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Impact of high CO_2 concentrations on marine life: Molecular mechanisms and physiological adaptations of pH and ion regulation in marine fish

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Impact of high CO_2 concentrations on marine life: Molecular mechanisms and physiological adaptations of pH and ion regulation in marine fish

Auswirkungen erhöhter CO_2-Konzentrationen auf das Leben im Meer: Molekulare Mechanismen physiologischer Anpassungen der pH- und Ionenregulation mariner Fische

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List of abbreviations

AE	Cl^-/HCO_3^--exchanger (**A**nion **E**xchanger)
ATP	**A**denosine **tri**-**p**hosphate
AWI	**A**lfred **W**egener **I**nstitute
CCS	**C**arbon **C**apture and **S**torage
DIC	**D**issolved **I**norganic **C**arbon
DTT	**D**i**t**hio**t**hreitol
EST	**E**xpressed **S**equence **T**ag
HA	$\mathbf{H^+}$-**A**TPase
IPCC	**I**ntergovernmental **P**anel on **C**limate **C**hange
ITR	**I**nverted **T**erminal **R**epeats
MAPK	**M**itogen-**A**ctivated **P**rotein **K**inase
MHC	**M**ajor **H**istocompatibility **C**omplex
MRC	**M**itochondrion-**r**ich **C**ell
NBC	$\mathbf{Na^+}/HCO_3^-$ (**bi**carbonate)-**c**otransporter
NHE	$\mathbf{Na^+}/\mathbf{H^+}$-**e**xchanger
NKA	$\mathbf{Na^+}/\mathbf{K^+}$-**A**TPase
NKCC	$\mathbf{Na^+}/\mathbf{K^+}/\mathbf{2Cl^-}$-contransporter
P_{CO_2}	$\mathbf{CO_2}$ partial **p**ressure
ppm	**p**arts **p**er **m**illion
PCR	**P**olymerase **C**hain **R**eaction
RLM-RACE	**R**NA **L**igase **M**ediated - **R**apid **A**mplification of **c**DNA **E**nds
RT	**R**everse **T**ranscription
SMR	**S**tandard **M**etabolic **R**ate
TCA	**T**ri**C**arboxylic **A**cid
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol

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Summary

The world's oceans serve as a buffer system for atmospheric CO_2 concentrations. However, the buffer capacity of the oceans is limited, and the imbalance caused by the additional anthropogenic CO_2 input has already led to a measurable acidification of the oceans. Certainly, these physicochemical changes affect marine organisms and their ecosystems. Within limits, fish are able to acclimate to an elevated CO_2 concentration (hypercapnia) and the accompanying pH decrease by regulating their internal ion composition and acid-base parameters. The aim of this thesis was to study the impact of CO_2 on the mechanisms of ion regulation and on energy metabolism, as well as the patterns of genetic regulation during acute (24 hours) and long-term (six weeks) acclimation to hypercapnia (10,000 ppm CO_2) in marine fish. The experiments focused on the gills, where over 90 % of the ion regulation takes place.

Elevated CO_2 concentrations have no obvious impact on the standard metabolic rate of the whole animal, as demonstrated in the North Sea eelpout *Zoarces viviparus*. The resting rate, which was evaluated from oxygen consumption measurements, remained stable over four days of hypercapnic incubation. Furthermore, no unusual behavioral or other stress indicators were detectable, suggesting a perfect acclimation capacity of the fish - at least for short time periods.

Compensatory capacity of energy metabolism was demonstrated in isolated gills of the North Sea eelpout and of two Antarctic nototheniid species, *Notothenia coriiceps* and *Gobionotothen gibberifrons*. In this thesis, a setup for analysis of metabolic rates in isolated gills was established to enable measurements of their oxygen consumption under hypercapnic conditions. Although metabolic rate remained constant, energy allocation shifted significantly in the gills of the notothenioids. With specific inhibitors the energy demand for ion regulation (Na^+/K^+-ATPase), protein and RNA biosynthesis could be evaluated. All three processes required more energy under hypercapnia. The extra costs may be covered by an increase in mitochondrial efficiency and energy savings on other physiological processes not directly involved in the stress response.

Acclimation in ion regulatory processes was examined in the gills of *Z. viviparus* under hypercapnia for an incubation period of six weeks. The capacity of Na^+/K^+-ATPase (the "workhorse" of ion regulation) as well as its protein and mRNA level increased steadily, a finding in line with the increased energy demands for the respective processes in the gills of Antarctic fish. Further ion transporters driven by Na^+/K^+-ATPase activity were studied at transcriptional levels: Na^+/H^+-exchanger (NHE1), Cl^-/HCO_3^--exchanger (AE1) and Na^+/HCO_3^--cotransporter (NBC1). The two members of the HCO_3^--transporter family were isolated and characterized for the first time in gills of marine teleost fish. The expression patterns of individual ion transporters support a biphasic model of branchial ion regulation under hypercapnia: Within the initial

regulatory phase, when the fish are reversing the respiratory acidosis, the associated net secretion of protons and accumulation of bicarbonate are favored by the downregulation of respective transporters (NHE1, NBC1 and AE1) and simultaneous increase of Na^+/K^+-ATPase capacity. Towards the end of the acclimation phase a long-term increase of Na^+/K^+-ATPase and Na^+/HCO_3^--cotransporter levels maintain the new ion equilibrium

For a more comprehensive picture, the acclimation response of the total branchial transcriptome was examined in *Z. viviparus* after 24 hours of hypercapnia. 1600 differentially regulated genes were identified by the generation of normalized, subtractive cDNA libraries. The patterns observed support and broaden the model of ion regulation in the initial acclimation phase (see above). Moreover, other processes responsive to hypercapnia were identified, such as signal transduction cascades, stress and immune responses and cellular maintenance. In line with energy allocation patterns, shifts in metabolic fluxes towards biosynthetic processes like gluconeogenesis and RNA/DNA synthesis were detected, while mitochondrial capacity seemed to remain stable.

Although the fish are able to acclimate to elevated CO_2 concentrations, the increased energy demand for ion regulation and the shifts in metabolic fluxes might diminish energy availability for other crucial components of the energy budget like growth and reproduction. The key processes and candidate genes identified in the present thesis may serve as sensitive biomarkers for future studies that consider realistic ocean acidification scenarios. In fish, but also in less tolerant aquatic animals, substantial knowledge of affected mechanisms will help elaborating a comprehensive picture of future consequences of ocean acidification on the ecosystem level.

Zusammenfassung

Ohne die CO_2 Aufnahme durch die Ozeane wäre der vom Menschen verursachte CO_2-Anstieg in der Atmosphäre und damit das Ausmaß der globalen Erwärmung bereits jetzt um vieles höher. Der zusätzliche CO_2-Eintrag führt jedoch zu Verschiebungen im natürlichen Carbonat-Gleichgewicht und damit zu einer Versauerung des Meerwassers. Diese physikochemischen Änderungen werden sich unweigerlich auf die Organismen und Lebensgemeinschaften der Meere auswirken. Fische reagieren auf die erhöhte CO_2-Konzentration (Hyperkapnie) und den dadurch erniedrigten pH-Wert, indem sie Störungen ihres Ionen- und Säure-Base-Gleichgewichts kompensieren. Ziel dieser Arbeit war es, die Mechanismen der Ionenregulation und deren Auswirkungen auf den Energiestoffwechsel, sowie die zugrunde liegende Genregulation während der Anpassung an Hyperkapnie (10 000 ppm CO_2) in marinen Fischen zu untersuchen. Da die Ionenregulation des Fisches zu über 90 Prozent in den Kiemen stattfindet, konzentrierten sich die Untersuchungen hauptsächlich auf dieses Organ.

Am Beispiel der Nordsee-Aalmutter *Zoarces viviparus* wurde gezeigt, dass die Erhöhung der CO_2-Konzentration keinen direkt ersichtlichen Einfluss auf den Grundstoffwechsel des Ganztieres hat. Die Ruhestoffwechselrate, die anhand von Sauerstoffverbrauchsmessungen ermittelt wurde, blieb unverändert, als die Tiere für einen Zeitraum von vier Tagen hyperkapnischen Bedingungen ausgesetzt wurden. Auch in ihrem Verhalten ließen die Fische keine Auffälligkeiten oder sichtbaren Anzeichen von Stress erkennen, so dass sie zumindest durch kurzeitige Hyperkapnie nicht beeinträchtigt zu sein scheinen.

In einem für diese Arbeit weiterentwickelten Versuchsaufbau wurde der Sauerstoffverbrauch von isolierten, perfundierten Kiemen unter Hyperkapnie gemessen. Sowohl in den Kiemen der Nordsee-Aalmutter, als auch der zwei antarktischen Notothenioiden, *Notothenia coriiceps* und *Gobionotothen gibberifrons*, hatte die erhöhte CO_2-Konzentration keinen Einfluss auf den metabolischen Grundumsatz. In den Kiemen der antarktischen Fische wurde jedoch eine gravierende Verschiebung in der Energieverteilung festgestellt: der Energiebedarf für die Ionenregulation (Na^+/K^+-ATPase), Proteinbiosynthese und RNA-Synthese, der mit Hilfe spezifischer Hemmstoffe ermittelt wurde, war unter Hyperkapnie signifikant erhöht. Der Mehrbedarf für diese Prozesse könnte durch eine Erhöhung der Mitochondrieneffizienz und durch Einsparung an anderen physiologischen Prozessen gedeckt werden, die nicht direkt an der Stressantwort beteiligt sind.

Die Akklimation der Ionenregulationsprozesse unter Hyperkapnie wurde in einer Langzeitstudie in Kiemen der Aalmutter *Z. viviparus* näher untersucht. Über einen Zeitraum von sechs Wochen hat sich die Kapazität der Na^+/K^+-ATPase (dem „Motor“ der Ionenregulation)

sowie ihr Protein- und mRNA-Gehalt stetig erhöht, im Einklang mit dem erhöhten Energiebedarf für die entsprechenden Prozesse in den antarktischen Fischen. Auch weitere Transporter, die von der Na^+/K^+-ATPase angetrieben werden, wurden auf transkriptioneller Ebene untersucht: Na^+/H^+-Austauscher (NHE1), Cl^-/HCO_3^--Austauscher (AE1) und Na^+/HCO_3^--Cotransporter (NBC1). Die beiden Hydrogencarbonat-Transporter wurden dafür zum ersten Mal in marinen Knochenfischen isoliert und charakterisiert. Anhand der Expressionsmuster der Transporter wurde ein zweiphasiges Modell für die Ionenregulation unter Hyperkapnie postuliert. In der anfänglichen regulatorischen Phase, in der die Fische der respiratorischen Azidose entgegenarbeiten, wird die Sekretion von Protonen und Akkumulation von Hydrogencarbonat durch verringerte Transkriptionsraten entsprechender Transporter (NHE1, NBC1 und AE1) bei gleichzeitig ansteigender Na^+/K^+-ATPase-Kapazität unterstützt. Für das neue Ionengleichgewicht am Ende der Akklimationsphase ist eine dauerhafte Erhöhung der Transkriptmenge von Na^+/K^+-ATPase und Na^+/HCO_3^--Cotransporter erforderlich.

Um ein vollständigeres Bild der Akklimation zu erhalten, wurden die Aalmutter-Kiemen auf der Ebene des gesamten Transkriptoms untersucht. Die Konstruktion zweier normalisierter, subtraktiver cDNA-Banken ermöglichte die Identifizierung von 1600 Genen, die nach 24-stündiger Hyperkapnie differentiell reguliert werden. Anhand der Genexpressionsmuster konnte das Ionenregulationsmodell für die frühe Akklimationsphase (siehe oben) bestätigt und weiter ausgebaut werden. Außerdem wurden weitere Prozesse identifiziert, die an der Hyperkapnie-antwort beteiligt sind, z.B. in der Signaltransduktion, der Stress- und Immunantwort und dem Zellmanagement. Im Einklang mit den Verschiebungen im Energiebudget zeichnete sich im Stoffwechsel eine Verschiebung in Richtung biosynthetischer Prozesse ab (Gluconeogenese, RNA/DNA-Synthese), während die Mitochondrienkapazität unverändert schien.

Auch wenn die Fische zunächst keine offensichtlichen Schwierigkeiten mit der Anpassung an erhöhte CO_2-Konzentrationen zeigen, kann der erhöhte Energieaufwand für die Ionenregulation und die Verschiebung der Stoffwechselflüsse zu einer Verringerung der Energiereserven für Wachstum und Reproduktion führen. Die Schlüsselprozesse und Kandidatengene, die in der vorliegenden Arbeit identifiziert wurden, können als sensitive Biomarker für weitere Studien im Hinblick auf die zukünftig erwarteten Szenarien der Ozeanversauerung dienen. Ein grundlegendes Verständnis der betroffenen regulatorischen Mechanismen in Fischen, und auch in weniger toleranten Wasserlebewesen, kann dabei helfen, die Folgen der zukünftigen Ozeanversauerung für das Ökosystem zu ermessen.

1 Introduction

1.1 Ocean acidification

The impact of anthropogenic carbon dioxide emissions on global warming has become an issue of wide scientific interest and research in the last decades. The increasing atmospheric CO_2 levels not only contribute to the greenhouse effect, but also affect the world's oceans (IPCC, 2007). The natural carbon cycle involves a balance of CO_2 fluxes between the atmosphere and the terrestrial and marine biospheres. In the water all dissolved inorganic carbon (DIC) belongs to a natural bicarbonate-buffer system, forming an equilibrium between dissolved free carbon dioxide ($CO_{2(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) according to the following equation:

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \Leftrightarrow CO_3^- + 2H$$

Consequently, elevated CO_2 partial pressures in the water, also denoted as environmental hypercapnia, lead to an increase of protons and thus a drop of the water pH. Caldeira and Wicket (2003) first described this process as ocean acidification. Since the beginning of the industrialization in the early 19th century there has been a steady rise in anthropogenic CO_2 emissions. Primarily due to fossil fuel consumption and deforestation, the atmospheric CO_2 level has increased from 280 ppm (preindustrial) to currently 385 ppm (IPCC, 2007). In the ocean surface layers a 30 % increase in proton concentration has already occurred, which corresponds to a significant pH decrease in the seawater from the natural range of 7.6 - 8.2 to values between 7.5 - 8.1 (Haugan & Drange, 1996; Caldeira & Wickett, 2003; Seibel & Walsh, 2003). Climate models predict a rise in CO_2 concentrations to values between 490 and 1130 ppm by the end of this century [according to different categories of emission and stabilization scenarios (IPCC, 2000, 2007). With undiminished usage of all the world's estimated fossil fuel resources of about 5000 gigatonnes of carbon, atmospheric CO_2 concentrations may even rise to more than 1900 ppm by the year 2300 (Caldeira & Wickett, 2003). This increase will cause a pH reduction in the ocean surface layers of 0.30 to 0.46 units by the year 2100 (Caldeira & Wickett, 2005) and may reach up to 0.77 units in 2300 [(Caldeira & Wickett, 2003; Zeebe *et al.*, 2008), see.Figure 1-1].

The disequilibrium in the natural bicarbonate-buffer system directly affects the ability of calcifying marine organisms to produce calcerous skeletal structures due to the decreasing concentrations of the natural carbon mineral forms aragonite and calcite (Riebesell *et al.*, 2000; Gazeau *et al.*, 2007; Ridgwell *et al.*, 2007; Fabry *et al.*, 2008; Iglesias-Rodriguez *et al.*, 2008). With "business-as-usual" emissions, the Southern Ocean will be aragonite undersaturated by 2100,

which will threaten the organisms that rely on aragonite for maintaining their shells, such as pteropods (planctonic snails). Thus, the whole ecosystem will be at risk, as pteropods play an important role in the food web and carbon cycling (Orr *et al.*, 2005).

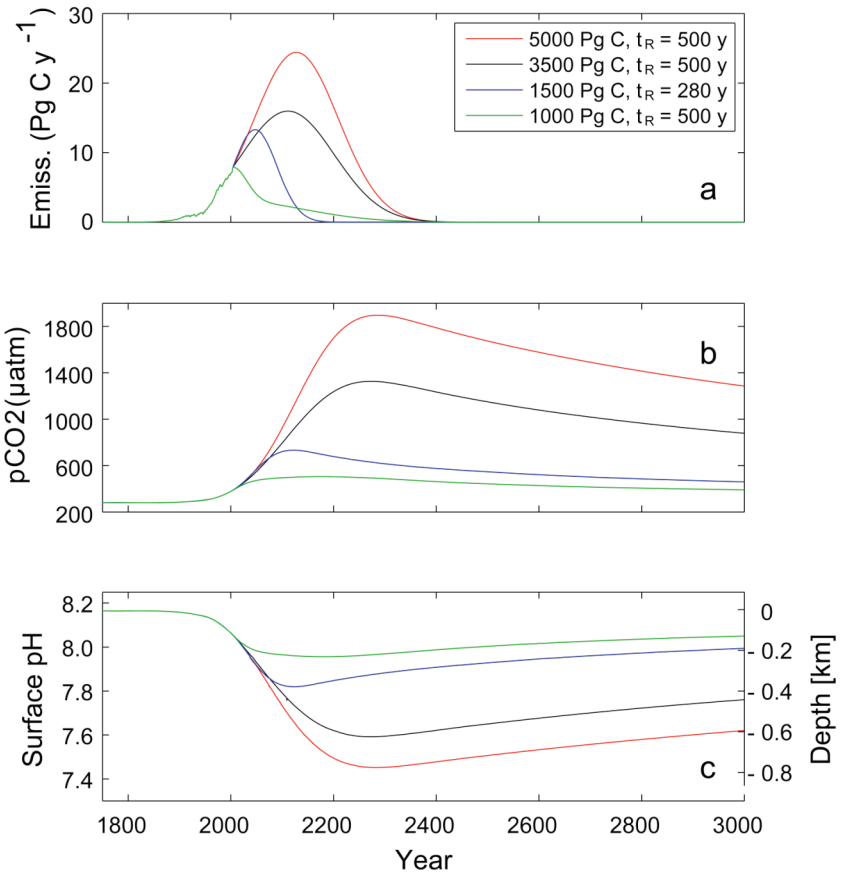

Figure 1-1: Anthropogenic CO_2 emission scenarios
CO_2 emission (**a**) in Petagrams Carbon per year (1 PgC = 1 Gigatonne C; t_R = release time) and respective development of CO_2 partial pressures (**b**) and ocean surface pH (**c**). **Red** line represents "business-as-usual" with combustion of all remaining fossil fuel [see also (Caldeira & Wickett, 2003)]. **Green** line reflects immediate emission stop. Scenarios are based on historic emission data and future expected total emissions and release time after Zeebe *et al.* (2008).

The deprivation of the food web by the loss of calcification capabilities of key species is not the only factor threatening the marine ecosystems; the pH drop may also cause disturbances in the acid-base regulation, ventilatory system, energy budget and metabolism of major marine life forms [e.g. (Claiborne & Heisler, 1983; Toews *et al.*, 1983; Larsen *et al.*, 1997; Jensen *et al.*, 2000; McKenzie *et al.*, 2002; Brauner *et al.*, 2004; Miles *et al.*, 2007; Pane & Barry, 2007)]. In the long run, this may have severe consequences for the animals' resilience and may impair growth and reproduction rates [for reviews see (Pörtner *et al.*, 2004; Pörtner, 2008)].

1.2 Past and future CO_2 concentrations

The present CO_2 concentrations are the highest measured for the last 650,000 years according to ice core data (IPCC, 2007). During the last glacial and interglacial periods CO_2 and other greenhouse gas concentrations (e.g. methane, nitrous oxide) oscillated within relatively narrow lower and upper limits. The anthropogenic increase accompanying the industrialization since 1860 was rapid and extreme and is still accelerating (see Figure 1-2).

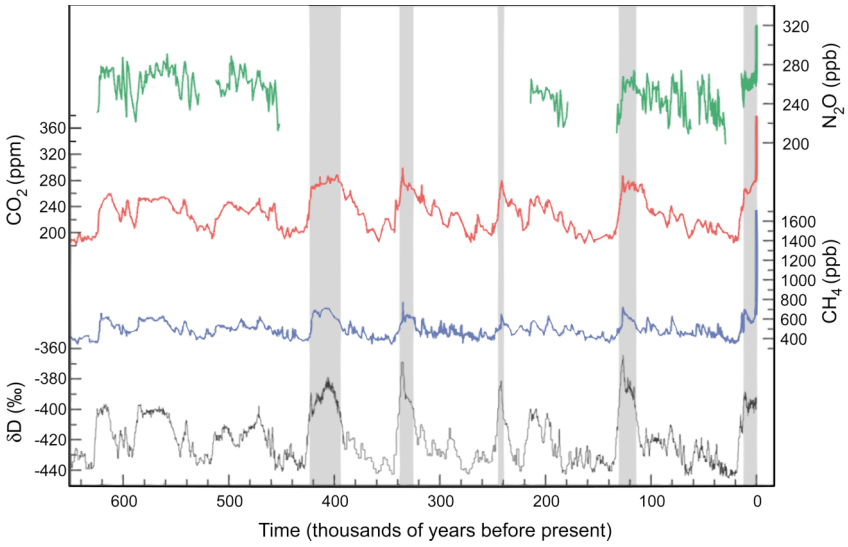

Figure 1-2: Glacial - Interglacial ice core data
Concentrations of the greenhouse gases CO_2, methane (CH_4) and nitrous oxide (N_2O) over the last 650,000 years (until the middle of the Pleistocene, Quaternary period) from air trapped in ice cores and from recent atmospheric measurements. Variation in deuterium (δD) in Antarctic ice reflects local temperature. Shaded bands indicate interglacial warm periods [from (IPCC, 2007)]

By now ongoing global change has led to increasing temperature, rising sea levels and ocean acidification. The long-term consequences of this anthropogenic impact on the earth's environment are completely unpredictable. Going back 250 million years in time, the boundary between Permian and Triassic periods is marked by earth's greatest mass extinction event, which eliminated up to 92 percent of all marine species. This extinction event was probably caused by the interplay of global warming, anoxia, toxic sulfide and hypercapnia (Knoll *et al.*, 2007). As deficits were highest amongst calcifying species (e.g. corals and echinoderms), Bambach and Knoll concluded, that hypercapnia may even have been the key trigger (Bambach *et al.*, 2002; Knoll *et al.*, 2007). More recent events of global change in the Cenozoic era, such as the Paleocene-Eocene Thermal Maximum (PETM) about 55 mio years ago, are also marked by a rapid increase in temperature and CO_2 concentration. During that period, a major extinction of foraminifera occurred probably due to an oxygen deficiency in the oceans (Zachos *et al.*, 2008). Analysis of these events in earth history are important for a deeper understanding of the complex interactions between climate and carbon cycling and for modeling future climate scenarios.

Since becoming aware of the CO_2 problem, the idea is under current discussion to stabilize the atmospheric CO_2 concentration by carbon capture and storage (CCS). The CO_2 emitted by power plants should be captured, compressed and sequestered into underground geological formations, such as oil or coal seams, aquifers or saline-filled basalt formations. Besides geological storage, ocean storage (e.g. in the deep-sea) would also be possible. Since this idea first came up over 30 years ago (Marchetti, 1977), sequestration in the deep sea has been developed and investigated (Herzog *et al.*, 1991; Haugan & Drange, 1992; Brewer *et al.*, 1999; Brewer *et al.*, 2004). Different methods for sequestering the CO_2 in the ocean are being discussed, as illustrated in Figure 1-3. However, the biological impact of ocean CO_2 storage needs thorough investigation. Depending on the mode of injection, the CO_2 partial pressure could, at least locally,

increase significantly and lead to the acidification of the water over hundreds of kilometers around the release sites (Caulfield *et al.*, 1997). Especially the current studies on consequences for deep-sea animals (Tamburri *et al.*, 2000; Thistle *et al.*, 2006) agree in that the consequences of controlling atmospheric CO_2 by ocean storage should be carefully assessed. It may "help reducing the impact on surface ocean biology, but at the expense of deep-ocean biology" (IPCC, 2005). In the ongoing discussion, the supporters of ocean storage demand field-verification to allow responsible decisions about this issue (Adams & Caldeira, 2008). To prevent potentially negative effects, the OSPAR Commission for the Protection of the Marine Environment of the North-East Atlantic (http://www.ospar.org) has already adopted a decision to „Prohibit the Storage of Carbon Dioxide Streams in the Water Column or on the Sea-bed" which entered into force on January 15th 2008.

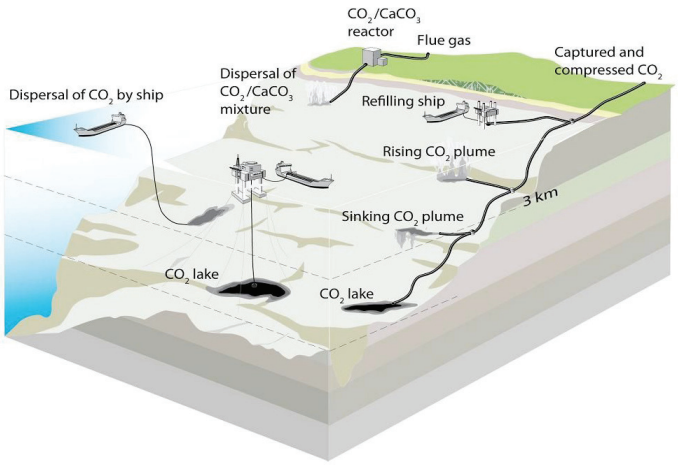

Figure 1-3: Methods of ocean storage
Compression and injection of CO_2 by ships or pipelines into 3000 m depth, where CO_2 is denser than water and will form lakes. Injection of CO_2 into 1000 m depths where it will be diluted in the water. Conversion of CO_2 to calcium carbonate (by using limestone) and dispersion in the ocean, where it will dissolve as calcium and bicarbonate ions [from (IPCC, 2005)].

1.3 Coping with acidification

Some marine life forms and even whole communities have evolved facing natural CO_2 variations - e.g. in the sediment, in tidal rock pools, in hypoxic ground water and near hydrothermal vents [see (Pörtner *et al.*, 2004; Pörtner, 2008)]. Nevertheless, with a more or less constant CO_2 partial pressure (P_{CO_2}) and a resulting surface pH of 8.2 ± 0.2 in the pelagic region during the last 7.5 Mio years (Spivack *et al.*, 1993), most marine animals are adapted to stable CO_2 concentrations and pH values. Nevertheless, marine fish can buffer acid-base disturbances and compensate their internal cellular and blood pH values within certain species-specific limits (Heisler, 1986a, 1993).

The mechanisms for pH defense include metabolic interconversion of acids and bases (Pörtner, 1989), passive buffering in intra- and extracellular compartments and active proton-equivalent ion exchange (Heisler, 1986a; Walsh & Milligan, 1989). For acute pH changes, the buffering by non-bicarbonate buffers is the quantitatively most important defense mechanism, especially in the intracellular fluids. These buffer substances have dissociation constants near

physiological pH and comprise protein side chains like histidine or cysteine, terminal NH_2 groups and organic and inorganic phosphates (Somero, 1985; Heisler, 1986a). The predominant buffers in the extracellular fluids are erythrocytic hemoglobin and plasma bicarbonate. Due to the low hematocrit and therefore hemoglobin levels together with low HCO_3^- concentration in the fish blood compared to terrestrial animals, the extracellular buffer capacity amounts to only 10 % of the intracellular value (Heisler, 1986a). Ventilatory compensation of pH disturbances by exhaling CO_2 is also limited because of the low CO_2 diffusion gradient between the organism and the water (Piiper & Scheid, 1992). Therefore, fish rely mainly on branchial ion transport processes to maintain their ion- and acid-base balance. While intestines (Wilson *et al.*, 1996) and kidneys (Wood *et al.*, 1999) contribute to proton equivalent ion transfer, the primary organ for this are the gills, where over 90 % of acid-base regulation takes place (Heisler, 1986b).

1.4 Acid-base regulation in the fish gill

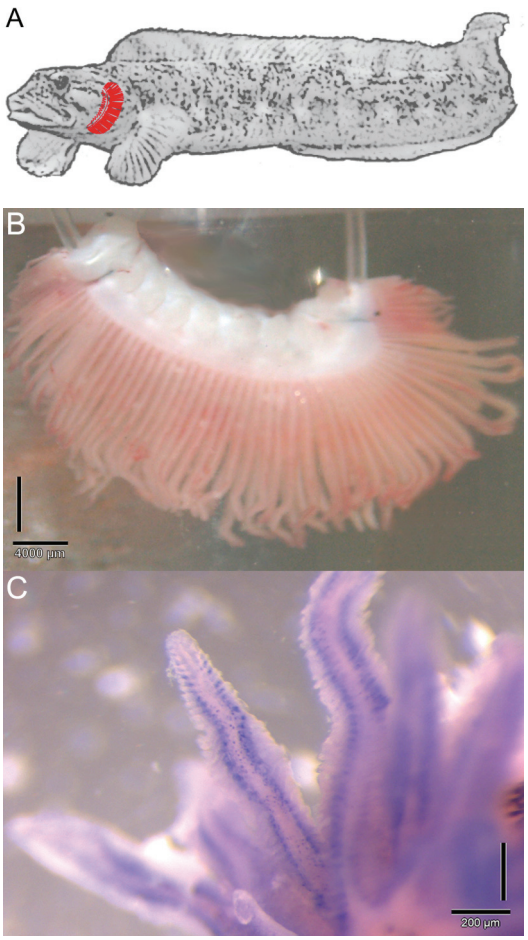

Figure 1-4: Fish gill anatomy
A: Schematic view of gill location in North Sea eelpout (*Z. viviparus*) **B:** Isolated gill arch from Antarctic Humped rockcod (*G. gibberifrons*; picture by Z. Zittier) **C**: Labeled mitochondrion-rich cells (blue) in gill filaments from Antarctic eelpout (*P. brachycephalum*; fish provided by H. Windisch; picture by R. Kiko and M. Lucassen, unpublished)

The gill epithelium consists of six cell types: pillar, mucous, neuroepithelial, pavement (PVCs), mitochondrion-rich cells (MRCs) and undifferentiated cells. The most abundant cells (>90 %) are the PVCs, which are important for gas exchange, while the MRCs are likely the main site for energy production, pH- and osmoregulation (Wilson & Laurent, 2002; Evans *et al.*, 2005).They are located mainly at the afferent side of the gill filaments (the anchoring point) and along the main lamellae of the filaments. In seawater teleosts, NaCl is secreted via the MRCs, which is why they are also referred to as chloride cells. The driving force for the salt extrusion against the higher external salt concentration is provided by the Na^+/K^+-ATPase, which has a very high abundance in mitochondrion-rich cells, emphasizing the high energy demand of ion regulation. This ion pump thus serves as a cellular marker for these cells (see Figure1-4).

Na^+/K^+-ATPase is the main ion pump energizing the cells for pH homeostasis. Maintenance of intra- and extracellular pH is achieved by electroneutral exchange of H^+ or NH_4^+ with Na^+, and of HCO_3^- with Cl^- across the epithelia. A pH reduction - either induced externally (e.g. by environmental hypercapnia) or internally (e.g. by metabolic acidosis) - is compensated for by the accumulation of bicarbonate in the blood, while electroneutrality is ensured by the concomitant drop of the chloride level (Heisler, 1993; Pörtner *et al.*, 2004).

The function of Na^+/K^+-ATPase (**NKA**) in fish gills was first examined in acclimation studies of euryhaline killifish to seawater (Epstein *et al.*, 1967). Since then, many different branchial ion transporters have been identified and analyzed, but the complex interplay of these proteins regulating ion- and pH balance is still not completely understood [see (Marshall, 2002; Evans *et al.*, 2005; Perry & Gilmour, 2006)]. Ion transport processes in fish gills are summarized in the following according to present knowledge (see Figure 1-5).

Under hypercapnia, environmental CO_2 diffuses into the cell via the apical membrane. Within the cell it is hydrated by carbonic anhydrase (**CA**) to form HCO_3^- and H^+. Bicarbonate ions can be transported basolaterally into the blood or secreted apically via respective transporters (see below) Protons can be extruded from the gill into the surrounding water to compensate for the acidosis. In freshwater fish, active proton secretion by an apical V-type H^+-ATPase (**HA**) is probably coupled to the import of Na^+ ions via an ENaC (epithelial Na^+-channel)-like channel (Lin & Randall, 1991; Clarke & Potts, 1998). In marine fish gills, the utilization of the Na^+ gradient - due to the hyperosmotic seawater - may be favored to extrude protons by apical Na^+/H^+-exchanger isoforms [**NHE2, NHE3** (Claiborne *et al.*, 1999; Tresguerres *et al.*, 2005)]. A further isoform of the Na^+/H^+-exchanger (**NHE1**) has been identified in marine fish, which is

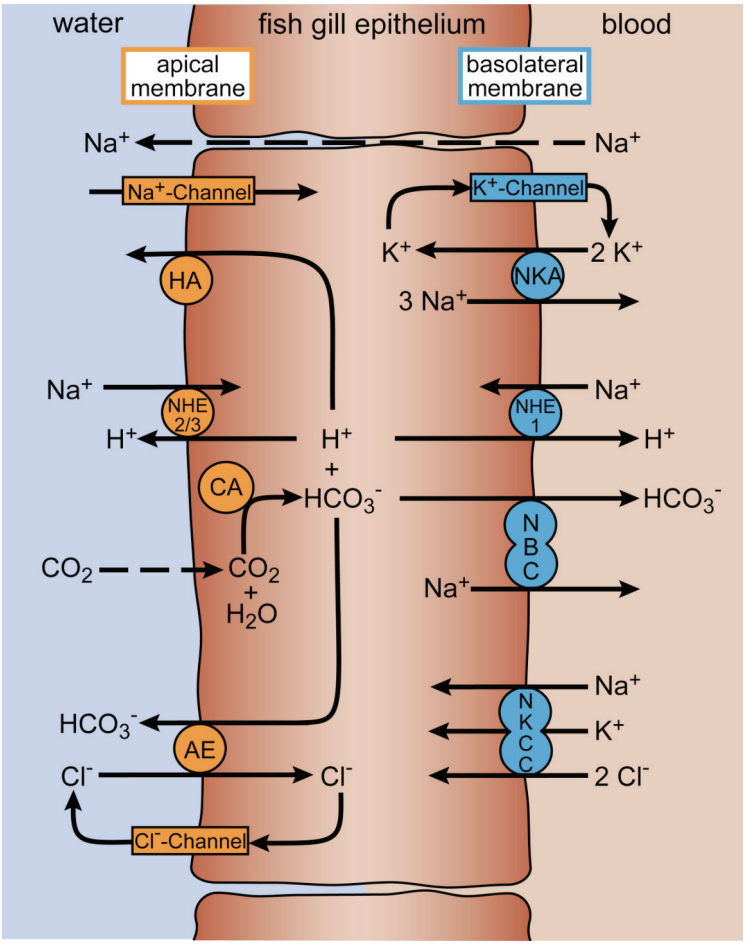

Figure 1-5: Gill ion transport in teleost fishes
State-of-the-art model of ion transport processes in gill epithelial cells [modified after (Claiborne *et al.*, 2002; Evans *et al.*, 2005)].

probably located in the basolateral membrane (Claiborne *et al.*, 1999). However, both Na^+/H^+-exchangers and H^+-ATPases exist in marine as well as freshwater fish and their different contributions or functional roles still needs further investigation [reviewed by (Evans *et al.*, 2005)]. For enhanced net acid secretion, the apical proton efflux is accompanied by basolateral export of HCO_3^-. A Na^+/HCO_3^--cotransporter (**NBC**) is located in the basolateral membrane of a freshwater fish, exporting Na^+ and HCO_3^- ions into the extracellular space (Hirata *et al.*, 2003). A role of NBC1 for intracellular pH regulation under hypercapnia was also postulated in freshwater fish (Perry *et al.*, 2003b). Another bicarbonate transporter, the Cl^-/HCO_3^--exchanger (named **AE** for anion exchanger) has been demonstrated for the apical membrane of freshwater fish, where it exports HCO_3^- against Cl^- (Wilson *et al.*, 2000). In that way, AE functions in base secretion, so that a reduced efflux of HCO_3^- via this transporter was postulated to support pH regulation under hypercapnia induced acidosis (Heisler, 1993; Claiborne *et al.*, 2002). At the beginning of the current thesis, the role for the bicarbonate transporters relied on data from freshwater fish only, so the identification and characterization of these transporters had to be confirmed for marine fish.

Marine fish have to secrete salt (mainly NaCl) against the inward diffusion gradient of hyperosmotic seawater. The mechanisms of osmoregulation have been studied extensively during the last decades and are generally accepted as follows (Figure 1-5): Na^+, K^+ and Cl^- ions from the plasma enter the epithelial cell via a basolateral $Na^+/K^+/2Cl^-$-cotransporter. The basolateral Na^+/K^+-ATPase pumps 3 Na^+ in exchange with 2 K^+ ions into the extracellular space. K^+ ions are recycled back into the extracellular space by basolateral K^+-channels, while Cl^- ions are secreted apically by Cl^--channels. In this way a positive transepithelial potential difference is generated that drives efflux of plasma Na^+ ions via leaky tight junctions into the surrounding water [reviewed by (Claiborne *et al.*, 2002; Evans *et al.*, 2005)].

1.5 Energy maintenance

By utilizing ATP for the generation of ion gradients, Na^+/K^+-ATPase is one motor for ion and pH regulation. Thus, the energy consumption of the sodium pump largely represents the energy demand of ion and pH regulation, which may account for 7 - 10 % of the animal's total energy turnover under resting conditions (Mommsen, 1984; Perry & Walsh, 1989; Gibbs & Somero, 1990; Boeuf & Payan, 2001). In gills, Na^+/K^+-ATPase makes up for 30 % of the total energy turnover (Stagg & Shuttleworth, 1982; Morgan & Iwama, 1999) and is therefore one of the main energy consumers. Besides, cellular maintenance depends on a number of ATP consuming processes like protein biosynthesis, RNA and DNA synthesis, Ca^{2+}-transport, signal transduction, metabolic substrate cycles (e.g. carbohydrate, amino acid or fatty acid degradation or

biosynthesis), gluconeogenesis, cellular movement (e.g. contraction) and urea synthesis [reviewed by (Rolfe & Brown, 1997)]. Besides substrate-level phosphorylation (in early stages of carbohydrate catabolism) ATP is mainly synthesized by mitochondrial F_1F_O-ATP-Synthase. As the respired oxygen is closely linked to the generated ATP via the respiratory chain, oxygen consumption is a direct measure of energy turnover (ATP synthesis and utilization) of an animal. The oxygen consumption under resting, non-feed conditions also represents the organisms' standard metabolic rate (SMR).

Fish are able to compensate well for pH-disturbances. Even under relatively severe hypercapnia (10,000 ppm) they recover their internal pH values quickly, usually within 24 hours (Perry, 1982; Toews *et al.*, 1983; Heisler, 1993; Larsen *et al.*, 1997; Michaelidis *et al.*, 2007). However, little is known about the energetic costs of acid-base regulation. Invertebrates are usually much more CO_2 sensitive than fish due to their lack of sufficient internal pH compensation (Pörtner, 2008). While they are struggling with acid-base status, other vital cellular processes are reduced, as shown for calcification (Gazeau *et al.*, 2007), growth rate in mussels (Michaelidis *et al.*, 2005) and protein biosynthesis in a sipunculid worm (Langenbuch *et al.*, 2006). These severe impairments may result in reduced growth and reproduction rates and consecutively a high sensitivity of these animal phyla toward CO_2. Although fish seem to be able to raise enough energy for their ion and acid-base regulation, they may also have to take it at the expensure of other processes. On longer time scales, this may have consequences for their growth and reproduction rates as well.

1.6 Concept of the thesis

The present thesis set out to improve our understanding of responses to elevated CO_2 levels in marine fish. High CO_2 concentrations of 10,000 ppm have been used consistently in a mechanistic approach to clearly identify the essential processes responding to hypercapnia. The thesis centers on ion regulation and associated energy requirements in fish gills. It thereby ranges from the organismic to the molecular level, including the underlying patterns of transcriptomic regulation.

In particular, the following questions were elaborated:

- **How, and to what extent, are gill ion transporters involved in acclimation to hypercapnia?**

  In fish, environmentally induced acid-base disturbances are balanced by transepithelial ion transport processes, mainly in the gills. In this part of the thesis, the individual gene expression of ion transporters (Na^+/K^+-ATPase, Na^+/H^+-exchanger, Na^+/HCO_3^--cotransporter, Cl^-/HCO_3^--exchanger) was examined in the gills of *Zoarces viviparus*. Here, the two members of the bicarbonate transporter family (Na^+/HCO_3^--cotransporter and Cl^-/HCO_3^--exchanger) were demonstrated for the first time in marine fish gills. During acclimation to hypercapnia over six weeks, a time resolved analysis of the transporters at transcriptional, as well as translational and functional levels was carried out. This led to model development for the acute/short-term and long-term adjustments of the protein inventory.

- **Does hypercapnia cause a shift in energy budget of fish gills?**

  The compensation of acid-base disturbances by ion transport processes may require energy that has to be provided by the organism. A shift in the total energy requirement may become visible in the standard metabolic rate (SMR), as determined for common eelpout (*Z. viviparus*) under normo- *vs.* hypercapnic conditions. To specify the effects on gills, an isolated perfused gill model was developed and used for SMR measurements of eelpout gills, as well as of gills from two Antarctic notothenioid species (*Notothenia coriiceps* and *Gobionotothen gibberifrons*). Furthermore, the contribution of individual metabolic processes to fuel the demand of hypercapnia acclimation in notothenioids could be elaborated by using specific inhibitors.

- **How do regulatory adjustments of the transcriptome contribute to the hypercapnia response?**

  The acclimation of physiological processes to changing environmental factors is based on the differential regulation of specific genes. Identification of these genes and the respective changes in gene expression can provide new insights into the acclimation process by revealing the various metabolic pathways, signal cascades and cellular processes involved. In this project, normalized and subtractive cDNA libraries from the gills of common eelpout (*Z. viviparus*) have been generated to identify up- and downregulated genes within the regulatory phase of hypercapnia exposure (after 24 hours). The identified gene groups may point to key processes in hypercapnia acclimation and serve as candidate genes, which may be tested for their response to ocean acidification scenarios and in other animal phyla.

2 Materials & methods

2.1 Animals

Specimens from the common eelpout *Zoarces viviparus* (Linnaeus, 1758) were used for the main parts of this thesis. *Z. viviparus* belongs to the family of Zoarcidae (subfamily Zoarcinae) within the order of perciform fish (Anderson, 1994). The cosmopolitan, mostly benthic zoarcidae comprise almost 260 species which are distributed over all the world's oceans from the Arctic to the Antarctic (Nelson, 2006). *Z. viviparus* lives in shallow (0 - 40 m), coastal areas from the Irish Sea and the English Channel, in the North Sea and the Baltic Sea and northwards to the Northeast Atlantic, White Sea and Barents Sea (Figure 2-1). Its diet consists of polychaets, crustaceans and molluscs (Knijn *et al.*, 1993). It is ovoviviparous, with fertilization taking place in the late summer and birth of the offspring in the winter (Götting, 1976).

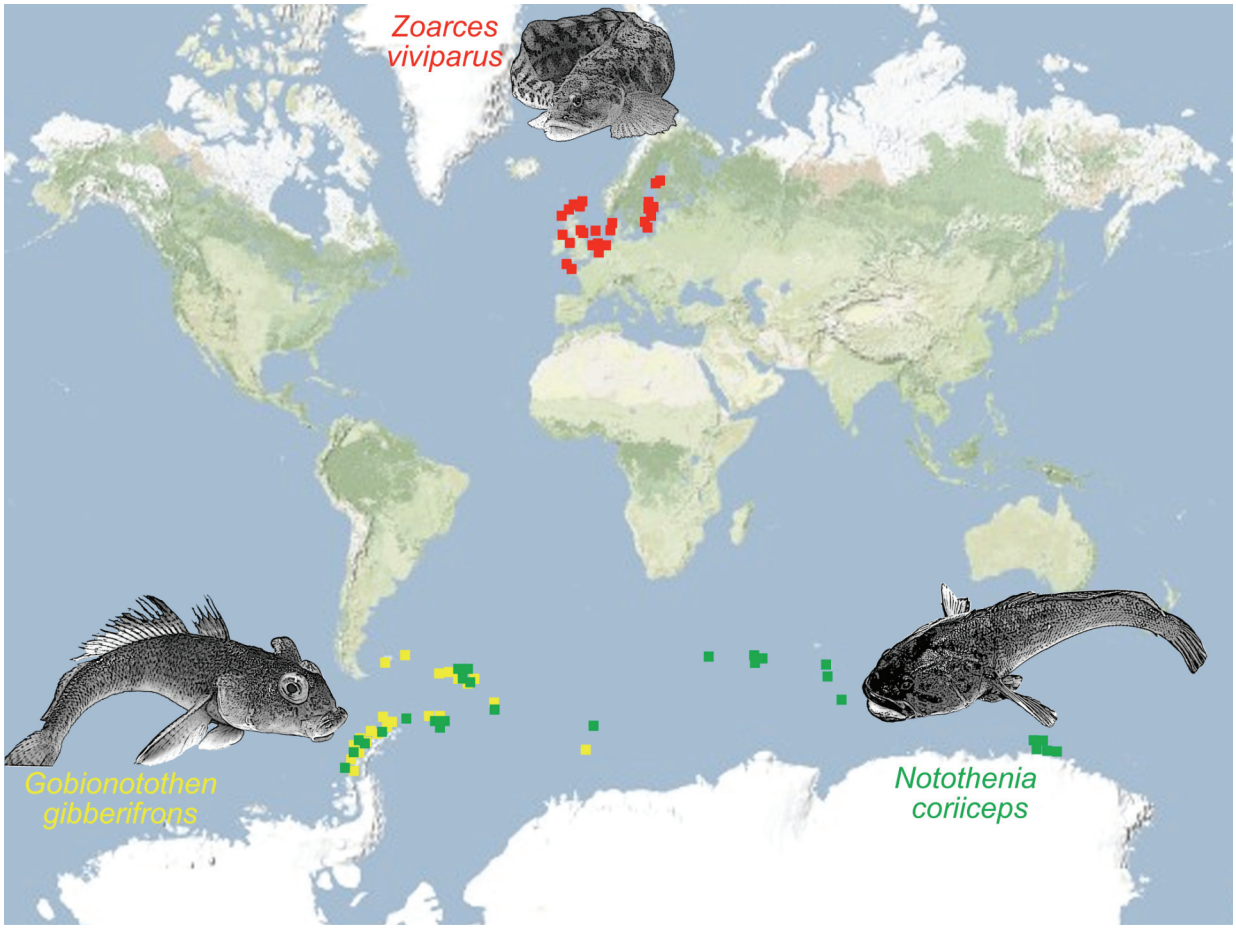

Figure 2-1: Fish species distribution pattern
Global distribution patterns of *Z. viviparus* (**red**), *N. coriiceps* (**green**) and *G. gibberifrons* (**yellow**). Map and data are derived from FishBase (http://www.fishbase.org). Picture of *Z. viviparus* has been taken in the aquarium at the Alfred-Wegener-Institute Bremerhaven. Pictures of notothenioids have been taken by Z. Zittier during the Antarctic expedition XXIII/8 on board of RV 'Polarstern'.

For one of the projects in this thesis two Antarctic notothenиid species have been investigated: *Notothenia coriiceps* (Richardson, 1844) and *Gobionotothen gibberifrons* (Lönnberg, 1905). The family of Nototheniidae belongs to the suborder Notothenioidei, which dominates the Antarctic fish fauna, comprising about 50 % of all individuals (Eastman, 2005). *N. coriiceps* probably exists throughout the Antarctic seas on the continental shelf at depths up to 500 m. It has been demonstrated in the western Ross Sea, Antarctic Peninsula, islands of the Scotia Arc to South Georgia, Weddell Sea, Bouvet Island, and the sub-Antarctic islands of the Indian Ocean sector (Figure 2-1). *G. gibberifrons* can be found near Scotia Arc and Heard Island, near the southern Shetland, southern Sandwich, and southern Orkney Islands and near South Georgia Island at depths of up to 430 m (Figure 2-1). While *G. gibberifrons* is a more sluggish benthic fish and feeds on various infauna, *N. coriiceps* is considered to have a more active, benthopelagic life style, preying also on smaller fish and krill (Casaux *et al.*, 1990; Gon & Heemstra, 1990).

For publications I and III common eelpouts *Z. viviparus* were caught and obtained from the Nordseeaquarium Büsum in the German bight (Figure 2-2) in May 2005. At the Alfred Wegener Institute (AWI) in Bremerhaven (Germany) they were kept in a flow-through aquarium system in aerated seawater at 10 ± 0.5 °C and a pH of 8.1 ± 0.05 (habitat conditions) under a 12 hour day/night cycle before the beginning of the experiments in October 2005. The fish were fed with North Sea shrimps *ad libidum* twice a week. The last meal was 5 days before tissue sampling and 2 days before oxygen consumption measurements. At sampling time the animals had overall weights of 40.4 ± 21.6 g and lengths of 20.2 ± 3.0 cm. For additional gill respiration measurements, eelpouts had been caught with bottom traps in the German bight near Helgoland (Figure 2-2) in April 2004 and April 2005 and were kept in the aquarium system at the AWI. Animals were raised to overall weights of 170.8 ± 86.4 g and lengths of 30.1 ± 3.6 cm before experimentation in the Winter of 2007/08.

Specimens of *G. gibberifrons* (672 - 1169 g, 39.5 - 46.0 cm) were collected during an Antarctic summer expedition with RV 'Polarstern' from 11/2006 to 2/2007 [ANT XXIII/8; (Gutt, 2008)] from bottom trawls at depths of 60 - 490 m at the Antarctic Peninsula near Elephant and Joinville Island (Figure 2-2). *N. coriiceps* (211 - 557 g, 26.0 - 34.5 cm) were caught by the crew of Jubany station near King George Island (Figure 2-2) 3 - 4 weeks prior to experimentation and fed once a week with fish meat. Fish were transported to the ship by helicopter. On board, all animals were kept in an air-conditioned container with an aquarium system and aerated natural seawater at 0.0 ± 0.5°C temperature. The fish were observed for 2 to 5 days prior to experimentation to ensure that they were in a healthy condition. The fish were not fed during that time.

Figure 2-2: Sampling areas of fish species
Z. viviparus (**red**) was caught in the German bight in the North Sea, *N. coriiceps* (**green**) and *G. gibberifrons* (**yellow**) were caught in the Southern ocean at the Antarctic Peninsula. Maps were derived from Google Maps (http://maps.google.de/maps).

All experiments were conducted following German legislation. An approval of the work was issued by competent German authority (Freie Hansestadt Bremen, reference number 522-27-11/2-0; date: 2002-11-28).

2.2 Hypercapnia acclimation experiment

A six-week long-term experiment under hypercapnia was conducted with *Z. viviparus*. For this purpose animals were transferred from the control aquarium to another system containing 10 ± 0.5°C cold seawater equilibrated with 10,000 ppm CO_2. The gas mixture was provided by a mass flow controller (MKS instruments Deutschland GmBH, München, Germany) through mixing of 1 % CO_2 and 99 % air. The resulting pH in the water was controlled with a pH-Meter (340i, WTW, Weilheim, Germany) equipped with a SenTix 81 electrode (WTW) calibrated between pH 7 and 10 according to NBS (National Bureau of Standards) scale. The pH value remained stable at 6.9 ± 0.05 over the whole acclimation period. The fish that served as the control group had also been transferred to another tank 24 h before sampling to take possible effects of handling into account. Groups of 8-9 fishes were sampled at control (0 h hypercapnia) and the designated time points, 8 h, 24 h, 48 h, 4 d, 7 d, 14 d and 42 d after the onset of hypercapnia. The animals were consecutively caught, stunned by a blow to the head and after weight and length determination killed by cutting their spine. Fish were dissected quickly; gill and other tissue samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

2.3 Whole animal respiration

Z. viviparus were used for the determination of standard metabolic rates (SMR) under control conditions and under short-term hypercapnia (10,000 ppm CO_2) over 4 days by using intermittent closed respirometry (see publication I). Animals weighing 37.7 ± 6.2 g (n = 11) were incubated in cylindrical perspex chambers, which were perfused with freshly aerated seawater at a controlled temperature of 10°C ± 0.2°C. For the generation of hypercapnic conditions, a gas mixture of 1 % CO_2 and 99 % air was used. Respirometry runs were performed twice a day by generating a closed–loop circulation. Oxygen consumption rates were measured with oxygen optodes and calculated from linear declines in chamber oxygen partial pressure over time using the following formula

$$M(O_2) = \Delta P(O_2)\ \beta(O_2)\ V\ w^{-1}$$

where $\mathbf{M(O_2)}$ is the oxygen consumption rate [$\mu mol(O_2)\ g^{-1}\ h^{-1}$], $\mathbf{\Delta P(O_2)}$ is the gradient of oxygen decrease over time in the chamber [kPa], $\mathbf{\beta(O_2)}$ is the oxygen capacity of water [$\mu mol(O_2)\ l^{-1}\ kPa^{-1}$] at the respective salinity and temperature after Boutilier *et al.* (1984), **V** is the volume of the closed chamber loop [l] and **w** is the animal weight [g].

2.4 Isolated perfused gill respiration

A tissue model for measuring oxygen consumption of isolated, perfused gills was developed after Lyndon (1994) and Morgan & Iwama (1999). The experiments with *G. gibberifrons* and *N. coriiceps* gills were carried out onboard RV 'Polarstern' during the Antarctic summer expedition ANTXXIII/8 from 11/2006 to 2/2007 (Gutt, 2008). Afterwards, experiments with *Z. viviparus* gills were conducted at the Alfred Wegener Institute in Bremerhaven. In both cases, gill SMR was determined under control and hypercapnic (10,000 ppm CO_2) conditions.

2.4.1 Isolated perfused gill preparations

Fish were stunned by a blow to the head or by application of 0.3 g l^{-1} MS 222 (3-Aminobenzoic Acid Ethyl Ester Methansulfonate, Sigma-Aldrich, Taufkirchen, Germany) to the water. They were infused intravenously with heparin (500 U $100g^{-1}$) to avoid blood clotting. After about 10 minutes of heparin exposure animals were taken out of the water and killed by cutting their spine. The gill arches were dissected quickly and placed in ice-cold saline, where they were cleared from blood and cannulated. To maximize sample number, the first three gill arches from each side were used for isolated perfused gill preparations, whereas the filaments of the fourth arches were frozen and stored as tissue samples at -80°C. For cannulation, polyethylene tubing was fed through the gill arches (inner diameter 0.38 - 0.86 mm for notothenioids, 0.16 mm for eelpout)

and at both sides fixed with perma-hand silk suture. An opening in the tubing over the length of the arch enabled perfusion (for details see publication II). Preparation of up to six gill arches per fish was completed within 60 - 75 minutes. Prepared gill arches were used for respiration measurements directly after preparation (see 2.4.2), or were kept in cold saline, while being perfused with a pulsatile flow of saline provided by a peristaltic pump. Working temperatures were according to the fishes' habitat temperatures, 0°C for Antarctic notothenioids and 10°C for North Sea eelpout. Composition of the saline for the respective fish species is shown in Table 2-1. The bicarbonate concentration was chosen to match a pH compensated condition in the blood under hypercapnia, when bicarbonate has accumulated to compensate for the extracellular acidosis (Heisler, 1993).

Table 2-1: Saline composition for isolated perfused gill preparations.

Contents are given in mmol l^{-1}. pH was adjusted to 7.9

	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	HCO_3^-	PO_4^-	HPO_4^{2-}	Glucose
Notothenioids	148.4	131	5.2	2	1.3	0.3	27	0.2	0.7	5.6
Eelpout	191.5	164.5	2.5	2	2	2	30	0.05	0.7	5

2.4.2 Oxygen consumption measurements

For details about the respiration system setup see publication II. In summary, the respiration chambers were filled with filtered (0.2 µm), freshly aerated seawater. For normocapnic controls, compressed air was humidified in a gas wash bottle. A gas mixture of 1 % CO_2 in air was used to generate hypercapnic conditions. The respective pH values in Antarctic water were 8.0 ± 0.05 for normocapnic and 6.9 ± 0.05 for hypercapnic seawater at 0°C. In North Sea water, values were 8.15 ± 0.05 and 6.85 ± 0.05 (NBS scale).

After cannulation, the gill arch was placed into the chamber and connected to the perfusion system. Cooling jackets around the respiration chambers perfused by a thermostat ensured constant temperatures with an accuracy of ± 0.1°C. A second thermostat set to lower temperatures was used to cool the whole perfusion system by means of a cooling coil.

Gills were perfused with a pulsatile flow of saline (flow rates 0.035 to 0.27 ml/min) by use of a peristaltic pump. The efferent pressure head could be adjusted to a value of 2 - 3 kPa. Oxygen content was measured online in the chamber with an oxygen micro-sensor (needle type, 140 µm, PreSens, Regensburg, Germany) and before and after the perfusate by two flow-through cell oxygen mini-sensors coupled with temperature sensors (FTC-PSt3, PreSens). Oxygen sensors

were calibrated to 0 % with a saturated sodium-dithionite-solution and to 100 % in humid air according to the manufacturer's protocol.

Gill oxygen consumption was calculated as described by Lyndon (1994) with the following formula:

$$M(O_2) = [(P_a - P_e)\,\alpha O_2)\,vfl + \Delta P_{ch}\,\alpha O_2)\,V]\,w^{-1}$$

where $\mathbf{M(O_2)}$ is the oxygen consumption rate [µmol (O_2) g^{-1} h^{-1}], $\mathbf{P_a}$ **and** $\mathbf{P_e}$ are the afferent and efferent oxygen contents of the perfusate [kPa]; $\boldsymbol{\alpha(O_2)}$ is the oxygen capacity of the water [µmol (O_2) l^{-1} kPa^{-1}] at the respective salinity and temperature after Boutilier *et al.* (1984), **vfl** is the flow rate [l h^{-1}], $\mathbf{\Delta P_{ch}}$ is the oxygen decrement over time in the chamber [kPa h^{-1}], **V** is the chamber volume [l] , and **w** is the fresh weight of the gill [g].

2.4.3 Application of inhibitors

In gills of *N. coriiceps* and *G. gibberifrons*, specific inhibitors were used to evaluate the energy allocation for three important cellular ATP-consuming processes under hypercapnia (see publication II). Protein synthesis was inhibited with cycloheximide, which inactivates peptidyl transferase activity of the cytosolic ribosomal 60S subunit (Obrig et al., 1971). Actinomycin D was used to block DNA-primed RNA synthesis by forming a stable complex with double stranded DNA (Kirk, 1960; Sobell, 1985). Na^+/K^+-ATPase activity as a measure for ion regulation was specifically inhibited by Ouabain (Wheeler & Whittam, 1962; Whittam, 1962).

2.5 Molecular biology

2.5.1 RNA isolation

Total RNA from gill tissue was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For mRNA quantification by real-time PCR (polymerase chain reaction, see 2.5.3), complete removal of DNA was ensured by DNase digestion with the DNA-*free* kit (Applied Biosystems, Darmstadt, Germany). Integrity of the RNA was controlled with a RNA 6000 Nano LabChip assay (Agilent

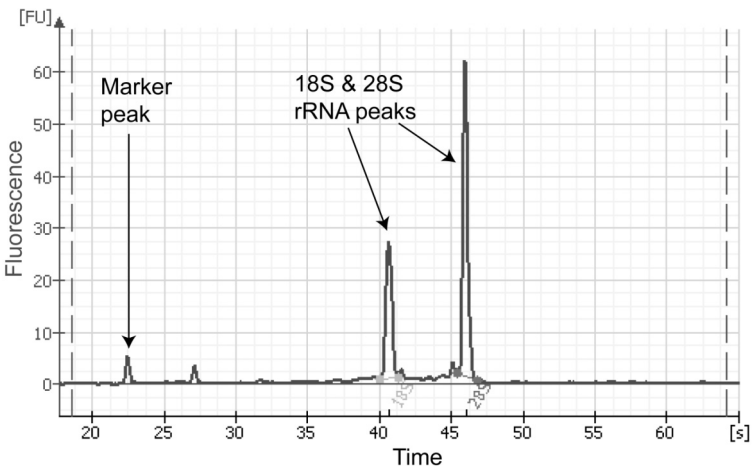

Figure 2-3: Total RNA sample from *Z. viviparus* gill tissue
Quality of total RNA is approved by intact 18S and 28S rRNA peaks (ratio 1.5) and minimal low molecular weight contamination and noise.

Technologies, Waldbronn, Germany, see Figure 2-3). From the total RNA, poly(A)-selected mRNA was isolated with the Oligotex mRNA mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA and mRNA concentrations were determined spectrophotometrically and a measure of quality was the ratio of A_{260}/A_{280}, which was always > 2.0.

2.5.2 Cloning and sequencing of bicarbonate transporters

The cDNA sequences of two bicarbonate transporters, the Na^+/HCO_3^--cotransporter (NBC1) and the Cl^-/HCO_3^--exchanger (anion exchanger AE1), were identified for gene expression studies. Different primer pairs (see table in Appendix 7.1) for NBC1 and AE1 were constructed from conserved regions in previously published sequences obtained from GenBank (NBC1: *Oncorhynchus mykiss*, AF434166; *Tribolodon hakonensis*, B055467; AE1: *O. mykiss*, X61699, *Oreochromis mossambicus*, AAQ89898). The resulting overlapping sequence fragments were subjected to GenBank BLAST search at the National Center for Biotechnology Information (NCBI) at the National Library of Medicine (NLM), National Institutes of Health (NIH) website. BLAST searches were performed using the BLASTX algorithm (Altschul *et al.*, 1997). For NBC1, the identified sequence was used to construct gene specific primers (see table in Appendix 7.1) for completing the sequence by RACE-technique (rapid amplification of cDNA ends) with the First Choice RLM (RNA ligase mediated)-RACE kit (Ambion/Applied Biosystems, Frankfurt, Germany). For reverse transcription (RT) 0.2 µg mRNA (see 2.5.1) were incubated for 65 min at 42°C with 1 U μl^{-1} reverse transcriptase (Superscript RT, Invitrogen, Karlsruhe, Germany), 6 pmol of respective gene specific reverse primer, 0.625 mM of each dNTP, 10 mM DTT (Dithiothreitol), 2.5 mM $MgCl_2$, 50 mM KCl and 20 mM Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol)-HCl (pH 8.4). The reaction was terminated by heat denaturation of the enzyme for 20 min at 70°C. PCR reactions to amplify RT-products were performed in a gradient cycler (TGradient, Biometra, Göttingen, Germany) as follows: 1 cycle of 94°C for 4 min, followed by 33 cycles of 94°C for 40 s, 53.1-62.9°C for 1 min and 72°C for 1 min (final elongation at 72°C for 8 min). The reaction was performed with 0.5 U μl^{-1} Taq-Polymerase (Eppendorf, Hamburg, Germany) or SuperTaq Plus polymerase (Ambion/Applied Biosystems) for sequences with an expected length > 1,000 bp in 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 pmol μl^{-1} primer pair, 50 mM KCl and 20 mM Tris-HCl (pH 8.4). PCR products were separated by 1.3 % agarose gel electrophoresis. Fragments of appropriate size were purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned with the TOPO TA cloning kit (plasmid vector pCR4-TOPO, chemically competent TOP10 *E.coli*; Invitrogen) according to the manufacturers' protocols. 6 - 8 clones were cultured overnight in LB medium with ampicillin (100 µg ml^{-1}). Plasmids were isolated with the QIAprep Spin Miniprep kit (Qiagen) and checked for inserts by

restriction digestion with *Eco*RI (Invitrogen) and agarose gel electrophoresis. Plasmids containing inserts of appropriate size were sent to Eurofins MWG GmbH (Martinsried, Germany) for sequence determination with T3 or T7 sequencing primers.

The sequence fragments were aligned to full length sequence of NBC1 and the partial sequence of AE1 (Figure 2-4). Sequences can be obtained from GenBank under following accession numbers: NBC1: GenBank EU552533, AE1: GenBank EU552535. The software MacVector 9.5 was used for primer design, sequence alignments and analysis and AssemblyLign 1.0.9c for assemblage of overlapping fragments.

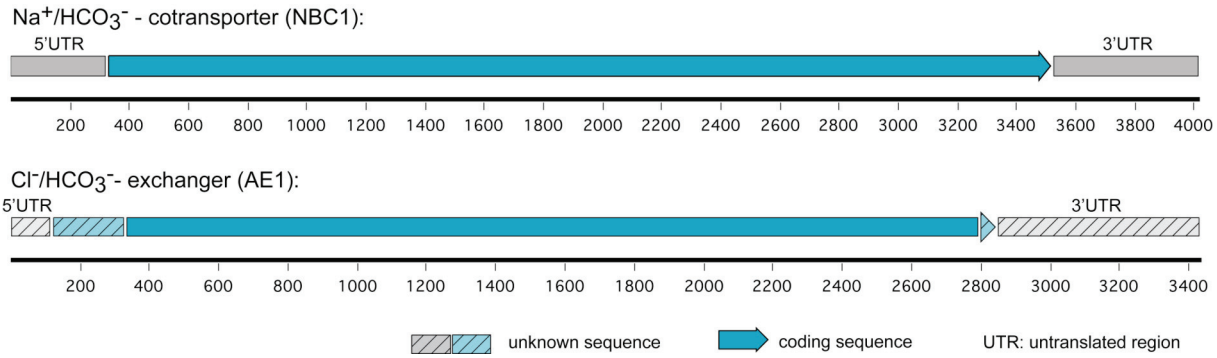

Figure 2-4: Scheme of bicarbonate cotransporter sequences
Basepair lengths of untranslated (grey) and coding regions (blue) of the cDNA sequences. The complete sequence of Na^+/HCO_3^--cotransporter mRNA, and the main part of the coding region of Cl^-/HCO_3^--exchanger was identified. Hatched areas show the putative lengths of the missing parts of the AE1 cDNA.

2.5.3 mRNA quantification by real-time PCR

DNA-free total RNA (see 2.5.1) was transcribed into cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany) to serve as a DNA template for quantitative real-time PCR performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Darmstadt, Germany, for primer table and detailed PCR protocol see publication I). mRNA expression values were evaluated relative to β-actin. The suitability of such "housekeeping" genes depends on experimental conditions, animal species and tissue type (Olsvik *et al.*, 2005; Hibbeler *et al.*, 2008). For *Z.viviparus* gill tissue, expression of β-actin did not change significantly over time to the extent that the genes under study did, which is why β-actin was considered to be an applicable endogenous control. For the calculation of relative expression levels of target genes under conditions x compared to control o, the comparative Ct method was used according to the following formula:

$$2^{(Ct_{target,\,x} - Ct_{\beta\text{-actin},\,x}) - (Ct_{target,\,o} - Ct_{\beta\text{-actin},\,o})}$$

where Ct corresponds to the threshold cycle number.

2.5.4 Whole cell and membrane extracts

For the generation of whole cell extracts, 50-100 mg of frozen gill tissue were quickly disrupted in ice-cold extraction buffer by hand homogenizing and Ultra Turrax treatment (see publication I for details). After removing of the cell debris by low-speed centrifugation, the supernatant was used as crude cell extract for protein quantification and enzyme activity measurements of the Na^+/K^+-ATPase (see 2.5.5; 2.5.6). Membrane enrichment was achieved by high-speed centrifugation of the crude homogenate. The sedimented membrane fraction was resuspended in a minimal volume of extraction buffer and used for protein quantification of the Na^+/H^+-exchanger (see 2.5.5). Total protein contents of crude and membrane extracts were determined according to the method of Bradford (1976) using bovine serum albumin as standard.

2.5.5 Protein quantification by Western Blotting

From the crude and membrane extracts the proteins were fractionated by polyacrylamide gel electrophoresis after Laemmli (1970) and transferred to PVDF (Polyvinylidene Fluorid) - membranes (see publication I for details). Briefly, non-specific protein binding sites were saturated with milk-powder proteins dissolved in a blocking buffer. Blots were incubated with primary antibodies for immunodetection of Na^+/K^+-ATPase and Na^+/H^+-exchanger, respectively, at 4°C overnight. After thorough washing, blots were incubated with the secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase). Antibody binding was visualized by chemiluminescence and detected and quantified with a cooled CCD (charge coupled device) camera system. One randomly chosen sample was used on every gel for normalization of different immunoblots. For quantification, a protein concentration was used in a range, where the signal changed linearly with antibody binding.

2.5.6 Na^+/K^+-ATPase activity assay

Na^+/K^+-ATPase activity was measured in gill crude extracts (2.5.4) after a method by Allen & Schwarz (1969). In this enzyme test, ATP hydrolysis is coupled to the oxidation of NADH by the auxiliary enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) according to the following scheme:

$$\text{ATP} \xrightarrow{\text{ATPase, } Na^+,\ K^+,\ Mg^{2+}} \text{ADP} + P_i$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow{\text{pyruvate kinase, } Mg^{2+}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NAD(P)H} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{Lactate} + NAD^+$$

After starting the reaction by adding the homogenate sample to the reaction mixture (see publication I), the oxidation of NADH was followed photometrically by measuring the decrease

of extinction at λ = 339 nm. The fraction of Na^+/K^+-ATPase activity in total ATPase activity was determined by inhibition of Na^+/K^+-ATPase with 5 mM ouabain added to the assay. With an extinction coefficient for NADH of ε = 6.31 mM^{-1} cm^{-1}, enzyme activity was evaluated as μmol ATP consumed per g tissue fresh weight per hour.

2.6 Suppression subtractive hybridization

mRNA (see 2.5.1) from *Z. viviparus* gill tissue was used for the construction of subtractive cDNA libraries to compare the gene expression pattern after 24 hours of hypercapnia with the normocapnic control (see 2.2). Both, a forward and a reverse subtracted, normalized library were generated using the BD PCR-Select cDNA Subtraction Kit (BD Biosciences, Heidelberg, Germany), which is based on Diatchenko and co-workers (1996), according to manufacturer's instructions. With this method, the suppression subtractive hybridization (SSH), specifically those genes can be identified, which are up- or downregulated under a certain condition.

Figure 2-5 shows the principle of the SSH technique: mRNA is reverse-transcribed into cDNA and digested with a restriction enzyme to obtain handy fragments. They are separated into two groups and ligated with special adaptors (1 and 2). The cDNA from the controls (driver) is then hybridized in excess with the target cDNA (tester). Thus, only upregulated genes remain as single strands (forward subtraction). Similarly, by swapping driver and tester, the downregulated genes are obtained (reverse subtraction). During the hybridization, high abundant cDNA fragments also form homo-hybrids with the same adaptors on both ends due to the faster reaction kinetics. In a second hybridization, groups with adaptors 1 and 2 are mixed together, so that tester-tester-hetero-hybrids are forming. In the following amplification, the PCR-suppression effect (Siebert *et al.*, 1995) allows the normalization of genes with respect to their abundance. The terminally ligated adapters contain repeated sequences (ITRs, inverted terminal repeats), which form pan-like structures of the cDNA strands and inhibit amplification by PCR. Therefore, the highly abundant sequences with identical ITRs from the first hybridization step are only marginally amplified, whereas the fragments with different ITRs are amplified in the normal, exponential manner (Figure 2-5)

The differentially expressed cDNAs were cloned using the pGEM-T Easy cloning kit (Promega, Mannheim, Germany) for blue/white screening (see publication II for details). For each library, 1056 white colonies were selected, cultured and shipped to GATC Biotech (Konstanz, Germany) for sequence determination.

Sequences were processed with programs of the Staden Package (http://staden.sourceforge.net: PreGap4, Gap4) and subjected to GenBank BLASTX search.

Based on the Gene Ontology project (The Gene Ontology Consortium, 2000), positive GenBank matches were assigned to functional categories (see publication II).

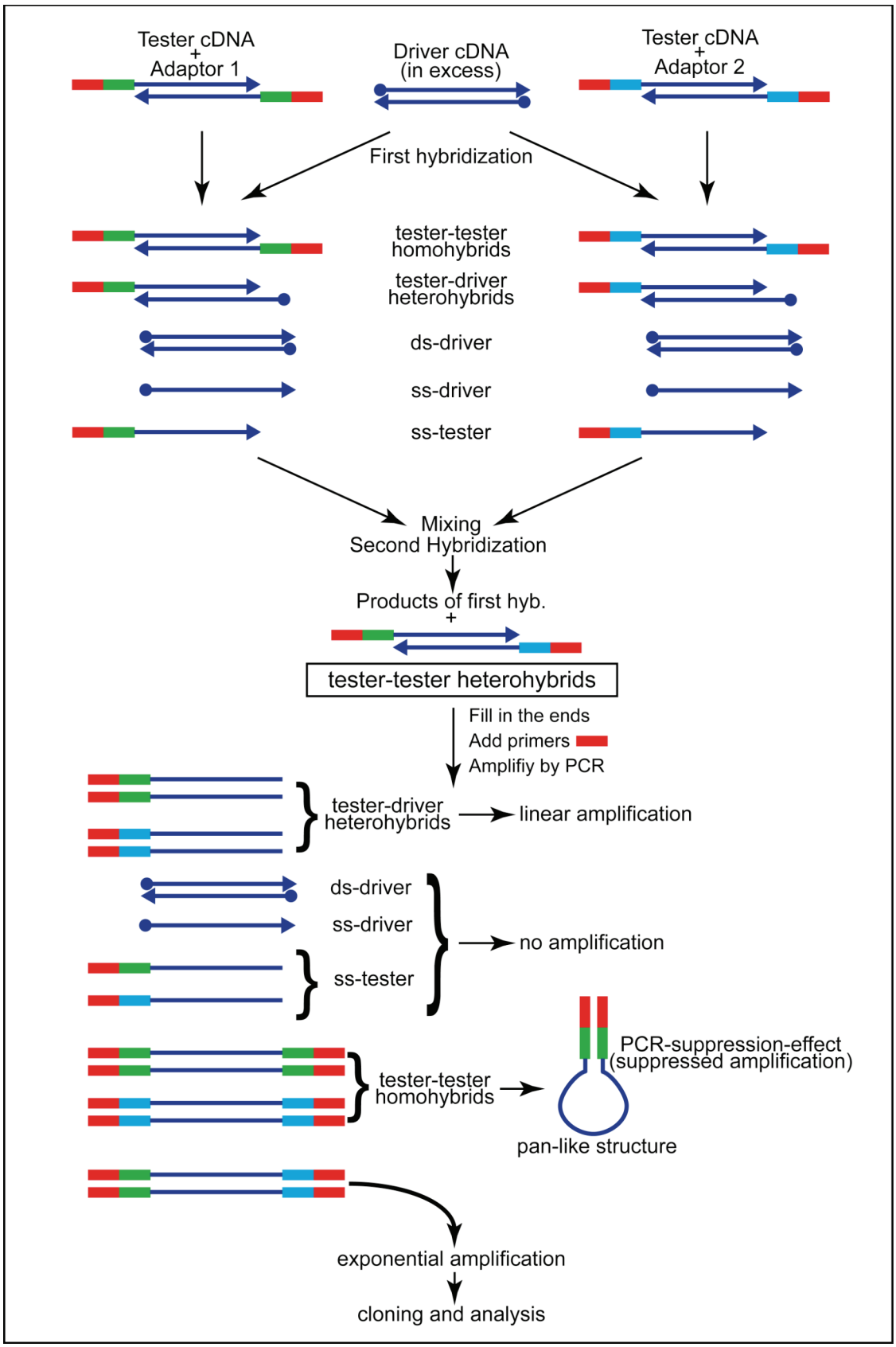

Figure 2-5: Suppression subtractive hybridization reaction scheme
After BD PCR-Select cDNA Subtraction Kit User Manual (No. 637401, 12/2004)

2.7 Statistics

The amount of gill tissue from *Z. viviparus* that was necessary for assessing the molecular data in publication I was limited. Therefore, from each treatment group of 8-9 fish two or four samples were pooled from two individual fish of the same size and sex. The pooled sample was treated as $n = 1$ in the statistical analysis. Outliers were identified at the 95 % significance level using Nalimov's test (Noack, 1980) and removed. Statistical significance was tested with the remaining samples at the $p < 0.05$ level using analysis of variance (one-factor ANOVA) and the *post-hoc* Dunnett's test for comparing the samples from different time points of hypercapnia incubation to the 0 h control sample. Values were expressed as means ± standard error of the mean (s.e.m.).

For SMR measurements in publication I, two-factor ANOVA and the post-hoc Student-Newman-Keuls' test for all pairwise multiple comparisons were used to test for significant influences of the factor time and the factor hypercapnia on whole animal oxygen consumption. Values were expressed as means ± s.e.m.

The oxygen consumption rates of isolated gills under control and hypercapnic conditions (publication II, Additional Results) were expressed as means ± s.e.m. Statistical significance of differences between control and hypercapnic groups was tested at the $p < 0.05$ level using Student's t-test.

For the determination of energy budgets in publication II, effects of inhibitors on individual gill arches were measured and mean values were evaluated for each group in the case of *G. gibberifrons* (control, cycloheximide, actinomycin D, ouabain). For *N. coriiceps* each gill arch was used as its own control prior to application of one inhibitor. Means ± s.e.m. were derived from percent inhibition data per group. Again, statistical significance of differences between control and hypercapnic groups was tested at the $p < 0.05$ level using Student's t-test.

3 Publications

List of publications and my contribution towards them

Publication I

Katrin Deigweiher, Nils Koschnick, Hans O Pörtner and Magnus Lucassen (2008)

Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia

American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 295 (5): R1660-R1670

The concept of this study was developed by ML, HOP and myself. I conducted the experiments supported by NK. I analyzed the data and wrote the manuscript, which was revised together with ML and HOP.

Publication II

Katrin Deigweiher, Timo Hirse, Magnus Lucassen, Christian Bock and Hans O Pörtner (2009)

Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids

Journal of Comparative Physiology B - Biochemical, Systems, and Environmental Physiology (submitted)

I developed the concept and design of the experiments in cooperation with TH, supported by ML and HOP. TH and I carried out the experiments on board 'RV Polarstern', supported by CB. I analyzed the data and wrote the manuscript, which was revised by CB, ML and HOP.

Publication III

Katrin Deigweiher, Hans O Pörtner and Magnus Lucassen (2009)

Differential gene expression in gills of marine eelpout under hypercapnia

Comparative Biochemistry and Physiology - Part D. Genomics and Proteomics (submitted)

Together with ML, I elaborated the concept and outline of this study. I carried out the experiments and wrote the manuscript, supported by ML in data analysis. The manuscript was revised in cooperation with ML and HOP.

PUBLICATION I

Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia

K Deigweiher, N Koschnick, H O Pörtner & M Lucassen

2008

American Journal of Physiology:
Regulatory, Integrative and Comparative Physiology

295: R1660-R1670

Am J Physiol Regul Integr Comp Physiol 295: R1660–R1670, 2008.
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Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia

Katrin Deigweiher, Nils Koschnick, Hans-Otto Pörtner, and Magnus Lucassen

Alfred Wegener Institute for Polar and Marine Research, Marine Animal Physiology, Bremerhaven, Germany

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Deigweiher K, Koschnick N, Pörtner H, Lucassen M. Acclimation of ion regulatory capacities in gills of marine fish under environmental hypercapnia. *Am J Physiol Regul Integr Comp Physiol* 295: R1660–R1670, 2008. First published September 17, 2008; doi:10.1152/ajpregu.90403.2008.—The preservation of ion balance and pH despite environmental fluctuations is essential for the maintenance of vital cellular functions. While several ion transporters contribute to acid-base regulation in fish, the involvement and expression of key transporters under hypercapnia remain to be established. Here, two members of the HCO_3^- transporter family (Na^+/HCO_3^- cotransporter NBC1 and Cl^-/HCO_3^- exchanger AE1) were described for the first time in gills of marine fish. Benthic eelpout *Zoarces viviparus* were acclimated to 10,000 ppm CO_2. Hypercapnia did not affect whole animal oxygen consumption over a period of 4 days. During a time series of 6 wk NBC1 mRNA levels first decreased by about 40% (8 to 24 h) but finally increased about threefold over control. mRNA expression of AE1 decreased transiently by 50% at *day 4* but recovered to control levels only. Reduced mRNA levels were also found for two Na^+/H^+ exchangers (NHE1A, NHE1B) during the first days (by 50–60% at *days 1* and *2*), followed by restoration of control levels. This pattern was mirrored in a slight decrease of NHE1 protein contents and its subsequent recovery. In contrast, Na^+-K^+-ATPase mRNA and protein contents, as well as maximum activity, rose steadily from the onset of hypercapnia, and reached up to twofold control levels at the end. These results indicate shifting acclimation patterns between short- and long-term CO_2 exposures. Overall, ion gradient-dependent transporter mRNA levels were transiently downregulated in the beginning of the disturbance. Upregulation of NBC1 on long timescales stresses the importance of this transporter in the hypercapnia response of marine teleosts. Long-term rearrangements include Na^+-K^+-ATPase at higher densities and capacities, indicating a shift to elevated rates of ion and acid-base regulation under environmental hypercapnia.

Zoarces viviparus; Na^+-K^+-ATPase; Na^+/HCO_3^- cotransporter; Cl^-/HCO_3^- anion exchanger; Na^+/H^+ exchanger; real-time polymerase chain reaction

VARIOUS ASPECTS OF ORGANISMAL acid-base regulation across fish gill epithelia have been studied extensively in the past decades, and studies of the patterns of ion and pH regulation in response to environmental stimuli have a long history (16, 19). Ion transport mechanisms have been under investigation since the discovery of the role of fish gill Na^+-K^+-ATPase in adaptation to seawater by Epstein et al. (15). A large variety of ion transporters in fish gills has been described (for reviews, see Refs. 16, 32, 40). With rising concern about the impact of anthropogenic CO_2 emissions on ocean physicochemistry and marine organisms (reviewed by Ref. 43), the response of the ion transport machinery to ambient hypercapnia (increased CO_2 partial pressure) and ocean acidification has attracted renewed interest. CO_2 concentrations in atmosphere and ocean surface waters are expected to increase from current levels of 380 ppm to a maximum of 1,130 ppm until the end of the century (23a). Deep-sea carbon dioxide sequestration ventures as proposed more than 30 years ago (30) would expose marine animals to even higher CO_2 partial pressures. Identification of the mechanisms affected by elevated CO_2 levels in marine animals is crucial in understanding ecosystem level effects of ocean acidification.

In fish, acid-base regulation under elevated CO_2 levels is achieved by a direct or indirect net accumulation of bicarbonate, accompanied by an equimolar loss of anions, mostly Cl^-. An involvement of the bicarbonate transporter family seems likely (19, 27, 50). So far, two kinds of bicarbonate transporters have been described for gills of freshwater fish, the Cl^-/HCO_3^- exchanger (named AE for anion exchanger) and the Na^+/HCO_3^- cotransporter (NBC1), both from the solute carrier (SLC) 4 gene family (16, 44). AE1 has been localized in the apical membrane of gill epithelial cells (53), mediating Cl^- import correlated with HCO_3^- secretion. Thus, HCO_3^-/Cl^- exchange may play an important role during alkalosis but might also be operative during hypercapnia-induced acidosis as proposed earlier (7, 19). In the cyprinid *Osorezan dace*, inhabiting an extremely acidic lake, NBC1 has been localized in the basolateral membrane, mediating export of Na^+ and HCO_3^- to the extracellular space (22). It remains to be established whether these transporters are functionally expressed in seawater fish.

Direct secretion of protons may contribute to reduce the acid load under hypercapnic conditions. Again, the mechanisms involved may differ between freshwater and seawater fish. Studies in freshwater fish have proposed active export of protons by an apical V-type H^+-ATPase, coupled to an import of Na^+ ions via an ENaC (epithelial Na^+-channel)-like channel (10, 28). In seawater fish, use of the Na^+/H^+ exchanger and the existing Na^+ gradient is energetically cheaper. Both the proton ATPase and sodium/proton exchange exist in seawater, as well as in freshwater fish gills but may participate in different proportions. The required extrusion of Na^+ ions is mediated by the basolateral Na^+-K^+-ATPase, transporting Na^+ against K^+ into the extracellular space. Together with the secretion of Cl^- ions via apical Cl^--channels, a positive transepithelial potential is generated, which is thought to drive the diffusion of Na^+ ions through leaky tight junctions into the surrounding water. This process may be paralleled by the activity of a basolateral

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$Na^+/K^+/2Cl^-$ cotransporter, which mediates the import of these ions from the extra- into the intracellular space. Recycling of K^+ ions is achieved by basolateral K^+-channels (for reviews, see Refs. 7 and 16).

Previous studies have examined the responses of freshwater and marine fish to hypercapnia for up to 20 days, focusing on general patterns and mechanisms involved in acid-base regulation (8, 9, 27, 33, 37, 50). In the present study, we identified essential transport proteins involved in the hypercapnia response of the marine eelpout *Zoarces viviparus*. The viviparous eelpout with its low dispersal range has become a model organism to monitor and study the response to climate change and associated environmental factors (42). We studied transcriptional and translational patterns, as well as the functional capacity of Na^+-K^+-ATPase as a key enzyme driving most energy-dependent ion transport processes, including those involved in acid-base regulation over a period of 6 wk. Furthermore, we characterized two members of the bicarbonate transporter family, namely NBC1 and AE1, for the first time in marine fish gills. Together with Na^+/H^+ exchanger (NHE1), the expression of these ion gradient-dependent transporters was assessed. As in previous investigations 10,000 ppm of CO_2 has been used in the present study to elucidate essential and responsive molecular processes under more extreme levels of hypercapnia. Future studies will need to elaborate the role of such mechanisms under CO_2 concentrations as predicted by climate scenarios (23a).

MATERIALS AND METHODS

Animals and experimental procedures. Common eelpout (*Z. viviparus*) were obtained from the Nordseeaquarium Büsum (Germany) in May 2005 and kept in a flow-through aquarium system at 10°C under a 12:12-h day-night cycle. They were fed twice a week with North Sea shrimps ad libitum prior and during the acclimation period but not during the last 5 days before sampling. For incubations under hypercapnia, the animals were transferred to another aquarium system containing seawater equilibrated with 10,000 ppm CO_2, resulting in a drop in water pH from control levels of 8.1 to 6.9. The gas mixture was provided by a mass flow controller (MKS Instruments Deutschland, München, Germany) through mixing of 1% CO_2 and 99% air. pH measurements were performed with a pH-Meter (340i, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) equipped with a SenTix 81 electrode (WTW) calibrated between pH 7 and 10, according to the National Bureau of Standards. In order to take possible effects of handling into account, the control group was also transferred to another tank 24 h before sampling. Groups of 8 or 9 fishes were sampled at every designated time point (0 h control, 8 h, 24 h, 48 h, 4 days, 7 days, 14 days, 42 days). Animals were caught consecutively and stunned by a blow to the head. After weight and length determinations (overall means 40.4 ± 21.6 g, 20.2 ± 3.0 cm), the fish were killed by cutting their spine as close to the cranium as possible. From this cut, the cranium was opened and the brain was dissected first. Thereafter, gill and other tissue samples were dissected quickly, frozen in liquid nitrogen and stored at −80°C until further analysis. Tissues were not perfused before freezing, because the contribution of blood to whole gill expression levels has been previously shown to be negligible (38, 46). Furthermore, other possibly blood-contaminated tissues like liver did not show any mRNA expression of NHE1a or NHE1b in *Z. viviparus* at all (M. Lucassen, N. Koschnick, E. Sokolov, H. Pörtner, unpublished data). Handling and killing of the fish were conducted in conformity with the recommendations of the American Veterinary Medical Association. An approval of the work was issued by competent German authority (Freie Hansestadt Bremen, reference number 522-27-11/2-0; date: 2002-11-28).

mRNA expression of ion transporters. Total RNA from gill tissue was isolated according to the RNeasy Mini Kit (Qiagen, Hilden, Germany), and complete removal of DNA was ensured by DNase digestion with the DNA-free kit (Applied Biosystems, Darmstadt, Germany). Integrity of the RNA was controlled with a RNA 6000 Nano LabChip assay (Agilent Technologies, Waldbronn, Germany), and the concentration was determined spectrophotometrically (BioPhotometer Eppendorf, Hamburg, Germany).

For expression studies, cDNA sequences of Na^+-K^+-ATPase (ATN-A1) of two isoforms of the Na^+/H^+ exchanger (NHE1A, NHE1B) and of β-actin (26) as endogenous control were used from earlier studies (M. Lucassen, N. Koschnick, E. Sokolov, H. Pörtner, unpublished data; accession numbers: ATN-A1: EU810373, NHE1A: EU810375, NHE1B: EU810376) For the NBC1, the entire cDNA sequence was isolated from *Z. viviparus* using a combined approach of reverse transcriptase-linked PCR with conserved primers and rapid amplification of cDNA ends (RACE), as described earlier (31). In the same way, a partial cDNA sequence from zoarcid Cl^-/HCO_3^- exchanger (AE1) was obtained (for primers, see Supplemental Table 1 in the online version of this article). PCR reactions were performed in a gradient cycler (TGradient, Biometra, Göttingen, Germany) as follows: 1 cycle of 94°C for 4 min, followed by 33 cycles of 94°C for 40 s, 53.1–62.9°C for 1 min, and 72°C for 1 min (final elongation at 72°C for 8 min). Separation, cloning, and analyses of PCR fragments were performed as described by Mark et al. (31). The sequences have been submitted to GenBank and can be obtained under the following accession numbers: NBC1: EU552532 and AE1: EU552533.

Total RNA from all specimens was transcribed into cDNA with the high-capacity cDNA archive kit (Applied Biosystems, Darmstadt, Germany) and used as a template for quantitative real-time PCR performed on an ABI 7500 Real Time PCR System (Applied Biosystems). Primers and Taqman probes for real-time PCR were designed by using the Primer Express software (ver. 3.0, Applied Biosystems; see Table 1).

PCR reactions contained 0.2 to 1 ng of cDNA, 900 nM of each primer, 200 nM of each probe and 2x TaqMan PCR Master Mix (Applied Biosystems). Real-time PCR reactions were performed, according to the standardized protocol. All samples were run in triplicate, after each PCR condition had been optimized, with cDNA concentrations ranging about five orders of magnitude according to the manufacturer's instructions. To ensure that background or genomic DNA contaminations were negligible, no-template controls and no-reverse-transcribed-RNA controls were run, respectively. mRNA expression values of the different transporters were normalized against the "housekeeping" gene β-actin. Recent studies on the suitability of "housekeeping" genes emphasized the necessity of testing the expression stability of these genes (21, 35). Depending on experimental conditions, animal species and tissue type, not every "housekeeping" gene can serve as an applicable endogenous control. In case of *Z. viviparus* gill tissue, expression of β-actin did not change significantly over time to the extent that the genes under study did. For the calculation of relative expession levels under condition "n" compared to condition "control" (0), the comparative Ct method was used: {formula: $2^{[(Ct_{transporter,n} - Ct_{\beta\text{-}actin,n}) - (Ct_{transporter,0} - Ct_{\beta\text{-}actin,0})]}$}, where Ct corresponds to the threshold cycle number.

Protein quantification. Frozen gill tissue samples were quickly homogenized with a conical glass tissue grinder in 10 volumes of ice-cold buffer [50 mM imidazole, pH 7.4, 250 mM sucrose, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1% (wt/vol) deoxycholate, protease inhibitor cocktail from Sigma-Aldrich, Taufkirchen, Germany; Cat. No. P 8340] followed by Ultra Turrax treatment (3 × 10 s). Cell debris was removed by centrifugation for 10 min at 1,000 *g* and 0°C. One-half of the supernatant was used as a crude extract for Na^+-K^+-ATPase activity measurements and protein quantification, and the other half was used for membrane preparation by centrifugation for

Table 1. *Primer and probe sequences used for qRT-PCR*

Fragment	Term	Sequence	Position	Length
ATN-A1	Forward	CCCTTCAACTCCACCAACAAA	485–505	131
	Probe	6-FAM-ATCAGCTCTCCGTCCAC-MGB	507–523	
	Backward	AGCAGCGGTCCAAAATCCT	617–599	
NHE1A	Forward	GGACGATCAATGAGGAAATCCA	1169–1190	85
	Probe	6-FAM-ACCCAGTTCCTTGACCAC-MGB	1192–1209	
	Backward	AATGTCCTCGATTCCAGTTAGCA	1255–1233	
NHE1B	Forward	GAGCCGCTGTTTGTTTTCGT	600–619	85
	Probe	6-FAM-CGTACCTCTCCGCTGAG-MGB	634–650	
	Backward	TGCCGGACAGGTGGAAGA	686–669	
AE1	Forward	TGATTGCTGTGGATATTTGCATT	1677–1699	59
	Probe	6-FAM-AGGACGCTTACACCC-MGB	1701–1715	
	Backward	CCTTTTGGCACAACCAGTTTC	1737–1717	
NBC1	Forward	CTGGCAACTCGTCAGACCTTT	2209–2229	61
	Probe	6-FAM-TGATGTCGCCCTGCTTA-MGB	2231–2247	
	Backward	TGACAGGCAGATCAGTACGGTTT	2271–2249	
β-Actin	Forward	GGAGAAGATGACCCAGATCATGTT	91–114	98
	Probe	6-FAM-CCATGTACGTTGCCATC-MGB	135–151	
	Backward	AGTGGTACGACCAGAGGCATACA	190–168	

The positions of the primers and probes correspond to the following genes: AE1, GenBank EU552535; NBC1, GenBank EU552533; β-Actin, GenBank AY227658; ATN-A1, GenBank EU810373; NHE1A, GenBank EU810375; NHE1B, GenBank EU810376.

1 h at 20,000 *g* and 0°C. The sedimented membrane fraction was resuspended in 2 volumes of extraction buffer and was used for NHE1 protein quantification. Total protein contents of crude and membrane extracts were determined according to the method of Bradford (2), using BSA as the standard.

For immunoblotting, 8 μl of crude extracts for Na^+-K^+-ATPase and 20 μl of membrane extracts for Na^+/H^+ exchanger were used. Proteins were fractionated by SDS-PAGE on 10% polyacrylamide gels, according to Laemmli (26), and transferred to PVDF membranes (Bio-Rad, Munich, Germany), using a tank blotting system (Bio-Rad). Blots were preincubated for 1 h at room temperature in TBS-Tween buffer [TBS-T, 50 mM Tris·HCl, pH 7.4, 0.9% (wt/vol) NaCl, 0.1% (vol/vol) Tween20] containing 5% (wt/vol) nonfat skimmed milk powder. As primary antibody for the Na^+-K^+-ATPase α-subunit, the α5 monoclonal antibody (culture supernatant: 1:100), originally developed against the chicken α-subunit by D. M. Fambrough (John Hopkins University, Baltimore, MD), was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Development, maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa. For NHE1 mouse monoclonal antibody 4E9 raised against porcine NHE1 (45), (Chemicon, Temecula, CA) was used in the same buffer (2 μg in 5 ml). Blots were incubated with primary antibodies at 4°C overnight. After washing with TBS-T, blots were incubated for 1 h with goat anti-mouse IgG antibody (horseradish peroxidase conjugated; Pierce, Rockford, IL) diluted 1:2,000 in TBS-T containing 5% nonfat skimmed milk powder. Protein signals were visualized by using the ECL Western blotting detection reagents (GE Healthcare, Munich, Germany) and recorded by a LAS-1000 charge-coupled device camera (Fuji, Tokyo, Japan). Signal intensity was calculated using the AIDA Image Analyzer software (ver. 3.52, Raytest, Straubenhardt, Germany), and results were expressed as values normalized to the 0 h control. One randomly chosen sample was used on every gel for normalization of different immunoblots. Membrane preparations were used to determine the optimal concentration ratio for antigen over primary and secondary antibody. For quantification, an intermediate protein concentration was used in a range, where the signal changed linearly with antibody binding.

Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity was measured in gill crude extracts (see *Protein quantification*) in a coupled enzyme assay with pyruvate kinase (PK) and lactate dehydrogenase (LDH) using the method of Allen and Schwarz (1). The reaction was started by adding the sample homogenate to the reaction buffer containing 100 mM imidazole, pH 7.4, 80 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 0.24 mM Na-($NADH_2$), 2 mM phosphoenolpyruvate, about 12 U/ml PK and 17 U/ml LDH, using a PK/LDH enzyme mix (Sigma-Aldrich). The oxidation of NADH coupled to the hydrolysis of ATP was followed photometrically at 10°C in a DU7400i spectrophotometer (Beckman Coulter, Krefeld, Germany) over a period of 10 min, measuring the decrease of extinction at $\lambda = 339$ nm. The fraction of Na^+-K^+-ATPase activity in total ATPase (TA) activity was determined by the addition of 5 mM ouabain to the assay. Enzyme activity was calculated using an extinction coefficient for NADH of $\varepsilon = 6.31$ $mM^{-1}\cdot cm^{-1}$ and given as micromoles consumed ATP per gram tissue fresh weight (fwt) per hour.

Standard metabolic rate. Animals were starved for 2 days prior to and during the experiments. Standard metabolic rates (SMR) were determined using intermittent closed respirometry. Briefly, animals weighing 37.7 ± 6.2 g ($n = 11$) were incubated in cylindrical perspex chambers (diameter = 3 cm, length = 25 cm) for a period of 10 days. For control measurements and during prehypercapnia and posthypercapnia treatments, chambers were perfused with freshly aerated (100% air) seawater from a reservoir, using a peristaltic pump (ISM 404B, Ismatec, Wertheim-Mondfeld, Germany) and gas-tight tubing (Tygon T4406-23, Saint-Gobain Performance Plastics, Charny, France). For hypercapnia a gas mixture of 1% CO_2 and 99% air was provided by a gas mixing pump (Wösthoff, Bochum, Germany). Water flow rates of 60 to 66 ml/min ensured chamber oxygen partial pressures of approximately 18 to 20 kPa between measurements. Temperature was maintained at 10°C (± 0.2°C) by placing the four replicate chambers in a temperature-controlled water bath. Respirometry runs were performed twice a day (11:00 AM, 4:00 PM), by creating a closed-loop circulation. Oxygen partial pressures were measured using a fiber-optic oxygen sensing system (Oxy-4 micro, PreSens, Regensburg, Germany) with oxygen optodes (needle-type microsensors, PreSens) incorporated into the closed loop. Within 10 to 12 min, animals usually had reduced chamber oxygen levels to 14–16 kPa. Oxygen consumption rates were calculated from linear declines in chamber oxygen partial pressure with time using the following formula: $M(O_2) = \Delta P(O_2)\ \beta(O_2)\ V\ w^{-1}$, where $M(O_2)$ is the oxygen consumption rate [$\mu mol(O_2)\cdot g^{-1}\cdot h^{-1}$], $\Delta P(O_2)$ is the gradient of oxygen decrease over time in the chamber [kPa], $\beta(O_2)$ is the oxygen capacity of water [$\mu mol(O_2)\cdot l^{-1}\cdot kPa^{-1}$], V is the volume of the closed chamber loop (liters), and w is the animal weight (g).

Statistics. Values were expressed as means ± SE. Because of the limited amount of gill tissue available from some animals, two or four

among the total amount of samples for each treatment were pooled from two individual fish of same size and gender. The pooled sample was treated as $n = 1$ in the statistical analysis. Outliers were identified at the 95% significance level using Nalimov's test and were removed. Statistical significance was tested with the remaining samples at the $P < 0.05$ level using one-factor ANOVA and the post hoc Dunnett's test for comparing the samples from different time points of hypercapnia incubation to the 0 h control sample. Where indicated, additional ANOVA tests were performed to identify significant changes during the early or long-term phase of hypercapnia exposures. For SMR measurements, two-factor ANOVA and the post hoc Student-Newman-Keuls' test for all pairwise multiple comparisons have been used to test for significant influences of the factor time and the factor hypercapnia on whole animal oxygen consumption.

RESULTS

All molecular and functional results were obtained from measurements in gill tissue samples taken during the hypercapnia (10,000 ppm CO_2) trial at 0 h (control), 8 h, 24 h, 48 h, 4 days, 7 days, 14 days, and 42 days.

Ion transporter mRNA expression. The mRNA expression levels of the Na^+/H^+ exchanger first decreased at the onset of hypercapnia but recovered to control levels thereafter and remained unchanged until the end of the incubation period (fig. 1*A*). The dominant isoform NHE1A, which was generally found expressed at 10-fold higher levels than isoform 1B, decreased by about 60% during the first 2 days and recovered slowly thereafter, while isoform 1B showed an initial 50% decrease and was already fully recovered after 2 days (Fig. 1*A*). The Cl^-/HCO_3^- anion exchanger AE1 showed an expression pattern with minimal expression after *day 4* (50% below control levels, albeit marginally and only significant when testing 4 days against control and 42 days) followed by full recovery to control values (Fig. 1*B*). After an initial, slight decline within the first 2 days (40%), Na^+/HCO_3^--cotransporter mRNA was stabilized until *week 2* but was upregulated to about 300% after 6 wk of acclimation (Fig. 1*C*). In contrast to gradient-dependent ion transporters, mRNA expression of Na^+-K^+-ATPase increased significantly during the first 24 h after the onset of the CO_2 treatment and was maintained—with the exception of *day 4*—at a significantly higher level of about 200% for the remaining time period (Fig. 1*D*).

Ion transporter protein levels. Na^+-K^+-ATPase and Na^+/H^+ exchanger protein levels were determined by immunoblotting (fig. 2). After cell fractionation, both antibodies detected a single, clear band depicting the expected size in the membrane fraction; no signals were detectable in the cytosol (data not

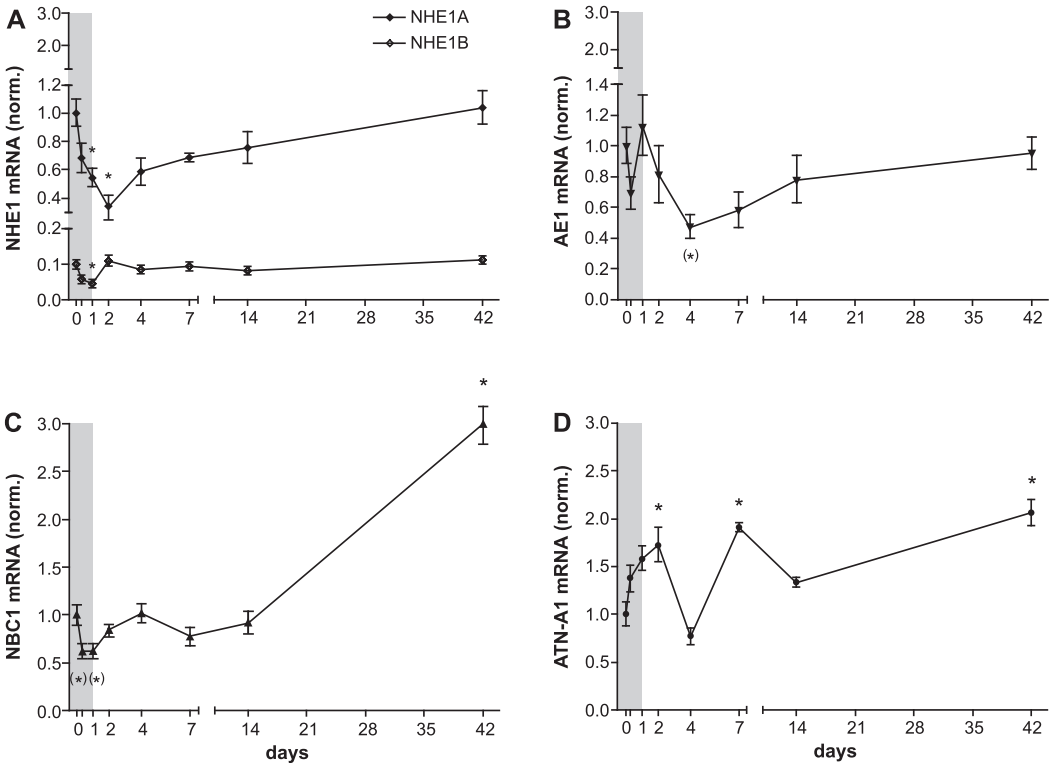

Fig. 1. Gill ion transporter gene expression during 6 wk of exposure to 10,000 ppm hypercapnia. *A*: Na^+/H^+ exchanger (NHE1) isoforms A and B. *B*: Cl^-/HCO_3^- anion exchanger (AE1), *C*: Na^+/HCO_3^- cotransporter (NBC1), *D*: Na^+-K^+-ATPase (ATN-A1). Expression is given relative to the expression of β-actin and is normalized to the respective 0 h controls. Significant differences compared to the 0 h control group ($P < 0.05$) are indicated by asterisks (one-factor ANOVA). Asterisks in parentheses indicate significant differences to controls according to one-factor ANOVA for the acute (24 h) or long-term phase (up to 6 wk) of acclimation. The acute phase is depicted by gray shading. Values are expressed as means ± SE (n = 5–7).

shown). For Na^+-K^+-ATPase, in some samples, degradation products became visible due to the sensitivity of the detection system, which account for up to 5% of the main signal (not included into the quantification). Because of the high abundance of Na^+-K^+-ATPase, the respective antibody reaction was quantified in crude extracts, while NHE1 was determined in isolated membrane fractions. Since antibody NHE 4E9 was raised against porcine NHE1, it is reasonable to assume that this antibody did not differentiate between the two zoarcid NHEs, which are both closely related to mammalian isoform 1. Protein levels tended to follow the patterns of mRNA levels and were slightly decreased within the first 2 days, albeit only significantly for *day 1* when using ANOVA for this early time frame (Fig. 3*A*). Thereafter, NHE protein levels returned back to control values. Na^+-K^+-ATPase protein levels were found significantly increased after 2 days of hypercapnia and were maintained about 25 to 35% above control levels until the end of the trial (Fig. 3*B*).

Na^+-K^+-ATPase activity. Functional capacities of Na^+-K^+-ATPase rose steadily during the hypercapnia trial. The maximum activity of Na^+-K^+-ATPase was determined at 114.6 ± 16.2 μmol ATP·g_{fwt}^{-1}·h^{-1}. From *day 2* onward, levels were significantly increased and reached a 60% higher activity level at the end of the 6-wk trial (Fig. 4).

Standard metabolic rate. Oxygen consumption rates of *Z. viviparus* (n = 7) during acute hypercapnia are presented in Fig. 5. Starting from significant elevated values of about 2.5 μmol (O_2)·g^{-1}·h^{-1}, oxygen consumption stabilized after 2.5 days at about 1.5 μmol (O_2)·g^{-1}·h^{-1}. The onset of hypercapnia, indicated by the pH drop from 8.1 to 6.9, had no influence on SMR, which remained unchanged for 4 days, and also during 3 days of recovery under normocapnia at 1.54 ± 0.21 μmol (O_2)·g^{-1}·h^{-1} (means ± SD). In two control animals, the same stabilization pattern during the first 3 days became apparent, with a constant respiration rate of 1.55 ± 0.24 μmol (O_2)·g^{-1}·h^{-1} for the following 8 days (means ± SD). Generally, eelpouts are sluggish fish and an excellent model for SMR measurements, since they prefer to hide in small caves or tubes without moving during normal maintenance, as also reported earlier (52). In the present study, the observed activity levels were minimal during the whole experimentation.

DISCUSSION

Acid-base and associated ion regulation in fish gills comprise about 90% of the organism's total acid-base regulation (7, 16, 18, 19, 41). Studies of the genetic basis and molecular structure of essential ion transporters have focused on the involvement of specific ion transporters in acid-secretion such as Na^+/H^+ exchanger, Na^+/HCO_3^- exchanger, V-type H^+-ATPase, and a putative H^+/K^+-ATPase, or on the contrasting role of Cl^-/HCO_3^- exchangers for base secretion (5, 6, 13, 39, 49, 51).

In marine teleosts (*Perciformes*, *Salmoniformes*, *Anguilliformes*), hypercapnia levels such as those applied in the present study lead to a transient drop of extracellular and intracellular pH values (27, 33, 37, 50). Blood pH is usually fully restored to control levels within 10–24 h [with the exception of *Sparus aurata*, in which a small, but significant decrease in pHe was still visible at *day 3* (33)]. pH compensation may involve net proton release but ultimately leads to the net accumulation of bicarbonate in all investigated species (27, 33, 37, 50).

Responses of the Na^+-K^+-ATPase. Na^+/K^+-ATPase is expected to be the key enzyme providing the driving force for many gradient-dependent transport processes in the membranes of fish gills. Although the molecular regulation of Na^+-K^+-ATPase has been studied in fish with regard to osmoregulation, temperature, and development (reviewed in

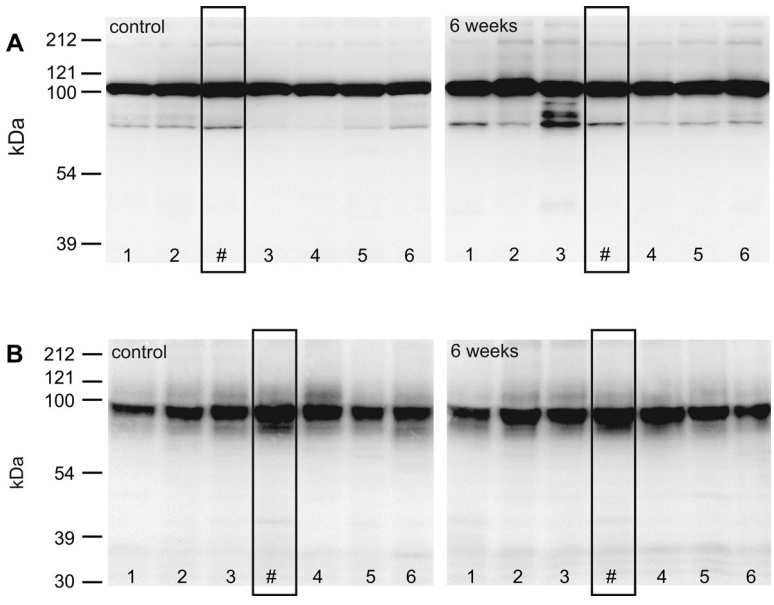

Fig. 2. Quantification of Na^+-K^+-ATPase (*A*) and Na^+/H^+ exchanger (*B*) protein levels in control animals (n = 6) or after 6 wk of hypercapnia (10,000 ppm) (n = 6). Distinct immunoreactive bands were observed for α−ATN-A1 and NHE1 with monoclonal antibodies corresponding to molecular masses of about 105 kDa and about 90 kDa, respectively. Sample 3 (6 wk) showed significant degradation of the Na^+-K^+-ATPase protein and was removed according to the outlier test. A reference sample (#) was used to equalize signal intensities from different blots.

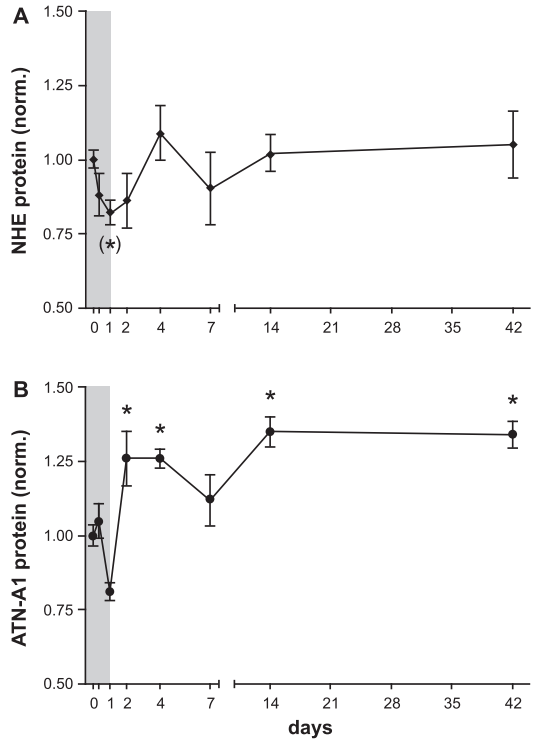

Fig. 3. Gill ion transporter protein abundance under 10,000 ppm hypercapnia. Expression is normalized to the respective 0 h controls. *A*: Na^+/H^+ exchanger (NHE1), *B*: Na^+-K^+-ATPase α-subunit (ATN-A1). Significant differences compared to the 0 h control group ($P < 0.05$) are indicated by asterisks (one-factor ANOVA). Asterisks in parentheses indicate significant differences according to one-factor ANOVA for the early phase of acclimation, which is depicted by gray shading. Values are expressed as means ± SE (n = 5–7).

Ref. 32), only a few studies have examined its response to hypercapnia. Seidelin et al. (47) found Na^+-K^+-ATPase mRNA levels of developing smolts (Atlantic salmon) reduced under short-term hypercapnia (4 days, 20,000 ppm CO_2), while its enzymatic activity remained unaffected. In contrast, Ishimatsu et al. (24) reported a significant increase of Na^+-K^+-ATPase activity in Japanese flounder after exposure to 1% and 5% hypercapnia (unpublished data cited in Ref. 24).

In the gills of *Z. viviparus* mRNA expression, protein abundance and functional capacities of Na^+-K^+-ATPase were upregulated during 6 wk of hypercapnia. The present data on the control Na^+-K^+-ATPase capacities are identical to earlier measurements in this species [120.4 ± 9.2 μmol ATP·g_{fwt}^{-1}·h^{-1} (n = 8); K. Deigweiher and M. Lucassen, unpublished data], sampled 3 mo earlier. Thus, the Na^+-K^+-ATPase capacities are stable over time, if no further treatment is applied, and the observed responses in the present study can be clearly attributed to the hypercapnia stress. The transcriptional response of Na^+-K^+-ATPase took place within 2 days after the onset of hypercapnia. While mRNA increased immediately, the protein level seemed to initially decrease and respond to the mRNA increments with a delay of about 24 h. At *day 4*, mRNA had returned to control levels, which was apparently mirrored in a decreasing protein level at *day 7*. Thereafter, mRNA and protein levels increased to 200 and 140% of control levels, respectively, and remained elevated until the end of the 6-wk acclimation period.

Similar regulatory patterns involving a delay between the transcriptional and functional upregulation of Na^+-K^+-ATPase activity in fish gills have been seen after salinity transfer experiments. In the gills of Atlantic salmon smolts, there was a direct increase of Na^+-K^+-ATPase mRNA expression after 24 h, followed by an increase in activity after 11 days, when mRNA levels had transiently returned to control levels. Values started to increase again at the end of the trial after 25 days (11). During short-term hyposmotic shock in the milkfish *Chanos chanos,* Lin et al. (29) found within an "adjustive phase" significantly increased Na^+-K^+-ATPase activity (after 3 h) and elevated protein contents (after 12 h). After return of these values to control levels within 24 h, mRNA expression increased only within the "regulatory phase" (48–96 h), followed by another increase of protein abundance and activity.

Overall, higher functional capacities of Na^+-K^+-ATPase support ion- and acid-base status under environmental hypercapnia. This increment is apparently achieved by progressively higher transcription and translation levels of the respective gene, involving continuous upregulation during the long-term treatment (6 wk). The regulatory stimulus for upregulation remains unclear. The similarity of the patterns observed in the present study to those seen under osmotic stress indicates that gene expression may respond to changes in demand rather than, for example, transient hypercapnia-associated acidosis. Na^+-K^+-ATPase provides an ion gradient, which directly drives transporters involved in proton equivalent ion exchange. The elevated demand on steady-state acid-base regulation may thus trigger the upregulation of Na^+-K^+-ATPase.

Implications of hypercapnia for gradient-dependent ion transporters. Gene expression of gradient-dependent ion transporters was regulated differently from that of Na^+-K^+-ATPase. In general, a short-term repression in the beginning was followed by a restoration until the end of the hypercapnia trial, with the exception of a long-term accumulation of Na^+/HCO_3^- cotransporter transcripts after 6 wk. Our findings are in accordance with the mechanisms generally found for marine fish under environmental hypercapnia. During the initial stage of acclimation, the pH drop in response to CO_2 accumulation is compensated for by nonbicarbonate buffering, which leads to a limited rise in plasma bicarbonate levels. Further pH compensation is probably achieved by net importing bicarbonate from the environment via epithelial ion transporters, since higher environmental bicarbonate concentrations support the recovery rate (19). In *Scyliorhinus stellaris*, environmental HCO_3^- concentration was even revealed as the limiting factor for acid-base relevant ion transfer (19, 20). Additionally, higher proton export rates would support higher serum levels of bicarbonate originated from endogenous sources. Increments in extracellular bicarbonate levels usually involve a decrease of Cl^- levels (9, 27). Therefore, existence of a Cl^-/HCO_3^- exchange mechanism at the apical membrane was postulated (19). A Na^+/HCO_3^- cotransporter in the basolateral membrane may support net bicarbonate transport from epithelial cells into the blood. However, Cl^-/HCO_3^- exchanger (49, 53) and Na^+/HCO_3^- cotransporter (22, 39) have only been characterized in the gills of freshwater fish so far.

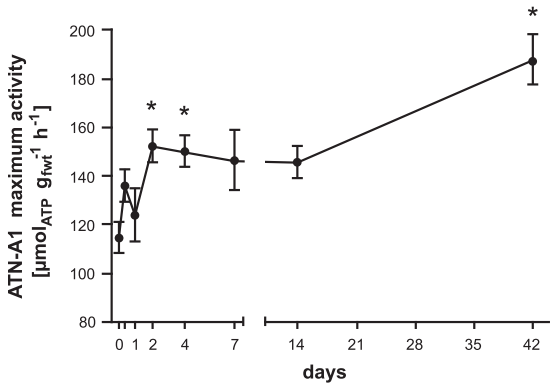

Fig. 4. Specific gill Na^+-K^+-ATPase activity under hypercapnia treatment (10,000 ppm). Activity is given in micromoles ATP per gram fresh weight (fwt) per hour. Significant differences compared to the 0 h control group ($P < 0.05$) are indicated by asterisks (one-factor ANOVA). Values are means ± SE (n = 5–7).

The role of the Na^+/HCO_3^- cotransporter in acid-base regulation has been explored in limnic fish. In the Osorezan dace (*Tribolodon hakonensis*), which live in a naturally extremely acidic lake, the protein has been localized by immunostaining at the basolateral membrane of gill chloride cells using a primary antibody against a C-terminal fraction of NBC1 (22). Also, mRNA expression increased within 5 days after transfer to low-pH water (pH 3). Similarly, NBC1 mRNA expression was increased under acute hypercapnia (10,000 ppm, 6 h) in gills of the rainbow trout *O. mykiss* (39). In marine *Z. viviparus*, however, NBC1 mRNA level was 50% downregulated during the first 24 h of hypercapnia (Fig. 1*C*). After the onset of hypercapnia, nonbicarbonate buffering and the accumulation of metabolically produced bicarbonate may reduce the need for HCO_3^- transport into the plasma. Furthermore, if bicarbonate accumulates in the epithelial cells, the hydration of CO_2 and dissociation to protons and bicarbonate are downregulated. This would minimize the production of H^+ in the cells, which supports the capacity for pH recovery (see Fig. 6).

Extended hypercapnia exposure led to the restoration of NBC1 mRNA within 2 wk and to a threefold higher expression at the end of the trial, indicating a differentiated response to acute vs. long-term hypercapnic conditions. As with Na^+-K^+-ATPase and NHE1, the change in message likely results in changing protein levels. In case of the Na^+/HCO_3^- cotransporter, elevated protein contents would support maintenance of elevated bicarbonate levels (Fig. 6*B*), as described for several fishes (9, 27, 33, 50). The threefold increment of NBC1 mRNA at the end of the trial emphasizes an important role of this transporter in steady-state acid-base regulation under long-term hypercapnia.

Since HCO_3^- accumulation under hypercapnia is usually accompanied by a decrease of Cl^- concentration, a role for gill Cl^-/HCO_3^- exchange has been postulated (see introduction). However, AE1 mRNA expression in *Z. viviparus* was only slightly affected, with a trend to decrease initially under hypercapnia, reaching a 50% decrease at *day 4*, after which time it recovered slowly to control levels until the end of the trial (Fig. 1*B*). This pattern is not in line with a rising importance of Cl^-/HCO_3^--exchange under hypercapnia. As AE1 is abundantly expressed in fish erythrocytes (17, 23), a contribution of blood mRNA to total tissue mRNA expression level might be possible. For NHE1A or NHE1B, blood contamination of the patterns seen in gills in the present study is likely negligible (see MATERIALS AND METHODS). Furthermore, Perry et al. (38) found no obvious contribution of blood mRNA to total tissue mRNA expression levels of H^+-ATPase, although also being expressed in blood cells. The existence and location of AE1 have been identified at least in freshwater fish gills (53). Thus, a functional role of AE1 in marine fish gills seems likely, but the localization and thus the transepithelial transport direction

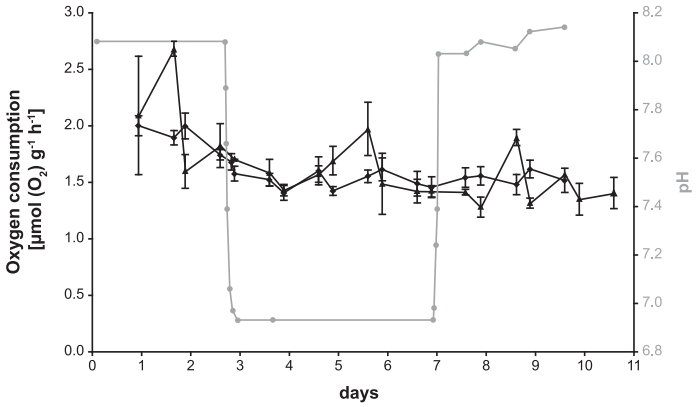

Fig. 5. Standard metabolic rate (SMR) of *Zoarces viviparus* under short-term hypercapnia (10,000 ppm). The left y-axis shows SMR as micromoles O_2 consumed per gram fish weight per hour. The right y-axis shows the change in water pH during the hypercapnia trial. After 3 days of normocapnia, CO_2 concentrations were increased for 4 days (indicated by the pH drop from 8.1 to 6.9; gray circles). Thereafter, recovery under normocapnia was monitored for 3 days. Values are expressed as means ± SE (n = 7; diamonds). Respiration rates of two control animals (triangles: median ± SD) were measured over a period of 11 days under normocapnia. Two-way ANOVA with the factors time and hypercapnia revealed significantly elevated oxygen consumption levels for the first two time points compared to the remaining time period, but there was no significant influence of hypercapnia exposure.

might be completely different. If AE1 in seawater fish gills is located apically, our findings would be in line with a role for the exchanger in base release. As base release is likely reduced during the transient acidosis, the putative decrement of AE1 content would cause lower Cl^- influx and support net bicarbonate uptake by reducing HCO_3^- loss (Fig. 6*A*). Alternatively, AE1 might be localized basolaterally in marine fish. Although this localization could support higher plasma bicarbonate and lower chloride levels under hypercapnia, the expression of this transporter remained at control levels after 6 wk. Therefore, a role of AE1 for direct basolateral Cl^-/HCO_3^- exchange under hypercapnia seems unlikely, and the regulation pattern under acute and long-term hypercapnia would be most consistent with a role for apical AE1.

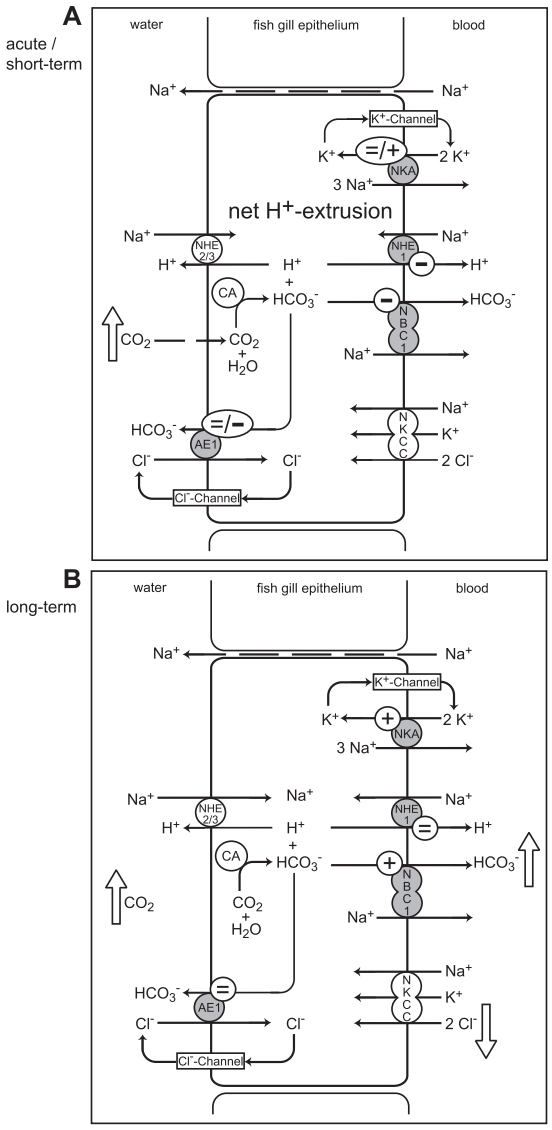

Fig. 6. Preliminary model of acute/short-term (*A*) and long-term (*B*) adjustments to environmental hypercapnia in the gills of seawater teleosts [modified after Claiborne et al. (7) and Evans et al. (16)]. CO_2 is hydrated by carbonic anhydrase (CA) after diffusive entry resulting in HCO_3^- and H^+. *A*: Acute pH compensation is achieved by nonbicarbonate buffering together with net H^+ extrusion supported by transitional downregulation (−) of the basolateral Na^+/H^+ exchanger (NHE1) and Na^+/HCO_3^- cotransporter (NBC1). Na^+-K^+-ATPase (NKA) expression is maintained and then upregulated (=/+). A delayed downregulation (=/−) of apical Cl^-/HCO_3^- anion exchanger (AE1) supports the maintenance of higher bicarbonate levels in the cell and plasma when extracellular pH is already restored. During long-term compensation (*B*), net accumulation of extracellular HCO_3^- is supported by an increase (+) in the abundance of basolateral NBC1 and the maintenance (=) of AE1 and NHE1 levels compared to controls. Net Cl^- decrease in blood may be mediated by a basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and apical Cl^- channels. NHE1 is operating at control levels under long-term steady state conditions. Net proton extrusion is possibly achieved by apical NHE2/3. The driving force for the new steady state is provided by elevated Na^+-K^+-ATPase levels in the basolateral membrane. Excess Na^+ can diffuse via leaky tight junctions into the surrounding water.

Two mechanisms have emerged in recent years for the net release of protons from the organism via the gills: ATP-consuming proton extrusion via a V-type H^+-ATPase, electrochemically coupled to a Na^+ channel, and electroneutral Na^+/H^+ exchange via a gradient-driven ion transporter. The latter might be more important in marine fish, because the intrusion of Na^+ would be favored by the inward gradient of Na^+. In both cases the extra Na^+ load would have to be compensated for by Na^+-K^+-ATPase (for reviews, see Refs. 7, 16, 41). For *Z. viviparus*, two isoforms of the Na^+/H^+ exchanger have been isolated with degenerated primers for NHE1. Both isoforms differ by about 20% (comparable to the interspecies differences within teleosts) and clearly belong to the NHE1 isoform family when compared with NHE2 or NHE3. No mRNA expression of NHE1A or NHE1B could be detected in liver or white muscle of *Z. viviparus* (M. Lucassen, N. Koschnick, E. Sokolov, H. Pörtner, unpublished data). Thus, further isoform differentiation may have taken place in the teleost branch, possibly accounting for the specific requirements within the marine environment. For both isoforms, mRNA expression was decreased by about 50% during the first days of hypercapnia, followed by a recovery to control values until the end of the trial. Isoform 1A was expressed 10-fold higher than isoform 1B, and the recovery of the former took about 2 days longer. The protein abundance of NHE1 was measured by immunoblotting with an antibody that has been successfully used in several other studies to specifically identify NHE1 in marine and euryhaline teleosts and elasmobranchs (4, 6, 14). The decrease after 2 days (probably representing the more abundant isoform 1A) followed by restoration to control levels thus parallels the mRNA data.

The functional role of different NHE isoforms may be relevant to understand how downregulation of the Na^+/H^+ exchanger genes and of their protein expression in the early phase could help to overcome hypercapnic acidosis. So far eight distinct isoforms of the SLC9 gene family have been found in mammals (reviewed in Ref. 36). In marine fish, the existence of at least three isoforms (NHE1, NHE2, and NHE3) has been demonstrated by use of antibodies and/or sequencing (reviewed in Refs. 7 and 16). Recently, Edwards et al. (14)

studied NHE isoform expression in the gills of freshwater (FW) and seawater (SW) adapted euryhaline fish *Fundulus heteroclitus* after 1 h of hypercapnia treatment (10,000 ppm CO_2) and found an increase in the expression of NHE2 in FW fish, while in SW fish, both NHE1 and NHE3 were increased. These findings are not in line with our study, which is rather in line with an earlier study by the same group, in which a decrease in NHE1 after metabolic acidosis was found in the gills of marine sculpin. The authors postulated that NHE1 is localized at the basolateral membrane (6). Downregulation of NHE1 in the beginning would then favor net acid excretion, thereby counteracting the acute effects of hypercapnia, while isoform NHE2/3 at the apical membrane is maintained or even upregulated (Fig. 6*A*). Indeed, NHE2 has been localized in the apical membrane, and protein abundance remained constant after acute acidosis over 8 h in gills of the marine teleost *Myoxocephalus octodecemspinosus* (3). Further isoforms at the apical membrane might thus be involved in H^+ secretion. However, the existence of such isoforms remains unconfirmed for *Z. viviparus* despite analysis by means of different antibodies or by cDNA approaches.

Proposed working model of gill ion transporter regulation under hypercapnia. The present study provides insight into shifting roles of transporters in acid-base regulation during acclimation to environmental hypercapnia. Even though changes in protein levels might be less pronounced than responses of mRNA expression to hypercapnia, the shifts in mRNA levels found here for AE1 and NBC1 over time likely reflect the respective changes in protein levels. In line with available literature, we propose a biphasic acclimation mechanism to hypercapnia for *Z. viviparus*. The acute response within the gill, when the pH drop is being compensated for through nonbicarbonate buffering and stimulated ion exchange by the existing transporter inventory, occurs within minutes to hours. Within this recovery phase (usually completed within 10–24 h), a first response at the mRNA level takes place by downregulation of basolateral NHE1 (after 24 h) and NBC1 (after 8 h). Within the epithelial cells, this may support higher bicarbonate levels and subsequent inhibition of carbonic anhydrase on the one hand (NBC1) and reduced proton export into the plasma on the other (NHE1). Net H^+ extrusion by further transporters (e.g. NHE2 and NHE3) has to be postulated to explain the recovery of pH (Fig. 6*A*). After this initial compensation, the protein inventory is rearranged long term to meet the requirements of the new ion composition. During this acclimatory phase, when pH compensation is most probably completed, short-term downregulation of AE1 (*day 4*) may support the maintenance of the now required higher bicarbonate concentration (Fig. 6*A*). Accordingly, Na^+-K^+-ATPase as the driving force of ion and pH regulation is maintained from the beginning and then progressively upregulated under long-term hypercapnia up to 6 wk and beyond (Fig. 6, *A* and *B*). For Na^+-K^+-ATPase, a feedback regulation of mRNA and protein levels in the early acclimation phase became visible, as previously postulated under hyposmotic exposure (29). For maintaining the new steady-state of acid-base regulation, with elevated HCO_3^- and lower chloride levels in the serum, net proton release is likely reduced, and ion transport capacities are rearranged and adapted to the new requirements, with restoration of Cl^-/HCO_3^- exchanger AE1 and Na^+/H^+ exchanger NHE1 and upregulation of the Na^+-HCO_3^- cotransporter NBC1 (Fig. 6*B*).

Consequences of hypercapnia for whole animal performance? Whole animal oxygen consumption (Fig. 5), which was similar to values reported for *Z. viviparus* earlier (52), was not affected by short-term hypercapnia. Thus, severe impairments of the marine fish under hypercapnic conditions (10,000 ppm) were not detectable. Nonetheless, the expression patterns indicate rearrangements of the protein inventory during the early phase of hypercapnic exposure. It is assumed that the energy demand of ion and pH regulation is elevated if the main ion transporter—the Na^+-K^+-ATPase—operates at an elevated activity level. In isolated gill tissue and perfused gills, respectively, the energy demand of the Na^+-K^+-ATPase accounted for up to 30% of total oxygen consumption (34, 48). Theoretical calculations of the contribution of epithelial ion transport to whole animal oxygen consumption range from 0.5 to 15% in seawater fish (12, 25). Thus, the increased Na^+-K^+-ATPase capacities found in the present study appear to be too small to be detectable within SMR measurements. Furthermore, an increased energy demand of ion regulation might be compensated for through decreased rates of protein turnover, anabolism, or activity levels. As the observed levels of motor activity were minimal in these sluggish benthic fish under all experimental conditions applied, a response at that functional level can be excluded as unlikely. Energy allocation in hypercapnia-exposed animals would have to be determined to assess relative shifts in energy budget associated with hypercapnia. Negative effects on whole animal performance like those on growth are conceivable, as Na^+-K^+-ATPase is operating at a higher rate under hypercapnia.

Perspectives and Significance

Investigation of further transporters and other isoforms, as well as differentiation between cell types, will be required in future analyses to deepen the understanding of the complex interaction of ion transport mechanisms. Furthermore, the regulatory signals eliciting the shift in gene expression of ion transport mechanisms and their response to rates and steady-state parameters of ion and acid-base status require elaboration. The present study already points to NBC1 and Na^+-K^+-ATPase as key transporters supporting long-term ion and acid-base regulation under hypercapnia. The analyses of these transporters under the predicted CO_2 scenarios, especially in combination with proposed temperature increments may serve as sensitive markers for the detection of long-term effects on the animals' resilience.

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GRANTS

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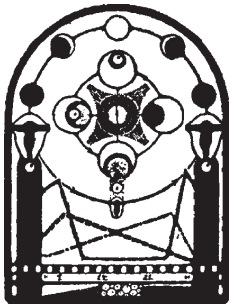

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Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids

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Hypercapnia induced shifts in gill energy budgets of Antarctic notothenioids

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Abstract

The mechanisms responsive to hypercapnia (elevated CO_2 concentrations) were investigated with a focus on energy turnover in isolated perfused gills of two Antarctic Notothenioids (*Gobionotothen gibberifrons, Notothenia coriiceps*). Branchial oxygen consumption of isolated gills was measured under normo- *vs.* hypercapnic conditions (10,000 ppm CO_2). The fractional costs of ion regulation, protein and RNA synthesis in the energy budgets were determined using specific inhibitors. While overall gill energy turnover was maintained under hypercapnia in both species as well as in a temperate zoarcid (*Zoarces viviparus*), fractional energy consumption by the examined processes rose drastically in *G. gibberifrons* (100-180%), and somewhat less in *N. coriiceps* gills (7-56%). In conclusion, high CO_2 concentrations induce cost increments in epithelial processes, however, at maintained overall rates of branchial energy turnover.

key words: isolated perfused gill respiration, *Gobionotothen gibberifrons*, *Notothenia coriiceps,* Ouabain, Cycloheximide, Actinomycine D

Introduction

Antarctic ecosystems with very stable biotic and abiotic factors have evolved after Antarctic waters were isolated by formation of the circum-Antarctic current in the late Oligocene (about 25 Mio years ago). The Antarctic fish fauna is dominated by the suborder Notothenioidei, with about 50% of all individuals belonging to the family Nototheniidae (Eastman 2005). No fossil record is available, but the Notothenioidei probably have appeared in the Eocene [38 Mio yrs ago (Eastman 1993)] and began to diversify in isolation on the Antarctic continental shelf (Di Prisco 2000). These fish have adapted to permanently cold temperatures and are considered to be highly stenothermal (Pörtner 2006; Somero and De Vries 1967; Somero et al 1998). Unless acclimation occurs, they may be most sensitive to rising temperatures associated with global change (Pörtner et al 2004). Sensitivity to fluctuations in other environmental factors like salinity or changing CO_2 and oxygen levels may be similarly high. The stress response in Antarctic fish generally differs from other teleosts: the release of catecholamines (like adrenalin) is reduced and the secondary stress response (e.g. hematocrit level, heart rate) seems to be under cholinergic rather than adrenergic control (Egginton and Davison 1998).

In marine animals, sensitivity to variable CO_2 tensions may be mirrored in the capacity for transepithelial ion and acid-base regulation (Pörtner 2008). In both marine invertebrates and fishes, cell and tissue functions are sensitive to disturbed acid-base parameters. In a study of hypercapnia effects on isolated hepatocytes of two Antarctic fish species, elevated CO_2 levels (10,000 ppm) per se did not change cellular energy turnover, however, the associated drop in extracellular pH led to an almost complete shutdown of protein synthesis (Langenbuch and Pörtner 2003). It is well known that marine fishes are capable to restore acid-base balance during exposure to hypercapnia *in vivo* (Heisler 1993; Larsen et al 1997; Michaelidis et al 2007; Perry 1982; Toews et al 1983) and are thereby able to circumvent pH induced metabolic depression. Ion regulation mechanisms involved in acid-base regulation have been investigated in a number of fish species (Claiborne and Evans 1992; Claiborne and Heisler 1983; Edwards et al 2005; Jensen et al 2000).

For a more detailed study of the functional response of the gills of Antarctic fishes to hypercapnia we chose two members of the family Nototheniidae, *G. gibberifrons* and *N. coriiceps*. While *G. gibberifrons* is a more sluggish benthic fish, feeding on various infauna, *N. coriiceps* is considered to have a more active, benthopelagic life style, preying also on smaller fish and krill (Casaux et al 1990; Gon and Heemstra 1990). We established an isolated perfused gill model for these species to study the impacts of hypercapnia on transepithelial acid-base regulation at the level of associated energy turnover. Gills are multifunctional and metabolically very active organs, not only enabling gas exchange, but also covering over 90% of the fishes' ion and acid-base

regulation and taking part in the elimination of nitrogenous waste [for review see (Evans et al 2005)]. The calculated energetic costs comprise about 7 - 10% of whole animal energy turnover (Boeuf and Payan 2001; Gibbs and Somero 1990; Mommsen 1984; Perry and Walsh 1989). These energetic costs may be influenced by temperature such that the response to hypercapnia may also be temperature dependent.

Using isolated perfused gills according to a method originally developed by Shuttleworth (1972), we measured oxygen consumption according to Lyndon (1994) as an indicator of branchial metabolic rate (SMR). By application of specific inhibitors we were able to calculate the contribution of ion transport rates (through Na^+/K^+-ATPase) or distinct metabolic processes like protein synthesis and RNA synthesis to the energy budget of the gills under normocapnic and hypercapnic conditions. Exposure to high CO_2 concentrations (10,000 ppm) was chosen for a clearly identifiable response of key processes and mechanisms. To our knowledge, this is the first study addressing hypercapnia-induced shifts in energy budgets of isolated perfused gill preparations of teleost fish. The findings support recent interpretations of functional shifts associated with gene expression shifts during long-term hypercapnia in gills of marine fish (Deigweiher et al 2008).

Materials & Methods

Animals

Specimens of *Gobionotothen gibberifrons (Loennberg;* 672 - 1169 g, 39.5 - 46.0 cm, n=16) were collected during an Antarctic summer expedition with RV 'Polarstern' in 2006/07 [ANT XXIII/8; (Gutt 2008)] from bottom trawls at depths of 60 - 490 m at the Antarctic Peninsula near Elephant and Joinville Island. *Notothenia coriiceps (Richardson;* 211 - 557 g, 26.0 - 34.5 cm, n=11) were caught with fish traps at Jubany station (King-George-Island) 3 - 4 weeks prior to experimentation and fed once a week with fish meat. Fish were transported to the ship by helicopter. On board, all animals were kept in an air-conditioned container in aquaria systems with aerated natural seawater at 0.0 ± 0.5°C. They were observed for 2 to 5 days prior to experimentation to ensure that they were in a healthy condition. The fish were not fed during that time.

Isolated perfused gill preparations

Experiments with the notothenioids were carried out onboard RV 'Polarstern'. For a maximal sample number, several gill arches from one fish were used for isolated perfused gill preparations as described in earlier studies (Decostere et al 2002; Smith et al 2006). In both species the first

three gill arches of each side were used, as their sizes and fractional masses of the respective whole basket mass were similar (see table 1). The filaments of each fourth gill arch were frozen and stored as tissue samples. Fish were stunned by a blow to the head and infused intravenously with heparin (500 U $100g^{-1}$). After about 10 minutes of heparin exposure animals were taken out of the aquarium and killed by cutting their spine. The gill arches were dissected quickly and placed in ice-cold saline, where they were cleared from blood and cannulated by using a polyethylene tubing (inner diameter 0.38 - 0.86 mm, Portex Ltd., Smiths Industries Medical Systems, Kent, UK). To enable easier cannulation, the outer ends of the arches were cut off. They were blotted dry, frozen in liquid nitrogen and stored at -80°C for later weight determination at the Alfred Wegener Institute in Bremerhaven. The tubing fed through the gill arch had an opening over the length of the arch to enable perfusion (Fig. 1). To prevent leakage, cannulae were sutured to both sides of the gill arch with perma-hand silk suture (4-0, Ethicon Inc., Johnson & Johnson, Somerville, New Jersey, USA). Preparation of up to six gill arches per fish was completed within one hour. Prepared gill arches were used for respiration measurements directly after preparation (see below), or were kept in ice-cold saline, while being perfused with a pulsatile flow of saline provided by a peristaltic pump (Reglo Digital MS-4/8, Ismatec Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany). After measurements, gill arches were blotted dry, frozen and stored at -80°C for later weight determination. Saline used for notothenioid fishes was composed as follows: 148.4 mM Na^+, 131 mM Cl^-, 5.2 mM K^+, 2 mM Ca^{2+}, 1.3 mM Mg^{2+}, 0.3 mM SO_4^{2-}, 27 mM HCO_3^-, 0.2 mM $H_2PO_4^-$, 0.7 mM HPO_4^{2-}, 5.6 mM D-Glucose (Gey's Balanced Salt Solution, Sigma-Aldrich, Taufkirchen, Germany). The ice-cold perfusate was briefly gassed with N_2 to lower its oxygen content to 50 - 70% air saturation, with HCO_3^- concentrations maintained at 25 ± 0.5 mM at a pH of 7.85 ± 0.5 (determined at the Alfred Wegener Institute in a gas chromatograph, 6890N, Agilent Technologies, Waldbronn, Germany). All pH measurements were performed with a pH-Meter (340i, WTW, Weilheim, Germany) equipped with a SenTix 81 electrode (WTW) calibrated between pH 7 and 10 according to NBS scale (National Bureau of Standards). The applied pH value was within the range of 7.54 - 7.85 reported earlier for notothenioid blood (Kunzmann 1991). The bicarbonate concentration was chosen to match pH compensated hypercapnic conditions in the blood, when bicarbonate has accumulated to compensate for the hypercapnia induced acidosis (Heisler 1993).

Oxygen consumption measurements

The respiration measurements used here were essentially those described by Lyndon (1994) and Morgan & Iwama (1999) with the following modifications: Custom-made cylindrical respiration chambers with an adjustable volume of 60 - 120 ml were used (Construction by E. Dunker,

Alfred Wegener Institute, Bremerhaven, Fig. 2). They were filled with 0.2 µm filtered, freshly aerated seawater. Air provided by a membrane pump (HP-100, Hagen Deutschland GmbH, Holm, Germany) and humidified was used for normocapnic controls. A gas mixture of 1% CO_2 in air, provided by a gas-mixing pump (Woesthoff Messtechnik GmbH, Bochum, Germany) generated hypercapnic conditions. The respective pH values at 0°C were 8.0 ± 0.05 for normocapnic and 6.9 ± 0.05 for hypercapnic seawater (NBS scale, pH determination see above).

After cannulation the gill arch was placed into the chamber. The polyethylene tubing was fed through holes in the lid and sealed with gas tight connector pieces (Microelectrode Holder Half-Cells, World Precision Instruments Inc., Sarasota, FL, USA), which had been screwed into the chamber lid (see Fig. 2). An oxygen micro-sensor (needle type, 140 µm, PreSens, Regensburg, Germany) used for oxygen consumption measurements in the chamber was inserted through a third hole in the lid. It was sealed with a rubber gasket, but still allowed extrusion of air bubbles while closing the chamber and checking for leakage (Figs 2, 3). Leaking gill arches were discarded.

A cooling jacket around the respiration chambers ensured a constant temperature of 0 ± 0.1°C by cooling with a thermostat (RP 845, Lauda GmbH, Lauda-Koenigshofen, Germany) set to -0.5°C. A second thermostat set to -16°C was used to keep the temperature of the perfusion system at 1 ± 1.0°C by means of a cooling coil. The respiration chamber was equipped with a magnetic stir bar to enable consistent mixtures of the water bath (Fig. 3). In an additional set of experiments, *G. gibberifrons* gill respiration rates were evaluated at a temperature of 6°C and compared to control conditions. These measurements allowed the evaluation of Q_{10} values from Van't Hoff's law.

Gills were perfused with a pulsatile flow of saline (flow rate 0.27 ml/min) by a peristaltic pump (Reglo Digital MS-4/8, Ismatec). The efferent pressure head could be adjusted with a lab stand set to 15 - 25 cm above the respiration chamber to maintain a pressure of 2 - 3 kPa. The pressure head was monitored by use of a pressure transducer (MLT 0699, AD Instruments GmbH, Spechbach, Germany) inserted into the efferent cannula. Online monitoring of oxygen content in the perfusate occurred before and after the gill arch, using two flow-through cell oxygen mini-sensors coupled with temperature sensors (FTC-PSt3, PreSens; Fig. 3). Oxygen consumption was recorded by use of oxygen meters (Microx TX3 for needle-type and Fibox 2 AOT for flow-through oxygen sensors, PreSens). All data were recorded using a PowerLab system (AD Instruments) and transferred to a PC using the software Chart 4.0 (AD Instruments). Prior to measurements, oxygen sensors were calibrated to 0% with a saturated sodium-dithionite-solution and to 100% in humid air.

Gill oxygen consumption was calculated as described by Lyndon (1994) with the following formula:

$$M(O_2) = [(P_a - P_e)\ \alpha(O_2)\ vfl + \Delta P_{ch}\ \alpha(O_2)\ V]\ w^{-1}$$

where $\mathbf{M(O_2)}$ is the oxygen consumption rate [µmol (O_2) g^{-1} h^{-1}], $\mathbf{P_a}$ **and** $\mathbf{P_e}$ are the afferent and efferent oxygen contents of the perfusate [kPa]; $\boldsymbol{\alpha(O_2)}$ is the oxygen capacity of the water [µmol (O_2) l^{-1} kPa^{-1}] at the respective salinity and temperature after Boutilier et al. (1984), **vfl** is the flow rate [l h^{-1}], $\mathbf{\Delta P_{ch}}$ is the gradient of oxygen decrease over time in the chamber [kPa h^{-1}], **V** is the chamber volume [l] , and **w** is the fresh weight of the gill [g].

Viability tests

Viability of the gill arches after preparation was determined through analyses of long-term respiration measurements. In an additional experiment blue colored saline (0.01% Trypan Blue, Sigma-Aldrich, Taufkirchen, Germany) was used to validate homogeneous supply of saline to the filaments in one exemplary gill arch from each species.

Analyses of branchial energy budget

Energy budget components were evaluated by inhibition of protein synthesis with cycloheximide, RNA synthesis with actinomycin D and ion regulation by inhibition of Na^+/K^+-ATPase with ouabain. All substances were dissolved in dimethyl sulfoxide (DMSO) and added to the perfusate. Final DMSO concentration did not exceed 1.6% and exclusive addition of DMSO to the saline did not affect oxygen consumption of the gills (data not shown). Cycloheximide specifically inhibits eukaryotic protein synthesis by inactivating peptidyl transferase activity of the cytosolic ribosomal 60S subunit (Obrig et al 1971). As in previous studies (Casey et al 2002; Mark et al 2005; Smith and Houlihan 1995), the concentration used was 5 mM. Actinomycin D was applied in a concentration of 3,2 µM (Morgan et al 1991; Smith and Houlihan 1995) to block DNA-primed RNA synthesis by forming a stable complex with double stranded DNA (Kirk 1960; Sobell 1985). Ouabain, which specifically inhibits Na^+/K^+-ATPase activity (Wheeler and Whittam 1962; Whittam 1962) was used at concentrations of 5 mM (Krumschnabel et al 1994; Mark et al 2005).

Data analysis and statistics

Oxygen consumption rates were determined under normocapnic and hypercapnic conditions. If not depicted differently, data are given as mean values ± standard error of the mean (s.e.m.). In the case of *G. gibberifrons*, effects of inhibitors on individual gill arches were measured and mean values were evaluated for each group (control, cycloheximide, actinomycin D, ouabain). For the

determination of control respiration rates the gill arches from 12 individual fish were used, and the normocapnic and hypercapnic group comprised 19 and 20 measurements, respectively. For the inhibitor-sensitive respiration rates 7 to 11 gill arches (= n numbers) from 3 to 4 individual fish were measured for each of the six groups. For *N. coriiceps* each gill arch was used as its own control prior to application of one inhibitor. Mean values ± s.e.m. were derived from percent inhibition data per group. Control respiration rates were evaluated from the gill arches of 11 individual fish in 27 and 28 measurements under normo- and hypercapnia, respectively. Thereby, each of the six inhibitor groups comprised 8 to 10 gill arches (= n numbers) from 4 to 5 individual fish. Statistical significance of differences between control and hypercapnic groups was tested at the $p < 0.05$ level using Students' t-tests.

Results

The long-term experiment confirmed the viability of gill arches in the respiration chamber: after 20 minutes of equilibration the respiration rate remained stable for at least 23 hours (Fig. 4). Generally, keeping prepared gill arches in the "waiting loop" for up to 2 hours prior to measurements did not diminish their respiratory performance. Homogeneous perfusion of the gills was controlled with trypan blue added to the perfusate. The dye reached the filament tips within 20 - 30 minutes after infusion. Only at the entrance region of the saline and at the outer filaments (beyond the tied suture) perfusion was not complete (about 25% of gill wet weight, Fig. 1).

Gill oxygen consumption rates were normalized to the weight of the complete individual gill arches, including cartilaginous arch and the non-perfused area. Mean oxygen consumption of *G. gibberifrons* isolated perfused gills at 0°C was 2.96 ± 0.10 μmol (O_2) g_{fwt}^{-1} h^{-1} (n = 19) under normocapnic conditions and did not change significantly under hypercapnia, when it was 2.70 ± 0.10 μmol (O_2) g_{fwt}^{-1} h^{-1} (n = 20). Gills of *N. coriiceps* had a significantly higher mean respiration rate of 4.05 ± 0.15 μmol (O_2) g_{fwt}^{-1} h^{-1} (n = 27) at 0°C, which was also not affected by hypercapnic exposure (3.94 ± 0.17 μmol (O_2) g_{fwt}^{-1} h^{-1}; n = 28).

Respiration rates of isolated gills from *G. gibberifrons* at 0° and after warming to 6°C, under both normo- and hypercapnic conditions, are depicted in figure 5. Upon warming, oxygen consumption rose from 2.68 ± 0.15 to 3.47 ± 0.19 μmol (O_2) g_{fwt}^{-1} h^{-1} (n = 6, normocapnia) and from 2.47 ± 0.13 to 3.07 ± 0.15 μmol (O_2) g_{fwt}^{-1} h^{-1} (n = 6, hypercapnia). Calculated Q_{10} values were 1.54 and 1.43, respectively. No significant difference could be observed between Q_{10} values under control and hypercapnic conditions.

Figure 6 shows the percent fractions of energy allocation to RNA and protein synthesis, as well as ion regulation in the isolated perfused gills. Generally, each of the three processes shared equal fractions of the energy budget under control conditions, between 6.9 and 11.8% in *G. gibberifrons* (Fig. 6A) and between 18.5 and 23.1% in *N. coriiceps* (Fig. 6B), leaving 71.4% and 39.0%, respectively, of the energy consumption unexplained. Under hypercapnic acidosis, the fractions of the individual processes increased significantly - with the exception of actinomycin D sensitive respiration in *N. coriiceps* - to values between 16.3% and 28.1% in *G. gibberifrons* (Fig. 6A) and between 20.8% and 29.7% in *N. coriiceps* (Fig. 6B). With unchanged metabolic rates, this increase occurred at the expense of the unexplained fraction, which was halved to 32.2 and 20.6%, respectively. Table 2 summarizes the shifts in the energy budgets of both fish for the individual processes: in *G. gibberifrons*, all three processes increased by a factor of 2 to 2.8, while in *N. coriiceps*, oxygen demand for RNA synthesis remained constant whereas it increased for protein synthesis and Na^+/K^+-ATPase-dependent ion exchange by a factor of 1.6 and 1.3, respectively.

Discussion

The aim of the present study was to identify some key processes and mechanisms associated with energy demand and responsive to hypercapnia in the gills of teleost and especially notothenioid fish. In the light of the putative relevance of acid-base regulation for defining the sensitivity of metabolic processes and the whole organism to hypercapnia (Pörtner 2008), gills were chosen as a tissue model in accordance with their major role in ion- and acid-base regulation. At the same time we are not aware of publications addressing the principle consequences of hypercapnia for the energy budget of teleost gills. Further interpretation must thus await availability of comparative information for other fish species.

Methodology

Isolated or perfused gill arches have been used to study gill ion transport processes for more than 40 years (Bellamy 1961; Richards and Fromm 1969; Shuttleworth 1972). Lyndon (1994) has developed a method to measure oxygen consumption of isolated gills and Morgan & Iwama (1999) used this technique to evaluate the costs of salt transport in the gills through application of specific inhibitors. Based on these studies, we have developed a system for measuring oxygen consumption of isolated gills by monitoring the oxygen levels in the respiration chamber as well as in the perfusate of the gill arch in real time. Homogenous supply of saline to the filaments and sustained viability of the gill arches was confirmed over periods of 24 hours (see Fig. 1, 4). The

setup has been constructed in a way that it can also be used on board of research vessels and on research stations.

One has to bear certain methodological constraints in mind when using inhibitors for the determination of individual energy consuming processes. The targeted metabolic processes are not necessarily independent of each other, even if they are usually treated as such, just as in this study. For example, inhibiting transcription will also cause an inhibition of translation due to the lack of mRNA transcripts. In general, specific blocking of individual ATPases may synergistically reduce "work" for other consumers, resulting in a further reduction of ATP production (and oxygen consumption) through negative feedback. Such synergistic interaction between processes might lead to overestimations of the energy demand of individual processes. Another problem is the possibility of unspecific side effects of inhibitors, which might also lead to overestimates. This has especially been discussed for cycloheximide, where dose-dependent inhibition has been reported (Bowgen et al 2007; Wieser and Krumschnabel 2001). However, we applied reasonable inhibitor concentrations and minimized variability in the experimental protocol. We found significant changes in the energy budget of the gills in response to hypercapnia unrelated to such inhibitor artifacts. These changes indicate functional shifts in this organ, even if the exact values of the individual fractions of energy consumption may remain elusive due to methodological constraints. Furthermore, the fractions of energy budget allocated to transcription, translation and ion regulation in this study were similar to those reported for various tissues and cell types of fish and mammals.

Energy turnover

Figure 5 summarizes the data for *N. coriiceps* at 0° and for *G. gibberifrons* at 0°C and 6°C as well as for *Z. viviparus* at 10°C (unpublished data) under normo- and hypercapnia compared to branchial energy turnover in marine flounder and seawater acclimated cutthroat trout at 10°C (Lyndon 1994; Morgan and Iwama 1999). Gill energy turnover in Antarctic fish is found well below the observed oxygen consumption rates of gills from temperate fish at their respective habitat temperature. Fitting an exponential curve according to Van't Hoff's law yielded Q_{10} values for *G. gibberifrons* of 1.54 and 1.43, somewhat below a Q_{10} value around 2 as expected for temperature dependent metabolism [e.g. (Clarke and Johnston 1999)]. One might speculate that the slightly higher MO_2 value of *N. coriiceps* gills at 0°C compared to the value expected from the curve fit (see Fig. 5) is due to metabolic cold compensation at tissue level according to the concept of MCA [metabolic cold adaptation, (Clarke 1983; Wohlschlag 1960)]. However, MCA has not been confirmed at the level of whole animal standard metabolic rate for *N. coriiceps* and other Antarctic notothenioids (Holeton 1974; Johnston et al 1991; Steffensen 2002). Holeton reported the only

study with data for both species used in our study and reported higher whole animal MO_2 for *N. coriiceps* than for *G. gibberifrons* (Holeton 1970). By scaling MO_2 to the respective mean body weights [scaling coefficient = 0.8 (Clarke and Johnston 1999; Holeton 1974)] and considering the weight fraction of the gill basket in total body weight, the expected fraction of gill MO_2 in whole animal metabolic rate can be evaluated (see Tab. 3). Accordingly, the measured fraction of gill respiration in resting SMR is higher than expected from its weight fraction and accounts for 6.45% in *G. gibberifrons* and, similarly, 6.30% in *N. coriiceps*. Values obtained in *Z. viviparus* were 4.6% (unpublished data). These values are comparable to values of 2.4 in seawater and 3.9% in freshwater trout (Morgan and Iwama 1999) and 6.64% in Atlantic cod (Johansen and Pettersson 1981); a much larger range between 11 - 31% was reported for seawater flounder (Lyndon 1994) .

Overall, gill oxygen consumption comprises an essential part of whole animal metabolism. As the gills are the organs primarily facing environmental conditions and disturbances, we consider them relevant indicators of the response of mechanisms involved in ion and acid-base regulation to hypercapnia.

Energy allocation

Under control conditions (normocapnia, pH = 8.0, 0°C) the mass specific rate of gill oxygen consumption was 40% higher in *N. coriiceps* than in *G. gibberifrons* ($p < 0.0001$), and the fractions of the main energy consuming processes were different in the two species. While translation, transcription and ion regulation accounted for 30% of the energy budget of *G. gibberifrons*, *N. coriiceps* gills used 60% of their total energy turnover for the same processes (Fig. 6). The remaining energy fraction can probably be attributed to other ATPases, metabolic processes and to the mitochondrial proton leak. In mammals, substrate bound energy is allocated to protein synthesis (25 - 30%), Na^+/K^+-ATPase (19 - 28%), Ca^{2+}-ATPase (4 - 8%), gluconeogenesis (7 - 10%), ureagenesis (3%) and actinomyosin ATPase (2 - 8%), leaving between 13 and 40% to remaining processes like RNA synthesis, substrate cycling (e.g. glucose / glucose-6-phosphate, phosphoenolpyruvate / pyruvate / oxaloacetate, acetate / acetylCoA) and signal transduction (Rolfe and Brown 1997). Furthermore, about 25% of the oxygen demand is associated with the proton leak (Nobes et al 1990; Rolfe and Brand 1996). No comprehensive data are available for teleost fish and their epithelia specialized on osmoregulation and ion exchange. Gills may additionally use proton ATPases, as demonstrated for salmonid fish in freshwater (Lin et al 1994; Perry et al 2000; Seidelin et al 2001; Sullivan et al 1995; Sullivan et al 1996). In cutthroat trout, bafilomycin-sensitive H^+-ATPase contributed significantly to the oxygen consumption of excised gill tissue (Morgan and Iwama 1999). In a recent study, we found two isoforms of V-type H^+-

ATPases upregulated in the gills of *Z. viviparus* after 24 hours of hypercapnia (unpublished data), indicating a role for branchial proton pumps in marine fish.

The phenomenon that ion regulation, protein and RNA synthesis in *N. coriiceps* gills consume larger fractions of the total energy budget than in *G. gibberifrons* also requires consideration. An explanation for this relatively large difference could be based on the different lifestyles of these fishes. While *G. gibberifrons* has been described as a sluggish, benthic fish, *N. coriiceps* is a benthopelagic species with a higher activity pattern, also preying on smaller fish (Casaux et al 1990; DeWitt et al 1990). This more active lifestyle results in a higher standard metabolic rate (Holeton 1974; Morris and North 1984; Zimmerman 1997) which is also associated with higher energy turnover in the gills as seen in this study. We can presently only speculate that the lower baseline ion exchange rate in gills from the sluggish *G. gibberifrons* implies a higher "idling" of the proton leak than in *N. coriiceps*. A high level of proton leak due to futile idling of metabolic pathways and mitochondria might support fast responses to changing ATP demand (Rolfe and Brand 1997), which in the gills would be associated with ion or acid-base regulation. The drastic rise in energy turnover of the tested processes under hypercapnia in *G. gibberifrons*, at the expense of a drop in residual oxygen demand and maintained overall branchial oxygen turnover, would be in line with these considerations (see below). Further investigations must address these important relationships.

The hierarchy of processes contributing to standard metabolic rate usually differs between tissues and the information available on energy allocation to individual processes in fish tissues is scarce. However, when comparing cellular data from mammals and fish, similar fractions of energy allocation were obtained for rat thymocytes and temperate fish hepatocytes, where RNA synthesis ranked second after protein synthesis and higher than Na^+/K^+-ATPase (Buttgereit and Brand 1995; Wieser and Krumschnabel 2001). Only in Antarctic fish hepatocytes Na^+/K^+-ATPase had a slightly higher energy demand than either protein or RNA synthesis (Mark et al 2005).

In *G. gibberifrons and N. coriiceps* gills, the highest energy fraction was consumed by Na^+/K^+-ATPase, reflecting the importance of branchial functions in ion regulation, which accordingly accounts for a significant fraction of standard metabolic rate (Boeuf and Payan 2001). Na^+/K^+-ATPase consumed 11.8 and 23.1% of gill metabolic rate, respectively (Fig. 6). In trout, ouabain sensitive respiration accounted for a fraction of 25% in freshwater acclimated gills (Morgan and Iwama 1999). 25% had also been found in gill tissue from seawater adapted flounder (Stagg and Shuttleworth 1982). In isolated cell systems, comparable values have been reported: 22% in rat cardiomyocytes (Casey et al 2002) and 24% in goldfish hepatocytes (Krumschnabel et al 1994). In Antarctic fish hepatocytes Na^+/K^+-ATPase comprised between 40

- 45% (Mark et al 2005). As mentioned above, the low value for *G. gibberifrons* might be associated with the lower metabolic rate and the sluggish life style of these fish.

The fraction of energy used by RNA synthesis (12.8 and 19.4% in *G. gibberifrons* and *N. coriiceps*, respectively, Fig. 6) under control conditions was within the range reported for isolated cells: 8% in human blood cells (Schmid et al 2000), 15% in rat thyme cells (Buttgereit and Brand 1995), 20% in rat cardiomyocytes (Casey et al 2002). Again, the highest values of 24 - 35% were reported in Antarctic fish hepatocytes (Mark et al 2005).

The estimated fractional costs of protein synthesis determined from cycloheximide sensitive oxygen consumption (6.9% and 18.5% in *G. gibberifrons* and *N. coriiceps*, respectively, Fig. 6) were lower than reported for isolated liver cells, where values varied between 27% in rat cardiomyocytes (Casey et al 2002), 20 - 37% in Antarctic fish hepatocytes (Langenbuch and Pörtner 2003; Mark et al 2005), 50% in goldfish hepatocytes (Krumschnabel et al 1994), and 60 - 90% in fish cell lines (Smith and Houlihan 1995). The fractional costs for whole animal protein synthesis range between 11% and 42% (Carter et al 1993; Houlihan et al 1988; Lyndon et al 1992). A large variability in protein synthesis costs has been reported in fish, depending on species and tissue type. Moreover, synthesis may become more efficient (lower aerobic costs) at higher rates [reviewed by (Houlihan et al 1995)]. Gills generally have a high protein turnover rate (in some fish species second only to liver and occasionally intestines) with a fractional contribution to whole animal protein synthesis between 2.5 and 18% per day [for review see (Lyndon and Houlihan 1998)].

Absolute rates and the fractional energy costs for transcription, translation and ion regulation reported here are among the lowest reported for tissues or cell types from different fish or mammals. In the light of low rates of energy metabolism of Antarctic fish (Johnston et al 1991), this indicates lower gill activity than in warm water fishes.

Impacts of hypercapnia on energy allocation

Total gill oxygen consumption remained constant in *G. gibberifrons* and *N. coriiceps*, as well as the temperate *Z. viviparus* under hypercapnia (see Fig. 5). However, in a more detailed study of the Antarctic notothenioids we observed significant shifts in the branchial energy budget. Hypercapnic exposure of *G. gibberifrons* gills induced a significant two- to threefold increase in the energy demand of all examined processes. As oxygen consumption remained constant, the fraction of energy available to residual processes (e.g. proton leak, ATPases) was reduced by about 50% (see Fig. 6, Tab.2). These effects were not as dramatic in *N. coriiceps*, where energy turnover used for translation and ion regulation was higher to begin with and increased less, but still significantly, 1.3 to 1.6 fold. At constant overall energy demand, residual energy turnover also

decreased by about 50%. These results clearly show, that - even if the gills' total energy demand is not affected by higher CO_2 concentrations - rearrangements of metabolic and ion exchange processes occur to match the new requirements for ion and acid-base regulation. A higher ion transport activity might lead to a higher protein turnover rate for Na^+/K^+-ATPase and several other transporters and enzymes involved in acid-base regulation. Indeed, a higher energy demand for protein synthesis was measured in the gills of both species. An increased translational activity might be supported by a higher transcriptional activity, which became evident only in the gills of *G. gibberifrons*.

In line with these results, Na^+/K^+-ATPase enzyme activity, as well as transcription and translation rates increased under hypercapnia in gills of the North Sea eelpout (Deigweiher et al 2008). Elevated transcription and/or translation levels of a number of ion channels and transporter proteins under hypercapnia has also been shown in gills of rainbow trout (Ivanis et al 2008; Perry et al 2000; Perry et al 2003; Shahsavarani and Perry 2006) and mummichog (Edwards et al 2005).

Extracellular pH and bicarbonate levels of the perfusate used in our study were reflecting the blood status typically observed in marine fish after compensation of a hypercapnic acidosis (Larsen et al 1997; Michaelidis et al 2007; Toews et al 1983). pH recovery under hypercapnia is usually accompanied by the accumulation of bicarbonate (Heisler 1993). Therefore, the observed shifts in energy allocation can be interpreted as reflecting the branchial response to long-term aquatic hypercapnia under whole organism conditions.

Furthermore, full compensation for the extracellular acidosis would explain why our present observations are in apparent contrast to those in isolated hepatocytes of Antarctic fish, where hypercapnic acidosis induced a decrease of cycloheximide sensitive respiration (Langenbuch and Pörtner 2003). The authors concluded that the liver cells were undergoing metabolic depression under non-compensated acidosis, as is known for invertebrates (Langenbuch and Pörtner 2002; Reipschläger and Pörtner 1996). In trout hepatocytes anoxia caused a 50% down regulation of protein synthesis. Interestingly, hepatocytes from anoxia-tolerant goldfish maintained protein synthesis rate under anoxia (Wieser and Krumschnabel 2001). However, the role of extracellular pH has not been addressed in both cases. Metabolic depression would indeed be a mechanism suitable to extend tolerance to environmental stressors like anoxia or hypercapnia. The present data indicate that such metabolic depression does not occur in branchial tissue under conditions of fully compensated extracellular acidosis.

The present finding of modified energy budget due to increased ion regulatory processes is in line with results obtained in salinity transfer studies, which may also have occurred under constant extracellular pH conditions. Interestingly, the fraction of ouabain sensitive respiration in

isolated cutthroat trout gills increased from 25% in the freshwater control group to 37% in seawater acclimated fish, associated with reduced branchial oxygen consumption in seawater acclimated cutthroat trout (Morgan and Iwama 1999). Conversely, in flounder gill tissue ouabain sensitive oxygen consumption increased from 25% in the seawater control group to 28% after freshwater acclimation (Stagg and Shuttleworth 1982).

Conclusion & Perspectives

The hypercapnia induced increments in energy demand of ion exchange, protein and RNA synthesis and the concomitant 50% decrease of residual energy turnover in gills of both Antarctic species, *G. gibberifrons* and *N. coriiceps,* suggests a strong shift in tissue energy budgets, emphasizing the relevance of branchial acid-base regulation in the response to hypercapnia. A large decrement in proton leakage may be involved, supporting a rapid response and enhanced energy efficiency. In light of very recent gene expression data, a change in energy demand of ATPases might also be involved. Insufficient support of other vital processes like metabolic substrate cycles, gluconeogenesis and cell signaling may occur in due course. Such shifts and their consequences for whole organism functioning under hypercapnia remain to be explored.

As comparative data are presently unavailable for hypercapnia effects on branchial energy budgets, it remains unclear, whether the present findings reflect a stronger response by polar fishes to hypercapnia. The question also arises whether these phenomena will become involved under expected scenarios of ocean acidification, with an increase of CO_2 concentrations from currently 380 ppm to about 1,000 ppm over the next 100 years (IPCC 2007). Such effects become more likely under combined conditions of ocean warming and acidification (Pörtner and Farrell 2008). However, high CO_2 concentrations such as those applied in the present study are relevant in the light of proposed scenarios of CO_2 disposal or storage (Caldeira et al 2005), where concentrations could rise drastically near injection or leakage sites (Caulfield et al 1997). Future projects need to address these questions of relevant and critical CO_2 concentrations as well as the impact of long-term hypercapnic acclimation of the fish on branchial energy budget.

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Ethical standards

All animal experiments were conducted following German legislation. An approval of the work was issued by competent German authority (Freie Hansestadt Bremen, reference number 522-27-11/2-0; date: 2002-11-28).

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Table 1: Comparison of tissue masses of first, second and third gill arches from *G. gibberifrons* and *N. coriiceps*. Fractional values are shown in comparison to the whole gill basket mass.

	1st	2nd	3rd
G. gibberifrons			
Masses of gill arches, g [means ± s.d.; (n arches)]	1.84 ± 0.35 (26)	1.88 ± 0.32 (29)	1.70 ± 0.30 (28)
Fraction of respective gill mass [means ± s.d.]	14 ± 1.9 %	14.2 ± 2.0 %	12.9 ± 1.8 %*)
N. coriiceps			
Masses of gill arches, g [means ± s.d.; (n arches)]	1.01 ± 0.30 (20)	1.06 ± 0.30 (20)	1.03 ± 0.24 (18)
Fraction of respective gill mass [means ± s.d.]	15.3 ± 2.1 %	16.0 ± 2.4 %	14.6 ± 2.1 %

*) value significantly lower than values for 1st and 2nd gill arches (p= 0.0192; 1way-ANOVA and Student-Newman-Keuls post test)

Table 2: Shifts in energy budgets of isolated perfused gills of *G. gibberifrons* and *N. coriiceps*. Percent values are those in comparison to control rates (100%)

	G. gibberifrons	*N. coriiceps*
Other processes[1)]	45 % -	53 % -
Protein synthesis[2)]	236 % +	156 % +
Ion regulation[3)]	198 % +	129 % +
RNA synthesis[4)]	284 % +	107 % =

[1)] non-inhibited respiration minus fractions of inhibited processes [2)] Cycloheximide sensitive respiration [3)] Ouabain sens. resp. [4)] Actinomycin D sens. resp.

Table 3: Weight and respiration values for whole animals and gill baskets from *G. gibberifrons* and *N. coriiceps*.

	G. gibberifrons	*N. coriiceps*
Body weight, g [means ± s.e.m.; (n animals)]	946 ± 25.3; (16)	385 ± 33.4; (11)
Whole gill weight, g [means ± s.e.m.; (n animals)]	13.4 ± 0.45; (16)	6.7 ± 0.60; (11)
Fraction of body weight	1.42 %	1.74 %
Whole animal MO_2, µmol O_2 g_{BW}^{-1} h^{-1} 0°C*)	0.62	1.08
Gill MO_2, µmol O_2 g_{GW}^{-1} h^{-1} 0°C [means ± s.e.m.; (n gill arches)]	2.83 ± 0.07 (39)	3.93 ± 0.11 (55)
Fraction of whole animal MO_2	6.45 %	6.30 %

*) Whole animal MO_2 values for *G. gibberifrons and N. coriiceps after* Holeton (1970) were standardized to the respective average body weights in this study using a scaling coefficient of 0.8 (Clarke and Johnston, 1999; Holeton, 1974) Note: Data are expressed per g_{BW}.

Figures

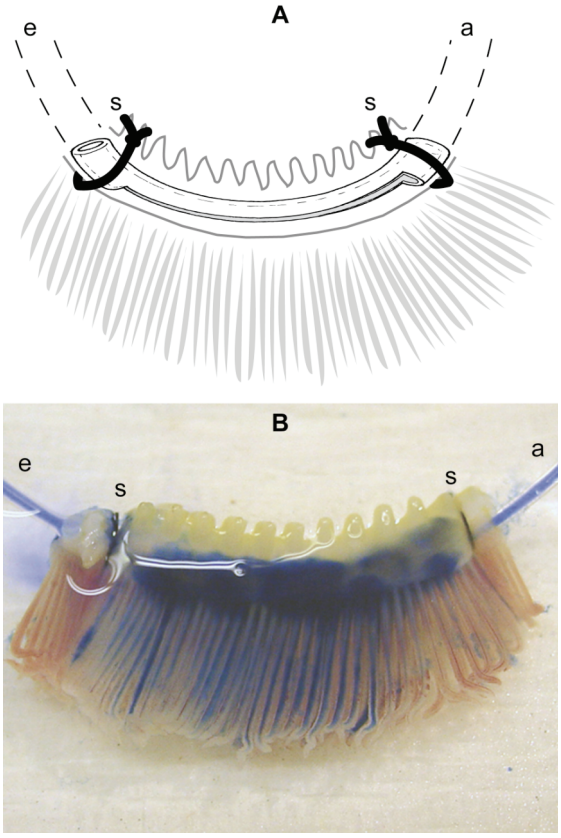

Figure 1: **Isolated gill arch perfusion**. **A:** Tubing with suitable diameters and a certain length of openings was used for the respective gill arches. Tubes were fixed and tightened with suture (**s**) on both ends. Afferent (**a**) and efferent (**e**) endings were led through gas tight connectors in the chamber lid and attached to the perfusion tubings. **B:** *N. coriiceps* gill arch during perfusion with blue colored saline. The picture was taken directly after removing the gill arch from the respiration chamber. The dye (0.01% Trypan Blue) had reached the filament tips after 20 - 30 minutes. Perfusion was not complete at the saline entrance region and saline could not reach the outer filaments, where the perfusion tubing had to be fixed with suture, but the main part of the perfused area shows homogenous supply of saline.

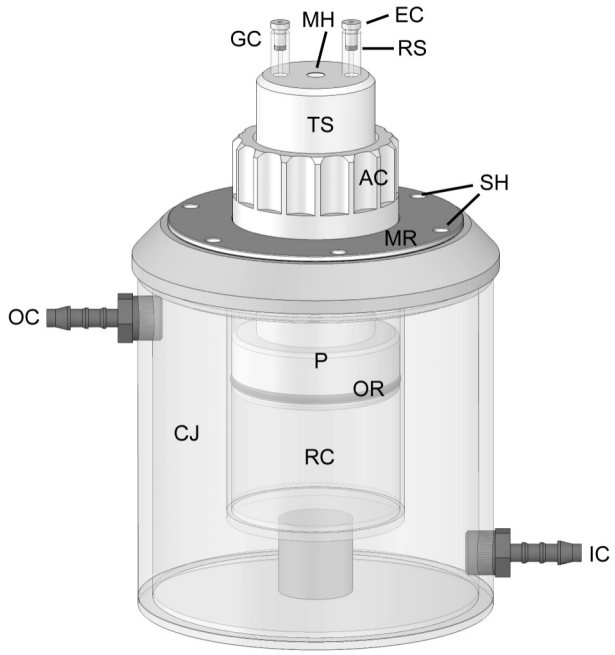

Figure 2: Technical drawing of the respiration chamber. A cooling jacket (**CJ**), supplied with circulating cooling fluid via tubing connectors (incoming, **IC** and outgoing, **OC**), enabled maintenance of low, stable temperatures in the respiration chamber (**RC**). The chamber lid consisted of a plunger (**P**) on a threaded spindle (**TS**) and was adjustable by a collar (**AC**). It was fitted with an o-ring (**OR**) for tightness to gas and fixed by a metal ring (**MR**) with six screws (**SH**: screw holes). The gill arch was suspended in the chamber by the perfusion tubing leading through holes in the chamber lid. The tubes could be constricted in the gastight connectors (**GC**) by compressing rubber seals (**RS**) with screwable end caps (**EC**). The oxygen micro-sensor could be inserted through a third hole in the lid (**MH**).

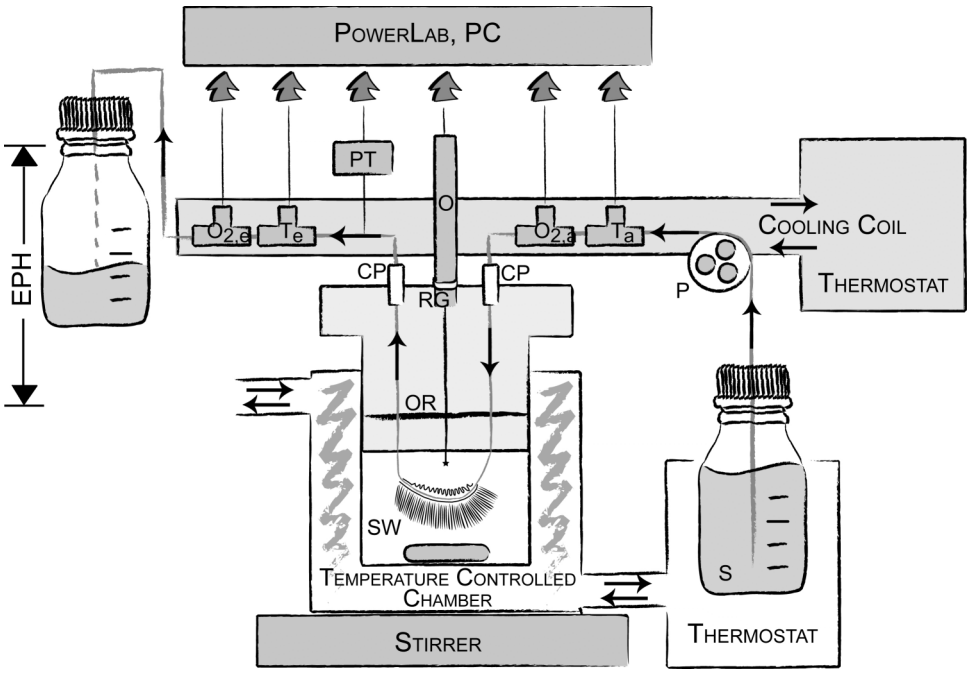

Figure 3: **Respiration setup** [modified after (Lyndon, 1994; Morgan and Iwama, 1999)]. **S**: saline, **P**: peristaltic pump, $\mathbf{T_{a/e}}$: afferent / efferent flow-through temperature-sensor, $\mathbf{O_{2a/e}}$: afferent / efferent flow-through oxygen sensor, **O**: oxygen micro-sensor, **PT**: pressure transducer, **RG**: rubber gasket, **CP**: gas tight perfusion tubing connector piece, **OR**: o-ring, **SW**: sea water, **EPH**: efferent pressure head

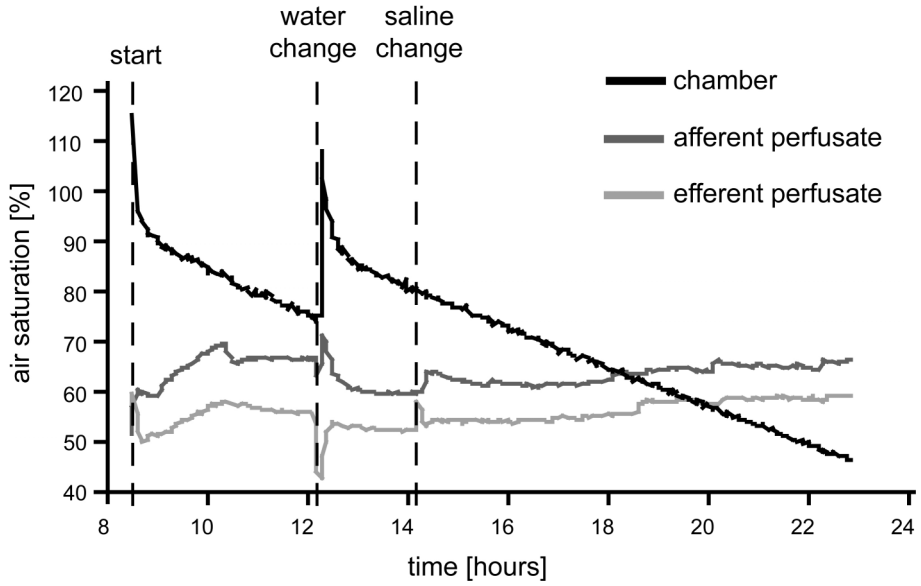

Figure 4: **Test of gill viability.** One gill arch had been stored in the waiting loop during measurements of one day and was placed into the respiration system 8 hours after its preparation. Seawater and saline were repeatedly exchanged during the measurement. Decreases in oxygen tensions in the chamber were following a linear regression over the whole time course [9 - 12 hours (before water change): r^2 = 0,9854; 13 - 22.5 hours (after water change): r2 = 0.9986. According to Run's test there was no significant deviation from linear regression]. The gill arch showed a stable respiration rate until 23 hours after gill preparation, when the experiment was abandoned.

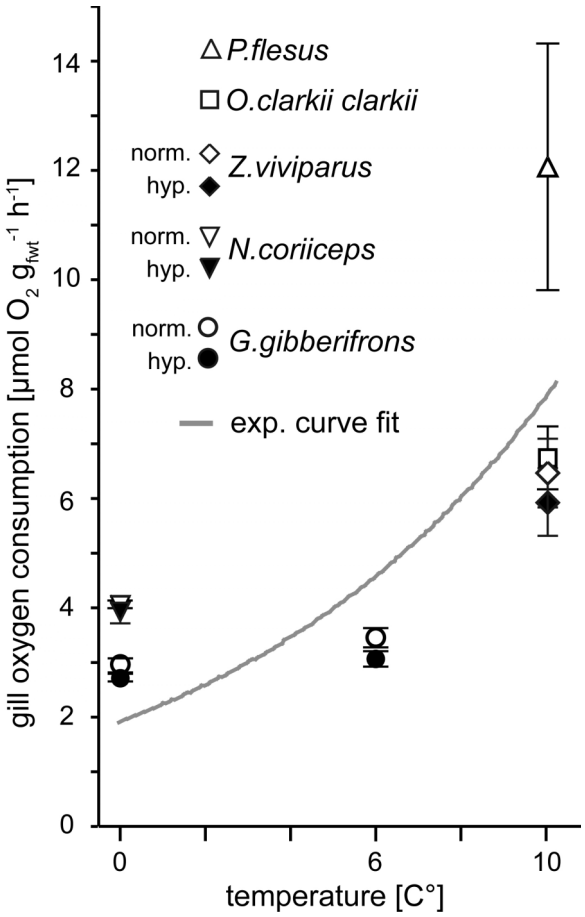

Figure 6: Oxygen consumption of isolated gills of *N. coriiceps, G. gibberifrons* (this study) and *Z. viviparus* (unpublished data) under normocapnia (hollow symbols) and hypercapnia (full symbols) compared with values reported for *Oncorhynchus clarki clarki* (Morgan and Iwama, 1999) and *Platichthys flesus* (Lyndon, 1994) in seawater at different temperatures. An exponential curve was fitted over the mean oxygen consumption values at the respective temperatures. Values are given as means ± standard error of the mean (s.e.m.).

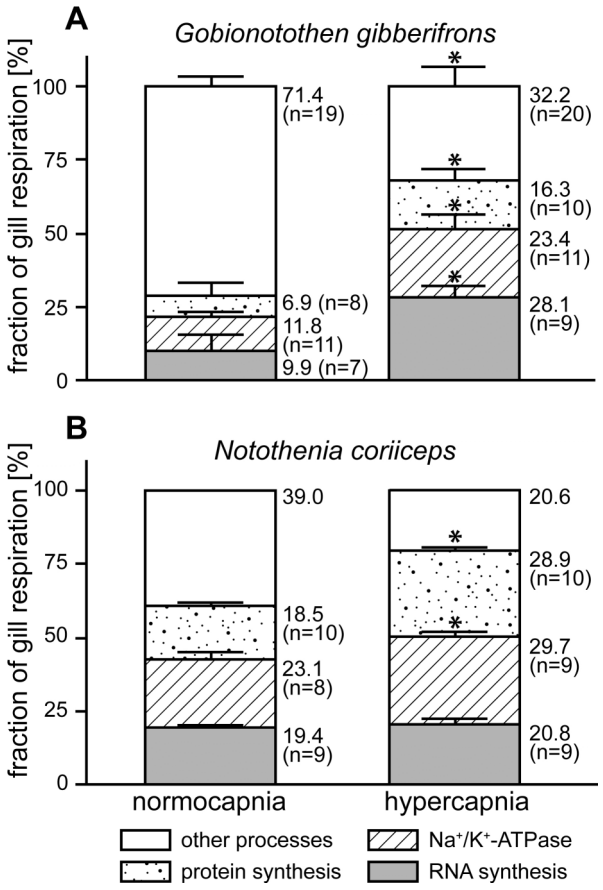

Figure 7: Energy budgets of *G. gibberifrons* (A) and *N. coriiceps* (B) gills under hypercapnia. Medium bicarbonate levels were elevated to compensate for the hypercapnia-induced acidosis. Asterisks indicate significant differences ($p < 0.05$) of the single processes under hypercapnia compared to normocapnic control values. Values are mean percentage fractions of gill respiration ± standard error of the mean (s.e.m.).

PUBLICATION III

Differential gene expression in gills of marine eelpout under hypercapnia

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Differential gene expression in gills of marine eelpout under hypercapnia

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running head: Suppression subtractive cDNA libraries from eelpout gills

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Abstract

Among marine animals, fish are believed to be relatively tolerant to elevated CO_2 concentrations due to their high capacity to regulate acid-base status. Nevertheless, fishes respond to elevated CO_2 tensions by acclimation and experience a change in branchial energy budget. The underlying adaptability of the transcriptome is defined by differential responses of common and specific gene clusters and networks. In this study, we isolated about 1,600 differentially expressed sequence tags (ESTs) from gills of marine eelpout *Z. viviparus* during the early stage of acclimation to hypercapnia (24 h, 10,000 ppm) by using the suppression subtractive hybridization technique. Responsive genes covered all vital cellular processes, from cell signaling and maintenance to metabolic processes to the general stress response including heat shock proteins. These changes in gene expression were accompanied by those of a large fraction of transcription factors and of genes encoding components of the transcription and translation apparatus. Expression patterns indicate a shift in metabolic carbon flux: Differentially regulated genes involved in lipid degradation, but also lipid biosynthesis may support enhanced turnover of lipids. Genes encoding for tricarboxylic acid cycle enzymes and pyruvate dehydrogenase seemed suppressed during early hypercapnia. At the same time, gluconeogenesis was apparently favored over glycolysis, probably due to the activation of the pentose phosphate shunt for provision of NADPH and ribuloses for RNA/DNA syntheses. A number of ion regulatory proteins were found upregulated, including Na^+/K^+-ATPase and transporters involved in acid-base regulation (Na^+/H^+-exchanger; H^+-ATPase, carbonic anhydrase isoforms). These data confirm and complement earlier studies on the response of ion regulatory mechanisms. The present study provides access to the first normalized and subtractive cDNA libraries from fish in response to hypercapnia, with a number of candidate genes that may serve as sensitive markers for future studies in fish and other marine species.

Key words: suppression subtractive hybridization (SSH), expressed sequence tags (EST), *Zoarces viviparus*, metabolism, ion regulation

Introduction

Marine animals, especially those in isolated water bodies, may be regularly exposed to transiently elevated CO_2 partial pressures (environmental hypercapnia). CO_2 usually accumulates associated with hypoxia events. Due to anthropogenic emissions the concentration of atmospheric CO_2 has increased from pre-industrial levels of 280 ppm to currently 385. Within this century, a maximum of about 1000 ppm can be expected (IPCC 2007). The atmospheric CO_2 equilibrates with the oceans and causes acidification; the concentration of protons in the surface waters has already increased by 30 % corresponding to a pH decrease of 0.1 units (Caldeira and Wickett 2003). CO_2 sequestration in the deep-sea, a concept proposed more than 30 years ago (Marchetti 1977) and previously investigated in field studies (Brewer et al. 1999; Brewer et al. 2004; Thistle et al. 2006), or leakage from geological storage would expose marine animals to even higher CO_2 partial pressures. The disturbance of ocean physicochemistry not only influences calcification processes (Gazeau et al. 2007; Ridgwell et al. 2007; Iglesias-Rodriguez et al. 2008), but also organismal ion and acid-base regulation [reviewed by (Pörtner et al. 2004; Pörtner 2008)]. The regulatory mechanisms involved in the CO_2 response are under thorough investigation and will help understanding the impacts of ocean acidification on marine ecosystems.

Marine teleost fish display sophisticated capabilities to regulate internal acid-base status; they usually restore intra- and extracellular pH values within short time periods when exposed to environmental hypercapnia (Toews et al. 1983; Heisler 1993; Larsen et al. 1997; Michaelidis et al. 2007). The role of specific ion transporters has been studied in gills, where whole organism ion regulation predominantly takes place (Perry et al. 2000; Seidelin et al. 2001; Edwards et al. 2005; Deigweiher et al. 2008; Ivanis et al. 2008). In these studies, hypercapnia was found to induce regulatory adjustments in transcription, translation and functional levels of several membrane transporters, indicating substantial rearrangements of the (membrane) protein inventory. The underlying signal transduction pathways promoting these responses remain to be established. In common eelpout the capacity of the central ion pump Na^+/K^+ ATPase was found increased in response to hypercapnia (Deigweiher et al. 2008). The observed responses may involve shifts in energy budget with consequences for cellular and whole organ functioning.

As changes in gene expression patterns are clearly involved in the acclimation process, assessing the entire transcriptome will provide a comprehensive overview of affected processes and pathways. Normalized, subtractive cDNA libraries, which reveal up- or downregulated genes, have been developed in order to identify such expression patterns. The suppression subtractive hybridization (SSH) technique first described by Diatchenko *et al.* (1996) has been established as a powerful tool in studies of the response of aquatic animals to pathogens (Bayne et al. 2001; Tsoi et al. 2004; Dios et al. 2007), pollutants (Marchand et al. 2006; Reynders et al. 2006; Hagenaars et

al. 2008), hypoxia (David et al. 2005), or salinity (Kalujnaia et al. 2007). SSH - sometimes in combination with differential screening techniques - thereby provides the highest probability in detecting differentially expressed genes even at low abundance. Differential screening alone also revealed responses to hypoxia and temperature stress in some fish species [e.g. cold-acclimation of zebrafish and carp (Tang et al. 1999) and hypoxia (Gracey et al. 2001) and heat-acclimation (Buckley et al. 2006) of goby]. The common goal in all of these approaches is the identification of (known or unknown) genes involved in the respective acclimation to stress, reflecting differential regulatory patterns in signal transduction, immune response, metabolism and stress response. Specific responses were identified upon exposure to several stressors: in acute phase reactants after pathogen exposure (Bayne et al. 2001; Tsoi et al. 2004), in respiratory or oxidative metabolic genes under hypoxia (David et al. 2005) or in genes involved in osmoregulatory processes due to salinity stress (Kalujnaia et al. 2007). Although transcription and translation are not necessarily tightly correlated with respect to timing and extent of the regulation pattern [i.e. (Deigweiher et al. 2008)], at least one of the transcriptome studies demonstrated that the induction of genes led to changes in the respective protein levels, confirming that transcriptional adjustments may elicit a rearrangement of the protein inventory (Reynders et al. 2006). Therefore, the identified genes not only lead to an understanding of acclimation and the mechanisms involved, but can also serve as genetic markers for environmental stressors (Marchand et al. 2006). Moreover, tissue specific transcriptome regulation patterns can reveal specific metabolic roles of the respective tissue (Gracey et al. 2001).

The present study is the first to identify differential gene expression patterns in fish gills under environmental hypercapnia (10,000 ppm). The common eelpout *Zoarces viviparus* was chosen as a model organism to monitor and study the response to climate change and associated environmental factors (Pörtner and Knust 2007). Furthermore, regulation of specific gill ion transporters at transcriptional and translational levels has previously been reported during time-dependent acclimation to hypercapnia (Deigweiher et al. 2008). The present data set comprising about 1,600 differentially expressed sequence tags complements these results. It demonstrates how a combination of hypothesis-driven functional and explorative genomic approaches can widen our understanding of organismal responses to environmental challenges.

Materials & Methods

Animals and acclimation experiment

Common eelpout (*Zoarces viviparus, Linnaeus*) were exposed to hypercapnia as described in our previous study (Deigweiher et al. 2008). In summary, fish were kept in flow-through aquarium

systems at 10°C under a 12 h day/night cycle. For the experiment they were transferred from control (380 ppm CO_2, pH 8.1) to hypercapnic conditions (10,000 ppm CO_2, pH 6.9). For this study, gill tissue samples were collected from controls and from specimens after 24 hours of hypercapnic exposure. Both sampling groups comprised 9 fish of both sexes and with overall body weights of 45.6 ± 21.0 g and lengths of 21.3 ± 2.5 cm. The tissue samples had been frozen in liquid nitrogen and stored at -80°C until further use. All experiments were conducted following German legislation. An approval of the work was issued by competent German authority (Freie Hansestadt Bremen, reference number 522-27-11/2-0; date: 2002-11-28).

SSH cDNA library construction

Total RNA from gill tissue was isolated according to the RNeasy Mini Kit (Qiagen, Hilden, Germany). Integrity of the RNA was controlled with a RNA 6000 Nano LabChip assay (Agilent Technologies, Waldbronn, Germany) and concentration was determined spectrophotometrically (BioPhotometer Eppendorf, Hamburg, Germany). For both groups a total of 245 µg RNA was pooled from all 9 specimens and used for poly(A)-selected mRNA isolation with the Oligotex mRNA mini Kit (Qiagen). The mRNA was concentrated by precipitation and determined spectrophotometrically (NanoDrop 1000, Peqlab Biotechnologie, Erlangen, Germany). 2.2 and 2.5 µg mRNA from the control and the 24h hypercapnia group, respectively, were used for generation of a forward and a reverse subtracted, normalized library by using the BD PCR-Select cDNA Subtraction Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. The subtracted cDNAs were used in PCR (using the BD Advantage cDNA polymerase mix, BD Biosciences) to amplify the differentially expressed sequences. The forward subtracted sample resulted in a cDNA library enriched with overexpressed/upregulated genes induced by hypercapnia, whereas reverse subtraction revealed the underexpressed/downregulated genes.

Cloning and sequencing

The differentially expressed cDNAs were cloned using a pGEM-T Easy cloning kit and high-efficiency competent *Escherichia coli* JM109 cells (Promega, Mannheim, Germany). After transformation, cells were plated onto LB agar plates with ampicillin (100 µg/ml), and X-gal (80 µg/ml) and IPTG (0.5 mM) for blue/white screening. For each library, 1056 white colonies were selected and grown overnight on 96-well plates in LB medium. Using LB freezing medium (containing 36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM trisodium citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, and 4.4 % glycerol) enabled storage of cultures at -80°C. For sequencing,

they were inoculated onto 96-well LB agar plates with ampicillin and shipped to GATC Biotech (Konstanz, Germany).

Sequence processing and gene identification

Sequence files were processed using programs of the Staden Package (http://staden.sourceforge.net). After removing vector and adapter sequences with Pregap4 v1.5 (Bonfield and Staden 1996), sequences were aligned with Gap v4.10 (Bonfield et al. 1995). The resulting sequences were subjected to GenBank BLAST search at the National Center for Biotechnology Information (NCBI) at the National Library of Medicine (NLM), National Institutes of Health (NIH) website. BLAST searches were performed using the BLASTX algorithm (Altschul et al. 1997). Those sequences with matches in GenBank were assigned to functional categories based on the Gene Ontology project (The Gene Ontology Consortium 2000). When only low BLAST homology was found (E-values > 1 x 10^{-5}), sequences were analyzed for possible open reading frames (ORFs) with the software MacVector (v10.0). So identified coding regions have been submitted to further BLASTX search. If the search for ORFs resulted in amino acid sequences shorter than 80 aa, no further analysis was carried out.

Results & Discussion

Acclimation capacity to environmental change is set by the genome and reflected in the transcriptome. Hypercapnia as an environmental stressor causes changes in the internal pH and ion equilibria in marine fish. Since gill epithelia accomplish more than 90 % of proton equivalent ion exchange involved in whole organism acid-base regulation, the gills were chosen to identify differential expression patterns in response to hypercapnia (10,000 ppm CO_2). Within the acute response phase, compensation of the acidosis is usually achieved within 10 - 24 hours by the accumulation of bicarbonate and equimolar loss of Cl^- (Perry 1982; Toews et al. 1983; Heisler 1993; Larsen et al. 1997; Michaelidis et al. 2007). On time scales up to six weeks a parallel study detected transcriptional responses in mRNA levels encoding essential membrane proteins, indicating rearrangement of the transcriptome to meet the new steady state requirements for the ion and acid-base status (Deigweiher et al. 2008). The 24-hour time point at the end of the acute pH compensation response was chosen for identification of differentially expressed sequence tags (ESTs).

Library characteristics

1003 sequence files were obtained from the forward (overexpressed) and 1040 files from the reverse subtracted library (suppressed genes) in 1056 clones. Sequence processing resulted in 806 upregulated and 795 downregulated contigs (contiguous pairs or sets of overlapping DNA fragments) with an average length of 526 and 544 base pairs, respectively. ESTs with significant homologies to sequences obtained from GenBank (E-values $< 1 \times 10^{-5}$), were classified according to the respective Gene Ontology (GO) terms (The Gene Ontology Consortium 2000). Sequences with lower homology (E-values $> 1 \times 10^{-5}$) were checked for open reading frames (ORFs). If the sequence was identified as a terminal region of a gene (flanked by an untranslated region), it could in most cases still be identified according to the high homology of the terminal region. If no identification was possible (despite existence of an ORF), gene products were classified as "unknown, hypothetical protein". In cases with significant homologies to other hypothetical or unnamed protein products from various vertebrates in GenBank, sequences were assigned to "identified protein with unknown function". In a few cases, where the molecular function has been identified (e.g. "Kelch-like 18 (Drosophila)" with "protein-binding" function), but in an unknown biological context or process, genes were assigned to the category "protein in unknown biological process". Those sequences, that did not reveal any homology and had no ORF resulting in a protein sequence of at least 80 amino acids, were depicted as "ORF < 80 aa".

The redundancy of genes in both libraries was low, emphasizing that the sequencing effort was appropriate for good coverage of hypercapnia induced patterns without being oversaturated. Although 1601 ESTs were finally analyzed, only a small fraction of clones occurred in both libraries (about 9 %) and were excluded as putative false positives. Most of these questionable clones were assigned to the immune response, in particular to major histocompatibility complex (MHC) classes I and II. Because of the polymorphic nature of these genes due to several alleles and loci it is impossible to differentiate whether these matches in both libraries represent the real pattern of differential expression of several loci or false positives. Nevertheless, the immune response seems to be essential during the early phase of environmental hypercapnia, given the large number of clones in both libraries (53 upregulated, 74 down) and the unusually large fraction of ambiguous hits (about 50 %) compared to other processes. Almost all other "false positive clones" represented genes known to be expressed at high abundance under normal maintenance conditions, like cellular structure proteins (e.g. cofilin, keratin, stathmin) and some, but not all proteins encoded on the mitochondrial genome (cytochrome c oxidase subunit 1, NADH dehydrogenase subunit 1; see the section *Metabolism and mitochondria* below). Subtraction of these genes might have been incomplete due to their high background expression level.

Figure 1 shows the distribution and classification of gene sequences for both, the up- and downregulated libraries. The fraction of sequences having no homology to other genes in GenBank is 33 and 27 % and in line with other fish cDNA libraries: *Onchorynchus mykiss* liver: 36 % (Bayne et al. 2001), *Paralichthys olivaceus* leucocytes: 27.7 % (Nam et al. 2000), *Sparus aurata* brain: 19.6 and 11.8 % (Dios et al. 2007), *Cyprinus carpio* liver: 13 % (Reynders et al. 2006). Subtracting the sequences with ORFs < 80 aa, the remaining fraction of unknown, hypothetical proteins was only 16 and 8 % for the up- and downregulated libraries, respectively. A certain number of genes might always remain unidentified from homology analyses due to the existence of about 30 % unique genes in the genome of an individual species. This has been concluded from completed genome projects of *Drosphila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* (Rubin et al. 2000). The human genome also comprises about 42 percent genes with currently unknown molecular functions (Venter et al. 2001). Then again, the number of unidentified genes from *Z. viviparus* might be reduced further depending on progress in ongoing sequencing projects focusing on vertebrates and especially fish species like *Danio rerio* (e.g. http://www.sanger.ac.uk/genbiol).

Hypercapnia induced changes in gene expression

The differential gene expression pattern after 24 hours of exposure to 10,000 ppm CO_2 included genes from various processes of different organizational levels. These genes have been sorted accordingly into ion and pH homeostasis, cellular maintenance, cell signaling, stress response, immune response, RNA/DNA metabolism, protein metabolism, energy metabolism and mitochondrial functions and transport processes. The complete lists of all up- and downregulated genes can be found in supplementary tables 1 and 2. In the following the most important findings for the different processes are described in more detail.

Ion and pH homeostasis

Gills are the main tissue for transepithelial ion exchange and acid-base regulation in fish. Previous work showed that the hypercapnic response comprises shifts in the expression of several ion transporters in *Z. viviparus* (Deigweiher et al. 2008). In perfect agreement with this study we found an EST of the α-subunit of the Na^+/K^+-ATPase, which probably represents the same transcript that we previously used for quantification of expression by real-time PCR. In our previous study the development of Na^+/K^+-ATPase mRNA levels over six weeks of hypercapnia was mirrored in parallel changes in protein and enzyme activity levels (Deigweiher et al. 2008). This clearly substantiates the regulation at transcriptional level seen in the present study. Furthermore, two different isoforms of the Na^+/K^+-ATPase β-subunit were found: upregulated β_1 and a

downregulated β_1-duplicate isoform β_{233} (Cutler et al. 2000). The β-subunits are known as regulatory subunits within the Na^+/K^+-ATPase complex and are involved in setting proper ATPase and transport functioning [e.g. (Scheiner-Bobis 2002; Liu and Askari 2006)]. The β_{233}-isoform seems to be gill-specific and was induced together with β_1 after salinity transfer in gills of freshwater acclimated eel (Cutler et al. 2000). The induction of β_1 and simultaneous repression of β_{233} in eelpout gills under hypercapnia requires further analysis to understand their regulatory role under hypercapnia. It is possible that, besides an upregulation of the catalytic transport unit α, further modification of enzyme functioning takes place through incorporation of different β-subunit isoforms (β_1 instead of β_{233}) to adjust the transport function of the holoenzyme.

Under control conditions, but especially during the acute response phase and under maintained hypercapnia, extrusion of protons via the gills enables pH control or recovery. For marine fishes Na^+/H^+-exchanger (NHE) isoform 2 and 3 are suggested to be located in the apical membrane for removing protons in exchange for sodium from the hyperosmotic environment [for review see (Claiborne et al. 2002)]. This view is in line with the upregulation of an EST of NHE2 in *Z. viviparus*. In addition, two ESTs encoding for different subunits of V-type H^+-ATPases were also found upregulated. These ATPases are seen responsible for proton extrusion in freshwater fish, but both proton export pathways are also operative in marine fishes [reviewed by (Evans et al. 2005)]. Altogether, the induction of these ion transporters reflects enhanced proton extrusion via branchial epithelia under hypercapnia.

In addition to the comparatively low number of ESTs coding for ion regulatory genes in both libraries, we found a considerable number of separate clones for carbonic anhydrase genes in both libraries. At least two isoforms were upregulated, whereas three different isoforms were downregulated. Eight isoforms of carbonic anhydrase are currently known to exist in vertebrates: three cytosolic (CA-I, CA-II and CA-III), two membrane-bound (CA-IV and CA-VII), one mitochondrial (CA-V), one salivary gland (CA-VI) and an as yet uncharacterized form (see http://pfam.sanger.ac.uk/family?acc=PF00194). The classification of present ESTs remains difficult, as transcripts are incomplete. EST sequences in our libraries show similarities to human CA II or IV isoforms and one EST is similar to a eukaryotic-type carbonic anhydrase domain (VB-like). Full-length sequencing of the respective clones will be necessary to differentiate between isoforms and their putative functional roles under hypercapnia. Nevertheless, our results indicate compensatory adjustment in carbonic anhydrase levels in response to the extra CO_2 load. In freshwater rainbow trout, for example, the cytosolic CA isoform (Esbaugh et al. 2005) was found upregulated 20-fold under acute hypercapnia [24 h, 8,000 ppm (Georgalis et al. 2006)]. These changes reflect that enhanced CO_2-hydration rates support the accumulation of extracellular bicarbonate for pH compensation. Bicarbonate accumulation is usually balanced by

falling chloride levels in blood serum [e.g. (Perry 1982; Toews et al. 1983; Larsen et al. 1997; Michaelidis et al. 2007)]. Accordingly, we identified two different chloride channels in the upregulated library. As a corollary, the present findings broaden the results and proposed working model of molecular and functional rearrangements in ion exchange of the hypercapnic fish gill (Deigweiher et al. 2008). However, further less obvious changes exist which may contribute to maintaining tissue and whole organism functioning under hypercapnic exposure.

Cell signaling & stress response

The largest fraction of genes up- or downregulated under hypercapnia involves those supporting signaling cascades, e.g. in cell cycle regulation and cellular development: G-protein signaling cascades, Ras- and Rho-family GTPase signaling, phosphatidylinositol-, MAPK-, NFκB- and Notch signaling. Further analysis of these genes will have to address their involvement in response to elevated CO_2 concentrations.

Evidence from vertebrate and invertebrate systems indicates that the activity of mitogen-activated protein kinases (MAPKs) is essential for heat shock protein (HSP) expression in response to various stressors like hypoxemia (Rafiee et al. 2003) and extreme temperature which likely also involves hypoxemia (Anestis et al. 2007). Several HSP families have been identified and assigned based on sequence similarity and molecular mass. Each family comprises proteins expressed constitutively (i.e. Hscs) and some induced in response to protein-denaturing stress [i.e. Hsps, (Basu et al. 2002)]. A number of heat-shock proteins were found differentially regulated in this study. The induction of two Hsp70 isoforms, Hsp90α and the respective binding proteins and chaperones (Hsp40) and the parallel downregulation of Hsp90β and Hsp40 homologues are unmistakable stress signals in the gills and indicate a complex differential response of distinct HSPs during the early regulatory phase. Furthermore, two upregulated genes from the signal transduction cluster could be clearly related to the stress response: oxidative stress responsive 1a protein [Ser/Thr protein kinase activity, (Tamari et al. 1999)] and stress-induced-phosphoprotein 1 [Hsp70/Hsp90-organizing protein, (Honore et al. 1992)]. Glutathione peroxidase (Gpx) and glutathione S-transferase (GST) are key enzymes of cellular detoxification systems against reactive oxygen species. In a salinity transfer experiment of seawater flounder, mRNA levels of Gpx and GST in liver were found increased with decreasing salinity, indicating oxidative stress (Choi et al. 2008). In this study, Gpx3 and GST rho - a teleost specific GST (Konishi et al. 2005) - were downregulated and GST alpha was upregulated, demonstrating some relevance to the hypercapnia response. Hypercapnia caused enhanced heat sensitivity of the edible crab *Cancer pagurus* mirrored in a reduction of haemolymph oxygen partial pressure, thereby narrowing the thermal tolerance window (Metzger et al. 2007). Thermal extremes were shown to cause

functional hypoxemia and oxidative stress in ectotherms (Pörtner 2002); it seems obvious that environmental stressors exacerbating hypoxemia or oxidative stress result in similar responses of the underlying molecular systems.

Immune response

As mentioned above, genes involved in the immune response were identified in both libraries. In addition to the large fraction of MHC class I and II proteins, some of the genes are associated with defense mechanisms of the immune system like inflammation [e.g. CC-chemokines CK1, SCYA 103 and 113; for chemokine classification in teleost fish see (Peatman and Liu 2007)] or B- and T-cell activation (e.g. antigens CD6, CD 48, CD80 and receptors CD247, Zap70). Others are part of the innate immune system (e.g. complement system components C3 and 9, toll-like receptor 3). Generally, the large number of immune relevant ESTs indicates readjustment of immune system components during acute hypercapnia exposure despite the ambiguous hits in both libraries (see section *Library characteristics* above). The interpretation of these patterns remains elusive and requires further investigations.

Cellular maintenance

Besides stress and immune responses, mechanisms contributing to cellular maintenance also displayed transcriptional responses under hypercapnia. The assigned sequences include genes for cell adhesion and junctions (e.g. catenin, claudin, desmoglein, integrin and thrombospondin) and cytoskeletal organization (e.g. actin, advilin, collagen, keratin and mysosin). Responses in cell proliferation and development were governed by the differential regulation of ESTs encoding caveolin, centromere protein Q, cornifelin, septin, envoplakin and radixin. Expression patterns revealed no obvious up- or downregulation of specific cellular processes. The involvement and functional contribution of different enzyme classes requires further analysis. A number of genes involved in apoptosis (e.g. caspases, deoxyribonucleases I and II) or the respective signaling (pycard, rock1, programmed cell death 6) were identified in both libraries. This differential expression pattern may indicate adjustments in the balance between apoptosis and mitosis. The downregulation of several caspase genes and inductors of apoptosis in eelpout gills under hypercapnia is suggestive of inhibition of apoptosis.

DNA replication and transcription

The regulation of the transcriptome also involves DNA and RNA metabolism. A few genes involved in DNA metabolism like in replication [e.g. histone chaperone SET (Gamble and Fisher 2007), replication factors] and repair [e.g. RAD23 (Ng et al. 2003), ERCC-8/CSA (Kamiuchi et

al. 2002)] were found mainly in the downregulated library. This might indicate a lower cell cycle activity, but this aspect requires further analysis.

The number of ESTs related to transcriptional regulation was second to the one encoding signal transduction components in the two libraries. Some were linked to RNA processing and splicing [e.g. splicing factors, nuclear ribonucleoproteins, ribonucleases, helicases (DEAH/DEAD box polypeptides)]. The majority encoded for regulatory proteins like transcription factors, RNA/DNA binding motifs and zinc finger proteins. Due to the diversity of these processes and the genes involved, and due to the many hits in both libraries, we cannot predict an overall up- or downregulation of the whole transcription apparatus. However, the sheer number of transcription-related genes points towards a shift in the regulatory processing of the transcriptome during the early phase of hypercapnia. This likely involves the induction of some genes and the simultaneous repression of others (illustrated, for example, by the differential regulation of zinc finger proteins, known as sequence-specific DNA binding motifs of transcription factors: the upregulation of zinc finger motifs no. 208 and 452 and downregulation of no. 598 and 760 probably modifies the transcription of different gene products by induction and repression, respectively). This is in line with the differential regulation of specific ion transporter genes in our previous study, where an upregulation of Na^+/K^+-ATPase and a downregulation of bicarbonate and proton exchangers occurred during the acclimation phase (Deigweiher et al. 2008).

Protein metabolism: translation, modification and degradation

Similar to RNA/DNA metabolism, the protein biosynthesis machinery in the eelpout gill was also modulated under hypercapnia. The differential expression of various ribosomal protein- and translation initiation factor-coding genes (eIF3, eIF4) and three repressed tRNA-synthetase genes (lysyl, threonyl and valyl) again indicate complex acclimation of the translational machinery. A number of differentially expressed genes are involved in post-translational protein modifications like glycosylation (e.g. ribophorin 1, dolichyl-phosphate mannosyl-transferase) and crosslinking (e.g. transgluatminase). Furthermore, we found ESTs involved in protein localization like signal peptide processing (e.g. furin, signal peptide peptidase) and protein targeting (e.g. SEC63, receptor transporter protein 1, translocase of inner mitochondrial membrane, vacuolar protein sorting). Specific protein degradation is necessary for the maintenance of the protein inventory. In protein catabolism we identified genes responsible for ubiquitination, the targeting of proteins for degradation. This process involves three enzyme classes, which were all found differentially expressed in the two libraries: E1 type ubiquitin-like modifier activating enzymes (1, 3 and 7), E2 type ubiquitin conjugating enzyme (E2N-like), E3 type ubiquitin ligase (nedd4a). Protein

degradation itself was apparently regulated by modified expression of genes related to proteasomes (e.g. proteasome subunits alpha and beta type, assembly chaperone 2) and various proteases (e.g. cathepsin, prostasin, marapsin). Again, the differential expression of these gene products might enable the directed degradation or preservation of specific proteins necessary for the acclimation to hypercapnia during the early phase.

Metabolism and mitochondria

Several metabolic pathways were represented in the differentially regulated libraries, indicating shifts in metabolic fluxes. Several components of the lipid degradation pathway were affected during the early response to hypercapnia. Figure 2 shows the expression of enzymes in lipid degradation and β-oxidation from triacylglycerol to acetyl-coenzyme A (acetyl-CoA), with upregulated lipase, short-chain acyl-CoA dehydrogeanse and enoyl-CoA-hydratase, and downregulated acyl-CoA-synthetase, carnitine-O-palmitoyl-transferase, medium-chain acyl-CoA-dehydrogenase and 3-ketoacyl-CoA-thiolase. Whereas short-chain fatty acid oxidation might have been activated, some EST hits may indicate a repression of long-chain fatty acid catabolism. However, the libraries did not display similar changes in the expression of genes encoding enzymes involved in fatty acid biosynthesis, except for two long-fatty-acid elongation-genes (upregulated trans-2,3-enoyl-CoA-reductase and downregulated "elongation of very long chain fatty-acids like"). If β-oxidation prevails under hypercapnic conditions, the generated acetyl-CoA would enter the tricarboxylic acid cycle to undergo final oxidation and support ATP generation. This requires fatty acid and carbohydrate metabolism in equilibrium, due to the demand for oxaloacetate. Otherwise, gluconeogenesis and/or the production of ketones should dominate. Indeed, ketogenesis is supported by upregulated 3-hydroxybutyrate-dehydrogenase (see Fig.2). Furthermore, the resulting equilibrium between glycolysis and gluconeogenesis seems to favor gluconeogenesis: Fructose-2,6-bisphosphate is one important regulator of this pathway, as it activates the 6-phosphofructo-1-kinase (PFKI), which catalyses the phosphorylation from fructose-6-phosphate to fructose-1,6-bisphosphate (one of the ATP consuming steps in glycolysis). Here, the generation of fructose-2,6-bisphosphate (and therefore glycolysis) was possibly inhibited as expression of the respective enzyme (6-phosphofructo-2-kinase, PFKII) was downregulated. Moreover, expression of an important pacemaker in gluconeogenesis, phosphoenol-pyruvate carboxykinase (PEPCK), which catalyses the transformation from oxaloacetate to phosphoenol-pyruvate, was upregulated (see Fig.2).

Within the TCA cycle, the expression of alpha- and gamma NAD^+-dependent isocitrate dehydrogenase subunit genes was found downregulated (see Fig. 2). Isocitrate dehydrogenase is known as an important rate-limiting enzyme for the TCA cycle, the subsequent accumulation of

citrate would inhibit PFKI and thus glycolysis in a feedback loop. Finally, the induction of pyruvate-dehydrogenase (DH) kinase indicates inhibition of the pyruvate-DH complex by phosphorylation. The cleavage of pyruvate into CO_2 and acetyl-CoA is the ultimate irreversible step in carbohydrate catabolism. Inhibition of glycolysis is also reflected in the protection of glycogen stores [which are not very large in fish gills anyway, (Mommsen 1984)], as gene expression of glycogen phosphorylase and phosphoglucomutase (conversion of glycogen into glucose-6-phosphate) was downregulated.

Under control conditions, gluconeogenesis in fish gills is not measurable, but has been postulated to be activated during stress response; this might involve activation of the pentose phosphate shunt, which provides NADPH and ribuloses for RNA/DNA synthesis and other synthesis pathways [reviewed by (Mommsen 1984)]. Indeed, expression of 6-phosphogluconolactonase, the second step in the pentose phosphate shunt, was found upregulated under hypercapnia (see Fig.2). The rate of this pathway is strictly controlled by the $NADP^+$/NADPH ratio in the cells. As the present data indicate lower flux through the TCA cycle, and moreover, reduced expression of mitochondrial-membrane bound nicotinamide nucleotide transhydrogenase, the other source for generation of NADPH from TCA-cycle generated NAD^+, the pentose phosphate shunt may be favored during the early regulatory phase according to the need for NADPH in anabolism. Clearly, metabolic regulation by differential gene expression patterns needs further evaluation in functional studies and measurements of specific metabolites. As a corollary, the sum of key metabolic enzymes found in both libraries already reveals a shift in metabolite flux favoring anabolic pathways.

Within oxidative metabolism, genes encoding the complexes of oxidative phosphorylation were differentially expressed as well (see table 1). Each complex was represented by several genes, with expression found up- or down regulated. Mitochondrial regulation is complicated by the need for coordinated expression of nuclear- and mitochondrial-encoded genes. Two mitochondrial-encoded subunits (NADH-DH subunit 1 and Cytochrome c oxidase subunit 1) were found in both libraries (see above section *Library characteristics*); but although mitochondrial genes are transcribed as one transcript (Clayton 1991), other subunits were only found upregulated indicating some specific posttranscriptional modulation of the different mRNA levels. Cytochrome c oxidase (complex IV) is thought to be the flux limiting step of the respiratory chain, and its nuclear encoded subunit V has been postulated as a pacemaker for its transcriptional regulation (Fukuda et al. 2007). Two isoforms of this subunit were identified in the downregulated library. This may indicate an overall downregulation of mitochondrial capacity. In line with this conjecture, a shift from aerobic to a more anaerobic phenotype became visible in muscle of *S. aurata* upon hypercapnia (Michaelidis et al. 2007). However, cytochrome c

oxidase and citrate synthase capacities remained constant in *Z. viviparus* gills after 14 days of hypercapnic exposure (Penno 2006). In our parallel study we found higher capacities of Na^+/K^+ ATPase, the workhorse of ion and pH regulation, indicating a higher energy demand upon hypercapnia (Deigweiher et al. 2008), which might rely on aerobic energy production. Thus, the complex expression pattern of several mitochondrial and nuclear encoded genes of the components of oxidative phosphorylation may indicate a fine-tuning of mitochondrial functions at unchanged capacities of mitochondrial respiration. The parallel induction of important genes of the oxidative stress response (see above) may then reflect an imbalance in oxidative phosphorylation during the early phase of hypercapnic response with higher rates of reactive oxygen species formation, which elicit antioxidative defense.

Conclusions & Perspectives

The differential gene expression patterns observed in the gills of *Z. viviparus* after 24 hours of hypercapnia clearly reflect an acclimation process broadly affecting all vital cellular processes. Upregulation of stress response proteins and of the defense reactions of the immune system are indicative of a general stress state of the gill tissue. Furthermore, shifts in metabolic flux may be elicited by the induction of gluconeogenesis probably supporting the pentose phosphate shunt for the generation of NADPH and ribose for RNA/DNA component synthesis. The large fraction of genes related to signal transduction offers a solid background for the detection of regulatory pathways involved in the hypercapnic responses of metabolism and ion regulation. The differential regulation of Na^+/K^+-ATPase, other ion exchange mechanisms and of several carbonic anhydrases may reflect the changing demands of ion and acid-base regulation (Deigweiher et al. 2008). As a perspective, the present study emphasizes how combined hypothesis-driven functional and explorative, genomic approaches are suitable to deepen our understanding of an organism's response to environmental challenges. Furthermore, it offers a large set of candidate genes that may serve as reliable markers when assessing the impact of more moderate CO_2 concentrations as expected in future ocean acidification scenarios (IPCC 2007), not only in organisms with high capacities of acid-base regulation like fish, but also in more sensitive marine species as among invertebrates.

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Table 1: Up- and downregulated genes involved in components of oxidative phosphorylation and their coding localization

Component	Gene	Localization	up	down
complex I	*NADH-DH subunit 1*	*mitochondrion*	+	+
	NADH-DH subunit 2	mitochondrion	+	
	NADH-DH Fe-S protein 7	nucleus	+	
	NADH-DH 1β, sub complex 5	nucleus		+
	NDUFA2: assessory subunit complex I	nucleus	+	
complex II	Ubiquinol-cytochrome c reductase core protein II	nucleus		+
	ubiquinol-cytochrome C reductase complex-like	nucleus		+
	Cytochrome b (2 ESTs)	mitochondrion	+	
complex IV	*Cytochrome c oxidase subunit 1*	*mitochondrion*	+	+
	Cytochrome c oxidase subunit 3	mitochondrion	+	
	Cytochrome c oxidase subunit Vab	nucleus		+
	Cytochrome c oxidase subunit Vaa	nucleus		+
ATP synthase	F1 complex α subunit 1	nucleus	+	
	F1 complex γ subunit	nucleus	+	
	Fo complex subunit b isoform 1	nucleus		+
	Fo complex subunit c (subunit 9)	nucleus		+

Figures

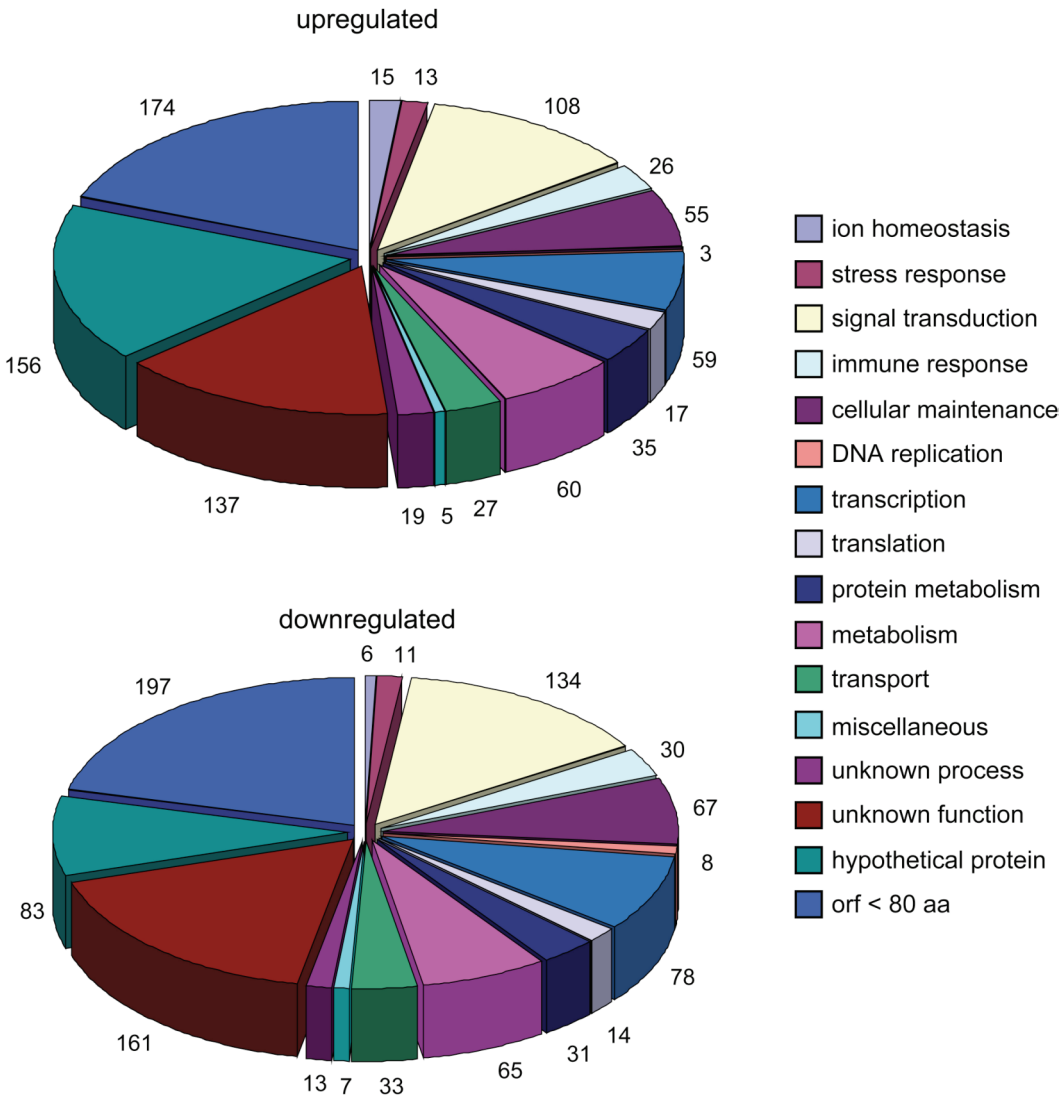

Figure 1: Pie chart diagram of differentially regulated ESTs under hypercapnia. Distribution of upregulated (above) and downregulated (below) genes within cDNA libraries of gill tissue from *Z. viviparus* after 24 hours of hypercapnia acclimation identified by suppression subtractive hybridization. Digits indicate the numbers of ESTs that were attributed to the respective known or unknown functions (see legend).

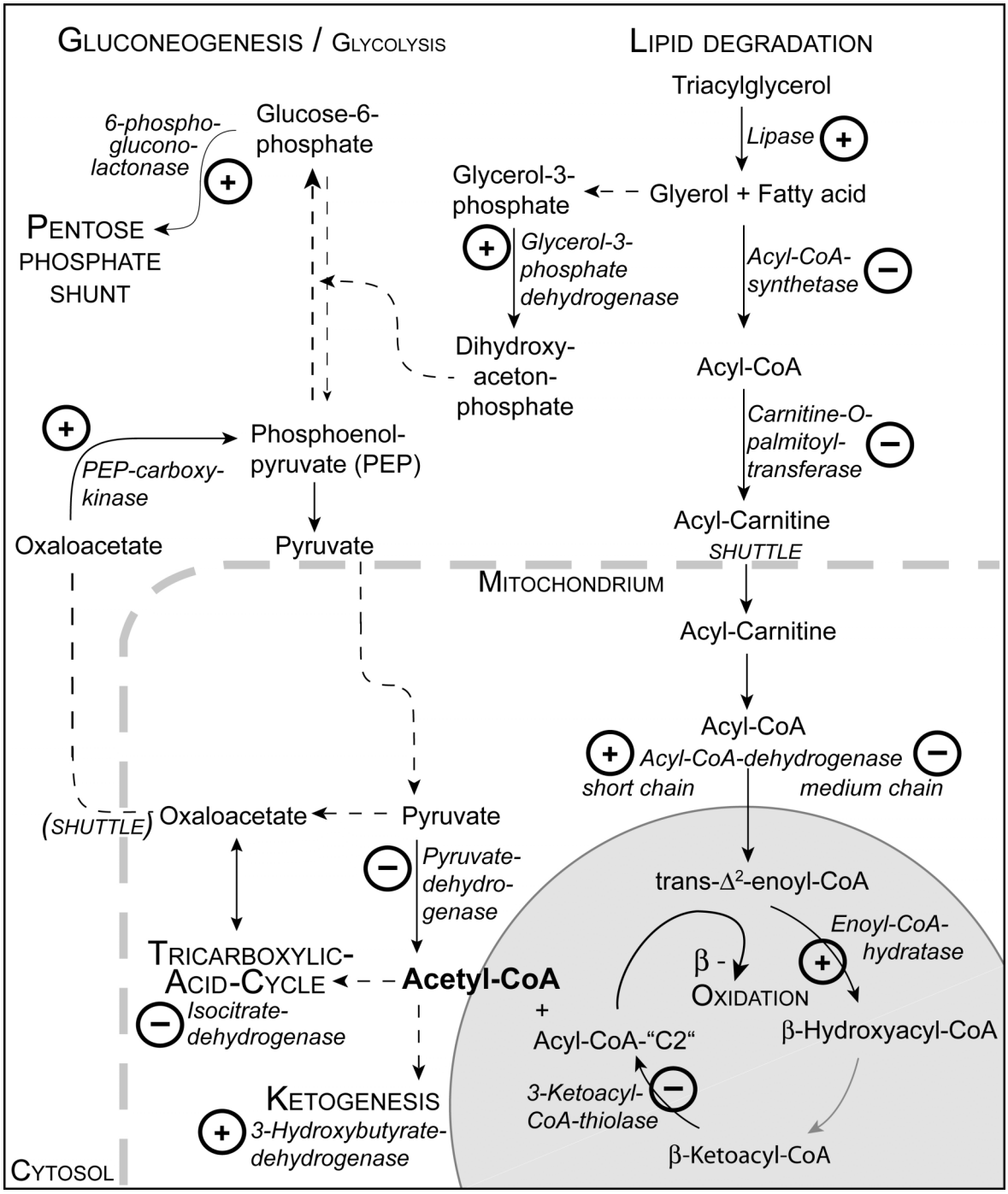

Figure 2: **Metabolic fluxes in gills after 24 hours of hypercapnia**. Genes involved in the pathways of carbohydrate and lipid metabolism are illustrated as upregulated (+) or downregulated (-). Gluconeogenesis was favored over glycolysis (see text for individual genes involved), probably leading to the fuelling of the pentose phosphate shunt and inhibition of the tricarboxylic acid (TCA) cycle. Acetyl-CoA generated by the enhanced lipid degradation that cannot enter the TCA cycle, is probably redirected into ketogenesis for energy supply.

4 Additional results

4.1 Isolated, perfused eelpout gill respiration

The setup developed for respiration measurements of isolated, perfused fish gills (see publication II) has also been used for gills of North Sea eelpouts *Z. viviparus*. Although the eelpouts used for this study were about 4 times bigger than the fish used for publications I and III, their gill size was still at the lower limit for the perfusion system (see Figure 4-1).

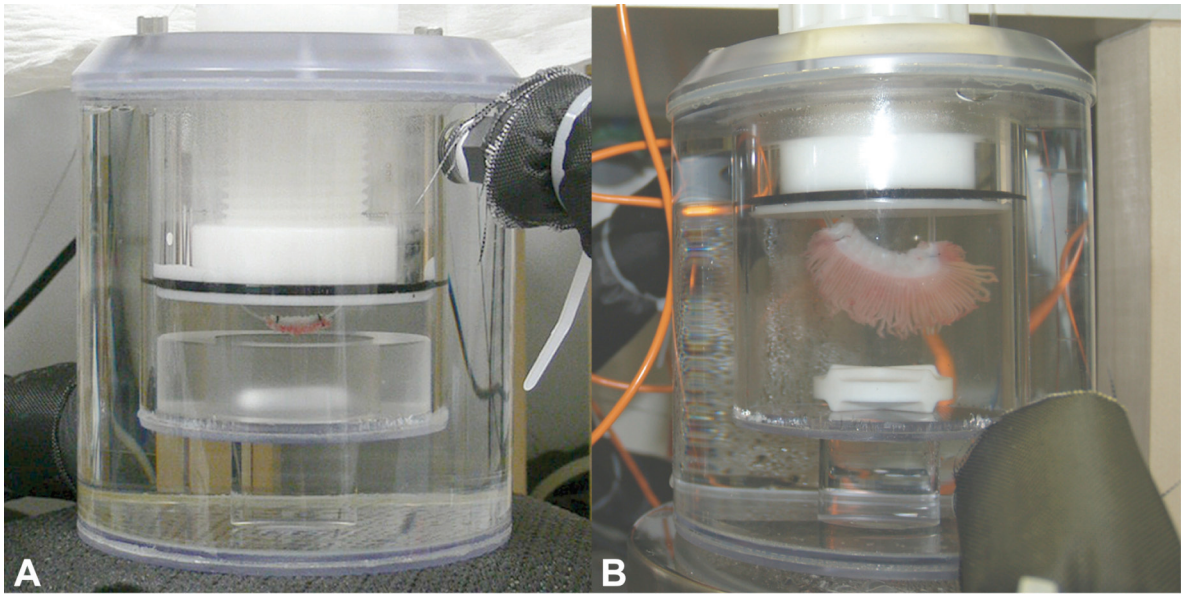

Figure 4-1: Size comparison of isolated perfused gill respiration measurements
Gill arch of North Sea eelpout (**A**) and of Antarctic notothenioid (**B**) demonstrated in identical respiration chambers. Chamber volume for eelpout gill measurement was further reduced by insertion of an opaque plastic ring.

Respiration values under control (normocapnia, pH 8.15, 10°C) and under hypercapnic conditions (10,000 ppm CO_2, pH 6.85, 10°C) were obtained from gill arches of 8 individual fish. Control MO_2 was 6.46 ± 0.45 µmol (O_2) g_{fwt}^{-1} h^{-1} (n = 17) and did not change significantly ($p > 0.05$) under hypercapnia, where it was 5.94 ± 0.45 µmol (O_2) g_{fwt}^{-1} h^{-1} (n = 14). Infusion of blue colored saline (0.01 % Trypan Blue, Sigma-Aldrich, Taufkirchen, Germany) demonstrated sufficient and homogenous gill perfusion. The dye usually reached the filament tips within 30 minutes. Only the outer filaments, where the perfusion tubing had to be fixed by suture, remained underperfused (see Figure 4-2).

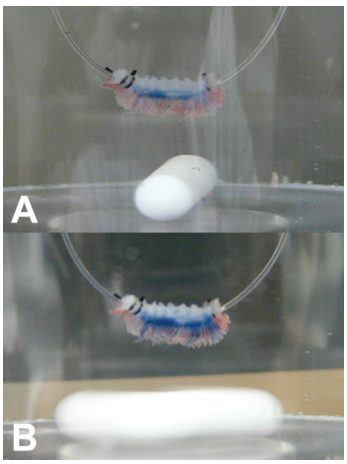

Figure 4-2: Perfusion efficiency
Eelpout gill arch during perfusion with blue colored saline (0.01 % Trypan Blue) after 10 (**A**) and 30 (**B**) minutes.

For comparison of oxygen consumption of isolated gills with values from whole animals, oxygen consumption rates of *G. gibberifrons* and *N. coriiceps* reported by Holeton (1970) and of *Z. viviparus* (publication I) had to be standardized to the average body weights of the animals used in the isolated gill studies (see Table 4-1). Following formula was applied using a scaling coefficient of 0.8 (Holeton, 1974; Clarke & Johnston, 1999):

$$MO_2(x) = MO_2(y)\left(\frac{BW(y)}{BW(x)}\right)^{1-A}$$

where $\mathbf{MO_2}$ is the oxygen consumption rate [µmol (O_2) g^{-1} h^{-1}], **BW** is the body weight [g] and **A** is the scaling coefficient (0.8).

Table 4-1: Scaling of whole animal MO_2 with respect to body weight

	Average fish weight (Y) [g]	Whole fish MO_2*) (Y) [µmol (O_2)$g^{-1}h^{-}$]	Average fish weight (X) [g]	Standardized MO_2*) (X) [µmol (O_2)$g^{-1}h^{-}$]
G. gibberifrons	470[1)]	0.72[1)]	946[3)]	0.62[3)]
N. coriiceps	1000[1)]	0.9[1)]	385[3)]	1.08[3)]
Z. viviparus	40[2)]	1.54[2)]	160	1.42

*) Note: Data are expressed per g_{BW}; [1)] Holeton (1970), [2)] publication I, [3)] publication II

Gill respiration values for eelpout in this study were in line with data from other isolated gill preparations from teleost fish (Table 4-2):

Table 4-2: Comparison of respiration rates and body weights of whole animals and isolated gills from teleost fish.

	Fish weight [g]	Whole fish MO_2 *) [µmol (O_2)$g^{-1}h^{-}$]	Gill weight [g]	Fract. gill weight [%]	Gill MO_2 [µmol (O_2)$g^{-1}h^{-}$]	Fract. gill MO_2 [%]	Ref.
G. morhua 15°C	450-1200	2.43		3.8	4.25	6.6	e
O. clarki clarki 10°C	310 ± 28	3.64 ±0.15	3.95 ± 0.43	1.27	6.7 ± 0.6	2.3	c
P. flesus 10°C	179 ± 25	0.97	4	2.23	12.06 ± 2.39	27.7	d
Z. viviparus 10°C	160 ± 80	1.42 ± 0.19	1.65 ± 0.21	1.06	6.46 ± 0.45	4.6	b
G. gibberifrons 0°C	946 ± 25	0.62[a)]	13.4 ± 0.45	1.42	2.83 ± 0.07	6.5	b
N. coriiceps 0°C	385 ± 33	1.08[a)]	6.7 ± 0.60	1.74	3.93 ± 0.11	6.3	b

*) Note: Data are expressed per g_{BW}; [a)] Holeton (1970) [b)] this thesis, [c)] Morgan & Iwama (1999), [d)] Lyndon (1994) [e)] Johansen & Pettersson (1981)

5 Discussion

The response of marine fish to high environmental CO_2 concentrations was examined with a focus on gills as the main organs involved in ion- and pH regulation. The present thesis is based on three main projects described in the introduction and realized in publications I to III, addressing the question of hypercapnia response on the organismic, molecular and genomic level. Results from the three publications and additional data will be reviewed in the following and discussed in the context of acclimation to hypercapnia in fishes.

5.1 Metabolic consequences of hypercapnia

As demonstrated in publication I, common eelpouts maintained a stable standard metabolic rate (SMR) when exposed to hypercapnia over 3 days (publication I, fig 5). This is in line with most studies in fish, where changes in P_{CO_2} generally did not affect resting oxygen consumption. However, in some elasmobranch species [*Scyliorhinus stellaris* (Randall *et al.*, 1976), *Raja ocellata* (Graham *et al.*, 1990)] and also in freshwater trout (Thomas *et al.*, 1983) a transient increase in respiration rate has been observed [reviewed by (Ishimatsu *et al.*, 2005)]. Moreover, in an earlier study of the Antarctic eelpout *Pachycara brachycephalum* an increase in SMR has been reported, using the same experimental setup as for the confamilial North Sea species in publication I (Burgard, 2004). This finding may reflect a higher sensitivity or responsiveness of polar fish to environmental challenges (like hypercapnia) compared to temperate fish [see also (Pörtner *et al.*, 2004; Pörtner *et al.*, 2005)]. Furthermore, long-term impairment of metabolic equilibria cannot be excluded as long as the mechanisms underlying acclimation are not completely understood. The restoration of internal pH and ion balance under environmental hypercapnia and especially the maintenance of a new equilibrium is most likely associated with an increase in energy demand resulting from higher ion transport activity driven by the main ion pump, the Na^+/K^+-ATPase (see also 5.2, 5.3). However, this additional energy demand does not involve a higher oxygen consumption rate. To shed light on the principles of the hypercapnia response in fish and on changes in energy turnover and ion transport processes, appropriate studies were designed and carried out within this thesis. As the gills are the organs primarily facing environmental conditions and disturbances, and are responsible for over 90 % of the ion and acid-base regulation in fishes (Heisler, 1986b), these organs were chosen for the examination of energy budgets, ion transport processes and the underlying genomic regulation (see publications I - III).

5.2 Gill energy turnover

Gills are multifunctional organs that are responsible for gas exchange, take part in nitrogenous waste elimination and are, as stated above, essential for ion and pH regulation [reviewed by (Evans et al., 2005)]. They display high metabolic costs, which have been calculated to about 7 - 10 % of whole animal standard metabolic rate in seawater fish (Boeuf and Payan, 2001; Gibbs and Somero, 1990; Mommsen, 1984; Perry and Walsh, 1989), despite accounting for only 1 - 3 % of the total body mass. Consequently, mass specific oxygen consumption is much higher than that of the entire animal. In the filaments - the most active parts of the gills - mass specific oxygen consumption can even be 5 - 10 times higher than the average of the whole animal. The fractional gill oxygen consumption rates obtained from isolated perfused gill measurements range from 4.6 % in North Sea eelpouts to 6.4 % in the Antarctic notothenioids (publication II and Table 4-2 in the Additional Results section). Data from other fish are reasonably in line with these fractional costs ranging from 2.4 % in seawater and 3.9 % in freshwater acclimated trout (Morgan and Iwama, 1999) to 6.64 % in Atlantic cod (Johansen and Pettersson, 1981) and 11 to 31 % in seawater acclimated flounder (Lyndon, 1994).

Gill metabolic costs comprise a more or less fixed fraction of the whole animal SMR. Therefore, species-specific differences in the total oxygen demand are also reflected by differences in gill respiration rates of the respective species. It has been demonstrated that the standard metabolic rate is positively correlated to the activity levels of polar fish (Holeton, 1974; Morris & North, 1984; Zimmerman, 1997). Among Antarctic fishes studied in publication II, *G. gibberifrons* is a sluggish, benthic fish with a low SMR (0.35 mmol O_2 h^{-1} for a 500 g fish[#]) while *N. coriiceps* is a more active, benthopelagic fish with a higher SMR (0.53 mmol O_2 h^{-1} for a 500 g fish[#]). Accordingly, *N. coriiceps* gills had a significantly higher oxygen consumption rate (3.9 µmol O_2 g^{-1} h^{-1}) than the gills of *G. gibberifrons* (2.8 µmol O_2 g^{-1} h^{-1}). Similarly, among temperate species, bottom-dwelling eelpout and flounder display lower total and gill metabolic rates than the more active benthopelagic cod and trout (see Table 4-2), confirming validity of the data from isolated gill measurements.

When exposing the isolated gills to hypercapnia, oxygen consumption was the same as under control conditions in both Antarctic notothenioids and in the North Sea eelpout. The saline used for gill perfusion had been chosen to resemble typical blood acid-base status of fish after complete pH recovery under hypercapnia, which is associated with elevated plasma bicarbonate concentrations (Perry, 1982; Toews *et al.*, 1983; Larsen *et al.*, 1997; Michaelidis *et al.*, 2007). Therefore, gill functional rates were considered to reflect the whole organism's metabolic

[#] Values (after Holeton, 1974) scaled to an average 500 g fish by using a scaling coefficient of 0.8

state after compensation of hypercapnia induced pH disturbances, and not during acute pH recovery. Comparable to whole animal SMR in eelpout (publication I), an increased energy demand in the gills for compensating and maintaining the new ion- and acid-base balance seems likely, but was apparently not achieved by a higher respiration rate. For a more detailed picture of the branchial energy allocation, experiments in publication II were carried out to identify energy consumption of three cellular key processes: RNA synthesis, protein synthesis and Na^+/K^+-ATPase activity as a measure for ion regulation. The fractional contribution of translation, transcription and ion regulation to overall energy turnover was different between the two species. Taken together, the three processes accounted for about 60 % of gill energy turnover in *N. coriiceps*, but only for about 30 % in *G. gibberifrons* (publication II, fig. 7). Thus, there is a large fraction of other processes using the remaining energy turnover, especially in *G. gibberifrons*. From mammalian studies it is known that mitochondria utilize up to 90 % of total oxygen uptake, 25 % of which is attributed to proton leakage (Nobes *et al.*, 1990; Rolfe & Brand, 1996). Metabolic processes like protein synthesis, RNA synthesis and Na^+/K^+-ATPase-dependent ion regulation use the ATP generated by mitochondrial F_1F_O-ATPase. Further substrate bound energy is consumed by Ca^{2+}-ATPases, metabolic substrate cycles (e.g. glucose/glucose-6-phosphate, phosphoenolpyruvate/pyruvate/oxaloacetate, acetate/acetylCoA), gluconeogenesis, ureagenesis, cell movement and signal transduction [reviewed by (Rolfe & Brown, 1997)]. In gills, H^+-ATPases might also contribute significantly to the energy budget (Morgan & Iwama, 1999), but for pH- and osmoregulatory epithelia and other fish tissues comprehensive data are not yet available. Therefore, the phenomenon that *G. gibberifrons* gills only consume 30 % of their energy budget for the processes studied, while *N. coriiceps* consumes 60 % for the same, can only be answered speculatively: It may be advantageous for *G. gibberifrons* that the low baseline cost for ion regulation and protein and RNA synthesis enables a higher idling capacity of proton leakage compared to *N. coriiceps*. This might support fast responses to changing ATP demand (Rolfe and Brand 1997) for induced ion and acid-base regulation under hypercapnia. It also has to be mentioned that measuring the individual processes in different gill samples and adding them up is prone to potential errors in the estimated total energy budget (see also Methodology section in publication II). However, the data obtained in Antarctic fish gills are comparable to cellular data from other fish species and mammals, and similar fractions of energy allocation were obtained. In both, *N. coriiceps* and *G. gibberifrons* gills the processes of ion regulation (Na^+/K^+-ATPase), protein synthesis and RNA synthesis shared more or less equal fractions of the energy budget, with a slightly higher energy demand for ion regulation. The importance of ion regulation in the gills is clearly emphasized when comparing these results to data from rat thymocytes and fish hepatocytes, where ATP demand of protein and RNA synthesis was higher than that of Na^+/K^+-

ATPase (Buttgereit & Brand, 1995; Wieser & Krumschnabel, 2001). Comparable data from isolated gill measurements were only available for Na^+/K^+-ATPase activity in temperate fish. These costs were 25 % for seawater flounder and freshwater trout (Stagg & Shuttleworth, 1982; Morgan & Iwama, 1999) compared to 23 % in *N. coriiceps* and 12 % in *G. gibberifrons* (see publication II fig. 7). It remains to be established whether the lower fraction in *G. gibberifrons* gills can be attributed to the generally lower metabolic activity of this fish species.

Nevertheless, when comparing the data from normocapnic controls with data from measurements under hypercapnia, striking differences were apparent. In both notothenioid species, the respiration rates attributed to the examined processes increased significantly (see publication II fig. 7). As overall energy demand remained constant, the fraction of oxygen available to residual processes (e.g. proton leak, Ca^{2+}- ATPases, metabolic substrate cycles) was reduced by about 50 %. The higher ion transport activity of the Na^+/K^+-ATPase (30 and 100 % increase in *N. coriiceps* and *G. gibberifrons*, respectively) was probably supported by overall increased rates of protein biosynthesis (60 and 200 % increase in *N. coriiceps* and *G. gibberifrons*, respectively). A higher translation rate may result from a higher transcription rate, which was evident at least in the gills of *G. gibberifrons* (180 % increase in RNA synthesis), though in *N. coriiceps* no increase was found. In line with these results, gill Na^+/K^+-ATPase enzyme activity, as well as transcript and protein levels also increased steadily during hypercapnia acclimation of the North Sea eelpouts (publication I). The induction of the transcription and translation apparatus in the notothenioid gills is most likely not only required for higher Na^+/K^+-ATPase capacities, but also for other gene products, which may belong to the ion transport machinery or other cellular processes (see also publication III). In line with the present results, upregulation of translation and transcription levels of several ion transporters has also been observed in gills of trout and mummichog under hypercapnia (Perry *et al.*, 2000; Perry *et al.*, 2003a; Edwards *et al.*, 2005; Shahsavarani & Perry, 2006; Ivanis *et al.*, 2008).

5.3 The molecular transport machinery

The balance of the ion and pH homeostasis requires the adjustment of respective ion transporters, probably on different regulatory levels. As already mentioned above, the key enzyme for the branchial ion transport in fish gills is Na^+/K^+-ATPase, which provides the driving force for all gradient dependent transport processes. The regulation of this enzyme has been comprehensively investigated on transcriptional, translational and functional levels in a variety of studies concerning osmoregulatory challenges, temperature acclimation and development [reviewed by (Marshall, 2002)]. But to this day, the data for its regulation under hypercapnia are sparse and contradictory: In developing Atlantic salmon, branchial Na^+/K^+-

ATPase mRNA levels were reduced after 4 days at 20,000 ppm CO_2, while enzyme activity remained constant (Seidelin *et al.*, 2001). In gills of Japanese flounder, activity increased after exposure to 10,000 and 50,000 ppm [unpublished data, cited in (Ishimatsu *et al.*, 2005)] and in gills of Atlantic cod its protein and activity levels increased 2-fold after 12 months of hypercapnia treatment at 6,000 ppm CO_2 (Melzner *et al.*, 2008).

In publication I, the regulation of Na^+/K^+-ATPase in the gills of *Z. viviparus* was investigated during six weeks of hypercapnia (10,000 ppm CO_2) covering the acute pH compensation phase (up to 24 hours) and thereafter before a new steady state was reached. The enzyme's mRNA, protein and activity levels were steadily upregulated from day 2 onward (publication I, Figs 1D, 3B & 4). Apparently, the higher functional capacity of the sodium pump satisfies the new requirements of ion and acid-base regulation. The increase in transport activity seems to be achieved by elevated transcription and translation levels of the respective gene, similarly to observations after salinity transfer experiments [e.g. (Epstein *et al.*, 1967; Jensen *et al.*, 1998; D'Cotta *et al.*, 2000; Tipsmark *et al.*, 2004; Lin *et al.*, 2006)]. Although the stimulus for the upregulation remains unclear, it seems to rely on the generally higher demand for ion transport driven by Na^+/K^+-ATPase.

Among the transporters that depend on the ion gradient generated by the sodium pump are the Na^+/H^+-exchanger, Na^+/HCO_3^--cotransporter and Cl^-/HCO_3^--exchanger. Expression levels of these transporters have also been studied in publication I during hypercapnia acclimation (fig. 1A-C). Since pH compensation under hypercapnia is achieved by a direct or indirect net accumulation of bicarbonate and an equimolar decrease of Cl^- ions (Perry, 1982; Toews *et al.*, 1983; Larsen *et al.*, 1997; Michaelidis *et al.*, 2007), a role for the bicarbonate transporters in acid-base regulation seems likely (Heisler, 1993). So far, the Na^+/HCO_3^--cotransporter (NBC) and Cl^-/HCO_3^--exchanger (AE), both belonging to the bicarbonate transporter family SLC4 (solute carrier 4), has only been characterized in freshwater fish gills (Wilson *et al.*, 2000; Hirata *et al.*, 2003; Perry *et al.*, 2003a).

Interestingly, while NBC1 mRNA level was increased short-term after external acidification in freshwater *Tribolodon hakonensis* (Hirata *et al.*, 2003) and under environmental hypercapnia in *Oncorhynchus mykiss* (Perry *et al.*, 2003a), it transiently decreased in *Z. viviparus* by 50 % during the first 24 hours of hypercapnia and increased thereafter up to threefold by the end of the acclimation period of six weeks (publication I, fig. 1C). In contrast, mRNA expression of AE1 was only slightly affected: it tended to decrease after 4 days of hypercapnia, but returned thereafter and remained at control levels before the end of the trial (publication I, fig. 1B). Two isoforms of the Na^+/H^+-exchanger 1 (NHE1A and NHE1B) showed a similar regulation pattern: Their mRNA level was decreased by about 50 % during the first days of hypercapnia and

returned to control levels before the end of the trial with about tenfold higher abundance of NHE1A compared to isoform 1B (publication I, fig. 1A). Additionally, the protein content of NHE1 was measured by immunoblotting (probably representing the more abundant isoform 1A) and reflected the change in mRNA levels (publication I, fig. 3A).

On the basis of the differential gene regulation pattern, a working model for acclimation of gill ion transporters under hypercapnia was elaborated in accordance with the mechanisms generally found for marine fish (publication I, fig. 6). Even though changes in protein levels may be less pronounced than responses of mRNA expression to hypercapnia, the shifts in mRNA levels found here for AE1 and NBC1 likely reflect the respective changes in protein levels, as confirmed for Na^+/K^+-ATPase and NHE1. Acclimation takes place in a biphasic process. In the acute response phase, which probably sets in within minutes, pH compensation is achieved by non-bicarbonate buffering and stimulates ion exchange by the existing transporter machinery. If bicarbonate accumulates in the epithelial cells, the need for HCO_3^- transport into the plasma (by NBC1) and the hydration of CO_2 and dissociation into protons and bicarbonate (by carbonic anhydrase) may be reduced. This would minimize the production of H^+ in the cells and therefore the exportation rates into the plasma (by basolateral NHE1).

These initial pH compensation processes, which are usually completed within 10 - 24 hours, may trigger the transient downregulation of NBC1 (after 8 h) and NHE1 (after 24 hours). Net H^+ extrusion by apically located transporters [e.g. NHE2/3, reviewed by (Claiborne *et al.*, 2002; Evans *et al.*, 2005)] has to be postulated to explain the recovery of pH. Indeed, under short-term hypercapnia (1 - 24 hours, 10,000 ppm CO_2), increased mRNA levels of NHE2 have been demonstrated in the gills of freshwater *Fundulus heteroclitus* (Edwards *et al.*, 2005) and *O. mykiss* (Ivanis *et al.*, 2008). NHE2 was also found upregulated in the differential gene expression study of *Z. viviparus* gill tissue after 24 hours of hypercapnia (publication III). As an alternative to Na^+/H^+-exchange, apical proton release can be achieved by a V-type H^+-ATPase which is electrochemically coupled to a Na^+-channel [e.g. (Lin & Randall, 1991; Fenwick *et al.*, 1999)]. Although this ATPase seems to be less important for seawater fish, where Na^+/H^+-exchangers are favored by the inward gradient of Na^+ ions, two subunits of H^+-ATPase were identified among the upregulated genes in publication III, indicating a possible role of this enzyme for hypercapnia regulation.

After completion of pH recovery, rearrangements of the ion transporter protein inventory seem likely to occur in the acclimatory phase. Within this period, short-term downregulation of AE1 was observed (after 4 days). To date, comparable data for the role of AE1 in fish gills is sparse. According to Wilson and co-workers (2000), it is positioned at the apical side in our proposed working model, where its decrease would cause lower Cl^- influx and

HCO_3^- efflux rates, supporting net bicarbonate accumulation and loss of chloride ions. Assuming that AE1 would be located in the basolateral membrane, it might actively contribute to the accumulation of bicarbonate and maintenance of its concentration in the blood by higher transport activity. But as its expression level was not upregulatd under hypercapnia, such a role for this transporter seems very unlikely, and the results of the present thesis are in line with its apical location.

On the contrary, NBC1 mRNA levels increased threefold after 6 weeks of hypercapnia, indicating a long-term regulatory role for this transporter. Here, increased transporter protein abundance - as indicated by elevated mRNA levels - would support the maintenance of elevated bicarbonate levels under new steady state conditions. Na^+/K^+-ATPase serves as motor for generating the new ion balance. Accordingly, the function of this enzyme was maintained from the beginning and progressively upregulated from day 2 onward up to 6 weeks of hypercapnia on mRNA, protein and enzyme activity levels.

Ion and pH regulation in the gills - as a common focus of all projects in this thesis - was also represented in the differential gene regulation pattern in *Z. viviparus* in publication III. In agreement with the results from publication I, an α-subunit of the Na^+/K^+-ATPase was found in the upregulated library (Figure 5-1). This subunit probably represents a fragment of the same transcript that was used for mRNA quantification by real-time PCR. Additionally, two separate isoforms of the Na^+/K^+-ATPase β-subunit were found (β_1 up- and β_{233} downregulated). Within the Na^+/K^+-ATPase complex, the β-subunits accomplish regulatory functions, facilitating proper ATPase and transport functions (Scheiner-Bobis, 2002; Liu & Askari, 2006). Together with the upregulation of the α-subunit, the utilization of different β-subunit isoforms (β_1 instead of β_{233}) seems to further modify the function of the Na^+/K^+-ATPase. Although the mRNA levels of the other ion transporters studied in publication I were transiently downregulated during the early acclimation phase, their gene transcripts have not been found in the downregulated library. This may be due to a higher sensitivity of the real-time PCR assay compared to the SSH technique, or to the unavoidable "gaps" in the trancscriptome study. Nevertheless, the postulated need for proton export by isoforms 2/3 of the Na^+/H^+-exchanger (NHE) has been confirmed by the finding of genes coding for NHE2 and two subunits of V-type H^+-ATPases in the upregulated library (see above). Furthermore, the process of chloride extrusion as an electroneutral compensation for the accumulation of bicarbonate during pH recovery may be facilitated by the upregulation of two genes coding for different chloride channels.

For a functional hypothetical model describing the (early) response of hypercapnia exposed fish gills, the involvement of carbonic anhydrases seems to be necessary. Accordingly, several separate clones for carbonic anhydrase (CA) genes have been identified, including at least two up-

and three downregulated isoforms (Figure 5-1). As no full-length transcripts exist, their classification is difficult, but similarities were found with human CA II and IV isoforms and with a eukaryotic-type carbonic anhydrase domain (VB-like). The characterization of these isoforms will only be possible by sequence analysis of the complete genes, but the presence of the carbonic anhydrases in the differentially regulated libraries shows the importance of these enzymes for the compensation of the additional CO_2 load under hypercapnia.

The regulatory pattern demonstrated in the eelpout gills may be assigned to marine teleost fish gills during hypercapnia response in general. Considering the shifts in the branchial energy budgets towards higher Na^+/K^+-ATPase activity and generally higher protein and RNA biosynthesis rates, at least the data for the Antarctic notothenioid fish are confirming this model.

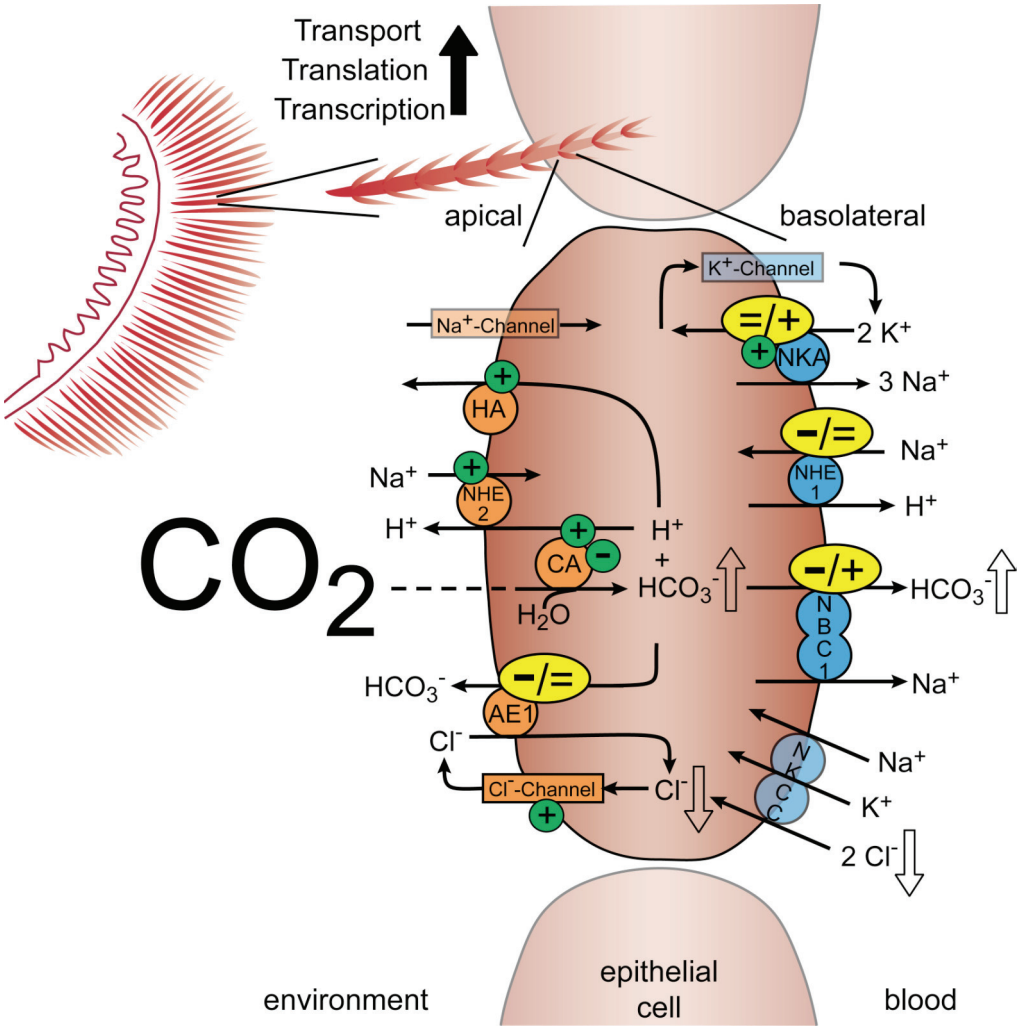

Figure 5-1: Working model for ion transport regulation under hypercapnia
The model summarizes the results of the present thesis. It is based on the proposed model in publication I (yellow, early regulatory/long-term acclimatory phase) and expanded by the transcriptome analysis of publication III (green, early regulatory phase). The black arrow at the top symbolizes the overall increased transport, translation and transcription activity demonstrated in publication II. Hollow arrows indicate changes in ion concentration levels. mRNA levels of the respective transporters are depicted as upregulated (+), downregulated (-) or unchanged (=). Abbreviations of enzymes: **HA:** H^+-ATPase, **NHE1/2**: Na^+/H^+-exchanger isoforms ½, **CA**: Carbonic anhydrase, **AE1**: Cl^-/HCO_3^--exchanger, **NKA**: Na^+/K^+-ATPase, **NBC1**: Na^+/HCO_3^--cotransporter, **NKCC**: $Na^+/K^+/2Cl^-$-cotransporter.

5.4 Tanscriptomic analyses

At this point, the question remains open which processes other than ion transport are involved in the hypercapnia response, and how they might be modified. First answers to this question were presented in publication III and will be discussed in the following. Analysis of the differentially expressed sequence tags (ESTs) from eelpout gill samples after 24 hours of hypercapnia (publication III) revealed gene responses from various cellular processes, including cell signaling, stress response, immune response, cellular maintenance, DNA replication and transcription, translation, protein metabolism, metabolism, mitochondrial functions and transport, as well as pH and ion regulation (see publication III, fig. 1 and Appendix 7.2 and 7.3 for the complete tables of the identified up- and downregulated genes).

In both libraries, the largest gene cluster could be assigned to cell communication and signal transduction. This was not surprising, as cellular communication - intra- as well as intercellular - is required for acclimation of the whole organism. However, the large number of differentially regulated genes in many important signal cascades (e.g. G-protein signaling, Ras- and Rho-family GTPases and mitogen-activated protein kinase (MAPK) pathway) indicates the involvement of numerous cellular functions and metabolic processes responsive to hypercapnia. Two of the upregulated signal transduction genes were clearly involved in the direct stress response [oxidative stress responsive 1a protein (Tamari *et al.*, 1999), stress-induced-phosphoprotein 1 (Honore *et al.*, 1992)]. Additionally, a number of differentially regulated heat-shock proteins and chaperones, known as general stress signals, were involved (Basu *et al.*, 2002). As part of the organism's stress or defense response, glutathione metabolism was enhanced; glutathione is an antioxidant important for the detoxification of reactive oxygen species. Furthermore, several immune related genes were identified in the two libraries, including major histocompatibility complex (MHC) proteins, antigens for B- and T-cell activation and members of the innate immune system. In addition to the apparent defense reactions against the environmental stressor, many genes involved in regular cell maintenance functions were modified at the transcriptional level, for example in cell adhesion, cytoskeletal organization and cell development and proliferation. Apoptosis seemed to be repressed under the given conditions through the downregulation of several caspase genes and apoptosis inductors. Possibly, the equilibrium between cell mitosis and cell death had to be readjusted under hypercapnia.

The acclimation process also became manifest in several metabolic pathways and seemed to cause shifts in metabolic fluxes. Lipid degradation and β-oxidation seemed to overweigh fatty acid biosynthesis. Apparently, the generated Acetyl-CoA (the end product of β-oxidation) was used for ketone body synthesis, while its usual conversion within the tricarboxylic acid (TCA) cycle was repressed, due to downregulation of isocitrate-dehydrogenase and the apparent shift in

carbohydrate metabolism from glycolysis to gluconeogenesis (see publication III, fig. 2). Although in fish gills gluconeogenesis is not measurable under normal conditions, it may be necessary during stress response for activation of the pentose phosphate shunt, which provides NADPH and ribuloses for RNA/DNA synthesis (Mommsen, 1984). In line with this, 6-phosphogluconolactonase (the second step in the pentose phosphate shunt) was upregulated in the eelpout gills. Overall, the sum of key metabolic enzymes found in both libraries indicates shifts in metabolite fluxes towards biosynthetic rather than catabolic processes during acclimation to hypercapnia.

Additionally, genes for the individual complexes of oxidative phosphorylation in mitochondrial energy metabolism were found differentially expressed. In this gene cluster, the observed regulation pattern revealed no clear shift towards up- or downregulation, probably due to the complex regulation of nuclear and mitochondrial encoded genes. However, subunit V of cytochrome C oxidase, which is probably regulating transcription of the holoenzyme (Fukuda *et al.*, 2007), was found downregulated. As cytochrome C oxidase (complex IX) is thought to be the rate-limiting step for the respiratory chain, this may indicate an overall downregulation of mitochondrial function. In line with this, a shift from aerobic to anaerobic metabolism was reported from functional enzymatic studies in muscle tissue of *Sparus aurata* (Michaelidis *et al.*, 2007) and *Z. viviparus* (Penno, 2006) during hypercapnia acclimation. However, this effect was not confirmed for gill tissue (Penno, 2006), and respiration data from isolated gills also showed a constant energy consumption rate under hypercapnia (publication II, Additional Results). Therefore, the present gene regulation pattern rather indicates a fine-tuning of the mitochondrial function at unchanged total capacities, which involves differential gene expression of individual subunits and/or isoforms of the single respiratory chain complexes.

A higher energy demand for translation and probably transcription under hypercapnia acclimation was postulated for gills of Antarctic notothenioids in publication II. The differential gene expression pattern in the eelpout gills support these findings, as the genes involved in the transcriptional processes per se represented the second biggest gene cluster in both, the up- and downregulated library (publication III, fig. 1). The large number of genes involved in transcriptional regulation (transcription factors, RNA/DNA binding motifs and zinc finger proteins) as well as RNA processing and splicing revealed a high transcription activity and regulation of the transcription apparatus itself during the early phase of hypercapnia acclimation. Similarly, a high translational activity became evident in protein metabolism, indicated by a number of differentially expressed translation initiation factors and ribosomal proteins. Furthermore, genes involved in post-translational protein modification and localization as well as protein-degradation (ubiquitination enzymes, proteasomes and proteases) were identified.

Overall, the modifications in RNA and protein metabolism illustrate the higher energy demand for these processes in the notothenioid gills. They also confirm upregulation of the ion transporters in the eelpout gills through higher transcriptional and translational activity of the respective genes. According to this it can be assumed that the differential genetic regulation for the other processes described above also become manifest at the functional level.

5.5 Conclusions & perspectives

At first glance, marine fishes seemed to be perfectly capable of dealing with high CO_2 concentrations, since neither visible stress symptoms such as unusual activity or opercular flare nor any disturbance in their metabolic rates were detectable at organismic and tissue level. Nevertheless, a closer analysis of the fishes' gills revealed a large number of energetic, cellular and genomic modifications during hypercapnia acclimation.

Significant shifts in the energy budget of isolated gills from the Antarctic notothenioids occurred with increasing energy demands for ion regulation, translation and transcription under elevated CO_2 concentrations. Full external pH compensation was mimicked by the experimental design, thus the higher energy demand in the isolated gill system most likely represents the early regulatory phase of the time series defined in publication I. A shift in energy allocation towards ion regulation seemed to be required for establishing the new ion equilibrium in the gills. Accordingly, capacities of Na^+/K^+-ATPase in common eelpout gills were found elevated under hypercapnia within the same time frame and also long-term, when a new steady state can be assumed. These findings indicate a common principle of hypercapnia response in marine fish. Therefore, similar shifts to those found in the Antarctic fish seem likely in the eelpout gill energy turnover, although they could not be experimentally assessed due to methodological restrictions. Moreover, assessing total enzymatic capacities and mRNA expression levels seem to be suitable tools for predicting functional tissue activities, as demonstrated for Na^+/K^+-ATPase.

The regulation of ion transport was not restricted to the sodium pump. A number of other ion transporters directly involved in proton or bicarbonate exchange have been found regulated at the transcriptional level. However, in contrast to Na^+/K^+-ATPase, these enzymes were all transporters that depend on ion gradients and cannot directly utilize ATP to increase their transport activity. Although post-translational modifications of these molecules may help in adapting their transport rates (e.g. by conformational changes, which can enable or disable entry of ions into the transport channel), their regulation by alternating mRNA content and finally protein number is likely. For Na^+/H^+-exchanger (NHE1), the correlation between transcript and protein level has been confirmed, and similarly, mRNA expression indicates the respective changes in protein number of Cl^-/HCO_3^--exchanger (AE1) and Na^+/HCO_3^--cotransporter

(NBC1). The genetic identification and characterization of these bicarbonate transporters in the present thesis confirmed their presence and role for hypercapnia acclimation in gills of marine teleost fish for the first time.

The working model for hypercapnia acclimation (presented in publication I) was expanded by the differential gene expression analysis in the eelpout gills in the early regulatory phase (publication III), where the apical Na^+/H^+-exchanger isoform 2 (NHE2) and two V-type H^+-ATPase (HA) isoforms, as well as Cl^- channels have been found upregulated after 24 hours of hypercapnia. Furthermore, differentially expressed gene sequences of carbonic anhydrase (CA) have been identified. The apparent adjustment of carbonic anhydrases by individually expressed isoforms stresses the importance of these enzymes for the hypercapnic response. Together, the observed changes at the transporter/enzyme level seem to aid pH recovery and the adjustment of HCO_3^- and Cl^- concentrations. At the presumed new steady state after six weeks, higher Na^+/K^+-ATPase (NKA) and NBC1 levels were necessary to meet the requirements of the changed ion and acid-base equilibrium, while AE1 and NHE1 were back at control levels (Figure 5-1).

Certainly, the acclimation process was not limited to ion and acid-base regulation. The transcriptome study revealed a number of other processes in the gills that are modified under hypercapnia. The direct stress response proteins, as well as the high number of immune defense related proteins were indicating a general stress state of the gill tissue. The expression pattern also demonstrated enhanced activity for cell communication and maintenance, transcription, translation and protein degradation as well as shifts in metabolic pathways. Within mitochondria, the gene expression pattern of enzyme complexes of the respiratory chain indicated functional adjustments rather than a directed up- or downregulation, representing an unchanged overall ATP production. This is in line with the stable oxygen consumption rates of the isolated gills under hypercapnia in this study and is confirmed by the unchanged mitochondrial capacities found in the gills of *Z. viviparus* after 14 days of hyercapnia (Penno, 2006). However, for the metabolic fluxes in carbohydrate and lipid pathways the picture was different. Gluconeogenesis was favored over glycolysis, probably to fuel the pentose phosphate shunt. The generation of NADPH and RNA/DNA ribose components by this pathway may support the increased transcription activity, as well as general biosyntheses of metabolites that rely on NADPH. Additionally, tricarboxylic acid cycle seemed downregulated, leading to a reduction of NADH production. Certainly, these processes still need to be confirmed by functional studies (e.g. by evaluating the enzymatic activities and measuring concentrations of the corresponding metabolic products). Nevertheless, the metabolic shifts towards biosynthetic rather than catabolic pathways at unchanged mitochondrial capacities are in line with the unchanged oxygen consumption in isolated gills during the early regulatory phase of hypercapnia acclimation of notothenioids and

eelpouts. As the shifts towards gluconeogenesis and pentose phosphate shunt consumes rather than produces energy, elevated mitochondrial efficiencies (e.g. by reducing the proton leak) have to be postulated. Other processes, which would occur under normal, well-fed conditions (e.g. lipid synthesis), may also be reduced. The observed modifications of the energy and metabolic turnover are probably not limited to the gills. Shifts in energy metabolism of muscle tissues from aerobic to anaerobic energy production have been reported after 2 to 3 weeks of hypercapnia acclimation in marine eelpout and seabream (Penno, 2006; Michaelidis *et al.*, 2007). A permanent redistribution of the animal's total energy budget may thus be necessary under long-term elevated CO_2 concentrations.

It remains to be examined in future studies, whether stenothermal polar fishes respond more strongly to hypercapnia than eurythermal fish. With respect to the increased energy demand for ion regulation and underlying translational and transcriptional activity, the data sets from temperate eelpout and Antarctic notothenioid gills indicate a common picture. However, a direct comparison of the energy budgets was not possible, as the eelpout gills turned out to be less suitable for isolated gill respiration measurements (due to their small size). Another fish model will have to be used to assess the gill energy allocation under hypercapnia in eurythermal species. For example, an adequate model species with appropriate gill size would be the Atlantic cod *Gadus morhua*, which has already been studied intensively with regard to environmental changes (Johansen & Pettersson, 1981; Larsen *et al.*, 1997; Pörtner *et al.*, 2001; Lucassen *et al.*, 2006). In addition to this, gill Na^+/K^+-ATPase activity and protein level were found increased by 100 % under long-term hypercapnia acclimation in this species [6,000 ppm CO_2 (Melzner *et al.*, 2008)].

Meaningful comparative studies rely on suitable model organisms. In this respect comparison of the present data of common eelpout should be extended to the confamilial Antarctic eelpout *P. brachycephalum*. This is of particular interest as whole animal respiration measurements had originally implied a more pronounced reaction of *P. brachycephalum* to hypercapnia, indicated by increased whole animal metabolic rates (Burgard, 2004). Moreover, the genetic similarity of these confamilial eelpout species is very high (96 - 99 % DNA sequence identity), such that most of the mRNA and protein quantification studies developed for *Z. viviparus* can be directly transferred to *P. brachycephalum*. Therefore, the enormous set of cDNA sequences obtained by the differential gene expression study in *Z. viviparus* can be used for transcriptional studies in both eelpout species. These genes can be used for completing the model of gill ion regulation under hypercapnia in marine fishes and reveal possible differences between species inhabiting stable versus fluctuating environmental conditions. Furthermore, the transcriptional studies of the new identified processes and genes involved in the hypercapnia response in the gills could be expanded to other tissues and different environmental stressors to

identify gene clusters and regulatory networks, which define the acclimation capacity and sensitivity of an organism. Moreover, the candidate genes found in fish may also serve as reliable markers for CO_2 sensitive processes in other marine (invertebrate) species, with probably lower regulatory capacities.

It must be considered that the CO_2 concentration of 10,000 ppm used consistently in this thesis is much higher than the expected environmental concentrations of about 1,000 ppm by the end of this century and also the maximal value of 1,900 ppm by the year 2300 (IPCC, 2007). However, significantly higher concentrations than 1,900 ppm occur at least locally at sites with volcanic activity in the deep-sea or in some shallow water habitats. Near carbon storage sites extremely high CO_2 concentrations can be expected as well. However, due to the unratable risks of ocean storage for the marine environment, it has been prohibited at least for the North-East Atlantic by the OSPAR commission (http://www.ospar.org).

The present thesis was designed as a pilot study providing essential mechanisms of hypercapnia responses for the first time in marine fish at molecular to organismic level, including the identification and characterization of bicarbonate transporters, the energy budgeting in isolated gills and the construction of differential cDNA libraries. The regulatory processes enabling acclimation to elevated CO_2 concentrations will be based on the same mechanisms, albeit that the operating expense will depend on the magnitude of the disturbance. On the basis of this thesis further studies on the key processes will have to reveal to what extent hypercapnia acclimation will affect the fishes under realistic scenarios. In addition, marine ecosystems are challenged by both ocean acidification and climate warming. The combined effects of several stressors may reduce the animals' tolerance windows (Pörtner & Farrell, 2008) and the resilience they would otherwise display against one stressor alone. Current studies indicate that common molecular mechanisms of stress responses may explain these synergistic effects. Experiments on the combined effects of CO_2 stress at elevated temperature are necessary for an understanding of the impacts of global change on marine fauna. Indeed, in studies on the crab *Cancer pagurus* the impact of hypercapnia led to a narrowing of the temperature tolerance window (Metzger *et al.*, 2007). The data presented for fish in this thesis provide a useful framework for future investigations.

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7 Appendix

7.1 Primer list used for sequencing of NBC1 and AE1

(Supplementary table 1 of publication I)

Fragment	Term	Sequence	Position	Length
NBC1-1	NBC1-F6	ACARGAGATGGAGTGGAAGGARAC	688-710	408
	NBC1-B13	TCGTTGAGGCTGTTGGAGGTYAGG	1095-1071	
NBC1-2	NBC1-F14	AGACCAAGAAGTCCAACCTGCG	959-980	1029
	NBC1-B33	TGAGTTTGTGCTCCTTCCTGTT	1987-1963	
NBC1-3	NBC1-F40	GCAGGGTGTGTTGGAGAGTTTC	1812-1833	586
	NBC1-B47	GTAGGTCCCCAGGAAAAGGATG	2397-2376	
NBC1-4	NBC1-F55	GTTATCTTCTGTGGWGTGGAYGCC	2499-2517	551
	NBC1-B60	ACTGGACRCCRTTGAGAGAKGC	3049-3028	
NBC1-5	NBC1-F60	GSTACCAYYTGGACCTGTTCTGG	2738-2760	454
	NBC1-B65	GACTTGAGGAYCCAYAGRAGGGC	3191-3169	
NBC1-6	NBC1-F02	CYGTCATCTCCATCGCCCACAT	2819-2840	532
	NBC1-B01	TTCTTCTTCYTCTTCTCATCCTCC	3350-3327	
NBC1-7	NBC1-F1n	TGTCTGCGATGAAGGGGAAGTG	393-414	696
	NBC1-B2n	AGGCTGTTGGAGGTGAGGTTTC	1088-1067	
NBC1-8	NBC1-F2n	TTTTTGATGTCGCCCTGCTTAC	2228-2249	498
	NBC1-B8n	TGAGTTTGTGCTCCTTCCTGTT	2725-2703	
NBC1-9	NBC1-F14	AGACCAAGAAGTCCAACCTGCG	959-980	1066
	NBC1-B1u	CCGTATTCTGTCTGGTGCTGGT	2024-2003	
NBC1-3'	NBC1-RACE-F10	GTATCTTTGTGTTCCTCCTGAC	2924-2945	
	NBC1-RACE-F14	CACTCAACGGTGTCCAGTTC	3032-3015	
NBC1-5'	NBC1-RACE-B1	TTCTTGGTCTGGTGGCGATGCTTC	724-754	
	NBC1-RACE-B2	CCTCCACCTTCTCCTCAAACTT	945-968	
AE1-1	AE1-F4	CCAAYGCCAMAACMAGAGGG	329-348	988
	AE1-B10	CATTGCCACGATGAGAACCAC	1316-1297	
AE1-2	AE1-F19	GCAGCATTGTGATTCCTCCGAC	1379-1398	507
	AE1-B24	ATGACRGCAGCMAGRACCTG	1885-1863	
AE1-3	AE1-F19	CGGARMAMATGATGGGCGTG	1379-1398	800
	AE1-B34	ACATRGARAGCAGGGCRGTGTTG	2197-2175	
AE1-4	AE1-F38	CGGARMAMATGATGGGCGTG	2088-2109	633
	AE1-B47	GGAAGATGAGGATGARGACMAGC	2812-2790	
AE1-5	AE1-F01	CAATGCSAGRGGMTGGTTTATC	1052-1031	557
	AE1-B01	MRGCRTCCAGACATTTCATTTCC	1608-1588	

The positions of the primers and probes correspond to the following genes:
AE1: GenBank EU552535; NBC1: GenBank EU552533.

7.2 List of upregulated genes in eelpout gills under hypercapnia

(Supplementary table 1 of publication III)

c is the number of contigs and **n** is the number of clones with sequences from the same hit.

Homology to known genes	Organism	Accession no./ RefSeq identifier	E-value	c	n
Ion homeostasis					
similar to Mid-1-related chloride channel 1	*Danio rerio*	XM_690250.3	4E-53	1	1
chloride intracellular channel a	*Danio rerio*	NM_199524.1	3E-58	1	3
Na^+/K^+ ATPase, beta subunit isoform 1b	*Danio rerio*	NM_131671.1	9E-08	1	2
Na^+/K^+ ATPase, alpha subunit isoform 1b	*Oncorhynchus mykiss*	NM_001124460.1	2E-54	1	1
NHE Na^+/H^+ exchanger isoform 2	*Oncorhynchus mykiss*	EF446605.2	7E-55	1	1
V-type H^+ ATPase, lysosomal V0 protein A3 (T-cell, immune regulator 1)	*Xenopus tropicalis*	BC090359.1	8E-52	1	1
V-type H^+ ATPase B subunit	*Oncorhynchus mykiss*	NM_001124597.1	1E-85	1	1
Carbonic anhydrases					
carbonic anhydrase (human CA IV-like)	*Dicentrarchus labrax*	AJ854106.1	2E-52	2	2
carbonic anhydrase IV-like, fragment	*Danio rerio*	EF591981.1	2E-45	3	3
Stress response					
Signalt transduction					
oxidative-stress responsive 1a (Ser/Thr protein kinase)	*Danio rerio*	NM_001098747.1	3E-07	1	1
stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	*Danio rerio*	NM_001007766.1	1E-18	1	1
Heat shock proteins					
DnaJ (Hsp40) homolog, subfamily B, member 1	*Danio rerio*	NM_001003571.1	7E-80	1	1
DnaJ (Hsp40) b11 protein	*Danio rerio*	BC066411.1	1E-42	1	2
heat shock 70kDa protein 8	*Danio rerio*	BX120005.6	2E-54	1	1
heat shock 70kDa protein binding protein	*Homo sapiens*	NM_003932.3	3E-65	1	1
heat shock cognate 70kDa protein	*Oncorhynchus mykiss*	P08108	1E-123	1	2
heat shock protein 90kDa alpha 2	*Danio rerio*	BC154423.1	2E-06	1	1
heat shock protein 90kDa alpha (cytosolic), class B member 1	*Danio rerio*	CU075928.10	1E-35	1	1
Cell detoxification against reactive oxygen species					
glutathione peroxidase 3 isoform 1	*Mus musculus*	NM_001083929.1	1E-50	1	1
glutathione S-transferase, alpha-like	*Danio rerio*	NM_213394.1	2E-28	1	1
Signal transduction, cell communication					
Regulation of cell cycle, development					
abl-interactor 1	*Danio rerio*	NM_200738.1	1E-06	1	1
B-cell translocation gene 1	*Danio rerio*	NM_200020.1	4E-63	1	1
carcinoembryonic antigen-related cell adhesion molecule 1	*Danio rerio*	NM_001113794.1	2E-10	1	1
casein kinase 2 alpha 1	*Danio rerio*	NM_131252.1	4E-110	1	1
similar to ceramide kinase	*Monodelphis domestica*	XM_001374991.1	9E-77	1	1
CTAGE (cutaneous T-cell lymphoma associated genes) family, member 5	*Danio rerio*	NM_200311.1	4E-21	1	1
cyclin G1	*Danio rerio*	AY423016.1	8E-88	1	1
delta C	*Danio rerio*	NM_130944.1	1E-16	1	1
similar to FtsJ (cell division protein) homolog 3	*Danio rerio*	XM_001920462.1	2E-38	1	1
hepatocyte growth factor-regulated tyrosine kinase substrate	*Danio rerio*	NM_199868.1	6E-42	1	1
similar to JAK2 (Janus kinase 2)	*Ornithorhynchus anatinus*	XM_001505887.1	4E-57	1	1
inhibitor of growth family, member 5a	*Danio rerio*	NM_198211.1	1E-13	1	1
integrin, alpha 6	*Danio rerio*	XM_001922966.1	2E-26	1	1
integrin, beta-like 1	*Danio rerio*	NM_001024072.1	1E-08	2	2
interleukin 15, like	*Danio rerio*	BC108058.1	2E-07	1	1
Mps One Binder kinase activator-like 3 isoform 3	*Homo sapiens*	NM_001100819.1	7E-102	1	1
pescadillo	*Xenopus laevis*	NM_001087088.1	2E-42	1	1
similar to plasminogen related growth factor receptor 3	*Danio rerio*	XM_001921257.1	3E-66	1	1
phospholipase D2	*Danio rerio*	XM_689557.3	7E-45	1	1
protein phosphatase 1, catalytic subunit, beta isoform-like	*Danio rerio*	NM_199916.1	1E-04	2	2
protein phosphatase 5, catalytic subunit	*Mus musculus*	CH466654.2	6E-79	1	1
protein phosphatase-2A, catalytic subunit-like	*Oreochromis mossambicus*	DQ465382.1	1E-57	1	1
probable RING-B-box-coiled coil protein	*Anguilla japonica*	AB086259.1	1E-39	2	2
Rho GDP dissociation inhibitor (GDI) alpha	*Danio rerio*	BC063968.1	3E-17	1	1
secreted immunoglobulin domain 4	*Danio rerio*	NM_001034182.2	9E-08	1	1
septin 5a	*Danio rerio*	NM_199988.1	4E-67	1	1
serine/threonine kinase a (Aurora B)	*Danio rerio*	NM_212566.1	8E-48	1	1
similar to small conductance calcium-activated potassium channel protein 3	*Danio rerio*	XM_691690.3	2E-36	1	1
sortilin 1, like	*Danio rerio*	NM_001122664.1	3E-82	1	1

tetraspanin 13	*Danio rerio*	NM_001005970.1	5E-76	1	1
thioredoxin interacting protein	*Danio rerio*	NM_200087.1	5E-76	1	1
vertebrate transmembrane 4 superfamily-like	*Danio rerio*	NM_001002748.1	5E-26	1	1
similar to vacuolar protein sorting-associated protein 8 hom.	*Danio rerio*	XM_001498641.1	6E-68	2	2
G-protein signal cascade					
amyloid beta (A4) precursor-like protein 2	*Danio rerio*	NM_001123012.1	9E-71	2	2
arrestin domain containing 2	*Danio rerio*	BC068345.1	2E-47	1	1
arrestin domain containing 3	*Danio rerio*	NM_001080029.1	2E-158	1	2
cell division cycle 42, like	*Danio rerio*	NM_199865.1	2E-101	1	1
dedicator of cytokinesis 9	*Homo sapiens*	CH471085.1	6E-121	1	1
G protein-coupled receptor 89A	*Homo sapiens*	NM_001097613.1	2E-91	1	1
similar to frizzled-8	*Bos taurus*	XM_869051.2	2E-01	2	2
Ras signaling					
novel protein similar to vertebrate Rap guanine nucleotide exchange factor 1	*Danio rerio*	CR933523.10	2E-44	1	1
RAS p21 protein activator 1	*Danio rerio*	XM_001341973.2	5E-60	1	1
RAB40c, member RAS oncogene family	*Danio rerio*	NM_001040348.1	3E-38	1	1
RAB5A, member RAS oncogene family	*Danio rerio*	NM_201485.1	7E-18	1	3
ADP-ribosylation factor-like 8	*Homo sapiens*	NM_178815.3	2E-16	1	1
Rho signaling					
similar to phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1	*Canis familiaris*	XM_543041.2	6E-01	1	1
similar to kalirin, RhoGEF kinase	*Danio rerio*	XM_689887.3	2E-80	1	2
ArhGEF7a protein	*Danio rerio*	BC095868.1	5E-24	1	1
ras homolog gene family, member T1a	*Danio rerio*	BC044431.1	7E-115	2	2
Signaling pathways					
Phosphatidylinositol signaling					
arrestin beta	*Danio rerio*	NM_214681.1	1E-51	1	1
diacylglycerol kinase alpha	*Homo sapiens*	AF064769.1	5E-29	1	3
granulin 1	*Danio rerio*	BC153543.1	2E-16	1	1
inositol (myo)-1(or 4)-monophosphatase 1	*Danio rerio*	NM_001002745.1	1E-57	1	1
inositol 1,3,4-triphosphate 5/6 kinase	*Danio rerio*	BC050497.1	1E-100	1	2
inositol 1,4,5-triphosphate receptor, type 1 iso 1	*Homo sapiens*	NM_001099952.1	9E-105	1	2
MAPK signaling					
mitogen-activated protein kinase kinase kinase 2 (MAP3K2)	*Homo sapiens*	NM_006609.3	6E-38	1	1
mitogen-activated protein kinase kinase kinase kinase 5 (MAP4K5)	*Homo sapiens*	CH471078.2	1E-11	1	1
SH2-domain-containing inositol 5-phosphatase 2a	*Danio rerio*	NM_001039804.1	2E-58	1	1
similar to inositol polyphosphate 4-phosphatase type II alpha	*Danio rerio*	XM_683397.3	7E-107	1	1
Protein kinases					
ADAM metallopeptidase domain 9	*Gallus gallus*	NM_001031396.1	6E-24	1	2
casein kinase II, beta subunit	*Mus musculus*	CH466666.2	6E-122	1	1
CDC-like kinase 2	*Danio rerio*	NM_001044879.1	3E-31	1	2
G kinase anchoring protein 1	*Danio rerio*	NM_213072.1	3E-36	1	1
phosphoinositide 3-kinase gamma	*Paralichthys olivaceus*	AY514674.1	2E-13	1	1
similar to protein kinase N2	*Danio rerio*	XM_695612.2	5E-09	1	2
similar to DCLK3 (Doublecortin- and CAM kinase-like 3)	*Danio rerio*	XM_001919282.1	3E-57	1	1
serum/glucocorticoid regulated kinase 1	*Danio rerio*	NM_199212.1	2E-37	1	1
Miscellaneous					
adiponectin receptor 2	*Danio rerio*	BC154261.1	2E-25	1	2
calmodulin 2	*Homo sapiens*	NM_001743.3	3E-79	1	1
chemokine-like factor superfamily 4 isoform 2	*Homo sapiens*	NM_181521.2	3E-11	1	1
COP9 constitutive photomorphogenic homolog subunit 4	*Danio rerio*	NM_205556.1	1E-59	2	2
ephrin A1	*Danio rerio*	NM_200783.2	8E-12	1	1
similar to GTPase IMAP family member 4 (Immunity-associated protein 4)	*Danio rerio*	XM_684195.2	2E-38	2	1
similar to GTPase, IMAP family member 9	*Danio rerio*	XM_001919559.1	4E-12	1	2
novel protein similar to vertebrate leupaxin	*Danio rerio*	BX901930.17	9E-43	1	1
protein tyrosine phosphatase, receptor type, K	*Homo sapiens*	CH471051.2	3E-94	1	2
Purinergic receptor P2X, ligand-gated ion channel	*Danio rerio*	BC162912.1	3E-34	1	1
semaphorin 4e	*Danio rerio*	NM_001083865.1	4E-17	1	1
Srl protein , sarcalumenin	*Danio rerio*	BC078198.1	2E-22	1	1
stromal membrane-associated protein 1	*Danio rerio*	NM_001077778.1	9E-124	1	1
transducin (beta)-like 2	*Danio rerio*	NM_212767.1	3E-30	1	1
translationally controlled tumor protein	*Danio rerio*	NM_198140.1	2E-30	1	2
WW domain containing adaptor with coiled-coil	*Homo sapiens*	CH471072.2	6E-01	1	1
Immune response					
butyrophilin-like 2 (MHC class II associated)	*Sus scrofa*	BX323846.2	2E-13	1	1
CD6 antigen	*Homo sapiens*	CH471076.1	1E-06	1	1
similar to CD48 antigen precursor (B-lymphocyte activation marker BLAST-1)	*Danio rerio*	XM_695682.3	1E-14	1	1
CD247 antigen like	*Danio rerio*	NM_001100107.1	5E-08	1	1
similar to CC chemokine SCYA103	*Danio rerio*	AB331767.1	2E-18	1	2

chemokine CK-1 precursor	*Onchorynchus mykiss*	NM_001124254.1	2E-08	1	1
complement component C3	*Anarhichas minor*	AJ309570.1	5E-140	1	3
complement component 9	*Danio rerio*	NM_001024435.1	2E-60	1	2
G7c protein (major histocompatibility complex class III)	*Homo sapiens*	NM_025258.2	1E-26	1	1
similar to guanylate binding protein 1, interferon-inducible, 67kDa	*Danio rerio*	XM_686432.3	4E-08	1	1
interferon induced transmembrane protein 3 (1-8U)	*Homo sapiens*	BC008417.1	2E-15	1	1
similar to NOD3 (nodulin) protein isoform 1	*Danio rerio*	XM_001923386.1	1E-82	1	1
peptidoglycan recognition protein 5	*Danio rerio*	BC093314.1	4E-34	1	1
selenoprotein S	*Danio rerio*	NM_001045334.1	6E-46	1	1
serum amyloid A-like 1	*Danio rerio*	NM_200135.2	9E-41	1	1
similar to small inducible cytokine A21	*Danio rerio*	XM_001336492.2	1E-12	1	1
similar to TAP binding protein-like	*Danio rerio*	XM_001341626.2	1E-13	1	1
uromodulin	*Mus musculus*	CH466531.1	4E-15	1	2
similar to V-set domain containing T cell activation inhibitor 1	*Danio rerio*	XM_001338054.2	2E-03	1	1
Src-like-adaptor 2	*Mus musculus*	BC052655.1	2E-46	1	1
similar to human ZAP70, zeta-chain associated protein kinase 70kDa	*Danio rerio*	BX511253.13	4E-42	1	1
Cellular maintenance					
Cell adhesion					
claudin i	*Danio rerio*	NM_131768.2	2E-13	1	1
cadherin-15	*Homo sapiens*	D83542.1	2E-14	1	1
similar to desmoglein 2	*Danio rerio*	XM_683420.2	2E-29	1	1
similar to hyaluronic acid binding protein 2 isoform 1, partial	*Danio rerio*	XM_681117.3	2E-28	1	1
lysosomal membrane glycoprotein 2	*Danio rerio*	NM_001013533.1	1E-16	1	1
thrombospondin 1	*Danio rerio*	XM_685303.3	3E-84	1	2
Cytoskeleton, motiliy					
cytoskeletal beta actin	*Ictalurus.punctatus*	DQ417126.1	6E-33	1	1
actinin, alpha 1 isoform c	*Homo sapiens*	NM_001130005.1	2E-34	1	1
ARP3 actin-related protein 3 homolog	*Danio rerio*	NM_001003944.1	3E-51	1	1
advillin	*Rattus norvegicus*	CH473950.2	4E-29	1	1
cullin 4B	*Xenopus tropicalis*	NM_001127419.1	2E-87	1	1
type I cytokeratin	*Danio rerio*	NM_131108.1	6E-25	1	3
kinectin 1 isoform a	*Homo sapiens*	NM_182926.2	7E-56	1	1
myosin, light polypeptide 9-like	*Danio rerio*	NM_214699.1	3E-90	1	1
motile sperm domain containing 2	*Mus musculus*	AL732426.6	2E+00	1	1
similar to titin (connectin), partial	*Danio rerio*	XM_001332950.2	3E-11	2	2
tropomyosin4-1	*Takifugu rubipres*	AB090370.1	2E-83	1	1
tropomyosin1-1	*Takifugu rubipres*	NM_001032571.1	2E-60	1	1
troponin	*Danio rerio*	NM_201093.1	6E-46	1	1
Cell proliferation, development					
similar to vertebrate aggrecan 1	*Danio rerio*	XM_001339918.1	2E-21	1	1
similar to alpha-tectorin	*Danio rerio*	XM_001344182.1	1E-41	1	1
caveolin 1 isoform a	*Danio rerio*	NM_212651.1	6E-42	1	1
centromere protein Q	*Gallus gallus*	NM_001044649.1	4E-06	1	1
similar to chondroitin sulfate N-acetylgalactosaminyl-transferase 1	*Danio rerio*	XM_001333443.2	1E-75	1	1
similar to CUB and zona pellucida-like domains 1	*Danio rerio*	XM_001340473.2	4E-20	3	3
ectodermal-neural cortex (with BTB-like domain)	*Danio rerio*	BC045427.1	4E-65	1	1
epithelial membrane protein-2-like	*Ictalurus punctatus*	DQ417173.1	2E-42	1	1
fukutin	*Danio rerio*	DQ826745.1	4E-26	1	1
hypothetical protein similar to human IGSF8	*Danio rerio*	XM_001331631.1	3E-16	1	1
similar to cornifelin (placenta-specific 8) -like	*Danio rerio*	XM_001338121.1	4E-38	1	1
septin 7 a	*Danio rerio*	BC097041.1	7E-34	1	1
spint1, serine peptidase inhibitor, Kunitz type 1	*Danio rerio*	NM_001111223.1	9E-50	1	1
similar to testican 3 (proteoglycan)	*Danio rerio*	XM_685403.2	5E-28	1	2
similar to Tripartite motif-containing protein 16 isoform 1	*Danio rerio*	XM_001334829.2	4E-98	1	1
Apoptosis					
caspase-1	*E.caballus*	NM_001081842.1	4E-09	1	2
clptm1 (cleft lip and palate transmembrane protein)-like prot.	*Danio rerio*	NM_001002380.1	6E-15	1	1
deoxyribonuclease I	*Oreochromis mossambicus*	AJ001305.1	2E-53	1	1
deoxyribonuclease II	*X.laevis*	NM_001093202.1	2E-04	1	1
Apoptosis signaling					
novel NACHT domain containing protein	*Danio rerio*	BX322597.3	4E-11	1	1
protein disulfide isomerase family A, member 3	*Homo sapiens*	CH471082.1	5E-09	1	1
pycard (PYC and CARD domain containing protein)	*Danio rerio*	AF231013.1	1E-06	1	1
rock1 (Rho-associated, coiled-coil containing protein kinase 1)	*Oryctolagus cuniculus*	NM_001082367.1	2E-40	1	2
synovial apoptosis inhibitor 1, synoviolin	*Danio rerio*	BX323077.15	2E-54	1	1
Tax1 (human T-cell leukemia virus type I) binding protein 1	*Danio rerio*	BC060918.1	7E-70	1	1
toll-like receptor adaptor molecule	*Takifugu rubripes*	NM_001113194.1	6E-09	1	1
Similar to Putative TRAF4-associated factor 1	*Danio rerio*	BC163181.1	8E-03	1	1
DNA replication, repair					
DNA replication factor	*Homo sapiens*	CH471184.2	1E-05	1	1

Oligonucleotide/oligosaccharide-binding fold-containing protein 2A	*Danio rerio*	Q5PRC7	4E-50	1	2
Transcription					
RNA processing					
cDNA sequence BC003993, isoform CRA_b	*Mus musculus*	NM_030560.3	2E-12	1	1
DEAD (Asp-Glu-Ala-Asp) box polypeptide 41	*Danio rerio*	NM_201045.1	9E-124	1	1
heterogeneous nuclear ribonucleoprotein D iso d	*Homo sapiens*	NM_001003810.1	3E-58	2	2
similar to ISY1 splicing factor homolog (*S. cerevisiae*)	*Monodelphis domestica*	XM_001378384.1	4E-86	1	2
ribonuclease Ok2	*Oncorhynchus keta*	AB061717.1	5E-41	1	2
similar to synaptotagmin binding, cytoplasmic RNA interacting protein, iso 1	*Ornithorhynchus anatinus*	XM_001510053.1	2E-24	1	1
THO (tyrosine hydroxylase) complex 4	*Homo sapiens*	CH471099.1	2E-14	1	1
similar to tuftelin interacting protein 11	*Danio rerio*	XM_001923713.1	1E-89	1	1
zinc finger, matrin type 5	*Danio rerio*	NM_001003771.1	4E-44	1	1
Regulation of transcription					
ADNP (activity dependent neuroprotective protein) homeobox 2	*Danio rerio*	NM_001098265.1	3E-18	1	1
basic transcription factor 3 isoform A	*Homo sapiens*	NM_001037637.1	3E-61	2	2
capicua homolog (Drosophila)	*Homo sapiens*	CH471126.1	6E-01	1	1
CCAAT/enhancer binding protein (C/EBP), delta	*Danio rerio*	BC045282.1	3E-24	1	1
Chromodomain helicase DNA binding protein 1	*Homo sapiens*	BC117134.1	2E-106	1	1
COMM (commissureless) domain containing 7	*Homo sapiens*	CH471077.2	9E-43	1	2
DEAH (Asp-Glu-Ala-His) box polypeptide 38	*Danio rerio*	NM_200876.1	8E-38	1	2
GATA-binding protein 3	*Danio rerio*	NM_131211.1	2E-83	1	1
grainyhead-like 1	*Danio rerio*	XM_001923728.1	1E-97	1	1
histone deacetylase	*Takifugu rubripes*	AF411956.1	8E-06	1	1
histone H3-like centromeric protein A	*Danio rerio*	Q803H4	8E-23	1	1
HMG (hich mobility group) box containing protein 1	*Homo sapiens*	AF019214.1	1E-64	1	1
homeo box (H6 family) 3	*Danio rerio*	NM_131634.1	8E-30	1	1
homeodomain interacting protein kinase 2	*Danio rerio*	NM_001099985.2	1E-118	1	1
host cell factor C1	*Mus musculus*	AL672002.14	3E+00	1	1
interferon regulatory factor 1	*Channa argus*	EF067849.1	2E-105	2	2
interferon regulatory factor 11	*Danio rerio*	BC085555.1	7E-48	2	2
LAG1 longevity assurance homolog 5	*Danio rerio*	NM_199628.1	8E-47	1	2
matrin 3-like	*Danio rerio*	NM_001122784.1	1E-24	1	1
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	*Danio rerio*	XM_684887.3	2E-03	2	2
PPAR (peroxisome proliferative activated receptor), gamma, coactivator-related 1	*Mus musculus*	AK122322.1	5E-01	1	1
similar to PPAR-alpha interacting complex protein 285	*Danio rerio*	XM_694159.3	1E-26	2	2
similar to poly (ADP-ribose) polymerase family, member 15	*Macaca mulatta*	XM_001105794.1	7E-35	1	2
RNA binding motif protein 25	*Homo sapiens*	CH471061.1	1E-37	1	1
similar to tubby like protein 4	*Danio rerio*	XM_686015.2	3E-01	1	1
TBP (TATA box binding protein)-associated factor 5	*Gallus gallus*	NM_001031235.1	7E-72	1	1
TBP-like 1	*Danio rerio*	BC085661.1	5E-08	1	2
teashirt zinc finger homeobox 3	*Homo sapiens*	AK291466.1	4E-05	1	1
transcription factor pu.2	*Danio rerio*	EU685253.1	1E-15	1	2
similar to putative transcription factor, partial	*Ornithorhynchus anatinus*	XM_001514206.1	9E-11	1	1
transposase	*Pleuronectes platessa*	AJ249085.1	1E-02	1	2
zinc finger homeodomain 4	*Homo sapiens*	CH471068.1	1E+00	1	1
zinc finger protein 598	*Danio rerio*	Q6PFK1	3E-02	1	1
zinc finger protein 760	*Mus musculus*	NM_001008501.1	2E-68	1	1
zinc finger, CCHC domain containing 17	*Danio rerio*	NM_200544.1	6E-15	1	1
Translation					
eukaryotic translation initiation factor 4 (eIF4), gamma 2a	*Danio rerio*	NM_001014289.2	8E-90	1	1
eIF4G-related protein NAT1B	*Danio rerio*	AB096101.1	1E-81	1	2
similar to putative eukaryotic translation initiation factor 3 (eIF3) subunit isoform 1	*Canis familiaris*	XM_548323.2	2E-53	1	1
G-rich RNA sequence binding factor 1	*Danio rerio*	NM_001045852.1	1E-20	1	1
ribosomal protein L3	*Solea senegalensis*	AB374542.1	6E-44	1	1
ribosomal protein L4 (60S)	*Pagrus major*	AY190725.1	2E-17	1	1
ribosomal protein L6 (60S)	*Pagrus major*	AY190726.1	3E-53	1	1
ribosomal protein L15 (60S)	*Danio rerio*	BX927163.32	3E-97	1	1
ribosomal protein L17 (mitochondrial)	*Danio rerio*	NM_001004683.1	4E-07	1	1
ribosomal protein L19	*Xiphophorus hellerii*	EU163944.1	3E-30	1	1
ribosomal protein L21	*Danio rerio*	NM_001002155.1	1E-53	1	1
ribosomal protein L23	*Homo sapiens*	NM_000978.3	5E-51	1	1
ribosomal protein S13	*Danio rerio*	NM_001002079.1	1E-16	1	1
ribosomal protein S15a	*Danio rerio*	NM_212762.1	2E-67	1	1
ribosomal protein Sa-like protein (40S)	*Sparus aurata*	AY550956.1	3E-49	1	1
similar to SECIS (sec insertion sequence) binding protein 2	*Monodelphis domestica*	XM_001375665.1	7E-40	1	1
Protein metabolism					
Protein processing and localisation					
aspartate beta-hydroxylase	*Danio rerio*	BC095638.1	9E-04	1	1

cytosolic sialic acid 9-O-acetylesterase homolog	*Homo sapiens*	NM_170601.3	3E-22	1	1
furin (paired basic amino acid cleaving enzyme)	*Danio rerio*	CR382344.9	8E-60	1	1
nuclear localization signal binding protein	*Mus musculus*	S79410.1	2E-10	1	1
procollagen-lysine 2-oxoglutarate 5-dioxygenase 3	*Danio rerio*	NM_001044343.1	4E-124	1	1
protein disulfide isomerase associated 4	*Danio rerio*	BC117629.1	1E-25	1	1
ribophorin I	*Danio rerio*	BC049303.1	5E-93	1	1
similar to receptor (chemosensory) transporter protein (RTP1)	*Homo sapiens*	BC034744.1	5E+00	1	2
novel protein similar to vertebrate transglutaminase 1	*Danio rerio*	AL929335.9	5E-69	1	1
zinc finger protein 313	*Oncorhynchus mykiss*	NM_001124518.1	8E-42	1	1
Protein degradation					
ATP-dependent metalloprotease FtsH1-like 1	*Danio rerio*	NM_001089514.1	8E-54	1	1
cathepsin B, a	*Danio rerio*	NM_213336.1	3E-31	1	2
cathepsin S, b.2	*Danio rerio*	BC096862.1	2E-21	1	1
coagulation factor II	*Danio rerio*	NM_213390.1	1E-42	1	1
ER degradation enhancer, mannosidase alpha-like 1	*Danio rerio*	NM_201189.1	1E-141	1	1
novel protein similar to ER degradation enhancer, mannosidase alpha-like 3	*Danio rerio*	NM_001045109.1	7E-06	1	1
nascent polypeptide-associated complex alpha subunit	*Danio rerio*	NM_173264.1	2E-65	1	1
prostasin-like	*Danio rerio*	NM_001105601.1	1E-50	1	1
proteasome 26S non-ATPase subunit 8	*Mus musculus*	NM_026545.3	4E-67	2	3
proteasome assembly chaperone 2	*Danio rerio*	NM_213032.1	5E-23	1	1
proteasome subunit, alpha type, 2	*Danio rerio*	NM_001024441.1	2E-22	1	2
proteasome subunit, alpha type, 5	*Danio rerio*	NM_205708.1	5E-40	1	2
pyroglutamyl-peptidase I	*Homo sapiens*	NM_017712.2	2E-63	1	1
transmembrane protease, serine 4	*Danio rerio*	NM_001077738.1	1E-08	1	1
ubiquitin ligase nedd4a	*Danio rerio*	NM_001034186.1	5E-52	1	1
ubiquitin-like modifier activating enzyme 3	*Danio rerio*	NM_213467.1	4E-107	1	2
ubiquitin specific protease 53	*Homo sapiens*	NM_019050.2	7E-67	1	1
Metabolism					
Lipid metabolism					
acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	*Danio rerio*	NM_001003743.1	4E-14	1	3
apolipoprotein AI precursor	*Platichthys flesus*	AJ844288.1	1E-12	1	1
14 kDa apolipoprotein	*Perca flavescens*	EU144048.1	1E-11	2	2
similar to butyrophilin, subfamily 2, member A2	*Danio rerio*	XM_680023.3	2E-05	1	1
enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	*Danio rerio*	NM_001004529.2	1E-40	1	1
glycerol-3-phosphate dehydrogenase	*Gadus morhua*	AY635584.1	3E-55	1	1
glycoprotein, synaptic 2 (trans-2,3-enoyl-CoA reductase)	*Danio rerio*	NM_201299.1	2E-07	1	1
similar to glycerophosphodiester phosphodiesterase domain containing 2	*Danio rerio*	XM_686560.3	6E-33	1	1
2-hydroxyacyl-CoA lyase 1	*Danio rerio*	NM_213085.1	1E-65	1	1
similar to 3-hydroxybutyrate dehydrogenase, type 2, partial	*Ornithorhynchus anatinus*	XM_001507478.1	4E-24	1	1
sphingolipid delta 4 desaturase/C-4 hydroxylase 2	*Danio rerio*	XM_689103.3	8E-102	1	1
sterol carrier protein-X/sterol carrier protein-2	*Homo sapiens*	U11297.1	1E-17	1	1
triacylglycerol lipase	*Pseudopleuronectes americanus*	AF512562.1	8E-29	1	1
Carbohydrate metabolism					
aldehyde dehydrogenase	*Danio rerio*	AF254955.1	3E-13	1	2
galactokinase 1	*Danio rerio*	NM_001006002.1	3E-02	1	1
GDP-mannose 4,6-dehydratase isoform	*Danio rerio*	NM_001102475.1	5E-49	1	1
glucose phosphate isomerase a	*Danio rerio*	BC083507.1	8E-41	1	1
novel protein similar to vertebrate hexosaminidase A (HEXA)	*Danio rerio*	BX571730.9	2E-06	1	1
mitochondrial phosphoenolpyruvate carboxykinase (PEPCK)	*Homo sapiens*	X92720.1	4E-01	1	1
novel protein similar to pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4)	*Danio rerio*	BX510640.4	5E-27	1	1
phosphorylase kinase, gamma 2	*Homo sapiens*	CH471192.1	2E-51	1	2
6-phosphogluconolactonase	*Homo sapiens*	NM_012088.2	2E-50	1	1
Miscellaneous					
asparagine-linked glycosylation 3 homolog (alpha-1,3-mannosyltransferase)	*Danio rerio*	NM_001020696.1	3E-17	1	2
aspartate aminotransferase, mitochondrial	*Oncorhynchus tshawytscha*	EF042601.1	5E-08	2	2
ATPase family, AAA domain containing 1b	*Danio rerio*	AL731788.8	4E-87	1	1
cysteine dioxygenase, type I	*Danio rerio*	NM_200741.1	7E-14	1	1
cytidine deaminase	*Danio rerio*	NM_205679.1	8E-39	1	2
cytochrome P450 2K5 (human 2C19)	*Oncorhynchus mykiss*	NM_001124742.1	2E-27	1	1
dihydrolipoamide branched chain transacylase E2	*Danio rerio*	NM_001013515.1	7E-24	2	2
dihydropyrimidine dehydrogenase	*Danio rerio*	NM_212893.1	2E-85	1	1
similar to HECT domain (Homologous to E6- ass. prot. C-Term.) containing 1	*Ornithorhynchus anatinus*	XM_001512255.1	2E-52	1	1
hydroxysteroid (17-beta) dehydrogenase 8	*Danio rerio*	NM_001005292.2	3E-76	1	2
hydroxysteroid (17-beta) dehydrogenase 14	*Homo sapiens*	NM_016246.2	9E-28	1	2
phosphatidylinositol glycan, class P	*Danio rerio*	NM_199712.1	4E-01	1	1
phosphatidylinositol glycan, class Q	*Danio rerio*	NM_199429.1	8E-29	1	2
SEC63-like protein	*Danio rerio*	NM_001002588.1	1E-93	1	1
spermidine/spermine N1-acetyl transferase 1	*Danio rerio*	NM_001093748.2	1E-05	1	1

Mitochondrial genes					
ATP synthase F0 subunit 8	*Pholis crassispina*	NC_004410.1	2E-15	1	1
ethylmalonic encephalopathy 1	*Xenopus tropicalis*	NM_001005706.1	5E-73	1	1
cytochrome c oxidase subunit III, mitochondrial	*Lycodes toyamensis*	NC_004409.1	3E-89	1	1
NADH hydrogenase subunit 2	*Neolamprologus nigriventris*	AY740392.1	2E-45	1	1
hyp. protein similar to NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2	*Danio rerio*	NM_001024420.1	7E-31	1	1
NADH dehydrogenase (ubiquinone) Fe-S protein 7 (NADH-CoQ reductase)	*Homo sapiens*	CH471139.2	8E-13	1	1
ubiquinol-cytochrome C reductase core protein II	*Danio rerio*	BC152592.1	6E-66	1	1
ubiquinol-cytochrome C reductase complex-like	*Ictalurus punctatus*	DQ407860.1	2E-27	1	1
Transport					
Ion transport					
novel protein similar to vertebrate B-box and SPRY domain containing (BSPRY)	*Danio rerio*	BX294186.9	7E-17	1	1
type II CAX cation/proton exchanger	*Danio rerio*	NM_001025507.3	5E-80	1	1
similar to P-type ATPase type 13A	*Apis mellifera*	XM_396194.3	6E+00	1	1
similar to P-type ATPase, class I, type 8B, member 1, part.	*Danio rerio*	XM_001920475.1	4E-103	1	1
similar to solute carrier family 4 (anion exchanger), member 1, adaptor protein	*Danio rerio*	XM_685743.3	5E-77	1	1
similar to transcobalamin-1 precursor	*Danio rerio*	BC162201.1	2E-17	1	1
transferrin	*Pagrus major*	AY335444.1	5E-43	2	2
Neurotransmitter					
taurine transporter	*Danio rerio*	NM_001037661.1	5E-150	1	1
solute carrier family 5 (choline transporter), member 7	*Homo sapiens*	BC111524.1	2E-01	1	2
Protein					
adaptor-related protein complex 1, sigma 2 subunit	*Homo sapiens*	CH471074.1	8E-48	1	1
archain 1	*Danio rerio*	NM_201459.1	1E-52	1	1
chromatin-modifying protein 4b	*Danio rerio*	Q7ZVC4	3E-07	1	1
chromatin modifying protein 2B	*Gallus gallus*	NM_001030792.1	3E-58	1	1
coatomer protein gamma 2-subunit	*Takifugu rubripes*	AJ251323.1	2E-27	1	1
importin 7	*Homo sapiens*	CH471064.2	7E-146	1	1
importin 9	*Danio rerio*	BC129156.1	4E-07	1	1
nucleoporin 107	*Danio rerio*	BC125812.1	2E-49	1	1
Ran-binding protein (importin) 7	*Danio rerio*	AY286403.1	5E-03	1	1
signal recognition particle receptor	*Gallus gallus*	NM_001039277.1	2E-47	1	1
Miscellaneous					
vacuolar protein sorting 4b	*Danio rerio*	NM_200906.1	1E-11	1	1
ATP-binding cassette, sub-family A (ABC1), member 12	*Danio rerio*	XM_681540.3	3E-55	1	1
solute carrier family 37 (glycerol-3-phosphate transporter), member 3	*Bos taurus*	NM_001075284.1	3E-42	1	1
metaxin 3	*Danio rerio*	BC094962.1	8E-47	1	1
similar to synaptogyrin 3	*Monodelphis domestica*	XM_001363198.1	4E-43	1	1
Rab acceptor 1 (prenylated)	*Danio rerio*	NM_214780.1	2E-21	1	1
Miscellaneous					
autophagy 3-like	*Danio rerio*	NM_200022.1	1E-47	1	1
mucin 5AC, oligomeric mucus/gel-forming	*Homo sapiens*	P98088	7E-25	1	1
reverse transcriptase-like protein	*Paralichthys olivaceus*	AY136821.1	4E-37	3	3
Unknown biological process					
anterior gradient 2 homolog	*Danio rerio*	NM_001123597.1	7E-71	1	1
similar to armadillo repeat containing 5	*Danio rerio*	XM_688555.2	3E-11	1	2
similar to bromodomain and PHD finger containing, 3	*Danio rerio*	XM_001922163.1	8E-30	1	1
similar to protein FAM (family with sequence similarity) 111A, partial	*Danio rerio*	XM_001923035.1	1E-06	1	1
Kelch-like 18 (Drosophila)	*Danio rerio*	BC071523.1	6E-84	1	1
Kelch-like 24 (Drosophila)	*Xenopus tropicalis*	NM_001078869.1	7E-83	1	1
NLR family, CARD domain containing 5	*Equus caballus*	XM_001915501.1	5E-25	1	1
similar to R3H domain (binds single-stranded nucleic acids)	*Rattus norvegicus*	XM_573442.2	1E-03	1	1
testis expressed 10	*Danio rerio*	NM_212705.1	1E-67	1	1
transmembrane emp24 protein transport domain containing 3	*Danio rerio*	NM_001128668.1	1E-11	1	1
similar to tetratricopeptide repeat domain 31	*Danio rerio*	XM_683503.3	3E-03	1	1
similar to ubiquitously transcribed tetratricopeptide repeat, X chromosome	*Danio rerio*	XM_692654.2	3E-63	1	1
similar to zymogen granule protein 16	*Macaca mulatta*	XM_001104596.1	5E-25	1	4
zinc finger protein 330	*Danio rerio*	NM_213373.1	1E-139	1	1
zinc finger, FYVE domain containing 27	*Gallus gallus*	NM_001039304.1	3E-22	1	1

7.3 List of downregulated genes in eelpout gills under hypercapnia

(Supplementary table 2 of publication III)

c is the number of contigs and **n** is the number of clones with sequences from the same hit.

Homology to known genes	Organism	Accession no./ RefSeq identifier	E-value	c	n
Ion homeostasis					
Na^+/K^+ ATPase, subunit beta-233	*Anguilla anguilla*	Q9I9C3	4E-24	1	1
Carbonic anhydrases					
carbonic anhydrase II-like	*Danio rerio*	NM_131110.1	3E-64	1	1
carbonic anhydrase IV-like, fragment	*Danio rerio*	EF591981.1	1E-25	1	3
novel protein with eukaryotic-type carbonic anhydrase domains (VB-like)	*Danio rerio*	BX005103.4	3E-11	1	1
Stress response					
Protein folding					
peptidylprolyl isomerase D (cyclophilin D)	*Danio rerio*	NM_001002065.1	1E-123	1	1
peptidylprolyl isomerase F	*Gallus gallus*	NM_001031226.1	5E-73	1	1
DnaJ (Hsp40)-like, subfamily B, member 6	*Paralichthys olivaceus*	DQ199620.1	1E-119	1	3
novel protein similar to vertebrate DnaJ (Hsp40) homolog, subfamily C, member 5	*Danio rerio*	BX511178.6	3E-73	1	1
heat shock protein 90kDa beta (glucose-regulated protein 94), member 1 (Hsp90)	*Danio rerio*	NM_198210.2	3E-129	1	1
von Hippel-Lindau binding protein 1	*Danio rerio*	NM_001020624.1	4E-24	1	3
Metabolism					
glutathione S-transferase rho	*Pagrus major*	AB158412.1	2E-78	1	1
Signal transduction, cell communication					
Regulation of cell cycle, development					
similar to abl-interactor 1	*Danio rerio*	XM_689325.3	9E-02	1	2
activin A receptor, type IB	*Danio rerio*	BC163377.1	1E-05	1	3
BRCA2 and CDKN1A interacting protein	*Danio rerio*	NM_001013475.1	2E-114	1	1
cadherin 1, epithelial	*Danio rerio*	NM_131820.1	2E-21	1	1
carcinoembryonic antigen-related cell adhesion molecule	*Danio rerio*	NM_001113794.1	3E-46	1	4
ephrin-A5	*Xenopus tropicalis*	NM_001016422.2	9E-06	1	1
similar to epigen	*Danio rerio*	XM_001344355.2	7E-14	1	1
growth factor, augmenter of liver regeneration	*Danio rerio*	NM_001089386.1	2E-07	1	2
latent transforming growth factor beta binding protein 2	*Pan troglodytes*	XM_510064.2	5E-01	1	1
integrin alpha 7	*Mus musculus*	NM_008398.2	7E+00	1	2
integrin, beta-like 1	*Danio rerio*	NM_001024072.1	1E-13	8	8
similar to integrin beta-7 precursor	*Rattus norvegicus*	XM_343336.3	3E-01	1	2
similar to interleukin 12 receptor beta 2.b	*Danio rerio*	XM_001923820.1	4E-27	1	1
mosin, light chain kinase	*Danio rerio*	BC163913.1	2E-33	1	1
neuregulin 2	*Homo sapiens*	CH471062.2	7E-11	1	1
p21-activated kinase 2	*Danio rerio*	NM_001002717.1	2E-78	1	1
novel protein similar to vertebrate plexin C1	*Danio rerio*	BX649503.3	1E-78	1	1
similar to quiescin Q6-like 1	*Danio rerio*	XM_682961.3	9E-11	1	1
ring finger protein 7	*Xenopus tropicalis*	NM_001114261.1	6E-32	1	1
septin 8a	*Danio rerio*	NM_001115117.1	1E-43	1	1
tetraspanin 14	*Mus musculus*	NM_145928	3E-109	1	4
novel protein similar to vertebrate tetraspanin 15	*Danio rerio*	BX950860.8	5E-73	1	2
thyroid hormone receptor interactor 10	*Rattus norvegicus*	NM_053920.1	6E-13	1	1
tyrosine-3-monooxygenase/tryptophan-5-monooxygenase activator, beta polypeptide	*Homo sapiens*	NM_003404.3	4E-60	1	2
tyrosine-3-monooxygenase/tryptophan-5-monooxygenase activator, beta polypeptide-like	*Danio rerio*	BC065346.1	1E-04	1	2
G-protein signal cascade					
beta-3a-adrenergic receptor	*Oncorhynchus mykiss*	NM_001124628.1	2E-47	1	1
C-C chemokine receptor family-like	*Danio rerio*	NM_001045027.1	8E-70	1	1
similar to dedicator of cytokinesis 8	*Danio rerio*	XM_001920072.1	1E-17	1	2
similar to G protein-coupled receptor 126 beta 1	*Danio rerio*	XM_001920792.1	2E-14	1	2
similar to G protein-coupled receptor 89A	*Danio rerio*	XM_684729.3	1E+00	1	1
similar to Probable G-protein coupled receptor 52	*Rattus norvegicus*	XM_001069712.1	1E+00	1	1
melanocortin 1 receptor	*Danio rerio*	NM_180970.1	8E+00	1	1
similar to Proteinase-activated receptor 3 precursor (PAR-3) (Thrombin receptor-like 2)	*Danio rerio*	XM_001340652.2	6E+00	1	1
similar to Regulator of G-protein signaling 5 (RGS5)	*Danio rerio*	XM_685411.3	9E-48	1	2
Ras signaling					
DENN/MADD domain containing 2D	*Homo sapiens*	CH471122.1	7E-13	2	2

fibroblast growth factor 2	*Danio rerio*	NM_212823.1	3E-03	1	1
novel protein similar to vertebrate TBC1 domain family, member 15 (TBC1D15)	*Danio rerio*	BX950194.9	3E-93	1	2
RAB1A, member RAS oncogene family	*Homo sapiens*	CH471053.2	2E-69	1	1
RAP1B, member of RAS oncogene family	*Danio rerio*	AY423018.1	3E-32	1	1
RasGEF domain family, member 1	*Homo sapiens*	BC121003.1	4E-05	1	1
Ras-related GTP binding C	*Danio rerio*	BC154154.1	1E-26	1	1
similar to Rap1 guanine-nucleotide exchange factor	*Danio rerio*	XM_001343204.1	2E-100	1	1
similar to RUN and TBC1 domain containing 1	*Gallus gallus*	XM_415919.2	5E-01	1	1
similar to TBC1 domain family, member 8 iso 2	*Bos taurus*	XM_864858.3	1E+00	1	1
similar to unc-51-like kinase 1	*Danio rerio*	XM_001922196.1	5E-36	1	1
Rho signaling					
similar to deleted in liver cancer 1	*Danio rerio*	XM_001921739.1	4E-39	1	1
FYVE, RhoGEF and PH domain containing 6	*Homo sapiens*	CH471054.1	8E-71	1	2
similar to rac/cdc42 guanine nucleotide exchange factor 6	*Danio rerio*	XM_681489.3	2E-46	1	1
ras homolog gene family, member Ad	*Danio rerio*	NM_001002445.1	2E-100	1	1
small GTPase RhoA	*Danio rerio*	NM_212749.2	7E-106	1	2
Rho GTPase activating protein 1	*Danio rerio*	NM_001017781.1	7E-18	1	1
similar to Rho GTPase-activating protein 27	*Danio rerio*	XM_689796.2	6E-48	1	1
Signaling pathways					
Phosphatidylinositol signaling					
phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide	*Mus musculus*	CH466572.1	3E-04	1	1
similar to p120-PI3K	*Danio rerio*	XM_687605.2	2E-92	1	1
similar to Phosphatidylinositol-4-phosphate 5-kinase-like protein 1	*Danio rerio*	XM_001923957.1	4E-27	1	1
phosphatidylinositol-specific phospholipase C, X domain containing 3	*Bos taurus*	NM_001103304.1	2E-02	1	3
MAPK signaling					
similar to Mitogen-activated protein kinase kinase kinase kinase 3 (MEK kinase kinase 3)	*Danio rerio*	XM_001921600.1	5E-12	1	1
mitogen-activated protein kinase kinase kinase 8	*Homo sapiens*	NM_005204.2	1E+00	1	1
NFkB signaling					
Nedd4 family interacting protein	*Oncorhynchus mykiss*	AJ544264.1	1E-78	1	2
mitochondrial ubiquitin ligase activator of NFKB 1	*Homo sapiens*	NM_024544.2	5E-06	1	2
Notch signaling					
deltex2	*Danio rerio*	NM_001127307.1	3E-18		3
notchless homolog 1	*Danio rerio*	NM_001020582.1	1E-38		1
notch homolog 3 variant	*Homo sapiens*	AB209447.1	2E-01		1
Protein kinases					
protein kinase C substrate 80K-H	*Danio rerio*	NM_201053.1	3E-73	1	1
family with sequence similarity 62 (C2 domain containing), member A	*Danio rerio*	XM_694639.3	3E-39	1	1
similar to testis expressed gene 14	*Rattus norvegicus*	XM_001081119.1	5E+00	1	2
microtubule associated serine/threonine kinase-like	*Equus caballus*	XM_001494482.1	7E-01	1	1
Miscellaneous					
akirin 2	*Danio rerio*	BC065319.1	3E-12	1	2
armadillo repeat containing 6	*Bos taurus*	NM_001098966.1	3E-15	1	1
ATPase, H+ transporting, lysosomal accessory protein 2	*Danio rerio*	NM_213023.1	2E-21	1	1
basigin	*Danio rerio*	BC056721.1	1E-96	1	1
casein kinase 1, delta	*Homo sapiens*	CH471099.1	8E-83	1	2
cysteine and glycine-rich protein 2 binding protein	*Danio rerio*	NM_001002699.2	1E-26	1	1
leucine rich repeat containing 1	*Homo sapiens*	NM_018214.4	2E-85	1	1
novel protein similar to sorting nexin 9	*Danio rerio*	BX324115.8	6E-87	1	1
prosaposin	*Danio rerio*	BC063994.1	7E-18	1	1
protein tyrosine phosphatase, non-receptor type 20 isoform 2	*Homo sapiens*	NM_015605.7	2E+00	1	1
S100 calcium binding protein A1	*Danio rerio*	NM_001089351.1	3E-21	1	1
S100 calcium binding protein V2	*Danio rerio*	NM_001080161.2	3E-28	1	1
signal transducing adaptor family member 2	*Danio rerio*	NM_198808.1	7E-41	1	1
similar to GTPase, IMAP family member 2	*Danio rerio*	XM_001332676.2	7E-13	1	1
similar to GTPase, IMAP family member 7	*Danio rerio*	XM_688704.3	8E-68	1	4
similar to GTPase, IMAP family member 8	*Danio rerio*	XM_001921448.1	2E-06	2	2
similar to membrane-spanning 4-domains, subfamily A, member 4 isoform a	*Macaca mulatta*	XM_001086681.1	3E-09	1	1
similar to ring finger protein 31, partial	*Danio rerio*	XM_690941.3	3E-08	1	3
similar to zinc finger, FYVE domain containing 16	*Danio rerio*	XM_001920883.1	7E-14	1	1
sorting nexin 25	*Rattus norvegicus*	CH473995.2	3E-86	1	2
hypothetical protein similar to tescalin	*Danio rerio*	NM_205693.1	4E-54	1	1
testis-expressed sequence 2 protein	*Homo sapiens*	Q8IWB9	2E-66	1	2
Immune response					
annexin A11a isoform 2	*Danio rerio*	NM_183410.1	2E-31	1	3
annexin A11b	*Danio rerio*	NM_181766.2	1E-76	1	2
barrier-to-autointegration factor	*Danio rerio*	NM_205562.1	3E-17	1	1
Similar to CC chemokine SCYA113	*Danio rerio*	BC162696.1	4E-11	2	6

CD80-like protein	*Oncorhynchus mykiss*	NM_001124414.1	3E-05	1	1
C-type lectin domain family 3, member B	*Xenopus tropicalis*	NM_001011424.1	5E-01	1	1
complement receptor-like	*Oncorhynchus mykiss*	NM_001124404.1	6E-04	1	1
endoplasmic reticulum aminopeptidase 2	*Homo sapiens*	BC065240.1	8E-73	1	2
F11 receptor	*Danio rerio*	NM_001004667.2	1E-21	1	1
Fc receptor, IgE, high affinity I, gamma polypeptide	*Danio rerio*	NM_001100106.1	1E-02	1	1
FYN binding protein (FYB-120/130)	*Homo sapiens*	CH471119.1	3E-07	1	1
similar to galectin 9	*Danio rerio*	XM_001921957.1	3E+00	1	1
similar to G7c protein (MHC class III)	*Danio rerio*	XM_680202.3	1E-52	1	1
interferon gamma inducible protein 30	*Danio rerio*	NM_001006057.1	5E-81	1	1
interferon-gamma receptor alpha chain	*Oncorhynchus mykiss*	NM_001124416.1	2E-02	1	1
IK cytokine	*Danio rerio*	NM_199589.1	1E-61	1	1
similar to leukocystatin; class II cystatin	*Monodelphis domestica*	XM_001382053.1	1E-12	1	1
mucosa associated lymphoid tissue lymphoma transloc. gene 1	*Danio rerio*	NM_152976.1	4E-106	1	2
neutrophil cytosolic factor 2	*Takifugu rubripes*	NM_001032682.1	1E-115	1	1
toll-like receptor 3	*Paralichthys olivaceus*	AB109396.1	2E-04	1	1
Cellular maintenance					
Cell adhesion					
catenin (cadherin-associated protein), alpha	*Danio rerio*	BC155815.1	6E-100	1	1
claudin 8c	*Takifugu rubripes*	AY554389.1	8E-45	1	1
similar to extracellular link domain-containing 1	*Danio rerio*	XM_684017.3	2E-05	1	1
F11 receptor	*Danio rerio*	NM_001004667.2	8E-10	1	1
similar to Fc fragment of IgG binding protein	*Danio rerio*	XM_001341078.2	1E-44	1	1
integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	*Gallus gallus*	NM_204146.1	6E-58	1	4
poliovirus receptor	*Rattus norvegicus*	NM_017076.2	9E-05	1	2
Cytoskeleton, cell motiliy					
actin related protein 2/3 complex, subunit 1A	*Danio rerio*	NM_201343.1	8E-80	1	1
actin related protein 2/3 complex, subunit 1B	*Danio rerio*	NM_213156.1	3E-33	1	1
similar to advillin	*Bos taurus*	XM_599021.3	8E-95	1	2
CLIP1 protein	*Homo sapiens*	BC047895.1	9E-03	1	1
collagen triple helix repeat containing 1	*Homo sapiens*	CH471060.1	4E+00	1	1
collagen, type I, alpha 3	*Danio rerio*	NM_201478.1	3E-05	1	1
dynein heavy chain-like	*Ictalurus punctatus*	DQ399421.1	6E-98	1	1
similar to dynein, axonemal, heavy chain 9, partial	*Danio rerio*	XM_001336050.2	6E-01	1	1
HSPC300	*Homo sapiens*	AF161418.1	1E-34	1	2
type I keratin E7	*Oncorhynchus mykiss*	NM_001124353.1	2E-06	2	2
kinesin family member C1	*Danio rerio*	NM_131206.1	2E-17	1	1
similar to kinesin family member 21A, partial	*Danio rerio*	XM_001920223.1	2E+00	1	1
microtubule-associated protein 1 light chain 3 beta	*Danio rerio*	NM_199604.1	1E-55	1	1
similar to myosin I beta	*Danio rerio*	XM_689729.3	2E-19	1	1
myosin IE	*Danio rerio*	XM_677757.3	3E-22	1	1
myosin VIb	*Danio rerio*	AY691328.1	6E-115	1	1
similar to Myosin-XVI	*Danio rerio*	XM_001920923.1	4E-01	1	1
similar to paralemmin 2	*Danio rerio*	XM_001340904.2	3E-03	1	1
similar to plectin 1 isoform 2	*Danio rerio*	XM_001923313.1	3E-66	1	2
WD repeat domain 1	*Danio rerio*	AY394939.1	3E-29	1	1
Wiskott-Aldrich syndrome-like	*Homo sapiens*	NM_003941	1E-09	1	1
Cell proliferation, development					
atrophin 1	*Mus musculus*	D87744.1	2E+00	1	1
envoplakin	*Danio rerio*	NM_001118897.1	7E-90	2	2
Golgi autoantigen, golgin subfamily a, 2	*Homo sapiens*	NM_004486.4	8E-01	1	2
leucine zipper and CTNNBIP1 domain containing	*Danio rerio*	NM_001002598.1	9E-86	1	1
mesoderm development candidate 2	*Homo sapiens*	NM_015154.1	4E-62	1	1
procollagen C-endopeptidase enhancer	*Danio rerio*	NM_001030181.2	5E-52	1	1
radixin isoform 1	*Danio rerio*	NM_001004296.1	1E-32	1	1
similar to rap2 interacting protein x isoform 1	*Gallus gallus*	XM_001233433.1	5E-11	1	1
Apoptosis					
CARD-like protein	*Danio rerio*	NM_001130402.1	5E-05	1	1
caspase a	*Danio rerio*	NM_131505.2	2E-09	1	2
caspase 3 (apoptosis-related cysteine protease b)	*Danio rerio*	NM_001048066.1	1E-04	1	1
caspase 10	*Paralichthys olivaceus*	AB247498.1	2E-76	1	2
deoxyribonuclease I-like 3	*Danio rerio*	NM_213153.1	4E-27	2	2
growth arrest and DNA-damage-inducible, alpha- like	*Danio rerio*	NM_200576.1	2E-66	1	1
similar to HtrA serine peptidase 2	*Danio rerio*	XM_001333269.2	7E-35	1	2
SH3-domain GRB2-like endophilin B1	*Danio rerio*	NM_001017617.1	1E-101	1	1
Apoptosis signaling					
B-cell receptor-associated protein 31	*Danio rerio*	NM_200092.1	6E-26	1	1
programmed cell death 6	*Danio rerio*	NM_200950.1	9E-13	2	2
similar to serine/threonine kinase 2 isoform 2	*Bos taurus*	XM_615401.4	2E-38	1	1
tumor protein, translationally-controlled 1	*Xenopus laevis*	NM_001086678.1	2E-28	1	3
DNA replication, repair					
CTF18, chromosome transmission fidelity factor 18 homolog	*Danio rerio*	NM_001110102.2	5E-68	1	2

DNA excision repair protein ERCC-8 (CSA)	*Danio rerio*	BC154184.1	4E-93	1	1
novel protein similar to vertebrate deoxyribonuclease I-like 2	*Danio rerio*	CU459089.7	1E-06	1	1
HMG2 (high mobility group 2) gene product	*Oncorhynchus mykiss*	L32954.1	5E-88	1	1
replication factor C (activator 1) 5	*Danio rerio*	NM_001003862.1	4E-58	1	1
RAD23b homolog	*Mus musculus*	NM_009011.4	1E+00	1	1
similar to histone chaperone SET	*Monodelphis domestica*	XM_001367588.1	4E-02	1	1
Transcription					
RNA processing					
AU RNA binding protein/enoyl-Coenzyme A hydratase	*Danio rerio*	NM_001003576.1	6E-48	1	1
HpaII tiny fragments locus 9c (tRNA methyltransferase)	*Danio rerio*	NM_199929.1	3E-83	1	1
M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein) isoform 2	*Danio rerio*	XM_704562.3	1E-95	1	1
RNA binding motif protein 5 isoform 5	*Pan troglodytes*	XM_001167452.1	6E-36	5	5
RNA binding motif protein 8A	*Homo sapiens*	NM_005105.2	1E-84	1	1
serine/arginine repetitive matrix 1	*Danio rerio*	NM_213442.1	4E-12	2	2
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	*Homo sapiens*	CH471065.1	1E-78	1	2
DEAD box polypeptide 18 (predicted)	*Papio anubis*	DP000513.1	4E-01	1	1
DEAD box polypeptide 49	*Xenopus tropicalis*	NM_204078.1	3E-42	1	1
similar to DEAH (Asp-Glu-Ala-His) box polypeptide40	*Danio rerio*	XM_693677.3	3E-77	1	1
similar to poly(A)-specific ribonuclease	*Monodelphis domestica*	XM_001375740.1	4E-80	1	1
small nuclear ribonucleoprotein polypeptide A'	*Salmo salar*	NM_001123704.1	5E-116	1	1
small nuclear ribonucleoprotein polypeptide E	*Danio rerio*	NM_201004.1	2E-44	1	1
splicing factor 3b, subunit 5	*Danio rerio*	NM_001002478.1	2E-31	2	2
UTP6, small subunit processome component, homolog	*Danio rerio*	NM_001111207.1	1E-77	1	1
Regulation of transcription					
activating transcription factor 1	*Danio rerio*	NM_199723.1	4E-42	1	2
ataxin-3	*Danio rerio*	AY956353.1	1E-63	1	1
basic leucine zipper and W2 domains 1a	*Danio rerio*	NM_199708.1	2E-69	1	1
cAMP responsive element binding protein 1	*Danio rerio*	NM_200909.1	6E-23	1	1
cAMP responsive element binding protein-like 2	*Homo sapiens*	CH471094.1	6E-10	1	1
chromatin accessibility complex 1-like	*Danio rerio*	NM_001013293.1	4E-41	1	1
D4, zinc and double PHD fingers, family 3	*Homo sapiens*	CH471061.1	2E-13	1	1
E74-like factor 3 (ets domain transcription factor, epithelial-specific), partial	*Danio rerio*	XM_684268.3	5E-31	2	2
forkhead box B2	*Homo sapiens*	NM_001013735.1	1E-05	1	1
interferon regulatory factor 2	*Siniperca chuatsi*	AY395717.1	3E-07	1	2
mediator complex subunit 7	*Danio rerio*	NM_199741.1	3E-35	1	1
myeloid/lymphoid or mixed-lineage leukemia; translocated to, 6 (MLLT6)	*Homo sapiens*	BC064612.1	4E+00	1	1
neuroblastoma myc-related oncogene 1	*Danio rerio*	NM_212614	1E-06	1	1
neuronal double zinc finger protein	*Homo sapiens*	AF052224.1	3E+00	1	1
novel protein similar to human high-mobility group protein 2-like 1 (HMG2L1)	*Danio rerio*	CR383684.7	4E-76	1	3
nuclear factor (erythroid-derived 2)-like 2	*Danio rerio*	BC152659.1	5E-25	1	1
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	*Danio rerio*	XM_001341675.2	6E-53	1	1
protein phosphatase 1, regulatory (inhibitor) subunit 7	*Danio rerio*	NM_001037386.1	4E-117	1	1
RNA binding motif protein 15B	*Homo sapiens*	BC139836.1	2E-01	1	1
runt-related transcription factor 1	*Homo sapiens*	BC110828.1	8E-14	1	3
similar to E3 SUMO-protein ligase PIAS1 (protein inhibitor of activated STAT protein 1)	*Danio rerio*	XM_687829.2	5E-35	1	2
similar to MORC family CW-type zinc finger protein 2	*Monodelphis domestica*	XM_001380493.1	9E-66	1	1
similar to metastasis associated 1	*Danio rerio*	XM_001333237.1	5E-78	1	1
similar to monocyte protein (MOP) 5	*Monodelphis domestica*	XM_001381548.1	8E-89	1	1
similar to MYC-associated zinc finger protein	*Danio rerio*	XM_001918941.1	4E-38	1	1
similar to poly (ADP-ribose) polymerase 14	*Danio rerio*	XM_001332545.1	2E-32	3	4
similar to transcriptional co-repressor Sin3A	*Gallus gallus*	XM_413695.2	1E-66	1	1
sirtuin (silent mating type information regulation 2 homolog) 5 (S. cerevisiae)	*Danio rerio*	NM_001002605.1	3E-03	1	2
suppressor of defective silencing 3 homolog (S. cerevisiae)	*Homo sapiens*	CH471054.1	7E-79	1	1
thioredoxin interacting protein	*Danio rerio*	NM_200087.1	6E-17	1	1
general transcription factor IIIC, polypeptide 4, 90kDa, partial	*Equus caballus*	XM_001499211.2	2E+00	1	1
putative homeodomain transcription factor 1	*Homo sapiens*	AL365321.16	2E-31	1	1
transcription factor MAFB (v-maf musculo-aponeurotic fibrosarcoma oncogene homolog B)	*Gallus gallus*	NM_001030852.1	2E-01	1	1
YY1 transcription factor b	*Danio rerio*	BC155321.1	3E-35	1	1
transposase	*Rana pipiens*	AY261371.1	8E-03	1	2
zinc finger protein 75 (D8C6)	*Equus caballus*	XM_001494693.2	3E+00	1	1
zinc finger, CCHC domain containing 9	*Danio rerio*	NM_198370.1	1E-35	1	1
zinc finger, MYM-type 2	*Homo sapiens*	AL137119.26	2E-01	1	1
similar to zinc finger protein 208	*Rattus norvegicus*	XM_001060419.1	7E+00	1	2
similar to zinc finger protein 452	*Danio rerio*	XM_001921928.1	2E-01	1	1
similar to zinc finger, ZZ-type containing 3	*Danio rerio*	NM_001100027.1	7E-51	1	1
similar to zinc-finger protein	*Danio rerio*	XM_688286.3	9E-11	1	1
Translation					

cylindromatosis (turban tumor syndrome)	*Danio rerio*	XM_679725.3	2E-07	1	1
density-regulated protein	*Danio rerio*	NM_001002697.2	4E-73	1	1
eukaryotic translation initiation factor 3 (eIF3), subunit 12	*Homo sapiens*	NM_013234.2	5E-54	1	2
eukaryotic translation initiation factor 4A (eIF4), isoform 3	*Homo sapiens*	D21853.1	3E-114	1	1
similar to eIF4E	*Monodelphis domestica*	XM_001364246.1	7E-105	1	1
pseudouridylate synthase 7	*Danio rerio*	BC133871.1	9E-26	1	2
ribosomal protein L30	*Danio rerio*	NM_200028.1	3E-25	1	1
ribosomal protein large P0-like protein	*Sparus aurata*	AY550965.1	1E-52	1	1
ribosomal protein S17 (40S)	*Siniperca chuatsi*	AY909424.1	2E-59	1	1
ribosomal protein S21	*Homo sapiens*	NM_001024.3	1E-37	1	1
lysyl-tRNA synthetase	*Danio rerio*	NM_001002386.1	8E-59	1	1
novel protein similar to threonyl-tRNA synthetase	*Danio rerio*	BX950187.14	1E-48	1	1
valyl-tRNA synthetase	*Takifugu rubripes*	P49696	2E-165	1	2
Protein metabolism					
Protein processing and localisation					
dolichyl-phosphate mannosyltransferase polypeptide 3	*Danio rerio*	NM_200809.2	1E-16	1	1
signal recognition particle 54	*Danio rerio*	NM_200988.1	2E-28	2	2
signal peptide peptidase 3	*Danio rerio*	NM_001015068.1	3E-39	1	1
similar to signal peptide, CUB domain, EGF-like 1	*Danio rerio*	BC152490.1	4E-03	1	1
translocase of inner mitochondrial membrane 17 homolog A	*Danio rerio*	NM_198367.1	5E-39	1	1
translocase of inner mitochondrial membrane 23 homolog	*Danio rerio*	NM_001105598.1	6E-23	1	2
vacuolar protein sorting 13D (yeast)	*Danio rerio*	XM_001919953.1	9E+00	1	1
Protein degradation					
ADAM metallopeptidase domain 19	*Bos taurus*	NM_001075475.2	7E+00	1	1
similar to dipeptidase 3	*Danio rerio*	XM_001345496.2	4E-49	1	1
N-glycanase 1	*Danio rerio*	NM_001020601.1	7E-23	1	1
similar to marapsin	*Danio rerio*	XM_001332964.2	7E-36	2	2
peptidase (mitochondrial processing) beta	*Danio rerio*	NM_001012496.1	2E-82	2	2
similar to prolyl endopeptidase, partial	*Danio rerio*	XM_001346625.2	2E-34	1	1
proteasome activator subunit 2	*Danio rerio*	NM_131374.1	3E-12	1	2
proteasome subunit, beta type, 11	*Danio rerio*	NM_131676.1	3E-50	1	1
SUMO2 (small ubiquitin-like modifier) protein	*Homo sapiens*	BC008450.1	7E-42	1	1
similar to suppression of tumorigenicity 14	*Danio rerio*	XM_680264.3	3E-55	1	2
similar to ubiquitin and ribosomal S27A fusion protein	*Danio rerio*	BC162814.1	1E-03	1	2
ubiquitin C variant	*Homo sapiens*	AB209782.1	3E-18	1	1
ubiquitin specific peptidase 47	*Homo sapiens*	CH471064.2	1E-101	1	1
ubiquitin-conjugating enzyme E2N-like	*Danio rerio*		1E-75	1	1
ubiquitin-like modifier activating enzyme 1	*Danio rerio*	NM_213062.1	2E-124	1	1
ubiquitin-like modifier activating enzyme 7	*Homo sapiens*	NM_003335.2	2E+00	1	1
similar to ubiquitin-activating enzyme E1C iso 1	*Ornithorhynchus anatinus*	XM_001510239.1	8E-111	1	1
Metabolism					
Lipid metabolism					
acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	*Danio rerio*	BX569796.6	3E-76	2	2
acyl-Coenzyme A synthetase short-chain family member 1	*Homo sapiens*	CH471133.3	3E-11	1	1
arachidonate 12-lipoxygenase	*Danio rerio*	NM_199618.1	2E-70	1	1
beta-1,4-N-acetyl-galactosaminyl transferase 1	*Danio rerio*	NM_001080570.1	2E-51	1	2
diazepam binding inhibitor isoform 2	*Homo sapiens*	NM_001079863.1	6E-20	1	1
elongation of very long chain fatty acids-like	*Danio rerio*	NM_199875.1	3E-65	1	2
low density lipoprotein receptor adaptor protein 1	*Homo sapiens*	CH471059.2	4E+00	1	1
mitochondrial trifunctional protein beta subunit (hadhb): 3-ketoacyl-CoA thiolase	*Danio rerio*	NM_200019.1	1E-57	1	2
myo-inositol-1 phosphate synthase-like	*Oreochromis mossambicus*	DQ465381.1	2E-08	1	1
phosphatidylserine synthase 1	*Homo sapiens*	BC004502.2	7E-37	1	2
similar to carnitine O-palmitoyltransferase I iso 1	*Danio rerio*	XM_001922906.1	4E-52	1	2
similar to phosphatidic acid phosphatase type 2c	*Danio rerio*	XM_687169.3	7E-13	1	1
Carbohydrate metabolism					
glycogen phosphorylase (muscle) A	*Danio rerio*	NM_001020628.1	1E-49	1	2
glycogen phosphorylase (isoform)	*Oreochromis mossambicus*	DQ081728.1	1E-39	1	1
phosphoglucomutase 3	*Homo sapiens*	CH471051.2	4E-16	1	1
glutamic-oxaloacetic transaminase (aspartate aminotransferase) 1, soluble	*Danio rerio*	XM_695567.3	1E-03	1	1
similar to glycosyltransferase 1 domain containing 1	*Danio rerio*	XM_001345359.2	8E-20	1	1
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	*Danio rerio*	BC060931.1	2E-107	1	1
isocitrate dehydrogenase 3 (NAD+) alpha	*Danio rerio*	BC049011.1	3E-54	1	2
isocitrate dehydrogenase 3 (NAD+) gamma	*Xenopus tropicalis*	NM_001030426.1	1E-43	1	2
Amino sugar metabolism					
N-acetylneuraminic acid synthase	*Takifugu rubripes*	AJ705104.1	2E-27	1	1
iduronate 2-sulfatase	*Homo sapiens*	CH471171.2	1E-21	1	1
putative cytidine monophospho-N-acetylneuraminic acid synthetase	*Takifugu rubripes*	NM_001078593.1	1E-53	1	2
Miscellaneous					
aldehyde dehydrogenase 3 family, member D1	*Danio rerio*	NM_173221.2	2E-16	1	1

similar to arachidonate lipoxygenase 3	*Danio rerio*	XM_001333998.1	2E-41	1	1
coenzyme Q6 homolog	*Xenopus tropicalis*	NM_001006828.2	3E-47	1	1
dCMP deaminase	*Danio rerio*	NM_001017639.1	4E-11	1	2
dihydrolipoamide dehydrogenase	*Danio rerio*	AY391421.1	8E-23	1	1
epididymal secretory protein E1	*Danio rerio*	NM_173224.1	7E-38	1	1
glutathione S-transferase	*Pagrus major*	AB158412.1	2E-78	1	1
glyoxylate reductase/hydroxypyruvate reductase	*Danio rerio*	NM_001020525.1	7E-27	1	1
similar to skeletal muscle and kidney-enriched inositol phosphatase	*Danio rerio*	XM_689458.3	6E-62	1	1
methylmalonyl Coenzyme A mutase	*Danio rerio*	NM_001099226.1	7E-61	1	1
NAD kinase	*Gallus gallus*	NM_001030870.1	1E-78	1	1
selenoprotein W2a	*Oreochromis mossambicus*	AY737049.1	8E-12	1	5
spermidine synthase		NM_003132.2	9E-43	1	1
sulfotransferase	*Danio rerio*	NM_214686.1	2E-12	1	1
thioredoxin	*Ictalurus punctatus*	Q9DGI3	1E-31	1	1
uroporphyrinogen III synthase	*Danio rerio*	AB180839.1	2E-37	1	1
Mitochondrial genes					
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	*Danio rerio*	NM_001005960.1	1E-84	1	1
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9)	*Danio rerio*	NM_131761.1	9E-36	1	1
cytochrome b	*Zoarces viviparus*	EU492073.1	2E-17	1	1
cytochrome c oxidase subunit Vaa	*Danio rerio*	NM_001024403.1	5E-64	1	2
cytochrome c oxidase subunit Vab	*Danio rerio*	BC059554.1	6E-27	1	1
electron-transfer-flavoprotein, beta polypeptide	*Danio rerio*	NM_212998.1	2E-40	1	1
putative ferric-chelate reductase 1		A4QP81	5E-12	1	1
NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5	*Danio rerio*	BC107623.1	2E-47	1	2
nicotinamide nucleotide transhydrogenase	*Danio rerio*	NM_214756.1	7E+51	1	1
ubiquinol-cytochrome c reductase complex chaperone, CBP3 homolog	*Gallus gallus*	NM_001006285.1	2E-26	1	1
Transport					
Ion					
Ammonium transporter Rh type B glycoprotein	*Takifugu rubripes*	NM_001032646.1	5E-101	1	1
Ammonium transporter Rhesus blood group associated glycoprotein	*Danio rerio*	BX276177.6	2E-48	1	1
similar to type IIb sodium-phosphate transporter	*Monodelphis domestica*	XM_001367042.1	3E+00	1	1
transient receptor potential cation channel, subfamily M, member 7	*Homo sapiens*	CH471082.1	7E-01	2	2
Neurotransmitter					
gamma-aminobutyric acid GABA(A) receptor associated protein	*Danio rerio*	AY582140.1	2E-44	1	1
gamma-aminobutyric acid receptor-associated protein-like	*Danio rerio*	NM_001002707.2	3E-45	1	1
synaptosomal associated protein	*Bos taurus*	NM_001083410.1	2E-46	1	1
synaptophysin-like protein	*Danio rerio*	NM_212669.1	7E-13	1	1
Protein					
adaptor protein complex AP-2, mu1	*Danio rerio*	NM_212577.1	2E-125	1	1
dopey family member 2	*Homo sapiens*	Q9Y3R5	7E-30	1	1
similar to nucleoporin 160kDa	*Monodelphis domestica*	XM_001364481.1	1E-49	1	1
pituitary tumor-transforming 1 interacting protein	*Danio rerio*	NM_001005941.1	6E-49	1	1
novel protein simlar to human and mouse vacuolar protein sorting 35 (yeast) (VPS35)	*Danio rerio*	AL928906.4	2E-109	1	1
Miscellaneous					
claudin 30d	*Takifugu rubripes*	AY554376.1	5E-13	1	1
collagen and calcium binding EGF domains 1	*Mus musculus*	CH466528.2	7E-03	1	3
endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and golgi 2	*Danio rerio*	NM_200407.1	1E-09	1	1
nuclear pore complex-associated protein TPR	*Danio rerio*	NM_001030123.1	8E-85	1	2
phosphoinositide-binding protein PIP3-E isoform 1	*Homo sapiens*	NM_001130699.1	2E-12	1	1
Rab9 effector protein with kelch motifs	*Gallus gallus*	NM_001080891.1	3E+00	1	1
solute carrier family 25 (mitochondrial carrier oxoglutarate carrier), member 11	*Mus musculus*	CH466596.1	1E-110	1	2
STARD3 N-terminal like	*Danio rerio*	NM_001002320.1	4E-47	1	3
ATP binding cassette TAP2a protein	*Oncorhynchus mykiss*	NM_001124243.1	1E-50	1	1
transmembrane protein 9	*Danio rerio*	BX544876.8	8E-33	1	1
similar to zymogen granule protein 16	*Macaca mulatta*	XM_001104596.1	1E-22	1	1
Miscellaneous					
mucin 2, oligomeric mucus/gel-forming	*Homo sapiens*	M74027.1	3E-18	2	3
mucin-2 precursor (intestinal mucin-2)	*Homo sapiens*	Q02817	8E-07	1	2
PSMC3 interacting protein	*Danio rerio*	NM_001002124.1	3E-83	1	2
Unknown biological process					
coiled-coil domain containing 43	*Danio rerio*	NM_200658.1	9E-71	1	1
similar to coiled-coil domain containing 136	*Danio rerio*	XM_689879.3	2E-01	1	2

Ctr9, Paf1/RNA polymerase II complex component, homolog	*Danio rerio*	NM_001083583.1	1E-40	1	1
GRAM domain containing 3	*Homo sapiens*	NM_023927.1	2E-03	1	1
immunoglobulin-like domain containing receptor 1	*Danio rerio*	NM_001004642.1	2E-82	1	2
similar to S19 binding protein	*Danio rerio*	XM_001339171.1	9E-05	1	1
TatD DNase domain containing 1	*Homo sapiens*	CH471060.1	2E-97	1	2
THAP domain containing 9 protein	*Homo sapiens*	BC131722.1	1E+00	1	1
similar to transmembrane protein 14A	*Ornithorhynchus anatinus*	XM_001516482.1	9E-32	1	1
similar to tripartite motif-containing protein 16 (Estrogen-responsive B box protein)	*Danio rerio*	XM_681577.2	1E-09	1	1

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Erklärung gem. § 5 (1) Nr. 3 PromO

(vom 14. März 2007)

Ich erkläre hiermit,

1. dass ich mich vor dem jetzigen Promotionsverfahren keinem anderen Promotionsverfahren unterzogen habe

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Katrin Deigweiher