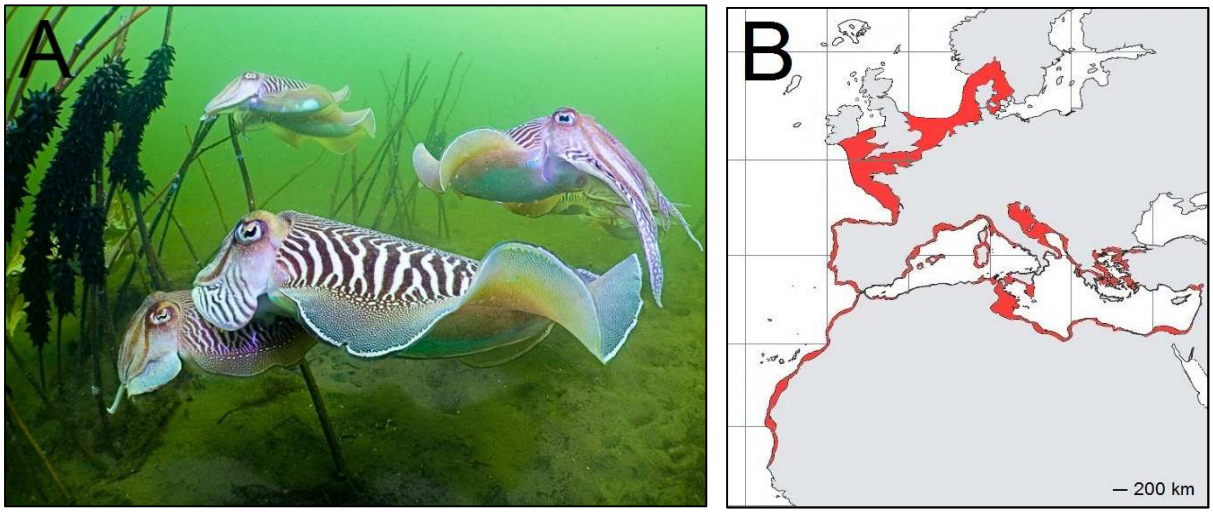

Fig. 2.1: Habitus and distribution of the common cuttlefish *Sepia officinalis*. A: Cuttlefish during mating in the Oosterschelde, Netherlands. The zebra-like banding pattern indicates maturity. Black egg clutches are visible on the left side of the picture. © Joris van Alphen. B: Distribution map of *S. officinalis*. Red areas represent regions where the common cuttlefish can be encountered. Figure adopted from Jereb & Roper (2005) and changed considering the findings of von Boletzky (1983).

The cuttlefish lives on the continental shelf in depths reaching from the surface down to 150 (rarely 200) m (von Boletzky 1983, Jereb & Roper 2005). The species is also found in brackish waters and can tolerate salinities down to 27 psu (Mangold-Wirz 1963). *Sepia officinalis* is a eurythermal species exposed to broad temperature ranges (von Boletzky 1983), which differ between populations. For example, cuttlefish from the English Channel experience temperatures of 9-17.5℃ (Bocaud-

Camou & Boismery 1991), whereas specimen from the Mediterranean are exposed to 10-30℃ ambient temperatures (Artegiani et al. 1 997, Mark pers. comm.).

The common cuttlefish has a demersal lifestyle and prefers sandy or muddy bottoms. It often burrows in the ground leaving only the eyes above the surface (Jereb & Roper 2005). *S. officinalis* performs hovering movements using the lateral fins for propulsion. The calcified internal shell, the cuttlebone, contains gas filled chambers and acts as a floatation device. Jet propulsion is only used as an escape response or during attacks on prey. The diet consists of various taxa of crustaceans, polychaetes, molluscs (also other cephalopods) and fishes (Castro & Guerra 1990).

The embryonic development of the cuttlefish is temperature dependent and lasts 80-90 days at 15℃ (von Boletzky 1983). The development time decreases with increasing temperature. At 15℃, maturity is reached after 14 – 18 month at a size varying from 6 – 8 cm mantle length in males to 11 – 25 cm mantle length in females (von Boletzky 1983). Adults of temperate regions can grow to a size of 50 cm and a weight of 2 kg, whereas subtropic adults stay smaller (30 cm, 2 kg) (Jereb & Roper 2005). Mature specimens display a zebra-like banding pattern, which is especially pronounced in males (Fig. 2.1A). *S. officinalis* can reach an age of up to two years but dies after the first reproduction (semelparity) (von Boletzky 1983). Spawning takes place in shallow coastal waters, where eggs are attached to seaweeds, rocks or other solid structures (Fig. 2.1A) (Jereb & Roper 2005). The common cuttlefish is an important species for commercial fisheries and is sold as high quality food (Jereb & Roper 2005). The species is also considered suitable for larger scale breeding in aquaculture, thus providing a potential fast-growing high quality food source (Sykes et al. 2006). Stock sizes are poorly known but populations are not considered threatened (Dunn 1999, Wang et al. 2003).

In the present study, the data of juvenile cuttlefish from two populations was investigated (see 2.2). *S. officinalis* exposed to hypoxia (HOx) or the respective control conditions (C_HOx) originated from eggs collected near Caen (France) at the English Channel in June 2010 (Thonig 2011). *S. officinalis* exposed to hypercapnia (HCa) or the respective control conditions (C_HCa) originated from eggs collected in the Venice Lagoon (Mediterranean) in May/June 2009 (Strobel 2011). *S. officinalis* exposed to simultaneous hypoxia + hypercapnia (H+H) or the respective control conditions (C_H+H) originated from eggs collected in the Venice Lagoon (Mediterranean) in 2011 (present study).

2.2 Study design & experimental set-up

The effects of hypoxia and hypercapnia on cuttlefish physiology were investigated in a manipulative laboratory experiment. In this experiment, specimens of *S. officinalis* were exposed to hypoxia and hypercapnia or kept under control conditions. Incubation was carried out during the time of this master thesis (10.02.2012-10.09.2012). The samples of this experiment were compared to samples derived from cuttlefish exposed to either hypoxia (Thonig 2011) or hypercapnia (Strobel 2011) or the respective control conditions in earlier experiments. All incubations were carried out in the section “Integrative Ecophysiology” at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven, Germany.

2.2.1 Hypoxia & hypercapnia

Cuttlefish were kept either under hypoxia and hypercapnia (H+H: O_2 = 61 ± 6% air saturation, CO_2 = 0.11 ± 0.02 kPa) or under control conditions (C_H+H: O_2 = 96 ± 9% air saturation, CO_2 = 0.04 ± 0.002 kPa) for ~5 weeks (21.03.2012 – 03.05.2012). Values are presented ± standard deviation (SD). Both treatments included n = 24 individuals each. Cuttlefish body mass (at dissection date) was 16-24 g.

The cuttlefish of the different incubations were kept in two separate seawater recirculation systems. The experimental set-ups for both incubations were identical (except for the gas bubbling, see below) and each system had a total volume of ~1010 L. A system consisted of a reservoir tank (~660 L), a header tank above the incubation boxes and a collection tank below (Fig. 2.2). All three tanks were covered with lids to minimize gas exchange. The 8 incubation boxes (transparent PVC, 30 x 20 x 14 cm, 84 L) were put into 2 overflow basins with 4 boxes per basin. Each incubation box contained 3 individuals of *S. officinalis* and was covered with a lid (Fig. 2.3). The basins and the header tank were arranged in a rack and the racks of both systems were covered with black plastic foil. The creation of a dark environment minimizes stress for the cuttlefish (Denton & Gilpin-Brown 1961, Mark, pers. comm.).

The water was pumped (38 $L*min^{-1}$ & 40 $L*min^{-1}$ pumps, Eheim GmbH & Co. KG, Deizisau, Germany) from the collection tank into the reservoir tank and then into the header tank (Fig. 2.2). From the header tank the water flowed though PVC tubes into the incubation boxes. The overflow basins collected the water pushed out of the boxes. Finally, the water flowed from the basins back into the collection tank below. The header and the reservoir tank had spillovers to the reservoir and the collection

tank, respectively (Fig. 2.2). This maintained constant water levels in all tanks. The average water throughput in the incubation boxes was 1.07 $L*min^{-1}$ (H+H) and 1.17 $L*min^{-1}$ (C_H+H). The average water parameters are shown in Tab. 2.1.

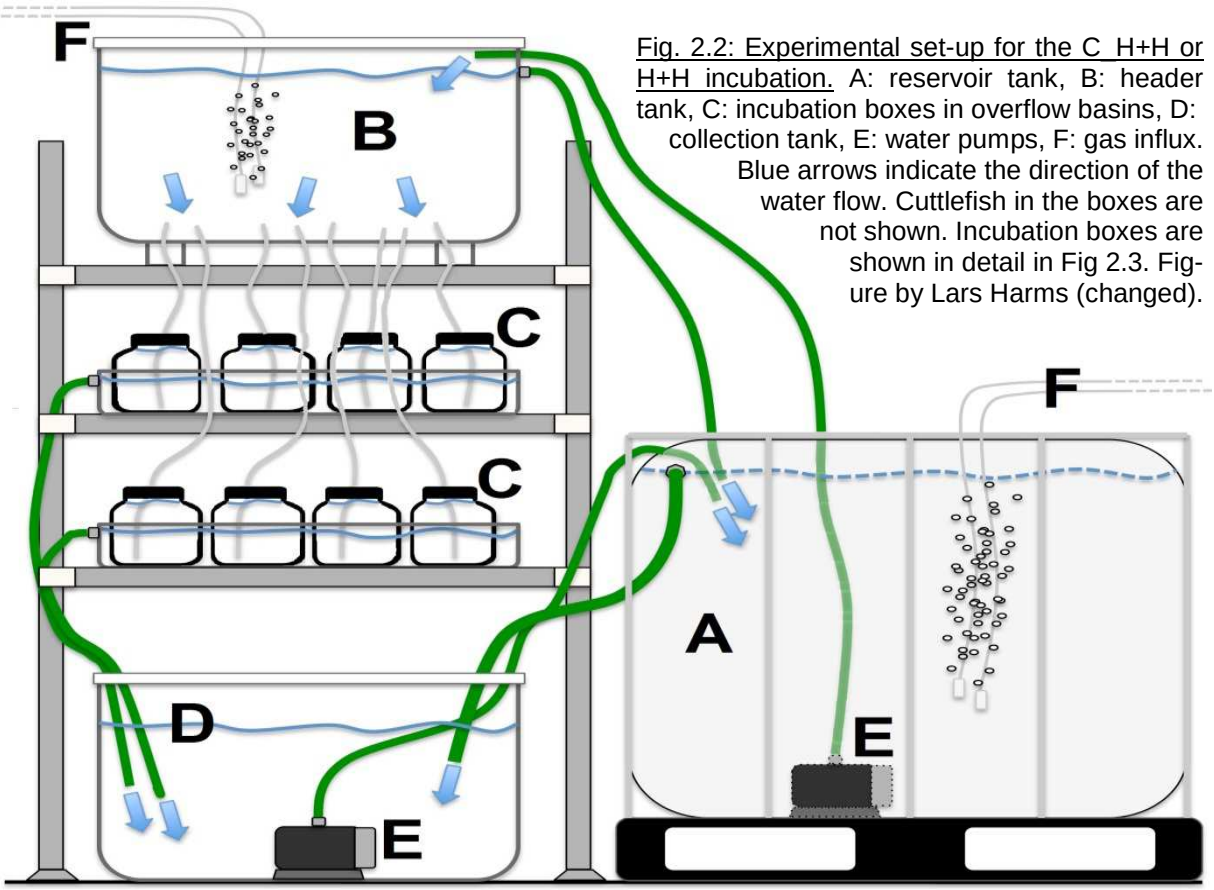

Fig. 2.2: Experimental set-up for the C_H+H or H+H incubation. A: reservoir tank, B: header tank, C: incubation boxes in overflow basins, D: collection tank, E: water pumps, F: gas influx. Blue arrows indicate the direction of the water flow. Cuttlefish in the boxes are not shown. Incubation boxes are shown in detail in Fig 2.3. Figure by Lars Harms (changed).

The reservoir and header tanks were constantly bubbled with a defined gas mixture of compressed air, CO_2 and N_2 to achieve the wanted O_2 and CO_2 concentrations in the respective system (Fig. 2.2). The gas for the C_H+H incubation was mixed by a gas mixing system (HTK Hamburg GmbH, Hamburg, Germany); the gas for the H+H treatment was mixed by another system (Multi-Channel Flow Ratio/Pressure Controller Type 647B, MKS Instruments Deutschland GmbH, München, Germany), which received premixed gas from the aforementioned HTK system and pure nitrogen (N_2). In the H+H treatment the interaction area between water and ambient air was reduced by the addition of buoyant plastic balls (Ø = 2 cm) to the overflow basins and the collection tank (Fig: 2.3).

Fig. 2.3: Four incubation boxes in an overflow basin. The boxes are covered with lids. The cuttlefish are visible on the bottom of the front boxes (brown). Each box was supplied by two water tubes from the header tank to increase the water throughput. The water surface of the overflow basin was covered with buoyant plastic balls (H+H incubation).

To avoid an accumulation of ammonia (NH_4^+) and nitrite (NO_2^-) in the system, the water was changed twice a week. For this purpose, the reservoir tank was uncoupled from the running system and the water in the tank was exchanged. After ~24 h of gas equilibration, the reservoir tank was coupled back to the system and the water in the header and collection tank was replaced. The cuttlefish were fed living grass shrimp (*Palaemonetes* cf. *varians*) once a week.

Tab. 2.1: Water parameters in the aquarium systems during the 5 weeks of incubation at H+H and C_H+H conditions (21.03.2012 – 03.05.2012). Mean values ± SD are shown. DIC: dissolved inorganic carbon. n: number of measurements for the respective parameter. The numbers of measurements were identical for both treatments. CO_2 was measured as [ppm] and then converted to [kPa] assuming standard atmospheric pressure of 101.325 kPa. Water pH was measured on the free H+ ion scale according to Hirse et al. (unpubl.).

Treatment	n	C_H+H	H+H
Temperature [℃]	12	15.9 ± 0.3	16.7 ± 0.2
Salinity [psu]	11	33.3 ± 1.2	33.1 ± 1.1
O_2 [% air saturation]	11	96 ± 9	61 ± 6
CO_2 [kPa]	8	0.042 ± 0.002	0.109 ± 0.017
water pH	11	8.15 ± 0.14	7.77 ± 0.07
DIC [µmol*L^{-1}]	7	2216 ± 41	2304 ± 70
NH_4^+ [mg*L^{-1}]	12	0.13 ± 0.04	0.12 ± 0.06
NO_2^- [mg*L^{-1}]	12	0.32 ± 0.18	0.36 ± 0.20

The water pH was determined using a pH-meter (pH 3310, WTW GmbH, Weilheim, Germany) with a glass electrode (Inlab Routine Pt1000®, Mettler-Toledo GmbH, Gießen, Germany). To account for temperature dependent fluctuations, pH values

were corrected with the values of Tris buffered synthetic seawater standard. Standard was obtained from the Scripps Institution of Oceanography (San Diego, USA). The pH standard was measured directly after the incubation water.

2.2.2 Hypoxia

In an experiment of Thonig (2011), *S. officinalis* (n = 7) was exposed to an oxygen concentration of 51 ± 4% air saturation at an ambient temperature of 15.3 ± 0.2℃ for an incubation period of 9 weeks (09.02.2011 – 12.04.2012). The CO_2 concentration was not monitored or regulated. Assuming equilibrium of CO_2 with the ambient air, the CO_2 partial pressure of the water was probably close to 0.04 kPa. The treatment was defined as (HOx). In parallel, cuttlefish (n = 8) were kept in a control incubation at 100% O_2 air saturation (no SD given) and the same temperature (C_HOx). Cuttlefish body mass (at dissection date) was 40-70 g. The cuttlefish were fed daily with living sand shrimp (*Crangon crangon*). After dissection, samples of funnel, systemic heart and branchial hearts were stored in Eppendorf tubes at -80℃ for further analysis.

2.2.3 Hypercapnia

In an experiment of Strobel (2011), *S. officinalis* (n = 5) was exposed to a CO_2 partial pressure of 0.12 ± 0.01 kPa at an ambient temperature of 16.1 ± 0.4℃ for an incubation period of 21 weeks (19.05.2010 – 13.10.2010). The O_2 concentration was 100% air saturation. The treatment was defined as (HCa). In parallel, cuttlefish (n = 5) were kept in a control incubation at 0.04 kPa CO_2 (no SD given) and the same temperature (C_HCa). Cuttlefish body mass (at dissection date) was 40-160 g. The cuttlefish were fed living or frozen sand shrimp (*C. crangon*) twice a week. After dissection, samples of funnel were stored in Eppendorf tubes at -80℃ for further analysis. Branchial heart samples were wrapped in labeled aluminum foil and stored in liquid nitrogen for further analysis (-196℃). S ystemic hearts were not sampled.

2.3 Sample processing & analysis

2.3.1 Sampling

The dissection procedure described here refers to the animals incubated at H+H or C_H+H conditions. There was no noteworthy difference to the dissection procedure

of the animals incubated at hypoxia (Thonig 2011) or hypercapnia (Strobel 2011), except that funnel instead of mantle tissue of the hypoxia or hypercapnia treated animals and the respective controls was sampled (Mark, pers. comm.). Each animal was dissected separately. An individual was removed from the incubation basin and placed into an opaque bucket containing water from the respective incubation with 3% ethanol. Anesthesia was not expected to affect the measured tissue parameters (Storey & Storey 1979), but caused a deoxygenation of the blood pigment hemocyanin due to the ceasing of ventilation (Mark pers. comm.). The dissection was started when the cuttlefish showed no more movement and reaction to stimuli (after ~4 min). The animal was dabbed with paper to remove water. Weight, total length and mantle length were recorded. The animal was then placed on ice with the ventral side pointing upwards. The mantle and the funnel were cut open along the anteroposterior axis without damaging the organs in the mantle cavity. A blood sample was drawn from the *Vena cava cephalica* with a 1 mL plastic syringe. The processing of the blood sample is described in chapter 2.3.2. Afterwards, tissue samples of branchial hearts (BH), systemic heart (SH) and mantle (M) were taken (in that order). Each tissue sample was freeze-clamped, wrapped in labeled aluminum foil and stored in liquid nitrogen (-196℃). The we ights of the systemic heart and the branchial hearts were recorded before storage. Finally, a cut through the brain along the anteroposterior axis was performed to kill the animal. The complete dissection procedure took 5 – 7 minutes. All individuals were processed in a uniform manner.

2.3.2 Blood acid-base parameters

This section refers only to the animals incubated at H+H or C_H+H conditions (see 2.2.1). Blood parameters were not analyzed in the hypoxia or hypercapnia treated cuttlefish and in the respective controls. The partial pressures of O_2 (PO_2) and CO_2 (PCO_2) in the blood of the *Vena cava cephalica* were determined. The blood pH (pH_e) in this vessel was measured and the blood bicarbonate (HCO_3^-) concentration was calculated from the aforementioned values. The samples were analyzed using a blood gas analyzer (BGA) with gas electrodes for O_2 and CO_2 as well as a pH-electrode (MT 33, Eschweiler GmbH & Co. KG, Kiel, Germany). Data was recorded with the software Chart™ (v. 5.4.1, ADInstruments GmbH, Spechbach, Germany). The BGA was adjusted to incubation temperature (see Tab. 2.1) with a thermostat and calibrated. Standardized gases (0% O_2 / 0.2% CO_2, 5% O_2 / 0.5% CO_2, pure N_2)

were used for the calibration of the O_2- and the CO_2-electrode. The gases were purchased from AIR LIQUIDE Deutschland GmbH, Düsseldorf. The pH-electrode was calibrated with IUPAC pH standards (I = 0.1 M, pH = 6.865, 7.413 & 9.180 at 25℃). The electrodes were flushed with milli-Q wat er between the pH standards. The signal intensities [mV] recorded during calibration were later used to convert the recorded data of the samples into PO_2, PCO_2 and pH_e.

A small volume of the freshly drawn blood sample was injected into the BGA, making sure that all electrodes were covered with sample. The rest of the sample was stored on ice. When the measured signals were stable, the BGA was flushed with seawater (2-3 times) and the next sample was injected. If possible, the blood of each individual was measured twice and the mean was used for further calculations. pH_e was determined using the measured values of the pH standards. If the blood volume of a single animal was insufficient, blood samples of more than one individual were pooled. In the result section, pooled samples are marked with asterisks (*) in tables but are not explicitly mentioned in the text.

Tab. 2.2: Concentrations of major ions in the blood of *Sepia officinalis*. Mean values of 7 juvenile cuttlefish. Ion concentrations are given as mmol*L^{-1}. Data obtained from Wittmann (unpubl.).

Ion species	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}
Concentration [mmol*L^{-1}]	445.24	11.98	47.52	9.42	506.10	1.63

PO_2 and PCO_2 were calculated as [kPa] with the values recorded from the standardized gases, the temperature and the ambient air pressure during the measurement. To determine the concentration of HCO_3^- blood pK'''_e and the CO_2 solubility α_{CO_2e} were calculated according to Heisler (1986). To this end, the blood molarity (M_e) was calculated using the concentrations of the major ions (see Tab. 2.2) as well as the hemocyanin concentration in cuttlefish blood. Blood protein concentration was assumed to be identical to blood hemocyanin concentration because of the problems of oxygen transport and increasing viscosity discussed in the introduction (see 1.2.1). Thus, a total blood protein concentration of 150 g*L^{-1} was assumed (Wichertjes et al. 1986, Zielinski et al. 2001, Strobel et al. 2012). The concentration of bicarbonate in the blood (HCO_3^-) was then calculated as described in equation (1) as [mmol*L^{-1}]:

$$HCO_3^- = \left(P_{CO_2} * \left(10^{pK'''-pHi} * \alpha_{CO_2} + \alpha_{CO_2}\right)\right) - \left(P_{CO_2} * \alpha_{CO_2}\right) \quad [\text{mmol*L}^{-1}] \qquad (1)$$

2.3.3 Intracellular acid-base parameters

Intracellular pH (pH_i) was determined using the homogenate method developed by Pörtner et al. (1990) in a slightly modified way. Modifications are marked in the text (*) and the procedure of Pörtner et al. is provided in brackets at the end of the respective sentence. The intracellular CO_2 partial pressure (PCO_2) and the intracellular bicarbonate concentration (HCO_3^-) could then be calculated. The described measurements and calculations were performed in 15 (10 for PCO_2 and HCO_3^-) arbitrarily chosen mantle samples of cuttlefish from the H+H incubation and in 10 arbitrarily chosen mantle samples from the C_H+H incubation. Acid-base parameters were also measured in branchial hearts from the HCa and the C_HCa incubation. The branchial heart samples were completely consumed by this measurement, because of the small amount of tissue available. pH_i was measured twice in each mantle sample. Due to the shortage in sample mass, brachial hearts samples of different HCa animals were pooled and pH_i was measured only once. From each pH_i measurement two samples were taken for CO_2 analysis. This resulted in two pH_i values and four PCO_2 values for each mantle sample and in one pH_i value and two PCO_2 values for each pooled branchial heart sample. To end up with one value for each parameter and animal (or animal-pool), the values derived from one sample were averaged and means were used for further statistics.

To avoid changes in pH_i during sample preparation and measurement, potassium fluoride (KF) and nitrilotriacetic acid (NTA) were added to remove Mg^{2+} and Ca^{2+} ions. These ions are essential for the activity of kinases and ATPases. As the cellular H^+-concentration is mainly affected by adenylate dependent reactions including the activity of the muscular myosin ATPase (Pörtner et al. 1990), the inhibition of the involved enzymes by ion removal should conserve pH_i. Medium parameters were adopted from the cephalopod *Illex illecebrosus* according to Pörtner et al. (1990).

Before the sample analysis, a weak buffer medium with 160 mmol*L^{-1} KF and 2.9 mmol*L^{-1} NTA was prepared. The two constituents were stored separately in doubled concentration (320 mmol*L^{-1} KF & 5.8 mmol*L^{-1} mmol*L^{-1} NTA) and mixed at a 1:1 ratio before usage. The buffer capacity of the medium was low to prevent effects on the measured pH_i values. The medium pH was adjusted to 0.5 units below the

maximum expected pH_i using 0.05 mol*L^{-1} NaOH and 0.1 mol*L^{-1} HCl. Due to deterioration, the mixed medium was not used longer than a week.

For the sample analysis, an empty vial (0.9 or 1.2 mL, depending on the amount of available tissue) was weighed (W1) (MC1 Analytic AC 210S, Sartorius AG, Göttingen, Germany). The vial was then filled with 200 µl of KF/NTA-medium and weighed again (W2). Tissue samples stored at -196℃ were ground to powder under liquid nitrogen (N_2). Depending on the vial size, 100-200 mg (0.9 mL vial) or 200-250 mg (1.2 mL vial) tissue powder were then transferred to the vial. Beforehand, the medium in the vial was frozen in liquid nitrogen to keep the tissue cold. The vial with the tissue was weighed again (W3). Afterwards, the vial was completely filled with medium and air bubbles were released by stirring with a preparation needle. The vial was closed making sure that no air was left inside and weighed for the last time (W4). The recorded weights were used later to calculate the tissue wet weight and the CO_2 concentration in the tissue (see below). The sample was vortexed briefly (Vortex-Genie 2, Scientific Instruments, Bohemia, USA) and homogenized by ultrasound* for 1 min at 0℃ (80% intensity & 50% cycle, Branson So nifier 450, Hielscher Ultrasound GmbH, Teltow, Germany) (Pörtner et al.: No ultrasound homogenizing). After centrifuging* (1 min, 11000 g, 0℃) the pH in the s upernatant was measured with a pH optode (PreSens Needle-Type-Housing-pH-Microsensor, PreSens GmbH, Regensburg, Germany) at incubation temperature (see Tab. 2.1) (Pörtner et al.: Centrifuging for 15 sec). Before measurement, the optode was calibrated using a pH-meter with a glass electrode and IUPAC pH standards (I = 0.1 M, pH 6.865 & 7.413 at 25℃) with the ionic strength adjusted to I = 0. 16 mol*L^{-1} by adding potassium chloride (KCl). Data were recorded with the analysis software Chart (v. 5.3, ADInstruments GmbH, Spechbach, Germany) and HView (v. 5.25b, PreSens GmbH, Regensburg, Germany). During the sample preparation and the measurement, exposure to ambient air was minimized. This should prevent changes of the CO_2 concentration in the sample thereby also protecting sample pH.

Directly after pH_i measurement, 2 x 200 µL of the supernatant were drawn with a gastight Hamilton syringe* and were injected into two sealed gas chromatography (GC) vials for analysis of the CO_2 content (Pörtner et al.: Injection of 50 µL into a Hamilton syringe containing 2 ml 0.01 mol*L^{-1} HCl. Syringe was shaken for at least 2 min). The GC-vials were prepared before the start of sample analysis and were filled with 3 mL of air-equilibrated 0.1 mol*L^{-1} HCl each. The CO_2 content of the vials was

then measured by gas chromatography (6890N Network GC System, Agilent Technologies GmbH, Böblingen, Germany). Together with the samples, the CO_2 content of the KF/NTA-medium was determined. For this, 2 x 200 µL of medium were injected directly into 2 GC-vials per day. The results of the gas chromatography (provided as area) had to be converted into CO_2 concentrations in the supernatant [mmol*L^{-1}]. For this, a calibration curve was created by measuring CO_2 standards reaching from 0 mmol*L^{-1} CO_2 to 11.36 mmol*L^{-1} CO_2. The calibration curve was created daily and was measured together with the samples and the medium. The linear equation of the calibration curve was later used to convert the results of the GC [Area] to CO_2 concentrations [mmol*L tissue water^{-1}] according to equation (6).
From the CO_2 concentration in the supernatant, the initial concentration in the tissue [mmol*L tissue water^{-1}] could be calculated using the weights recorded during sample preparation (see above). To do this, the tissue mass (m_{tissue}) and the volume of medium (V_{medium}) in the sample were calculated.

$$m_{tissue} = W3 - W2 \quad [g] \tag{2}$$

$$V_{medium} = (W2 - W1) + (W4 - W3) \quad [mL] \tag{3}$$

The values $W1$, $W2, W3$ and $W4$ represent the respective weights recorded during sample preparation. For each sample a dilution factor f was calculated to account for the addition of KF/NTA-medium.

$$f = \frac{\left((m_{tissue} * 0.8) + V_{medium}\right)}{(m_{tissue} * 0.8)} \tag{4}$$

The values of m_{tissue} and V_{medium} are derived from equations (2) and (3). The value of 0.8 [mL*g tissue^{-1}] reflects the relative amount of water in the tissue. The dilution factor is dimensionless. The gas chromatography depicts an amount of CO_2 as the area under a peak. To obtain the real CO_2 amount in a sample ($A_{corrected}$), the CO_2 present in the medium (A_{medium}) had to be subtracted from the initially measured amount of CO_2 (A_{sample}). A_{medium} is the mean area of the pure KF/NTA-medium of the respective run. The dilution of the medium caused by the water enclosed in the

tissue was considered by inserting a factor derived from m_{tissue} and V_{medium} into the equation.

$$A_{corrected} = A_{sample} - \left(\frac{V_{medium}}{((m_{tissue} * 0.8) + V_{medium})} * A_{medium}\right) \quad (5)$$

The area values (A_x) are dimensionless. The concentration of CO_2 in the tissue water (Ctw_{CO_2}) was then calculated using the values derived from equation (4) and (5), as well as the slope (s) and the y-intercept (b) from the linear equation of the calibration curve.

$$Ctw_{CO_2} = (s * A_{corrected} + b) * f \quad \text{[mmol*L tissue water}^{-1}\text{]} \quad (6)$$

To calculate the intracellular CO_2 partial pressure (P_{CO_2}) it was necessary to determine the dissociation constant (${pK'''}_i$) and the CO_2 solubility ($\alpha_{CO_2 i}$) in the sample. The equations for these calculations were derived from Heisler et al. (1986) assuming a protein concentration of 200 g*L^{-1} for the calculation of ${pK'''}_i$ (Pörtner et al. 1990). Intracellular ion concentrations were adopted from Robertson (1965). For the calculation of $\alpha_{CO_2 i}$, a molarity (M_i) of 0.560 mol*L^{-1} was assumed (Pörtner et al. 1990). *P*CO_2 could then be determined using the calculated values of ${pK'''}_i$ and $\alpha_{CO_2 i}$, the CO_2 concentration in the tissue water (eq. 6) and the measured pH_i (eq. 7). The partial pressure given in [mmHg] was converted to [kPa] as described in equation (8). The conversion factor is valid for standard air pressure at sea level (101.325 kPa).

$$P_{mmHg} = Ctw_{CO_2} * \left(10^{{pK'''}-pHi} * \alpha_{CO_2} + \alpha_{CO_2}\right)^{-1} \quad \text{[mmHg]} \quad (7)$$

$$P_{CO_2} = P_{mmHg} * 0.1333223684211 \quad \text{[kPa]} \quad (8)$$

The intracellular bicarbonate concentration (HCO_3^-) was calculated from $\alpha_{CO_2 i}$, Ctw_{CO_2} and P_{mmHg} according to equation (9) as [mmol*L tissue water^{-1}].

$$HCO_3^- = Ctw_{CO_2} - \left(\alpha_{CO_2 i} * P_{mmHg}\right) \quad \text{[mmol*L tissue water}^{-1}\text{]} \quad (9)$$

2.3.4 Preparation of PCA-extracts

The concentration of many cellular substances cannot directly be determined from tissue samples. Therefore, the wanted metabolites were extracted from the tissue using perchloric acid (PCA). From each available tissue sample an extract was prepared for subsequent analysis. Samples were pooled, if the tissue mass of a single sample was insufficient (see end of this section). A part of the tissue was ground to powder under liquid nitrogen and 250-300 mg of the powder were transferred to a 2.0 mL Eppendorf tube prefilled with 0.5 mL frozen 0.6 mol*L^{-1} PCA. The weights of the empty tube (W5) and the tube filled with 0.5 mL PCA (W6) were determined beforehand. After adding the tissue, the tube was weighed again (W7). The exact tissue mass was calculated (eq. 10) and PCA was added in the volume necessary to reach a tissue/PCA-ratio of 1:5 (eq. 11). The respective total volume of PCA was calculated according to equation (12).

$$m_{tissue} = W7 - W6 \quad [\text{g}] \tag{10}$$

$$V_{PCA\,added} = m_{tissue} * 0.5 - (W6 - W5) \quad [\text{mL}] \tag{10}$$

$$V_{PCA\,total} = (W6 - W5) + (W8 - W7) \quad [\text{mL}] \tag{12}$$

The cup was closed and weighed (W8). Then it was vortexed briefly (Vortex-Genie 2, Scientific Instruments, Bohemia, USA) and homogenized for 2 min at 0℃ by ultrasound (80% intensity, 50% cycle). Afterwards the cup was centrifuged at 14000 g and 0℃ for 1 min. The complete supernatant was transferred to a new, pre-weighed (W9) 2.0 mL Eppendorf tube. The volume of the supernatant was determined by weighing the tube again (W10). 5 mol*L^{-1} KOH was added in an amount matching 8% of the supernatant volume (eq. 13).

$$V_{KOH\,added} = (W10 - W9) * 0.08 \quad [\text{mL}] \tag{13}$$

Afterward, 5 mol*L^{-1} KOH was added in small steps till a pH of ~7.5 was obtained. The total amount of added KOH was recorded (V_{KOH}). The pH was checked with indicator paper (pH 6.4-8.0, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The changing of the pH caused a precipitation of perchlorate. The tube was stored

on ice for ~2 min to allow precipitation. The tube was centrifuged at 14000 g and 0℃ for 2 min. The supernatant was distributed to labeled 0.5 mL Eppendorf tubes, which were then stored at -80℃.

The PCA-extracts are used for several kinds of analyses. However, the results of these analyses always show the concentration of the respective substance in the extract (Ce_x). The initial concentration in the tissue (Ct_x) was calculated as described in equation (14).

$$Ct_x = Ce_x * \frac{((m_{tissue} * 0.8) + V_{PCA\,total} + V_{KOH})}{m_{tissue}} \quad [\mu mol*g\ tissue^{-1}] \qquad (14)$$

x represents the respective substance analyzed. The value of 0.8 [mL*g tissue^{-1}] reflects the relative amount of water in the tissue. The calculation of Ce_x depends on the analytical method and is described in the respective sections (see 2.3.5, 2.3.6 & 2.3.7). Ce_x was always calculated as [mmol*L^{-1}].

The tissue mass of systemic hearts and branchial hearts samples were too little to create a PCA-extract for each animal. Therefore, the systemic hearts or branchial hearts of different animals from one incubation were pooled to create an extract. The replicate numbers of systemic and branchial heart measurements are therefore smaller than those of mantle and funnel measurements (see results). Systemic and branchial heart samples or samples from different incubations were never mixed during pooling. In the results section, pooled samples are marked with asterisks (*) in tables but are not explicitly mentioned in the text.

2.3.5 Intracellular metabolite measurement by capillary electrophoresis

Intracellular metabolite concentrations were measured in mantle or funnel samples of all six incubations (H+H, C_H+H, HOx, C_HOx, HCa, C_HCa) as well as in systemic and branchial hearts of animals from the H+H, C_H+H, HOx and C_HOx incubations. Systemic and branchial hearts from the HCa and the C_HCa incubations were not investigated, as no tissue was available (see 2.2.3 & 2.3.3). Each sample was measured twice. To end up with one value for each animal (or animal-pool), the two values derived from one sample were averaged and means were used for further statistics.

The analyzed metabolites were adenylates (ATP, ADP, AMP), phospho-L-arginine (PLA), arginine (Arg), and octopine. Three-point calibration curves of the desired substances were created using standards with known metabolite concentrations. No calibrations curves could be created for PLA as no standard was available. The rough concentrations for PLA were calculated using the calibrations of former measurements. To accommodate possible differences between former and present measurements, the results of the other metabolites were compared between old and new measurements. The averaged factor of change was then applied to correct the PLA concentrations. Afterwards, the prepared PCA-extracts of the samples were unfrozen and diluted 1:4 with milli-Q water. Before analysis by capillary electrophoresis (CE), the samples were filtered and uric acid (2 $g*L^{-1}$ → 1:10 mixed with sample) was added as an internal standard. The separation was performed as described by Casey et al. (1999) using 40 $mmol*L^{-1}$ borate-buffer with additional 10 $mmol*L^{-1}$ NaCl for a better resolution of the ATP and ADP peaks (P/ACE^{TM} System MDQ capillary electrophoresis, Beckmann Coulter GmbH, Krefeld, Germany).

The results of the CE measurements were given as Area. The detection limits differed between the metabolites and corresponded to concentrations of ~1.6 $\mu mol*g\ tissue^{-1}$ (arginine), ~0.21 $\mu mol*g\ tissue^{-1}$ (octopine), ~0.25 $\mu mol*g\ tissue^{-1}$ (PLA) and ~0.04 $\mu mol*g\ tissue^{-1}$ (ATP, ADP, AMP). The exact detection limits depended on the tissue/medium ratio in the PCA-extracts and varied between the different samples. The metabolite concentrations in the PCA-extracts were calculated using the linear equations from the calibration curve of the respective metabolite [$mmol*L^{-1}$]. Finally, the metabolite concentration in the tissue [$\mu mol*g\ tissue^{-1}$] was calculated with the weights recorded during the extract preparation (see 2.3.4).

The concentrations of free ADP and free AMP were calculated as [$\mu mol*g\ tissue^{-1}$] according to Pörtner et al. (1996) using the measured pH_i values (see 2.3.3) and the measured concentrations of arginine, PLA, ATP (see above) and Pi (see 2.3.6). A free magnesium concentration of 1 $mmol*L^{-1}$ was assumed (Robertson 1965). The Gibbs free energy change of ATP hydrolysis ($\Delta G/\Delta \xi$) was calculated as [$kJ*mol^{-1}$] using the concentrations of free ADP and free AMP (Pörtner et al. 1996). Free ADP, AMP and Gibbs free energy could only be calculated in samples, for which pH_i values and the tissue concentrations of ATP, arginine, PLA and inorganic phosphate were available (see above). Thus, the parameters could only be calculated for the 15

mantle samples from cuttlefish incubated under hypoxia + hypercapnia (H+H) and for the 10 mantle samples from cuttlefish incubated under control conditions (C_H+H).

2.3.6 Enzymatic measurement of inorganic phosphate (P_i)

Inorganic phosphate concentration was measured in mantle/funnel samples of all six incubations (H+H, C_H+H, HOx, C_HOx, HCa, C_HCa) as well as in systemic and branchial hearts of animals from the H+H, C_H+H, HOx and C_HOx incubations. Systemic and branchial hearts from the HCa and the C_HCa incubations were not investigated as no tissue was available (see 2.2.3 & 2.3.3). Each sample was measured twice (see below). To end up with one value for each animal (or animal-pool), the two values derived from one sample were averaged and means were used for further statistics.

The concentration of P_i in the PCA-extract was determined by measuring the P_i-dependent NADH/NAD^+ turnover via photometry. The core reaction of this method is the conversion of glyceraldehyd-3-phosphate (GAP) to 1,3-bisphophoglycerate (1,3-bPG), which also involves P_i and NAD^+/NADH. The reaction is catalyzed by the enzyme glyceraldehyd-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12).

$$\text{GAP} + \text{NAD}^+ + \text{P}_\text{i} \xrightarrow{GAPDH} \text{1,3-bPG} + \text{NADH} + \text{H}^+$$

Due to the different optical properties of NAD^+ and NADH, the concentration change can be measured with a photometer at a wavelength of λ = 340 nm (LKB Biochrom 4060, Pharmacia AG, Uppsala, Sweden). Glyceraldehyd-3-phosphate, together with dihydroxyacetone phosphate (DHAP), is provided by the degradation of fructose-1,6-bisphosphate (Fru-1,6-bP) catalyzed by the enzyme aldolase (EC 4.1.2.13).

$$\text{Fru-1,6-bP} \xrightarrow{Aldolase} \text{DHAP} + \text{GAP}$$

To avoid an inhibition of the core reaction caused by the accumulation of products, supporting reactions are catalyzed by the enzymes phosphoglycerate kinase (PGK, EC 2.7.2.3) and hexokinase (EC 2.7.1.2). First, 1,3-bPG is removed by the acting of phosphoglycerate. The ATP produced by this reaction is then degraded by hexokinase.

$$\text{1,3-bPG + ADP} \xrightarrow{PGK} \text{3-phosphoglycerate + ATP}$$

$$\text{glucose + ATP} \xrightarrow{Hexokinase} \text{glucose-6-phosphate + ADP}$$

To measure the P_i concentration, a reaction mixture was prepared in a 0.5 mL micro cuvette (d = 1 cm). Before the start of the measurement, the respective solutions were mixed as follows:

- 400 µL buffer medium
  - TRA (200 mmol*L^{-1})
  - EDTA (0.5 mmol*L^{-1})
  - Glucose (2 mmol*L^{-1})
  - $MgCl_2$ (5 mmol*L^{-1})
  - Fru-1,6-bP (1.96 mmol*L^{-1})
- 40 µL NAD^+ (50 mmol*L^{-1})
- 40 µL ADP (10 mmol*L^{-1})
- 8 µL enzyme mix
  - Hexokinase (106 U*mL^{-1})
  - Aldolase (31 U*mL^{-1})
  - GAPDH (68 U*mL^{-1})
  - PGK (68 U*mL^{-1})
- 310 or 305 µL milli-Q water
- 10 or 15 µL sample

} 320 µL

The total volume of the mixture was 808 µL. Fructose-1,6-bisphosphate was added to the buffer medium directly before the preparation of the reaction mixture. The enzyme mix was prepared right before the measurement, too. The recording of the extinction was started ~5 min before the addition of the sample. The samples were prepared from PCA-extracts (see 2.3.4). From each PCA-extract, two replicates with different sample volumes (10 or 15 µL) were measured. This made it possible to determine whether the sample volume affects the extinction. When the sample is added to the reaction mixture, the P_i in the sample triggers the conversion of NAD^+ to NADH and the extinction increases. After the addition of the sample, the extinction was recorded till it became stable again. The whole measurement was performed at 37℃ to ensure proper enzyme functioning. Enzymes of *Saccharomyces cervisiae* (baker yeast) and *Oryctolagus cuniculus* (rabbit) were used. With the initial extinction

(E_0) and the extinction at the end of the reaction (E_1) the extinction change (ΔE) was determined according to equation (15). As P_i was the limiting constituent for the production of NADH (all other constituents were given in excess), ΔE directly depended on the P_i concentration in the sample (C_{P_i}).

One molecule P_i yields the production of one molecule NADH. Therefore, C_{P_i} could be calculated according to the Lambert-Beer Law (eq. 16).

$$\Delta E = E_1 - E_0 \tag{15}$$

$$C_{P_i} = \frac{\Delta E * V_{total}}{\varepsilon_{340} * d * V_{sample}} \quad [\text{mmol*L}^{-1}] \tag{16}$$

V_{total} is the total volume of the reaction mixture (808 µL) and V_{sample} is the added sample volume (10 or 15 µL). d is the distance the light has to pass through the reaction mix (1 cm). ε_{340} is the extinction coefficient of NADH at λ = 340 nm (6.3 L* mmol^{-1}*cm^{-1}). The detection limits was ΔE = 0.01, which corresponded to a concentration of ~0.68 µmol*g tissue^{-1}. The P_i concentration in the tissue [µmol*g tissue^{-1}] could then be calculated according to equation (14). In this case C_{P_i} corresponded to Ce_x (see 2.3.4).

2.3.7 Enzymatic measurement of succinate

The succinate concentration was measured in the mantle tissue of cuttlefish from the H+H and the C_H+H incubations. However, only a few samples were measured, due to the very low concentration of succinate in the samples (see 3.3.8). Succinate was not investigated in any other tissue or treatment, as the available amount of tissue was insufficient. Each sample was measured twice (see below). To end up with one value for each animal the two values derived from one sample were averaged and means were used for further statistics.

The substrate dependent turnover of NAD^+/NADH was also used for the photometrical determination of the succinate concentration in the PCA-extract. The measurement was performed using a commercial succinate test kit (Succinic acid UV-method, Cat. No. 10 176 281 035, R-Biopharm AG, Darmstadt). First, the succinate in the sample as well as coenzyme A (CoA) are used to transform inosine-5-triphosphate (ITP) to inosine-5-diphosphate (IDP). The reaction is catalyzed by the

enzyme succinyl-CoA synthethase (SCS, EC 6.2.1.4). The enzyme pyruvate kinase (PK, EC 2.7.1.40) then creates pyruvate from IDP and phosphoenolpyruvate (PEP). The pyruvate is involved in the NADH-converting core reaction, which is mediated by the enzyme L-lactate dehydrogenase (L-LDH, EC 1.1.1.27).

$$\text{succinate + ITP + CoA} \xrightarrow{SCS} \text{IDP + succinyl-CoA + } P_i$$

$$\text{IDP + PEP} \xrightarrow{PK} \text{ITP + pyruvate}$$

$$\text{pyruvate + NADH + } H^+ \xrightarrow{L-LDH} \text{L-lactate + } NAD^+$$

As all constituents (except succinate) were given in excess and one molecule of succinate yields the conversion of one molecule of NADH, the decrease in NADH concentration was directly dependent on the initial concentration of succinate. The change of the NADH concentration was measured at a wavelength of λ = 340 nm. The measurement was performed according to the instructions of the test kit. However, the total volume of the assay was quartered to reduce the consumption of sample material and chemicals. The data recording was started before the addition of the sample (120 or 140 µL). The addition of the sample caused a small drop in extinction due to the NADH-consuming activity of the L-LDH, which converts the pyruvate in the sample. When the extinction was stable again, the addition of SCS started the reactions. The extinction was then recorded till it became stable again. The whole measurement was performed at 37℃ to ensu re proper enzyme functioning. With the extinction before the addition of SCS (E_0) and the extinction at the end of the reaction (E_1) the extinction (ΔE) change was determined according to equation (17). The succinate concentration in the PCA-extract $C_{succinate}$ was then calculated according to the Lambert-Beer Law (eq. 18).

$$\Delta E = E_0 - E_1 \tag{17}$$

$$C_{succinate} = \frac{\Delta E * V_{total}}{\varepsilon_{340} * d * V_{sample}} \quad [\text{mmol*L}^{-1}] \tag{18}$$

V_{total} is the total volume of the reaction mixture (767.5 µL) and V_{sample} is the added sample volume (120 or 140 µL). d is the distance the light has to pass through the reaction mix (1 cm). ε_{340} is the extinction coefficient of NADH at λ = 340 nm (6.3 L* mmol^{-1}*cm^{-1}). The detection limits was ΔE = 0.016, which corresponded to a concentration of ~0.18 µmol*g tissue^{-1}. The succinate concentration in the tissue [µmol*g tissue^{-1}] could then be calculated according to equation (13). In this case $C_{succinate}$ corresponded to Ce_x (see 2.3.4).

2.4 Statistical analysis

The obtained data was analyzed using SigmaPlot 12.0 software (Systat Software GmbH, Erkrath, Germany). The data sets for each incubation, tissue and parameter were tested separately for outliers using Nalimov's outlier test.

Each data set was compared to the respective control (i.e. PLA H+H M was compared to PLA C_H+H M, ATP HOx BH was compared to ATP C_HOx BH and so on). The pair of data sets was checked for normal distribution of data points using the Shapiro-Wilk test and for homogeneity of variances using the Equal Variance test offered by the software. If both prerequisites were given, the data sets were compared with a Student's t-test (abbreviated as t-test in tables). Otherwise, a Mann-Whitney test was applied for comparison.

Graphics were created with Microsoft Excel (v. 14.0.6112.5000) and Microsoft Paint (v. 6.1).

3 Results

3.1 Blood acid-base parameters

There was a significant difference ($p < 0.001$) between the blood CO_2 partial pressures of the H+H and the C_H+H incubations. Whereas mean blood PCO_2 was 0.28 ± 0.05 kPa in the animals incubated under C_H+H conditions (n = 16* → Asterisk (*) indicates pooled sample), exposure to Hypoxia & hypercapnia (n = 16*) caused a rise (> 30%) of mean blood PCO_2 up to 0.37 (Fig. 3.1B).

The blood bicarbonate concentration was significantly higher in the H+H incubation than in the C_H+H incubation ($p < 0.001$). Compared to the control (n = 16*, mean = 3.20 ± 0.47 mmol*L^{-1}), the HCO_3^- concentration rose by ~20% (n = 16*, mean = 3.87 ± 0.56 mmol*L^{-1}) in the H+H incubation (Fig. 3.1C).

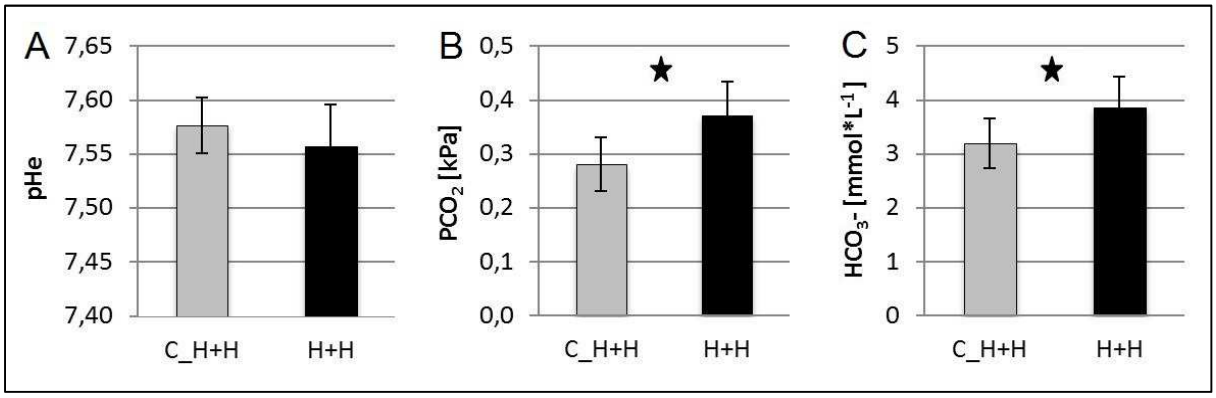

Fig. 3.1: Blood acid-base parameters. A: Blood pH (pH_e), B: blood CO_2 partial pressure (PCO_2), C: blood bicarbonate concentration (HCO_3^-). Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H) and in the respective control (C_H+H). Asterisks (*) indicate significant differences.

Blood pH (pH_e) did not differ significantly between H+H (n = 16*, mean = 7.56 ± 0.04) and C_H+H (n = 16*, mean = 7.58 ± 0.06) incubation (p = 0.108).

Blood oxygen partial pressures (PO_2) did not differ significantly between the control and the H+H treatment. The mean values ± SD for H+H and C_H+H were 2.75 ± 0.66 kPa and 2.48 ± 0.66 kPa, respectively. The values were mostly distributed between 2 and 3 kPa and never exceeded 4.1 kPa.

3.2 Intracellular acid-base parameters

The pH_i was 7.51 ± 0.03 in mantle tissue of the control (C_H+H) incubation. The exposure to Hypoxia & hypercapnia caused a significant decrease in mantle pH_i with

a mean of 7.46 ± 0.05 (Fig. 3.2A, Tab.3.1). There was no significant difference in the pH_i values of branchial hearts from the hypercapnia incubation (mean = 6.75 ± 0.16) and the respective control incubation (mean = 6.60 ± 0.01) (Fig. 3.2A, Tab. 3.1).

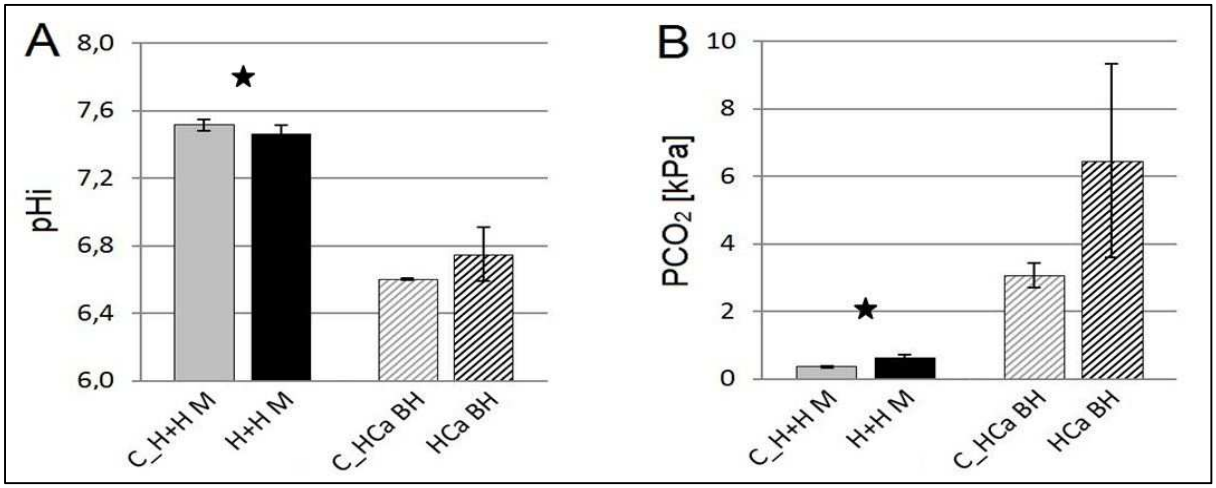

Fig. 3.2: Intracellular acid-base parameters. A: intracellular pH (pH_i), B: intracellular CO_2 partial pressure (PCO_2). Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HCa). M: mantle, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control. Results for the intracellular bicarbonate concentration are not shown (see Tab. 3.1).

Tab. 3.1: Comparison of intracellular acid-base parameters. Results for intracellular pH (pH_i), intracellular CO_2 partial pressure (PCO_2) and intracellular bicarbonate concentration (HCO_3^-) are shown. PCO_2 values are in [kPa], HCO_3^- values are in [mmol*L^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and respective control. Asterisks (*) indicate pooled samples.

	incubation	tissue	n	mean ± SD	statistics	p-value
pH_i	C_H+H	M	14	7.51 ± 0.03	t-test	< 0.001
	H+H		10	7.46 ± 0.05		
	C_HCa	F	2*	6.60 ± 0.01	M-W	0.333
	HCa		2*	6.75 ± 0.16		
PCO_2	C_H+H	M	9	0.36 ± 0.03	M-W	< 0.001
	H+H		9	0.63 ± 0.10		
	C_HCa	F	2*	3.07 ± 0.37	M-W	0.333
	HCa		2*	6.47 ± 2.86		
HCO_3^-	C_H+H	M	10	2.81 ± 0.18	M-W	0.838
	H+H		9	2.79 ± 0.45		
	C_HCa	F	2*	6.60 ± 0.01	M-W	0.333
	HCa		2*	6.75 ± 0.16		

PCO_2 was a significantly elevated in H+H incubated mantle tissue (0.63 ± 0.10 kPa) and almost doubled compared to the control (C_H+H), which had a PCO_2 of 0.36 ±

0.03 kPa (Fig.3.2B, Tab. 3.1). In the HCa incubation the PCO_2 of branchial hearts (6.47 ± 2.86 kPa) was not significantly different from the C_HCa incubation (mean = 3.07 ± 0.37 kPa) (Fig.3.2B, Tab.3.1).

The intracellular HCO_3^- concentration was 2.79 ± 0.45 mmol*L-1 under Hypoxia & hypercapnia conditions (H+H) and 2.81 ± 0.18 in the control (C_H+H) with no significant difference between the incubations (Tab. 3.1). In the HCa incubation, the HCO_3^- concentration was 6.75 ± 0.16 mmol*L^{-1}. In the control (C_HCa) the concentration was 6.60 ± 0.01 mmol*L^{-1}. The difference was not significant.

3.3 Intracellular metabolite concentrations

3.3.1 Arginine

Hypoxia & hypercapnia: The exposure to Hypoxia & hypercapnia (H+H) caused no significant changes in the arginine concentrations of mantle (M), systemic heart (SH) and branchial heart (BH) tissue (Tab 3.2). In both treatment and control, the concentration was ~17 µmol*g tissue^{-1} for the mantle, ~4.3 µmol*g tissue^{-1} for the systemic heart and ~1.9 µmol*g tissue^{-1} µmol*g tissue^{-1} (Fig. 3.3).

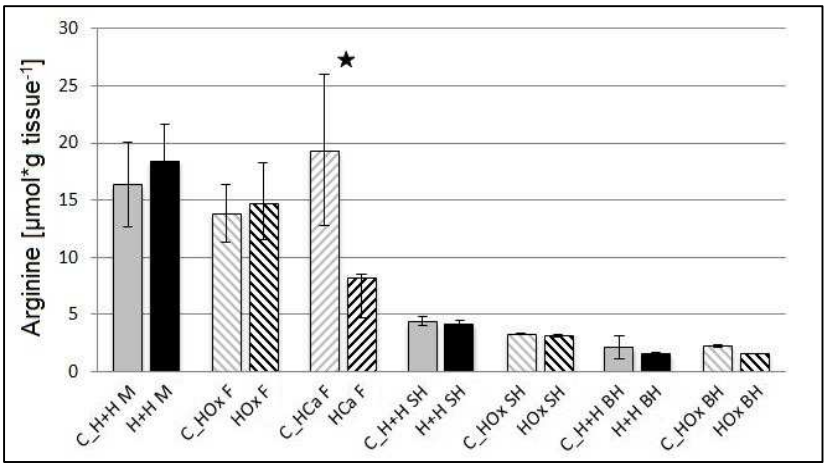

Fig. 3.3: Arginine tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypoxia: The exposure to hypoxia (HOx) caused no significant changes in the arginine concentrations of funnel (F), systemic heart and branchial heart tissue (Tab 3.2). In both treatment and control, the concentration was ~14 µmol*g tissue^{-1} for the funnel, ~3.2 µmol*g tissue^{-1} for the systemic heart and ~2 µmol*g tissue^{-1} µmol*g tissue^{-1} (Fig. 3.3).

Tab. 3.2: Comparison of arginine concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	C_H+H	21	16.40 ± 3.70	M-W	0.248
	H+H	22	18.46 ± 5.53		
F	C_HOx	8	13.82 ± 2.54	t-test	0.561
	HOx	7	14.70 ± 3.14		
F	C_HCa	5	19.39 ± 6.56	t-test	0.010
	HCa	5	8.30 ± 3.54		
SH	C_H+H	5*	4.40 ± 0.38	M-W	0.556
	H+H	4*	4.24 ± 0.21		
SH	C_HOx	2*	3.27 ± 0.05	M-W	1.0
	HOx	2*	3.24 ± 0.22		
BH	C_H+H	4*	2.14 ± 0.96	M-W	0.686
	H+H	4*	1.65 ± 0.03		
BH	C_HOx	2*	2.28 ± 0.09	M-W	0.333
	HOx	2*	1.60 ± 0.03		

Hypercapnia: While arginine was 19.38 ± 6.56 µmol*g tissue^{-1} in the control (C_HCa), the concentration was significantly reduced and more than halved in the HCa incubation (mean = 8.34 ± 3.54 µmol*g tissue^{-1}) (Fig. 3.3, Tab. 3.2).

3.3.2 Octopine

Hypoxia & Hypercapnia: No tissue (mantle, systemic heart, branchial heart) of the H+H incubated cuttlefish showed a significant difference in octopine concentrations compared to the C_H+H incubation (Tab.3.3). Average octopine concentration of H+H and C_H+H were ~0.6 µmol*g tissue^{-1} for the mantle, ~0.4 µmol*g tissue^{-1} for the systemic heart and ~0.25 µmol*g tissue^{-1} for the branchial hearts (Fig. 3.4).

Hypoxia: The funnel octopine concentration was significantly lower in the HOx incubation (mean = 2.79 ± 0.87 µmol*g tissue^{-1}) and ~60% of the C_HOx incubation (mean = 4.79 ± 0.69 µmol*g tissue^{-1}) (Fig. 3.4Tab. 3.3). There was no significant difference between HOx and C_HOx incubations for systemic heart (~2.4 µmol*g tissue^{-1}) and branchial hearts (~0.7 µmol*g tissue^{-1}) (Fig. 3.4, Tab. 3.3).

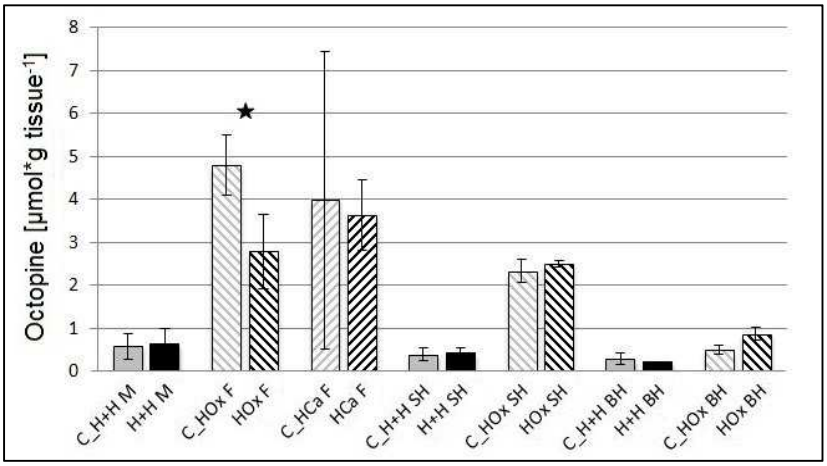

Fig. 3.4: Octopine tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypercapnia: Funnel octopine concentration was 3.63 ± 0.81 µmol*g tissue^{-1} in the HCa treatment and 3.98 ± 3.47 µmol*g tissue^{-1} in the respective control incubation (C_HCa) with no significant difference between the incubations (Fig. 3.4, Tab. 3.3).

Tab. 3.3: Comparison of octopine concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	C_H+H	20	0.58 ± 0.30	M-W	0.696
	H+H	22	0.64 ± 0.36		
F	C_HOx	6	4.79 ± 0.69	t-test	< 0.001
	HOx	8	2.79 ± 0.87		
F	C_HCa	5	3.98 ± 3.47	M-W	0.841
	HCa	5	3.63 ± 0.81		
SH	C_H+H	5*	0.38 ± 0.15	t-test	0.548
	H+H	5*	0.43 ± 0.10		
SH	C_HOx	2*	2.33 ± 0.26	M-W	0.667
	HOx	2*	2.49 ± 0.07		
BH	C_H+H	4*	0.28 ± 0.13	M-W	0.200
	H+H	4*	0.21 ± 0.003		
BH	C_HOx	2*	0.50 ± 0.10	M-W	0.333
	HOx	2*	0.86 ± 0.14		

3.3.3 Phospho-L-arginine (PLA)

Hypoxia & hypercapnia: The concentration of PLA in mantle, systemic heart and branchial hearts did not differ significantly between the H+H and the C_H+H incubations (Tab. 3.4). In both incubations, the PLA concentration was ~15.8 µmol*g

tissue^{-1} for the mantle, 0.35 µmol*g tissue^{-1} for the systemic heart and ~0.28 µmol*g tissue^{-1} for the branchial hearts (Fig. 3.5).

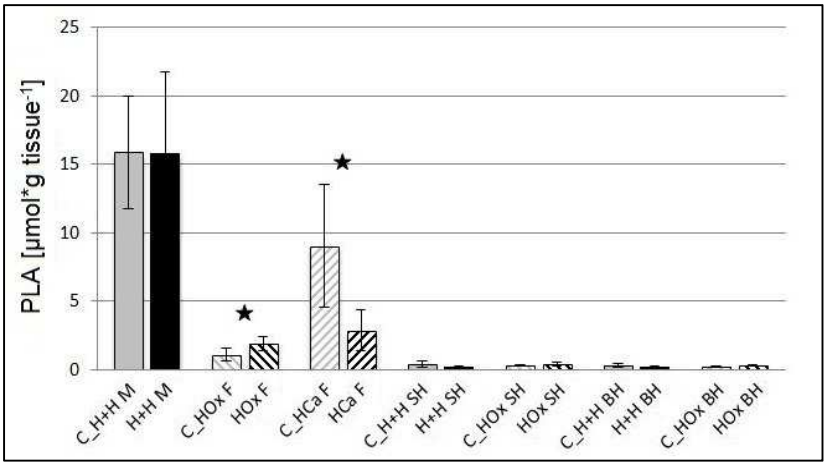

Fig. 3.5: PLA tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypoxia: Hypoxia treated funnel tissue (mean = 1.93 ± 0.49 µmol*g tissue^{-1}) had a significantly higher PLA concentration than funnel from the C_HOx incubation (mean = 1.13 ± 0.49 µmol*g tissue^{-1}) (Fig. 3.5, Tab.3.4). The PLA concentrations in systemic (~0.35 µmol*g tissue^{-1}) and branchial hearts (~0.34 µmol*g tissue^{-1}) did not differ significantly between incubations (Fig. 3.5, Tab.3.4).

Tab. 3.4: Comparison of PLA concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	C_H+H	22	15.88 ± 4.12	M-W	0.824
	H+H	22	15.82 ± 5.90		
F	C_HOx	7	1.13 ± 0.49	t-test	0.013
	HOx	6	1,93 ± 0.49		
F	C_HCa	5	9.05 ± 4.45	t-test	0.034
	HCa	4	2.88 ± 1.49		
SH	C_H+H	5*	0.43 ± 0.21	t-test	0.159
	H+H	4*	0.26 ± 0.001		
SH	C_HOx	2*	0.31 ± 0.06	M-W	0.667
	HOx	2*	0.45 ± 0.14		
BH	C_H+H	4*	0.33 ± 0.15	M-W	0.190
	H+H	5*	0.25 ± 0.01		
BH	C_HOx	2*	0.29 ± 0.01	M-W	0.333
	HOx	2*	0.39 ± 0.001		

Hypercapnia: The PLA concentrations of HCa treated cuttlefish funnel (mean = 2.88 ± 1.49 µmol*g tissue^{-1}) and the control (C_HCa) group (mean = 9.05 ± 4.45 µmol*g tissue^{-1}) differed significantly (ΔPLA = +6.62 µmol*g tissue^{-1}) (Fig. 3.5, Tab. 3.4).

3.3.4 ATP

Hypoxia & hypercapnia: Incubation at Hypoxia & hypercapnia (H+H) did not significantly change the tissue (mantle, systemic heart, branchial heart) concentration of ATP compared to the C_H+H incubation (Tab. 3.5). Average concentrations of ~3.6 µmol*g tissue^{-1} (mantle), ~0.5 µmol*g tissue^{-1} (systemic heart) and ~0.1 µmol*g tissue^{-1} (branchial heart) were found in H+H and C_H+H (Fig. 3.6).

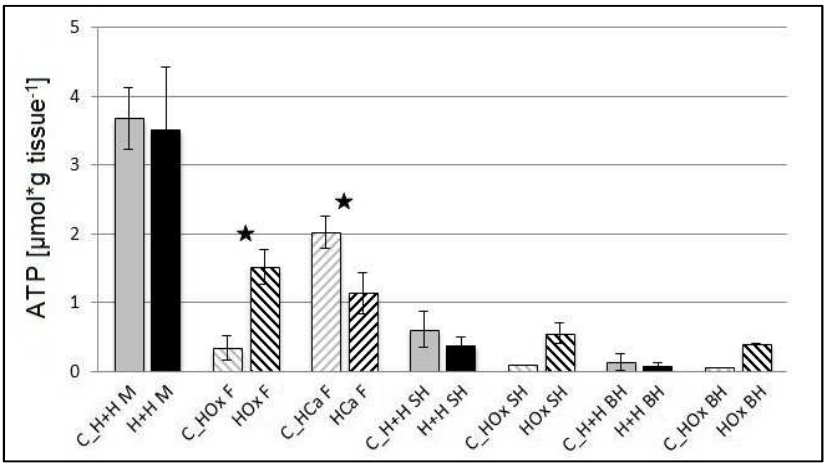

Fig. 3.6: ATP tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypoxia: Funnel ATP was significantly higher (4-fold increase) in the HOx incubation (mean = 1.52 ± 0.26 µmol*g tissue^{-1}) than in the C_HOx incubation (mean = 0.35 ± 0.18 µmol*g tissue^{-1}) (Fig. 3.6, Tab. 3.5). There was no significant difference in the ATP concentrations of HOx and C_HOx incubations for systemic heart (~0.3 µmol*g tissue^{-1}) and branchial hearts (~0.2 µmol*g tissue^{-1}) (Tab. 3.5).

Hypercapnia: Under HCa conditions, funnel ATP concentration (mean = 1.14 ± 0.30 µmol*g tissue^{-1}) was significantly reduced to 56% of the concentration under control (C_HCa) conditions (mean = 2.03 ± 0.23 µmol*g tissue^{-1}) (Fig. 3.6, Tab. 3.5).

Tab. 3.5: Comparison of ATP concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	C_H+H	22	3.68 ± 0.45	M-W	0.855
	H+H	21	3.52 ± 0.90		
F	C_HOx	7	0.35 ± 0.18	t-test	< 0.001
	HOx	6	1.52 ± 0.26		
F	C_HCa	4	2.03 ± 0.23	t-test	0.002
	HCa	5	1.14 ± 0.30		
SH	C_H+H	5*	0.61 ± 0.26	t-test	0.548
	H+H	5*	0.39 ± 0.13		
SH	C_HOx	2*	0.10 ± 0.004	M-W	0.333
	HOx	2*	0.56 ± 0.15		
BH	C_H+H	4*	0.14 ± 0.12	t-test	0.200
	H+H	5*	0.08 ± 0.04		
BH	C_HOx	2*	0.06 ± 0.005	M-W	0.333
	HOx	2*	0.40 ± 0.004		

3.3.5 ADP

Hypoxia & hypercapnia: Mantle ADP concentrations in the H+H incubation (mean = 1.34 ± 0.29 µmol*g tissue^{-1}) and the C_H+H incubation (mean = 0.95 ± 0.25 µmol*g tissue^{-1}) differed significantly (Fig. 3.7, Tab. 3.6). There was no significant difference in ADP concentrations of H+H and C_H+H incubations for systemic heart (~0.7 µmol*g tissue^{-1}) and branchial hearts (~0.7 µmol*g tissue^{-1}) (Fig. 3.7, Tab. 3.6).

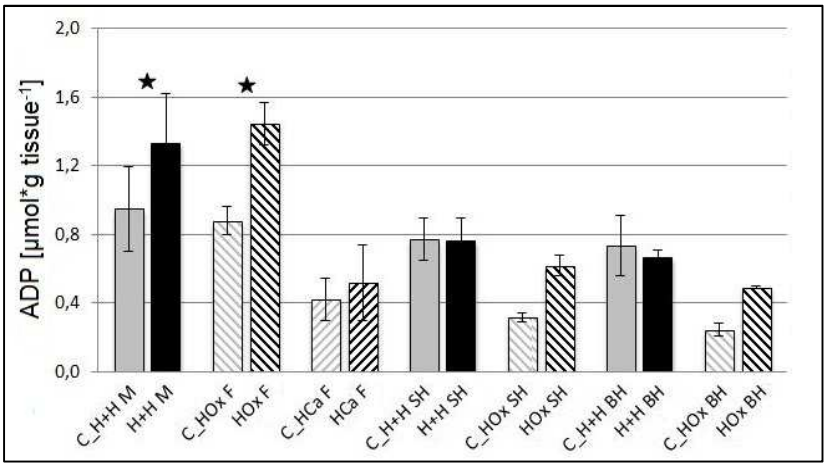

Fig. 3.7: ADP tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypoxia: In the hypoxia treatment (HOx; Fig. 3.7), the funnel ADP concentration was significantly higher (1.45 ± 0.12 µmol*g tissue^{-1}) than in the control (C_HOx) incubation (0.88 ± 0.08 µmol*g tissue^{-1}; ΔADP = +0.57 µmol*g tissue^{-1}; Tab. 3.6). Heart tissues were not significantly affected by hypoxia (Tab. 3.6). The average concentrations in both HOx and C_HOx were ~0.42 µmol*g tissue^{-1} for systemic heart and ~0.37 µmol*g tissue^{-1}, respectively (Fig. 3.7).

Tab. 3.6: Comparison of ADP concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	C_H+H	22	0.95 ± 0.25	t-test	< 0.001
	H+H	21	1.34 ± 0.29		
F	C_HOx	7	0.88 ± 0.08	t-test	< 0.001
	HOx	6	1.45 ± 0.12		
F	C_HCa	5	0.42 ± 0.13	t-test	0.419
	HCa	5	0.52 ± 0.22		
SH	C_H+H	5*	0.77 ± 0.12	t-test	0.939
	H+H	5*	0.76 ± 0.14		
SH	C_HOx	2*	0.32 ± 0.03	M-W	0.333
	HOx	2*	0.62 ± 0.06		
BH	C_H+H	4*	0.74 ± 0.18	t-test	0.429
	H+H	5*	0.67 ± 0.04		
BH	C_HOx	2*	0.25 ± 0.04	M-W	0.333
	HOx	2*	0.49 ± 0.01		

Hypercapnia: The ADP concentrations in cuttlefish funnel under hypercapnia (HCa) and in the respective control (C_HCa) did not differ significantly (Tab. 3.6). The average concentration for both incubations was ~0.7 µmol*g tissue^{-1} (Fig. 3.7).

3.3.6 AMP

Hypoxia & hypercapnia: Mantle AMP was significantly (~50%) higher in the H+H incubation (mean = 0.30 ± 0.12 µmol*g tissue^{-1}) in comparison to the C_H+H incubation (mean = 0.20 ± 0.11 µmol*g tissue^{-1}) (Fig. 3.8, Tab. 3.7). There was no significant difference between H+H and C_H+H incubations for systemic heart (~0.9 µmol*g tissue^{-1}) and branchial hearts (~1.0 µmol*g tissue^{-1}) (Fig. 3.8, Tab. 3.7).

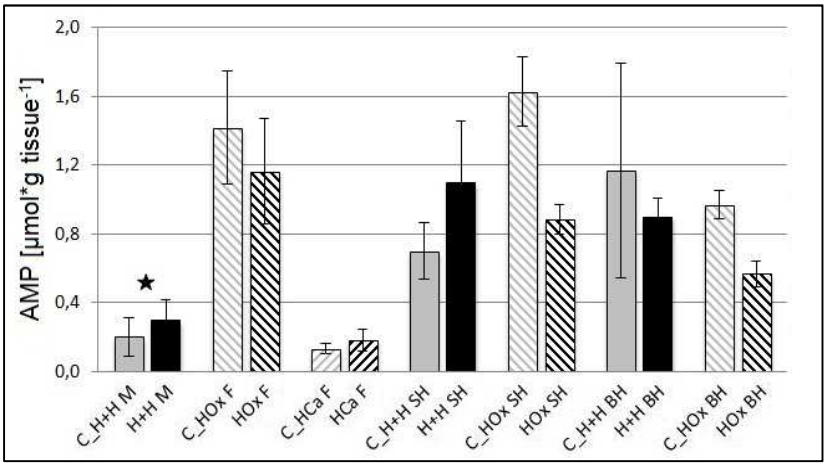

Fig. 3.8: AMP tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypoxia: Hypoxia (HOx) caused no significant changes in the AMP concentrations of funnel, systemic heart and branchial heart tissue (Tab 3.7). In both treatment and control, AMP was ~1.3 µmol*g tissue^{-1} for the funnel, ~1.4 µmol*g tissue^{-1} for the systemic heart and ~0.8 µmol*g tissue^{-1} for the branchial hearts (Fig. 3.8).

Tab. 3.7: Comparison of AMP concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	C_H+H	21	0.20 ± 0.11	M-W	0.003
	H+H	21	0.30 ± 0.12		
F	C_HOx	7	1.42 ± 0.33	t-test	0.159
	HOx	7	1.16 ± 0.31		
F	C_HCa	5	0.13 ± 0.03	t-test	0.152
	HCa	4	0.18 ± 0.06		
SH	C_H+H	5*	0.70 ± 0.17	t-test	0.051
	H+H	5*	1.10 ± 0.35		
SH	C_HOx	2*	1.63 ± 0.20	M-W	0.333
	HOx	2*	0.88 ± 0.09		
BH	C_H+H	5*	1.17 ± 0.62	M-W	1.0
	H+H	5*	0.90 ± 0.11		
BH	C_HOx	2*	0.97 ± 0.08	M-W	0.333
	HOx	2*	0.57 ± 0.07		

Hypercapnia: Funnel AMP concentrations were not significantly affected by hypercapnia (Tab. 3.7). In the HCa and the C_HCa incubations, the average AMP concentration was ~0.16 µmol*g tissue^{-1} (Fig. 3.8).

3.3.7 Free ADP, free AMP & Gibbs free energy

The concentration of free ADP in the mantle tissue of cuttlefish incubated under H+H conditions (mean = 0.32 ± 0.14 µmol*g tissue^{-1}) was not significantly different from the concentration under C_H+H conditions (mean = 0.29 ± 0.07 µmol*g tissue^{-1}) (Tab.3.8). The relative amount of free ADP in comparison to the total ADP concentration (see 3.3.5) was 24% under Hypoxia & hypercapnia and 30% under control conditions.

Tab. 3.8: Free ADP, free AMP and Gibbs free energy. Free ADP and free AMP values are in [µmol*g tissue^{-1}]. Gibbs free energy (ΔG/Δξ) values are in [kJ*mol^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, M: mantle, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control.

	incubation	tissue	n	mean ± SD	statistics	p-value
free ADP	C_H+H	M	9	0.29 ± 0.07	t-test	0.506
	H+H		14	0.32 ± 0.14		
free AMP	C_H+H	M	9	0.026 ± 0.010	t-test	0.260
	H+H		14	0.037 ± 0.025		
ΔG/Δξ	C_H+H	M	8	-56.00 ± 0.34	M-W	0.384
	H+H		15	-55.61 ± 1.08		

Free AMP concentration was 0.026 ± 0.010 µmol*g tissue^{-1} in mantle tissue from the H+H incubation and 0.037 ± 0.025 µmol*g tissue^{-1} in the C_H+H incubation, with no significant difference between the incubations (Tab.3.8). The percentage of free AMP compared to the total AMP concentration (see 3.3.6) was 12% in the treatment (H+H) and 13% in the control (C_H+H).

Gibbs free energy change of ATP hydrolysis (ΔG/Δξ) was not significantly affected by exposure to Hypoxia & hypercapnia (Tab.3.8). The mean values were -55.61 ± 1.08 kJ*mol^{1-} in the H+H incubation and -56.00 ± 0.34 kJ*mol^{1-} in the C_H+H incubation.

3.3.8 Inorganic phosphate (P_i)

Hypoxia & hypercapnia: The concentration of P_i in mantle (~19 µmol*g tissue^{-1}), systemic heart (1.3 µmol*g tissue^{-1}) and branchial hearts (~5 µmol*g tissue^{-1}) did not differ significantly between the H+H and the C_H+H incubations (Fig. 3.9, Tab. 3.9).

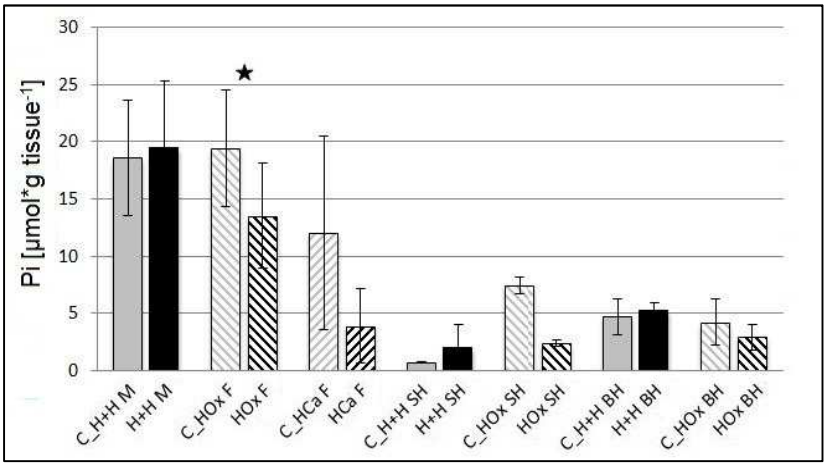

Fig. 3.9: Inorganic phosphate (P_i) tissue concentrations. Mean values ± SD are shown for the cuttlefish incubated under Hypoxia & hypercapnia (H+H), hypoxia (HOx) or hypercapnia (HCa) and for the respective controls (C_H+H, C_HOx, C_HCa). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts. Asterisks (*) indicate significant differences between treatment and control.

Hypoxia: The funnel P_i concentration in the HOx incubation (mean = 19.43 ± 5.11 µmol*g tissue^{-1}) was significantly lower (70% of control) than in the C_HOx incubation (mean = 13.55 ± 4.58 µmol*g tissue^{-1}) (Fig. 3.9, Tab. 3.9). P_i concentrations of the HOx and C_HOx incubations did not differ significantly for systemic heart (~5 µmol*g tissue^{-1}) and branchial hearts (~3.5 µmol*g tissue^{-1}) (Fig. 3.9, Tab. 3.9).

Tab. 3.9: Comparison of inorganic phosphate (P_i) concentrations in different incubations and tissues. Values are in [µmol*g tissue^{-1}]. n is the number of replicates. H+H: Hypoxia & hypercapnia, C_H+H: Hypoxia & hypercapnia control, HOx: hypoxia, C_HOx: hypoxia control, HCa: hypercapnia, C_HCa: hypercapnia control, M: mantle, F: funnel, SH: systemic heart, BH: branchial hearts, M-W: Mann-Whitney test. Red p-values indicate a significant difference between treatment and the respective control. Asterisks (*) indicate pooled samples.

tissue	incubation	n	mean ± SD	statistics	p-value
M	H+H	21	18.61 ± 5.03	t-test	0.603
	C_H+H	22	19.48 ± 5.79		
F	HOx	8	19.43 ± 5.11	t-test	0.046
	C_HOx	6	13.55 ± 4.58		
F	HCa	5	12.07 ± 8.48	t-test	0.171
	C_HCa	3	3.92 ± 3.22		
SH	H+H	4*	0.70 ± 0.05	M-W	0.286
	C_H+H	5*	2.03 ± 1.99		
SH	HOx	2*	7.44 ± 0.71	M-W	0.333
	C_HOx	2*	2.39 ± 0.26		
BH	H+H	5*	4.74 ± 1.58	t-test	0.537
	C_H+H	4*	5.29 ± 0.68		
BH	HOx	2*	4.25 ± 1.99	M-W	0.667
	C_HOx	2*	2.93 ± 1.13		

Hypercapnia: The P_i concentration was 3.92 ± 3.23 µmol*g tissue^{-1} in the HCa treatment and not significantly different from the control (C_HCa) incubation (mean = 12.07 ± 8.48 µmol*g tissue^{-1}) (Fig. 3.9, Tab. 3.9).

3.3.9 Succinate

A rise in succinate is associated with anaerobic metabolism, which can also be indicated by octopine. Hence, a rise of succinate can be predicted by elevated octopine concentrations (see 1.2.3). Therefore, succinate was only measured in the samples with the highest octopine concentrations. However, the intracellular succinate concentration in the mantle tissue of cuttlefish from the H+H treatment and the control (C_H+H) was below the detection limit in most of the investigated samples. Therefore, other tissue samples were not measured to save sample material.

Calibration could be performed down to a concentration of ~0.3 µmol*g tissue^{-1} (R^2 = 0.9993). Succinate could only be detected in one sample with a concentration of 0.19 µmol*g tissue^{-1}. Thus, succinate concentration of mantle tissues from the H+H and C_H+H incubations was probably below 0.2 µmol*g tissue^{-1} in all samples.

4 Discussion

Exposure of *Sepia officinalis* to simultaneous hypoxia and hypercapnia caused an increase in blood PCO_2 and bicarbonate (HCO_3^-), while blood pH (pH_e) was unaffected. A rise in mantle PCO_2 caused a drop in pH_i, but intracellular energy status and metabolite concentrations were mostly unaffected. Only mantle concentrations of total ADP and AMP increased during simultaneous hypoxia and hypercapnia.

Funnel tissue of cuttlefish exposed to hypoxia displayed elevated intracellular concentrations of phospho-L-arginine (PLA), ADP and ATP, whereas concentrations of octopine and inorganic phosphate (P_i) decreased. Hypoxia did not alter arginine and AMP concentrations in the funnel.

Hypercapnia caused a reduction of intracellular arginine, PLA and ATP levels in the funnel tissue of *S. officinalis*. All other intracellular metabolite concentrations remained unchanged regarding the funnel tissue.

Generally, systemic and branchial heart parameters were not significantly changed in any of the three treatments compared to their respective controls. There was also a trend towards reduced metabolite concentrations in the hearts compared to mantle or funnel tissue. The general problem of low replicate numbers and low statistical test power for the heart tissues is discussed in section 4.6.

4.1 Blood physiology

The increase of blood PCO_2 during hypoxia and hypercapnia (0.37 ± 0.06 kPa) compared to the control (0.28 ± 0.05 kPa) is in line with findings of earlier studies. Gutowska et al. (2010) observed that *Sepia officinalis'* blood PCO_2 increased from 0.22 ± 0.03 kPa to 0.98 ± 0.03 kPa during exposure to severe hypercapnia (0.6 kPa CO_2). The elevated blood PCO_2 is caused by the accumulation of CO_2 species in the blood. This is not only due to diffusion, but also reflects the cuttlefish's effort to maintain an outward directed CO_2 concentration gradient, which is needed to remove the metabolic CO_2 from the body fluids (Fabry et al. 2008, Melzner et al. 2012). Removal of CO_2 from the blood system may also be supported by hyperventilation (Gutowska et al. 2010, Schmidt et al. unpubl.), which is economical and does not severely affect metabolic rates and energy consumption (Melzner et al. 2006b, Gutowska et al. 2010). However, the CO_2 reduction effect of hyperventilation is rather

low due to the small concentration gradient between seawater and the organisms' body fluids (Scheid et al. 1989, Melzner et al. 2012).

It is likely that the observed rise in blood HCO_3^- concentration during hypoxia and hypercapnia (ΔHCO_3^- = +0.67 mmol*L^{-1}) is an active response of the cuttlefish to secure the blood oxygen transport of the hemocyanin. Elevated blood bicarbonate levels were detected in *S. officinalis* during hypercapnia (Gutowska et al. 2010) and during exercise (Pörtner et al. 1991). In both situations, the accumulation of vascular CO_2 from the environment or from increased cellular respiration rates could cause a decrease in pH_e and could thus impede O_2 binding by the pH-sensitive hemocyanin (Pörtner 1990, 1994). The accumulation of HCO_3^- counteracts this trend and helps to keep pH_e between pH values of 7.4 and 7.8. The cooperativity of *S. officinalis* hemocyanin is strongest in this range, which means that already small pH changes cause changes in O_2 affinity (Johansen et al. 1982a, Zielinski et al. 2001). This secures proper hemocyanin loading at the gills and unloading at the tissue. There are several potential sources for the bicarbonate that accumulates in the blood. During exercise, a release of HCO_3^- from the tissue could be observed in squids (Pörtner 1994, Pörtner & O'Dor 1994). Active ion transport by regulatory epithelia was identified as a source for bicarbonate in bivalves during hypercapnia (Lindinger et al. 1984). Hypercapnia induced dissolution of calcium carbonate ($CaCO_3$) was also found to increase blood HCO_3^- levels in bivalve species (Michaelidis et al. 2005a,b), but Gutowska et al. (2008) found elevated calcification rates in *S. officinalis* even during severe hypercapnia (0.6 kPa CO_2). If the increase in blood bicarbonate (and CO_2 in general; see above) presented in the present study was achieved by active ion transport, this ATP-intensive process would cause increased energy demands (Dubyak 2004, Melzner et al. 2012). This could reduce the survival time under unfavorable conditions, when the cuttlefish has to resort to storage- and time-limited anaerobic metabolism (see 1.2.3).

pH_e did not change during exposure to hypoxia and hypercapnia and this was partially due to the aforementioned accumulation of bicarbonate in the blood. However, the observed change in bicarbonate (ΔHCO_3^- = +0.67 mmol*L^{-1}) was small compared to the increase found by Gutowska et al. (2010) during 0.6 kPa hypercapnia (ΔHCO_3^- = +6.99 mmol*L^{-1}). This may be partially caused by the more severe hypercapnia, but despite this strong rise in bicarbonate, Gutowska et al. (2010) still found a significant reduction in pH_e. The relatively small rise in blood

HCO_3^- found in the present study seems sufficient to prevent a drop in pH_e, but the rise might was also supported by other factors. Another process that counteracted decreasing pH_e in the hypoxia and hypercapnia incubation was the uptake of H^+ ions by the hemocyanin during deoxygenation according to the Bohr-effect (Pörtner 1994). *Sepia officinalis* has a Bohr-coefficient between -1.15 and -1.5 $\Delta P_{50}/\Delta pH$ (P_{50} = PO_2 need for 50% O_2 saturation of the hemocyanin; Pörtner 1994), which means that per mol released O_2 1.15 to 1.5 mol protons are taken up (Lykkeboe et al. 1980, Pörtner 1990). During hypoxia, the hemocyanin O_2 saturation is reduced and H^+ is taken up instead, causing an increase in pH_e (Houlihan et al. 1982, Johansen et al. 1982a). In the present study, blood deoxygenation and thus H^+ uptake were probably artificially enhanced by anesthesia of the cuttlefish before dissection (see 2.3.1), but a similar effect should have occurred during the incubation under hypoxia and hypercapnia.

Although blood PO_2 could not be measured properly because of the aforementioned anesthesia, it might have been reduced under the given incubation conditions. A reduced blood PO_2 would ease the oxygen uptake at the gills and help to maintain the typically high ventilatory O_2 extraction rates (80% in *S. officinalis*; Melzner et al. 2006b), which are possible due to the uncoupling of ventilation and locomotion in cuttlefish (Wells & O'Dor 1991). It would also support the unloading of the hemocyanin at the tissue (Pörtner 1990, Melzner et al. 2007b). As O_2 affinity is rather dependent on pH than on PO_2 (Pörtner 1990), hemocyanin oxygen uptake at the gills would still be secured. An effect of hypercapnia on blood oxygen partial pressure is unlikely, because Gutowska et al. (2010) found a high venous blood PO_2 (~2 kPa) in cuttlefish exposed to 0.6 kPa CO_2.

Sepia officinalis is a good extracellular acid-base regulator, which can maintain proper functioning of its circulatory system down to a venous PO_2 < 1 kPa (Melzner et al. 2007b). The species withstands long-term exposure to hypoxia (50% air saturation; Thonig 2011) and even during a hypercapnia induced blood acidosis, the cuttlefish could keep blood PO_2 constant (Gutowska et al. 2010). Aside the aforementioned mechanism, *S. officinalis* can also strongly modify ventilation without severely increasing energy costs (Melzner et al. 2006b). As the effects of hypoxia and hypercapnia on pH_e might counteract each other (Johansen et al. 1982a, Truchot 1988), there might be only minor need for a compensatory increase of blood HCO_3^-.

Thus, the blood system of the cuttlefish has the potential to cope with future moderate changes in ocean oxygen and CO_2 partial pressures.

4.2 Tissue physiology

4.2.1 Effects of hypoxia (HOx)

The parameter changes in cuttlefish funnel tissue exposed to hypoxia strongly contrast the findings of other studies. Increased concentrations of PLA and ATP were found, whereas octopine and P_i decreased compared to the control group. Also, ADP was elevated in the treatment, but the changes in the other adenylates indicate this rise occurred mainly at the expense of AMP. Although Gibbs free energy could not be determined (pH_i values are missing for these incubations), an elevated [PLA]/([Arg]+[PLA]) ratio in the funnel implies that the energy state was higher in the treatment than in the control. Earlier studies on *S. officinalis* (Storey & Storey 1979), *L. brevis* (Zielinski et al. 2000) and the humboldt squid *Dosidicus gigas* (Rosa & Seibel 2008, Häfker & Seibel unpubl.) reported exactly opposite changes during hypoxia.

However, in these studies the cephalopods were usually exposed to short periods of strong hypoxia, which guaranteed the use of anaerobic metabolism and the respective changes in anaerobic metabolites (Pörtner 1987, Grieshaber et al. 1994). Even the “moderate” hypoxia applied to *S. officinalis* by Storey & Storey (1979) reflected a PO_2 of ~20% air saturation, which is still far below the long-term survival limit of ~50% air saturation in this species (Thonig 2011). Furthermore they used larger animals (75-135 g), which might be more vulnerable to hypoxia, due to their lower surface/volume ratio and the possible importance of cutaneous respiration (de Wachter et al. 1988, Johansen et al. 1982b, Pörtner 1994, Melzner et al. 2006b). The study of Zielinski et al. (2000) could help to resolve the conflict. Here, *L. brevis* was exposed to different oxygen tensions for two hours each to determine the critical PO_2 (characterized by the onset of anaerobic metabolism). A critical PO_2 of ~8 kPa (~42% air saturation) was determined and a decrease in PLA, ATP and Gibbs free energy was observed at a PO_2 below 6.6 kPa. However, an intracellular acidosis occurred already at an oxygen tension of 10.0 kPa (~52% air saturation).

Translating to *S. officinalis*, these findings could indicate that the hypoxia treated cuttlefish were incubated slightly above their critical PO_2 (~51% oxygen air saturation

≙ ~4 mg O_2*L^{-1}, see 2.2.2). Johansen et al. (1982b) and de Wachter et al. (1988) found even lower critical oxygen tension (2.6 mg O_2*L^{-1} and 3.2 mg O_2*L^{-1}, respectively) in similar sized cuttlefish at slightly higher temperatures (17℃ and ~19℃, respectively). However, those were short-ter m incubations (max 3 h) and the critical PO_2s for long-term survival were probably higher. At ~51% oxygen air saturation, the cuttlefish in the present incubation might have displayed a drop in pH_i, which could have caused a metabolic reduction (Pörtner 2002). A decrease of the metabolic activity (metabolic depression) could explain why the cuttlefish incubated under hypoxia apparently did even better than the respective control group. Although anesthesia does not directly affect parameters of several tissues (Storey & Storey 1979), the handling before dissection is always associated with minor stress that can cause alterations in tissue metabolite concentrations. If the hypoxia treated cuttlefish were in a state of metabolic depression, such metabolite changes would have been less pronounced compared to the control. Hypoxia has been identified as a trigger for metabolic depression in several cephalopod species (Wells 1979, Houlihan et al. 1982, Wells et al. 1992, Häfker & Seibel unpubl.), but unfortunately data on funnel pH_i in this experiment are missing to verify this assumption.

Neither systemic heart nor branchial hearts showed a change in any of the measured parameters. This could indicate that both tissues remained fully aerobic during hypoxia and cellular functioning was not visibly affected. For the branchial hearts that are more tolerant to hypoxia than the systemic heart as they receive O_2-poor venous blood this could hold true (Driedzic 1985, Schipp 1987). However, the systemic heart is considered obligatory aerobic and depends on a continuous supply with O_2-rich water from the gills (Driedzic 1985). Thus, as for the hypoxia and hypercapnia incubation, possible significant changes in metabolite concentrations might be invisible due to type 2 errors (see 4.6).

4.2.2 Effects of hypercapnia (HCa)

Sepia officinalis funnel tissue showed a decrease in the intracellular concentration of PLA during exposure to hypercapnia. Together with decreasing intracellular arginine levels and a reduction of the [PLA]/([Arg]+[PLA]) ratio, this could be a sign of anaerobic metabolism. Hypercapnia might have caused a reduction in pH_e and thus impeded proper O_2 transport to the tissue (Gutowska et al. 2010). The lack of oxygen in the tissue would then result in anaerobic metabolism. However, this is unlikely,

because a complete pH_e compensation was found in the hypoxia & hypercapnia incubation (see 4.2.3) and even severe hypercapnia (0.6 kPa CO_2) caused only minor reduction of pH_e (Gutowska et al. 2010). Additionally, the thin funnel is in close contact with the ambient water and might take up O_2 via cutaneous respiration (Pörtner 1994, Melzner et al. 2006b). Also, despite the reduced levels of PLA and arginine, the intracellular octopine concentration, which is a classical indicator for anaerobic metabolism, did not change compared to the control. A reason for this pattern might be that anaerobic metabolism took place and octopine was produced but immediately released into the bloodstream for oxidation in other tissues (Storey & Storey 1979). However, the results of the other incubations (see 4.2.1, 4.2.3) as well as earlier studies on squids (Pörtner et al. 1991, 1993) contradict this assumption. Increased blood octopine concentrations found by Storey & Storey (1979) may be caused by tissue infringements during dissection (Pörtner pers. comm.). Furthermore, the funnel is thin and has good access to ambient water, which means that it could rather act as a site of octopine oxidation than of octopine production. However, information on different octopine-dehydrogenase isoforms is missing for funnel tissue (Storey 1977). It might be possible that an increase in octopine concentration occurred during hypercapnia but could not be identified due to the strong variation of the results from the control group (mean = 3.98 ± 3.47 µmol*g $tissue^{-1}$). Nevertheless, it is questionable if hypercapnia actually did cause anaerobic metabolism in the funnel tissue. Gutowska et al. (2010) found a stable [PLA]/[P_i] ratio in *S. officinalis* exposed to severe hypercapnia (0.6 kPa CO_2), which indicated that no anaerobic metabolism took place here.

Another, although less likely, option is that the arginine produced from PLA mobilization was not channeled into anaerobic octopine production, but into amino acid catabolism instead (Hochachka & Fields 1982, Mommsen et al. 1982). The degradation of amino acids yields HCO_3^- and ammonium (NH_4^+), which can be excreted (Atkinson & Camien 1982, Pörtner et al. 1998). This process could help to buffer the intracellular acidosis that probably developed due to CO_2 accumulation under hypercapnia (Gutowska et al. 2010, Melzner et al. 2012, see 4.2.3). However, the peanut worm *Sipunculus nudus* showed decreased rates of NH_4^+ excretion together with metabolic depression during exposure to hypercapnia (Langenbuch & Pörtner 2002). For example, in the squid *I. illecebrosus* O_2 consumption and NH_4^+ excretion were linearly correlated, which indicates that the intracellular acidosis from

respiratory CO_2 production is (partially) buffered by the excretion of protons via ammonium (Hoeger et al. 1987). Although cephalopods are considered poor intracellular acid-base regulators (Pörtner & O'Dor 1994), *S. officinalis* displayed only a minor (but still significant) drop in pH_i during severe hypercapnia (Gutowska et al. 2010). Data on intracellular bicarbonate concentrations during exposure to hypercapnia would help to clarify the picture, but could not be measured due to a lack of sample material.

The reduced ATP concentration and a lowered [PLA]/([Arg]+[PLA]) ratio indicate that no metabolic depression occurred during exposure to hypercapnia (Gibbs free energy could not be determined due to lacking pH_i values). Hypercapnia is a common trigger for metabolic depression in several species of different animal groups including bivalves and gastropods (Rees & Hand 1990, Guppy & Withers 1999, Michaelidis et al. 2005b, Pörtner et al. 2005). But a hypercapnia-induced reduction of metabolic rate was neither found in *S. officinalis* (Gutowska et al. 2010) nor in other cephalopods (Rosa & Seibel 2008, Häfker & Seibel unpubl.). Instead, Gutowska et al. (2010) found elevated ventilation rates in *S. officinalis* during hypercapnia, which probably involve increased muscular activity of the funnel tissue. As hypercapnia did cause intracellular acidosis, but no metabolic reduction in cuttlefish (Gutowska et al. 2010), this contradicts the assumption that a drop in pH_i could be the trigger for metabolic depression in cuttlefish (see 4.2.1). However, intracellular acidosis may be only one of several factors involved in the triggering of metabolic depression (Reipschläger et al. 1997).

As for the other incubations, no changes appeared in systemic heart or branchial heart during hypercapnia. The effects of elevated ambient CO_2 und cephalopod hearts have not been studied yet and thus data is lacking for comparison. The fact that there were no differences in the heart metabolite concentrations between the hypercapnia incubation and the respective control group could have two meanings. Either both systemic and branchial hearts stayed fully aerobic during hypercapnia and maintained proper energy levels or differences could not be detected and type 2 errors were made (see 4.6).

4.2.3 Effects of simultaneous hypoxia & hypercapnia (H+H)

When applied separately, both hypoxia and hypercapnia caused clear changes in funnel tissue physiology. However, mantle tissue simultaneously exposed to both

stressors showed only minor changes of the intracellular parameters. This may be partially attributed to the different properties of the two tissues (see. 4.2.4), but the little changes observed under simultaneous hypoxia and hypercapnia may also reflect an interaction of both factors. Although a statistical comparison of the treatments was not possible because of the differences between the control groups, these interactions have to be examined in detail.

An elevated intracellular CO_2 partial pressure in the mantle indicates that the ambient hypercapnia did not only affect the blood system, but also proceeded to the cellular level. The small drop in mantle pH_i and a constant intracellular HCO_3^- concentration during hypoxia + hypercapnia indicate effective intracellular buffering. This minor change in pH_i contradicts the picture of cephalopods as poor intracellular acid-base regulators that mainly regulate their extracellular milieu (Pörtner & O'Dor 1994). A pH_i regulation by the degradation of amino acids and the production and excretion of NH_4^+ is unlikely, because this would include the accumulation of HCO_3^- (Atkinson & Camien 1982, Pörtner et al. 1998). A similar pattern of increasing intracellular PCO_2, decreasing pH_i and constant bicarbonate was observed in the high performance squid *I. illecebrosus* (Pörtner et al. 1991). Fabry et al. (2008) argued that the ability to accumulate intracellular HCO_3^- is an indicator for resistance to hypercapnia and accordingly several cephalopod species have low tolerance to elevated CO_2 compared to fishes with similar lifestyles (Pörtner et al. 2005). Nevertheless, *S. officinalis* showed only minor changes in mantle metabolite concentrations during exposure to hypoxia and hypercapnia. Compared to the results of the funnel tissue under hypoxia or hypercapnia, this observation could be correlated to the situation in the incubation boxes. The cuttlefish were lying on the bottom of the boxes most of the time, which means that the major region mantle was inactive while funnel is involved in ventilation and is permanently active (Bone et al. 1981). Thus there might was hardly any anaerobic metabolism in the mantle, that could cause metabolite accumulation and a reduction of pH_i. However, the funnel is thinner and the tissue has better access to oxygen from the ambient water by diffusion.

Mantle and hearts showed no changes in PLA, arginine, octopine and P_i. All these metabolites characteristically decrease (PLA) or increase (arginine, octopine) concentrations during anaerobic metabolism (Pörtner 1987). The fact that no (or hardly any) succinate could be detected furthermore supports the prediction that both cytosol and mitochondria of the investigated tissues stayed full aerobic during

hypoxia and hypercapnia (Pörtner 1987, Grieshaber et al. 1994, Finke et al. 1996). The prediction is further supported by the finding of constant [PLA]/([Arg]+[PLA]) ratios in all three tissues. The ratio is a measure for the energy status of a tissue and a shift would be an indicator for the depletion of energy storages during anaerobic metabolism (Pörtner et al. 1996). The presented findings are totally plausible as anaerobic metabolism is primarily designed to provide additional energy during short periods of insufficient O_2 supply (Pörtner 1987). Anaerobic metabolism hidden by the transfer of octopine from the mantle to the blood (Storey 1977, Storey & Storey 1979) is unlikely, as the sum of PLA, arginine and octopine did not differ between control and treatment and this process would still involve a decrease in PLA levels (Pörtner 1987).

The energy status of mantle systemic heart and branchial hearts was probably not affected by hypoxia and hypercapnia, because of constantly high levels of ATP and Gibbs free energy (in the mantle). This means that the intracellular acidosis did not severely affect energy metabolism, although it was neither buffered by a rise in HCO_3^- nor by an accumulation of inorganic phosphate (Pörtner 1987, 2002). This indicates either an effective non-bicarbonate non-phosphate buffering by protein H^+ uptake compound or a removal of protons from the intracellular space. Constant levels of free ADP and free AMP imply that enzyme activity was not greatly altered compared to the control incubation. The combination of a constant free ADP concentration and a drop in pH_i could mean that *S. officinalis* relies mostly on intracellular acidosis for the mobilization of PLA (Pörtner 2002). A similar pattern is found in the brief squid *Lolliguncula brevis*, which lives near the coast and regularly encounters hypoxic waters (Pörtner et al. 1996). In contrast, the longfin inshore squid *Loligo pealei* relies mainly on free ADP for PLA mobilization and is vulnerable to hypoxia (Pörtner et al. 1993). For the hearts, the results might be misleading, as small replicate numbers and low statistical test power increase the probability of type 2 (false negative) errors (see 4.6).

Although the energy status of the tissue was stable and thus, proper functioning of aerobic metabolism can be considered secured, there was still a significant increase in the mantle concentrations of total ADP and total AMP. This could mean that there was a slight shift in the adenylate ratio and that a small (insignificant) decrease of the large ATP pool caused significant augmentations to the relatively small pools of ADP and AMP. This shift might be a consequence of a reduced O_2 supply to the mantle

tissue or by effects of the reduced pH_i on enzyme function (Pörtner 2002). As there were no indicators for a disruption of blood oxygen transport (see 4.1), a drop in intracellular pH is the most likely explanation. Intracellular acidosis has been shown to reduce performance and thereby delay the depletion of energy reserves in the squid *L. brevis* as well as in the hypoxia-tolerant peanut worm *S. nudus* and could also help cuttlefish to survive long-term exposure to unfavorable conditions (Pörtner et al. 1996, 1998, Pörtner 2002).

A metabolic depression may be possible as intracellular acidosis is known to reduce muscular and metabolic performance (Pörtner 2002). Also, concurrence of anoxia and hypercapnia caused adenosine accumulation and metabolic depression in *S. nudus* (Reipschläger et al. 1997). Adenosine is a neuronal inhibitor, which is discussed as a mediator of a metabolic reduction induced by central nervous control (Reipschläger et al. 1997). Adenosine might also act directly on cellular functions, as cold induced ATP breakdown caused adenosine accumulation and reduction of mitochondrial energy production in the demersal fish *Zoarces viviparous* (Eckerle et al. 2008). Hypercapnia alone does not cause metabolic depression in *S. officinalis* (Gutowska et al. 2010), but the accumulation of intracellular CO_2 and the associated acidosis could support the reduction of performance during a hypoxia-induced metabolic depression (see 4.2.1) and help to conserve energy storages. Thus, a metabolic depression might have occurred, but data on O_2 consumption is lacking to secure this assumption. Anyway, the measured parameters imply that *S. officinalis* is able to withstand long-term exposure to the applied levels of hypoxia and hypercapnia. However, a reduction of animal performance is possible.

4.2.4 Comparison of tissues

Although a proper statistical assessment of the differences between the tissues was not possible due to strongly varying replicate numbers and heterogeneous variances, there are some general parameter patterns in the different tissues that can be discussed.

The concentration of the energy storage compounds PLA and ATP is generally lower in the funnel tissue than in the mantle tissue. This could reflect the different modes of activity. The funnel musculature is involved in ventilation, which depends on continuous aerobic energy production with a relatively small variation in energy demand (Melzner et al. 2006b). In contrast, the major fraction of the mantle, the

poorly vascularized central mantle displays mostly spontaneous but vigorous activity fueled by anaerobic metabolism (Bone et al. 1981). Thus, the mantle needs energy storages to provide fast energy during burst activity, while funnel tissue gets along with a smaller energy pool that is continuously refilled. Aerobic energy production may be supported by the thin structure of the funnel and ventilation, which flushes the funnel with ambient water and thus could enhance cutaneous respiration. However, the capacity of the funnel tissue for anaerobic energy production is lower than in the mantle.

The pattern observed in mantle and funnel becomes even more evident in the cuttlefish hearts. Both systemic heart and branchial hearts showed very low levels of PLA and ATP in all incubations. Together with the low concentrations of arginine and octopine compared to mantle or funnel, this indicates a very low anaerobic capacity for both heart types. As hearts have to work permanently to circulate the blood through the body, it makes sense that the tissues stay fully aerobic and permanently recycle the relatively small pool of ATP, using the continuous O_2 supply from the blood stream (Driedzic 1985). This is also indicated by the high mitochondria content of the hearts (Dykens & Mangum 1979, Oellermann et al. 2012). Similarly, the mitochondria content of central mantle (6.4% of muscle fiber profile), which performs short-term burst activity, is lower than the content in the mantle periphery (47% of muscle fiber profile), which performs continuous ventilatory mantle contractions (Bone et al. 1981). As the funnel is also involved in continuous ventilation, a high mitochondria content of the tissue is likely.

The actual ATP pool of heart tissues might be bigger than indicated by the concentrations measured as anesthesia stops heart contractions and thus, the O_2 supply to the hearts (see 2.3.1) and could have led to a fast conversion of ATP to ADP and AMP, which displayed higher levels. However, the cessation of heart contractions also means that energy demands and ATP turnover are reduced. Therefore, the found ATP concentrations could reflect the situation *in vivo*. Low arginine levels further support the finding that energy production in cephalopod hearts is rather fueled by carbohydrate degradation than by amino acid degradation (Mommsen et al. 1982, Driedzic et al. 1990). This coincides with the finding of Oellermann et al. (2012) that specimen of *S. officinalis*, which are living at higher temperatures and are more likely to experience hypoxia, prefer carbohydrates (pyruvate) over amino acids (proline) as energy substrate. Carbohydrates provide

more ATP per mol O_2 (see 1.2.2) and thus should be favored under oxygen limited conditions (Hochachka 1994, Oellermann et al. 2012).

A difference between the heart types could have been expected as the systemic heart receives O_2-rich blood from the gills, whereas branchial hearts are supplied with O_2-poor venous blood (Schipp 1987). Driedzic (1985) found cuttlefish branchial hearts to be more anoxia resistant than systemic hearts, which he considered obligatory aerobic. Also the systemic heart has higher ATP demands as it has to create higher pressures to circulate the blood through the body (Wells & Wells 1983, Driedzic 1985). Due to these findings it could be expected that the systemic heart shows more pronounced changes than the branchial hearts during hypoxia and/or hypercapnia. Driedzic et al. (1990) found similar ATPase activities in both heart types and assumed additional (yet unidentified) energy consuming processes in branchial hearts, but again it is most likely that differences between the tissues could not be identified due to the small replicate number resulting in a low test power. Different intracellular patterns may exist between the heart types, although detection was not possible.

4.3 The natural environment

According to the findings of the laboratory experiments, the investigated life stage of *S. officinalis* should be able to cope with the changes of environmental O_2 and CO_2 partial pressures expected from climate change (Caldeira & Wickett 2003, Meehl et al. 2007). However, in the natural environment there are additional factors that might change and can affect cuttlefish physiology.

The ambient PCO_2 applied in the experiments (0.1 kPa) reflects the expected change from the dissolution of atmospheric CO_2 in the ocean until the end of the century (Meehl et al. 2007). However, this scenario does not consider other factors that influence the CO_2 content of seawater. The degradation of organic matter in hypoxic waters sets free large amounts of CO_2. Melzner et al. (2012) calculated that if all oxygen is consumed (anoxia), neritic waters subjected to hypoxia can experience a PCO_2 between 1.7-3.4 kPa already today. Even if O_2 is not consumed completely, the combination of hypoxia and the additive effects of respiratory and atmospheric CO_2 accumulation would probably be more stressful than the conditions applied in the experiments. *Sepia officinalis* is able to withstand 0.6 kPa CO_2 for 48 hours, but studies investigating the effects of severe hypercapnia on long-term survival and

performance of the cuttlefish are scarce (Hu et al. 2011, Strobel et al. 2012). Studies on the effects of simultaneous long-term exposure to hypoxia and severe hypercapnia are missing.

Metabolic depression does extend the time of anaerobic metabolism due to the slower depletion of energy storages, but long-term survival would only be supported, if sufficient energy production can be sustained by aerobic metabolism. A decrease of O_2 and/or an increase of CO_2 beyond their respective critical thresholds can only be compensated by anaerobic metabolism for limited periods of time and would lead to death on the long term (Pörtner et al. 2005).

According to the concept of oxygen & capacity-limited thermal tolerance (OCLTT), the supply with oxygen, which can be affected by both hypoxia and hypercapnia, often sets the thermal limits for aerobic performance and distribution of a species (Frederich & Pörtner 2000, Pörtner 2010). *Sepia officinalis* has a broad aerobic temperature range reaching from 7℃ to 26.8℃ (Melz ner et al. 2006a). If it acted exclusively, the temperature increase expected from global warming would probably not strongly affect cuttlefish performance and survival in most populations (Boucaud-Camou & Boismery 1991, Artegiani et al 1997, Melzner et al. 2006b), but only shift them northwards. Nevertheless, in interaction with hypoxia and hypercapnia, increasing temperatures might have more severe consequences for *S. officinalis*. Melzner et al. (2006a) found that between 11℃ and 23℃ the oxygen consumption (MO_2) of cuttlefish increased exponentially according to a Q_{10} value of 2.5 (a temperature increase by 10℃ causes an increase of MO_2 by the factor 2.5). The increased energy demands were compensated by increased ventilation over the whole range of 7-26.8℃ (Melzner et al. 2006a), but the O_2 transport to the tissue depends on a proper functioning of the blood system. Generally, the O_2 affinity of the hemocyanin decreases with increasing temperature (Zielinski et al. 2001, Melzner 2007b). This supports the unloading at the tissue, but it can be disadvantageous, if other factors are involved. Hypoxia and hypercapnia have the potential to distort oxygen transport by the hemocyanin (see 4.1). Although hypoxia can increase pH_e and thus hemocyanin O_2 affinity, it can also cause a reduced blood PO_2. Together with the pH lowering effect of hypercapnia and elevated temperature (Reeves 1972) that reduce O_2 affinity, the complete loading of the hemocyanin at the gills could be threatened.

At the tissue level, a reduced respiratory CO_2 production due to metabolic depression could impair proper oxygen unloading and a general reduction of blood PO_2 would deteriorate the O_2 transport into the tissue. In cephalopods this factor might be critical as they lack myoglobin (Hochachka 1994). However, O_2 demands are also reduced during metabolic depression and a hypoxia-induced reduction of blood PO_2 (at constant pH_e) would also reduce the O_2 affinity of the hemocyanin and thus support unloading (Pörtner 1994, Zielinski et al. 2001). O_2 unloading is typically high in *S. officinalis* (≥ 80% O_2 used by tissue; Johansen et al. 1982a) but a disturbed release at the tissue would reduce the arteriovenous PO_2 gradient. This could in turn hinder the O_2 uptake at the gills that depends on a low venous PO_2 to function efficiently. Increased ventilation rates due to elevated temperatures and hypercapnia might counteract this problem as the supply with fresh (high PO_2) ambient water is increased and the PO_2 gradient between ambient water and blood is kept high. Although O_2 extraction rates can be lowered by hyperventilation (Melzner et al. 2006a,b, Gutowska et al. 2010) the total O_2 transport to the blood should be increased due to the bigger water volume that passes the gills. Nevertheless, during hypoxia the PO_2 of the ambient water is reduced and the smaller gradient could reduce oxygen uptake.

Hypoxia and hypercapnia will most likely narrow the thermal window of *S. officinalis*. Additionally, the scope for aerobically fuelled activities (behavior as well as internal processes) within the window will be reduced due to the redistribution of energy resources, which is necessary to cope with the changes (e.g. homeostasis, ventilation). Metabolic depression may allow long-term survival, but it reduces the scope for growth and reproductive capacity (Pörtner et al. 2005). Reduced activity also increases the vulnerability to predators and exacerbates hunting for food. Thus, even if cuttlefish individuals are able to survive long-term exposure to hypoxia, hypercapnia and elevated temperatures, negative effects of the population level can be expected (Pörtner et al. 2005, Pörtner 2010, Melzner et al. 2012).

4.4 Relevance

The common cuttlefish *S. officinalis* is a well investigated and easily cultivable cephalopod model species. Although there can be considerable physiological differences between cephalopod taxa (Pörtner et al. 1991, Rosa & Seibel 2008, Seibel 2007), there are also unifying principles (e.g. Bohr-effects < -1 or the major

metabolic pathways) that show similar reactions to environmental changes (Storey & Storey 1979, Zielinski et al. 2001, Rosa & Seibel 2008). Investigations on *S. officinalis* could thus also provide a chance for a better understanding of other cephalopods, which are more difficult to obtain or to breed in captivity.

Furthermore, *S. officinalis* is a good choice for experiments on the effects of climate change and anthropogenic influence. The species inhabits coastal waters (von Boletzky 1983), which are especially exposed to changing O_2 and CO_2 partial pressures, as well as rising temperatures and human activities (Diaz 2001, Diaz & Rosenberg 2008, Melzner et al. 2012). In this context, it is reasonable to point out the use of juvenile cuttlefish in the presented experiments. The juveniles grow in the coastal waters under fluctuating environmental conditions and, although they have to invest a large fraction of their energy into growth, they show higher tolerance to hypoxia than do adults (de Wachter et al. 1988). However, although mature cuttlefish spend most of their time in deeper waters with more stable ambient conditions (Jereb & Roper 2005), they also face changes in oxygen and CO_2 during the migrations to their coastal spawning grounds (Wang et al. 2003).

In general, it is reasonable to assess the ability of cephalopods to withstand long-term exposure to a combination of factors associated with climate change, as this is a more natural scenario than the exposure to changes of a single factor (e.g. only PCO_2). Although fish are a dominant group of higher marine animals (also with regard to fishery), the exploitation of fish stocks could lead to an expansion of competing cephalopods (Keyl et al. 2008, Vetter et al. 2008). Whereas fish landings are decreasing, cephalopod fishery is expanding (Caddy & Rodhouse 1998). Most cephalopods cannot be raised and cultivated in capture, but *S. officinalis* can be held in high densities and is (as most cephalopods) fast growing (von Boletzky 1983, Wells 1994). Thus it has the potential to provide significant amounts of high quality food produced in aquaculture (Sykes et al. 2006).

4.5 Synthesis

The obtained results indicate that juveniles of the common cuttlefish *Sepia officinalis* are able to withstand long-term simultaneous exposure to moderate levels of hypoxia (~60% air saturation) and hypercapnia (0.1 kPa CO_2) at an ambient temperature of ~16℃. Hypoxia alone caused an improvement of the f unnel tissue energy status that could indicate a hypoxia-induced metabolic depression. Exposure to hypercapnia

resulted in a decrease of the intracellular energy status of the funnel, which was probably accompanied by a reduction of pH_i. There were no signs of hypercapnia-induced metabolic depression in the funnel.

Hardly any changes occurred in cuttlefish during simultaneous exposure to hypoxia and hypercapnia. Blood PCO_2 was elevated, but pH_e was maintained stable due to a compensatory increase of the blood bicarbonate concentration and perhaps by a slight hypoxia-induced decrease of hemocyanin O_2 saturation and an accompanied H^+ uptake. Thus, O_2 transport to the tissue was secured although possibly slightly reduced. In the mantle tissue there might have been a reduction of the metabolic rate. However, this is not for certain, as the exact mechanisms of metabolic depression in cephalopods are still unknown.

No effects of hypoxia and/or hypercapnia could be detected in the systemic heart or the branchial hearts. This could indicate that these tissues have a strong resistance to the applied stressors, but it is most probable that present changes were concealed by type 2 errors (see 4.6). Generally, most metabolite concentrations were lower in the hearts than in the mantle or the funnel. Funnel concentrations were mostly below those of mantle tissue. This was interpreted as a reflection of the activity modes of the different tissues. Hearts (and partially also funnel) perform continuous work with constant aerobic power output. These tissues permanently recycle a small pool of energy compound to fulfill their low energy demands. The major fraction of the mantle displays short but vigorous activity supported by anaerobic metabolism, which relies mostly on cellular storages of energy metabolites (PLA, ATP) and accumulates end products (e.g. arginine, octopine, P_i).

4.6 Evaluation of Methods

Although anesthesia with ethanol does not affect tissue parameters (Storey & Storey 1979), it reduces blood oxygen content due to the cessation heart contractions and blood circulation. However, during blood sample transfer to the blood gas analyzer (BGA), the blood takes up oxygen again due to the high O_2 affinity of the hemocyanin. Measured blood PO_2 values of 2-3 kPa are close to the venous PO_2 *in vivo* (~1.7 kPa; Zielinski et al. 2001) and thus supporting the correctness of the other measured parameters (PCO_2, $[HCO_3^-]$, pH_i).

A general problem is the fact that the different incubations were not run simultaneously and were not completely identical with respect to the factors which

were not manipulated. Cuttlefish used in the hypoxia and hypercapnia experiment were smaller than those used in the experiments on hypoxia or hypercapnia and the sampled tissues were not identical for all experiments (mantle vs. funnel). The different properties of the tissues are discussed above (see 4.2.4), but also the different size (age) classes could have affected the measured parameters. Strobel et al. (2012) showed that the ratio of the different hemocyanin isoforms expressed by *S. officinalis* depends on temperature, ambient PCO_2 and also developmental stage. Physiological differences due to different ages or weights might have occurred and could involve energy resource allocation between the tissues (Rosa et al. 2004, Oellermann et al. 2012).

Another factor that was different between the experiments is the feeding rhythm. Cuttlefish were fed daily in the hypoxia experiment (Thonig 2011) and twice a week in the hypercapnia experiment (Strobel 2011), but only once a week in the hypoxia & hypercapnia experiment. However, in *S. officinalis* first physiological changes could be detected after seven days of starvation (Castro et al. 1992, Grigoriou & Richardson 2009). As the longest feeding interval was seven days (H+H experiment), effects caused by different feeding rhythms are unlikely. Nevertheless, the mentioned factors might be the reason for the deviations between the different control groups, which prohibited statistical comparisons between the different experiments.

The low replicate number especially for the heart tissues caused strongly fluctuating variances and low test powers. It was partially caused by the pooling of samples that was necessary due to the small mass of the heart tissues. The strong difference in replicate numbers and variances prohibited a comparison of the tissues within single incubations or between incubations. Because of the small tissue mass it was not possible to measure the acid-base parameters and succinate concentrations of all tissues. The missing information is needed to assess the separate effects of hypoxia and hypercapnia in bigger detail.

4.7 Outlook

The presented study proved that *S. officinalis* is able to withstand long-term moderate hypoxia and hypercapnia, but the mechanisms that enable the cuttlefish to survive under these conditions could not be identified in detail. A repetition of the presented experiments with incubation under hypoxia, hypercapnia or hypoxia and hypercapnia running in parallel with sampling of identical tissues should provide more comparable

results. In this context, an increase of the replicate number is appropriate to produce enough sample material for measuring all parameters (acid-base and metabolites) in all investigated tissues. Insights into blood physiology and metabolic depression could be augmented by measuring *in vivo* blood acid-base parameters and oxygen consumption rates of cannulated cuttlefish during exposure to hypoxia, hypercapnia or both.

More sample material could also be attained by using animals of larger size. Although mainly juveniles are exposed to the presented stressors, adult cuttlefish do also face hypoxia environmental changes during when migration to their spawning grounds (Wang et al. 2003, Jereb & Roper 2005). Ontogenetic changes in energy resource allocation as well as vulnerability to hypoxia and hypercapnia could be identified by incubating *S. officinalis* of different age (and size) classes under defined O_2 and CO_2 levels.

Considering the findings of Melzner et al. (2012), an intensification of the incubation conditions (especially with respect to hypercapnia) might be reasonable. A more authentic incubation scenario could be created by combining hypoxia and hypercapnia with increased temperature, the third major factor of oceanic climate change. Treating *S. officinalis* with stable levels of hypoxia and hypercapnia at different temperatures or varying the O_2 and CO_2 partial pressures for defined temperature levels would help to define the thermal window of the cuttlefish according to the “OCLTT” concept (Pörtner 2010). This would help to predict future changes in the distribution of *S. officinalis*. Investigating the combined effects of the aforementioned factors on cuttlefish embryos and hatchlings is also advisable, as eggs are deposited in the area that will probably be mostly affect by changes in temperature PO_2 and PCO_2 (Jereb & Roper 2005, Diaz & Rosenberg 2008).

Generally, long-term incubation experiments should be preferred over acute exposure in climate change research. Realistic critical limits of environmental factors only become evident, if an organism has enough time to either die or reach a new steady state (Pörtner et al. 2005). In the natural environment, most effects of climate change will be either long-lasting or persistent and future research should account for this.

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Declaration of authorship

Hereby, I declare that I wrote this Master thesis independently and without any external assistance. All information adopted from other sources (literally or in essence) is marked as such in the text and is summarized in the reference list.
The Master thesis has not been submitted to any other institution in the present or in a changed form.

N. Sören Häfker

Bremen

Affixes

A: Blood acid-base parameters of *S. officinalis* exposed hypoxia & hypercapnia (H+H) and the respective control conditions (C_H+H). Mean values of individuals (or individual pools) are shown. No° in dicates the animal number (more than one number indicate pooled samples). PO_2 and PCO_2 are in [kPa], HCO_3^- is in [µmol*g tissue^{-1}]. PO_2: blood O_2 partial pressure, PCO_2: blood CO_2 partial pressure, pH_e: blood pH, HCO_3^-: blood bicarbonate concentration. Asterisks (*) indicate outliers that were excluded from statistics.

Incubation	No°	PO_2	PCO_2	pH_e	HCO_3^-
C_H+H	1	1.37	0.22	7.59	2.50
	2,3	2.64	0.20	7.56	2.04*
	4	2.31	0.25	7.58	2.84
	5,6	2.25	0.22	7.59	2.49
	7	2.32	0.24	7.62	2.94
	8-10	3.93	0.28	7.54	2.77
	11,12	2.84	0.26	7.57	2.73
	13	1.95	0.35	7.59	3.95
	14	2.00	0.26	7.61	3.12
	15	2.56	0.30	7.55	3.03
	16	3.40	0.32	7.57	3.48
	17	2.17	0.30	7.61	3.63
	18	2.03	0.41*	7.51*	3.75
	19,20	4.25	0.33	7.54	3.25
	21	1.82	0.32	7.59	3.67
	22	3.30	0.37	7.54	3.71
	23	2.79	0.31	7.57	3.35
H+H	1,2	2.73	0.26	7.59	3.00
	3	2.78	0.28	7.59	3.16
	4	1.11*	0.31	7.61	3.76
	5,6	1.80	0.30	7.62	3.65
	7	4.01	0.35	7.49	3.10
	8	3.59	0.33	7.55	3.39
	9	2.66	0.32	7.58	3.49
	10,11	1.58	0.43	7.54	4.25
	12,13	3.10	0.41	7.53	3.96
	14,15	3.12	0.41	7.51	3.75
	16	2.43	0.41	7.57	4.51
	17	2.57	0.40	7.55	4.11
	18,19	3.33	0.44	7.50	3.95
	20	2.26	0.43	7.56	4.62
	21	2.16	0.40	7.58	4.50
	22	3.10	0.46	7.55	4.77

B: Intracellular acid-base parameters, free ADP & AMP and Gibbs free energy of *S. officinalis* exposed hypoxia & hypercapnia (H+H) and the respective control conditions (C_H+H) or to hypercapnia (HCa) and the respective control conditions (C_HCa). Mean values of individuals are shown. No° indicates the animal number (more than one number indicate pooled samples). PCO_2 is in [kPa], HCO_3^-, free ADP and free AMP concentrations are in [µmol*g tissue^{-1}], Gibbs free energy is in [kJ*mol^{-1}]. M: mantle, F: funnel, BH: branchial heart, pH_i: intracellular pH PCO_2: intracellular CO_2 partial pressure, HCO_3^-: intracellular bicarbonate concentration, ΔG/Δξ: Gibbs free energy change of ATP hydrolysis. Asterisks (*) indicate outliers that were excluded from statistics.

Incubation & Tissue		No°	pH_i	PCO_2	HCO_3^-	free ADP	free AMP	ΔG/Δξ
C_H+H	M	1	7.55	0.34	3.09	0.33	0.035	-55.85
		3	7.50	0.34	2.68*	0.52*	0.101*	-54.18*
		5	7.53	0.34	2.87	0.32	0.031	-55.94
		7	7.54	0.34	2.90	0.29	0.027	-56.15
		14	7.46	0.37	2.63	0.25	0.019	-56.27
		15	7.57	0.31	2.83	0.14	0.007	-57.75*
		17	7.49	0.38	2.93	0.34	0.039	-55.43
		18	7.48	0.38	2.89	0.36	0.035	-55.72
		20	7.55	0.28*	2.47	0.32	0.030	-56.13
		23	7.50	0.41	3.26*	0.22	0.016	-56.51
H+H	M	1	7.46	-	-	0.20	0.012	-56.74
		3	7.55	0.60	3.34	0.27	0.024	-56.24
		4	7.36	0.70	2.43	0.41	0.054	-54.47
		6	7.47	-	-	0.19	0.014	-56.29
		8	7.46	-	-	0.30	0.041	-54.77
		9	7.45	0.70	3.04	0.38	0.071	-54.04
		10	7.40	0.54	2.12	0.65*	0.117*	-53.88
		12	7.23*	1.19*	3.14	0.12	0.004	-57.16
		13	7.47	-	-	0.61	0.076	-55.09
		15	7.53	0.59	3.10	0.37	0.039	-55.78
		16	7.48	0.55	2.52	0.39	0.038	-55.70
		17	7.49	-	-	0.29	0.027	-55.94
		18	7.37	0.76	2.69	0.11	0.004	-57.43
		19	7.48	0.71	3.29	0.34	0.033	-55.76
		21	7.48	0.47	2.23	0.54	0.077	-54.82
C_HCa	BH	1,2	6.61	2.81	1.87	-	-	-
		3,4	6.60	3.33	2.16	-	-	-
HCa	BH	1,2	6.64	8.50	6.21	-	-	-
		3,5	6.86	4.45	5.31	-	-	-

C: Intracellular metabolite concentrations of *S. officinalis* exposed to control conditions in the hypoxia & hypercapnia experiment (C_H+H). Mean values of individuals are shown. No° indicates the animal number (more than one number indicate pooled samples). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, SH: systemic heart, BH: branchial hearts, Arg: arginine, Octo: octopine, PLA: phospho-L-arginine, P_i: inorganic phosphate. Asterisks (*) indicate outliers that were excluded from statistics.

Tissue	No°	Arg	Octo	PLA	ATP	ADP	AMP	P_i
M	1	14.07	0.22	17.35	3.76	0.51	0.11	14.14
	2	12.29	0.21	17.87	3.46	0.81	0.14	15.34
	3	21.91	0.93	13.25	3.25	1.14	0.34	25.29
	4	15.78	0.50	19.70	3.86	0.75	0.12	18.45
	5	9.12	0.28	11.60	3.87	0.77	0.13	11.77
	6	27.50*	0.95	7.58	2.97	1.56*	0.69*	35.78*
	7	15.16	0.33	21.17	3.87	1.13	0.14	17.41
	8	14.80	0.65	17.84	3.47	1.03	0.21	19.86
	9	22.85	0.78	5.44*	2.12*	1.44	0.62*	33.16*
	10	18.55	1.08	9.74	2.83	1.47	0.36	20.10
	11	17.98	0.51	10.39	2.75	0.94	0.47	23.25
	12	14.55	0.31	18.41	4.02	0.67	0.11	17.09
	13	15.78	1.11	16.97	4.21	0.98	0.19	16.89
	14	13.38	0.98	19.05	4.04	0.74	0.16	16.40
	15	7.61*	0.37	20.92	3.49	0.61	0.11	8.20
	16	19.22	0.28	14.33	3.83	1.05	0.26	19.32
	17	13.46	0.83	13.70	3.64	0.78	0.16	18.83
	18	16.78	0.75	19.18	4.38	0.89	0.10	21.31
	19	19.35	0.27	15.27	3.78	1.26	0.23	22.45
	20	14.67	0.46	20.39	4.17	0.93	0.16	17.06
	21	20.34	0.60	16.89	4.20	0.90	0.21	21.80
	22	22.45	1.41*	8.84	3.36	1.13	0.46	32.04
	23	11.84	0.40	18.91	3.67	0.96	0.11	13.90
SH	1-4	4.40	0.58	0.66	0.97	0.63	0.55	0.66
	5-9	4.02	0.29	0.29	0.36	0.69	0.85	0.78
	10-14	4.80	0.49	0.28	0.44	0.80	0.71	0.68
	15-18	4.03	0.22	0.26	0.49	0.77	0.87	0.68
	19-23	4.76	0.34	0.67	0.80	0.96	0.51	2.73*
BH	1-4	1.67	0.22	0.25	0.10	0.79	0.55	3.73
	5-9	6.41*	0.83*	0.97*	0.67*	2.68*	1.79	4.16
	10-14	3.58	0.47	0.56	0.31	0.94	1.88	6.77
	15-18	1.62	0.23	0.25	0.12	0.52	0.71	3.03
	19-23	1.69	0.22	0.26	0.04	0.69	0.92	5.98

D: Intracellular metabolite concentrations of *S. officinalis* exposed hypoxia & hypercapnia (H+H). Mean values of individuals are shown. No° indicates the animal number (more than one number indicate pooled samples). Concentrations are in [µmol*g tissue^{-1}]. M: mantle, SH: systemic heart, BH: branchial hearts, Arg: arginine, Octo: octopine, PLA: phospho-L-arginine, P_i: inorganic phosphate. Asterisks (*) indicate outliers that were excluded from statistics.

Tissue	No°	Arg	Octo	PLA	ATP	ADP	AMP	P_i
M	1	11.62	0.56	20.16	3.83	0.90	0.17	16.55
	2	20.08	0.64	7.69	2.22	1.89	0.63	23.76
	3	12.50	0.67	18.22	3.56	1.12	0.15	15.38
	4	19.82	2.23*	12.72	3.70	1.31	0.30	24.43
	5	10.57	0.95	6.32	1.73	0.99	0.38	11.17
	6	13.13	0.61	18.92	2.99	1.55	0.32	16.63
	7	21.14	0.66	8.88	2.89	1.63	0.44	25.33
	8	16.84	0.94	12.83	2.53	1.77	0.32	26.14
	9	13.85	0.23	7.74	2.43	1.03	0.28	13.42
	10	24.82	2.33*	12.71	4.23	1.34	0.27	27.14
	11	28.59	0.50	9.56	3.70	1.49	0.47	27.47
	12	11.04	0.29	20.33	4.00	0.90	0.14	10.60
	13	24.50	1.04	21.38	5.80*	1.10	0.17	22.63
	14	21.30	1.71	13.47	4.11	1.18	0.45	21.58
	15	18.11	0.83	21.63	4.18	1.55	0.27	18.87
	16	22.64	0.42	25.62	4.74	1.68	0.29	15.79
	17	15.42	0.28	19.40	3.83	1.23	0.21	15.57
	18	10.62	0.22	23.54	3.33	1.32	0.26	8.59
	19	17.51	0.50	20.31	4.30	1.61	0.28	17.82
	20	22.67	0.84	20.36	4.95	1.28	0.25	20.49
	21	22.73	0.27	17.82	4.47	1.16	0.27	21.27
	22	26.58	0.70	8.33	2.29	2.66*	0.82*	28.00
SH	1-4	3.95	0.41	0.26*	0.36	0.72	1.15	0.67
	5-8	4.28	0.53	0.26	0.36	0.79	1.08	5.12
	9-13	2.95*	0.33	0.26	0.47	0.79	0.70	2.95
	14-17	4.46	0.35	0.26	0.54	0.95	0.93	0.70
	18-22	4.27	0.54	0.26	0.21	0.57	1.66	0.69
BH	1-4	1.63	0.22	0.25	0.14	0.62	0.95	4.52
	5-8	1.65	0.21	0.25	0.04	0.73	0.78	7.80*
	9-13	1.70	0.22	0.26	0.04	0.68	0.82	6.00
	14-17	1.63	0.21	0.25	0.08	0.65	1.04	5.69
	18-22	1.51*	0.20*	0.24	0.10	0.65	0.93	4.95

E: Intracellular metabolite concentrations of *S. officinalis* exposed hypoxia (HOx) and the respective control conditions (C_HOx) or to hypercapnia (HCa) and the respective control conditions (C_HCa). Mean values of individuals are shown. No° indicates the animal number (more than one number indicate pooled samples). Concentrations are in [µmol*g tissue^{-1}]. F: funnel, SH: systemic heart, BH: branchial hearts, Arg: arginine, Octo: octopine, PLA: phospho-L-arginine, P_i: inorganic phosphate. Asterisks (*) indicate outliers that were excluded from statistics.

Incubation & Tissue		No°	Arg	Octo	PLA	ATP	ADP	AMP	P_i
C_HOx	F	1	15.48	4.95	1.48	0.47	0.89	1.32	24.21
		2	10.72	4.91	0.90	0.28	0.83	1.06	13.45
		3	14.55	5.00	0.42	0.09	0.56*	2.01	24.18
		4	9.78	5.03	4.29*	1.13*	0.84	0.36*	10.96
		5	13.52	5.31	0.71	0.17	0.74	1.70	21.31
		6	13.42	5.65	1.37	0.59	0.94	1.17	16.59
		7	17.24	3.80	1.86	0.46	1.00	1.31	21.82
		8	15.87	3.70	1.14	0.35	0.91	1.37	22.93
	SH	1.2.4.8	3.24	2.52	0.27	0.09	0.30	1.77	7.94
		3.5.6.7	3.31	2.15	0.35	0.10	0.34	1.49	6.94
	BH	1.2.4.8	2.34	0.43	0.30	0.06	0.22	0.91	2.84
		3.5.6.7	2.22	0.57	0.29	0.06	0.27	1.03	5.65
HOx	F	1	12.10	2.52	3.58*	1.71	1.37	0.82	9.40
		2	9.92	3.95	1.26	1.30	1.29	0.98	12.46
		3	16.39	5.39*	1.59	1.12	1.50	1.67	11.72
		4	17.51	2.23	1.79	1.55	1.94*	1.49	27.31*
		5	17.86	3.69	2.63	2.25*	1.62	1.10	22.38
		6	12.47	2.62	2.15	1.72	1.38	0.94	11.32
		7	16.64	1.71	2.17	1.74	1.54	1.16	13.99
	SH	1.4.5	3.40	2.45	0.35	0.45	0.58	0.95	2.57
		3.6.7	3.08	2.54	0.55	0.66	0.66	0.82	2.20
	BH	1.4.5	1.58	0.76	0.39	0.40	0.49	0.52	3.73
		3.6.7	1.62	0.96	0.39	0.40	0.50	0.62	2.13
C_HCa	F	1	5.11	0.72	2.69	0.18*	0.25	0.16	3.31
		2	11.76	3.17	11.47	2.07	0.52	0.15	14.98
		3	14.18	7.85	7.67	2.17	0.49	0.11	20.23
		4	6.68	0.76	14.63	1.69	0.32	0.10	2.75
		5	17.69	7.39	8.80	2.19	0.53	0.16	19.08
HCa	F	1	12.39	3.90	1.51	1.18	0.85	0.85*	-
		2	5.46	2.44	2.42	1.03	0.31	0.11	0.63
		3	10.52	4.31	3.07	1.24	0.62	0.27	7.08
		4	3.87	3.18	1.22	0.72	0.36	0.18	4.04
		5	9.29	4.32	4.79	1.54	0.45	0.19	-