Karen M. Hammer

Acid-base regulation and metabolite responses in shallow- and deep-living marine invertebrates during environmental hypercapnia

Thesis for the degree of Philosophiae Doctor

Trondheim, June 2012

Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biology

NTNU – Trondheim
Norwegian University of
Science and Technology

NTNU
Norwegian University of Science and Technology

Thesis for the degree of Philosophiae Doctor

Faculty of Engineering Science and Technology
Department of Engineering Design and Materials

ISBN 978-82-471-3654-6 (printed ver.)
ISBN 978-82-471-3655-3 (electronic ver.)
ISSN 1503-8181

Doctoral theses at NTNU, 2012:180

Printed by NTNU-trykk

Acknowledgements

This work was carried out at the Department of Biology at the Norwegian University of Science and Technology (NTNU), in collaboration with SINTEF Fisheries and Aquaculture and SINTEF Materials and Chemistry. Financial support was provided through the Framework 6 EU project CO_2ReMoVe. The work was initially supervised by Professor Karl Erik Zachariassen, who sadly passed away in 2009. Dr Erlend Kristiansen was also my supervisor through my Master's Degree and followed me until 2009. Left helpless and stranded my present supervisor Dr Sindre Pedersen came to my rescue. He has offered great support with his calm, patient and constructive approach to any problems that have appeared. Professor Bjørn Munro Jenssen also acted as my supervisor during the last two years. He reassured me that good things would come out of this work, and made an important contribution to the writing process during the final month. I owe them all a great thank you.

I would like to thank my co-authors Dr Trond Røvik Størseth and Dr Bjørn Henrik Hansen for their contributions to the papers and for their contagious enthusiasm regarding our work.

My colleagues and friends at SeaLab deserve a thank you for making it a great place to work. I would especially like to thank Dag Altin and Anders Olsen for all the great discussions about future plans, their help with all the unforeseen and odd little problems that show up when working with seawater in the lab, and because they are both so important and invaluable for the work that is carried out at SeaLab. Dr Maria Bergvik has been my office buddy, my great friend, and has even helped me collect most of the animals used in this work. You are awesome!

I am looking forward to spending more time with my family and friends now that the PhD regime has ended and I go back to being a normal….scientist again. Thank you for all your support and firm belief that I would be able to carry this through.

My dearest Håvard has now suffered for many years with his absent-minded and busy girlfriend. Thank you for your patience, your love, and the three course dinners served at any hour of the day. I love you.

Karen Marie Hammer
Trondheim, June 2012

Contents

Summary 1

Papers included in the thesis 3

1. Introduction 5

1.1 Carbon dioxide (CO_2) in seawater 5

1.1.1 Ocean acidification 6

1.1.2 Carbon capture and storage 7

1.1.3 Environmental hypercapnia 7

1.2. Acid-base homeostasis 8

1.3 Acid-base regulation 9

1.3.1 Acid-base regulation in bivalves 10

1.3.2 Extracellular acid-base regulation in teleosts and decapod crustaceans 11

1.3.3 Intracellular acid-base regulation 12

1.4 Physiological effects of elevated pCO_2 13

1.5 Deep-sea animals 13

Aim of study 15

2. Materials and methods 17

2.1 Choice of model species 17

2.2 Ethical considerations 17

2.3 Exposure system 19

2.3.1 Exposure conditions 19

2.3.2 Seawater carbonate chemistry 20

2.4 Analytical variables 20

2.4.1 Acid-base parameters 21

2.4.2 Quantitative PCR (qPCR) 21

2.4.3 Enzyme activity 22

2.4.4 ^{1}H-NMR metabolomics 22

3. Summary of papers 23

4. General Discussion 27

4.1 Extracellular acid-base status 27

4.1.1 The deep sea bivalve *A. excavata* 27

4.1.2 Shallow- and deep-living decapods (*C. maenas* and *P. borealis*) 29

4.2 Branchial ion transporting proteins 32

4. 3 Intracellular pH 35
4.4 Metabolic rate 36
4.5 Effects on extra-and intracellular metabolites 37
Conclusions and future perspectives 41
References 43
Paper I-IV

Summary

Global warming is considered to be the most adverse consequence from the increasing anthropogenic emissions of CO_2. However, in the marine environment additional problems related to the elevated levels atmospheric CO_2 may arise; the increased amount of CO_2 absorbed by the oceans may lead to a moderate, but consistent and global reduction in seawater pH due to the acidifying effect of CO_2, a phenomenon referred to as ocean acidification. Another potential problem may occur as a result of sub-seabed storage of anthropogenic CO_2, a disposal alternative introduced by the gas industry to mitigate CO_2 emissions to the atmosphere. Leakage from such storage sites could potentially cause a relatively local, but extreme acidification of the seawater near the leakage site. Both scenarios may create unfavourable conditions for marine organisms, and previous studies have reported that environmental hypercapnia (elevated pCO_2) may affect an array of physiological processes in marine organisms such as acid-base status and metabolic rate. Deep-living animals are considered to be particularly vulnerable to environmental hypercapnia due to their low metabolic rate and poor ability to counteract effects of environmental stressors.

To predict the possible outcome of the two scenarios described above it is important to understand the physiological mechanisms that marine organisms apply to handle the CO_2 stress. During conditions of elevated seawater pCO_2, the charge neutral CO_2 molecules permeate biological membranes and react with water in the body fluids resulting in the net formation of HCO_3^- and H^+. Thus, the primary effect of elevated pCO_2 is induction of body fluid acidosis. Acid-base regulation during acidosis is generally mediated by buffering compounds as well as acid elimination through direct removal of hydrogen ions (H^+) and/or accumulation of buffering bicarbonate ions (HCO_3^-).

In the current thesis the deep-sea bivalve *Acesta excavata*, the green shore crab *Carcinus maenas*, and the deep-water prawn *Pandalus borealis* were exposed to elevated seawater levels of pCO_2. The purpose was to study the responses of the different species to elevated pCO_2 and to compare the capacity of shallow- and deep-living animals to counteract CO_2-induced effects. To meet these objectives changes in acid-base relevant parameters (pH, pCO_2, $[HCO_3^-]$) and metabolic rate was studied in all three species, while gene expression and activity of ion regulating proteins as well as changes in the metabolome were determined in *C. maenas* alone.

Calcifying animals, such as bivalves, have been suggested to utilise HCO_3^- from the calcium carbonate shell to buffer acidosis. However, this buffering strategy may be restricted to closed systems such as during shell closure. Indeed, the findings in the present thesis indicate that shell dissolution does not occur in the deep-living bivalve *A. excavata* in response to CO_2-induced acidosis. Consequently, *A. excavata* does not seem to be able to compensate extra- or intracellular acidosis in response to severe

environmental hypercapnia, and experiences a drop in metabolic rate most likely induced by low body fluid pH. However, this species displays a relatively high non-bicarbonate buffering capacity, and may therefore be able to tolerate more moderate levels of CO_2 exposure than that experienced in the present study.

In decapod crustaceans extracellular pH-regulation occurs in the posterior gills by electroneutral ion exchange between the extracellular fluids and the surrounding seawater. The shore crab *C. maenas* was able to partially compensate extracellular acidosis by accumulating relatively high levels of HCO_3^- in response to elevated pCO_2, and the degree of compensation was dependent on the level of CO_2 exposure. The results from the present thesis suggest that this species can compensate acidosis without substantially increasing the acid-base regulatory capacity of the branchial ion transporting proteins. Surprisingly, the deep-water prawn *Pandalus borealis* exhibited similar abilities as the shore crab to counteract extracellular acidosis induced by elevated pCO_2. This was achieved by increasing the extracellular concentration of HCO_3^- to a similar degree as *C. maenas*. The findings indicate that this species display similar acid-base regulatory capacities as shallow-living decapods, thus nuancing the picture of the compensating capacities of deep-living animals. Acid-base regulation in both decapod species was achieved without affecting the osmolality of the extracellular fluid. This is in contrast to what has been reported for subtidal decapod crabs. The metabolic rate was not significantly affected in any of the two species, possibly due to their ability to maintain extracellular pH close to normal values.

While acid-base regulation in response to CO_2-induced acidosis has received increased scientific attention, only a very few studies have investigated responses of the metabolome to elevated pCO_2. ^{1}H-NMR metabolomics revealed that in the green shore crab CO_2 exposure induces a shift in the metabolic fingerprint in both hemolymph and extracts of gills and leg muscle. The shift is not the result of changes in metabolites involved in energy metabolism, as could be expected. Rather, it is due to a general decrease in the concentration of metabolites, particularly of important osmolytes such as the amino acids proline and glycine. The observed changes were most prominent after prolonged exposure, suggesting an exhaustive response rather than an active, compensatory mechanism. The results indicate that in response to elevated pCO_2 shore crabs experience symptoms resembling those of animals acclimated to condtions of reduced salinity. This may possibly suggests a disturbance of intracellular iso-osmotic regulation.

The present thesis indicates that *A. excavata* would be highly, and possibly permanently affected by severe CO_2 exposure associated with CO_2 leakage, while both the intertidal and deep-living decapods could tolerate relatively prolonged periods of quite severe hypercapnic conditions

Papers included in the thesis

I. **Hammer, K. M.**, Kristiansen, E., Zachariassen, K. E. (2011) Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius 1779) (Bivalvia; Limidae). *Marine Environmental Research* 72 (3): 135-142.

II. **Hammer, K. M.**, Pedersen, S. A., Hansen, B. H. Acid-base regulation and gene responses in the shore crab *C. maenas* during environmental hypercapnia. Submitted manuscript.

III. **Hammer, K. M.**, Pedersen S. A. The deep-water prawn *Pandalus borealis* displays a relatively pH regulatory capacity in response to CO_2-induced acidosis. Manuscript.

IV. **Hammer, K. M.**, Pedersen S. A., Størseth, T. R. Elevated levels of CO_2 changes the metabolic fingerprint in the shore crab *Carcinus maenas*. Preliminary accepted manuscript, *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*

Contributions:

[1]Paper I was initiated by KMH and EK in collaboration with KEZ, experiments were organised by KMH and EK and run by KMH, the analytical work, statistical analysis and writing was performed by KMH with comments from the other authors

[2]Paper II was initiated by KMH, experiments, analytical work, statistical analysis and writing was performed by KMH with comments from the other authors.

[3]Paper III was initiated by KMH in collaboration with SAP, experiments, analytical work, statistical analysis and writing was performed by KMH with comments from SAP.

[4]Paper IV was initiated by KMH in collaboration with TRS, experiments were performed by KMH, analytical work and statistical analysis was performed by KMH and TRS and writing was performed by KMH with comments from the other authors.

1. Introduction

There is a wide scientific consensus that the increase in the atmospheric level of CO_2 observed today is due to human activities, such as burning of fossil fuels, and that this increase is responsible for the observed global warming (Houghton 2005). The oceans are a natural buffer of atmospheric CO_2 and have absorbed approximately one third of the anthropogenic CO_2 emitted since the start of the industrial revolution (Sabine et al. 2004). Consequently, this has resulted in an increased partial pressure of CO_2 (pCO_2) and reduced pH in the sea. To reduce atmospheric emissions disposal alternatives such as carbon capture and storage (CCS) of anthropogenic CO_2 has been initiated. However, as leakage may occur, implementing sub-seabed CO_2 storage may further act to relocate the CO_2 problem from the atmosphere to the oceans.

To understand how elevated levels of CO_2 in the oceans may affect marine organisms and ecosystems it is important to both understand the properties of CO_2 in seawater, and how marine animals respond to these conditions.

1.1 Carbon dioxide (CO_2) in seawater

CO_2 is relatively soluble in seawater due to the formation of dissolved inorganic carbon (DIC) species, and the solubility increases with hydrostatic pressure, but decreases with increased salinity and temperature. When CO_2 reacts with water carbonic acid (H_2CO_3) is formed, followed by an almost immediate dissociation to bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and hydrogen (H^+) ions according to Eq 1:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \underset{}{\overset{K_1}{\rightleftharpoons}} HCO_3^- + H^+ \underset{}{\overset{K_2}{\rightleftharpoons}} CO_3^{2-} + 2H^+ \qquad (1)$$

where K_1 and K_2 are the first and second dissociation constants of H_2CO_3, respectively. Since carbonic acid amounts to less than 0.03 % of the concentration of aqueous CO_2, and the two molecules are chemically inseparable, the sum of the two chemical species is often denoted CO_2.

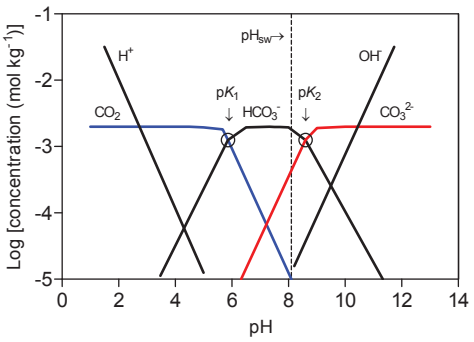

Fig. 1: Distribution of species diagram for the CO_2-HCO_3-CO_3^{2-} system in water. T = 25°C, S = 35. Circles indicate the pK values of carbonic acid at the given conditions, pH_{sw} denotes the typical pH of seawater Blue line indicates CO_2, black curved line indicates HCO_3^- and red line indicates CO_3^{2-}. (Modified from Zeebe and Wolf-Gladrow, 2001).

At surface seawater with a salinity of 35 ‰ and a temperature of 25°C the distribution of the DIC species CO_2, HCO_3^- and CO_3^{2-} is approximately 0.5 %, 86.5 % and 13 %, respectively (Zeebe and Wolf-Gladrow, 2001). As depicted in Fig. 1 bicarbonate is the dominant DIC at typical ocean pH of 8.1, while free CO_2 is only present at minute concentrations.

1.1.1 Ocean acidification

The CO_2-HCO_3^--CO_3^{2-} system, in addition to the total alkalinity, gives seawater a high buffering capacity with respect to pH (Zeebe and Wolf-Gladrow 2001). However, with the increasing atmospheric level more CO_2 is added to the seawater resulting in an increased concentration of H^+ (Fig.2, see 1). Consequently, a cumulative reduction of ocean pH by 0.1 units has been reported to take place during the past 200 years (Haugan and Drange 1996). This reduction in pH is referred to as ocean acidification. Model estimates indicate that assuming a "business-as-usual" scenario emission rate of anthropogenic CO_2 a reduction in mean ocean pH of 0.4 units may be expected within 2100, while a reduction by 0.7 pH units could occur within 2300 (Caldeira and Wickett 2003). Ocean acidification has also resulted in a decreased CO_3^{2-} concentration which is particularly critical for animals having calcium carbonate ($CaCO_3$) shells (Fabry 2008).

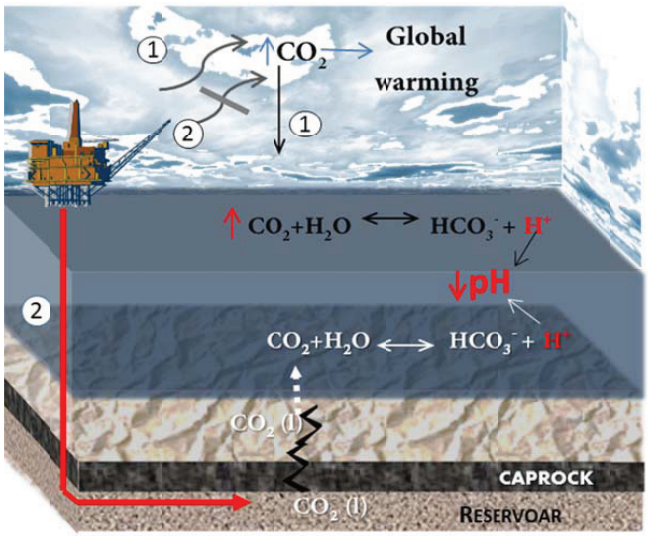

Fig .2. Schematic presentation of 1: Ocean acidification and 2: Carbon capture and storage (CCS) and potential leaks from geological storage of CO_2

1.1.2 Carbon capture and storage

Carbon capture and storage was originally developed by the oil industry to increase oil recovery in the producing wells, but is now a promising alternative to reduce future emissions of CO_2 into the atmosphere (IPCC, 2005). Sub-seabed geological storage involves injecting liquid CO_2 into porous rock formations, such as depleted oil or gas reservoirs, and in deep saline formations (Fig. 2, see 2). A sealed cap rock located over the storage site is required to prevent leakage into the ocean. CO_2 is generally injected 800 meters or more below the seafloor at ocean depths of approximately 300 meters. Here, the ambient temperature and hydrostatic pressure cause the CO_2 to remain in a liquid state (IPCC, 2005). About $1 \cdot 10^6$ tons of CO_2 has been captured and stored annually since the production started on Sleipner Vest in the North Sea in 1996 (Benson et al. 2005), and the geological formations in the North Sea area have a theoretical capacity for storing more than $8 \cdot 10^{11}$ tons of CO_2 (Turley et al. 2004).

Although geological storage of CO_2 is assumed to be relatively safe, leaks could pose a serious threat to the marine environment in the vicinity of a leakage site (Friedmann 2007). The probability of a leak occurring over a period of 1000 years has been estimated to be 34 %, and an average leak is estimated to comprise 0.2 % of the stored CO_2 (Turley et al. 2004). Benson et al. (2005) have suggested that leaks of geologically stored CO_2 may occur (1) through the pore system in caprocks if the capillary entry pressure is exceeded; (2) through openings in the caprock caused by the drilling of wells and (3) due to poorly completed pre-existing wells. Potential leaks from geological storages of CO_2 could affect the local seawater chemistry in a number of ways, the most important being the reduction of pH. In addition, displacement of other gases, such as oxygen, by diffusion into CO_2 bubble clouds could occur. The extent of the effects would depend on the amount of CO_2 released, and the rate, duration, and dispersal of the leak, as well as the reactions of CO_2 with the sediment (Turley et al. 2004).

1.1.3 Environmental hypercapnia

Although the normal ocean pCO_2 level in surface ocean waters is reported to be approximately 400 µatm (Melzner et al. 2009), it may vary considerably between different habitats. Elevated ambient pCO_2, also known as environmental hypercapnia, occurs naturally in many marine habitats For instance, the CO_2 levels near underwater volcanic vents are particularly high (Hall-Spencer et al. 2008; Tunnicliffe et al. 2009), while an elevated CO_2 level can be found in areas where upwelling brings CO_2 rich water from the deeper parts of the oceans to the surface (Feely et al. 2008). Also, in shelf areas, such as in the Western Baltic Sea, stratification followed by seasonal upwelling results in seasonally increased CO_2 levels in surface waters (Thomsen et al. 2010). Hypercapnic conditions may also form in tidal pools when respiration exceeds

photosynthesis in the night time, thus creating a build-up of pCO_2 in the ambient water (Truchot 1986).

1.2. Acid-base homeostasis

The physiological conditions in the body serve to create the optimum environment for biological processes. The maintenance of a more or less constant composition of the body fluids is termed homeostasis and is of vital importance and. Examples of homeostasis include the constancy of body temperature in mammals and birds, as well as the constancy of respiratory gases (O_2 and CO_2), pH, and osmotic pressure (Withers 1992).

Acid-base homeostasis concerns the balance between acids (H^+) and bases (OH^-). Hydrogen ions are formed when acids react with water. The activity of hydrogen ions (a_{H^+}) in a solution is usually expressed as the pH of the solution and, a low pH corresponds to a high $[H^+]$ according to Eq 2

$$pH = -\log a_{H^+} \qquad (2):$$

The pH scale ranges from 1 to 14, and the pH is defined as neutral if the concentration of H^+ equals that which results from the dissociation of water alone. At 25 °C pH 7 corresponds to neutral pH. However, the pH of pure water decreases with increased temperature by -0.017 units per °C, and a similar dependency of temperature is found in blood pH (Withers 1992). In addition to the effect of temperature, acid-base homeostasis in the body fluids may be offset by changes in temperature, high anaerobic activity and the pH of the surrounding medium (Heisler 1984). A condition of reduced body fluid pH is known as acidosis, while alkalosis is a condition of elevated pH.

The small size of the hydrated hydrogen ion (H_3O^+) causes it to bind stronger than larger ions (e.g. Na^+, K^+) to negatively charged molecules. They can be particularly damaging to proteins with even small changes in the concentration dramatically affecting the activity of enzymes. The explanation for this is that association by H^+ leads to an altered charge distribution of the enzyme, which further affects the enzyme-ligand combination rate (Madshus 1988; Somero 1986; Woodbury 1965).

The acids that are of highest physiological importance are carbonic acid and organic acids formed from aerobic and anaerobic metabolism. As described above, the concentration of H_2CO_3 is determined by the $[CO_2]$ and thus the pCO_2. Elevated pCO_2 in the body fluids, results in a condition referred to as respiratory acidosis. Accordingly, metabolic acidosis is caused by a build-up of acidic metabolites (Woodbury 1965).

Some ions affect the acid-base status under all conditions (H^+, OH^- and HCO_3^- ions), while others, such as buffer ions that change their dissociation when transferred between fluids of different pH values, are only acid-base relevant ions under certain conditions (Heisler 1984). The presence of buffers is one of the most important defences against body fluid acidosis. Buffers are compounds that minimize shifts in pH by reacting with exogenous H^+ (Roos and Boron 1981). They are weak acids or bases and include bicarbonate and non-bicarbonate compounds such as phosphates and imidazole groups of histidine residues in proteins (Burton 1978; Castellini and Somero 1981; Eberlee and Storey 1984).

Marine ectothermic animals have a relatively low pCO_2 of 0.3 to 0.6 kilopascals (kPa) compared to 5 to 8 kPa in the blood of terrestrial animals. However, marine organisms are able to maintain a pH close to that of air-breathing animals by having a correspondingly low concentration of HCO_3^-, an important buffer in the body fluids. To maintain a constant pH in the body fluids organisms must be able to eliminate surplus H^+ or OH^- produced in the body or originating from the environment (Heisler 1984; 1986).

1.3 Acid-base regulation

During conditions of environmental hypercapnia the body fluids of aquatic animals equilibrate rapidly with the environment, and the hydration of CO_2 leads to a net formation of HCO_3^- and H^+. To avoid detrimental effects caused by the reactive H^+, the animals must regulate the pH of the extra- and intracellular compartment. When mammals experience metabolic acidosis they respond by increasing their ventilation rate to increase CO_2 removal, thereby reducing the acid load in the body fluids (Fig. 3a). Compensation of respiratory acidosis, on the other hand, involves metabolic reabsorption of bicarbonate in the kidneys (Pitts and Lotspeich 1946). To compensate for the low concentration of O_2 in seawater aquatic ectotherms have relatively high basal ventilation rates. The high ventilation rate cause a moderate pCO_2 difference between the blood and seawater. Increasing the ventilation frequency to increase the elimination of CO_2, thus, offers a poor means of acid-base regulation. Marine ectotherms must therefore apply metabolic compensation to counteract acidosis (Heisler 1984).

The extracellular fluids have relatively low concentrations of pre-existing, buffers. Acidosis must therefore be counteracted by either excretion of H^+ directly or by accumulating buffers, such as HCO_3^- to neutralize H^+. The two means of acid elimination yield the same results with respect to pH compensation and in response to acidosis marine ectotherms are often able to fully or partially compensate extracellular pH by increasing the concentration of bicarbonate ions in their blood (Heisler 1986).

The relationship between pH and the concentrations of dissolved inorganic carbon (CO_2 and HCO_3^-) is given by the Henderson-Hasselbalch equation:

$$pH = pK_1 + \log \frac{[HCO_3^-]}{\alpha CO_2 pCO_2} \qquad (3)$$

where pK_1 is the first apparent pK value of carbonic acid, pCO_2 is the partial pressure and αCO_2 is the solubility of CO_2.

The compensation of extracellular pH during hypercapnic acidosis is best described by a pH-HCO_3^- plot (Davenport diagram) which depicts the simultaneous changes of pH and HCO_3^- with changes in pCO_2 (Fig. 3). If the time-dependent trajectory displays a straight line, where the decrease in pH is followed by a small increase in $[HCO_3^-]$ (solid arrow, bottom), there is no pH-compensation, and the increase in bicarbonate represents that formed from the dissociation of CO_2 (Fig 3b). However, if a decrease in pH is followed by an abrupt increase in HCO_3^-, and the pH increases or remains in a steady-state at a constant level of pCO_2, then the extracellular pH is restored through metabolic compensation (dotted upward arrow).

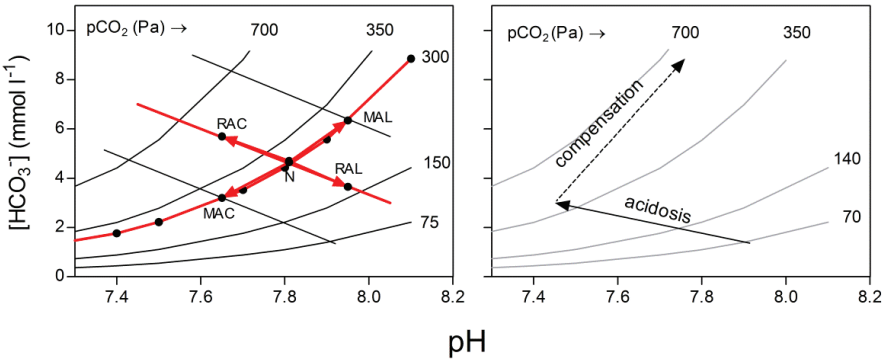

Fig. 3. A Davenport diagram A: Conditions of respiratory acidosis (RAC) and respiratory alkalosis (RAL), and metabolic acidosis (MAC) and metabolic alkalosis (MAL). B: Metabolic compensation through accumulation of HCO_3^- (dotted arrow) during respiratory acidosis. Isoplets indicate pCO_2.

1.3.1 Acid-base regulation in bivalves

Intertidal bivalves frequently experience acidosis during air exposure at low tide (Booth et al. 1984; Jokumsen and Fyhn 1982; Lindinger et al. 1984; Michaelidis et al. 2005; Walsh et al. 1984). Freshwater bivalves are hyper-osmotic to their medium and actively transport ions through epithelial transporters to maintain the extracellular osmolality well above that of the surrounding water. As a result these animals also have the ability to extrude H^+ or accumulate HCO_3^- through Na^+/H^+ and Cl^-/HCO_3^- exchangers, respectively, during acidosis (Byrne and Dietz 1997). In contrast, marine

bivalves are osmoconform, and there exist little evidence for the presence of such exchangers in marine species. Intertidal species often close their shell in response to environmental changes, thereby isolating themselves from the surrounding medium. Studies show that buffering by HCO_3^- originating from $CaCO_3$ of the shell seems to be their primary defence against extracellular acidosis experienced during valve closure episodes (Collip 1921; Crenshaw and Neff 1969; Dugal 1939; Jokumsen and Fyhn 1982; Lindinger et al. 1984). Shell formation and dissolution take place on and from the inner shell surface, respectively, and the process of precipitation and dissolution of $CaCO_3$ occur in the extrapallial fluid, which is in direct contact with the inner shell. During hypercapnic and metabolic acidosis H^+ reacts with $CaCO_3$ in the shell, yielding free calcium and bicarbonate ions (Booth et al. 1984):

$$CO_2 + H_2O + CaCO_3 \rightleftharpoons Ca^{2+} + 2HCO_3^- \quad (4)$$

While HCO_3^- neutralise H^+, the free calcium ions increase the strong ion difference in the hemolymph, thus reducing the acidosis. In addition, increased excretion of ammonia is suggested to increase the elimination of hydrogen ions in *M. edulis* (Booth et al. 1984; Lindinger et al. 1984; Walsh et al. 1984).

1.3.2 Extracellular acid-base regulation in teleosts and decapod crustaceans

The blood of fish and crustaceans contain haemoglobin and hemocyanins, respectively, and the oxygen carrying capacity of both respiratory pigments is sensitive to pH. It is therefore important for these animals to also regulate the extracellular pH in response to acidosis to avoid metabolic depression (Melzner et al. 2009). To counteract acidosis as a result of i. e. strenuous activity, these animals must have well-developed acid-base regulatory machinery. In both marine teleosts and decapod crustaceans the major site for acid-base regulation are the gills. The gills are also the site for gas exchange and osmoregulation. In decapods gas exchange mainly takes place in the anterior gills (gill 1-4), while the posterior gills (gill 6-9) contain mitochondria rich are the major site for both acid-base and osmoregulation (Wheatly and Henry 1992).

Branchial acid-base regulation occurs through electroneutral ion exchange where acid-base relevant ions (mainly H^+ and HCO_3^-) are exchanged for inorganic ions (Na^+, Cl^-) (Cameron 1986; Heisler 1984). This occurs through several ion transporting proteins, which are also important in the maintenance of osmolality in addition to their role in acid-base regulation (Fig 4). It has therefore been predicted that strong iono- and osmoregulating species will be most tolerant to ocean acidification as they are better equipped to counteract acid-base disturbances (Whiteley 2011). The Na^+/H^+ exchanger (NHE) extrudes hydrogen ions in exchange for sodium ions, while the Cl^-/HCO_3^- exchanger (CBE) is involved in the accumulation of bicarbonate ions in response to hypercapnic acidosis. Both proteins are located in the apical membranes of

the gill epithelial cells (Towle and Weihrauch 2001), and are thus in direct contact with the ambient seawater (see Fig. 4).

The energy needed to drive ion regulation derives from the activity of the basolateral enzyme Na^+/K^+-ATPase. This enzyme extrudes three Na^+ out of the cell in exchange for two K^+ per ATP (Skou and Esmann 1992), and is thereby involved in establishing the transmembrane sodium gradient (Ganong 1999). Ion-regulation is thought to be rather costly as the activity of Na^+/K^+-ATPase has been found to constitute up to 40 % of total energy expenditure (Leong and Manahan 1997). Another enzyme that may play a role in ion regulation is the vesicular-type (V-Type) H^+-ATPase. This enzyme is important for ion regulation in freshwater animals, and has been found to play a large role in ammonia excretion in marine decapods (Towle and Weihrauch 2001; Weihrauch et al. 2004).

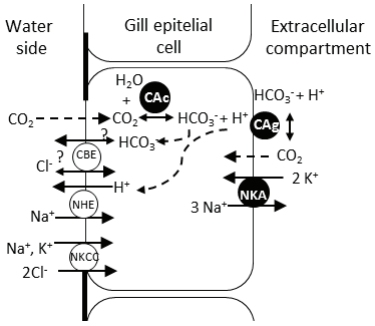

Fig. 4. Ion regulation in the gills of decapod crustaceans modified from Towle et al. (2001).

In mammals the enzyme carbonic anhydrase is found in the red blood cells where it catalyses the reversible dissociation of carbonic acid to HCO_3^- and H^+ to facilitate CO_2 removal from the tissues and transport. In decapod crustaceans two isoforms, membrane-associated (CA_g) and cytoplasmic carbonic anhydrase (CA_c) are found in high concentrations in the gills. While CA_g converts bicarbonate and hydrogen ions from the extracellular fluids to CO_2 and facilitate CO_2 excretion, CA_c catalyses the dissociation of CO_2 to bicarbonate ions which can further be used as counter ions for absorption of Cl^- from the surrounding medium. Both enzymes are thus believed to play important roles in both osmoregulation and acid-base regulation in fish and decapods (Burnett et al. 1981; Gilmour and Perry 2009; Henry and Cameron 1983).

1.3.3 Intracellular acid-base regulation

Since the majority of metabolic processes occur inside the cells the maintenance of intracellular acid-base homeostasis is considered to be of even higher importance than maintaining a constant extracellular pH (Madshus 1988). Animals employ two ways of compensating intracellular acidosis. The first line of defence is the intracellular non-

bicarbonate buffers (phosphates and imidazole groups of histidine residues) which bind and neutralize some of the surplus H^+. (Castellini and Somero 1981). Non-bicarbonate buffering occurs rapidly and only masks H^+ to reduce pH changes. To restore original pH the H^+ must be eliminated and this is achieved through ion transport over the cell membrane. This involves extrusion of H^+ and accumulation of HCO_3 into and from the extracellular compartment (or organelles), respectively through electroneutral ion exchange, mediated by the activity of Na^+/K^+ ATPase (Roos and Boron 1981; Thomas 1977).

1.4 Physiological effects of elevated pCO_2

Environmental hypercapnia is reported to induce a whole array of physiological effects, such as disturbance of acid-base balance and calcification, reductions in growth rates, reproduction and energy turnover, dissolution of shells and exoskeletons in calcifying animals, metabolic depression, narcosis and ,if persistent, death in marine invertebrates (Gutowska et al. 2010a; Gutowska et al. 2010b; Lindinger et al. 1984; Michaelidis et al. 2005; Miles et al. 2007; Pörtner et al. 2004; Pörtner et al. 1998; Reipschlager and Pörtner 1996; Ries et al. 2009; Spicer et al. 2007; Wickins 1984; Wood et al. 2008). The tolerance to CO_2 varies between species and also depends on the life-stage of the animal (Kikkawa et al. 2004; Kurihara 2008), and mortality increases with increased levels of CO_2 and time of exposure (Langenbuch and Pörtner 2004; Spicer et al. 2007). Studies have shown that CO_2 can be more toxic to aquatic animals than strong acids yielding the same pH, most likely owing to the high permeability of CO_2 and H_2CO_3 compared to the charged hydrogen ions (Jacobs 1920; Kikkawa et al. 2004). Thus, the physiological effects of elevated pCO_2 cannot be studied by simply lowering the pH using strong acids.

1.5 Deep-sea animals

The deep sea is usually defined as beginning at the shelf break which coincides with the transition from shallow-water fauna of the shelf to the deep-sea fauna and is characterized as a physically stable environment (Thistle 2003). Deep-sea animals are believed to be more sensitive to increased ocean levels of CO_2 than shallow-living animals and pelagic animals with high metabolic rates and capacities for high-burst swimming/movement. This assumption is based on the observations that deep-living animals often are found to have reduced metabolic rates and intracellular buffering capacities compared to shallow-living and active species (Castellini and Somero 1981; Seibel et al. 1997; Seibel and Walsh 2003). A poorer ability of deep-sea animals to regulate pH during hypercapnic conditions compared to in shallow-living has been confirmed for decapod crabs (Pane and Barry 2007). However, some deep-sea animals inhabit areas with naturally high CO_2 levels and low pH. The vent mussel

Bathymodiolus brevior can be found both at locations having naturally high pCO_2 due to volcanic activity, as well as locations with normal CO_2 levels (Tunnicliffe et al. 2009).

Aim of study

The main objective of this thesis was to increase the general mechanistic understanding of physiological processes involved in response to CO_2–induced acidosis in different species, and to provide more information of how deep-living species, which are believed to be particularly vulnerable to increased seawater pCO_2, are affected and how they handle these conditions.

To meet these aims the following investigations were made:

1. Study the changes in acid-base parameters (pCO_2, pH, and $[HCO_3^-]$) in the deep-sea bivalve *A. excavata* (Paper I) and in shallow- and deep-living decapod crustaceans (Paper II and III, respectively) in response to time-dependent (Paper I-III) and level-dependent (Paper II) exposure to elevated seawater pCO_2.

2. Investigate the effects of elevated seawater pCO_2 on the metabolic rate in shallow- (Paper II) and deep-living invertebrate species (Paper I and III)

4. Investigate CO_2-induced changes in gene expression of ion-regulating proteins in the posterior gills of *C. maenas* (Paper II), as well as changes in the activity of the ion-regulating enzyme Na^+/K^+-ATPase in both *C. maenas* and *P. borealis* (Paper II-III).

5. Study the time- and level-dependent CO_2-induced changes in the composition of extracellular and intracellular metabolites in *C. maenas* using ^{1}H-NMR metabolomics in combination with multivariate analysis (Paper IV).

2. Materials and methods

2.1 Choice of model species

The species chosen for this thesis represent animals adapted to a life in the intertidal zone and species that are found in deeper water. This comparative approach was adopted to compare the responses of the intertidal species to those of deep-living animals.

The deep sea bivalve *Acesta excavata*

The giant file shell *A. excavata* is a large, deep-living bivalve which has been found at ocean depths down to 3200 meters, but is most commonly found at depths between 200 to 800 meters (Freiwald et al. 2005; Järnegren and Altin 2006). It is found in association with the cold-water coral *Lophelia pertusa*, but also occurs in areas close to, and inside pockmarks along the Norwegian Coast (Hovland 2005). Rather than to conduct experiments with shallow-living species the results obtained in Paper I were compared to the results from previous studies. Animals were collected in the Trondheimsfjord by use of an ROV (Fig. 5).

The green shore crab *Carcinus maenas*

The shore crab *C. maenas* inhabits the intertidal zone and experiences daily fluctuations in abiotic factors such as temperature, salinity, pO_2 and pCO_2. *C. maenas* is a relatively strong osmoregulating species that can maintain body fluid osmolality well above seawater values under conditions of reduced salinity (Henry et al. 2002). Previous studies have revealed that this species has a high tolerance to hypercapnic conditions (Truchot 1975). *C. maenas* was therefore chosen as a model species for this thesis to reveal the defensive mechanisms of decapod crustaceans to elevated levels of CO_2. Animals were collected at local beaches in the Trondheimsfjord, Norway (Fig 6).

The deep-water prawn *Pandalus borealis*

The deep-water prawn *Pandalus borealis* is found at ocean depths from 50-500 meters, depending on the temperature of the surroundings (Shumway et al. 1985). *P. borealis* is a benthic, stenohaline species, and was chosen for this thesis to study how deep-living decapod species is affected by elevated pCO_2 and their ability to regulate the acid-base status in response to CO_2-induced acidosis. The animals were collected in the Åsenfjord by use of a shrimp trawl equipped with a barrel at the bottom end to minimize damage to the animals (Fig.7).

2.2 Ethical considerations

Decapod crustaceans are included in the Animal Welfare Act, and the experiments regarding *C. maenas* and *P. borealis* were approved by the Norwegian National Animal Research authority.

Fig. 5 Collection of *A. excavata* in the Trondheimsfjord, Norway using the ROV Minerva, and the research vessel MS Gunnerus (Photo: Audun H. M. Hagen). The natural environment of *A. excavata* (Photo: Johanna Järnegren).

Fig. 6. Collection of shore crabs by use of crayfish pods at local beaches in the Trondheimsfjord, Norway, and sampling of hemolymph (Photo Håvard Egge).

Fig. 7. Collection of the deep-water prawn *P. borealis* aboard the shrimp boat Bremsnes. To minimize damage to the animals a barrel was attached in the bottom end of the trawl. (Photo Håvard Egge).

2.3 Exposure system

Figure 8 shows the exposure system used in Papers I, II and IV, while Fig. 1 in Paper III gives a schematic overview of the set up used for *A. excavata*. The exposure system used in all experiments were similar in the way that rather than to equilibrate seawater using premixed CO_2 gas mixtures, CO_2 was added to the system in the form of seawater saturated with 100% CO_2 gas (~pH 4.8). The CO_2-saturated seawater was further mixed with fresh, normocapnic seawater at a set ratio in a CO_2 header tank to yield the pCO_2 chosen for the different studies. The CO_2 water was then pumped to a distribution chamber which supplied the exposure water to the different exposure chambers. All systems were semi flow-through, as the exposure water was recirculated in addition to the continuous addition of fresh seawater.

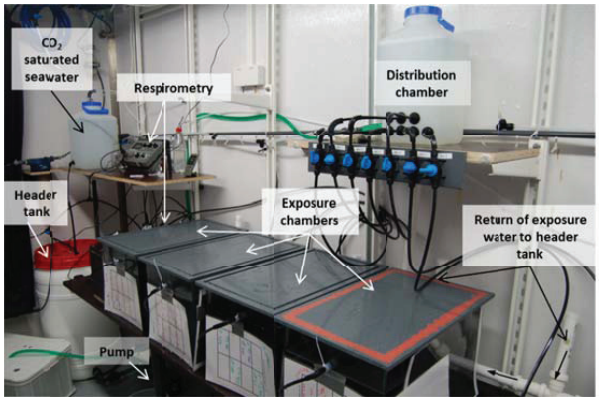

Fig. 8. Exposure set-up used in Paper I, II and IV

2.3.1 Exposure conditions

Table 1. Exposure conditions in the different experiments

	A. excavata Paper I	*C. maenas* Paper II & IV	*P. borealis* Paper III
Temperature Salinity	8ºC 35 ‰	11ºC 35 ‰	7ºC 35 ‰
Control	24 h pH_{NBS} 8.12 pCO_2 = 530 µatm	168 h pH_{NBS} 8.08 pCO_2 = 488 µatm	168 h pH_{NBS} 8.06 pCO_2 = 511 µatm
Time-dependent	0.5-96 h pH_{NBS} 6.33 pCO_2 = 33000 µatm	1-672 h pH_{NBS} 6.94 pCO_2 = 7573 µatm	12-384 h pH_{NBS} 6.85 pCO_2 = 9100 µatm
Level-dependent		336 h pH_{NBS} 7.40, 6.94, 6.62, 6.33 pCO_2 = 2674, 7.573,16020, 30743 µatm = 'pH 7.4,6.9, 6.6 and 6.3 group'	

2.3.2 Seawater carbonate chemistry

To determine the seawater carbonate chemistry it is necessary to measure at least two relevant parameters: In all experiments seawater pH was measured potentiometric using the NBS scale (pH_{NBS}). In addition, pCO_2 was determined using semi-log linear relationship between pH and pCO_2 in Paper I (see below for hemolymph), while total CO_2 (TCO_2) and total alkalinity (TA) were measured Paper II and IV, and TA was measured in Paper III. The measured values were used to calculate pCO_2 using the CO2SYS software (Pierrot et al. 2006).

2.4 Analytical variables

The analytical variables determined for the different species are described below in Table 2.

Table 2. Overview of parameters studied in the different species

Parameter	*A. excavata* Paper I	*C. maenas* Paper II and IV	*P. borealis* Paper III
(pCO_2, pH, $[HCO_3^-]$)$_e$	X	X	X
pH_i	X		X
Non-bicarbonate buffering capacity	X		
Extracellular osmolality		X	X
O_2 consumption	X	X	X
Ammonia excretion	X	X	X
Gene expression		X	
NKA activity		X	X
GDH activity			X
^{1}H-NMR metabolomics		X	

$_e$ = extracellular, $_i$ = intracellular, faded: measured but not included in paper

2.4.1 Acid-base parameters

pH

The acid-base parameters pH, pCO_2 and $[HCO_3^-]$ were determined to investigate how the animals were affected by CO_2-induced acidosis and whether they were able to counteract the acidosis. Extracellular pH was measured directly on hemolymph (Paper I-III), while intracellular pH was measured on extracts of muscle tissue containing metabolic inhibitors according to the method of Pörtner et al. (1990) in Paper I and III.

TCO_2 and pCO_2, $[HCO_3^-]$

In Paper I the pCO_2 in the hemolymph of *A. excavata* was determined based on the semi-log linear relationship between pCO_2 and pH. Samples of hemolymph were equilibrated using two different CO_2 tensions, and the resulting equation was used to calculate pCO_2 in the hemolymph from the original pH (Fig.9).

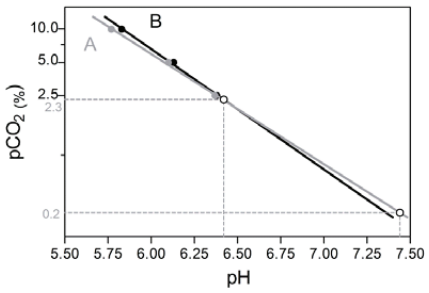

Fig. 9. pH/log pCO_2 line for hemolymph at 8 ºC. A: control (normocapnic) animal and B: hypercapnic animal

In Paper II and III total CO_2 of hemolymph was measured using a commercial analyser (Corning 965). pCO_2 and $[HCO_3^-]$ were calculated by rearranging the Henderson-Hasselbalch equation (Eq 3) and using the pK values and solubility of CO_2 given by Truchot (1976).

2.4.2 Quantitative PCR (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) is an established technique for determining the number of specific mRNA transcripts in biological samples. This allows us to study if the expression of specific genes is affected by endogenous or exogenous changes, and may therefore indicate if there is an increased or decreased need for the protein the gene encodes. The genes studied in the present study are

those encoding known ion transporting proteins found in the epithelial cells of posterior gills in *C. maenas* and that are thought to be involved in acid-base regulation

2.4.3 Enzyme activity

Increased gene expression, or even increased synthesis of an enzyme does not automatically mean that the enzyme activity increases. Many enzymes normally remain in an inactive state, and need to go through modulatory processes, such as phosphorylation, to become active. Thus, the only way to truly determine the activity of an enzyme is to measure it directly. The enzymes investigated in the present study were Total-ATPases and Na^+/K^+-ATPase, the driving force for ion regulation, in gills of *C. maenas* and *P. borealis* (Paper II and III) and glutamate dehydrogenase, a deaminating enzyme, in the abdominal muscle of *P. borealis* (Paper III).

2.4.4 ^{1}H-NMR metabolomics

Metabolomics is defined as the study of the repertoire of small metabolites in biological systems such as cells, tissues and body and plant fluids (Bundy et al. 2009; Viant 2007; Viant et al. 2003). The small metabolites are both endogenous molecules involved in or resulting from primary and intermediary metabolism, and exogenous compounds, such as drugs, other xenobiotics, and their intermediates (Kaddurah-Daouk et al. 2008), and the combination of all the metabolites within a cell is called the metabolome (Bundy et al. 2009; Viant 2007; Viant et al. 2003). Metabolic fingerprinting is a global, high throughput, rapid analysis to provide sample classification. It can be used as a screening tool to discriminate between samples from different biological status or origin, e.g. with respect to case/control or diseased or healthy organisms. Metabolic profiling is the identification and quantification of metabolites (Ellis et al. 2007) By studying the changes in the metabolome in response to a given stressor it is possible to find biomarkers of stress, and to map out the metabolic pathways affected by the stressor. In the present thesis ^{1}H-nuclear magnetic resonance (NMR) spectroscopy in combination with multivariate analysis, and quantification of single metabolites were applied to the water soluble fractions from extracts of hemolymph and tissues of walking leg and posterior gills of *C. maenas*.

3. Summary of papers

Paper I: Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius 1779) (Bivalvia; Limidae)

The aim of Paper I was to investigate how a deep-living bivalve, *A. excavata*, was affected by exposure to conditions that could arise from leakage from subsea storage of anthropogenic CO_2. To study the effects of severe environmental hypercapnia (pH_{NBS} 6.3, pCO_2 = 33000 µatm) acid-base status and metabolic rate of the animals was determined. In addition, extracellular Ca^{2+} concentration, ammonia excretion, and intracellular non-bicarbonate buffering capacity was determined to identify possible compensatory mechanisms used in response to CO_2-induced acidosis. The result was a significant drop in extracellular pH to a value just above the pH of the ambient seawater. The concentration of neither bicarbonate nor calcium ions increased, suggesting that dissolution of the $CaCO_3$ shell to release buffering HCO_3^- did not take place. Intracellular pH dropped significantly to 0.3 pH units below control values, but was still kept well above the pH of the ambient extracellular fluid. This may be explained by the intracellular non-bicarbonate buffering capacity which was comparable, and even higher than what has been previously found in shallow-living bivalves. While the metabolic rate of *A. excavata* plummeted in response to hypercapnic exposure it increased again over time to values not significantly different from controls towards the end of the experiment. Animals displayed a small increase in ammonia excretion, and the combined results for oxygen consumption and ammonia excretion reflects a decreased O:N ratio, and may suggest an increased role of nitrogen metabolism in hypercapnic animals. Failure to regain extracellular pH after recovery suggests a high degree of impairment from exposure to elevated levels of CO_2. However, the study revealed that although *A. excavata* was heavily affected by severe environmental hypercapnia, the increase in metabolic rate towards the end of exposure may indicate that it has higher tolerance to sever environmental hypercapnia than expected for a deep-sea species.

Paper II: Physiological responses of the shore crab *Carcinus maenas* in response to elevated levels of pCO_2

The aim of Paper II was to investigate the compensating responses of the shore crab *Carcinus maenas* to elevated levels of pCO_2 by studying the changes in acid-base status as well as gene expression of the branchial ion regulating machinery. To meet these objectives animals were subjected to time-dependent (1-672 hours, pH_{NBS} = 6.94, pCO_2 = 7573 µatm) and level-dependent (pH_{NBS} 7.40, 6.94, 6.62 and 6.33, pCO_2 = 2673, 7573, 16020 and 30743 µatm, for 2 weeks) exposure to environmental hypercapnia.

The study revealed that when challenged by elevated pCO_2 *C. maenas* was able to partially compensate an initial drop in extracellular pH by increasing the concentration of buffering HCO_3^-. The degree of extracellular acidosis was dependent on the level of exposure, but only animals exposed to the highest level of CO_2 displayed significantly reduced extracellular pH compared to control animals. The metabolic rate of animals was only to some extent affected by time of exposure, but was generally reduced with level of exposure. *C. maenas* displayed a small and transient decrease in extracellular osmolality, but neither time of CO_2 exposure nor level of pCO_2 induced significant changes in osmolality. Both gene expression of ion-regulating proteins, and Na^+/K^+-ATPase (NKA) activity was expected to increase to increase the acid-base regulatory capacity of the gills in response to elevated pCO_2. The gene expression of some proteins relevant for ion regulation, such as membrane-associated (CA_g) and cytoplasmic (CA_c) carbonic anhydrase and the $Na^+/K^+/2Cl^-$ cotransporter was significantly increased with time of exposure to pH_{NBS} 6.94. The gene expression of NKA and the Na^+/H^+ exchanger was only slightly increased with both time and level of exposure, and the activity of NKA was unchanged, and even reduced compared to control animals with both time of exposure and level of pCO_2. Level-dependent exposure only resulted in a significantly reduced gene expression of CA_g in the pH 7.4 group, while the gene expression of the remaining proteins was generally, but not significantly increased with level of exposure. The findings of this paper indicate that *C. maenas* does not need to substantially increase the acid-base regulatory capacity of the gills to achieve partial compensation of extracellular acidosis during prolonged exposure to elevated pCO_2. A partial rather than full compensation of extracellular pH may reduce the energetic costs associated with ion regulation, but may also serve to reduce the influence of acid-base regulation on extracellular osmolality, as suggested in previous studies.

Paper III: The deep-water prawn *Pandalus borealis* displays a relatively high pH-regulatory capacity in response to CO_2-induced acidosis

Deep-living animals are believed to have lower tolerance to, and poorer defence mechanisms against the effects of elevated pCO_2 compared to shallow-living species. The aim of Paper III was therefore to gain a better understanding of the compensatory capacity of deep-water species by studying how *P. borealis*, a deep-living (50-500 m) and stenohaline species respond to environmental hypercapnia (pH_{NBS} 6.86, pCO_2 = 9,000 µatm). Time-dependent CO_2 exposure (0.5-16 days) did not result in a large initial drop in extracellular pH. Rather, *P. borealis* settled at a new steady pH approximately 0.15 units below control pH. Partial compensation of acidosis, comparable to that found for shallow-living species, was achieved by increasing the concentration of HCO_3^- by up to three times the concentration of control animals. Intracellular pH was maintained at control values, and even increased to values significantly higher than control pH after eight days before returning towards control

values at the end of exposure. Extracellular osmolality and oxygen consumption was not significantly affected by the CO_2 exposure. Ammonia excretion rate increased with time in the initial phase of CO_2 exposure and was significantly higher than controls in the group exposed for one day before returning towards control rates again towards the end of the exposure period. The activity of the deaminating enzyme glutamate dehydrogenase (GDH, E.C. 1.4.1.3) in the abdominal muscle of *P. borealis* was generally, but not significantly higher in exposed animals, and did not correlate with ammonia excretion. Accordingly, GDH does not seem to play an important role in acid-base regulation in this species. The activity of both Na^+/K^+-ATPase and total ATPases was generally higher in exposed animals but not significantly increased compared to controls. This study suggests that during prolonged exposure to elevated levels of CO_2 *P. borealis* is able to establish a new steady-state extracellular pH in a similar manner as observed for shallow-living species. The compensation is achieved through a substantial accumulation of bicarbonate ions and suggests that *P. borealis* has a higher acid-base regulatory capacity than what is expected for a deep-living species.

Paper IV: Elevated levels of CO_2 changes the metabolic fingerprint in the shore crab *Carcinus maenas*

The aim of Paper IV was to gain a better understanding of the defence mechanisms applied against CO_2-induced acidosis by investigating the effects of elevated pCO_2 on the metabolome of a marine invertebrate that frequently experiences environmental hypercapnia in its natural habitat. This was done by running ^{1}H-NMR spectroscopy on water soluble extracts of hemolymph and tissues of leg muscle and gills from *C. maenas* exposed to time-dependent (1-672 hours, pH_{NBS} = 6.93, pCO_2 = 7,600 µatm) and level-dependent exposure (pH_{NBS} 7.4, 6.9, 6.6 and 6.3, pCO_2 = 2,500, 7,600, 16,500 and 30,000 µatm, for 2 weeks) followed by partial least squares regression analysis of data. Time-dependent exposure revealed that in gills and hemolymph a significant change in the metabolic fingerprint could only be found after prolonged exposure (4 weeks), while for muscle tissue changes were only apparent after short-term exposure (48 hours). Level-dependent exposure revealed that the metabolic fingerprint of animals subjected to elevated levels of ambient pCO_2 could be discriminated from controls, and the rank order of separation was in accordance with the exposure level in all sample types investigated. Separation of the groups was caused by a general decrease in the level of metabolites, particularly among important osmolytes in exposed animals. More specifically this meant a decreased level of amino acids such as glycine and proline, while the effects on quaternary ammonium compounds varied somewhat between the different sample types. The results suggested that the observed changes reflected an exhaustive effect rather than active defence mechanisms. The changes also indicated that exposure to elevated pCO_2 disturb intracellular osmoregulation, causing symptoms similar to that observed in response to exposure to

seawater of reduced salinity. The explanation for this may be that acid-base regulation and intracellular iso-osmotic osmoregulation occur, at least to some part, through similar ion transporting proteins and disturbance of acid-base homeostasis may thus affect the osmotic status of the cells.

4. General Discussion

The main objective of the present thesis was to investigate how shallow- and deep-living invertebrates are affected by elevated seawater pCO_2, and how they may counteract CO_2-induced stress. To study compensating mechanisms a strategy often adopted in physiology is to induce a strain on the system by inflicting a high degree of exposure. The chosen exposure levels have previously been reported to induce physiological responses in marine teleosts and invertebrates. The lowest exposure level applied in the present thesis has been predicted to occur by year 2300, assuming a 'business-as-usual' CO_2 emissions scenario (Caldeira and Wickett 2003) The most severe exposure levels applied (Paper I and II) are similar to what have been reported to occur in association with natural CO_2 seeps (Vetter and Smith 2005).

4.1 Extracellular acid-base status

4.1.1 The deep sea bivalve *A. excavata*

Bivalves often isolate themselves from the surroundings by closing the shell in response to fluctuations in environmental conditions (e.g. changes in salinity, air exposure, contaminants). Shell closure may lead to both hypercapnic and metabolic acidosis due to reduced gas exchange with the surroundings, and increased anaerobic metabolism, respectively (Booth et al. 1984). In response, shallow-living bivalves can mobilise bicarbonate from the inner part of the $CaCO_3$ shell to counteract the acidosis (Burnett 1988; Crenshaw and Neff 1969; Jokumsen and Fyhn 1982). However, during conditions of elevated seawater pCO_2 bivalves do not necessarily remain closed for prolonged periods of time, and the HCO_3^- formed from shell dissolution may consequently be lost and become unavailable for the animals.

The relatively small changes observed in extracellular Ca^{2+} concentration indicated that severe hypercapnic exposure (pH 6.35, pCO_2~33000 µatm) did not induce pronounced shell dissolution in the deep-living bivalve *A. excavata* (Paper I). Accordingly, no associated increase in extracellular HCO_3^- concentration was found, and the extracellular pH dropped to a level just above the pH of the ambient seawater (closed circles, Fig 10a). Partial compensation of extracellular acidosis during hypercapnic exposure has previously been demonstrated in intertidal bivalves of the genus *Mytilus* in experiments using closed setups (Lindinger et al. 1984; Michaelidis et al. 2005). The blue mussel *M. edulis* is able to partially compensate extracellular acidosis even when subjected to pCO_2 levels of 17000 and 34000 µatm (closed triangles, Fig10a) (Lindinger et al. 1984). However, in closed exposure systems any HCO_3^- produced from the dissolution of the $CaCO_3$ shell is retained in the exposure water and can thus be reabsorbed by the animals and used to buffer surplus H^+.

Thomsen et al. (2010) found that when using a flow-through system, with no recirculation, *M. edulis* were not able to increase the extracellular HCO_3^- concentration and could consequently not compensate extracellular acidosis at relatively moderate pCO_2 levels (1400 and 4000 µatm, open squares Fig 10a) as animals.

The results of Paper I thus point in two directions: Either *A. excavata* has a lower capacity for acid-base regulation compared to intertidal bivalves, or *A. excavata* could not utilize the HCO_3^- dissolved from the shell because it was lost to the seawater due to water exchange.

It should however be noted that in a another study using a flow-through system the sea urchin *Psammechinus miliaris* achieved a small, but transient compensation of the extracellular pH when exposed to elevated levels of CO_2. In that particular study, shell dissolution was found to be higher during moderate than during severe hypercapnia (Miles et al. 2007), suggesting that shell dissolution in calcifying animals is dependent on the pH of the water, with dissolution being weaker during more severe hypercapnia. It is thus possible that shell dissolution may occur in *A. excavata* during more moderate hypercapnic conditions.

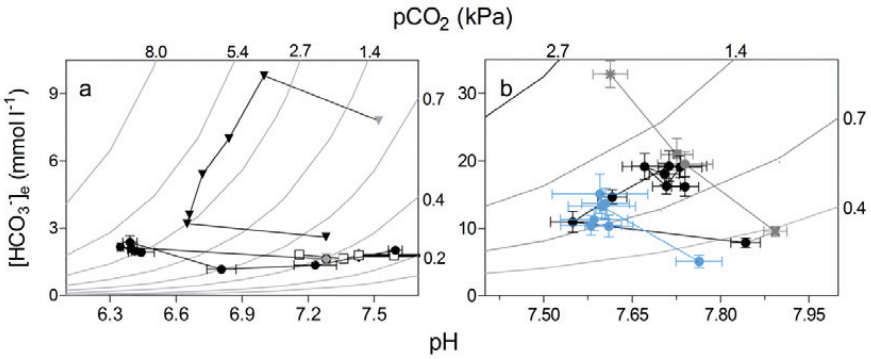

Fig 10. Davenport diagram showing changes in extracellular pH, pCO_2 and bicarbonate in (a) *Acesta excavata* (closed circles, Paper I) and *Mytilus edulis* adopted from Lindinger et al. (1984) (closed triangles, pCO_2=34,000 µatm) and Thomsen et al. (2010) (open squares, 1400 pCO_2=400 -1400 µatm) to elevated pCO_2 and 24 h recovery (grey symbols) , and (b) changes in. *Carcinus maenas* subjected to time- (closed circles) and level-dependent (grey) exposure (Paper II), and *Pandalus borealis* (blue) in response to time-dependent hypercapnic exposure (Paper III). The start points of the trajectories represent controls.

The relatively low concentration of hemolymph Ca^{2+} observed in control *A. excavata* (Fig 2d, Paper I) compared to that previously reported for shallow-living bivalve species (Lindinger et al. 1984; Michaelidis et al. 2005) could indicate that the composition of the shells in deep-sea bivalves differ from the shells of intertidal bivalves, which experience daily fluctuations in body fluid pH. However, Tunnicliffe et

al. (2009) showed that populations of the deep-sea mussel *Bathymodiolus brevior* are able to survive and produce $CaCO_3$ shells at locations with naturally high levels of CO_2 (pH 5.4–7.3). The shells were, however, substantially thinner and growth was slower in these animals compared to what was observed in a population living in normocapnic habitats (pH>7.8). Post-deposition dissolution in *B. brevior* occurred at the inner parts of the shell, suggesting that similar to shallow living species, deep-sea bivalves may use bicarbonate originating from the shell as a buffer during acidosis.

Although no animals died during the experiment *A. excavata* was not able to return extracellular pH back to normal values after 96 hours of recovery in normocapnic conditions (Fig 2b, Paper I), thus indicating that animals may not be able to regain normal physiological health status following a potential leakage from CO_2 reservoirs

4.1.2 Shallow- and deep-living decapods (*C. maenas* and *P. borealis*)

Decapod crustaceans distinguish themselves from most invertebrates by often displaying partial or complete compensation of respiratory acidosis (Cameron 1978; Pane and Barry 2007; Spicer et al. 2007). Paper II showed that in response to environmental hypercapnia (pH_{NBS} 6.94, pCO_2=7573 µatm) *C. maenas* experienced an initial drop in extracellular pH, approximately 0.3 pH units below that of control animals (Fig 10b, black closed circles). Although the pH rapidly increased again it was not fully compensated and remained approximately 0.15 pH units below control levels throughout the rest of the exposure period (4 weeks). In contrast to the results for *C. maenas*, no large initial drop in extracellular pH was observed in CO_2 exposed *P. borealis* (pH_{NBS} 6.86 and pCO_2 = 9,000 µatm Paper III). However, these differing results were most likely due to the different timing of the first sampling in the two studies. In Paper II the first sample was collected after one hour, whereas in Paper III the first sampling was conducted after 12 hours of exposure. Previous studies have shown that 12 hours is sufficient time for decapod crustaceans to reach a new steady-state pH in response to hypercapnic exposure (Spicer et al. 2007; Truchot 1975). It is therefore likely that at the time of the first sampling the extracellular pH of *P. borealis* had already reached the new steady-state pH level which was approximately 0.15 pH units below control values (Fig. 10b, blue circles). Thus, this suggests that the deep-water prawn displayed a similar degree of compensation as the intertidal *C. maenas*

The compensatory increase in extracellular pH observed in both *C. maenas* and *P. borealis* was achieved through an increase in the extracellular bicarbonate concentration. Both species more than doubled the concentration of extracellular bicarbonate in the initial phase of exposure. *P. borealis* even displayed bicarbonate concentrations three times higher than controls after one day of exposure. The impressive increase in extracellular bicarbonate suggests that the deep-water prawn *P. borealis* exhibits an acid-base compensatory capacity that is similar to the intertidal

shore crab (Paper II and III). This observation is somewhat surprising as deep-living animals have generally been considered to have reduced capacities to regulate body fluid pH in response to stress (Seibel and Walsh 2001). In fact, no significant increase in extracellular bicarbonate concentration, and thus no compensation of extracellular pH was found in the deep-sea crab *Chionecetes tanneri* when exposed to similar conditions as those experienced by *P. borealis* in Paper III (Pane and Barry 2007). The different responses in the two species may be due to the fact that *C. tanneri* is found at much greater depths than *P. borealis* (1000 m versus 50-500 m, respectively) where the environment is even more stable.

Although *C. maenas* and *P. borealis* displayed similar acid-base regulatory capacities this does not necessarily mean that the two species achieve this in exactly the same manner. *C. maenas* is a hyperregulating species with high iono regulatory capacity and is therefore expected to have high abilities to counteract changes in blood pH. *P. borealis* is considered to be a stenohaline species (Shumway et al. 1985) and it could therefore be expected that this species lacks the well-developed ion-regulatory mechanisms necessary for high level accumulation of bicarbonate ions. The shore crab is also regularly subjected to hypercapnic exposure during low tide, while the environment of *P. borealis* is generally more stable. However, one explanation for the high acid-base regulatory capacity may be related to its swimming activities. *P. borealis* displays a high speed flight response which may necessitate a high capacity for anaerobic work. *P. borealis* may thus need a well-developed acid-base regulatory machinery to handle the acidosis experienced in association with the flight responses on a regular basis. Thus, *P. borealis* may be pre-adapted to severe environmental hypercapnia. It is, however, also possible that the partial compensation displayed by *P. borealis* was a result of the high level of exposure applied in Paper III, and that complete compensation may achieved in this species during a lower level of exposure. The partial rather than full pH compensation observed in the shore crab may however reflect an energy conserving strategy.

Level-dependent CO_2 exposure (pH_{NBS} 7.40 – 6.33, pCO_2~2673 – 30743 µatm) of *C. maenas* revealed that compensation of acidosis is dependent on the level of exposure (Paper II), and only the group exposed to the highest exposure level displayed significantly reduced extracellular pH (Fig 10b, grey symbols). Both pCO_2 and bicarbonate concentrations increased exponentially with level of exposure, while the pH decreased in a corresponding manner. The concentrations of bicarbonate ions corresponded to both level of exposure and hemolymph pCO_2, and thus, offer a good explanation for the degree of acid-base regulation observed in the different groups.

Ion- and osmoregulating crabs such as *C. maenas* are generally thought to display higher abilities to counteract acid-base disturbances than osmoconforming species, due to their well-developed ion exchange mechanisms (Whiteley 2011). However, Spicer et

al. (2007) found that, contrary to *C. maenas*, the osmoconforming velvet swimming crab *Necora puber* could achieve full compensation of extracellular pH at hypercapnic conditions down to a seawater pH of 6.7. *N. puber* increased its hemolymph bicarbonate concentration by four times the concentration of control animals, which was much higher than that observed for *C. maenas* according to the CO_2 exposure level, and suggests that *N. puber* has a higher ability for bicarbonate accumulation. The different responses may be related to the different lifestyles of the two decapods. Active species, such as *N. puber*, need high oxygen-carrying capacities and often experience metabolic acidosis. They therefore have high extra- and intracellular levels of non-bicarbonate buffers, giving them a greater capacity for passive compensation of acidosis than slow-moving species, such as the green shore crab (Whiteley 2011). On the other hand, *C. maenas* often hide under rocks during low tide with reduced water available for gas exchange, and thus also often experience hypercapnic and metabolic acidosis. Since *N. puber* cannot take up bicarbonate from the surroundings , it is possible that the it originates from the catabolism of carboxylic amino acids as suggested by Langenbuch and Pörtner (2002).

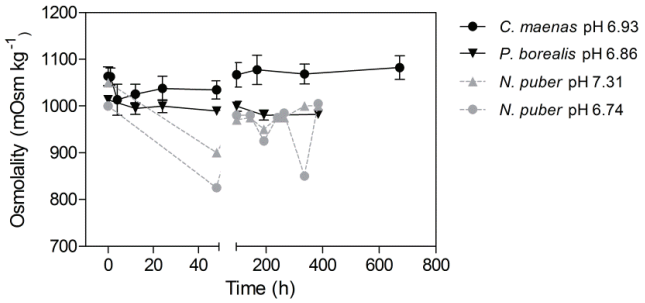

Fig. 11. Development in extracellular osmolality over time in *Carcinus maenas* (black circles), *Pandalus borealis* (black triangles) and *Necora puber* (grey symbols, adopted from Spicer et al. (2007)) in response to elevated pCO_2.

Previous studies have shown that changes in salinity can affect acid-base status (Truchot 1981; Whiteley et al. 2001) and that CO_2 stress may affect the osmolality of the body fluids (Spicer et al. 2007). This indicates that there is a close connection between acid-base regulation and osmoregulation. To follow up these observations the osmolality of both *C. maenas* and *P. borealis* was determined in Paper II and III, respectively (Fig 11). While *C. maenas* experienced a small, transient, decrease in extracellular osmolality, *P. borealis* displayed a small but consistent reduction of osmolality with time of exposure. However, the reductions in osmolality observed in both species were not significant, and small compared to the transient drops displayed

by the subtidal crab *N. puber* in response to elevated pCO_2 levels (Spicer et al. 2007) (see Fig 11.). The authors suggested that part of the reduction in osmolality observed in *N. puber* could be explained by the exit of chloride used as counter ions for bicarbonate accumulation, but that loss of Cl^- was not sufficient to explain the entire decrease in osmolality.

A possible explanation for the different development in osmolality observed for *C. maenas* and *N. puber* (Fig 11) may be due to the different osmoregulatory strategies used by the two species. While *N. puber* is an, *C. maenas* regulates the osmolality of its body fluids by actively absorbing ions during exposure to low salinities (Henry et al. 2002; Siebers et al. 1972). Whereas, *N. puber* was able to fully re-establish extracellular pH back to control values, the extracellular pH of *C. maenas* was only partially compensated. This may indicate that for osmoregulating decapods, such as *C. maenas*, there exists a trade-off between osmoregulation and acid-base regulation. A trade-off between extracellular acid-base regulation and osmoregulation for decapod crabs has previously been suggested by Cameron and Iwama (1987). The results in Paper III indicate that *P. borealis* has adopted a similar strategy as *C. maenas*. Rather than allowing a large drop, *P. borealis* regulates the osmolality of the extracellular environment in response to hypercapnic exposure. The small decrease observed may be due to the severity of the exposure.

4.2 Branchial ion transporting proteins

When hyperregulating decapod crabs are subjected to conditions of reduced salinity, they often respond by immediately increasing the activity of Na^+/K^+-ATPase (Castilho et al. 2001; Holliday 1985; Neufeld et al. 1980) and carbonic anhydrase (Henry et al. 2002; Serrano and Henry 2008) in the posterior gills to increase the absorption of Na^+ and Cl^- from the environment (Burnett 1985). In addition, the gene expression of electroneutral ion transporters has been found to increase in response to hypo-osmotic conditions (Jayasundara et al. 2007; Luquet et al. 2005; Towle and Weihrauch 2001). Since decapod crustaceans are thought to use similar ion-regulating proteins for osmoregulation and acid-base regulation it was expected that both gene expression of ion regulating proteins and the activity of NKA would increase in response to hypercapnic exposure.

Figure 12 depicts changes in the gene expression of ion-regulating proteins in the gills of *C. maenas* in response to time-and level-dependent exposure to elevated pCO_2 (Paper II). A significantly up-regulated gene expression was found for some of the proteins (CA and NKCC) in the time-dependent exposure. However, exposure to different levels of pCO_2 did not result in significantly altered expression of the genes investigated, with the exception of the significantly down-regulated mRNA levels of

membrane associated carbonic anhydrase (CA_g) in the group exposed to the lowest level of pCO_2 (pH 7.4 group).

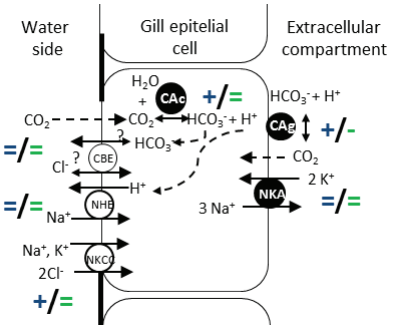

Fig. 12. Changes in gene expression and enzyme activities of ion regulating proteins in posterior gills of *Carcinus maenas* in response to time-dependent (blue) and level-dependent (green) exposure to elevated pCO_2. Significant increase and decrease are denoted by + and -, respectively. Non-significant changes are denoted =. Question marks indicate possible inward and, outward flux of HCO_3^- and Cl^-, respectively.

Gene expression of the Cl^-/HCO_3^- exchanger (CBE) fluctuated around control levels with time of exposure. Exposure to pH 7.4 resulted in a small, but not significant down-regulation of CBE, while the mRNA levels of the remaining groups were similar to controls. The results were in accordance, with those of Fehsenfeld et al (2011), who found a significant down-regulation of CBE expression in *C. maenas* at pH 7.2 and pCO_2 3000 µatm. This suggests that there is not an increased need for this ion exchanger in response to hypercapnic conditions.

Although level-dependent CO_2 exposure only elicited a general, but non-significant increase in mRNA levels of the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), gene expression increased consistently and significantly with time of exposure. The NKCC of *C. maenas* is located in the apical membrane of the gill epithelial cells (Towle and Weihrauch 2001) and is important for the uptake of Na^+ and Cl^- from the surrounding seawater (Luquet et al. 2005; Riestenpatt et al. 1996). One possible explanation for the induction of increased NKCC gene expression in *C. maenas* may be an increased demand for Cl^- absorption to replenish any loss of Cl^- caused by electroneutral uptake of HCO_3^-. Although increased gene expression has been reported in rats experiencing metabolic acidosis (Ikebe et al. 2001) NKCC has not been previously proposed as potentially important for acid-base regulation in crabs. The results from the present study indicate that NKCC may be involved in decapod acid-base regulation.

The mRNA levels of both cytoplasmic and membrane-associated carbonic anhydrase (CA_c and CA_g, respectively) were significantly increased compared to control levels with time of exposure. CA is known to play a crucial role in systemic acid–base regulation in fish by providing acid–base equivalents for exchange with the

environment (Georgalis et al. 2006; Gilmour and Perry 2009). In rainbow trout exposed to elevated pCO_2 inhibition of CA has been reported to impair acid excretion (Georgalis et al. 2006). Perry et al. (2010) found that both CA activity and protein concentration was significantly increased in the marine teleost plainfin midshipman (*Porichthys notatus*) when exposed to environmental hypercapnia. The role of CA in decapods is thought to be similar to that found in teleosts. For instance, when the red rock crab *Cancer productus* were injected with the CA inhibitor acetazolamide acidosis was intensified during air exposure due to an impairment of CO_2 excretion (McMahon et al. 1984).

Previous studies on marine decapods have suggested that under normal salinity conditions the concentration and activity of CA_c is low, but that exposure to conditions of reduced salinity induces in an increased synthesis and activity of the enzyme to increase the uptake of Na^+ and Cl^- (Henry et al. 2002; Serrano and Henry 2008). The results from Paper II may, thus, suggest that environmental hypercapnia induces a similar response (i.e. increased CA activity) and that increased synthesis of both CAc and CA_g is important to maintain pH at a tolerable level during hypercapnic acidosis.

Na^+/K^+-ATPase (NKA) is considered to be the driving force for branchial ion-regulation in decapod crustaceans (Melzner et al. 2009; Whiteley 2011). It was therefore expected that both gene expression and the activity of this enzyme would increase to enhance the acid-base regulatory capacity in response to environmental hypercapnia, as previously observed in fish (Deigweiher et al. 2008). However, in *C. maenas* the NKA mRNA levels did not vary significantly from controls. Furthermore, the observed changes in NKA mRNA levels were characterised by large variations within both the controls and the exposure groups. In accordance with the results from gene expression analysis, the activity of NKA was not significantly affected in hypercapnic *C. maenas* (Paper II). In comparison, a small, but non-significant increase in NKA activity was found in gills of the deep-water prawn *P. borealis* in response to hypercapnic exposure (Paper III). Thus, both species managed to partially compensate their extracellular pH without substantially increasing the activity of NKA. Pane et al (2008) observed that NKA activity in the gills of the crab *Cancer magister* was significantly decreased in response to short-term hypercapnic exposure. This may suggest that decapod crabs have naturally high NKA activities and do not need to increase NKA activity in response to CO_2-induced acidosis. This suggestion is supported by the findings of Henry et al. (2002) who reported that the NKA activity of *C. maenas* was high during control conditions, and that reduced salinity did not induce an increase in activity.

The relatively low gene expression of ion regulating proteins found in Paper II are in accordance with the results of Fehsenfeld et al. (2011). They did not report of large changes in the gene expression of branchial ion transporters in *C. maenas* in response

moderate hypercapnic exposure. In that particular study, however, microarray analysis revealed that the gene expression of other proteins involved in transmembrane transport was significantly up-regulated. The authors reported that the gene expression responses displayed by *C. maenas* were similar to that elicited by hypo-osmotic acclimation (Towle et al. 2011), confirming the close relationship between acid-base regulation and osmoregulation.

Overall this suggests that *C. maenas* have sufficient levels of branchial ion-regulating proteins and NKA activity during normal conditions, and therefore does not need to increase the ion-regulatory capacity of the gills to maintain a tolerable acid-base status during environmental hypercapnia. The findings do, however, suggest an increased need for the enzyme CA during hypercapnic exposure, and that NKCC may be involved in acid-base regulation in decapod crabs.

4. 3 Intracellular pH

While the homeostasis of extracellular pH is important to maintain the integrity of respiratory pigments, the maintenance of intracellular pH is considered to be of even higher importance due to the pH-sensitivity of metabolic processes (Somero 1986; Woodbury 1965). It is therefore more common to find a full compensation of intracellular pH than of extracellular pH in marine invertebrates (Michaelidis et al. 2005; Pörtner et al. 1998).

Although the intracellular pH of *A. excavata* decreased by 0.3 pH units during CO_2 exposure, it still remained at a level well above extracellular and seawater pH (Fig 3, Paper I). This could be explained by the relatively high intracellular non-bicarbonate buffering capacity observed in the adductor muscle of *A. excavata*, which was similar to what has been previously reported for intertidal bivalves (Eberlee and Storey 1984; Morris and Baldwin 1984). The intracellular non-bicarbonate buffering capacity represents buffers such as phosphates and imidazole groups of histidine residues in proteins which bind and neutralize some of the surplus H^+ (Castellini and Somero 1981; Eberlee and Storey 1984). However, non-bicarbonate buffering only masks protons during acidosis, and thus reduce pH changes compared to a non-buffered system. To restore pH during hypercapnic condtions animals must eliminate the protons by ion exchange. This involves extrusion of H^+ into the extracellular compartment (or organelles) and/or accumulation of HCO_3^- from the extracellular compartment. This occurs through transmembrane ion transporters similar to those found in the gills of crustaceans and fish (Claiborne et al. 2002; Zange et al. 1990).

The control animals of *P. borealis* (Fig 1d, Paper III) displayed a relatively low intracellular pH in the abdominal muscle compared to what has been reported in a previous study (Sartoris and Pörtner 1997). To confirm these results the intracellular

pH of a number of control animals were measured after the end of the experiment, resulting in the same mean pH value of 7.05. The intracellular pH fluctuated somewhat during exposure and was generally equal to, and even higher than the intracellular pH of control animals. This indicated that *P. borealis* is able to maintain, and even increase, intracellular pH during hypercapnic exposure. This suggests that *P. borealis* also displays a well-developed ion exchange mechanisms for intracellular pH regulation.

4.4 Metabolic rate

Environmental hypercapnia has been shown to affect metabolic rate in marine invertebrates, but the reported responses vary among different species (Gutowska et al. 2008; Michaelidis et al. 2005; Pörtner et al. 1998; Reipschlager and Pörtner 1996; Thomsen and Melzner 2010; Wood et al. 2008). Reduction in metabolic rate is associated with energy conservation, and a decrease in intracellular pH is one of the five general characteristics found to induce metabolic depression (Guppy and Withers 1999). A decrease in oxygen consumption in response to hypercapnic conditions has been found in several invertebrate species (Michaelidis et al. 2005; Pörtner et al. 1998; Reipschlager and Pörtner 1996).

The metabolic rate of *A. excavata* dropped significantly in animals exposed to severe hypercapnic conditions (Fig. 5, Paper I). This was in line with the large pH drop observed in this species, which most likely resulted in a reduced activity of metabolic enzymes and, thus, a reduced metabolic rate. As the hypercapnic conditions experienced by this deep-living species were very severe, the increased oxygen consumption observed during prolonged exposure may indicate a delayed response in which compensatory processes are activated.

As found in teleost fish and cephalopods, decapods have respiratory pigments to obtain a high oxygen carrying capacity of the blood in order to support a high metabolic activity. Because of the pH-sensitive of these respiratory pigments, it is important for these animals to regulate extracellular pH in response to metabolic acidosis, i.e. during strenuous activity (Melzner et al. 2009). The metabolic activity is upheld and these animals usually do not experience metabolic depression during hypercapnic conditions, possibly due to their ability to regulate extracellular pH (Deigweiher et al. 2008; Gutowska et al. 2008). Accordingly, time-dependent exposure of *C. maenas* to hypercapnic seawater with pH_{NBS} 6.94 did not cause any significant changes in the metabolic rate (Fig. 1d, Paper II).

Likewise, no significant changes in metabolic rate were found in the time-dependent exposure of *P. borealis* to pH_{NBS} 6.85 (Fig. 3a, Paper III). However, it should be noted

that in this particular study, the metabolic rates of exposed animals were generally lower than for control animals. The lowest metabolic rate for *P. borealis* was found in the later parts of exposure when the mean rate was only half of control rates. This may reflect an exhaustive effect from the exposure.

The metabolic rate of *C. maenas* did however decrease according to the level of exposure from pH_{NBS} 7.40 – 6.62, with metabolic rates being significantly reduced in the pH 6.6 group (Paper II). The metabolic rate of the pH 6.3 group on the other hand was increased compared to the other exposure groups. The increase observed in the pH 6.3 group may reflect an increased need for activation of energy demanding ion pumps at this severe exposure level, to assist in a compensatory increase in bicarbonate accumulation. The pH 6.3 group was indeed found to have the highest NKA activity of all of the exposure groups, at levels similar to controls. Also, the gene expression of several of the investigated ion transporting proteins was highest in this group. The decreased metabolic rates in the remaining exposure groups may have reflected the slightly reduced extracellular pH, or may be an indication of energy conservation during unfavourable conditions.

Ammonia excretion increased only moderately in *A. excavata* (Fig. 5b, Paper I) and *C. maenas* (not shown) but significantly in *P. borealis* in response to hypercapnic exposure, indicating increased protein metabolism and decreased O:N ratio (Fig. 3b, Paper III). Ammonia (NH_3) is a weak base and can neutralize excess protons by forming NH_4^+ at low pH (Roos and Boron 1981). In decapods, ammonium is believed to be excreted through the NHE (in place of H^+) and possibly by the involvement of V-type H^+-ATPase (Mangum et al. 1976; Weihrauch et al. 2004) Lindinger et al. (1984) suggested that ammonia excreted as NH_4^+ could serve as an important mechanism of acid excretion in hypercapnic *M. edulis*. It is, thus, possible that ammonia excretion also plays a role in acid-base regulation in *P. borealis*.

4.5 Effects on extra-and intracellular metabolites

To gain a more in depth knowledge on extra- and intracellular responses ^{1}H-NMR metabolomics was applied to study how the metabolite composition in hemolymph and tissues of *C. maenas* is affected by elevated pCO_2 (Paper IV). As mentioned above, environmental hypercapnia has been found to have varying effects on the metabolic rate in different species. It was therefore hypothesised that this would be reflected by changes in the composition of metabolites relevant for energy metabolism (i.e. glucose, succinate, α-ketoglutarate, etc.). It was also hypothesised that the metabolic response of the different tissues could reveal mechanisms relevant to cope with CO_2-induced stress.

By applying partial least squares regression analysis on the data obtained from ^{1}H-NMR spectroscopy, it was revealed that the metabolic fingerprint of hypercapnic animals could be separated from that of control animals for all three sample types investigated (hemolymph, muscle and gill tissues). Furthermore, the rank order of separation was more or less in accordance with the level of CO_2 exposure. That is, the metabolic fingerprint of the group exposed to the highest level of CO_2 was more different from that of control animals than the fingerprint of the second highest exposure level, etc. Further, results from the time-dependent exposure revealed that changes were manifested after prolonged exposure (2-4 weeks) in hemolymph and gills, while for muscle tissue the observed changes occurred in the short term phase of the exposure (48 h).

Only a few metabolites relevant for energy metabolism could be successfully identified, and these metabolites did not display significant changes in concentration with time or level of exposure to elevated CO_2. However, a significant decrease in the concentration of lactate was found in both hemolymph and gills of animals subjected to level-dependent exposure, possibly reflecting a decreased anaerobic metabolism in exposed animals. The observed changes in metabolic fingerprint were mainly due to altered levels of important osmolytes, such as the amino acids glycine and proline, and quaternary ammonium compounds such as glycine-betaine, homarine, dimethylamine and trigonelline. With a few exceptions the general trend was a decrease in the concentration of intracellular osmolytes in response to CO_2 exposure.

The majority of studies on intracellular metabolites in marine invertebrates have been in relation to changing salinities. Marine invertebrates are generally iso-osmotic, or slightly hyper-osmotic, to the ambient seawater. While the osmolality of the extracellular fluids is mostly made up of inorganic ions (i.e. Na^{+}, Cl^{-}, etc.), amino acids and quaternary ammonium compounds are additionally important in maintaining the osmotic pressure in the cells. Osmoregulating species, such as *C. maenas*, maintain the osmolality of the extracellular fluids at values substantially above that of the ambient water under hypo-osmotic conditions. Under these conditions the cells rapidly decrease the concentrations of intracellular osmolytes (amino acids, sugars, etch) to re-establish iso-osmotic conditions with the extracellular fluids, and thereby minimize shrinkage (Henry et al. 2002; Siebers et al. 1972).

The results from Paper IV may indicate that exposure to hypercapnic conditions may compromise the intracellular osmoregulation in *C. maenas*, resulting in symptoms resembling those observed in animals exposed to conditions of reduced salinity (Siebers et al. 1972). Long-term intracellular acid-base regulation occurs through ion transporting proteins similar to those found in the gills of crabs (Claiborne et al. 2002; Zange et al. 1990) Although extracellular osmolality of hypercapnic *C. maenas* was maintained possibly at the expense of a decreased pH (Paper II), it is possible that the

maintenance of intracellular pH surpasses the need to maintain intracellular osmolality at normal levels due to the detrimental effect of low pH on enzymes (Somero 1986), thus resulting in symptoms similar to hypo-osmotic stress.

The fact that the changes in the metabolic profile were manifested after prolonged exposure suggests that the changes may have been due to an exhaustive effect of exposure rather than induction of intracellular defence mechanisms. This indicates that prolonged hypercapnic exposure may disturb intracellular iso-osmotic regulation in *C. maenas*. Although a similar study was not performed on the hemolymph and tissues of *P. borealis* a similar effect could be expected for this species, as it displayed the same general pattern in extracellular acid-base regulation as *C. maenas*.

The results of the present thesis thus indicate that short-term exposure to severe hypercapnic conditions that may arise during a potential leakage from sub-seabed CO2 reservoirs elicits detrimental effects, and possibly irreparable damage to the deep-sea bivalve *A. excavata*. The shallow- and deep-living decapods *C. maenas* and *P. borealis*, however, seem to have relatively high tolerance to, and are to a large extent able to counteract acidosis associated with high seawater pCO_2. This is seemingly achieved without increasing the ion-regulatory capacity in *C. maenas*. However, the results may suggest that this species experiences disturbance of intracellular iso-osmotic regulation in response to prolonged hypercapnic exposure.

Conclusions and future perspectives

Conclusion 1. The lack of significant increase in extracellular Ca^{2+} concentrations indicated that no shell dissolution had taken place in *A. excavata* in response to severe environmental hypercapnia. Accordingly, this species was not able to accumulate bicarbonate to compensate extra- and intracellular acidosis.

Conclusion 2. Elevated pCO_2 initially induced metabolic depression in *A. excavata* but the metabolic rate returned towards normal values at the end of exposure. This may suggest an initiation of compensating mechanisms to counteract the effects of acidosis.

Future perspectives. The exposure level applied in Paper I was very high, and may possibly have masked any compensatory mechanisms employed by this species. It would therefore be interesting to also study the responses of *A. excavata* at more moderate levels of CO_2. The hydrostatic pressure at a hypothetical CO_2 leakage site could affect the seawater chemistry but may also affect the physiology of animals living there. It would therefore be interesting to mimic the *in situ* conditions to reveal the combined effects elevated pCO_2 and high pressure.

Conclusion 3. The combined results of Paper II and III revealed that the deep-water prawn display a similar ability to counteract extracellular acidosis as the shore crab in response to relatively high levels of CO_2. In both species this was accomplished by substantially increasing the concentration of the buffer HCO_3^-. Although *P. borealis* is not a typical deep sea species the findings of the present thesis challenge the general comprehension that deep-living animals have lower acid-base regulatory capacities compared to shallow-living species.

Conclusion 4. Both *C. maenas* and *P. borealis* displayed partial rather than full compensation of extracellular acidosis, and Paper II revealed that for *C. maenas* the degree of acidosis depends on the level of pCO_2. Acid-base regulation and osmoregulation are assumed to occur through similar ion transporting proteins and may therefore interfere with each other; however, the extracellular osmolality was not significantly affected by $CO_{2\,e}$ exposure in neither of the two species. The finding in Paper II and III may indicate that there exists a trade-off between pH- and osmoregulation, as has been suggested in previous studies.

Future perspectives. It would be interesting to conduct further studies on the acid-base regulatory capacity of other deep-living species, by exposing the animals to different levels of CO_2, ranging from those predicted for ocean acidification scenarios to those reported from natural CO_2 seeps to get a broader perspective on the CO_2 tolerance and acid-base regulatory abilities of deep-living species.

Conclusion 5. Decapod crustaceans often increase the ion-regulatory capacity of gill epithelial cells in response to changes in salinity, and it was therefore hypothesised that a similar response would be induced by environmental hypercapnia. Paper II suggested that *C. maenas* seemingly does not increase the acid-base regulatory capacity

substantially, with the exception of increased gene expression the $Na^+/K^+/2Cl^-$ cotransporter and carbonic anhydrase. The latter may reflect increased synthesis and activity of the enzyme. This suggest that *C maenas* has sufficient capacity in the ion transporting protein suite to maintain acid-base homeostasis at a steady-state level.

Conclusion 6. In accordance with the previous results for teleost fish and cephalopods, environmental hypercapnia did not induce significant changes in the oxygen consumption rate of neither *C. maenas* nor *P. borealis* at pH 6.9. However, metabolic rate was reduced according to level of exposure in *C. maenas*, possibly due to reduced extracellular pH.

Future perspectives. It would be interesting to conduct a study similar to Paper II where the response of the branchial ion transporting protein suite of both deep-living and subtidal, osmoconforming decapods is investigated.

Conclusion 6 Environmental hypercapnia was found to induce changes in the metabolic fingerprint of shore crabs. Prolonged exposure to several levels of CO_2 gave a decrease in important intracellular osmolytes, such as the amino acids proline and glycine, quaternary ammonium compounds, in addition to a decrease in the level of lactate. This indicates that elevated levels of CO_2 induces a disturbance of intracellular iso-osmotic regulation, an effect similar to what has been observed in animals subjected to hypo-osmotic conditions. We hypothesize that this may be due to a trade-off between osmoregulation and acid-base regulation, and that unlike the findings for the extracellular fluids intracellular pH is maintained at the expense of intracellular osmolality.

Future perspectives. It was hypothesised that exposure to environmental hypercapnia would elicit changes in metabolites involved in energy metabolism. It be interesting to run a similar study on extracts of the hepatopancreas, a tissue with high metabolic activity. It would also be of interesting to study CO_2-induced responses in the metabolome of other, less tolerant species.

References

Benson S, Cook P, Anderson J, Bachu S, Nimir HB, Basu B, Bradshaw J, Deguchi G, Gale J, von Goerne G, al. e (2005) Chapter 5: Underground geological storage, Executive summary IPCC Special report on Carbon dioxide Capture and Storage. Prepared by Working group III of the Intergovernmental Panel on Climate Change. New York

Booth CE, McDonald DG, Walsh PJ (1984) Acid-base balance in the sea mussel *Mytius edulis*. I. Effects of hypoxia on air-exposure on hemolymph acid-base status. Marine Biology Letter 5:347-358

Bundy J, Davey M, Viant M (2009) Environmental metabolomics: a critical review and future perspectives. Metabolomics 5 (1):3-21. doi:10.1007/s11306-008-0152-0

Burnett LE (1985) The function of carbonic anhydrase in crustacean gills. In: Gilles R, Gilles-Baillien M (eds) Transport processes, iono- and osmoregulation. Springer-Verlag, Berlin, pp 159-168

Burnett LE (1988) Physiological responses to air exposure: Acid-base balance and the role of branchial water stores. American Zoologist 28 (1):125-135. doi:10.1093/icb/28.1.125

Burnett LE, Woodson PBJ, Rietow MG, Vilicich VC (1981) Crab gill intra-epithelial carbonic anhydrase plays a major role in haemolymph CO_2 and chloride ion regulation. Journal of Experimental Biology 92 (1):243-254

Burton RF (1978) Intracellular buffering. Respiration Physiology 33 (1):51-58. doi:10.1016/0034-5687(78)90083-x

Byrne R, Dietz T (1997) Ion transport and acid-base balance in freshwater bivalves. Journal of Experimental Biology 200 (3):457-465

Caldeira K, Wickett ME (2003) Oceanography: Anthropogenic carbon and ocean pH. Nature 425 (6956):365-365

Cameron JN (1978) Effects of hypercapnia on blood acid-base status, NaCl fluxes, and trans-gill potential in freshwater blue *Callinectes sapidus*. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 123 (2):137-141. doi:10.1007/bf00687841

Cameron JN (1986) Acid-base equilibria in invertebrates. In: Heisler N (ed) Acid-base regulation in animals. Elsevier Science Publisher BV, Amsterdam, pp 357-389

Cameron JN, Iwama GK (1987) Compensation of progressive hypercapnia in channel catfish and blue crabs. Journal of Experimental Biology 133 (1):183-197

Castellini MA, Somero GN (1981) Buffering capacity of vertebrate muscle: Correlations with potentials for anaerobic function. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 143 (2):191-198. doi:10.1007/bf00797698

Castilho PC, Martins IA, Bianchini A (2001) Gill Na+,K+-ATPase and osmoregulation in the estuarine crab, *Chasmagnathus granulata* Dana, 1851 (Decapoda, Grapsidae). Journal of Experimental Marine Biology and Ecology 256 (2):215-227. doi:10.1016/s0022-0981(00)00315-4

Claiborne JB, Edwards SL, Morrison-Shetlar AI (2002) Acid–base regulation in fishes: cellular and molecular mechanisms. Journal of Experimental Zoology 293 (3):302-319. doi:10.1002/jez.10125

Collip JB (1921) A further study of the respiratory processes in *Mya arenaria* and other marine Mollusca. Journal of Biological Chemistry 49 (2):297-310

Crenshaw MA, Neff JM (1969) Decalcification at themantle-shell interface in molluscs. American Zoologist 9 (3):881-885. doi:10.1093/icb/9.3.881

Deigweiher K, Koschnick N, Pörtner HO, Lucassen M (2008) Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am J Physiol-Regul Integr Comp Physiol 295 (5):R1660-R1670. doi:10.1152/ajpregu.90403.2008

Dugal L-P (1939) The use of calcareous shell to buffer the product of anaerobic glycolysis in *Venus mercenaria*. Journal of Cellular and Comparative Physiology 13 (2):235-251. doi:10.1002/jcp.1030130210

Eberlee JC, Storey KB (1984) Buffering capacities of the tissues of marine molluscs. Physiological Zoology 57 (5):567-572

Ellis DI, Dunn WB, Griffin JL, Allwood JW, Goodacre R (2007) Metabolic fingerprinting as a diagnostic tool. Pharmacogenomics 8 (9):1243-1266. doi:10.2217/14622416.8.9.1243

Fabry VJ (2008) Marine Calcifiers in a High-CO_2 Ocean. Science 320 (5879):1020-1022. doi:10.1126/science.1157130

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B (2008) Evidence for upwelling of corrosive "acidified" water onto the continental shelf. Science 320 (5882):1490-1492. doi:10.1126/science.1155676

Fehsenfeld S, Kiko R, Appelhans Y, Towle DW, Zimmer M, Melzner F (2011) Effects of elevated seawater pCO_2 on gene expression patterns in the gills of the green crab, *Carcinus maenas*. Bmc Genomics 12. doi:48810.1186/1471-2164-12-488

Freiwald A, Roberts JM, Correa ML, Hall-Spencer J, Taviani M (2005) Distribution and habitats of *Acesta excavata* (Bivalvia: Limidae) with new data on its shell ultrastructure. In: Freiwald A (ed) Cold-Water Corals and Ecosystems. Erlangen Earth Conference Series. Springer Berlin Heidelberg, pp 173-205. doi:10.1007/3-540-27673-4_9

Friedmann SJ (2007) Geological Carbon Dioxide Sequestration. Elements 3 (3):179-184. doi:10.2113/gselements.3.3.179

Ganong WF (1999) Review of medical physiology. Appleton & Lange, Stamford, Conn.

Georgalis T, Perry SF, Gilmour KM (2006) The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology 209 (3):518-530. doi:10.1242/jeb.02018

Gilmour KM, Perry SF (2009) Carbonic anhydrase and acid-base regulation in fish. Journal of Experimental Biology 212 (11):1647-1661. doi:10.1242/jeb.029181

Guppy M, Withers P (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews 74 (01):1-40. doi:doi:null

Gutowska M, Melzner F, Langenbuch M, Bock C, Claireaux G, Pörtner H (2010a) Acid–base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 180 (3):323-335. doi:10.1007/s00360-009-0412-y

Gutowska M, Melzner F, Pörtner H, Meier S (2010b) Cuttlebone calcification increases during exposure to elevated seawater CO_2 in the cephalopod *Sepia officinalis*. Marine Biology 157 (7):1653-1663. doi:10.1007/s00227-010-1438-0

Gutowska M, Pörtner H, Melzner F (2008) Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO2. Marine Ecology Progress Series 373:303-309. doi:10.3354/meps07782

Hall-Spencer JM, Rodolfo-Metalpa R, Martin S, Ransome E, Fine M, Turner SM, Rowley SJ, Tedesco D, Buia M-C (2008) Volcanic carbon dioxide vents show ecosystem effects of ocean acidification. Nature 454 (7200):96-99. doi:http://www.nature.com/nature/journal/v454/n7200/suppinfo/nature07051_S1.html

Haugan PM, Drange H (1996) Effects of CO_2 on the ocean environment. Energy Conversion and Management 37 (6–8):1019-1022. doi:10.1016/0196-8904(95)00292-8

Heisler N (1984) Acid-Base Regulation in Fishes. In: Hoar WS, Randall DJ (eds) Fish Physiology, vol Volume 10, Part 1. Academic Press, New York, pp 315-401

Heisler N (1986) Buffering and transmembrane ion transfer processes. In: Heisler N (ed) Acid-base regulation in animals. Elsevier Science Publisher BV, Amsterdam, pp 3-47

Henry RP, Cameron JN (1983) The role of carbonic anhydrase in respiration, ion regulation and acid-base balance in the aquatic crab *Callinectes sapidus* and the terrestrial crab *Gecarcinus lateraliss*. Journal of experimental biology 103 (1):205-223

Henry RP, Garrelts EE, McCarty MM, Towle DW (2002) Differential induction of branchial carbonic anhydrase and Na^+/K^+ ATPase activity in the euryhaline crab, *Carcinus maenas*, in response to low salinity exposure. Journal of Experimental Zoology Part A: Comparative Experimental Biology 292 (7):595-603

Holliday CW (1985) Salinity-induced changes in gill Na, K-ATPase activity in the mud fiddler crab, *Uca pugnax*. Journal of Experimental Zoology 233 (2):199-208. doi:10.1002/jez.1402330206

Houghton J (2005) Global warming. Reports on Progress in Physics 68 (6):1343

Hovland M (2005) Pockmark-associated coral reefs at the Kristin field off Mid-Norway In: Freiwald A, Roberts JM (eds) Cold-Water Corals and Ecosystems. Erlangen Earth Conference Series. Springer Berlin Heidelberg, pp 623-632. doi:10.1007/3-540-27673-4_30

Ikebe M, Nonoguchi H, Nakayama Y, Tashima Y, Tomita K (2001) Upregulation of the Secretory-Type Na+/K+/2Cl--Cotransporter in the Kidney by Metabolic Acidosis and Dehydration in Rats. Journal of the American Society of Nephrology 12 (3):423-430

Jacobs MH (1920) To what extent are the physiological effects of carbon dioxide due to hydrogen ions? American Journal of Physiology 51 (2):321-331

Jayasundara N, Towle DW, Weihrauch D, Spanings-Pierrot C (2007) Gill-specific transcriptional regulation of Na+/K+-ATPase α-subunit in the euryhaline shore crab *Pachygrapsus marmoratus*: sequence variants and promoter structure. Journal of Experimental Biology 210 (12):2070-2081. doi:10.1242/jeb.004309

Jokumsen A, Fyhn HJ (1982) The influence of aerial exposure upon respiratory and osmotic properties of haemolymph from two intertidal mussels, *Mytilus edulis* L. and *Modiolus modiolus* L. Journal of Experimental Marine Biology and Ecology 61 (2):189-203

Järnegren J, Altin D (2006) Filtration and respiration of the deep living bivalve *Acesta excavata* (J.C. Fabricius, 1779) (Bivalvia; Limidae). Journal of Experimental Marine Biology and Ecology 334 (1):122-129

Kaddurah-Daouk R, Kristal BS, Weinshilboum RM (2008) Metabolomics: A Global Biochemical Approach to Drug Response and Disease. Annual Review of Pharmacology and Toxicology 48 (1):653-683. doi:doi:10.1146/annurev.pharmtox.48.113006.094715

Kikkawa T, Kita J, Ishimatsu A (2004) Comparison of the lethal effect of CO2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Marine Pollution Bulletin 48 (1-2):108-110

Kurihara H (2008) Effects of CO2-driven ocean acidification on the early developmental stages of invertebrates. Marine Ecology Progress Series 373:275-284. doi:10.3354/meps07802

Langenbuch M, Pörtner HO (2002) Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia:

identifying effective acid-base variables. Journal of experimental biology 205 (8):1153-1160

Langenbuch M, Pörtner HO (2004) High sensitivity to chronically elevated CO2 levels in a eurybathic marine sipunculid. Aquatic Toxicology 70 (1):55-61. doi:10.1016/j.aquatox.2004.07.006

Leong P, Manahan D (1997) Metabolic importance of Na+/K+-ATPase activity during sea urchin development. Journal of Experimental Biology 200 (22):2881-2892

Lindinger MI, Laurén DJ, McDonald G (1984) Acid-base balance in the sea mussel *Mytilus Edulis*. III. Effects of environmental hypercapnia on intra- and extracellular acid-base balance. Marine Biology Letter 5:371-381

Luquet CM, Weihrauch D, Senek M, Towle DW (2005) Induction of branchial ion transporter mRNA expression during acclimation to salinity change in the euryhaline crab *Chasmagnathus granulatus*. Journal of Experimental Biology 208 (19):3627-3636. doi:10.1242/jeb.01820

Madshus IH (1988) Regulation of intracellular pH in eukaryotic cells. Biochemical Journal 250 1-8

Mangum CP, Silverthorn SU, Harris JL, Towle DW, Krall AR (1976) The relationship between blood PH, ammonia excretion and adaptation to low salinity in the blue crab *Callinectes sapidus*. Journal of Experimental Zoology 195 (1):129-136. doi:10.1002/jez.1401950112

Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner HO (2009) Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences 6 (10):2313-2331

Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Marine Ecology Progress Series 293:109-118. doi:10.3354/meps293109

Miles H, Widdicombe S, Spicer JI, Hall-Spencer J (2007) Effects of anthropogenic seawater acidification on acid-base balance in the sea urchin *Psammechinus miliaris*. Marine Pollution Bulletin 54 (1):89-96

Morris GM, Baldwin J (1984) pH buffering capacity of invertebrate muscle: correlations with anaerobic muscle work. Molecular Physiology 5:61-70

Neufeld GJ, Holliday CW, Pritchard JB (1980) Salinity adaption of gill Na, K-ATPase in the blue crab, *Callinectes sapidus*. Journal of Experimental Zoology 211 (2):215-224. doi:10.1002/jez.1402110210

Pane EF, Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Marine Ecology Progress Series 334:1-9. doi:10.3354/meps334001

Pane EF, Grosell M, Barry JB (2008) Comparison of enzyme activities linked to acid-base regulation in a deep-sea and a sublittoral decapod crab species. Aquatic Biology 4 (1):23-32. doi:10.3354/ab00094

Perry S, Braun M, Genz J, Vulesevic B, Taylor J, Grosell M, Gilmour K (2010) Acid–base regulation in the plainfin midshipman (*Porichthys notatus*): an aglomerular marine teleost. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 180 (8):1213-1225. doi:10.1007/s00360-010-0492-8

Pierrot D, Lewis E, Wallace DWR (2006) MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.

Pitts RF, Lotspeich WD (1946) Bicarbonate and the renal regulation of acid-base balance. American Journal of Physiology -- Legacy Content 147 (1):138-154

Pörtner H, Langenbuch M, Reipschläger A (2004) Biological Impact of Elevated Ocean CO_2 Concentrations: Lessons from Animal Physiology and Earth History. Journal of Oceanography 60 (4):705-718. doi:10.1007/s10872-004-5763-0

Pörtner HO, Boutilier RG, Tang Y, Toews DP (1990) Determination of intracellular pH and PCO2 after metabolic inhibiton by fluoride and nitrilotriacetic acid. Respiration Physiology 81 (2):255-273

Pörtner HO, Reipschlager A, Heisler N (1998) Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. Journal of Experimental Biology 201 (1):43-55

Reipschlager A, Pörtner HO (1996) Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. Journal of Experimental Biology 199 (8):1801-1807

Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. Geology 37 (12):1131-1134. doi:10.1130/g30210a.1

Riestenpatt S, Onken H, Siebers D (1996) Active absorption of Na^+ and Cl^- across the gill epithelium of the shore crab *Carcinus maenas*: voltage-clamp and ion-flux studies. Journal of Experimental Biology 199 (7):1545-1554

Roos A, Boron WF (1981) Intracellular pH. Physiol Rev 61 (2):296-434

Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL, Wanninkhof R, Wong CS, Wallace DWR, Tilbrook B, Millero FJ, Peng T-H, Kozyr A, Ono T, Rios AF (2004) The Oceanic Sink for Anthropogenic CO2. Science 305 (5682):367-371. doi:10.1126/science.1097403

Sartoris FJ, Pörtner HO (1997) Temperature dependence of ionic and acid-base regulation in boreal and arctic Crangon crangon and Pandalus borealis. Journal of Experimental Marine Biology and Ecology 211 (1):69-83

Seibel BA, Thuesen EV, Childress JJ, Gorodezky LA (1997) Decline in pelagic cephalopod metabolism with habitat depth reflects differences in locomotory efficiency. Biological Bulletin 192 (2):262-278

Seibel BA, Walsh PJ (2001) Potential impacts of CO2 injection on deep-sea biota. Science 294 (5541):319-320. doi:10.1126/science.1065301

Seibel BA, Walsh PJ (2003) Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. Journal of Experimental Biology 206 (4):641-650. doi:10.1242/jeb.00141

Serrano L, Henry RP (2008) Differential expression and induction of two carbonic anhydrase isoforms in the gills of the euryhaline green crab, *Carcinus maenas*, in response to low salinity. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 3 (2):186-193. doi:10.1016/j.cbd.2008.02.003

Shumway SE, Perkins HC, Schick DF, Stickney AP (1985) Synopsis of biological data on the pink shrimp *Pandalus borealis* Krøyer, 1834. FAO [Food and Agriculture Organization of the United Nations] Fisheries Synopsis 144:1-57

Siebers D, Lucu C, Sperling KR, Eberlein K (1972) Kinetics of osmoregulation in the crab *Carcinus maenas*. Marine Biology 17 (4):291-303. doi:10.1007/bf00366739

Skou JC, Esmann M (1992) The Na,K-ATPase. Journal of Bioenergetics and Biomembranes 24 (3):249-261. doi:10.1007/bf00768846

Somero GN (1986) Protons, osmolytes, and fitness of internal milieu for protein function. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 251 (2):R197-R213

Spicer J, Raffo A, Widdicombe S (2007) Influence of CO_2-related seawater acidification on extracellular acid-base balance in the velvet swimming crab *Necora puber*. Marine Biology 151 (3):1117-1125. doi:10.1007/s00227-006-0551-6

Thistle D (2003) The deep-sea floor: an overview. In: Tyler, P. A. (ed) *Ecosystems of the world: Ecosystems of the deep oceans*. Elsevier. 28: 5-38. Ecosystems of the deep oceans. Elsevier, Amsterdam

Thomas RC (1977) The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. The Journal of Physiology 273 (1):317-338

Thomsen J, Gutowska MA, Saphorster J, Heinemann A, Trubenbach K, Fietzke J, Hiebenthal C, Eisenhauer A, Kortzinger A, Wahl M, Melzner F (2010) Calcifying invertebrates succeed in a naturally CO2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences 7 (11):3879-3891. doi:10.5194/bg-7-3879-2010

Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Marine Biology 157 (12):2667-2676. doi:10.1007/s00227-010-1527-0

Towle DW, Henry RP, Terwilliger NB (2011) Microarray-detected changes in gene expression in gills of green crabs (*Carcinus maenas*) upon dilution of environmental salinity. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 6 (2):115-125. doi:10.1016/j.cbd.2010.11.001

Towle DW, Weihrauch D (2001) Osmoregulation by gills of euryhaline crabs: Molecular analysis of transporters. American Zoologist 41 (4):770-780. doi:10.1668/0003-1569(2001)041[0770:obgoec]2.0.co;2

Truchot J-P (1975) Action de l'hypercapnie sur l'état acide-base du sang chez le crabe *Carninus maenas* (L.) (Crustacé Décapode). Comptes rendus hebdomadaires des séances de l'Académie des sciences Série D: Sciences naturelles 280 (3):311-314

Truchot J-P (1986) Changes in the hemolymph acid-base state of the shore crab ,*Carcinus maenas*, exposed to simulated tidepool condtions. The Biological Bulletin 170 (3):506-518

Truchot JP (1976) Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L): carbon dioxide solubility coefficient and carbonic acid dissociation constants. Journal of Experimental Biology 64 (1):45-57

Truchot JP (1981) The effect of water salinity and acid-base state on the blood acid-base balance in the euryhaline crab, *Carcinus maenas* (L.). Comparative Biochemistry and Physiology Part A: Physiology 68 (4):555-561. doi:10.1016/0300-9629(81)90361-3

Tunnicliffe V, Davies KTA, Butterfield DA, Embley RW, Rose JM, Chadwick Jr WW (2009) Survival of mussels in extremely acidic waters on a submarine volcano. Nature Geosci 2 (5):344-348

Turley C, Nightingale P, Riley N, Widdicombe S, Joint I, Gallienne C, Lowe D, Goldson L, Beaumont N, Mariotte, al. e (2004) Literature Review: Environmental impacts of a gradual or catastrophic release of CO_2 into the marine environment following carbon dioxide capture. The UK Department for Environment, Food and Rural affairs, London

Vetter EW, Smith CR (2005) Insights into the ecological effects of deep ocean CO_2 enrichment: The impacts of natural CO_2 venting at Loihi seamount on deep sea scavengers. Journal of Geophysical Research 110 (C9):C09S13. doi:10.1029/2004jc002617

Viant MR (2007) Metabolomics of aquatic organisms: the new 'omics' on the block. Marine Ecology Progress Series 332:301-306. doi:10.3354/meps332301

Viant MR, Rosenblum ES, Tjeerdema RS (2003) NMR-Based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health. Environmental Science & Technology 37 (21):4982-4989. doi:10.1021/es034281x

Walsh PJ, McDonald DG, Booth CE (1984) Acid-base balance in the sea mussel *Mytilus edulis*. II. Effects of hypoxia and air-exposure on intracellular acid-base status. Marine Biology Letter 5:359-369

Weihrauch D, Morris S, Towle DW (2004) Ammonia excretion in aquatic and terrestrial crabs. Journal of Experimental Biology 207 (26):4491-4504. doi:10.1242/jeb.01308

Wheatly MG, Henry RP (1992) Extracellular and intracellular acid-base regulation in crustaceans. Journal of Experimental Zoology 263 (2):127-142. doi:10.1002/jez.1402630204

Whiteley N (2011) Physiological and ecological responses of crustaceans to ocean acidification. Marine Ecology Progress Series 430:257-271. doi:10.3354/meps09185

Whiteley NM, Scott JL, Breeze SJ, McCann L (2001) Effects of water salinity on acid-base balance in decapod crustaceans. Journal of Experimental Biology 204 (5):1003-1011

Wickins JF (1984) The effect of hypercapnic sea water on growth and mineralization in penaied prawns. Aquaculture 41 (1):37-48

Withers PC (1992) Comparative animal physiology. Comparative animal physiology.

Wood HL, Spicer JI, Widdicombe S (2008) Ocean acidification may increase calcification rates, but at a cost. Proc R Soc B-Biol Sci 275 (1644):1767-1773. doi:10.1098/rspb.2008.0343

Woodbury JW (1965) Regulation of pH. In: Ruch TC, Patton HD (eds) Physiology and biophysics. W. B. Saunders Company, Philadelphia,

Zange J, Grieshaber MK, Jans AW (1990) The regulation of intracellular pH estimated by 31P-NMR spectroscopy in the anterior byssus retractor muscle of *Mytilus edulis* L. Journal of Experimental Biology 150 (1):95-109

Zeebe RE, Wolf-Gladrow D (2001) CO_2 in seawater: equilibrium, kinetics, isotopes. Elsevier, Amsterdam

Paper I

Marine Environmental Research 72 (2011) 135–142

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius, 1779) (Bivalvia; Limidae)

Karen M. Hammer [a,*], Erlend Kristiansen [a,b], Karl Erik Zachariassen [a]

[a] Department of Biology, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
[b] Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark

ARTICLE INFO

Article history:
Received 19 January 2011
Received in revised form
20 May 2011
Accepted 17 July 2011

Keywords:
Acesta excavata
Deep-living bivalve
Hypercapnia
CO_2
pH
Acid–base
Metabolism
Calcium

ABSTRACT

The option of storing CO_2 in subsea rock formations to mitigate future increases in atmospheric CO_2 may induce problems for animals in the deep sea. In the present study the deep-sea bivalve *Acesta excavata* was subjected to environmental hypercapnia (pHSW 6.35, $P_{CO_2} = 33{,}000$ μatm) corresponding to conditions reported from natural CO_2 seeps. Effects on acid–base status and metabolic rate were related to time of exposure and subsequent recovery. During exposure there was an uncompensated drop in both hemolymph and intracellular pH. Intracellular pH returned to control values, while extracellular pH remained significantly lower during recovery. Intracellular non-bicarbonate buffering capacity of the posterior adductor muscle of hypercapnic animals was significantly lower than control values, but this was not the case for the remaining tissues analyzed. Oxygen consumption initially dropped by 60%, but then increased during the final stages of exposure, which may suggest a higher tolerance to hypercapnia than expected for a deep-living species.

1. Introduction

The reported global warming observed in the past century has been attributed to the release of carbon dioxide (CO_2) from the burning of fossil fuels into the atmosphere. As a consequence, governments and the industry are committed to reduce the emissions of this greenhouse gas.

Subsea geological storage of CO_2 is considered a potentially attractive means of reducing anthropogenic emissions and involves injecting liquefied CO_2 into porous rock formations deep below the seafloor for permanent disposal. Cap rocks above the storage sites are expected to prevent the CO_2 from migrating to the above water column (Metz et al., 2005). This has been successfully performed for more than 10 years in the Utsira formation, at the Sleipner oil field in the North Sea. Although this is assumed to be a reasonably safe way of permanent CO_2 disposal there is an estimated 34% chance of a leak occurring from the storage site within 1000 years (Turley et al., 2004).

CO_2 acts as a weak acid when dissolved in water. Measurements from the Pacific Ocean showed that the average water pH in the close vicinity of a natural CO_2 seep was approximately 6.3 (Vetter and Smith, 2005) compared to approximately 8.1 in seawater equilibrated against the atmosphere.

CO_2 is considered to be a greater threat to aquatic animals than strong acids, which dissociate completely and act primarily through their hydrogen ions (Jacobs, 1920; Kikkawa et al., 2004). In contrast to hydrogen ions, the CO_2 (and carbonic acid, H_2CO_3) molecule is charge-neutral, which allows it to freely penetrate biological surfaces (e.g. cell membranes, skin). Consequently, CO_2 equilibrates between the water and the extra- and intracellular compartments of aquatic organisms. Once inside the body a fraction of the CO_2 molecules reacts with the extracellular water and form hydrogen ions, causing a reduction in pH. A lowering of body fluid pH due to elevated levels of CO_2 is referred to as hypercapnic acidosis. Such hypercapnic acidosis is known to induce metabolic depression in invertebrates, perturbations in growth, reproduction, development and energy turnover, dissolution of shells and exoskeletons and reduced calcification in calcifying animals as well as narcosis and death (Lindinger et al., 1984; Wickins, 1984; Wheatly and Henry, 1992; Reipschlager and Pörtner, 1996; Pörtner et al., 1998; Barker and Elderfield, 2002; Hayashi et al., 2004; Michaelidis et al., 2005; Gazeau et al., 2010; Waldbusser et al., 2011).

Aquatic animals are known to counteract acidosis through accumulation of bicarbonate ions, which neutralize excess hydrogen ions, and direct extrusion of hydrogen ions. This is

* Corresponding author. NTNU SeaLab, Brattørkaia 17 C, 7010 Trondheim, Norway. Tel.: +47 73596356; fax: +47 73596311.
E-mail address: karen.hammer@bio.ntnu.no (K.M. Hammer).

0141-1136/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marenvres.2011.07.002

achieved through ion exchange between the intra- and extracellular compartment, and between the extracellular fluids and the environment (Claiborne et al., 2002). The latter processes occurs mainly in the gills of fish and crustaceans (Wheatly and Henry, 1992; Claiborne et al., 2002) while bivalves accumulate bicarbonate ions through dissolution of their calcium carbonate ($CaCO_3^-$) shells (Lindinger et al., 1984). In addition, the presence of intracellular non-bicarbonate buffers (e. g. proteins, phosphates) minimizes changes in cellular pH by reacting with the exogenous hydrogen ions (Roos and Boron, 1981).

Animals living in the intertidal zone are known to be highly tolerant to changes in environmental conditions as they are daily exposed to variations in abiotic factors such as salinity, temperature and oxygen levels. They also frequently experience both metabolic acidosis (accumulation of acidic end products from anaerobic metabolism) and respiratory (hypercapnic) acidosis (retention of CO_2) during air exposure at low tide when gas exchange is limited by the absence of water (Burnett, 1988). In contrast the environmental conditions in the deeper parts of the oceans are believed to be rather stable. Deep-sea animals do not normally encounter large fluctuations in the CO_2 content of the water, as its concentration in the deep sea is solely determined by microbial activity (Heisler, 1986). They are therefore expected to have lower tolerability to large changes in the surrounding medium, and they are reported to have poorer buffering capacities and acid–base regulation mechanisms than shallow-living animals (Castellini and Somero, 1981; Seibel et al., 1997; Seibel and Walsh, 2001; Pane and Barry, 2007).

Acesta excavata is a large, deep-living bivalve inhabiting the North East Atlantic, from the southern parts of Iceland down to Senegal and Morocco. It has been observed at depths ranging from 33 to 3200 m, but is most commonly found at depths between 200 and 800 m. In the Trondheimsfjord this species lives under relatively stable conditions, with temperatures ranging between 6 and 8 °C, and salinity levels of 34–35 S (López Correa et al., 2005; Järnegren and Altin, 2006). *A. excavata* is reported to have the second lowest respiration rate, as well as the second highest clearance rate of all bivalves studied so far. Järnegren and Altin (2006) suggested that this is an adaptation to the low and irregular food supply in the deep sea.

Few studies have focused on the effects of hypercapnia on deep-sea animals (Pane and Barry, 2007; Tunnicliffe et al., 2009). Here we report on physiological effects of severe environmental hypercapnia in *A. excavata* in order to assess the effects of possible leaks from subsea geological storage of CO_2 on this deep-living species.

2. Materials and methods

2.1. Collection and storage of animals

Specimens of *A. excavata* (5.06–17.27 g soft body dry mass) were collected from the cold-water reefs at Røberg and Trolla (215–300 m) in the Trondheimsfjord by use of an ROV in September 2006 and May 2007. Animals were held in 30 L plastic trays (<20 animals per tray) continuously supplied with fresh seawater from 70 m depth, and regulated to a constant temperature of 8 °C. They were fed weening feed *ad libitum* once a week (Gemma Micro Diamond, Skretting). Animals were starved for one week prior to the experiments. All experiments were performed at a water and ambient temperature of 8 °C in climate controlled rooms.

2.2. Experimental setup

The experiments were carried out using a semi-recirculation system holding a total volume of approximately 120 L, where the entire volume was exchanged every 12 h, as outlined in Fig. 1.

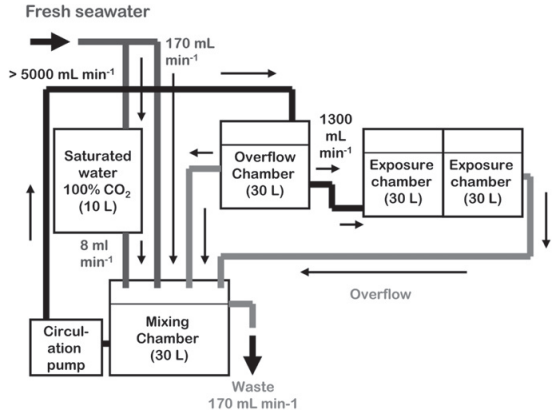

Fig. 1. Overview of the exposure setup used in the present study. Dark grey lines indicate supply of fresh seawater to the system, black lines indicate recirculated water going into the different chambers, and light grey lines indicate overflow of water from the chambers and water leaving as waste. Flow rates of water to the different chambers are indicated. Respiration chambers replaced one exposure chamber during respirometry.

During the exposure experiments seawater saturated with CO_2 gas (IND, AGA) was introduced into a mixing chamber at a rate of 8 mL min^{-1} (Fig. 1). At the same time a constant volume of fresh seawater (170 mL min^{-1}) was supplied to the mixing chamber, resulting in a volume equivalent to that of the entire circulating water volume of the system being exchanged within 12 h. The mixed water was distributed via an overflow chamber to two exposure chambers, and finally flowed back into the mixing chamber through outlets at the top of the exposure chambers. The experiments were started when seawater pH was stable, i.e. after about 4 days of equilibration.

During the experiments normocapnic seawater had a pH of 8.12 ± 0.05 and a P_{CO_2} of 530 ± 60 μatm, while seawater equilibrated with CO_2 had a mean pH of 6.35 ± 0.01 and P_{CO_2} of 32,881 ± 655 μatm. The small fluctuations observed were due to reduced flow from the CO_2 cylinder, and pH was reestablished after adjustments of the gas flow. The readings of P_{CO_2} and pH were stable indicating that the experimental setup in this study was an effective method to equilibrate seawater with CO_2.

In order to study the effects of CO_2 on *A. excavata* animals were placed in the exposure chambers described above and exposed to hypercapnic conditions for different lengths of time (0.5, 1, 4, 12, 24, 48 or 96 h). The ability of animals to recuperate after 96 h of exposure was studied by moving exposed animals to an aquarium with normocapnic conditions for recovery for 1, 4, 12, 24 or 96 h. Animals in recovery were kept in chambers that were identical to the exposure chambers and provided with normocapnic water which had approximately the same water flow as hypercapnic animals.

Each group was made up of 5 or 6 animals with the exception of the group exposed for 96 h, which was made up of 8 animals. Control animals were kept in the same chambers as described for recovery for approximately 24 h before samples were collected.

2.3. Oxygen consumption and ammonia-N excretion

Oxygen consumption and ammonia-nitrogen excretion was determined in closed respirometers. The respiration chambers consisted of ~2.5 L vacuum desiccators with a Dissolved Oxygen

Probe (DOP) (S120, Qubit systems) or an YSI Dissolved Oxygen Meter (Model 58) with an YSI Self-Stirring BOD probe (5905) placed in the outlet of the cover. Each chamber contained one animal at a time resting on a perforated disk of stainless steel, and a stirring magnet at the bottom. The chambers were placed in water baths, at 8 °C, situated on top of magnetic stirrers.

In between measurements the chambers were connected to the circulating system described above by the use of Dreschel adjustable bottle heads (MF 27/3/13, Quickfit, Barloworld Scientific, Ltd), which gave a flow rate of 310 mL min^{-1} in each chamber.

Ammonia-nitrogen excretion was determined as the difference in ammonia-nitrogen concentration in water samples collected at time zero and samples collected after 1 h from the respiration chambers. The concentration of ammonia-nitrogen (NH_3–N) in seawater was determined according to the indophenol blue method (Norwegian Standard 4746, 1975). The samples were filtered (GF/F, Whatman), and stored in PP centrifuge tubes at −20 °C prior to analysis.

In all cases animals were allowed to acclimate in the respirometers for at least 1 h prior to measurements and sampling. Oxygen consumption and ammonia-nitrogen excretion was related to the dry mass of the animal.

2.4. Acid–base parameters

Samples of hemolymph and tissues were collected immediately following removal of animals from the exposure chamber. Hemolymph samples were obtained from a sinus located on the ventral side of the posterior adductor muscle by the use of a 1 mL disposable plastic syringe. Hemolymph for determination of Ca^{2+} concentration was stored under liquid paraffin at −20 °C. Samples of the posterior adductor and pedal retractor muscle, foot, gills and mantle were collected and stored at −20 °C. Dry mass of the whole soft body tissue was determined after drying at 105 °C for 48 h (AE 50, Mettler Toledo).

The partial pressure of CO_2 (P_{CO_2}) in seawater and hemolymph was determined from the linear relationship between the logarithm of P_{CO_2} and pH (Astrup, 1956). The validity of this technique was tested by equilibrating samples of seawater and hemolymph with 3 known CO_2 tensions (seawater: 5, 10 and 100%; hemolymph: 2.5, 5 and 10%) giving Eq. (1) for seawater and 2 for hemolymph,

$$y = -1.0114x + 10.934, \quad R^2 = 0.9999 \tag{1}$$

$$y = -1.0001x + 6.7842, \quad R^2 = 0.9967 \tag{2}$$

where x is the P_{CO_2} in units of μatm and kPa, respectively. The resulting regression lines demonstrated that this method was applicable for determination of both seawater and hemolymph P_{CO_2}.

The pH of 50 μL samples of hemolymph were determined immediately after sampling following removal of the animal from the exposure chamber and again after equilibration with two known CO_2 tensions (2.5 and 5% or 5 and 10%, HiQ, Linde Gas, AGA). The pH of the equilibrated samples was plotted against their respective known P_{CO_2}, and the original P_{CO_2} of the samples could then be determined from their original pH. All pH measurements were carried out using a Radiometer Analytical MeterLab standard pH meter (PHM210), with a combined micro pH electrode (pHC3359-8, Radiometer Analytical MeterLab®) calibrated with Radiometer Analytical buffers (NBS).

The concentration of extracellular bicarbonate was calculated using a modification of the Henderson–Hasselbalch equation (Heisler, 1986):

$$\left[HCO_3^-\right] = 10^{pH-pK} \times \alpha_{CO_2} P_{CO_2} \tag{3}$$

where αCO_2 is the solubility of CO_2 in the hemolymph. The values of αCO2 and pK at 8 °C were calculated according to formulas proposed by Heisler (1986) to be 0.054 mmol L^{-1} $mmHg^{-1}$ and 6.18, respectively. In these calculations the molarity of dissolved species and ionic strength of seawater (M = 1.033 mmol kg^{-1}, I = 0.6995) were taken to represent these values in the hemolymph. The hemolymph protein concentration was determined by using the Bradford reagent (B 6916, Sigma) with bovine serum albumin (P0834, Sigma) as standards.

Intracellular pH (pH_i) of the posterior adductor muscle was determined according to the homogenate method developed by Pörtner et al. (1990). A thin (~2 mm) sample of the posterior adductor muscle was rapidly packed in aluminium foil and flash-frozen in liquid N_2 and manually ground to a powder in liquid N_2. The powder was then dissolved in 1 mL ice-cold media containing reagents which inhibit homogenate metabolism (160 mmol L^{-1} kF, 1 mmol L^{-1} NTA). Following brief mixing and centrifugation (5000 g for 1 min) the resultant pH of the supernatant was taken to represent the pH_i of the tissue.

Intracellular non-bicarbonate buffering capacity was measured in tissue samples of the posterior adductor and pedal retractor muscle, foot, gills and mantle from control animals and animals exposed to hypercapnia for 96 h as described by Castellini and Somero (1981). Tissue samples of about 0.5 g were homogenized (Heidolph DIAX 900) on ice in normal saline (0.9% NaCl) at a ratio of 1:20. After equilibration to ambient temperature the homogenate was titrated manually with NaOH between pH 6 and 7. Buffering capacity is defined as the μmoles of base needed to change the pH of the homogenate by one pH unit per gram wet weight of tissue (Castellini and Somero, 1981) and is denoted in units of slykes (β).

The concentration of calcium in hemolymph was determined using an atomic absorption spectrometer (PerkinElmer AAnalyst™ 2100). Native hemolymph (5 μl) was diluted in 40 mL 0.1 M HNO_3 with 0.1% La_2O_3 in acid-washed polyethylene bottles prior to analysis. Concentrations were measured at 422.7 nm and standard curves were made from $Ca(NO_3)_2$ (Analytical Standard, Spectrosol®, BDH Limited) diluted with the abovementioned solvent.

2.5. Statistical analyses

One-way analyses of variance (ANOVA) were performed with Dunnett's post test. Tukey's post tests were used on data concerning intracellular non-bicarbonate buffering capacity. Correlation of data was tested by using the Spearman correlation test. All tests were two-tailed, and the level of significance was set at $p \leq 0.05$. All values are presented as means with standard error of means (±s.e.m). The statistical analyses were performed using Graph Pad Prism 5.0 (GraphPad Software, San Diego California USA).

3. Results

Fig. 2 illustrates the changes in extracellular acid–base status (A-C) and Ca^{2+} concentration (D) with time of exposure to severe environmental hypercapnia and subsequent normocapnic recovery. Mean values of extracellular P_{CO_2} and pH (pH_e) in control animals were 0.204 ± 0.014 kPa and 7.60 ± 0.03, respectively (Fig. 2A and B). In the initial stages of exposure the animals experienced a rapid increase in hemolymph P_{CO_2} and a corresponding decrease in hemolymph pH to values close to those of the seawater, both being statistically different from control values. After 96 h P_{CO_2} was 3.442 ± 0.204 kPa and pH was 6.35 ± 0.01. Thus, no

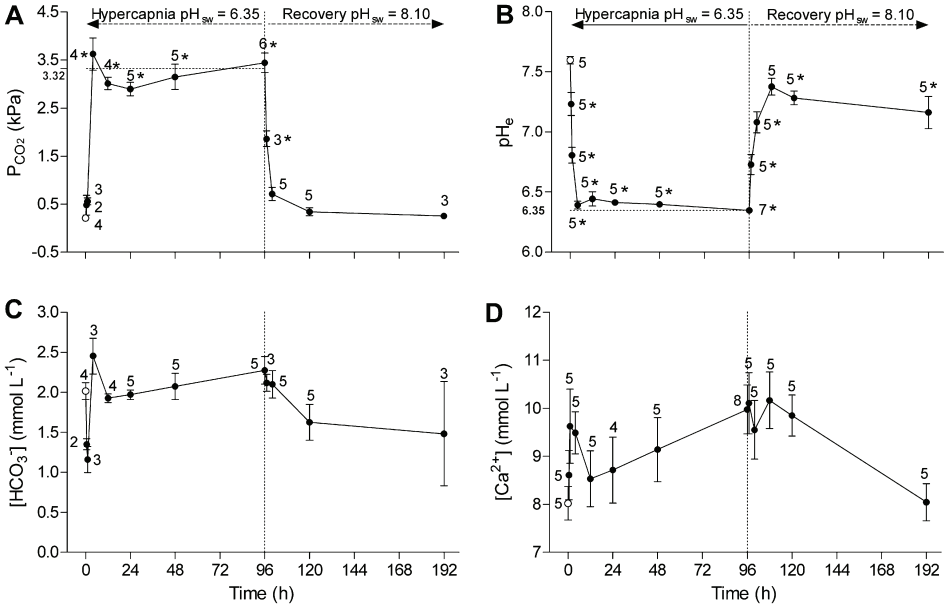

Fig. 2. Hemolymph acid–base parameters (P_{CO_2}, pH, $[HCO_3^-]$) and $[Ca^{2+}]$ of *Acesta excavata* during control conditions (open circles), severe hypercapnia ($t = 0.5$–96 h), and recovery in normocapnic water ($t > 96$ h). Broken horizontal lines in A and B indicate respective seawater values during hypercapnia. pH_{sw} denotes the pH of seawater. Values are means ± s.e.m. Numbers are *n*. Asterisks indicate values significantly different from control values ($p \leq 0.05$).

compensation of extracellular acidosis was observed. Extracellular P_{CO_2} and pH returned towards control values during recovery. However, pH_e continued to be significantly different from control values after 96 h of recovery.

Mean extracellular $[HCO_3^-]$ in control animals was 2.01 ± 0.11 mmol L^{-1}. During exposure the concentration of bicarbonate decreased rapidly and had dropped to 1.16 ± 0.16 mmol L^{-1} after 1 h (Fig. 2C). The highest value was found after 4 h when extracellular $[HCO_3^-]$ was 2.45 ± 0.23 mmol L^{-1}. This value was significantly higher than for animals exposed for 1 h, though not from control animals. Bicarbonate concentrations decreased slightly after animals were placed in recovery.

The mean hemolymph concentration of free calcium in control animals was 8.02 ± 0.35 mmol L^{-1} (Fig. 2D). A rapid increase in $[Ca^{2+}]$ within the first hour of exposure was followed by a decline, again followed by a gradual increase from 12 to 96 h. Extracellular $[Ca^{2+}]$ was elevated compared to control values throughout the exposure period and only gradually returned towards control values within 96 h of recovery, but was at no time significantly different from control values. Hemolymph $[Ca^{2+}]$ was not significantly correlated to $[HCO_3^-]$ ($p = 0.305$, $r = 0.155$).

Intracellular pH (pH_i) of the posterior adductor muscle as a function of time of exposure and recovery is illustrated in Fig. 3. Mean pH_i of control animals was 7.30 ± 0.03. During exposure intracellular pH declined rapidly and was significantly lower than in control animals after 4 h, with a mean value of 7.01 ± 0.05. No compensation of intracellular pH was observed during exposure. During recovery intracellular pH rapidly returned towards control values.

Fig. 4 illustrates the intracellular non-bicarbonate buffering capacities in the different types of tissues of *A. excavata* in control animals and in animals subjected to 96 h exposure. The buffering capacities varied significantly between the different types of tissues, with the highest value of 42.73 ± 1.69 slykes found for the posterior adductor muscle. The pedal retractor and foot showed significantly lower buffering capacities with 24.78 ± 3.33 and 19.75 ± 0.96 slykes, respectively. The lowest buffering capacities were found in the mantle and gills with 12.5 ± 1.36 and 9.18 ± 0.37 slykes, respectively. Following severe hypercapnia for 96 h the posterior adductor muscles had a mean buffering capacity of 33.95 ± 1.17 slykes, which was significantly lower than in control animals. There did not, however, seem to be a trend of the remaining tissues of hypercapnic animals having lower buffering capacities than normocapnic animals.

The oxygen consumption ranged between 0.282 and 2.42 mL O_2 h^{-1} in control animals, and between 0.114 and 1.13 mL O_2 h^{-1} in hypercapnic animals. The time-dependent changes in mass-specific oxygen consumption during control conditions and severe hypercapnia are depicted in Fig. 5A. Control animals had

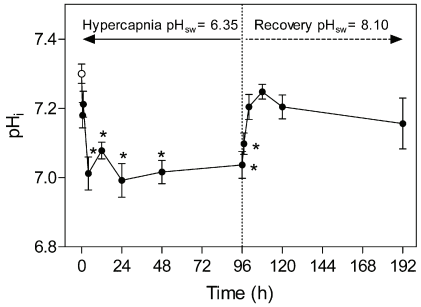

Fig. 3. Changes in intracellular pH of *Acesta excavata* during control conditions (open circle), severe hypercapnia and recovery. Values are means ± s.e.m, $n = 5$ or 6. Asterisks indicate values significantly different from control values ($p \leq 0.05$). pH_{sw} denotes the pH of seawater.

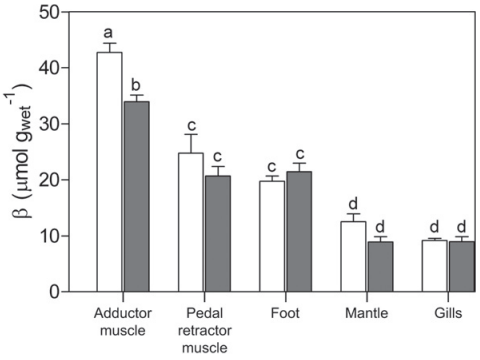

Fig. 4. The buffering capacities of different tissues in *Acesta excavata* exposed to control conditions (white bars) and severe hypercapnia for 96 h (dark bars). Values are means ± s.e.m, $n = 10$ in control animals and $n = 6$ in hypercapnic animals. Different letters indicate values significantly different from each other ($p \leq 0.05$).

a mean oxygen consumption of 0.063 ± 0.004 mL O_2 h^{-1} per gram dry tissue mass. Oxygen consumption declined rapidly during exposure. The lowest value was recorded after 4 h of exposure when oxygen consumption was 0.025 ± 0.003 mL O_2 h^{-1} g^{-1}.

The oxygen consumption started to increase slightly after 12 h of exposure, and was no longer significantly different from control values after 48 h. After 96 h mean oxygen consumption was only 29% lower than in control animals.

The mass-specific ammonia-N (NH_3–N) excretion increased rapidly, but not significantly, compared to control values during severe environmental hypercapnia (Fig. 5B). After 4 h of exposure the ammonia-nitrogen excretion had increased to 9.95 ± 1.14 μg h^{-1} g^{-1}, corresponding to a 63% increase relative to the mean control value. The excretion rate then decreased slightly and levelled off after 12 h. After 96 h of exposure the mean NH_3–N excretion was only 22% higher than control values.

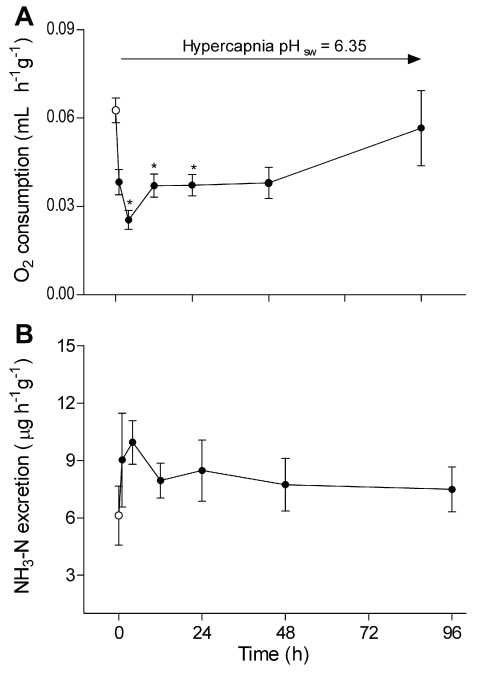

Fig. 5. Oxygen consumption (A) and ammonia-nitrogen excretion (B) in *Acesta excavata* measured during control conditions, and severe environmental hypercapnia. Open circles represents the mean of all control values. Values are means ± s.e.m, $n = 3–6$. Asterisks indicate values significantly different ($p \leq 0.05$) from control values.

4. Discussion

As reported for other marine invertebrates, environmental hypercapnia resulted in acidosis in the hemolymph of *A. excavata*. The severity of the exposure caused the extracellular pH to drop to that of hypercapnic seawater and no compensation of hemolymph pH or accumulation of bicarbonate ions was observed during exposure. Previous studies on other species have mostly involved exposure to moderate hypercapnia (P_{CO_2} = 10,000 μatm or less). Under these conditions complete compensation of extracellular acidosis is frequently observed in fish (Heisler, 1984, 1986), and marine invertebrates are often able to partially counteract acidosis through accumulation of bicarbonate ions (Lindinger et al., 1984; Pörtner et al., 1998; Michaelidis et al., 2005; Miles et al., 2007; Pane and Barry, 2007; Gutowska et al., 2010).

Considering the natural habitat of *A. excavata* this species was expected to have a much lower tolerability compared to the intertidal species mentioned above. In a comparative study Pane and Barry (2007) found that in contrast to the shallow-living Dungeness crab, the deep-sea crab *Chionoecetes tanneri* showed only a slight compensation of hemolymph pH during moderate hypercapnia, probably reflecting a general trend of poorer acid–base regulatory abilities in deep-sea animals as compared to more shallow-living species.

Partial compensation of extracellular acidosis during exposure to hypercapnic conditions has previously been demonstrated in intertidal mussels of the genus *Mytilus* in experiments using closed recirculation setups. The Mediterranean bivalve *M. galloprovincialis* was able to maintain extracellular pH slightly above that of seawater when exposed to long-term moderate hypercapnia (Michaelidis et al., 2005), and *M. edulis* was able to partially compensate extracellular acidosis even when subjected to P_{CO_2} levels of 13 and 26 mmHg (17000 and 34000 μatm) (Lindinger et al., 1984). The authors of both studies concluded that acidosis was counteracted by accumulation of bicarbonate from dissolution of the calcium carbonate shell.

During shell closure intertidal bivalves first use up the oxygen remaining in cells and hemolymph, resulting in a rise in P_{CO_2} due to limited gas exchange to the surroundings (Booth et al., 1984). The bivalves then switch to anaerobic metabolism, causing a build-up of acidic end products, and a subsequent decrease in body fluid pH. This acidosis is to some extent buffered by hydrolysis of $CaCO_3$ from the shell, as HCO_3^- react with excess H^+ and calcium ions increase the strong ion difference. The net reaction, however, is a formation of CO_2, which again leads to a decrease in pH. When the bivalves are re-submerged they rid themselves of the excess CO_2 and pH is reestablished (Crenshaw and Neff, 1969; Jokumsen and Fyhn, 1982; Burnett, 1988). When using a closed setup for CO_2 exposure, any bicarbonate or calcium ions that are dissolved from the shells remain in the system, and can be used to buffer body fluid acidosis.

Thomsen et al. (2010) found that when using a flow-through system *M. edulis* was not able to compensate extracellular pH when exposed to moderate P_{CO_2} levels (1400 and 4000) μatm and no significant increase in extracellular HCO_3^- was measured. Control animals of *A. excavata* had significantly lower concentrations of extracellular free calcium compared to values reported in *M. edulis* and *M. galloprovincialis* (Lindinger et al., 1984; Michaelidis et al., 2005). In addition, no significant shell dissolution or

accumulation of bicarbonate ions was observed in hypercapnic animals. The results of the present study may therefore be in accordance with those of Thomsen et al. (2010) that no compensation of pH occurs in bivalves exposed to hypercapnia when they are placed in an open system.

In a different study using a flow-through system the sea urchin *Psammechinus miliaris* achieved slight and transient compensation of extracellular pH when exposed to elevated levels of CO_2. Here, shell dissolution was higher during moderate than severe hypercapnia (Miles et al., 2007). These results may suggest that shell dissolution in calcifying animals is dependent on the pH of the water, with dissolution being weaker during more severe hypercapnia. This could indicate that shell dissolution is an active rather than a passive process. This is not unlikely as the enzyme carbonic anhydrase is reported to have a role in the shell formation in some bivalves (Freeman and Wilbur, 1948). In *P. miliaris* mortality was more conspicuous when exposed to severe hypercapnic conditions (Miles et al., 2007). Although *A. excavata* was strongly affected by hypercapnia no animals died during the experiment.

The fluctuations seen in the hemolymph content of Ca^{2+} may be a consequence of changes in the relative volumes of the intra- and extracellular compartments due to lowered energy production during exposure. It is well known that the partitioning of dominant osmolytes, such as amino acids and sodium, is a consequence of energy expenditure. As the aerobe metabolism of these animals was severely affected by hypercapnia the distribution of major osmolytes may have been affected and resulted in changes in the hemolymph volume. Previous studies have shown that the transmembrane gradient of sodium, calcium and many amino acids of *M. edulis* are sensitive to organic chemicals (Børseth et al., 1992, 1995; Zachariassen et al., 1996).

The lower level of hemolymph Ca^{2+} in this study may also indicate that the shells of deep-sea bivalves differ somewhat from the shells of intertidal bivalves which experience daily fluctuations in body fluid pH as well as shell dissolution due to the changing tide.

Although deep-sea animals are thought to have low tolerance to fluctuations in the environment, numerous species are known to thrive in reducing environments such as hydrothermal vents. These are areas where volcanic activity gives enriched gas tensions and create conditions of hypercapnia and low pH. In their study Tunnicliffe et al. (2009) compared the shells of two populations of the vent mussel *Bathymodiolus brevior*, one found at a location having naturally low pH and high P_{CO_2}, and the other from a location where pH was above 7.88. Both shell thickness and daily growth increments of the 'low pH group' were half of the 'high pH group'. As seen in intertidal bivalves, dissolution of shells seemed to occur on the inner shell surface, suggesting a compensatory response. The high age of the individuals (<40 years), may suggest that the 'low pH' bivalves have adapted to a life in this challenging environment. The authors suggested that although the mussels had a high tolerance to the acidic environment, higher vulnerability to predators due to reduced shell thickness may be a secondary effect to exposure to hypercapnic environments. Natural predators such as crabs were absent from the 'low pH' location, possibly due to the acidic environment. Since acid–base status of the animals was not studied, it is not known whether they are able to compensate for body fluid acidosis, or if they have adapted to a naturally acidic internal environment.

The intracellular buffering capacity varied significantly between the different tissues in *A. excavata*, with the highest value found for the posterior adductor muscle. This tissue-specific difference was in agreement with the results from studies involving both marine vertebrates and invertebrates, as muscles capable of burst glycolytic function or prolonged anaerobic work generally display higher buffering capacities than other tissues (Castellini and Somero, 1981; Eberlee and Storey, 1984; Morris and Baldwin, 1984). Deep-sea animals generally have lower non-bicarbonate buffering capacities than shallow-water or pelagic species due to lower metabolic rates (Castellini and Somero, 1981; Seibel et al., 1997). The results from this study, however, shows that *A. excavata* has similar, and in some cases higher buffering capacity compared to intertidal bivalves (Eberlee and Storey, 1984; Morris and Baldwin, 1984; Walsh et al., 1984).

Hypercapnia induced significant reductions in the buffering capacities of the posterior adductor muscle. No significant CO_2-induced changes were found in the other tissues tested. The reason for the reduction in the buffering capacity of the posterior adductor muscle during hypercapnia is not clear. It could, however, be a result of increased catabolism of proteins, or reduced rate of protein synthesis, as proteins are important intracellular buffers (Roos and Boron, 1981). Catabolism of amino acids generates bicarbonate in addition to ammonia (Atkinson and Bourke, 1984), and the decreased non-bicarbonate buffering capacity in the posterior adductor muscle may reflect an attempt to actively generate HCO_3^- through this mechanism.

Although not significant, there appeared to be an increase in ammonia-nitrogen excretion when animals were subjected to hypercapnic compared to control conditions. Increases in ammonia-nitrogen excretion during hypercapnia have previously been reported in the intertidal bivalve *M. edulis* and *M. galloprovincialis* (Lindinger et al., 1984; Michaelidis et al., 2005) and Lindinger et al. (1984) suggested that ammonia could play a role in acid excretion in *M. edulis*. The observed increase may indicate an increased utilization of amino acids or proteins as metabolic substrates in *A. excavata* during hypercapnia, consistent with the observed lowering of the non-bicarbonate buffering capacity of the posterior adductor muscle following exposure.

The mass-specific oxygen consumption for control animals was somewhat lower than what has previously been reported for *A. excavata* (Järnegren and Altin, 2006). This is most likely attributed to the different treatments of the animals during the experiment. In this study animals were starved one week prior to the experiment, while Järnegren and Altin (2006) fed the animals to keep them active and filtering when oxygen consumption was recorded. Feeding is known to affect oxygen consumption in animals (Thompson and Bayne, 1972).

A large decline in oxygen consumption was expected as hypercapnia is known to induce metabolic depression in marine invertebrates such as the marine sipunculoid *S. nudus* (Reipschlager and Pörtner, 1996) and the bivalve *M. galloprovincialis* (Michaelidis et al., 2005). Langenbuch and Portner (2002) found that oxygen consumption of hypercapnic isolated body wall tissue of *S. nudus* was reduced by 45% at an extracellular pH of 6.70. Hypercapnia initially caused a substantial reduction in the mass-specific oxygen consumption rate compared to control values. Although maintained below that of control animals, the mean oxygen consumption returned towards control values at the end of the experiment. The increased oxygen consumption by *A. excavata* during prolonged exposure may indicate a higher tolerance to extreme acidosis of the body fluids than what was expected. It could also be a delayed response in which metabolic processes are activated.

Thomsen and Melzner (2010) found that growing *M. edulis* for 2 months at different levels of CO_2 elicited an increase rather than reduction in oxygen consumption compared to control values. They suggested that the reduced shell growth observed in their study was due to synergistic effects of increased cellular energy demand and nitrogen loss rather than metabolic depression.

In another mollusk, the cephalopod *Sepia offcinalis*, no changes in metabolic rate was observed, the authors concluding that species

having naturally high metabolic rate and activity are pre adapted to high CO_2 as a protection at high energetic activities (Gutowska et al., 2008).

An increase in both metabolic rate and calcification rate in response to elevated CO_2 was found when the brittlestar *Amphiura filiformis* was subjected to hypercapnic exposure. This was however followed by a reduction in muscle biomass, reflecting an expensive means of compensation (Wood et al., 2008).

Metabolic depression during body fluid acidosis has mainly been attributed to reductions in intracellular pH (Busa and Nuccitelli, 1984; Michaelidis et al., 2005), but studies show that in the sipunculoid *S. nudus* oxygen consumption is affected by extracellular pH rather than intracellular pH. Whether the metabolic reduction observed in *A. excavata* is due to pH_e rather than pH_i is not clear from the results in this study.

The coincidental decrease in oxygen consumption and the increase in ammonia-nitrogen excretion may suggest a reduction in the O/N ratio of *A. excavata*, indicating a higher dependence on amino acid catabolism as a source of energy (Pörtner et al., 1998; Langenbuch and Portner, 2002). This is in accordance with studies conducted on *M. edulis* (Thomsen and Melzner, 2010).

5. Conclusion

In this study exposure of the deep-sea bivalve *A. excavata* to severe environmental hypercapnia induced extra- and intracellular acidosis that remained uncompensated during exposure. Oxygen consumption dropped significantly during the initial phase, but then approached control values at the end of exposure. The intracellular non-bicarbonate buffering capacity were similar to and, in some tissues, higher than those reported for intertidal bivalves, and the buffering capacity of the posterior adductor muscle was significantly lower in hypercapnic animals compared to control animals. Although animals were highly affected by exposure to hypercapnia the acid–base parameters returned towards control values and no mortality was observed in exposed animals. This study suggests that *A. excavata* displays higher tolerance to severe environmental hypercapnia than what may be expected for deep-sea animals. However, Tunnicliffe et al. (2009) found evidence that permanent exposure to similar conditions causes reduced growth rates and shell thickness in mussels adapted to a life at deep-sea vents, and such long-term effects may also develop in *A. excavata*.

Acknowledgements

This study was funded by VISTA, the research cooperation between The Norwegian Academy of Science and Letters and Statoil. The authors are grateful to NTNU SeaLab for use of laboratories and equipment and to Dag Altin and Anders Olsen for practical assistance.

References

Astrup, P., 1956. A simple electrometric technique for the determination of carbon dioxide tension in blood and plasma, total content of carbon dioxide in plasma, and bicarbonate content in "separated" plasma at a fixed carbon dioxide tension (40 mm Hg). Scandinavian Journal of Clinical & Laboratory Investigation 8, 33–43.

Atkinson, D.E., Bourke, E., 1984. The role of ureagenesis in pH homeostasis. Trends in Biochemical Sciences 9, 297–300.

Barker, S., Elderfield, H., 2002. Foraminiferal calcification response to glacial-interglacial changes in atmospheric CO_2. Science 297, 833–836.

Booth, C.E., McDonald, D.G., Walsh, P.J., 1984. Acid–base balance in the sea mussel *Mytius edulis*. I. Effects of hypoxia on air-exposure on hemolymph acid–base status. Marine Biology Letters 5, 347–358.

Børseth, J., Aunaas, T., Einarson, S., Nordtug, T., Olsen, A., Zachariassen, K., 1992. Pollutant-induced depression of the transmembrane sodium gradient in muscles of mussels. Journal of Experimental Biology 169, 1–18.

Børseth, J.F., Aunaas, T., Denstad, J.-P., Nordtug, T., Olsen, A.J., Schmid, R., Skjærvø, G., Zachariassen, K.E., 1995. Transmembrane sodium energy gradient and calcium content in the adductor muscle of *Mytilus edulis* L. in relation to the toxicity of oil and organic chemicals. Aquatic Toxicology 31, 263–276.

Burnett, L.E., 1988. Physiological responses to air exposure: acid-base balance and the role of branchial water stores. American Zoologist 28, 125–135.

Busa, W.B., Nuccitelli, R., 1984. Metabolic regulation via intracellular pH. American Journal of Physiology 246, R409–R438.

Castellini, M.A., Somero, G.N., 1981. Buffering capacity of vertebrate muscle: correlations with potentials for anaerobic function. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 143, 191–198.

Claiborne, J.B., Edwards, S.L., Morrison-Shetlar, A.I., 2002. Acid–base regulation in fishes: cellular and molecular mechanisms. Journal of Experimental Zoology 293, 302–319.

Crenshaw, M.A., Neff, J.M., 1969. Decalcification at themantle-shell interface in molluscs. American Zoologist 9, 881–885.

Eberlee, J.C., Storey, K.B., 1984. Buffering capacities of the tissues of marine molluscs. Physiological Zoology 57, 567–572.

Freeman, J.A., Wilbur, K.M., 1948. Carbonic anhydrase in molluscs. Biological Bulletin 94, 55–59.

Gazeau, F., Gattuso, J.P., Dawber, C., Pronker, A.E., Peene, F., Peene, J., Heip, C.H.R., Middelburg, J.J., 2010. Effect of ocean acidification on the early life stages of the blue mussel *Mytilus edulis*. Biogeosciences 7, 2051–2060.

Gutowska, M., Melzner, F., Langenbuch, M., Bock, C., Claireaux, G., Pörtner, H., 2010. Acid–base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 180, 323–335.

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

Hayashi, M., Kita, J., Ishimatsu, A., 2004. Comparison of the acid-base responses to CO_2 and acidification in Japanese flounder (*Paralichthys olivaceus*). Marine Pollution Bulletin 49, 1062–1065.

Heisler, N., 1984. Acid–Base regulation in fishes. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press, New York, pp. 315–401.

Heisler, N., 1986. Buffering and transmembrane ion transfer processes. In: Heisler, N. (Ed.), Acid–base regulation in animals. Elsevier Science Publisher BV, Amsterdam, pp. 3–47.

Jacobs, M.H., 1920. To what extent are the physiological effects of carbon dioxide due to hydrogen ions? American Journal of Physiology 51, 321–331.

Järnegren, J., Altin, D., 2006. Filtration and respiration of the deep living bivalve *Acesta excavata* (J.C. Fabricius, 1779) (Bivalvia; Limidae). Journal of Experimental Marine Biology and Ecology 334, 122–129.

Jokumsen, A., Fyhn, H.J., 1982. The influence of aerial exposure upon respiratory and osmotic properties of haemolymph from two intertidal mussels, *Mytilus edulis* L. and *Modiolus modiolus* L. Journal of Experimental Marine Biology and Ecology 61, 189–203.

Kikkawa, T., Kita, J., Ishimatsu, A., 2004. Comparison of the lethal effect of CO_2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Marine Pollution Bulletin 48, 108–110.

Langenbuch, M., Portner, H.O., 2002. Changes in metabolic rate and N excretion in the marine invertebrate *Sipunculus nudus* under conditions of environmental hypercapnia: identifying effective acid-base variables. Journal of Experimental Biology 205, 1153–1160.

Lindinger, M.I., Laurén, D.J., McDonald, G., 1984. Acid-base balance in the sea mussel *Mytilus Edulis*. III. Effects of environmental hypercapnia on intra- and extracellular acid-base balance. Marine Biology Letters 5, 371–381.

López Correa, M., Freiwald, A., Hall-Spencer, J., Taviani, M., 2005. Distribution and habitats of *Acesta excavata* (Bivalvia: Limidae) with new data on its shell ultrastructure. In: Freiwald, A. (Ed.), Cold-Water Corals and Ecosystems. Springer Berlin Heidelberg, pp. 173–205.

Metz, O., Davidson, H.C., de Coninck, M.L., Meyer, L.A., 2005. Summary for policymakers and Technical summary, IPCC Special Report on Carbon dioxide Capture and Storage, Prepared by Working Group III of the Intergovernmental Panel on Climate Change, New York, pp. 1–49.

Michaelidis, B., Ouzounis, C., Paleras, A., Pörtner, H.O., 2005. Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Marine Ecology Progress Series 293, 109–118.

Miles, H., Widdicombe, S., Spicer, J.I., Hall-Spencer, J., 2007. Effects of anthropogenic seawater acidification on acid-base balance in the sea urchin *Psammechinus miliaris*. Marine Pollution Bulletin 54, 89–96.

Morris, G.M., Baldwin, J., 1984. pH buffering capacity of invertebrate muscle: correlations with anaerobic muscle work. Molecular Physiology 5, 61–70.

Pane, E.F., Barry, J.P., 2007. Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Marine Ecology Progress Series 334, 1–9.

Pörtner, H.O., Boutilier, R.G., Tang, Y., Toews, D.P., 1990. Determination of intracellular pH and PCO_2 after metabolic inhibiton by fluoride and nitrilotriacetic acid. Respiration Physiology 81, 255–273.

Pörtner, H.O., Reipschlager, A., Heisler, N., 1998. Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. Journal of Experimental Biology 201, 43–55.

Reipschlager, A., Pörtner, H.O., 1996. Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. Journal of Experimental Biology 199, 1801–1807.

Roos, A., Boron, W.F., 1981. Intracellular pH. Physiological Reviews 61, 296–434.
Seibel, B.A., Thuesen, E.V., Childress, J.J., Gorodezky, L.A., 1997. Decline in pelagic cephalopod metabolism with habitat depth reflects differences in locomotory efficiency. Biological Bulletin 192, 262–278.
Seibel, B.A., Walsh, P.J., 2001. Potential impacts of CO_2 injection on deep-sea biota. Science 294, 319–320.
Thompson, R.J., Bayne, B.L., 1972. Active metabolism associated with feeding in the mussel *Mytilus edulis* L. Journal of Experimental Marine Biology and Ecology 9, 111–124.
Thomsen, J., Gutowska, M.A., Saphorster, J., Heinemann, A., Trubenbach, K., Fietzke, J., Hiebenthal, C., Eisenhauer, A., Kortzinger, A., Wahl, M., Melzner, F., 2010. Calcifying invertebrates succeed in a naturally CO_2-rich coastal habitat but are threatened by high levels of future acidification. Biogeosciences 7, 3879–3891.
Thomsen, J., Melzner, F., 2010. Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Marine Biology 157, 2667–2676.
Tunnicliffe, V., Davies, K.T.A., Butterfield, D.A., Embley, R.W., Rose, J.M., Chadwick Jr., W.W., 2009. Survival of mussels in extremely acidic waters on a submarine volcano. Nature Geosci 2, 344–348.
Turley, C., Nightingale, P., Riley, N., Widdicombe, S., Joint, I., Gallienne, C., Lowe, D., Goldson, L., Beaumont, N., Mariotte, et al., 2004. Literature Review: environmental impacts of a gradual or catastrophic release of CO2 into the marine environment following carbon dioxide capture. In: F.a.R.A.D (Ed.), Report to the UK Department for Environment, p. 57. The UK Department for Environment, Food and Rural affairs London.
Vetter, E.W., Smith, C.R., 2005. Insights into the ecological effects of deep ocean CO_2 enrichment: the impacts of natural CO_2 venting at Loihi seamount on deep sea scavengers. Journal of Geophysical Research 110, C09S13.
Waldbusser, G., Voigt, E., Bergschneider, H., Green, M., Newell, R., 2011. Biocalcification in the Eastern Oyster (*Crassostrea virginica*) in relation to long-term trends in Chesapeake Bay pH. Estuaries and Coasts 34, 221–231.
Walsh, P.J., McDonald, D.G., Booth, C.E., 1984. Acid-base balance in the sea mussel *Mytilus edulis*. II. Effects of hypoxia and air-exposure on intracellular acid-base status. Marine Biology Letters 5, 359–369.
Wheatly, M.G., Henry, R.P., 1992. Extracellular and intracellular acid-base regulation in crustaceans. Journal of Experimental Zoology 263, 127–142.
Wickins, J.F., 1984. The effect of hypercapnic sea water on growth and mineralization in penaied prawns. Aquaculture 41, 37–48.
Wood, H.L., Spicer, J.I., Widdicombe, S., 2008. Ocean acidification may increase calcification rates, but at a cost. Proceedings of the Royal Society B: Biological Sciences 275, 1767–1773.
Zachariassen, K., Olsen, A., Aunaas, T., 1996. The effect of formaldehyde exposure on the transmembrane distribution of free amino acids in muscles of *Mytilus edulis*. Journal of Experimental Biology 199, 1287–1294.

Paper II

Is not included due to copyright

Paper III

Is not included due to copyright

Paper IV

Elevated seawater levels of CO_2 changes the metabolic fingerprint of tissues and hemolymph from the green shore crab *Carcinus maenas*

Karen M. Hammer[a*], Sindre A. Pedersen[a], Trond R. Størseth[b]

[a] Department of Biology, Norwegian University of Science and Technology, NTNU, Trondheim, Norway

[b] SINTEF Fisheries and Aquaculture, Marine Resources Technology, Trondheim, Norway

[*]Corresponding author:

Tel.: +4773596356 fax +4773596311
Address: NTNU SeaLab
Brattørkaia 17 C
7010 Trondheim
Norway
E-mail address: karen.hammer@bio.ntnu.no

Abstract

Carbon dioxide (CO_2) acts as a weak acid in water and the increasing level of CO_2 in the atmosphere leads to ocean acidification. In addition, possible leakage from sub-seabed storage of anthropogenic CO_2 may pose a threat to the marine environment. ^{1}H-NMR spectroscopy was applied to extracts of hemolymph, gills and leg muscle from shore crabs (*Carcinus maenas*) to examine the metabolic response to elevated levels of CO_2. Crabs were exposed to different levels of CO_2 –acidified seawater with pH_{NBS} 7.4, 6.6 and 6.3 (pCO_2 ~ 2,600, 16,000 and 30,000 µatm, respectively) for two weeks (level-dependent exposure). In addition, the metabolic response was followed for up to 4 weeks of exposure to seawater pH_{NBS} 6.9 (pCO_2~7,600 µatm). Partial least squares regression analysis of data showed an increased differentiation between metabolic fingerprints of controls and exposed groups for all sample types with increasing CO_2 levels. Difference between controls and animals subjected to time-dependent exposure appeared after 4 weeks in hemolymph and gills, and after 48 hours of exposure in leg muscle. Changes in metabolic profiles were mainly due to a reduced level of important intracellular osmolytes such as amino acids (glycine, proline), while the level of other metabolites varied between the different sample types. The results are similar to what is observed in animals exposed to hypo-osmotic stress and may suggest disturbances in intracellular iso-osmotic regulation. The results may also reflect increased catabolism of amino acid to supply the body fluids with proton-buffering ammonia (NH_3). Alternatively, the findings may reflect an exhaustive effect of CO_2 exposure.

Keywords: Carbon dioxide; Hypercapnia; *Carcinus maenas*; Metabolomics; CO_2 storage Ocean acidification

1. Introduction

The ocean and the atmosphere exchange carbon in the form of CO_2, and the increasing atmospheric level of CO_2 observed today is therefore affecting the ocean. In seawater, CO_2 is hydrated to form carbonic acid (H_2CO_3) which dissociates into bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and hydrogen ions (H^+). Seawater has a high buffering capacity against the acidic effect of CO_2 due to the presence of dissolved inorganic carbon species and its total alkalinity (Zeebe and Wolf-Gladrow 2001). However, as the CO_2 level increases the pH of seawater is reduced, a phenomenon referred to as ocean acidification (Doney et al. 2009). Mitigating actions such as carbon capture and storage (CCS) have been put into practice to reduce anthropogenic CO_2 emissions to the atmosphere. Although sub-seabed storage sites are believed to offer safe containment of the captured CO_2, leaks may occur causing damage to the natural environment in the proximity of the leakage sites (Damen et al. 2006; Turley et al. 2004). Studies from natural CO_2 seeps indicate that burst leaks could lead to extreme CO_2 levels and changes in pH close to the leakage point (Vetter and Smith 2005), while currents may transport and disperse the acidic water to larger areas. However, the more plausible scenario of small and prolonged leaks would be a constant acidification at the leakage area (Turley et al. 2004).

Due to the charge neutral nature of the CO_2 molecule it easily penetrates biological surfaces such as cell membranes (Gutknecht et al. 1977). Thus, under conditions of elevated seawater levels of CO_2 (environmental hypercapnia) hydration of CO_2 in the body fluids of aquatic ectotherms results in an increased proton concentration and extra- and, intracellular acidosis. Previous works have mainly examined the effects of hypercapnia on acid-base homeostasis and metabolic rate (Gutowska et al. 2010a; Hammer et al. 2011; Michaelidis et al. 2005; Pane and Barry 2007; Pörtner et al. 1998; Reipschlager and Pörtner 1996; Thomsen and Melzner 2010), development and growth (Dupont et al. 2008; Gazeau et al. 2010; Kurihara 2008; Michaelidis et al. 2005) and calcification (Gutowska et al. 2010b; Ries et al. 2009). Environmental hypercapnia has been shown to affect all of the above mentioned factors and the effects vary between species and life stages of the animals (Gazeau et al. 2010; Kikkawa et al. 2004; Pane and Barry 2007).

The shore crab, *Carcinus maenas*, is an osmoregulating, euryhaline decapod which is native to the shores of Europe and North-Africa, but has in the last centuries been distributed to most continents of the world due to increased human activities such as international shipping. Its

success is most likely due to a high tolerance to fluctuations in environmental factors such as temperature, salinity, and oxygen level (McGaw et al. 2011). *C. maenas* frequently experiences both hypercapnic (CO_2-induced) and metabolic acidosis due to restricted gas exchange and anaerobic metabolism, respectively, during low tide (Truchot 1986). Studies have shown that when subjected to hypercapnic conditions it is able to rapidly return extracellular pH towards normal values by accumulating bicarbonate ions (Truchot 1975).

Metabolomics is a tool which allows simultaneous measurements of multiple metabolites of low molecular weight. By using methods such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), followed by appropriate statistical analysis that typically employs multivariate or other repeated univariate tests, one may reveal changes in the metabolic profile, rather than in single metabolites in response to drugs, toxicants or disease (Bundy et al. 2009; Viant 2007). In recent years, an increasing number of studies have applied this tool to reveal the metabolic responses of organisms to environmental and anthropogenic stressors, a field known as environmental metabolomics (Schock et al. 2010; Tuffnail et al. 2009; Viant 2007; Zhang et al. 2011a; Zhang et al. 2011b). In addition, there has been an increased application of environmental metabolomics in studies concerning marine invertebrates (Schock et al. 2010; Tuffnail et al. 2009; Viant et al. 2003; Wu and Wang 2011; Zhang et al. 2011a; Zhang et al. 2011b).

In this study, we have employed ^{1}H-NMR metabolomics on the polar extracts of hemolymph, gill and muscle tissue of the shore crab *C. maenas* in order to investigate how the metabolome is affected by environmental hypercapnia. As this species is known to frequently experience, and have a high tolerance to hypercapnic conditions (Truchot 1986) it was chosen as a model species to possibly elucidate mechanisms involved in the handling of CO_2-induced effects. The pCO_2 levels chosen for the present study are relatively high in order to induce a physiological strain on the animals. The levels range from those expected from worst case ocean acidification scenarios (Caldeira and Wickett 2003) to levels reported from natural CO_2 seeps (Vetter and Smith 2005). The latter may represent conditions that could arise during possible leakage from sub-seabed CO_2 storage sites.

1

2

2. Materials and methods

2. 1 Collection and maintenance of animals

Carcinus maenas (67 males and 24 females, 7-70 g wet mass) were collected in the Trondheimsfjord, Norway, from June to September 2009. Animals were transported directly to the holding facility in polystyrene containers with cooler bricks.

Prior to the experiments animals were kept in aquaria (30-50 L) with flow-through seawater holding a temperature of 11°C in a climate controlled room. The crabs were allowed to acclimate to laboratory conditions for a minimum of 14 days before being subjected to experimental conditions. The animals were fed frozen blue mussels twice a week during storage, but were starved for 48 hours prior to exposure to ensure that all animals were at a similar starting point with respect to stomach filling. Due to the length of the exposures animals were hand fed blue mussels, and allowed to eat for 15 minutes twice a week during the experiments. Prior to removal from the exposure chambers, all animals were starved for 48 hours.

The experiments were conducted according to the Animal Welfare Act and approved by the National Animal Research Authority (fdu.no, FOTS reference no. 1845).

2.2 Seawater chemistry measurements

All seawater pH measurements were carried out using a Radiometer Analytical MeterLab standard pH meter (PHM210), with a combined micro pH electrode (pHC3359-8, Radiometer Analytical MeterLab®) which was calibrated using Radiometer Analytical buffers (NBS). Seawater pH is denoted pH_{NBS} according to the NBS scale.

Total seawater CO_2 (TCO_2) was measured on 50 µL subsamples using a Corning 965 TCO_2 analyzer. The instrument was calibrated using dilutions (2.5-45 mM) of 2 g L^{-1} CO_2 standard (Reagecon). Calibrations were performed frequently every day. However, for the samples in the level-dependent exposure total alkalinity (TA) was used to calculate pCO_2. The apparent activity coefficient of H^+ (f_{H+}) was determined by four-point titration of 20 ml seawater by 5 ml standard acid with normalities in the range of 0.01-0.016N HCl which was used to calculate total alkalinity of seawater (Anderson et al. 1999). TA measurements gave the same results as TCO_2 measurements. Concentrations of seawater carbonate species were calculated from the measured data using the CO2SYS software (Pierrot et al. 2006) with the dissociation constants for NBS scale of Merbach et al. (1973) which have been refit by Dickson and Millero (1987).

2.3 Experimental setup

2.3.1 Exposure system

The exposure system, depicted in Fig 1, was a semi-flow through system with a total volume of approximately 300 L. Seawater saturated with CO_2 (pH_{NBS} 4.88) was produced through aeration with pure CO_2 gas (Yara, Mapcon). The CO_2 saturated seawater was mixed with fresh seawater at a set ratio in a CO_2 header tank to yield the desired exposure conditions. The mixed CO_2-seawater was pumped to a distribution chamber, which further distributed the exposure water to the individual exposure chambers. Excess water in the exposure chambers exited through an outlet at the bottom of the exposure chambers and flowed back into the header tank. Surplus water was eliminated through an overflow outlet.

The exposure chambers were 30 L glass aquariums with lids. In order to prevent physical interactions between the crabs, six separate cells were made from PVC for each aquarium with one crab placed in each cell. The cells were placed on top of a perforated PVC sheet elevated above the floor of the chambers to facilitate cleaning, and prevent animals from blocking the outlet. Water was supplied to each individual cell by connecting a manifold with six outlets to the inlet of the aquarium. Exposure chambers were cleaned every 14 days.

2.3.2 Time-dependent exposure (pH 6.9)

Several previous studies investigating the physiological responses to elevated pCO_2 in marine teleosts and invertebrates have applied exposure levels close to pCO_2 = 10,000 µatm , (~pH 6.85) to induce strain on the animals (Deigweiher et al. 2008; Pane and Barry 2007; Pörtner et al. 1998). The exposure conditions chosen for the time-dependent exposure was therefore close to these CO_2 levels (pH_{NBS} 6.94 ± 0.02 and pCO_2 = 7,573 ± 252 µatm). Crabs were subjected to these hypercapnic conditions for a period of 1 to 672 hours, to study responses related to time of exposure.

2.3.3 Level-dependent exposure (pH 7.4, 6.6 and 6.3)

Level-dependent responses to elevated pCO_2 was studied by exposing animals to levels ranging from worst case scenarios of ocean acidification (Caldeira and Wickett 2003) to levels reported from natural CO_2 seeps (Vetter and Smith 2005) which may represent conditions that may arise during a possible leakage from anthropogenic CO_2 reservoirs. Exposure to the three other CO_2 levels was done by creating a CO_2-seawater solution in the header tank which represented the highest exposure level (pH_{NBS} 6.33 ± 0.01, pCO_2 = 30,743 ± 691). This "stock solution" was diluted with fresh seawater to create the two remaining exposure levels (pH_{NBS} 6.62 ± 0.01, pCO_2 = 16,020 ± 393 µatm, and pH_{NBS} 7.40 ± 0.03, pCO_2

= 2674 ± 162 µatm). Animals were subjected to level-dependent exposure for two weeks (336 h). The different exposure groups are hereby referred to according to the pH of the exposure water. That is: the pH 7.4, pH 6.9, pH 6.6 and pH 6.3 group. The animals representing the pH 6.9 group were held under the experimental conditions explained above for the time series experiment.

Control animals were concurrently held in a near identical semi-flow through system supplied with normocapnic water (pH_{NBS} 8.08 ± 0.02, pCO_2 = 488 ± 14 µatm) instead of hypercapnic water.

Each exposure group was made up of 6 to 12 animals, with a similar distribution with respect to the size of the crabs between the groups.

2.4 Treatment of samples and data

2.4.1 Hemolymph

Hemolymph was collected from the infrabranchial sinus on unanaesthetised animals by puncturing the arthrodial membrane at the base of the fourth walking leg using a 0.5 mL ice-chilled Hamilton gas tight syringe pre-rinsed with ice-cold Crab ringer solution. Samples were spun down (12,000 rpm, 4 min) to remove hemocytes and stored at -20°C. Thawed hemolymph was filtered using pre-washed (2 x 0.1 M NaOH and 8 x dH_2O) centrifuge filters (Nanosep® 30 K red) at 10,000 g for 10 min at 4°C. The filtrate was frozen at -80°C, later lyophilized and stored at -80°C until analysis.

2.4.2 Gill and muscle tissue

Animals were killed by destroying the ventral ganglion and dissection was carried out on ice in a climate controlled room (10°C). Posterior gills (gill arch 9), and muscle tissue from one walking leg was collected immediately after the animal was killed. Tissues were flash frozen in liquid nitrogen and stored at -80°C. Samples were later lyophilized and treated according to a modified method of Wu et al. (2008), which is based on the extraction method of Bligh and Dyer (1959).

Briefly, lyophilized gill tissue (2-40 mg dry mass) was homogenized in 800 µL 2:1 methanol-water using a Precellys 24 bead-based homogenizer (Bertin Technologies). The entire homogenate was then transferred to 1.8 mL glass vials to which 400 µL chloroform and 175 µL distilled water was added. The sample was vortexed for 2 x 30 sec, left on ice for 15 min,

and spun down (2,000 g for 5 min). Following centrifugation the polar phase was transferred to a fresh tube, dried in a vacuum centrifuge for 1 h at 30°C and lyophilized for at least 24 h.

Lyophilized muscle tissue (11-59 mg dry mass) was homogenized in 1.5 mL 2:1 methanol-dH_2O. As it proved difficult to collect the entire muscle homogenate the sample was spun down (5,700 rpm, 0°C, 10 min) and the supernatant (~550 µL) was used instead. Apart from this the supernatant of muscle tissue was treated as described for gill tissue.

2.4.3 NMR sample preparation and acquisition of data

Gill extracts were dissolved in 550 µl of D_2O added hydrazine (10 mM) as a pH indicator and 2.2-Dimethyl-2-silapentane-5-sulfonate (DSS, 5 mM) as an internal standard. Sodium azide (NaN_3, 0.2 % w/v) was added to inhibit growth of bacteria. An aliquot of 500 µl was transferred to 5 mm NMR tubes and used for NMR spectroscopy in a Bruker DRX 500 NMR spectrometer resonating at 500.07 MHz which was fitted with a BBO-probe. For each sample 512 free induction decays with a spectral width of 6009 Hz were collected into 48076 data points. Water suppression was achieved using the Bruker noesygpgr1d pulse sequence.

Hemolymph samples and muscle extracts were dissolved in 200 µl D_2O (PBS buffered to pH 7.4) with 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP, 1 mM) and transferred to Bruker 3mm NMR tubes. NMR was performed with a Bruker DRU 600 US+ NMR spectrometer fitted with a BBO probe. For each sample 128 FIDs with a spectral width of 12019Hz were collected into 64K data points.

2.4.4 Multivariate analysis and quantification of NMR data

For multivariate analysis in Matlab (R2010b, MathWorks, Inc.), the ProMetab_v3_3 software was used to import data from TOPSPIN 1.3. The residual water resonance was omitted from import. The TMAO/betaine peak at 3.27 ppm was removed from the spectra before multivariate analyses. Information on betaine was still retained in the resonance at 3.90 ppm. The data were binned with a resolution of 0.005 ppm leaving 1900 data points for analysis. Principal components analysis (PCA) and partial least squares (PLS) regression analyses were performed in PLS-Toolbox 6.0 (Eigenvector Research, Inc.) within Matlab. The analyses were performed on both binned data (hemolymph and gills) and binned data subjected to a generalized log transformation (muscle) (Purohit et al. 2004). PLS models were cross validated by the venetian blinds cross validation in PLS-toolbox and a permutation test was performed on the models to test for over fitting.

For quantification in Chenomx (Chenomx, Inc) the Bruker FID file was processed in the Chenomx processor before quantification in the Chenomx profiler. For gill data, hydrazine was used for pH calibration, and for muscle and hemolymph the pH was set to 7.4 ± 0.05. Homarine is not included in the Chenomx library and was therefore quantified by integrating the homarine singlet (4.4 ppm) using TOPSPIN 1.3. The concentrations of metabolites were normalized for pre-extraction dry weight of tissues and hemolymph to reduce the variability due to differences in sample mass and thus, also the mass of the crab.

2.5 Statistics

All data were tested for normality using the Kolmogorov-Smirnov test. Data with non-normal distribution were tested using Kruskal-Wallis with Dunn's *post hoc* test. Changes in concentrations of individual metabolites between controls and time groups displaying significantly different LV was tested using Mann-Whitney. The level of significance was set at $p \leq 0.05$. All values are presented as means with standard error of means (± SEM).

3. Results

Figure 2 (A-C) shows typical ^{1}H-NMR spectra of polar metabolites obtained from hemolymph (A), muscle (B) and gill (C) extracts from *C. maenas*. According to these spectra the polar extracts of small organic metabolites mostly consists of amino acids (glycine, proline, glutamate, etc), quaternary ammonium derivatives (glycine-betaine, homarine and trigonelline) and glycolytic products (lactate). Peak assignments were based on chemical shifts and peak multiplicity, as well as the use of Chenomx NMR software suite. Identification of metabolites was further confirmed by ^{1}H, ^{1}H-COSY and ^{1}H, ^{13}C-HSQC 2D NMR in muscle and hemolymph extracts.

The ^{1}H-NMR spectral data sets of all extracts were initially subjected to PCA and some classification was found. However, PLS regression analysis gave a better description of the changes in the metabolome in response to hypercapnic exposure, and a clearer separation between exposure groups and controls was found.

3.1 Level-dependent exposure

Separation between controls and exposed animals was particularly conspicuous in the level-dependent exposure. The rank order of separation seemed to be more or less in accordance with the degree of exposure for all sample types (Fig 3, 4 and 5A).

3.1.1 Hemolymph

Scores from PLS regression of hemolymph extracts of animals exposed to different levels of pCO_2 are given in Fig. 3A. All exposure groups showed significantly different scores compared to the control group, and the rank order of separation occurred according to exposure level. As seen in Fig 3B the changes were most prominent along Latent variable 1 (LV1), where a higher level of lactate, taurine, glycine and glycine-betaine were found in control animals compared to exposed animals. Glutamine and lysine were predominantly higher in exposed compared to control animals (Fig. 3C). Along LV2 most metabolites were higher at negative values, indicating a higher level in exposed animals (Fig. 3D). The results for LV1 were in accordance with the results found after quantification of metabolites using the Chenomx software suite (Table 1). However, the large variations within the groups made it difficult to identify statistically differences in metabolite concentration. Lactate was found at a significantly lower level in the pH 7.4 group compared to control animals, while glycine was lower in both the pH 7.4 and pH 6.6 group. Leucine and glutamine was significantly higher in the pH 6.6 group, while in the pH 6.3 group lysine, N6-acetyllysine, and leucine were found to be significantly higher than in control animals.

3.1.2 Muscle

Compared to hemolymph an even clearer separation of the different groups was observed in the level-dependent experiment (Fig. 4A). A significant difference between scores of control animals and all four exposure groups was found along LV1 (Fig. 4B). According to the loading plot the level of alanine, arginine, proline, glutamate, glutamine and glycine was higher in controls compared to exposed animals, while malonate, taurine, glycine-betaine and homarine levels were higher in exposed animals (Fig. 4B). Quantification of metabolites revealed that the concentration of glycine was significantly lower in the pH 7.4 and pH 6.6 groups, proline was significantly lower in the pH 6.3 group, and threonine was significantly lower in the pH 6.6 group compared to controls (Table 2). A significantly higher concentration of trigonelline was found in the pH 6.6 and pH 6.3 group compared to controls.

3.1.3 Gill

The results for gill extracts are given in Fig. 5A-C. The score plot revealed that only the two highest exposure groups could be significantly separated from controls along LV1, while the pH 7.4 group was significantly different from controls along LV2 (Fig. 5B). From the loading plot it appeared that alanine, proline, glutamine, glutamate, dimethylamine, choline, glycine and homarine occurred at higher levels in control animals, while the levels of arginine, lysine, malonate, acetylcholine, taurine, and glycine-betaine were higher in exposed animals (Fig.

5C). Quantification revealed that the levels of glycine, sarcosine and dimethylamine were significantly lower in the pH 7.4 group compared to control animals. Lactate was significantly lower in the pH 6.6 group, while proline and trigonelline was lower in the pH 6.6 and pH 6.3 group compared to control animals (Table 3). The level of creatine in gills was significantly higher compared to controls in the pH 7.4 and pH 6.6 group.

3.2 Time-dependent exposure

Fig 6 describes the time-dependent trajectory of change in scores of the different sample types. It was apparent that in the short term phase (1-48 h) of exposure the changes in the scores progressed differently than in the "long-term" phase (96-672 h). Only hemolymph samples of animals subjected to 672 hours of exposure could be significantly separated from controls. Quantification of data revealed that separation of the 672 hour group was due to a significant increase in leucine, lysine and glutamine, while lactate was significantly reduced compared to controls (Table 1).

For muscle extracts the scores of animals subjected to 4 and 48 hours of exposure were somewhat separated from controls. No significant changes in metabolite concentrations was found in the 4 hour group, while proline was significantly lower compared to controls in the 48 hour group (Table 2).

PLS regression of gill extracts revealed that only the group subjected to 672 hours of exposure was significantly separated from controls (Fig 6C). Here, a number of metabolites were reduced compared to controls, while only taurine was significantly elevated (Table 3).

4. Discussion

The effects of elevated seawater CO_2 on marine organisms have received increased scientific attention in the recent years. Here we present one of the first studies to apply ^{1}H-NMR based metabolomics to investigate the effects of environmental hypercapnia. The objective was to study effects on the metabolic fingerprint and possibly elucidate biomarkers and/or protective mechanisms in *C. maenas* subjected to CO_2 stress.

The results of level-dependent exposure show that when using PLS regression on ^{1}H-NMR data it is possible to distinguish tissue and hemolymph extracts of hypercapnic *C. maenas* from those of control animals. In addition, some degree of separation could be identified

between the different exposure groups, and the rank order of separation was more or less in accordance to the level of exposure.

Only a few metabolites relevant for energy metabolism were identified. The observed changes in metabolic profiles were typically caused by important osmolytes such as amino acids and quaternary ammonium compounds. Marine invertebrates are generally slightly hyper-osmotic to their surroundings, and while the major osmolytes in the extracellular fluids are inorganic ions (Cl^-, Na^+) the osmotic pressure inside the cells is also regulated by organic osmolytes such as amino acids and quaternary ammonium compounds (Pierce 1982; Shoffeniels and Gilles 1970). This is most likely because high concentrations of inorganic ions can exert deleterious effects on enzymes. In fact, the organic osmolytes present in the highest levels (i.e. glycine, glycine-betaine and proline) are compatible solutes and can even enhance the performance of enzymes (Bowlus and Somero 1979).

Although the hemolymph generally contains very low levels of metabolites, changes may occur if metabolites are excreted into the hemolymph as it flows through the soft body tissues. The hemolymph level of glycine followed a similar pattern as observed in leg muscle and gills, with significantly lower levels in exposed animals compared to controls. The significantly higher level of hemolymph glutamine in exposed animals compared to controls may possibly reflect increased excretion from the cells as slight decreases in both muscle and gill glutamine was observed.

The concentrations of the essential amino acids leucine and lysine increased with increasing CO_2 level, possibly due to increased protein degradation with level of exposure. Gilles and Shoffeniels (1969) found that concentrations of essential amino acids of decapod nerves can be regulated by modifying the permeability of the cell membranes in response to changing salinities, suggesting a role in intracellular iso-osmotic regulation. Lysine has previously been found to play a role in iso-osmotic regulation in the hemolymph of the giant freshwater prawn *Macrobrachium rosenbergii* (Huong et al. 2001), and the changes observed in this study may thus reflect osmotic disturbance in hypercapnic *C. maenas*.

In the hemolymph of the blue crab, *Callinectes sapidus*, subjected to oxidative stress, Shock et al. (2010) found an increase in glucose and lactate levels, which are reliable indicators of biological stress and anaerobic respiration, respectively. While no change was found in hemolymph glucose level in the present study, a significant decrease in lactate was found in exposed animals compared to controls. This was unexpected, and also in disagreement with

the results of Pane and Barry (2007) who found a significant increase in lactate levels in Dungeness crabs (*Cancer magister*) subjected to short term environmental hypercapnia. Perhaps these findings suggest that the increase in lactate during hypercapnic exposure is transient, and do not occur during prolonged exposure.

A significant increase in trigonelline was found in muscle tissue. Trigonelline belongs to the quaternary ammonium compounds of which many are known to exert protective effects on enzymes when animals and plants are subjected to salt and temperature stress (Bowlus and Somero 1979). The concentration of proline was decreased in all exposure groups, and significantly lower compared to controls in the pH 6.3 group. The level of glycine fluctuated with level of exposure, but was significantly lower in the pH 7.4 and 6.6 group while the two remaining groups displayed levels similar to controls. A similar trend was found for succinate and while no groups were significantly different from controls, the succinate level of the pH 7.4 group was significantly lower compared to the pH 6.3 group (not shown). This may suggest that the level of CO_2 has an effect on the Krebs cycle.

In gill extracts of hypercapnic *C. maenas* there was a decrease in the concentrations of a majority of the metabolites, the most prominent reductions being for proline, glycine, trigonelline, dimethylamine and sarcosine. While the former four are important osmolytes (Shoffeniels and Gilles 1970), sarcosine is both the precursor and byproduct of glycine synthesis and catabolism (Bloch and Schoenheimer 1940), and may simply reflect glycine metabolism. The concentration of creatine increased significantly with level of exposure. However, the biological role of creatine in crustaceans is unclear. As no large difference in creatine phosphate level was found it is difficult to link this to changes in energy reserves.

The large contribution of glycine-betaine in the loading plot indicated a large increase with exposure to CO_2 in the gills. Although glycine-betaine increased some, its impact was somewhat reduced after quantification. This was possibly due to differences in the size of the animals within the groups, as the level of glycine-betaine has been found to be higher in larger animals (Størseth et al. 2009). The increase in glycine-betaine may be explained by an increased synthesis from glycine to exert protective effect on enzymes during CO_2 stress.

Changes in metabolic fingerprints were only apparent after long term exposure in hemolymph and gills, however, for muscle extracts changes were found in one of the short-term exposure groups. While the time-dependent changes in hemolymph and muscle were in accordance with the results from the level-dependent exposure, the gills of the four week exposure group

displayed a different pattern. Here, a pronounced decrease in a number of metabolites, including important osmolytes (proline, glutamate, arginine, alanine, etc.) and metabolites involved in energy metabolism (ATP/ADP, acetate, malonate), was accompanied by a large increase in taurine. Taurine is an important compatible osmolyte in marine crustaceans and fish (Huxtable 1992), and the increase may reflect a compensation for the loss of other osmolytes.

To our knowledge, the only other study to have employed ^{1}H-NMR spectroscopy to investigate the effects of hypercapnia is that of Lannig et al. (2010). They studied the synergistic effects of increased temperature and ocean acidification (pH 7.7) on oysters, *Crassostrea gigas*, and observed a depletion of alanine and ATP levels in mantle, while succinate was significantly increased in gills and hepatopancreas. Their results suggested a shift in the metabolic pathways towards increased gluconeogenesis due to an impairment of glycolysis. The results of the present study give no indication of such a change in energy metabolism, perhaps with the exception of gills in *C.maenas* subjected to prolonged exposure.

Although there was some variation between the metabolic responses of the different sample types, a common trait was a decrease in glycine and proline in exposed animals. Glycine and proline are, in addition to alanine, the two most important amino acids when it comes to intracellular iso-osmotic regulation in marine decapod crustaceans (Siebers et al. 1972). During hypo- and hyper-osmotic conditions the concentrations of these compounds are typically decreased and increased, respectively (Cobb et al. 1975; Shinagawa et al. 1995). While increased concentration is associated with increased *de novo* synthesis, a decrease is thought to reflect increased protein or amino acid catabolism. Cobb et al. (1975) found that only glycine and proline levels decreased in the shrimp *Penaeus stylirostris* at reduced salinity. The results of this study thus suggest that exposed animals experience effects similar to those seen during hypo-osmotic exposure, such as cell swelling. Decrease in proline has also been reported in starving tiger prawns (Smith and Dall 1991). Although the animals in this study were fed during the experiment, decrease in proline may indicate an impaired health status of exposed animals.

Previous studies have shown that acid-base regulation is associated with osmoregulation in crustaceans, as transport of hydrogen ions and bicarbonate ions occur through transport proteins that simultaneously transport sodium and chloride ions, respectively, into the cells (Cameron 1978; Truchot 1981; Wheatly and Henry 1992; Whiteley 2011; Whiteley et al.

1995). Thus, if acid-base regulation compromises the osmoregulation of the extracellular fluids, the result may be osmotic stress on the cells. In a parallel study we did, however, not find a significant decrease in the extracellular osmolality of hypercapnic *C. maenas* (Hammer et al. submitted manuscript). A decreased extracellular osmolality could have explained the symptoms of hypo-osmotic exposure found in the present study. Intracellular iso-osmotic regulation and acid-base regulation in decapods does, however, occur through ion transporting proteins similar to those found in the gills. It is, therefore, possible that the maintenance of intracellular pH during acidosis impairs intracellular iso-osmotic regulation.

The initial objective of this study was to reveal mechanisms involved in acid-base regulation and other CO_2 relevant processes. In mammals, acid-base regulation of metabolically produced acids mainly occurs in the kidneys, where ammonia (NH_3) produced from the deamination of glutamine and/or glutamate acts as a buffer for excess hydrogen ions. This process involves the enzymes phosphate-dependent glutaminase (PDG), which drives the formation of glutamate and ammonia from glutamine in the presence of phosphates, and glutamate dehydrogenase (GDH), which catalyzes the formation of α-ketoglutarate and ammonium in the presence of nicotineamide adine dinucleotide, respectively (Atkinson and Bourke 1984; Goldstein 1967).

No evidence of a similar mechanism for *C. maenas* could be found in response to CO_2-induced acidosis in the present study. If this mechansism was active, one would expect a reduction in glutamine, and possibly glutamate in tissue extracts of CO_2 exposed animals, as well as an increase in α-ketoglutarate. The former was found as both amino acids, particularly glutamine, was present at slightly lower levels in exposed animals. However, no significant difference compared to control animals was found after quantification. Also, we were not able to identify α-ketoglutarate in the NMR spectra of any of the samples. Perhaps a different result could be found if a similar study was performed on a different tissue, such as the hepatopancreas, which may exhibit higher activities of carbohydrate metabolism pathways (Giles et al. 1976).

Previous studies have, however, reported that both conditions of reduced salinities and environmental hypercapnia may result in increased ammonia excretion due to increased catabolism of deaminating amino acids. For instance, during hypo-osmotic condtions, it has been suggested that the NH_3 produced from deamination of amino acids bind to H^+ ions, and the resulting NH_4^+ is used as a counter ion to absorb Na^+ from the seawater (Mangum et al.

1976). During acidosis the same process is suggested to aid in acid excretion through neutralization and removal of H^+ (Lindinger et al. 1984). An increased catabolism of amino acids, such as glycine and threonine, to buffer H^+ in hypercapnic animals could thus possibly explain the reduced level of amino acid found in the present study.

The changes in metabolic profile in hypercapnic *C. maenas* seem to indicate an exhaustive stress effect rather than to reflect defensive mechanisms. Although changes in individual metabolites occurred, it was not possible to link these changes to specific biochemical pathways. This was either because no changes in relevant precursors or end products were found, or because the precursors/products could not be identified in the ^{1}H-NMR spectra. The argument of exhaustive effects is strengthened by the fact that no changes could be found in the early parts of the time-dependent exposure, with the exception of the muscle extracts of the 48 hour group.

C. maenas is known to have a high tolerance to environmental fluctuations, and was therefore expected to display some regulatory mechanisms in response to hypercapnia. However, it was not possible to identify any mechanisms from the results of this study. The results suggest that *C. maenas* settles at a new steady-state in response to physiological stress, possibly as an energy saving strategy. In fact, a recent study has shown that *C. maenas* does not increase the expression of its branchial ion regulatory system substantially in response to hypercapnic acidosis at pCO_2 = 4,000 μatm (Fehsenfeld et al. 2011). In the same study it was found that the gene expression pattern coincided with gene expression patterns from *C. maenas* subjected to hypo-osmotic conditions (Towle et al. 2011).

Conclusion

The results of this study did not reveal any new, or identify previously known mechanisms against CO_2-induced acidosis. Rather, the results indicated that prolonged exposure to elevated levels of CO_2 may impair intracellular iso-osmotic regulation, as a general decrease in the majority of intracellular osmolytes was found. This could possibly be a result of trade-offs between osmoregulation and acid-base regulation, as suggested in previous studies. It is also possible that the observed decrease in certain amino acids reflected increased catabolism, serving to produce ammonia to buffer surplus protons. The results could alternatively reflect an exhaustive effect of prolonged exposure to CO_2 stress. The study also revealed that ^{1}H-NMR spectroscopy based metabolomics can be used to separate animals exposed to environmental hypercapnia from control animals when PLS regression is applied to the data, thus working as a biomarker of exposure. While no obvious responses to hypercapnic exposure were found in the sample types used in the present study, other tissues, such as the hepatopancreas may reveal changes in important pathways involved, e.g. in carbohydrate metabolism. Also, investigating the metabolic responses of species less tolerant to hypercapnic conditions may reveal interesting results, as they would be expected to be more dependent on protective mechansism against the perturbing effects of CO_2.

Acknowledgements

This work was financed by the CO2ReMoVe project. The work was conducted at NTNU Centre of Fisheries and Aquaculture and SINTEF Fisheries and Aquaculture, both located at SeaLab in Trondheim. We would like to thank Anders Olsen, Dag Altin and Dr Erlend Kristiansen for practical assistance.

References

Anderson LG, Turner DR, Wedborg M, Dyrssen D (1999) Determination of total alkalinity and total dissolved inorganic carbon. In: Grasshoff K, Kremling K, Ehrardt M (eds) Methods of Seawater Analysis. Wiley-VCH Weinheim, pp 127-148

Atkinson DE, Bourke E (1984) The role of ureagenesis in pH homeostasis. Trends in Biochemical Sciences 9 (7):297-300

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37 (8):911-917

Bloch K, Schoenheimer R (1940) The biological demethylation of sarcosine to glycine. Journal of Biological Chemistry 135 (1):99-103

Bowlus RD, Somero GN (1979) Solute compatibility with enzyme function and structure: Rationales for the selection of osmotic agents and end-products of anaerobic metabolism in marine invertebrates. Journal of Experimental Zoology 208 (2):137-151. doi:10.1002/jez.1402080202
Bundy J, Davey M, Viant M (2009) Environmental metabolomics: a critical review and future perspectives. Metabolomics 5 (1):3-21. doi:10.1007/s11306-008-0152-0
Caldeira K, Wickett ME (2003) Oceanography: Anthropogenic carbon and ocean pH. Nature 425 (6956):365-365
Cameron JN (1978) Effects of hypercapnia on blood acid-base status, NaCl fluxes, and trans-gill potential in freshwater blue *Callinectes sapidus*. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 123 (2):137-141. doi:10.1007/bf00687841
Cobb BF, Conte FS, Edwards MA (1975) Free amino acids and osmoregulation in penaeid shrimp. Journal of Agricultural and Food Chemistry 23 (6):1172-1174. doi:10.1021/jf60202a015
Damen K, Faaij A, Turkenburg W (2006) Health, Safety and environmental risks of underground CO_2 storage – overview of mechanisms and current knowledge. Climatic Change 74 (1):289-318. doi:10.1007/s10584-005-0425-9
Deigweiher K, Koschnick N, Pörtner H-O, Lucassen M (2008) Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. Am J Physiol Regul Integr Comp Physiol 295 (5):R1660-1670. doi:10.1152/ajpregu.90403.2008
Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Research Part A Oceanographic Research Papers 34 (10):1733-1743
Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO_2 problem. Annual Review of Marine Science 1 (1):169-192. doi:doi:10.1146/annurev.marine.010908.163834
Dupont S, Havenhand J, Thorndyke W, Peck L, Thorndyke M (2008) Near-future level of CO2-driven ocean acidification radically affects larval survival and development in the brittlestar *Ophiothrix fragilis*. Marine Ecology Progress Series 373:285-294. doi:10.3354/meps07800
Fehsenfeld S, Kiko R, Appelhans Y, Towle DW, Zimmer M, Melzner F (2011) Effects of elevated seawater pCO_2 on gene expression patterns in the gills of the green crab, *Carcinus maenas*. Bmc Genomics 12. doi:48810.1186/1471-2164-12-488
Gazeau F, Gattuso JP, Dawber C, Pronker AE, Peene F, Peene J, Heip CHR, Middelburg JJ (2010) Effect of ocean acidification on the early life stages of the blue mussel *Mytilus edulis*. Biogeosciences 7 (7):2051-2060. doi:10.5194/bg-7-2051-2010
Giles IG, Poat PC, Munday KA (1976) Purification and properties of pyruvate kinase from the hepatopancreas of *Carcinus maenas*. Biochem J 153 (1):127-134
Gilles R, Schoffeniels E (1969) Isosmotic regulaton in isolated surviving nerves of *Eriocheir sinensis* Milne Edwards. Comparative Biochemistry and Physiology 31 (6):927-939
Goldstein L (1967) Pathways of glutamine deamination and their control in the rat kidney. American Journal of Physiology -- Legacy Content 213 (4):983-989
Gutknecht J, Bisson MA, Tosteson FC (1977) Diffusion of carbon dioxide through lipid bilayer membranes: effects of carbonic anhydrase, bicarbonate, and unstirred layers. The Journal of General Physiology 69 (6):779-794. doi:10.1085/jgp.69.6.779
Gutowska M, Melzner F, Langenbuch M, Bock C, Claireaux G, Pörtner H (2010a) Acid–base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 180 (3):323-335. doi:10.1007/s00360-009-0412-y
Gutowska M, Melzner F, Pörtner H, Meier S (2010b) Cuttlebone calcification increases during exposure to elevated seawater CO_2 in the cephalopod *Sepia officinalis*. Marine Biology 157 (7):1653-1663. doi:10.1007/s00227-010-1438-0
Hammer KM, Kristiansen E, Zachariassen KE (2011) Physiological effects of hypercapnia in the deep-sea bivalve *Acesta excavata* (Fabricius, 1779) (Bivalvia; Limidae). Marine Environmental Research 72 (3):135-142
Huong DTT, Yang W-J, Okuno A, Wilder MN (2001) Changes in free amino acids in the hemolymph of giant freshwater prawn *Macrobrachium rosenbergii* exposed to varying salinities:

relationship to osmoregulatory ability. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 128 (2):317-326
Huxtable RJ (1992) Physiological actions of taurine. Physiological Reviews 72 (1):101-163
Kikkawa T, Kita J, Ishimatsu A (2004) Comparison of the lethal effect of CO2 and acidification on red sea bream (*Pagrus major*) during the early developmental stages. Marine Pollution Bulletin 48 (1-2):108-110
Kurihara H (2008) Effects of CO2-driven ocean acidification on the early developmental stages of invertebrates. Marine Ecology Progress Series 373:275-284. doi:10.3354/meps07802
Lannig G, Eilers S, Pörtner HO, Sokolova IM, Bock C (2010) Impact of ocean acidification on energy metabolism of oyster *Crassostrea gigas* - changes in metabolic pathways and thermal response. Marine Drugs 8:2318-2339
Lindinger MI, Laurén DJ, McDonald G (1984) Acid-base balance in the sea mussel *Mytilus Edulis*. III. Effects of environmental hypercapnia on intra- and extracellular acid-base balance. Marine Biology Letter 5:371-381
Mangum CP, Silverthorn SU, Harris JL, Towle DW, Krall AR (1976) The relationship between blood PH, ammonia excretion and adaptation to low salinity in the blue crab *Callinectes sapidus*. Journal of Experimental Zoology 195 (1):129-136. doi:10.1002/jez.1401950112
McGaw I, Edgell T, Kaiser M (2011) Population demographics of native and newly invasive populations of the green crab *Carcinus maenas*. Marine Ecology Progress Series 430:235-240. doi:10.3354/meps09037
Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnology and Oceanography 18 (6)
Michaelidis B, Ouzounis C, Paleras A, Pörtner HO (2005) Effects of long-term moderate hypercapnia on acid-base balance and growth rate in marine mussels *Mytilus galloprovincialis*. Marine Ecology Progress Series 293:109-118. doi:10.3354/meps293109
Pane EF, Barry JP (2007) Extracellular acid-base regulation during short-term hypercapnia is effective in a shallow-water crab, but ineffective in a deep-sea crab. Marine Ecology Progress Series 334:1-9. doi:10.3354/meps334001
Pierce SK (1982) Invertebrate cell volume control mechanisms: A coordinated use of intracellular amino acids and inorganic ions as osmotic solute. The Biological Bulletin 163 (3):405-419
Pierrot D, Lewis E, Wallace DWR (2006) MS Excel Program Developed for CO2 System Calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee.
Purohit PV, Rocke DM, Viant MR, Woodruff DL (2004) Discrimination models using variance-stabilizing transformation of metabolomic NMR data. OMICS: A Journal of Integrative Biology 8 (2):118-130. doi:10.1089/1536231041388348
Pörtner HO, Reipschlager A, Heisler N (1998) Acid-base regulation, metabolism and energetics in *Sipunculus nudus* as a function of ambient carbon dioxide level. Journal of Experimental Biology 201 (1):43-55
Reipschlager A, Pörtner HO (1996) Metabolic depression during environmental stress: the role of extracellular versus intracellular pH in *Sipunculus nudus*. Journal of Experimental Biology 199 (8):1801-1807
Ries JB, Cohen AL, McCorkle DC (2009) Marine calcifiers exhibit mixed responses to CO_2-induced ocean acidification. Geology 37 (12):1131-1134. doi:10.1130/g30210a.1
Schock T, Stancyk D, Thibodeaux L, Burnett K, Burnett L, Boroujerdi A, Bearden D (2010) Metabolomic analysis of Atlantic blue crab, *Callinectes sapidus*, hemolymph following oxidative stress. Metabolomics 6 (2):250-262. doi:10.1007/s11306-009-0194-y
Shinagawa A, Suzuki T, Konosu S (1995) Preliminary studies on the effects of salinity on intracellular nitrogenous osmolytes in various tissues and hemolymph of the Japanese spiny lobster, *Panulirus Japonicus* (Von Siebold, 1824). Crustaceana 68 (2):129-137
Shoffeniels E, Gilles R (eds) (1970) Osmoregulation in aquatic arthropods vol Arthropoda, Part A. Chemical zoology, vol V. Academic Press, N.Y.
Siebers D, Lucu C, Sperling KR, Eberlein K (1972) Kinetics of osmoregulation in the crab *Carcinus maenas*. Marine Biology 17 (4):291-303. doi:10.1007/bf00366739

Smith DM, Dall W (1991) Metabolism of proline by the tiger prawn *Penaeus esculentus*. Marine Biology 110 (1):85-91. doi:10.1007/bf01313095
Størseth TR, Attramadal K, Øie G, Reitan KI, Galloway TF, Olsen Y (2009) Application of ^{1}H-NMR metabolomics to study cod larvae (*Gadus morhua* L). Paper presented at the Larvi '09 – Fish & Shellfish Larviculture Symposium, Gent,
Thomsen J, Melzner F (2010) Moderate seawater acidification does not elicit long-term metabolic depression in the blue mussel *Mytilus edulis*. Marine Biology 157 (12):2667-2676. doi:10.1007/s00227-010-1527-0
Towle DW, Henry RP, Terwilliger NB (2011) Microarray-detected changes in gene expression in gills of green crabs (*Carcinus maenas*) upon dilution of environmental salinity. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 6 (2):115-125. doi:10.1016/j.cbd.2010.11.001
Truchot J-P (1975) Action de l'hypercapnie sur l'état acide-base du sang chez le crabe *Carninus maenas* (L.) (Crustacé Décapode). Comptes rendus hebdomadaires des séances de l'Académie des sciences Série D: Sciences naturelles 280 (3):311-314
Truchot J-P (1986) Changes in the hemolymph acid-base state of the shore crab ,*Carcinus maenas*, exposed to simulated tidepool condtions. The Biological Bulletin 170 (3):506-518
Truchot JP (1981) The effect of water salinity and acid-base state on the blood acid-base balance in the euryhaline crab, *Carcinus maenas* (L.). Comparative Biochemistry and Physiology Part A: Physiology 68 (4):555-561. doi:10.1016/0300-9629(81)90361-3
Tuffnail W, Mills G, Cary P, Greenwood R (2009) An environmental ^{1}H NMR metabolomic study of the exposure of the marine mussel *Mytilus edulis* to atrazine, lindane, hypoxia and starvation. Metabolomics 5 (1):33-43. doi:10.1007/s11306-008-0143-1
Turley C, Nightingale P, Riley N, Widdicombe S, Joint I, Gallienne C, Lowe D, Goldson L, Beaumont N, Mariotte, al. e (2004) Literature Review: Environmental impacts of a gradual or catastrophic release of CO_2 into the marine environment following carbon dioxide capture. The UK Department for Environment, Food and Rural affairs, London
Vetter EW, Smith CR (2005) Insights into the ecological effects of deep ocean CO_2 enrichment: The impacts of natural CO_2 venting at Loihi seamount on deep sea scavengers. Journal of Geophysical Research 110 (C9):C09S13. doi:10.1029/2004jc002617
Viant MR (2007) Metabolomics of aquatic organisms: the new 'omics' on the block. Marine Ecology Progress Series 332:301-306. doi:10.3354/meps332301
Viant MR, Rosenblum ES, Tjeerdema RS (2003) NMR-Based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health. Environmental Science & Technology 37 (21):4982-4989. doi:10.1021/es034281x
Wheatly MG, Henry RP (1992) Extracellular and intracellular acid-base regulation in crustaceans. Journal of Experimental Zoology 263 (2):127-142. doi:10.1002/jez.1402630204
Whiteley N (2011) Physiological and ecological responses of crustaceans to ocean acidification. Marine Ecology Progress Series 430:257-271. doi:10.3354/meps09185
Whiteley N, Naylor J, Taylor E (1995) Extracellular and intracellular acid-base status in the freshwater crayfish, Austropotamobius pallipes between 1 and 12^{o}C. Journal of Exp Biol 198 (2):567-576
Wu H, Southam AD, Hines A, Viant MR (2008) High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. Analytical Biochemistry 372 (2):204-212
Wu H, Wang W-X (2011) Tissue-specific toxicological effects of cadmium in green mussels (*Perna viridis*): Nuclear magnetic resonance-based metabolomics study. Environmental Toxicology and Chemistry 30 (4):806-812. doi:10.1002/etc.446
Zeebe RE, Wolf-Gladrow D (2001) CO_2 in seawater: equilibrium, kinetics, isotopes. Elsevier, Amsterdam
Zhang L, Liu X, You L, Zhou D, Wang Q, Li F, Cong M, Li L, Zhao J, Liu D, Yu J, Wu H (2011a) Benzo(a)pyrene-induced metabolic responses in Manila clam *Ruditapes philippinarum* by proton nuclear magnetic resonance (^{1}H NMR) based metabolomics. Environmental Toxicology and Pharmacology 32 (2):218-225
Zhang L, Liu X, You L, Zhou D, Wu H, Li L, Zhao J, Feng J, Yu J (2011b) Metabolic responses in gills of Manila clam *Ruditapes philippinarum* exposed to copper using NMR-based metabolomics. Marine Environmental Research 72 (1-2):33-39

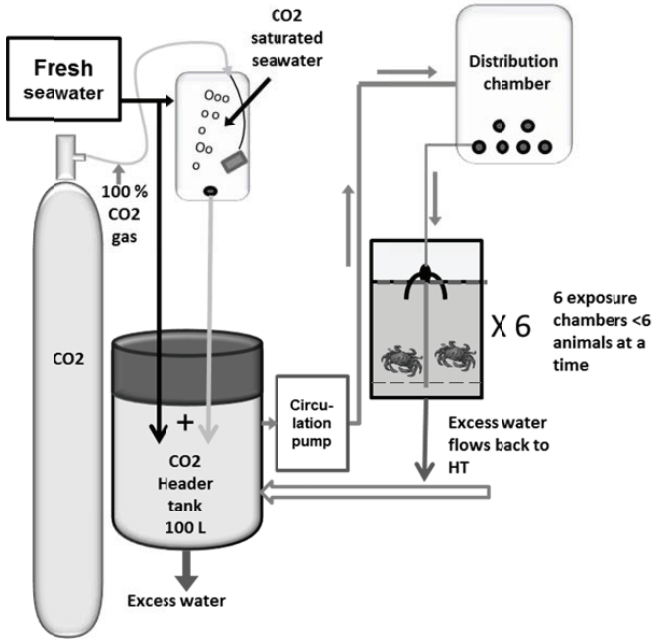

Fig. 1. Schematic overview of the CO_2 exposure set-up. CO_2 exposure water was created by mixing seawater saturated with 100% CO_2 gas with fresh, normocapnic (normal CO_2) seawater in a set ratio, depending on the desired exposure level. Arrows indicate the direction of water movement in the system. Black arrows indicate normocapnic seawater while grey, straight arrows indicate hypercapnic seawater.

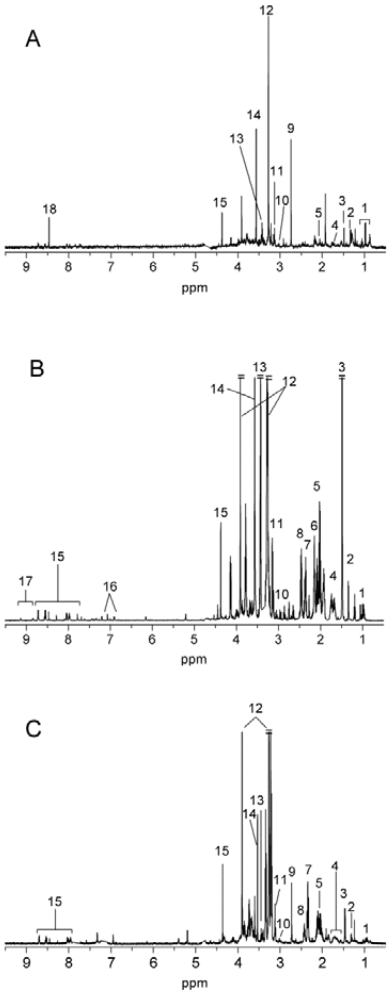

Fig. 2. Representative one-dimensional ^{1}H-NMR spectra of polar extracts in hemolymph (A), walking leg muscle (B) and gill (C) from *Carcinus maenas* held at normocapnic (control) conditions. Key to metabolites: 1 branched-chain amino acids (valine, isoleucine and leucine), 2 lactate, 3 alanine, 4 arginine, 5 proline, 6 methionine, 7 glutamate, 8 glutamine, 9 dimethylamine/sarcosine, 10 lysine, 11 malonate, 12 betaine, 13 taurine , 14 glycine, 15 homarine, 16 tyrosine, 17 trigonelline, 18 formate

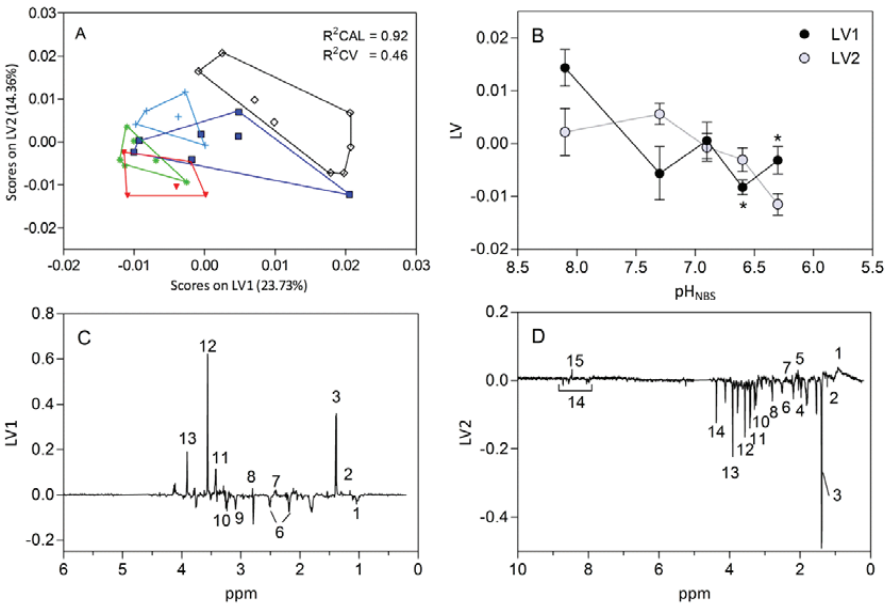

Fig. 3. Partial Least-Squares (PLS) regression model of data from hemolymph of *Carcinus maenas* showing (A) separation between controls (◊) and exposed to hypercapnic water with pH 7.4 (+), pH 6.9 (■), pH 6.6 (*) and pH 6.3 (▼) for two weeks; (B) dose-dependent changes according to LV1 (black circles) and LV2 (grey circles) where values are means ± SEM, n = 6-9, and asterisks indicate values significantly different from controls ($p \leq 0.05$); and loading plots for LV1 (C) and LV2 reflecting the changes in metabolites with exposure. Keys: (1) branched-chain amino acids (isoleucine, leucine and valine), (2) lactate, (3) alanine, (4) arginine, (5) proline (6) glutamine, (7) glutamate, (8) dimethylamine, (9) lysine, (10) glycerophosphocholine, (11) taurine, (12) glycine, (13) betaine, (14) homarine, (15) formate.

Table 1. Relative changes in metabolite concentrations in the hemolymph of *Carcinus maenas* exposed to elevated levels of CO_2

Metabolites	**pH**				**Time (h) pH 6.9**
	7.4	**6.9**	**6.6**	**6.3**	**672 h**
Osmoregulation					
Alanine	-	-	-	↓	-
Betaine	↓	-	-	-	-
Glutamine	-	-	↑	-	↑
Glycine	↓	-	↓	-	-
Proline	↓	↓	↓	↓	↓
Leucine	-	-	↑	↑	↑
Lysine	-	-	↑	↑	↑
N6-acetyllysine	↑	↑	↑	↑	-
Anaerobic metabolism					
Lactate	↓	↓	↓	-	↓

↑ = significantly increased, ↓ = significantly decreased and ↑ and ↓ = non-significant changes in metabolite concentrations ($p \leq 0.05$).

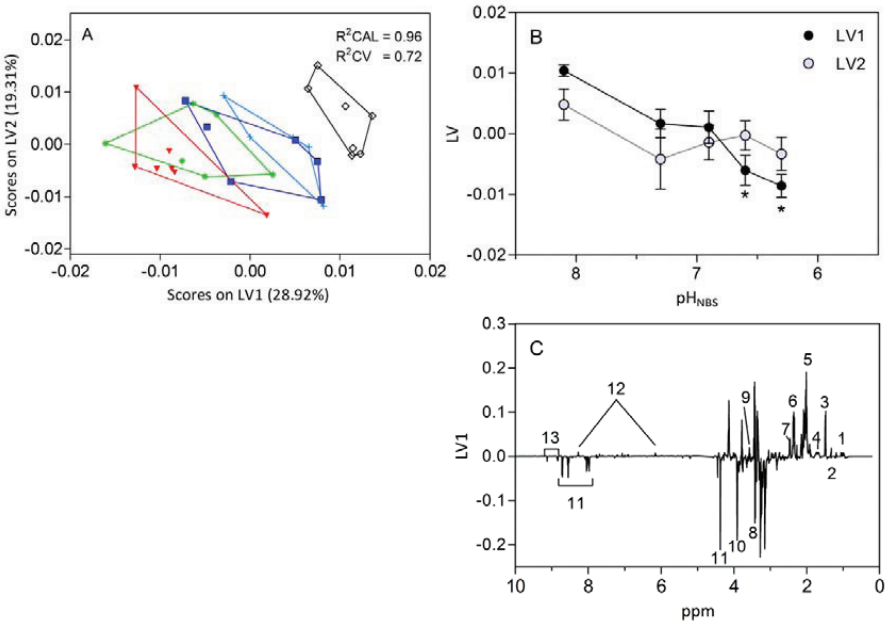

Fig. 4. Partial Least-Squares (PLS) regression model of gLog data from leg muscle extracts of *Carcinus maenas* showing (A) separation between controls (◊) and animals exposed to hypercapnic water with pH 7.4 (+), pH 6.9 (■), pH 6.6 (*) and pH 6.3 (▼) for two week; (B) dose-dependent changes according to LV1 (black circles) and LV2 (grey circles) where values are means ± SEM, n = 6-9, and asterisks indicate values significantly different from controls ($p \leq 0.05$); and loading plots for LV1 (C) reflecting the changes in metabolites with exposure. Keys: (1) branched-chain amino acids (isoleucine, leucine and valine), (2) lactate, (3) alanine, (4) arginine, (5) proline (6) glutamine, (7) glutamate, (8) taurine, (9) glycine, (10) betaine, (11) homarine, (12) ATP/ADP, (13) trigonelline.

Table 2. Relative changes in metabolite concentrations in muscle tissue of *Carcinus maenas* exposed to elevated levels of CO_2

Metabolites	**pH**				**Time (h) pH 6.9**
	pH 7.4	**pH 6.9**	**pH 6.6**	**pH 6.3**	**48 h**
Osmoregulation					
Alanine	↓	-	↓	-	↓
Betaine	-	-	↑	↑	-
Glutamine	-	-	-	-	-
Glycine	↓	-	↓	-	↑
Proline	↓	↓	↓	↓	↓
Glutamate	↓	↓	↓	↓	-
Homarine	↑	↑	↑	↑	-
Trigonelline	↑	↑	↑	↑	-
Threonine	↓	↓	↓	↓	-
Anaerobic metabolism					-
Lactate	-	-	-	↑	-
Energy metabolism					-
Glucose	-	-	-	-	-
Succinate	↓	-	↓	-	-

↑ = significantly increased, ↓ = significantly decreased and ↑ and ↓ = non-significant changes in metabolite concentrations ($p \leq 0.05$).

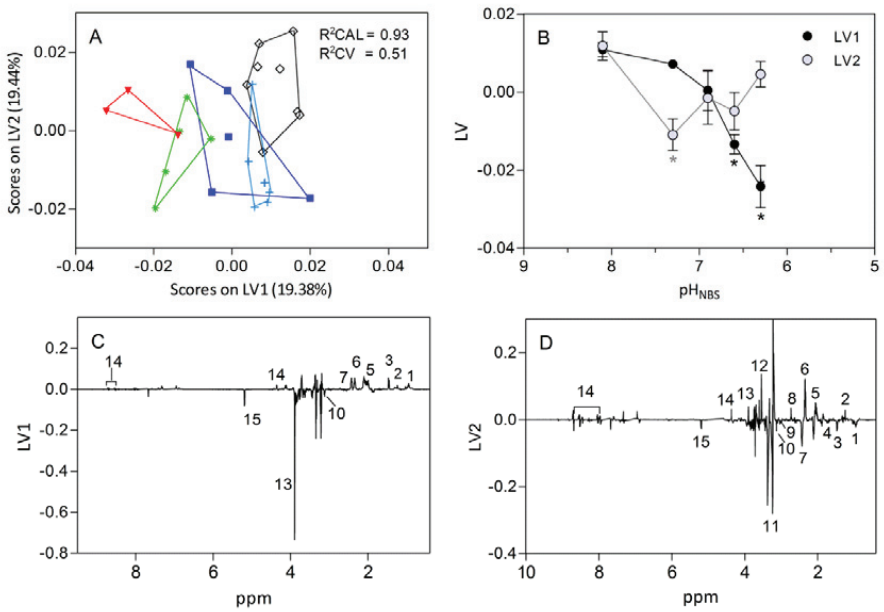

Fig. 5. Partial Least-Squares (PLS) regression model of data from gill extracts of *Carcinus maenas* showing (A) separation between controls (◊) and animals exposed to hypercapnic water with pH 7.4 (+), pH 6.9 (■), pH 6.6 (*) and pH 6.3 (▼) for two weeks (B) dose-dependent changes according to LV1 (black circles) and LV2 (grey circles) where values are means ± SEM, n = 6-9, and asterisks indicate values significantly different from controls ($p \leq 0.05$); and loading plots for LV1 (C) and LV2 (D) reflecting the changes in metabolites with exposure. Keys: (1) branched-chain amino acids (isoleucine, leucine and valine), (2) lactate, (3) alanine, (4) arginine, (5) proline, (6) glutamate, (7) glutamine (8) dimethylamine, (9) lysine (10) malonate, (11) taurine, (12) glycine, (13) betaine, (14) homarine, (15) unidentified compound (5.2 ppm).

Table 3. Relative changes in metabolite concentrations in gill tissue of *Carcinus maenas* exposed to elevated levels of CO_2

Metabolites	**pH**			**Time (h) pH 6.9**
	pH 7.4	**pH 6.6**	**pH 6.3**	**672**
Osmolytes				
Alanine	-	↓	-	↓
Arginine	↑	-	-	↓
Betaine	-	↑	↑	↓
Glutamine	↑	↓	↓	↓
Glutamate	↓	↓	↓	↓
Glycine	↓	↓	↓	↓
Proline	↓	↓	↓	↓
Dimethylamine	↓	↓	↓	-
Homarine	↓	↓	↓	
Methionine	-	-	↓	↓
Leucine	-	↓	↓	↓
Sarcosine	↓	↓	↓	-
Taurine				↑
Trigonelline	↓	↓	↓	↓
Valine	↑	↓	-	↓
Anaerob metabolism				
Lactate	↓	↓	↓	↓
Energy metabolism				
ATP	-	-	↓	↓
Creatine	↑	↑	↑	↓
Acetate	-	-	-	↓
Glucose	-	↑	↑	-
Malonate	-	↓	-	↓

↑ = significantly increased, ↓ = significantly decreased and ↑ and ↓ = non-significant changes in metabolite concentrations ($p \leq 0.05$).

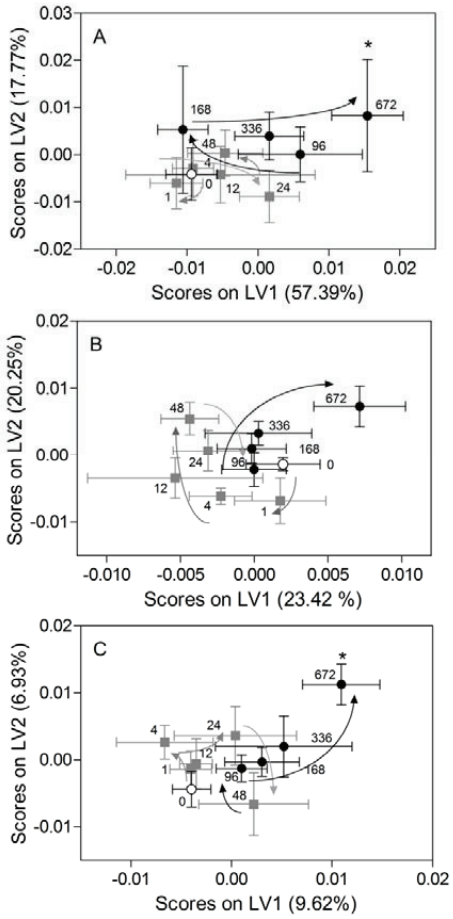

Fig. 6. Time-based trajectory plot of scores from hemolymph (A), leg muscle (B) and gill (C) extracts of *Carcinus maenas* subjected to control conditions (open circle) and short-term (1-48 h, grey squares) and prolonged (96-672 h, closed circles) exposure to environmental hypercapnia (pH_{NBS} 6.94, pCO_2 = 7,600 µatm). Numbers indicate exposure times, and arrows indicate directions of changes. Asterisks denote values significantly different from controls according to LV1. Values are means ± SEM, n = 3-9.

Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos Zoology	Breeding events of birds in relation to spring temperature and environmental phenology
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation
1984	Eivin Røskaft	Dr. philos Zoology	Sociobiological studies of the rook *Corvus frugilegus*
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos Zoology	Biochemical genetic studies in fish
1985	John Solem	Dr. philos Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains
1985	Randi E. Reinertsen	Dr. philos Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds
1986	Bernt-Erik Sæther	Dr. philos Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach
1986	Torleif Holthe	Dr. philos Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna
1987	Helene Lampe	Dr. scient Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires
1987	Olav Hogstad	Dr. philos Zoology	Winter survival strategies of the Willow tit *Parus montanus*
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway

1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
1987	Bjørn Åge Tømmerås	Dr. scient. Zoolog	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction
1988	Hans Christian Pedersen	Dr. philos Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care
1988	Tor G. Heggberget	Dr. philos Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure
1988	Marianne V. Nielsen	Dr. scient Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*)
1988	Ole Kristian Berg	Dr. scient Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.)
1989	John W. Jensen	Dr. philos Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth
1989	Helga J. Vivås	Dr. scient Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose *Alces alces*
1989	Reidar Andersen	Dr. scient Zoology	Interactions between a generalist herbivore, the moose *Alces alces*, and its winter food resources: a study of behavioural variation
1989	Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture
1990	Bengt Finstad	Dr. scient Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season
1990	Hege Johannesen	Dr. scient Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung
1990	Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990	Arne Johan Jensen	Dr. philos Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmion (*Salmo salar*) and brown trout (*Salmo trutta*): A summary of studies in Norwegian streams
1990	Tor Jørgen Almaas	Dr. scient Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues
1990	Magne Husby	Dr. scient Zoology	Breeding strategies in birds: Experiments with the Magpie *Pica pica*
1991	Tor Kvam	Dr. scient Zoology	Population biology of the European lynx (*Lynx lynx*) in Norway
1991	Jan Henning L'Abêe Lund	Dr. philos Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular
1991	Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991	Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants
1991	Trond Nordtug	Dr. scient Zoology	Reflctometric studies of photomechanical adaptation in superposition eyes of arthropods
1991	Thyra Solem	Dr. scient Botany	Age, origin and development of blanket mires in Central Norway

1991	Odd Terje Sandlund	Dr. philos Zoology	The dynamics of habitat use in the salmonid genera *Coregonus* and *Salvelinus*: Ontogenic niche shifts and polymorphism
1991	Nina Jonsson	Dr. philos	Aspects of migration and spawning in salmonids
1991	Atle Bones	Dr. scient Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992	Torgrim Breiehagen	Dr. scient Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher
1992	Anne Kjersti Bakken	Dr. scient Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (*Phleum pratense* L.)
1992	Tycho Anker-Nilssen	Dr. scient Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins *Fratercula arctica*
1992	Bjørn Munro Jenssen	Dr. philos Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks
1992	Arne Vollan Aarset	Dr. philos Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993	Geir Slupphaug	Dr. scient Botany	Regulation and expression of uracil-DNA glycosylase and O^6-methylguanine-DNA methyltransferase in mammalian cells
1993	Tor Fredrik Næsje	Dr. scient Zoology	Habitat shifts in coregonids.
1993	Yngvar Asbjørn Olsen	Dr. scient Zoology	Cortisol dynamics in Atlantic salmon, *Salmo salar* L.: Basal and stressor-induced variations in plasma levels ans some secondary effects.
1993	Bård Pedersen	Dr. scient Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993	Ole Petter Thangstad	Dr. scient Botany	Molecular studies of myrosinase in Brassicaceae
1993	Thrine L. M. Heggberget	Dr. scient Zoology	Reproductive strategy and feeding ecology of the Eurasian otter *Lutra lutra*.
1993	Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993	Kåre Haugan	Dr. scient Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994	Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (*Gallinago media*): Male mating success and female behaviour at the lek
1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994	Nils Røv	Dr. scient Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant *Phalacrocorax carbo carbo*
1994	Annette-Susanne Hoepfner	Dr. scient Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (*Rubus idaeus* L.)
1994	Inga Elise Bruteig	Dr. scient Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers
1994	Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994	Morten Bakken	Dr. scient Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, *Vulpes vulpes*

1994	Arne Moksnes	Dr. philos Zoology	Host adaptations towards brood parasitism by the Cockoo
1994	Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss *Dicranum majus* Sm. as influenced by nitrogen supply
1994	Torbjørn Forseth	Dr. scient Zoology	Bioenergetics in ecological and life history studies of fishes.
1995	Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions
1995	Hanne Christensen	Dr. scient Zoology	Determinants of Otter *Lutra lutra* distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink *Mustela vision*
1995	Svein Håkon Lorentsen	Dr. scient Zoology	Reproductive effort in the Antarctic Petrel *Thalassoica antarctica*; the effect of parental body size and condition
1995	Chris Jørgen Jensen	Dr. scient Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995	Martha Kold Bakkevig	Dr. scient Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport
1995	Vidar Moen	Dr. scient Zoology	Distribution patterns and adaptations to light in newly introduced populations of *Mysis relicta* and constraints on Cladoceran and Char populations
1995	Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the *Schistidium apocarpum* complex in Norway and Sweden
1996	Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; inpact fish-bacterial interactions on growth and survival of larvae
1996	Ola Ugedal	Dr. scient Zoology	Radiocesium turnover in freshwater fishes
1996	Ingibjørg Einarsdottir	Dr. scient Zoology	Production of Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*): A study of some physiological and immunological responses to rearing routines
1996	Christina M. S. Pereira	Dr. scient Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation
1996	Jan Fredrik Børseth	Dr. scient Zoology	The sodium energy gradients in muscle cells of *Mytilus edulis* and the effects of organic xenobiotics
1996	Gunnar Henriksen	Dr. scient Zoology	Status of Grey seal *Halichoerus grypus* and Harbour seal *Phoca vitulina* in the Barents sea region
1997	Gunvor Øie	Dr. scient Bothany	Eevalution of rotifer *Brachionus plicatilis* quality in early first feeding of turbot *Scophtalmus maximus* L. larvae
1997	Håkon Holien	Dr. scient Botany	Studies of lichens in spurce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters
1997	Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming
1997	Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture
1997	Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher
1997	Torgeir Nygård	Dr. scient Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitors

1997 Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper *Cinclus cinclus* in southern Norway
1997 Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (*Hylobius abietis*), analysed by gas chromatography linked to electrophysiology and to mass spectrometry
1997 Rolv Lundheim	Dr. scient Zoology	Adaptive and incidental biological ice nucleators
1997 Arild Magne Landa	Dr. scient Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation
1997 Kåre Magne Nielsen	Dr. scient Botany	An evolution of possible horizontal gene transfer from plants to sail bacteria by studies of natural transformation in *Acinetobacter calcoacetius*
1997 Jarle Tufto	Dr. scient Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997 Trygve Hesthagen	Dr. philos Zoology	Population responces of Arctic charr (*Salvelinus alpinus* (L.)) and brown trout (*Salmo trutta* L.) to acidification in Norwegian inland waters
1997 Trygve Sigholt	Dr. philos Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (*Salmo salar*) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997 Jan Østnes	Dr. scient Zoology	Cold sensation in adult and neonate birds
1998 Seethaledsumy Visvalingam	Dr. scient Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins
1998 Thor Harald Ringsby	Dr. scient Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998 Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (*Alces alces*) population: consequences of harvesting in a variable environment
1998 Sigurd Mjøen Saastad	Dr. scient Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity
1998 Bjarte Mortensen	Dr. scient Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro
1998 Gunnar Austrheim	Dr. scient Botany	Plant biodiversity and land use in subalpine grasslands. – A conservtaion biological approach
1998 Bente Gunnveig Berg	Dr. scient Zoology	Encoding of pheromone information in two related moth species
1999 Kristian Overskaug	Dr. scient Zoology	Behavioural and morphological characteristics in Northern Tawny Owls *Strix aluco*: An intra- and interspecific comparative approach
1999 Hans Kristen Stenøien	Dr. scient Bothany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)
1999 Trond Arnesen	Dr. scient Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway
1999 Ingvar Stenberg	Dr. scient Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker *Dendrocopos leucotos*
1999 Stein Olle Johansen	Dr. scient Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis

1999	Trina Falck Galloway	Dr. scient Zoology	Muscle development and growth in early life stages of the Atlantic cod (*Gadus morhua* L.) and Halibut (*Hippoglossus hippoglossus* L.)
1999	Marianne Giæver	Dr. scient Zoology	Population genetic studies in three gadoid species: blue whiting (*Micromisistius poutassou*), haddock (*Melanogrammus aeglefinus*) and cod (*Gradus morhua*) in the North-East Atlantic
1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*
1999	Ingrid Bysveen Mjølnerød	Dr. scient Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001	Jan Ove Evjemo	Dr. scient Zoology	Production and nutritional adaptation of the brine shrimp *Artemia* sp. as live food organism for larvae of marine cold water fish species
2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems

2001	Ingebrigt Uglem	Dr. scient Zoology	Male dimorphism and reproductive biology in corkwing wrasse (*Symphodus melops* L.)
2001	Bård Gunnar Stokke	Dr. scient Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002	Ronny Aanes	Dr. scient	Spatio-temporal dynamics in Svalbard reindeer (*Rangifer tarandus platyrhynchus*)
2002	Mariann Sandsund	Dr. scient Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and the Ral GTPase from *Drosophila melanogaster*
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar

2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*)
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	ph.d Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	ph.d Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	ph.d Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	ph.d Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Anders Gravbrøt Finstad	ph.d Biology	Salmonid fishes in a changing climate: The winter challenge
2005	Shimane Washington Makabu	ph.d Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation

2006 Kari Mette Murvoll	ph.d Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006 Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006 Nils Egil Tokle	ph.d Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006 Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006 Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006 Johanna Järnegren	ph.d Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006 Bjørn Henrik Hansen	ph.d Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006 Vidar Grøtan	ph.d Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006 Jafari R Kideghesho	ph.d Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006 Anna Maria Billing	ph.d Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006 Henrik Pärn	ph.d Biology	Female ornaments and reproductive biology in the bluethroat
2006 Anders J. Fjellheim	ph.d Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006 P. Andreas Svensson	ph.d Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007 Sindre A. Pedersen	ph.d Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007 Kasper Hancke	ph.d Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007 Tomas Holmern	ph.d Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007 Kari Jørgensen	ph.d Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007 Stig Ulland	ph.d Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, (*Mamestra brassicae* L.) (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007 Snorre Henriksen	ph.d Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007 Roelof Frans May	ph.d Biology	Spatial Ecology of Wolverines in Scandinavia
2007 Vedasto Gabriel Ndibalema	ph.d Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania

2007	Julius William Nyahongo	ph.d Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	ph.d Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	ph.d Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning
2007	Anne Skjetne Mortensen	ph.d Biology	Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008	Brage Bremset Hansen	ph.d Biology	The Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008	Jiska van Dijk	ph.d Biology	Wolverine foraging strategies in a multiple-use landscape
2008	Flora John Magige	ph.d Biology	The ecology and behaviour of the Masai Ostrich (Struthio camelus massaicus) in the Serengeti Ecosystem, Tanzania
2008	Bernt Rønning	ph.d Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, (*Taeniopygia guttata*)
2008	Sølvi Wehn	ph.d Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008	Trond Moxness Kortner	ph.d Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (*Gadus morhua*): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008	Katarina Mariann Jørgensen	Dr.Scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008	Tommy Jørstad	ph.d Biology	Statistical Modelling of Gene Expression Data
2008	Anna Kusnierczyk	ph.d Bilogy	*Arabidopsis thaliana* Responses to Aphid Infestation
2008	Jussi Evertsen	ph.d Biology	Herbivore sacoglossans with photosynthetic chloroplasts
2008	John Eilif Hermansen	ph.d Biology	Mediating ecological interests between locals and globals by means of indicators. A study attributed to the asymmetry between stakeholders of tropical forest at Mt. Kilimanjaro, Tanzania
2008	Ragnhild Lyngved	ph.d Biology	Somatic embryogenesis in *Cyclamen persicum.* Biological investigations and educational aspects of cloning
2008	Line Elisabeth Sundt-Hansen	ph.d Biology	Cost of rapid growth in salmonid fishes
2008	Line Johansen	ph.d Biology	Exploring factors underlying fluctuations in white clover populations – clonal growth, population structure and spatial distribution
2009	Astrid Jullumstrø Feuerherm	ph.d Biology	Elucidation of molecular mechanisms for pro-inflammatory phospholipase A2 in chronic disease

2009	Pål Kvello	ph.d Biology	Neurons forming the network involved in gustatory coding and learning in the moth *Heliothis virescens:* Physiological and morphological characterisation, and integration into a standard brain atlas
2009	Trygve Devold Kjellsen	ph.d Biology	Extreme Frost Tolerance in Boreal Conifers
2009	Johan Reinert Vikan	ph.d Biology	Coevolutionary interactions between common cuckoos *Cuculus canorus* and *Fringilla* finches
2009	Zsolt Volent	ph.d Biology	Remote sensing of marine environment: Applied surveillance with focus on optical properties of phytoplankton, coloured organic matter and suspended matter
2009	Lester Rocha	ph.d Biology	Functional responses of perennial grasses to simulated grazing and resource availability
2009	Dennis Ikanda	ph.d Biology	Dimensions of a Human-lion conflict: Ecology of human predation and persecution of African lions (*Panthera leo*) in Tanzania
2010	Huy Quang Nguyen	ph.d Biology	Egg characteristics and development of larval digestive function of cobia (*Rachycentron canadum*) in response to dietary treatments -Focus on formulated diets
2010	Eli Kvingedal	ph.d Biology	Intraspecific competition in stream salmonids: the impact of environment and phenotype
2010	Sverre Lundemo	ph.d Biology	Molecular studies of genetic structuring and demography in *Arabidopsis* from Northern Europe
2010	Iddi Mihijai Mfunda	ph.d Biology	Wildlife Conservation and People's livelihoods: Lessons Learnt and Considerations for Improvements. Tha Case of Serengeti Ecosystem, Tanzania
2010	Anton Tinchov Antonov	ph.d Biology	Why do cuckoos lay strong-shelled eggs? Tests of the puncture resistance hypothesis
2010	Anders Lyngstad	ph.d Biology	Population Ecology of *Eriophorum latifolium*, a Clonal Species in Rich Fen Vegetation
2010	Hilde Færevik	ph.d Biology	Impact of protective clothing on thermal and cognitive responses
2010	Ingerid Brænne Arbo	ph.d Medical technology	Nutritional lifestyle changes – effects of dietary carbohydrate restriction in healthy obese and overweight humans
2010	Yngvild Vindenes	ph.d Biology	Stochastic modeling of finite populations with individual heterogeneity in vital parameters
2010	Hans-Richard Brattbakk	ph.d Medical technology	The effect of macronutrient composition, insulin stimulation, and genetic variation on leukocyte gene expression and possible health benefits
2011	Geir Hysing Bolstad	ph.d Biology	Evolution of Signals: Genetic Architecture, Natural Selection and Adaptive Accuracy
2011	Karen de Jong	ph.d Biology	Operational sex ratio and reproductive behaviour in the two-spotted goby (*Gobiusculus flavescens*)
2011	Ann-Iren Kittang	ph.d Biology	*Arabidopsis thaliana* L. adaptation mechanisms to microgravity through the EMCS MULTIGEN-2 experiment on the ISS:– The science of space experiment integration and adaptation to simulated microgravity
2011	Aline Magdalena Lee	ph.d Biology	Stochastic modeling of mating systems and their effect on population dynamics and genetics
2011	Christopher Gravningen Sørmo	ph.d Biology	Rho GTPases in Plants: Structural analysis of ROP GTPases; genetic and functional studies of MIRO GTPases in *Arabidopsis thaliana*

2011	Grethe Robertsen	ph.d Biology	Relative performance of salmonid phenotypes across environments and competitive intensities
2011	Line-Kristin Larsen	ph.d Biology	Life-history trait dynamics in experimental populations of guppy (*Poecilia reticulata*): the role of breeding regime and captive environment
2011	Maxim A. K. Teichert	ph.d Biology	Regulation in Atlantic salmon (*Salmo salar*): The interaction between habitat and density
2011	Torunn Beate Hancke	ph.d Biology	Use of Pulse Amplitude Modulated (PAM) Fluorescence and Bio-optics for Assessing Microalgal Photosynthesis and Physiology
2011	Sajeda Begum	ph.d Biology	Brood Parasitism in Asian Cuckoos: Different Aspects of Interactions between Cuckoos and their Hosts in Bangladesh
2011	Kari J. K. Attramadal	ph.d Biology	Water treatment as an approach to increase microbial control in the culture of cold water marine larvae
2011	Camilla Kalvatn Egset	ph.d Biology	The Evolvability of Static Allometry: A Case Study
2011	AHM Raihan Sarker	ph.d Biology	Conflict over the conservation of the Asian elephant (*Elephas maximus*) in Bangladesh
2011	Gro Dehli Villanger	ph.d Biology	Effects of complex organohalogen contaminant mixtures on thyroid hormone homeostasis in selected arctic marine mammals
2011	Kari Bjørneraas	ph.d Biology	Spatiotemporal variation in resource utilisation by a large herbivore, the moose
2011	John Odden	ph.d Biology	The ecology of a conflict: Eurasian lynx depredation on domestic sheep
2011	Simen Pedersen	ph.d Biology	Effects of native and introduced cervids on small mammals and birds
2011	Mohsen Falahati-Anbaran	ph.d Biology	Evolutionary consequences of seed banks and seed dispersal in *Arabidopsis*
2012	Jakob Hønborg Hansen	ph.d Biology	Shift work in the offshore vessel fleet: circadian rhythms and cognitive performance
2012	Elin Noreen	ph.d Biology	Consequences of diet quality and age on life-history traits in a small passerine bird
2012	Irja Ida Ratikainen	ph.d Biology	Theoretical and empirical approaches to studying foraging decisions: the past and future of behavioural ecology
2012	Aleksander Handå	ph.d Biology	Cultivation of mussels (*Mytilus edulis):*Feed requirements, storage and integration with salmon (*Salmo salar*) farming
2012	Morten Kraabøl	ph.d Biology	Reproductive and migratory challenges inflicted on migrant brown trour (*Salmo trutta* L) in a heavily modified river
2012	Jisca Huisman	ph.d Biology	Gene flow and natural selection in Atlantic salmon
2012	Maria Bergvik	ph.d Biology	Lipid and astaxanthin contents and biochemical post-harvest stability in *Calanus finmarchicus*
2012	Bjarte Bye Løfaldli	ph.d Biology	Functional and morphological characterization of central olfactory neurons in the model insect *Heliothis virescens*.
2012	Karen Marie Hammer	ph.d Biology	Acid-base regulation and metabolite responses in shallow- and deep-living marine invertebrates during environmental hypercapnia