Signale und molekulare Mechanismen der Temperaturanpassung mitochondrialer Funktionen bei marinen Fischen

Signals and molecular mechanisms of temperature adaptation of mitochondrial functions in marine fish

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Contents

Summary V
Zusammenfassung VII

1 Introduction 1

1.1 The concept of thermal adaptation 1
1.2 Mitochondria 3
1.3 Signals for temperature adaptation 6
1.4 Biological model system 8
1.5 Thesis outline 10

2 Material and Methods 11

2.1 Animals 11
2.2 Cell culture 11
2.2.1 Isolation of hepatocytes 11
2.2.2 Cell culture conditions 12
2.2.3 Cellular viability 14
2.3 Enzyme activities 14
2.3.1 Cytochrome c oxidase (COX) 14
2.3.2 Citrate synthase (CS) 14
2.4 Protein quantification 15
2.5 Adenosine concentrations 15
2.6 RNA isolation and quality control 16
2.7 Sequence determination 17
2.7.1 Reverse transcription and polymerase chain reaction 17
2.7.2 Rapid amplification of cDNA ends 19
2.8 Quantification of specific mRNA 21
2.8.1 Construction of radiolabeled probes 21
2.8.2 Ribonuclease Protection Assay 21
2.9 Statistical analysis 23

3 Results 25

3.1 Development of a primary liver cell culture for *Z. viviparus* 25
3.1.1 Basic incubation conditions and culture medium 25
3.1.2 Adjustments of media composition 30
3.1.3 Verification of the culture conditions 33

3.2 Temperature effects on hepatocytes 37
3.2.1 In vivo temperature acclimation 37
3.2.2 Temperature effects on enzyme activities in isolated hepatocytes 38
3.2.3 Temperature effects on RNA and protein contents of isolated cells 39
3.2.4 Effects of in vitro incubation temperature on transcript levels 40
3.3 Effects of extracellular pH in isolated hepatocytes 43
3.4 Impact of adenosine on thermal acclimation 45
3.4.1 Adenosine levels during cold exposure in vivo 45
3.4.2 Adenosine effects on enzyme activities 46
3.4.3 Adenosine effects on total RNA and protein contents 47
3.4.4 Adenosine effects on specific mRNA expression 48
3.4.5 Impact of adenosine receptor antagonists and agonists 50
3.4.6 Cellular adenosine consumption 51
3.5 Influence of stress hormones on cultured hepatocytes 53
3.5.1 Hormone concentrations 53
3.5.2 Effects of epinephrine and cortisol on enzyme activities 55
3.5.3 Effects of epinephrine and cortisol on total RNA and protein contents 56
3.5.4 Effects of epinephrine and cortisol on mRNA expression 57
3.5.5 Seasonal variation of hormone response 59
4 Discussion 61
4.1 Primary liver cell culture for *Z. viviparus* 61
4.2 Temperature acclimation in isolated cells 64
4.3 Extracellular pH as a signal for thermal adaptation 66
4.4 The role of adenosine in thermal adaptation 67
4.4.1 Adenosine levels during in vivo cold exposure 67
4.4.2 Adenosine effects at the cellular level 68
4.5 Hormonal contributions to thermal adaptation 71
4.5.1 The impact of cortisol on metabolic adjustments 72
4.5.2 Epinephrine effects on energy metabolism 73
4.6 Conclusions and perspectives 76
5 References 79
Danksagung 95

Abbreviation list

8-PT	8-phenyltheophylline
ado	adenosine
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AR	adrenoreceptor
ATP	adenosine 5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
cDNA	complementary DNA
Ci	Curie (1 Ci = 37 10^9 Bq)
CISY	citrate synthase mRNA probe
cor	cortisol
COX	cytochrome *c* oxidase
COX2	cytochrome *c* oxidase subunit 2
COX4	cytochrome *c* oxidase subunit 4
cpm	counts per minute
CR	corticosteroid receptor
CRE	corticoid responsive element
CS	citrate synthase
ctrl	control
DMEM	Dulbecco's modified eagle medium
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
epi	epinephrine
h	hour(s)
HEPES	N-(2-Hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)
HSI	hepatosomatic index
L-15	Leibovitz L-15 medium

min	minute(s)
mRNA	messenger RNA
MOPS	3-(N-Morpholino)propanesulfonic acid
MS222	3-amino-benzioc-methanosufonate
NECA	5'-(N-ethylcarboxamido)adenosine
norm.	normalized
NRF	nuclear respiratory factor
PAGE	polyacrylamid gelelectrophoresis
PCR	polymerase chain reaction
pH_e	extracellular pH
pK	dissociation constant
RLM-RACE	RNA ligase mediated rapid amplification of cDNA ends
RNA	ribonucleic acid
RPA	ribonuclease protection assay
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SE	standard error of means
T	temperature
TCA	trichloracetic acid
tRNA	transfer RNA

Summary

Temperature has a large impact on the velocity of biochemical and enzymatic processes and hence is a key factor defining the performance of ectothermic organisms. In marine fish, temperature acclimation is well known to induce adjustments of mitochondrial capacities and functions. This thesis sets out to elucidate the cellular signals and molecular mechanism involved in thermal adaptation of mitochondrial functions in marine fish.

The basis for these mechanistic studies was laid by the establishment of a cellular system for the common eelpout *Zoarces viviparus*, a model organism to investigate thermal acclimation in fish. Primary culture developed for eelpout hepatocytes supported viability and physiological integrity of the cells for several days. Adjustments of mitochondrial functions occurred in response to varying ambient conditions, reaching steady state levels within the available time period. Primary culture of eelpout hepatocytes thus provided a suitable tool to investigate the mechanism involved in thermal adaptation.

On this groundwork, the involvement of systemic signals in temperature-dependent mitochondrial adjustment was investigated by monitoring mRNA expression and capacities of the mitochondrial key enzymes citrate synthase (CS) and cytochrome c oxidase (COX). *In vivo* temperature acclimation of *Z. viviparus* to 4 and 11°C resulted in an increase of CS activities in hepatocytes in the cold, while COX activities and the mRNA expression of the respective genes remained unaffected. In contrast, *in vitro* cold incubation of liver cells from warm acclimated animals left both mitochondrial enzymes unchanged and warm incubation of hepatocytes prepared from cold acclimated fish induced a simultaneous decrease of the activities of both enzymes and a decline of COX mRNA expression. The lack of cold acclimation in isolated liver cells and the differences between warm acclimation patterns *in vivo* vs. *in vitro* indicates the involvement of (a) systemic signal(s) in the induction or modulation of thermal adaptation.

Therefore, the impact of several potential effectors on thermal adaptation of mitochondrial functions was studied. Adenosine may act as such a signal, since it is known to be a general indicator for bioenergetic disturbances. In line with a potential role for this metabolite in thermal adaptation, adenosine levels in serum and liver of *Z. viviparus* increased within 24 h of *in vivo* cold incubation and were still elevated after 3 days in liver. Adenosine treatment of isolated hepatocytes caused a reduction of COX activities, but induced an increase of COX mRNA expression. These effects were not receptor mediated,

suggesting a diffusive entry and intracellular action of the metabolite. Adenosine may inhibit the translation of COX mRNA resulting in reduced COX activities, which in turn may cause a compensatory increase in COX mRNA levels. Thus it may act as a modulator in thermal adaptation by removing excess COX activities during warm acclimation or preventing its build-up during cold incubation.

Temperature changes might also involve a stress response in ectothermic animals. Therefore the impact of epinephrine and cortisol on mitochondrial functions was elaborated. Cortisol treatment of isolated eelpout hepatocytes increased the mRNA expression of CS and of the nuclear encoded, but not of the mitochondrial encoded, COX subunit. Enzyme activities were not affected. This resembles the situation during the early phase of cold acclimation described for *Z. viviparus* and suggests an involvement of cortisol in the induction of cold acclimation. The response of isolated hepatocytes to epinephrine significantly depended on the season in which the experiment was performed. The catecholamine revealed almost no effects during summer incubations, but was found to increase activities of both enzymes during experiments performed in winter. Thus a potential role for epinephrine may be restricted to acclimatisation in winter.

In conclusion, the findings of the present thesis confirm the requirement of systemic signals for the induction and modulation of thermal adaptation. They furthermore indicate a role for adenosine and the stress hormones epinephrine and cortisol in specific parts of this process.

Zusammenfassung

Die Temperatur hat großen Einfluss auf die Geschwindigkeit biochemischer und enzymatischer Prozesse, daher definiert sie als einer der Schlüsselfaktoren die Leistungsfähigkeit ektothermer Organismen. Eine Temperatur-Akklimation mariner Fische hat im Allgemeinen eine Anpassung mitochondrialer Kapazitäten und Funktionen zur Folge. Die vorliegende Arbeit untersucht die zellulären Signale und molekularen Mechanismen, die bei der Temperaturanpassung mitochondrialer Funktionen bei marinen Fischen involviert sind.

Die Basis für diese Untersuchungen wurde durch die Etablierung eines Zellsystems für die Aalmutter *Zoarces viviparus* geschaffen, die einen Modell-Organismus in der Erforschung der Temperaturanpassung bei Fischen darstellt. Die primäre Zellkultur, die für Hepatocyten der Aalmutter entwickelt wurde, erhielt die Überlebensfähigkeit sowie die physiologische Integrität der Zellen über mehrere Tage. Eine Anpassung mitochondrialer Funktionen zeigte sich als Reaktion auf veränderte Umgebungsbedingungen, wobei ein stabiles Niveau (*steady state*) innerhalb der verfügbaren Zeitspanne erreicht wurde. Die Primärkultur von Hepatocyten aus der Aalmutter lieferte folglich ein geeignetes Werkzeug, um die Mechanismen der Temperaturanpassung zu untersuchen.

Auf dieser Grundlage wurde die Beteiligung systemischer Signale bei der temperatur-abhängigen Anpassung der Mitochondrien untersucht; diese wurde anhand der mRNA-Expression sowie der Kapazitäten der mitochondrialen Schlüsselenzyme Citrat-Synthase (CS) und Cytochrom-c-Oxidase (COX) verfolgt. Die Temperatur-Akklimation von *Z. viviparus* bei 4 und 11°C führte zu einer Zunahme der CS-Aktivität in den Leberzellen in der Kälte, während die COX-Aktivität und die mRNA-Expression der zugehörigen Gene nicht beeinflusst wurden. Im Gegensatz dazu blieben beide Enzyme während der Kälteinkubation von Hepatocyten aus warm-angepassten Tieren unverändert. Eine Wärmeinkubation von Leberzellen, die aus kalt-angepassten Fischen gewonnen wurden, führte zu einer simultanen Abnahme der Aktivitäten beider Enzyme und einem Rückgang der COX-mRNA-Expression. Das Fehlen einer Kälte-Akklimation in isolierten Leberzellen und die Unterschiede zwischen der Wärme-Akklimation *in vivo* und *in vitro* weisen auf eine Beteiligung systemischer Signale bei der Induktion und Modulation der Temperaturanpassung hin.

Daher wurde der Einfluss verschiedener potenzieller Effektoren auf die Temperaturanpassung mitochondrialer Funktionen untersucht. Adenosin kann als solch ein Signal dienen, da es als genereller Indikator für bioenergetische Störungen gilt. Im Einklang mit der möglichen Rolle des Metabolits bei der Temperaturanpassung stiegen die Adenosin-Konzentrationen im Serum und der Leber von *Z. viviparus* nach 24 Stunden Kälteinkubation in vivo an und waren in der Leber auch noch nach 3 Tagen erhöht. Eine Behandlung isolierter Leberzellen mit Adenosin führte zu einer Abnahme der COX-Aktivität, induzierte allerdings eine Zunahme der COX-mRNA-Expression. Diese Effekte waren nicht rezeptorvermittelt, was eine diffusionsgetriebene Aufnahme und intrazelluläre Wirkung des Metabolits nahe legt. Adenosin könnte die Translation der COX-mRNA blockieren, was eine Reduktion der COX-Aktivität zur Folge hätte. Diese wiederum könnte einen kompensatorischen Anstieg der COX-mRNA-Menge auslösen. Adenosin könnte daher als Modulator bei der Temperaturanpassung wirken, indem es überschüssige COX-Aktivitäten während der Wärme-Akklimation abbaut oder während der Kälte-Akklimation deren Aufbau verhindert.

Temperaturveränderungen können außerdem eine Stress-Antwort bei ektothermen Tieren auslösen. Daher wurde der Einfluss von Cortisol und Epinephrin auf die mitochondrialen Funktionen untersucht. Eine Behandlung isolierter Hepatocyten aus der Aalmutter erhöhte die mRNA-Expression der CS und der kernkodierten – nicht jedoch der mitochondrial-kodierten – COX-Untereinheit. Die Enzymaktivitäten waren nicht beeinflusst. Dies ähnelt der Situation, die für die frühe Phase der Kälte-Akklimation von *Z. viviparus* beschrieben wurde, und weist auf eine Beteiligung von Cortisol bei der Induktion der Kälte-Akklimation hin. Die Reaktion isolierter Hepatocyten auf Epinephrin wies eine deutliche Abhängigkeit von der Jahreszeit auf, in der die Experimente durchgeführt wurden. Das Katecholamin zeigte nahezu keine Wirkung während der Sommerinkubationen. Es führte jedoch in den Experimenten während der Wintermonate zu einem Anstieg der Aktivitäten beider Enzyme. Eine mögliche Rolle von Epinephrin könnte daher auf die Akklimatisation im Winter beschränkt sein.

Die Ergebnisse der vorliegenden Arbeit bestätigen die Notwendigkeit systemischer Signale für die Induktion und Modulation der Temperaturanpassung. Weiterhin legen sie nahe, dass Adenosin sowie die Stresshormone Cortisol und Epinephrin in bestimmten Teilen dieses Prozesses mitwirken.

1 Introduction

During the last decade, it has become more and more accepted that there is an impact of climate change on organisms and ecosystems. Hence the ability of animals to tolerate or to adapt to the changing temperature regime became a matter of interest. This thesis was designed to elucidate potential signals and regulatory pathways that are involved in the induction of thermal acclimation of mitochondria, the cellular power plants. It should thereby contribute to a mechanistic understanding of thermal tolerance and adaptation in marine vertebrates and invertebrates.

1.1 The concept of thermal adaptation

Ectothermic organisms do not possess the ability to actively regulate their body temperature, and especially marine animals are directly exposed to the stimulating or limiting effects of temperature at all physical and biological processes. Thus, temperature is a key factor to shape the performance of individual organisms and, as a consequence, the biogeography of populations or entire species. In the marine habitat a broad range of environmental temperatures has been recorded across latitudes, reaching from -1.9°C in polar to about 40°C in tropical areas. In contrast to endothermic birds or mammals, no marine poikilotherm is distributed over the whole temperature range, leading to the conclusion that thermal tolerance windows differ between ectothermic species. Originally defined by the lethal temperature limits of an organism, characterization of the thermal tolerance window of a species nowadays differentiates between different phases of limitation and effects at various levels of biological organization (Pörtner 2001; 2002a). The first level of thermal limitation at the whole organism level is set by *pejus* temperatures, which indicate the limits for an optimum performance of an organism. Beyond this optimum range, survival is still possible, however, energy-consuming processes like growth or reproduction are progressively compromised. Therefore, the upper and the lower *pejus* temperature relate to the ecological distribution limits of a species (cf. Pörtner, 2001; Pörtner and Knust 2007). The width of the thermal tolerance window resembles the temperature regime in the habitat of a species. Organisms inhabiting environments with constant temperatures are usually assumed to possess a narrow tolerance window and referred to as stenothermal. In contrast to stenothermal organisms, eurythermal animals are subject to more or less extensive temperature alterations and are able to cope with these, by maintaining a broad tolerance window. Furthermore, most

temperate zone ectotherms are able to shift their thermal tolerance windows in order to adapt to long-term challenges such as seasonal temperature changes. This process is referred to as acclimatisation or, when induced in the laboratory under experimental conditions, as acclimation (Clarke, 1983).

First evidence for the ability of ectothermic organisms to adapt to temperature changes was obtained at the level of oxygen consumption. Due to the decelerating effects of low temperatures on all metabolic functions, cooling of poikilothermic animals leads to a decrease of overall oxygen consumption. When the period of cold exposure is extended, oxygen consumption increases and reverts to or at least approximates the initial level, resulting in perfect or partial compensation of temperature effects, respectively (Precht, 1958). A similar adaptation pattern, but in the opposite direction, can also be observed during warming of eurythermal organisms. Thermal acclimation on the level of oxygen consumption has been reported for many marine ectotherms throughout the animal kingdom, including annelids (Sommer and Pörtner, 2002), molluscs (Sokolova and Pörtner, 2003), crustaceans (Robert and Gray, 1972) and fish (Van Dijk et al., 1999). However, thermal compensation occurs at all levels of biological organisation. Temperature acclimation of fish has been shown to influence the structure and function of individual tissues e.g. by changing the relative proportion of muscle fibre types (Sidell, 1980), altering mitochondrial density and capacity (St-Pierre et al., 1998), changing the lipid composition of cellular membranes (Cossins, 1994) or affecting the expression of isozymes (Shaklee et al., 1977).

As recently demonstrated, the limits of thermal tolerance are set at the highest level of biological organisation, the functioning of the whole organism. Limitation occurs through the onset of a mismatch between oxygen demand and the capacity of oxygen supply with a key role for ventilation and circulation. These limits coincide with a loss in individual growth performance and in species abundance and hence shape the well being of a species in the field (Pörtner and Knust, 2007). Nonetheless, the basis of thermal tolerance windows and their shifts during temperature acclimation is laid at the molecular level, with a key role for mitochondria (Pörtner et al., 2005). Accordingly, the present study focuses on the level of mitochondrial functions to elucidate the impact of temperature and the underlying mechanisms for thermal adaptation in marine ectotherms.

1.2 Mitochondria

Mitochondria are cellular organelles present in all eukaryotes and are suggested to originate from prokaryotic cells capable of oxidative phosphorylation, which entered into symbiosis with simple eukaryotic host cells (Sagan, 1967). Mitochondria feature a separate genome, discrete ribosomes and as descendants from gram-negative bacteria, are surrounded by two membranes. The mitochondrial outer membrane is permeable for smaller molecules and ions, whereas the inner membrane is impermeable by passive diffusion, but contains the components of the respiratory chain and the enzyme complex responsible for ATP synthesis. The inner membrane, with a surface enlarged by extensive folding into cristae, encloses the mitochondrial matrix, which accommodates the components of the Krebs cycle, including pyruvate dehydrogenase, and the enzymes involved in the oxidation of lipids and amino acids. Accordingly, all metabolic pathways responsible for energy supply, except for glycolysis, are located in mitochondria (Lehninger, 1998). As a primary site of ATP production, mitochondria came into the focus of investigations of thermal adaptation. Observations of mitochondrial densities by electron microscopy showed that the volume fraction of fish muscle fibres occupied by mitochondria increased after seasonal cold acclimation (Johnston and Maitland, 1980; Egginton and Sidell, 1989; Sänger, 1993) and even more so in permanently cold adapted polar fish when compared to temperate zone species with a comparable ecology (Johnston et al., 1988; 1998). This process increases the aerobic capacity of the cell. A parameter that contributes to shape the capacity of aerobic energy metabolism of cells or tissues is the aerobic capacity of individual mitochondria. As compared to evolutionary cold adaptation where the capacity of mitochondrial respiration remains uncompensated (Johnston et al., 1998; Guderley, 1998), seasonal acclimation has been shown to increase mitochondrial capacity (per unit mitochondrial protein) in fish white and red muscle (Guderley and Johnston, 1996, St-Pierre et al., 1998). Similarly, total liver mitochondrial respiration rate of temperate eelpout *Zoarces viviparus* increased after cold acclimation and was found to be higher than in the polar relative *Pachycara brachycephalum* acclimated to the same temperature (Lannig et al., 2005).

A fast and effective way to estimate the overall mitochondrial capacity is the measurement of mitochondrial enzyme capacities, with two key-enzymes usually being analysed, namely citrate synthase (CS) and cytochrome *c* oxidase (COX). CS catalyses the initial

step of the Krebs cycle, the condensation of oxaloacetate and acetyl-CoA, provided by the oxidation of carbohydrates, lipids and amino acids, to citrate, which is gradually reoxidized to oxaloacetate, while providing reducing equivalents for the respiratory chain (figure 1). Thus it represents the link between the different catabolic pathways on the one hand and aerobic ATP production on the other hand. The second key-enzyme COX catalyses the terminal step of the respiratory chain, the reduction of oxygen to H_2O and hence represents the aerobic capacity of cells or tissues (figure 1).

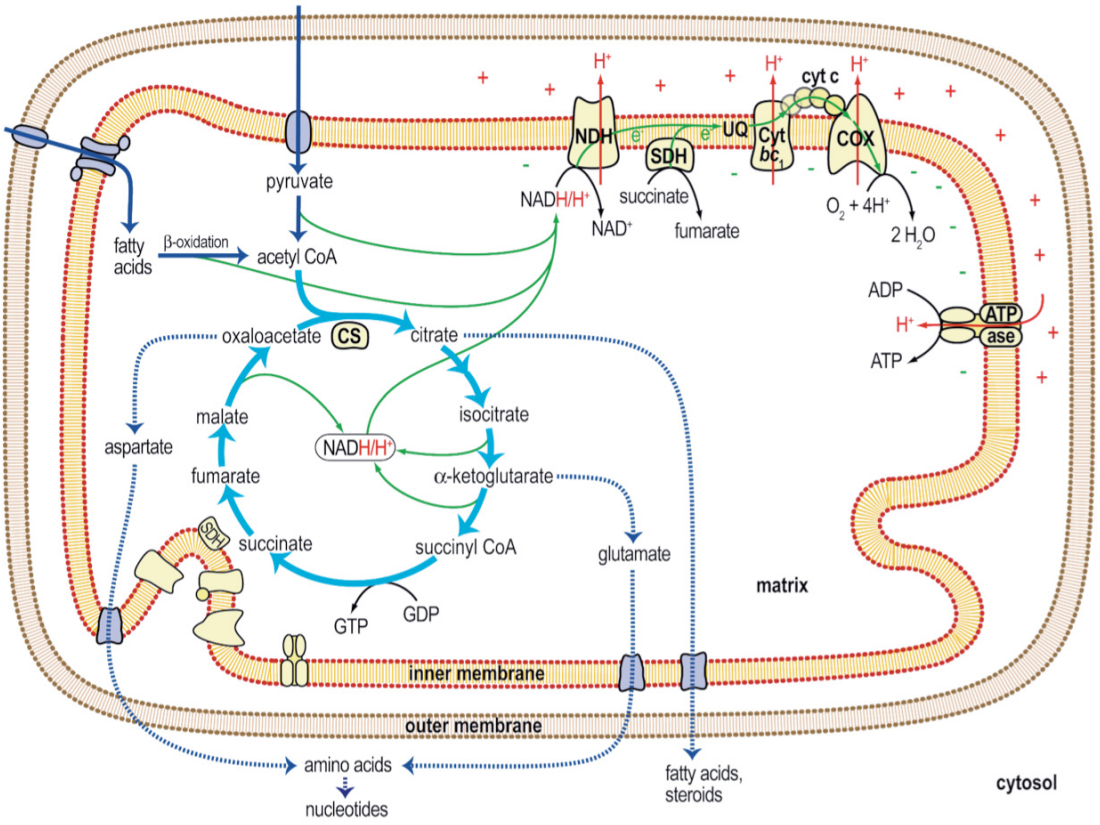

Figure 1: Schematic overview of the major pathways of energy metabolism in mitochondria. Blue arrows indicate the transport of pyruvate and fatty acids into mitochondria and further oxidation to acetyl CoA. The Krebs cycle is depicted by light blue arrows with the emanating anabolic pathways indicated by dashed arrows. Electron transfer to $NADH/H^+$ and through the electron transport chain (NDH: NADH-dehydrogenase; SDH: succinate dehydrogenase; UQ: ubiquinone; Cyt bc_1: cytochrome bc_1-complex; cyt c: cytochrome *c* and COX: cytochrome *c* oxidase) is illustrated by green arrows. Proton pathways in the respiratory chain and through the ATP synthetase complex (ATPase) are indicated by red arrows.

As an advantage over measurements of mitochondrial respiration rates, determination of relative changes in both enzymes also indicate changes of mitochondrial functions, since many intermediate products of the Krebs cycle act as precursors for various anabolic pathways (figure 1), e.g. the biosynthesis of lipids, steroids, particular amino acids and nucleotides (Lehninger, 1998). Furthermore, the determination of aerobic scope through

mitochondrial enzyme capacities allows dealing with samples stored frozen, which enables an inter-individual comparison of different tissues. For white and red muscle the increase of mitochondrial respiration rates during seasonal cold adaptation is commonly reflected at the enzymatic level, with higher capacities of both enzymes in cold than in warm acclimated fish (Battersby and Moyes, 1998; St-Pierre et al., 1998; Lannig et al., 2003; Lucassen et al., 2003; 2006). However, tissues other than muscle may display different patterns of enzyme activities with thermal acclimation. In the liver of temperate eelpout and cod, CS activities were elevated in the cold, whereas the activities of COX remained largely unaltered (Lannig et al., 2003; Lucassen et al. 2003; 2006). The increased ratio of CS over COX activity indicates removal of intermediate products from the Krebs cycle and may also indicate functional adaptation of mitochondria to tissue specific demands in the cold, most likely to enhanced lipid synthesis (Pörtner, 2002b). In trout liver, the incorporation of tritium into fatty acids was found to increase with lower acclimation temperatures, indicating elevated lipid biosynthesis (Hochachka and Hayes, 1962; Hazel and Sellner, 1979). Fatty acids are the main energy store in fish (Pitcher and Hart, 1982; Urich, 1990) and lipid fractions were shown to increase in the liver of cold acclimated eelpout and cod (Brodte et al., 2006; Eliassen and Vahl, 1982), with a consequence for liver sizes, indicated by elevated hepatosomatic indices (HSI).

Similar to cold acclimation, endurance exercise leads to increased aerobic capacities in fish muscle (Anttila et al., 2006; McClelland et al., 2006). In mammals shivering thermogenesis (Puigserver et al., 1998; Wu et al., 1999) and hormonal treatment (Nelson et al., 1984; Luciakova and Nelson, 1992) cause increased aerobic capacities. A decrease of mitochondrial capacities can also be observed following long-term exposure to low oxygen conditions in fish (Johnston and Bernard, 1982; Van der Meer et al., 2005). Mitochondrial biogenesis is complicated by the fact that the genes encoding for the enzymes of oxidative phosphorylation are located on both the mitochondrial and the nuclear genome. For instance, COX of vertebrates usually consists of 13 subunits, whereof three (I, II and III) are located on the mitochondrial genome (Grossman and Lomax, 1997). Several transcription factors such as nuclear respiratory factors (e.g. NRF-1) and the class of nuclear co-activators (e.g. PGC-1) are suggested to participate in the coordination of the expression of respiratory genes (Scarpulla, 2002). However, the primary effector(s) of the gene expression pathway(s) that alter mitochondrial capacities or functions during some or

all physiological challenges still need to be established (Leary and Moyes, 2000; Hood, 2001).

1.3 Signals for temperature adaptation

Several hypotheses about the nature of potential effectors that trigger mitochondrial proliferation during thermal adaptation of ectothermic organisms exist. As described above, mitochondrial capacities change unidirectionally with acclimation temperature within the thermal tolerance window, hence it was postulated that the triggering signal also has to operate unidirectionally, i.e. it should be increased at low temperatures and decreased at high temperatures, or vice versa (Pörtner, 2002a). This assumption draws attention to a potential role of extra- and intracellular pH in the coordination of thermal adaptation. The maintenance of cellular and especially enzyme functions requires a conservation of the protonation equilibrium of proteins. As the only amino acid featuring a dissociation-constant (pK) close to physiological pH values, histidine is the critical component in this relationship. The pK of the imidazole side chain of histidine exhibits a temperature-dependent change of, in average, -0.018 units per °C that matches the shift of extra- and intracellular pH with body temperature observed in many poikilotherms (Hochachka and Somero, 2002). Based on this observation, the alphastat hypothesis was introduced (Reeves, 1972), postulating that ectothermic organisms regulate the pH of their body fluids in order to maintain the protonation degree of imidazole groups.

Another common hypothesis is the involvement of higher systemic signals and in particular hormones (Umminger, 1978) that are known to control a wide range of physiological changes, including growth, maturation and reproduction. Although the endocrine system of teleost fish, in contrast to mammals, has not been thoroughly investigated, it has been suggested that basal effects are very similar in all vertebrates (Schmidt-Nielsen, 1999). Two classes of hormones are good candidates to participate in the process of thermal adaptation. On the one hand, hormones released by the thyroid gland are well known to exert profound effects on aerobic energy metabolism of mammals (cf. Goglia et al., 1999; Weitzel et al., 2003), but were also shown to increase the activities of CS in several tissues of catfish (Tripathi and Verma, 2003b) and of COX in mullet (LeRay et al., 1970). Thus, thyroid hormones might be involved in the regulation of temperature adaptation in poikilotherms. On the other hand, temperature shifts represent an acute stress situation for ectothermic animals, moving stress hormones into focus. In fish,

the stress response is mainly mediated by the catecholamines epinephrine and norepinephrine, which are produced by chromaffin cells of the head kidney almost immediately following the stress impulse, and the corticosteroid cortisol, which is released by the interrenal axis with slight delay compared to the catecholamines (cf. Wendelaar Bonga, 1997). Although the plasma levels of these hormones rapidly return to the initial concentrations when the stressor becomes chronic (Wendelaar Bonga, 1997), effects in the target tissues might persist, as already shown for the effect of cortisol in fish (Vijayan et al., 1991). The literature available on the effects of stress hormones on aerobic capacity of fish tissues and cells is only sparse. However, daily cortisol injections for one week have been reported to increase CS activities in the liver, brain and muscle of catfish (Tripathi and Verma, 2003a).

Finally, there is an attractive hypothesis that the bioenergetic disturbances themselves, induced by the rate limiting impact of temperature on physical and biochemical processes, trigger the adjustment of mitochondrial capacities. Several bioenergetic factors are discussed, including oxygen availability, nucleotides, phosphate, and fatty acids (Leary and Moyes, 2000); and only recently nitric oxide (NO) emerged to be a promising candidate in mammals (Nisoli et al., 2004). Of particular interest concerning temperature might be the metabolite adenosine, which was mostly examined in the context of hypoxic or anoxic conditions (Lutz and Kabler, 1997; Reipschläger et al., 1997; Renshaw et al., 2002). In general, adenosine is predominantly produced when energy demand exceeds energy supply, as a result of the breakdown of cytosolic ATP to AMP, followed by dephosphorylation to adenosine (Phillips and Newsholm, 1979). Since it is released from the cell by nucleoside transporters (Meghji et al., 1989), it can be distributed to the whole body and hence can act as a signal eliciting acute suppression of energy metabolism (cf. Buck, 2004). Adenosine exerts various physiological effects, including a reduction of protein synthesis rate (Tinton et al., 1995), a stimulation of anaerobic glycolysis (Lutz and Nilsson, 1997) and also a decrease of oxygen consumption in trout hepatocytes (Krumschnabel et al., 2000). These changes appear suitable to balance mismatches in energy demand and supply during periods of low energy availability. Hence adenosine is often denominated a "retaliatory metabolite" (Newby et al., 1990) and might also be involved in reverting any imbalances induced by temperature effects.

1.4 Biological model system

The analysis of the effect of single biotic or abiotic parameters on energy metabolism requires a well-controlled biological model system that allows the experimental adjustment of individual factors. These requirements can be met by both *in vivo* models, e.g. during application of different temperatures or the injection of biochemical reagents under controlled experimental conditions, or by *in vitro* systems like primary cell cultures. Compared to *in vivo* models for mechanistic studies, cell cultures offer several advantages, from ethical and economical considerations due to lower demand for living animals, to scientific benefits, such as better control of environmental impact factors and reduced influence of individual variations by the possibility to apply several conditions in parallel experiments. For mechanistic studies, with the main focus on thermal adaptation of aerobic energy metabolism in fish, isolated liver cells are a suitable *in vitro* model. The liver of fish is, as in other vertebrates, characterized by a high aerobic scope and, as described above, exhibits clear evidence for temperature adaptation at the molecular (enzyme capacities), cellular (lipid stores) and whole organ levels (HSI). Furthermore, short- and long-term studies of isolated hepatocytes have become quite popular in the last decades, thus a wide range of literature exists about isolation and culture techniques, even for fish.

A first method to isolate high yields of viable hepatocytes was presented in 1969 for mammalian liver (Berry and Friend, 1969). The study attracted wide attention and only a few years later, techniques were introduced to maintain isolated cells for several days or weeks (Bissell et al., 1973). Cell culture allowed hepatocytes to recover from stress, induced by the isolation procedure and extended the range of potential applications to the study of more long-term, time-dependent effects. In the late 1970's the method to isolate viable liver cells was applied in research using lower vertebrates. The first adaptation for teleosts was presented for goldfish (Birnbaum et al., 1976) and was followed by many protocols for several, mainly fresh-water fish species (Hazel and Prosser, 1979; Moerland and Sidell, 1981), and more recently, also for marine teleosts (Morrison et al., 1985; Walsh, 1987). Finally, techniques for maintaining isolated fish liver cells over periods of several days up to weeks were introduced (Koban, 1986, Blair et al., 1990). However, teleost fish are the most diverse group among vertebrates, with over 25 000 species and a great ecological and physiological variety. Accordingly, there is no uniform protocol for

the maintenance of fish hepatocytes, but cell isolation methods and culture conditions have to be established for each new species.

Figure 2: The common eelpout *Zoarces viviparus* (L.).

For the present thesis, the common eelpout *Zoarces viviparus* (Linnaeus, 1758) was chosen for the study of mechanisms of temperature adaptation in ectotherms (figure 2). This eurythermal fish has been the subject of ecological, physiological and molecular investigations (Hardewig et al., 1998; Lucassen et al., 2003; Brodte et al., 2006) and has become a model organism to study thermal acclimation and limitation in the recent years (Pörtner and Knust, 2007). *Zoarces viviparus* belongs to the subfamily Zoarcinae, a member of the family Zoarcidae (eelpouts) within the order Perciformes (Anderson, 1994) and inhabits benthic coastal areas in boreal climates from the southern Wadden Sea and the Baltic Sea (Vetemaa, 1998) up to the White Sea (Knijn et al., 1993). Individuals examined in the present study were collected in the German Bight of the North Sea and in the Kiel Bight of the Baltic Sea where they experience annual temperature fluctuations of 4 – 16°C (Fischer, 2003) and 0 – 14°C (Dippner, 1999), respectively. The common eelpout leads a mostly sedentary lifestyle and feeds on polychaetes, crustaceans and molluscs (Knijn et al., 1993). It displays an ovoviviparous reproduction cycle, where ovulation and internal fertilization occur in late summer and the offspring is released in the winter (Goetting, 1976).

1.5 Thesis outline

The key objective of this thesis is to contribute to the understanding of regulatory mechanisms involved in the thermal adaptation of ectothermic animals. The study focuses on the above-presented hypothesis that adjustments of mitochondrial capacities are induced by a primary effector, associated with the functional state of the organism. To investigate this assumption, the following aspects have been addressed:

1. Development of a primary cell culture for eelpout hepatocytes.

The establishment of an *in vitro* cellular system for the model organism *Zoarces viviparus* should provide the basis for well-controlled mechanistic studies and allow for the high-performance screening of potential signals. As a precondition the long-term viability and physiological integrity of eelpout liver cells had to be established for a time period sufficient to induce a change in mitochondrial capacities.

2. Can adjustments of mitochondrial functions observed *in vivo* be induced *in vitro* with temperature as a single factor?

The comparison of the capacities of mitochondrial enzymes after temperature acclimation of whole animals (*in vivo*) and isolated cells (*in vitro*) was designed to test for the basic ability of liver cells to thermally adapt without any higher systemic input and, alternatively, for the requirement of an additional signal.

3. Does the application of potential effectors induce changes of mitochondrial functions?

The determination of mitochondrial enzyme activities of isolated hepatocytes after application of various effectors at different incubation temperatures was performed to detect alterations of mitochondrial capacities or functions and should hence detect potential signals that participate in the regulation or modulation of thermal adaptation.

4. Does temperature or the application of potential signals affect the expression pattern of mitochondrial enzymes?

The expression of citrate synthase and of a mitochondrial and a nuclear encoded subunit of cytochrome *c* oxidase was measured to observe shifts of mitochondrial capacities and functions at the transcript level. This part of the thesis was designed to provide insight into the coordination of mitochondrial biogenesis and the level of biological organisation at which the different effectors operate.

2 Material and Methods

2.1 Animals

The study was carried out with common eelpout *Zoarces viviparus* from the North Sea. All animals used for the preparation of hepatocytes were kept in the aquarium of the Alfred-Wegener-Institute, Bremerhaven, at a salinity of 30‰ and constant temperatures under a 12:12 h light-dark cycle for at least three months to guarantee full acclimation. They were fed once per week with live *Crangon crangon*. Due to limited availability of North Sea eelpout, basic cell culture conditions were established using Baltic Sea specimens obtained from the Kieler Förde in summer 2001, kept at 11.0 ± 0.5°C and a salinity of 18‰. Further adjustments of culture conditions were carried out with *Z. viviparus* collected in March 2002, maintained at 11.0 ± 0.5°C. A first series of experiments meant to test techniques and investigate feasibility was carried out with these animals acclimated to 4.0 or 11.0 ± 0.5°C in December 2003/January 2004. A second incubation series was carried out during the following year with animals collected in April 2004 and acclimated to either 4.0 or 11.0 ± 0.5°C, before experiments were carried out from July to September 2004.

Adenosine concentrations were determined in tissues of laboratory-born offspring (1st generation) of common North Sea eelpout *Zoarces viviparus*, caught in the German Bight near Helgoland. Fish were raised in the aquarium of the Alfred-Wegener-Institute, Bremerhaven at 10.0 ± 0.5°C and 30‰ salinity under a 12:12 h light-dark cycle and fed twice per week with small shrimps (*Neomysis integer, Crangon crangon)*. Animals (mass: 13.5 ± 1.2 g) were acutely transferred to 4.0 ± 0.5°C and sampled before (t = 0) and after 1 and 3 days of cold incubation.

2.2 Cell culture

2.2.1 Isolation of hepatocytes

Hepatocytes were isolated following a procedure modified after Mommsen et al. (1994). During summer experiments, for each cell culture two fish from the same acclimation temperature were prepared simultaneously to obtain a sufficient number of cells. Animals were anaesthetised with 0.5 $g \cdot l^{-1}$ (3-amino-benzoic-methanosulfonate) and killed by a cut through the spinal cord. The liver of the first fish was carefully excised, weighted and immediately transferred to ice-cold solution 1 (magnesium-free Hank's medium, containing, in mM, 240 NaCl, 10 HEPES, 5.5 glucose, 5.4 KCl, 4.2 $NaHCO_3$, 0.4 KH_2PO_4,

0.3 Na_2HPO_4; pH 7.4) and perfused through the *Vena hepatica* with solution 1 to remove blood cells. The second liver was prepared accordingly. Subsequently both livers were transferred to one vial and each organ was perfused two times with 2 $ml \cdot g^{-1}$ fresh weight ice-cold collagenase solution (solution 1 + 1% bovine serum albumin (BSA) + 750 U ml^{-1} collagenase type IV). Between perfusions livers were gently massaged for about 10 min. After finely discerping the tissue, the suspension was gently shaken on ice for 60 min. Finally, it was filtered through 250 μm mesh-size gauze, and hepatocytes were collected by centrifugation (4 min at 70 g, 0°C) and washed by repeated centrifugation (2 min at 70 g, 0°C) in solution 1 containing 1% BSA to remove collagenase, lipids and erythrocytes. Cells were resuspended in culture medium (Leibovitz L-15 + 103 mM NaCl + 10 mM HEPES + 1% BSA + 5 mM glucose + 1% penicillin/streptomycin; pH 7.8) and shaken on ice until being dispersed for primary cell culture. Cell density and viability were determined in a Fuchs-Rosenthal haemocytometer dish by trypan-blue exclusion as described in chapter 2.2.3.

2.2.2 Cell culture conditions

The adjustment of culture conditions for liver cells from *Z. viviparus* is described in chapter 3.1 of the results section. Given below are the final conditions applied for eelpout hepatocytes.

For primary cell culture sub-samples of 2 million viable cells were portioned in uncoated polystyrene 6-well plates (Nunc, Roskilde, Denmark), each well containing 2 ml of culture medium. Incubation occurred under air without an exchange of culture medium, but the addition of 5 μmol glucose per 10^6 cells and day.

During initial investigations, carried out in winter, cells isolated from cold acclimated eelpout were incubated at different medium pH values of 7.8 (control conditions), 7.4, 7.2 and 6.5. Cells were incubated for up to 96 h at either 4.0 or 11.0 ± 0.1°C under control conditions or at 4.0 ± 0.1°C for dissenting media pH. The pH of the culture medium was adjusted at 4°C for all incubations using HCl and NaOH. In a further incubation series during this winter, hepatocytes prepared from warm acclimated *Z. viviparus* were incubated at 11.0 ± 0.1°C for up to 120 h under control conditions (ctrl) or under the addition of 0.1 μmol adenosine (ado), 0.1 nmol cortisol (cor) or 0.1 nmol epinephrine (epi) per 10^6 cells and day. Cells were sampled after 48 h and, subsequently, once every 24 h.

Hepatocytes isolated from 4 and 11°C acclimated eelpout in summer were cultured at 4.0 and 11.0 ± 0.1°C. At each temperature cells were incubated under control conditions or treated with 0.1 μmol adenosine, 0.1 or 10.0 nmol cortisol or epinephrine per 10^6 cells directly after dispersion and once every 24 h thereafter. Due to limited cell numbers available from cold-acclimated fish, cortisol incubation at 11°C had to be omitted for this group. To investigate the potential role of adenosine receptors, one group of cells was incubated for 30 min with 0.1 μmol 8-phenyltheophylline (8-PT) per 10^6 cells, a selective adenosine A_1-receptor antagonist, always prior to addition of adenosine. In another group adenosine was replaced with 0.1 μmol 5'-(N-ethylcarboxamido) adenosine (NECA) per 10^6 cells, a non-selective adenosine receptor agonist. Samples were collected after 48 and 72 h of incubation.

The specifications of the effectors, agonist and antagonists used for both incubation series are summarized in table 1.

Table 1: List of reagents applied to the hepatocytes. Given are the concentration and solvents of the stock solutions and the final concentrations used in cell culture. Reagents were added once every 24 h in a volume of 10 μl·ml^{-1} cell culture; if required solutions were prediluted in culture medium.

reagent name	stock solution	final concentration
Epinephrine	10 mM in 1 M NaOH	0.1 μM, 10 μM
Cortisol	10 mM in Ethanol	0.1 μM, 10 μM
Adenosine	10 mM in 0.05 M HCl	100 μM
8-Phenyltheophylline	10 mM in 0.1 M NaOH	100 μM
5'-(N-Ethylcarboxamido)adenosine	10 mM in 0.05 M HCl	100 μM

For the sampling of hepatocytes culture dishes were transferred on ice without shaking, and 1 ml of culture medium was removed. Cells were resuspended in the remaining culture medium and precipitated by centrifugation (2 min at 1000 g, 0°C). After residual medium was carefully removed, cells were immediately frozen in liquid nitrogen. Since a new steady state was established within the first 48 h of incubation under all tested conditions for both enzyme activities (cf. chapter 3.1.3) and no differences were found between time points (multi-factorial ANOVA), values determined in samples drawn after 48 – 120 h were pooled and treated as replicates.

2.2.3 Cellular viability

Viability of hepatocytes was determined by trypan-blue exclusion. For freshly isolated hepatocytes, a sub-sample of the obtained cell suspension was diluted with 4 volumes of culture medium and combined with 1:6 volumes of 0.4% trypan-blue (Sigma, Steinheim, Germany). To monitor viability during cell culture, hepatocytes were resuspended in their culture medium and mixed with 0.4% trypan-blue. Numbers of cells that excluded or took up the dye were counted in a Fuchs-Rosenthal haemocytometer dish.

2.3 Enzyme activities

Both mitochondrial enzymes, cytochrome c oxidase (COX) and citrate synthase (CS) were isolated using the same extraction protocol, modified after Lucassen et al. (2003). Frozen samples of hepatocytes were combined with 150 μl of ice-cold extraction buffer (20 mM Tris-HCl, 1 mM EDTA, 0.1% Tween 20, pH 7.4) and thawed on a Vortex Genie2 (Scientific Industries, New York, NY, USA) during shaking for 2 min at highest speed.

2.3.1 Cytochrome c oxidase (COX)

COX activity was determined according to Moyes et al. (1997) in 20 mM Tris-HCl pH 8.0, containing 0.5 % Tween 20 and 0.05 mM reduced cytochrome *c*. Cytochrome *c* was reduced by adding abundant sodium dithionate and purified by G-25 gel filtration. Oxidation of cytochrome *c* was monitored by the decrease in extinction at λ = 550 nm in a thermostatted spectrophotometer (DU 7400, Beckman, Fullerton, CA, USA) at 20°C. COX activity was determined from the slope obtained within the first 1.5 min after starting the reaction by the addition of homogenate sample. Autoxidation of cytochrome *c* was recorded for each assay within at least 2 min prior to sample addition and subtracted from the slope. For the calculation of enzyme activity in units per 10^6 cells an extinction coefficient (ε_{550}) for cytochrome *c* of 19.1 mM^{-1} cm^{-1} was used.

2.3.2 Citrate synthase (CS)

CS activity was determined following Sidell et al. (1987) in 75 mM Tris-HCl, pH 8.0 with 0.25 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), 0.4 mM acetyl CoA and 0.4 mM oxaloacetate. Prior to measurement, homogenates were sonicated for 5 min at 0°C in a bath sonicator (Branson, Danbury, CT, USA) with a duty cycle of 50% at 360 W. The formation of DTNB-S-CoA was monitored by following the increase in extinction at

$\lambda = 412$ nm in a spectrophotometer at 20°C. Enzyme activity in units per 10^6 cells was calculated from the slope using the extinction coefficient of 13.6 mM^{-1} cm^{-1} for the dye complex.

2.4 Protein quantification

Cellular protein content was measured in the enzyme extracts using the Bradford method (Bradford, 1976) at a wavelength of λ = 595 nm. Extinction was determined in a spectrophotometer (Biochrom 4060, Pharmacia, Cambridge, UK) after 5 min of incubation at room temperature. Protein concentrations were calculated using a standard curve created with BSA in the range of 0 – 10 μg protein/ml.

2.5 Adenosine concentrations

Adenosine concentrations were determined in medium samples from adenosine treated cells, collected during cell culture always prior to the addition of fresh adenosine and in the liver and blood serum of acutely cold exposed *Z. viviparus*.

Blood was collected from fish anaesthetised with 0.5 $g \cdot l^{-1}$ MS-222 by opening the caudal vein. Livers were excised, immediately frozen in liquid nitrogen and animals were killed by a cut through the spinal cord. Blood was stored on ice for 4 h to allow coagulation, centrifuged for 10 min at 5,000 *g* and the serum was transferred into fresh reaction tubes. Preliminary tests confirmed the adequacy of this sampling procedure, which kept adenosine levels close to or below detection limits in unstressed animals.

Serum samples were mixed with 0.2 volumes ice cold TCA (15% trichloracetic acid), sonicated for 1 min at 0°C in a bath sonicator (Branson, Danbury, CT, USA) and centrifuged (4 min at 16,000 g, 0°C) to precipitate proteins. The supernatants were neutralized with 4 volumes tri-n-octylamine/1,1,2-trichlortrifluorethane mix (1:4), centrifuged for 3 min at 16,000 g, 0°C, and the upper phase was collected. Frozen liver samples were pulverized under liquid nitrogen, suspended in 3.5 volumes ice cold TCA and processed as described for serum samples, but the pH of the extracts was adjusted to 9.0 – 9.4 with 2 M NaOH. Medium samples were mixed with 3.5 volumes ice cold TCA (15%), sonicated for 1 min at 0°C in a bath sonicator and further processed according to liver samples.

Adenosine was determined by capillary electrophoresis (P/ACE™ System MDQ, Beckman, Fullerton, CA, USA) using a method modified after Casey et al. (1999). Extracts were combined with 0.4 mM uric acid as an internal standard and filtered through a 0.2 μm syringe filter. Samples were separated on a 50 μm diameter uncoated fused silica capillary with a current of 30 kV at 40°C. Adenosine peaks were identified by migration time and sample spiking. Adenosine concentrations were calculated from the area ratio of adenosine/uric acid using a calibration curve created with concentrations between 0.5 – 50 μM ($y = 870.667\ x - 0.069$; $r^2 = 0.9989$) adenosine for serum and liver samples and between 4.4 – 44.4 μM adenosine for medium samples ($y = 1096.159\ x - 0.896$; $r^2 = 0.9976$).

2.6 RNA isolation and quality control

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). Frozen samples of $2 \cdot 10^6$ hepatocytes were lysed in 700 μl RLT buffer provided with the kit during shaking on a Vortex Genie2 for 2 min at highest speed and further processed as described in the manufacturer protocol for animal cells. RNA from frozen liver samples was isolated according to the manufacturer's protocol for animal tissues. Total RNA was eluted in RNase free TE-buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and concentrations were quantified in a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany).

The quality of RNA was monitored by A_{260}/A_{280} ratios with values always > 1.8. Additionally, the integrity of hepatocyte RNA was verified by formaldehyde agarose gel electrophoresis (1.0% agarose, 20 mM MOPS, 8 mM sodium acetate, 1 mM EDTA, pH 8.0, 12.3 M formaldehyde) according to Sambrock et al. (1989) (figure 3).

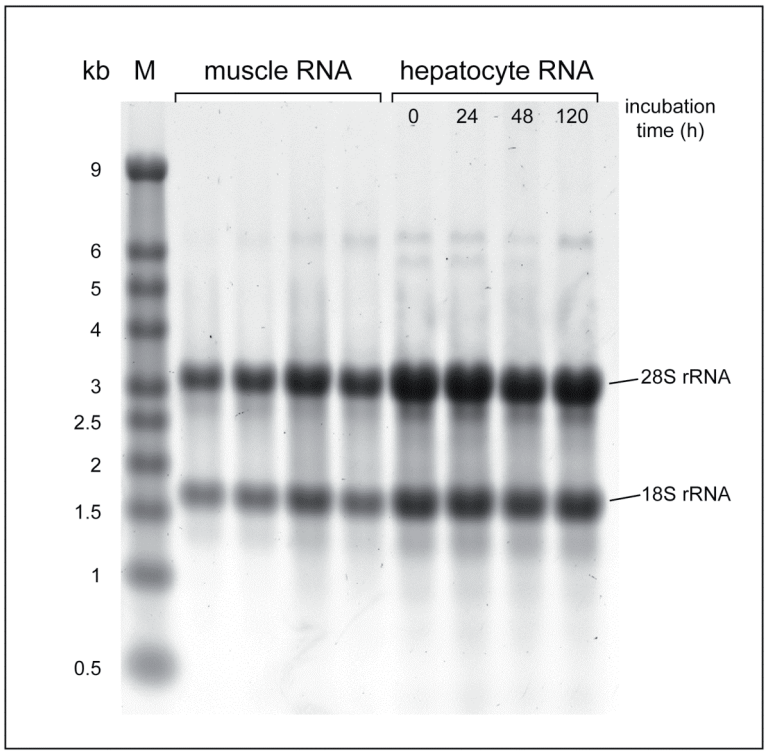

Figure 3: Formaldehyde agarose gel of total RNA isolated from eelpout hepatocytes. RNA was isolated from hepatocytes directly after preparation and after 24 – 120 h of incubation under control conditions. Muscle RNA of verified quality obtained from North Sea eelpout (provided by A. Schmidt) was added for comparison. 10 μg sample was loaded per lane. 2 μg of the RNA Millennium™ marker (Ambion, Austin, TX, USA) was applied as a size standard (M). The lengths of the standard fragments and the 28S and 18S fragment of the ribosomal RNA (rRNA) are indicated.

2.7 Sequence determination

2.7.1 Reverse transcription and polymerase chain reaction

Reverse transcription of mRNA and amplification by polymerase chain reaction (RT-PCR) was conducted as described by Lucassen et al. (2003). mRNA was isolated from total liver RNA of *Z. viviparus* using the Oligotex kit (Qiagen, Hilden, Germany) according to the users protocol, and eluted in Oligotex elution buffer (5 mM Tris HCl, pH 7.5) to an end concentration of ~ 0.1 μg ml^{-1}. Primers for RT-PCR (table 2) were designed using the MacVector 7.2 program package (Accelrys, Oxford, UK), homologous to the cDNA sequence available for *Thunnus obesus* cytochrome *c* oxidase subunit IV (COX4) (Hüttemann, 2000; GenBank accession no. AF204870). 0.4 μg mRNA were used for the reverse transcription, carried out with 400 U Superscript RT (Invitrogen, Karlsruhe, Germany) and 6 pmol of the reverse primer in 20 mM Tris HCl, pH 8.4, 50 mM KCl, 10 mM DTT, 2.5 mM $MgCl_2$ and 0.625 mM of each desoxynucleotide (dNTP). The reaction was incubated for 1 h at 37°C and terminated by 20 min of heating at 70°C. The

following PCR reaction was performed with 1.5 mM $MgCl_2$ using the degenerated primer pairs from table 2 in a gradient cycler (Tgradient, Biometra, Göttingen), applying 32 cycles of 45 sec denaturation at 94°C, 2 min annealing at 57 ± 6°C and 1 min elongation at 72°C. PCR fragments were separated by gel electrophoresis (1.5% agarose, 45 mM Tris-borate, pH 8.3, 1 mM EDTA) according to Sambrock et al. (1989) and purified using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Fragments were cloned with the TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany), according to the users manual. Plasmids were extracted from over night culture with the Qiaprep Spin Miniprep kit (Qiagen) and tested for the presence and size of inserts by restriction digestion with *Eco*RI (Invitrogen, Karlsruhe, Germany). DNA sequences of positive clones were determined by MWG-Biotech (Ebersberg, Germany) and analysed using MacVector 7.2.

Table 2: Primer list for RT-PCR and RACE. All sequences are written from 5' to 3'. The positions correspond to the position of the following genes: *Z. viviparus* cytochrome *c* oxidase subunit IV (COX4, COX4-3'; COX4-5'; COX4-a/b/c) (GenBank accession no. EF175142) and subunit II (COX2-a) (Lucassen et al., 2003; GenBank accession no. AY227660).

Fragment	Term	Sequence	Position	Length
COX4	Cox4-F5-M Cox4-B4	CTGAAGGAGAAGGAGAAGG CRGTGAARCCGATRAAGAAC	232-250 378-359	157
COX4-3'	Cox4-RACE-F1 Cox4-RACE-F2	TGAAGGAGAAGGAGAAGGGC CTGTGGTTGGAGGGATGTTC	233-252 353-372	517
COX4-5'	Cox4-RACE-B1 Cox4-RACE-B2 Cox4-RACE-B3	GCGGTACAATCCAATCTTCTCCTC CAACCACAGATTTCCACTCTTGC CGATAAAGAACATCCCTCCAACCAC	297-274 361-339 379-355	297
COX4-a	Cox4-F6 Cox4-B5	TGGGACTACGAAAACAAGCAGTG GCAATGAACATAAGAGGAAGAGGC	526-548 654-677	175
COX4-b	Cox4-F7 Cox4-B5	ACCCATAAGACCATAACCCACTTG GCAATGAACATAAGAGGAAGAGGC	562-588 654-677	139
COX4-c	Cox4-F8 Cox4-B6	CCTTCGCCTTGTTGGAAAACG TTCTCCTTCTCCTTCAGGGACAC	66-86 248-226	183
COX2-a	Cox2-F3 Cox2-B2	TTATCCTTATCGCCCTGCCC CATCAGCGGAGACTAAAACG	145-164 399-380	255

2.7.2 Rapid amplification of cDNA ends

The 5'- and 3'-terminal ends of COX4 mRNA were identified using the RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) kit (Ambion, Austin, TX, USA) according to the manufacturer's manual. Gene-specific backward primers for 5'-RACE and forward primers for 3'-RACE (table 2) were designed homologous to the partial sequence determined for COX4 of *Z. viviparus* by RT-PCR. Forward primers for the amplification of the 5'-terminus and backward primers for the 3'-terminus, corresponding to the adapter sequence, were provided with the kit. For PCR reaction 35 cycles of 45 sec denaturation at 94°C, 90 sec annealing at 57 ± 6°C and 1 min elongation at 72°C were applied. Cloning and analysis of positive clones was performed as described for RT-PCR fragments.

The derived cDNA sequence and the deduced amino acid sequence for COX4 of *Z. viviparus* (figure 4) was compared to the coding sequences and protein sequences of four other vertebrates available in the GenBank database using MacVector 7.2. The nucleotide and the protein sequences of COX4 were highly conserved within fish (*Z. viviparus*, *T. obesus* and *D. rerio*), with identities between 73 – 85% and 74 – 82%, respectively, but also shared more than 60% of identity with the human (*H. sapiens)* or frog (*X. laevis*) sequence (table 3).

Table 3: Interspecies comparison of cDNA and protein sequences of COX4. Percentage identities of nucleic acid (blue) and the deduced peptide sequence (yellow) were determined by comparison of the COX4 sequence obtained for *Z. viviparus* (GenBank accession no. EF175142) and the COX4 sequences available for *Thunnus obesus* (GenBank accession no. AF204870), *Danio rerio* (GenBank accession no. NM214701), *Xenopus laevis* (GenBank accession no. BC078463) and *Homo sapiens* (GenBank accession no. M21575).

	Z. viviparus	*T. obesus*	*D. rerio*	*X. laevis*	*H. sapiens*
Z. viviparus		85%	73%	62%	66%
T. obesus	82%		77%	65%	67%
D. rerio	74%	79%		64%	64%
X. laevis	60%	65%	63%		64%
H. sapiens	60%	60%	63%	65%	

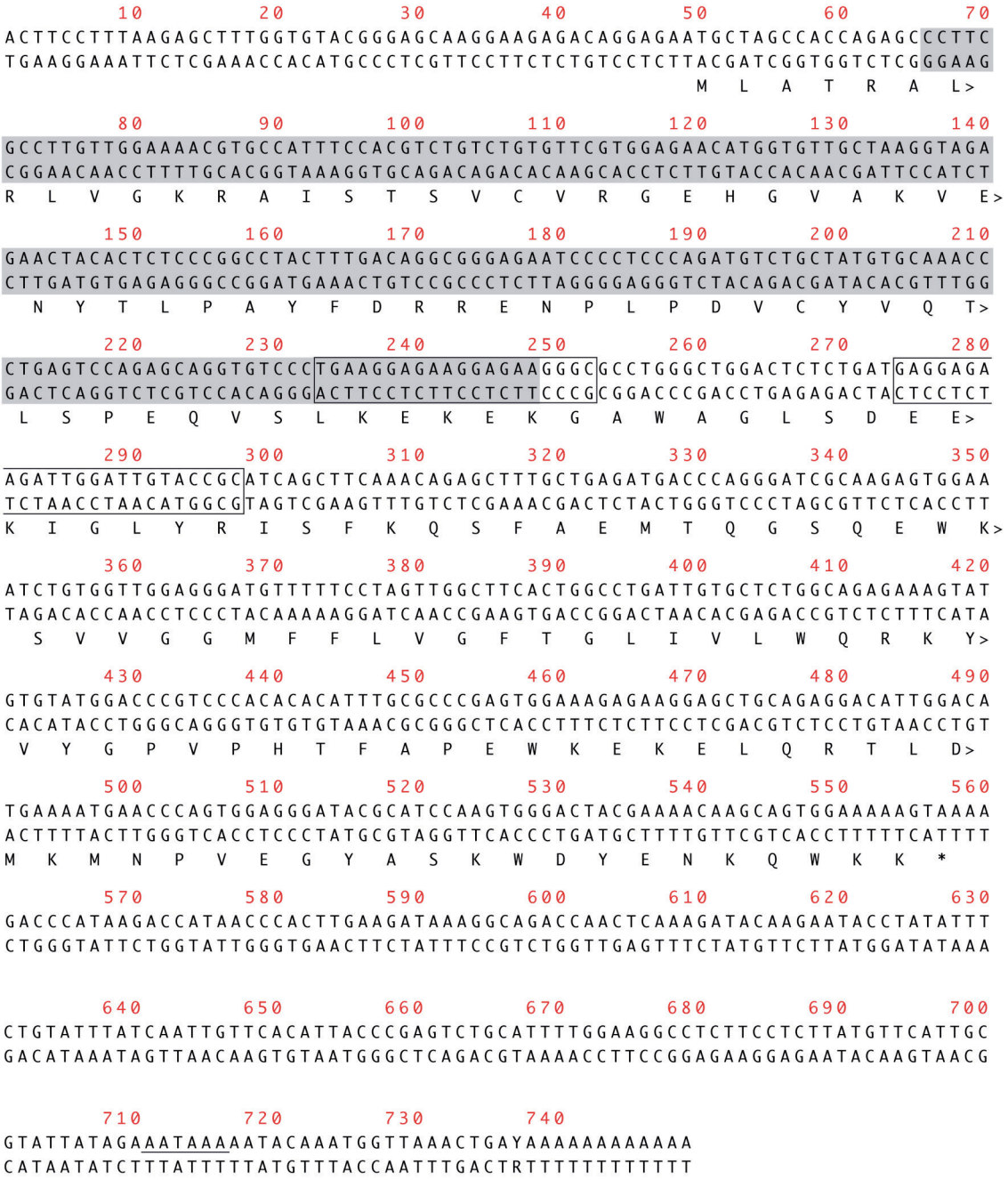

Figure 4: Full-length cDNA sequence of cytochrome *c* oxidase subunit 4 (COX4) from *Z. viviparus*. The consensus sequence was determined by alignment of RT-PCR and RACE fragments and resulted in 1 open reading frame (49 – 558). The deduced amino acids are given below the cDNA sequence. The binding positions of the primers Cox4-RACE-F1 (233 – 252) and Cox4-RACE-B1 (274 – 297) used for RACE-PCR are boxed; the polyadenylate signal is underlined. The fragment used as a probe for the RPA is highlighted in grey.

2.8 Quantification of specific mRNA

2.8.1 Construction of radiolabeled probes

For construction of a reliable COX4-specific probe, three primers pairs for fragment sizes between 139 and 183 bp were designed corresponding to the determined sequence (table 2). RT-PCR was performed as described above, but with an annealing temperature of 52°C for COX-a and COX-b. According to a preliminary test of all three probes, the 183 bp fragment (COX4-c) was applied for determination of COX4 mRNA levels. For COX2, a 255 bp fragment was isolated from an existing 507 bp fragment (Lucassen et al. 2003; accession no.: AY227660) by PCR with the primers given in table 2, applying the PCR conditions described before with an annealing temperature of 55°C. Existing fragments were used for the construction of CISY (369 bp; accession no.: AY382597) and β-actin (215 bp; accession no.: AY227657) probes (Lucassen et al. 2003; Mark et al., 2006).

To obtain linearised templates for the construction of antisense probes, PCR clones were digested with *Pst*I (CISY, COX2) or *Not*I (COX4, β-actin), depending on the direction of the insert within the plasmid. *Pst*I digested fragments were treated with Klenow fragment and dNTPs (Invitrogen, Karlsruhe, Germany) to remove protruding 3' ends. [α-^{32}P]UTP labelled probes were generated using the MAXIscript kit (Ambion, Austin, Texas) and purified by polyacrylamid gelelectrophoresis (PAGE) under denaturating conditions (6% acryl amide gel, 8 mM urea, 90 mM Tris-borate, pH 8.3, 2 mM EDTA) according to Lucassen et al. (2003). Equal intensities for protected fragments were achieved by adjustment of specific radioactivity, applied as follows: 1000 Ci/mmol for CISY and COX4 and 45 Ci/mmol for COX2 and β-actin.

2.8.2 Ribonuclease Protection Assay

The amounts of gene-specific RNA transcripts were quantified via ribonuclease protection assay (RPA) using the RPA III kit (Ambion, Austin, Texas) with β–actin as an internal standard to correct for loading differences. Due to small sample sizes for hepatocytes, the assay was adjusted to low RNA amounts (2 μg) following the user's manual. The amount of each radio-labelled probe was reduced to 20000 cpm per sample and hepatocyte RNA was supplied with an equal quantity of yeast RNA, to improve the formation of pellets. Sample RNA was hybridized simultaneously to all antisense probes at 42°C. RNase treatment was performed with an RNaseA/T1 dilution of 1:100. RNA:RNA hybrids were

precipitated and separated by denaturating PAGE as described above (2.8.1). Radioactivity was detected and quantified in a phosphor storage imaging system (FLA-5000, Fuji, Tokyo, Japan) using the AIDA software package (raytest, Straubenhardt, Germany). A representative example is given in figure 5.

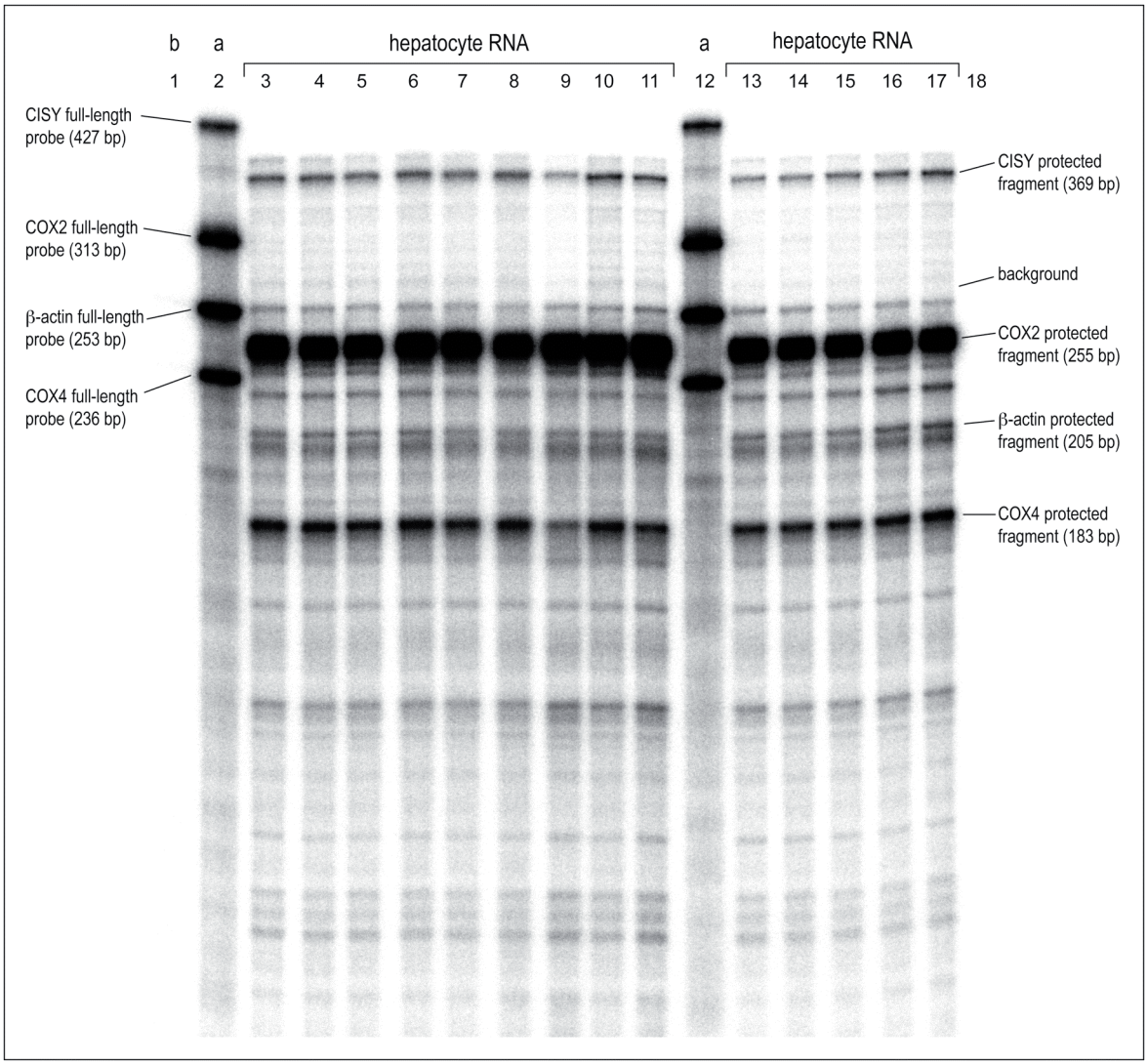

Figure 5: Representative picture of an RNase Protection assay performed with hepatocyte RNA samples. Probes for CISY, COX2, COX4 and β-actin, protected from RNase digestion by hybridization to hepatocyte RNA (lane 3 – 11, 13 – 17) and the length of the protected fragment are indicated on the right. The undigested full-length probes (a), coprecipitated with unspecific yeast RNA, are marked on the left. To verify the efficiency and specificity of the assays, probes incubated with unrelated yeast RNA were digested according to the hepatocyte samples (b). A background radiation equal to the probe area was determined for each lane closely above each protected fragment and subtracted from the probe intensity.

2.9 Statistical analysis

Statistical significance was tested at the $P \leq 0.05$ level. Prior to analyses, data were tested for Gaussian distribution and equal variances. Outliers were identified using Nalimov's test at the 95% significance level (Noack, 1980).

Time-dependence of cellular survival and differences between incubation conditions for establishment of primary cell culture conditions was analysed using one-factor ANOVA and multi-factorial ANOVA, respectively, and the Student-Newman-Keuls test as the post hoc test. Differences in freshly isolated hepatocytes were analysed using unpaired t-test. The impact of *in vivo* acclimation temperature in primary cell culture was observed with multi-factorial ANOVA. Repeated measures ANOVA and the post hoc Student-Newman-Keuls test were applied to analyse the effects of different treatments in each acclimation group. Adenosine concentrations were analysed using one-factor ANOVA and the post hoc Student-Newman-Keuls test. Linear regressions and squared correlation coefficients were calculated using SigmaStat 3.0. Unless stated otherwise, data are given as means ± range ($n = 2$) for development of cell culture (chapter 3.1) and as means ± SE ($n = 3 – 11$) for incubation experiments and adenosine measurements.

3 Results

3.1 Development of a primary liver cell culture for *Z. viviparus*

Preparation of hepatocytes was performed according to a protocol established for the Antarctic eelpout *Pachycara brachycephalum* (Langenbuch and Pörtner, 2003; Mark et al., 2005). The method worked efficiently for common eelpout *Zoarces viviparus*. Although the cell yields exhibited a great variation between preparations, hepatocytes were of good condition and showed only little uptake of trypan-blue dye. Cellular viability directly after isolation was always > 90%, providing the basis for the primary culture of eelpout hepatocytes.

In contrast to isolation techniques, there was no literature available on long-term maintenance of eelpout hepatocytes. Since the great ecological and physiological diversity of teleost fish prevents the adoption of protocols suitable for other species, the appropriate culture conditions for eelpout had to be established.

3.1.1 Basic incubation conditions and culture medium

Due to limited availability of North Sea eelpout, the development of cell culture condition was carried out on hepatocytes isolated from Baltic Sea specimens. Initial conditions were defined as follows: Media were complemented with 20 mM HEPES and 1% penicillin/streptomycin according to a protocol for European flounder (Winzer et al., 2002). Medium pH was adjusted to 7.8 at 4°C, resulting in a pH of 7.6 at 11°C. According to their original acclimation temperature, cells were cultured at 11.0 ± 0.1°C under moistured air atmosphere in the dark. Since hepatocytes tended to reaggregate to larger cell-clusters within a few hours, cells were portioned directly after preparation and cultured in 6-well polystyrene plates. $1 \cdot 10^6$ cells were applied per well, the minimum number required for photometric measurements of enzyme activities, and incubated in 2 ml of culture medium in a semi-static approach, with half of the medium volume being exchanged once every 48 h.

Medium osmolality

In a first attempt, the basic ability of eelpout hepatocytes to survive under *in vitro* conditions was tested using Dulbecco's modified eagle medium (DMEM), a classic medium originally developed for culturing mammalian cells (Dulbecco and Freeman,

1959). Due to the higher osmolality in extracellular fluids of marine teleosts than of terrestrial vertebrates the impact of an increased medium osmolality was also tested. Therefore, cells were maintained in basic DMEM, containing 110 mM NaCl, or in DMEM supplemented with 100 mM NaCl according to the isolation buffer (see chapter 2.2.1), resulting in a final concentration of 210 mM. Survival of hepatocytes was monitored by the number of viable cells, determined by trypan-blue exclusion.

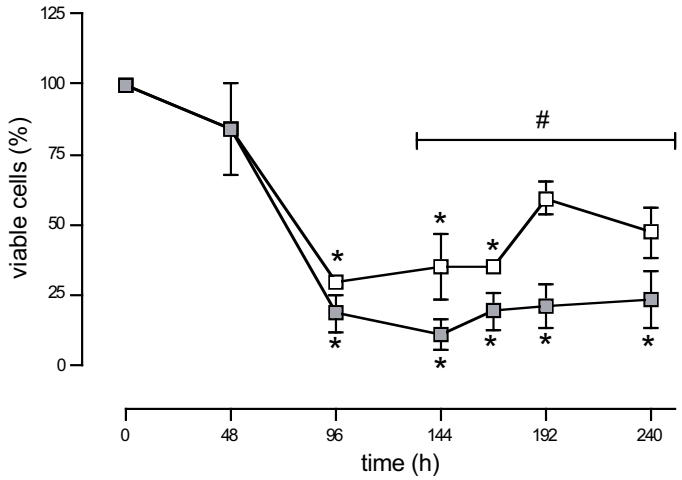

Figure 6: Influence of extracellular osmolality on the survival of hepatocytes from *Z. viviparus*. Cells were incubated at 11°C in basic DMEM (shaded squares) or NaCl-supplemented DMEM (open squares). Numbers of viable cells, determined by trypan-blue exclusion, are given in percent of the initial number. * Significant difference from initial value; # significant difference from culture in basic DMEM after 144 – 240 h (repeated measures ANOVA). Data are given as mean ± range (n = 2).

For both, the basic and the NaCl-supplemented DMEM no significant loss of viable cells was observed within the first 48 h of incubation. After 96 h the number of viable cells decreased significantly under both culture conditions by 69 – 87% below the initial number of hepatocytes (t = 0). During longer incubation periods, the number of viable cells cultured in basic DMEM remained on a significantly lowered level compared to initial controls. NaCl-enrichment of DMEM improved survival during extended incubation periods. The number of viable cells found after 144 – 240 h of incubation was significantly higher than in cells cultured with low-osmolality DMEM for the same period (figure 6). Accordingly, in subsequent experiments all media were supplemented with NaCl to a final concentration of 220 – 240 mM NaCl.

Cellular density

In a second approach the influence of cellular density on the survival rate of hepatocytes was investigated. Therefore, hepatocytes were incubated at densities of 0.5, 1.0 and $2.0 \cdot 10^6$ cells per well. In contrast to the previous experiment, incubations were carried out in HepatoZYME™ (Gibco, Karlsruhe, Germany), a specialized medium with proprietary unknown formulation developed to support the phenotypic expression in mammalian hepatocytes, to test for an improvement of cellular survival. In addition to cellular viability, total RNA content per cell was determined to monitor the physiological integrity of the cells.

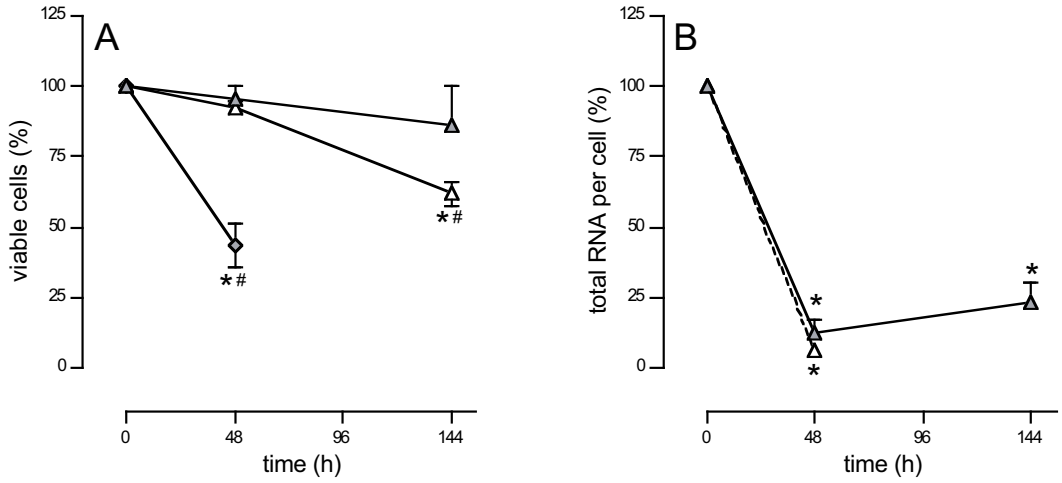

Figure 7: Effect of cellular density on the viability of hepatocytes from *Z. viviparus*. Number of viable cells (A) and total RNA amounts (B) were determined in hepatocytes cultured at 11°C and a density of 0.5 (shaded diamonds), 1.0 (open triangles) or $2.0 \cdot 10^6$ cells/well (shaded triangles). * Significant difference from initial value; # significant difference from incubations at other densities. Data are given in percent of the initial value as mean ± range (n = 2).

Survival of hepatocytes clearly depended on cell densities (figure 7A). When cultured at the lowest density of $0.5 \cdot 10^6$ cells per well, the number of viable cells decreased significantly by 57 ± 11% within the first 48 h of incubation. Higher densities significantly improved cellular survival and no significant decrease of cell numbers occurred after 48 h of cell culture. Hepatocytes cultured at a density of $1.0 \cdot 10^6$ cells per well displayed a significant loss of viable cells by 38 ± 4%. Long-term cellular survival was further and significantly improved after 144 h at the highest density of $2.0 \cdot 10^6$ cells per well.

Total RNA amounts per cell extracted from hepatocytes maintained at the higher densities of 1.0 and $2.0 \cdot 10^6$ cells per well, decreased significantly within the first 48 h by 87 – 94% and remained at this low level until the end of the incubation (figure 7B).

Comparison of culture media

Although HepatoZYME™ improved the long-term cellular viability over the effect of DMEM, the decline of total RNA quantities indicates a major loss of cellular functional rates. Thus, a further incubation series was carried out comparing the conservation of cellular viability and physiological functions, during culture in three different media: DMEM, HepatoZYME™ and Leibovitz L-15 medium (L-15), all enriched with NaCl and supplemented with 20 mM HEPES. According to the results of the preliminary experiment, incubations were performed applying $2.0 \cdot 10^6$ hepatocytes per well.

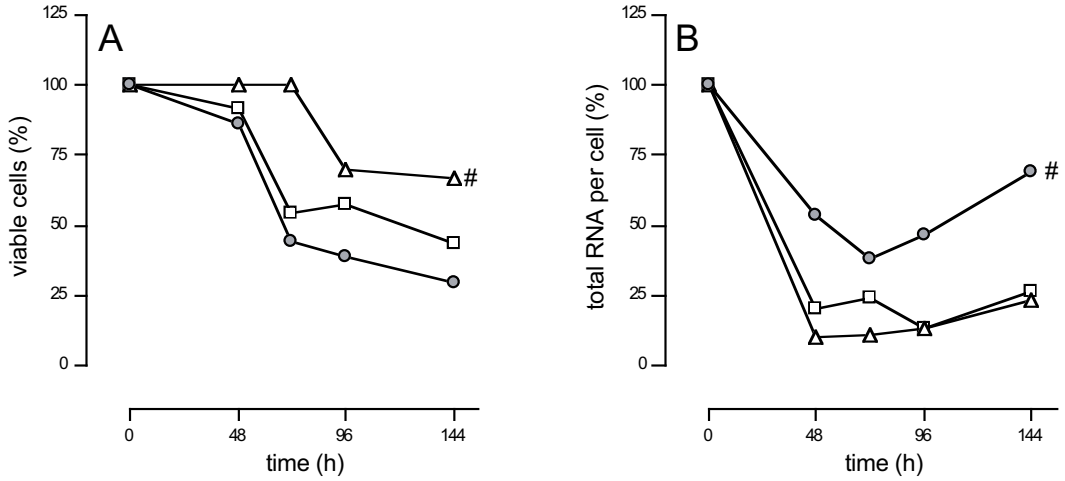

Figure 8: Comparison of different media for the primary culture of hepatocytes from *Z. viviparus*. Number of viable cells (A) and total RNA amounts per cell (B) were determined in hepatocytes incubated at 11°C in HepatoZYME™ (open triangles), DMEM (open squares) or L-15 (shaded circles). # Significant difference from other media (repeated measures ANOVA). Data were derived from one preparation and given in percent of the initial value.

Similar to the previous incubation series (figure 6, 7A), cellular viability was well conserved during the first 48 h of incubation in all three media (figure 8A). During further incubation, the number of viable cells was significantly higher in incubations with HepatoZYME™ than in the other media. In contrast, total RNA quantities referred to the counted number of viable cells were significantly higher in hepatocytes cultured in L-15 medium than in DMEM or HepatoZYME™. Cellular RNA concentrations decreased on

average by 48 ± 4%, while cells maintained in the two latter media lost 79 – 86% of their initial RNA content (figure 8B).

In an additional experiment, carried out with HepatoZYME™ and L-15 under the same conditions as before, the capacities of the mitochondrial key-enzyme citrate synthase (CS) were determined during cell culture as an indicator of metabolic capacity. To test for a potential correlation between physiological status and cellular survival in both media, CS activities and total RNA amounts per sub-sample were plotted against the number of viable cells determined under the same conditions.

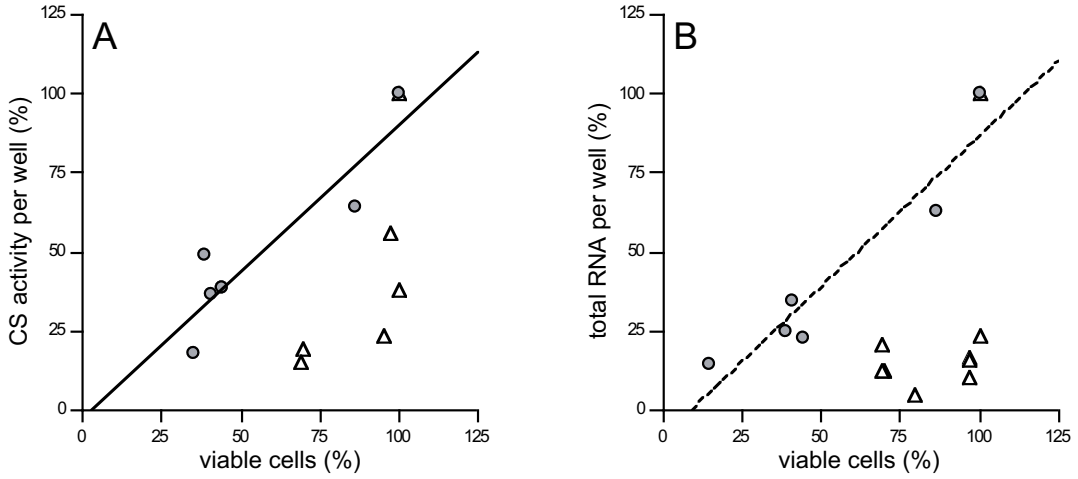

Figure 9: Relationship between viability and physiological status of eelpout hepatocytes cultured in different media. CS activity (A) and total RNA amounts per sub-sample (B) were determined in hepatocytes cultured in HepatoZYME™ (open triangles) or L-15 (shaded circles) for up to 144 h and plotted against the number of viable cells at the respective time point. Data are given in percent of the initial value (n = 6 – 9). Significantly linear correlations were obtained for L-15 for CS-activity ($y = 0.925\ x - 2.22$; $r^2 = 0.8350$; solid line), and total RNA amounts ($y = 0.950\ x - 8.12$; $r^2 = 0.8987$; dashed line).

In cells cultured with Leibovitz L-15 medium, statistical analysis revealed that CS activities (figure 9A) as well as RNA contents (figure 9B) in total sub-samples during incubation were linearly correlated with the number of viable cells determined at the respective time point ($p = 0.004$ for both). The slopes of the regression lines determined for CS activity (0.925 ± 0.184; $r^2 = 0.835$) and total RNA amounts (0.950 ± 0.160; $r^2 = 0.899$) did not significantly differ from the line of identity, which indicates congruency of cell number and metabolic rate. This suggests, that CS activities and total RNA quantities in each viable cell are conserved during incubation in L-15 medium. Accordingly, the decrease of metabolic capacity over time can mainly be ascribed to cellular mortality. In

contrast, the decrease in CS activities and total RNA levels per well that occurred in cells cultured in HepatoZYME™ did not correlate with the number of viable cells (figure 9).

3.1.2 Adjustments of media composition

As reported above, Leibovitz L-15 medium was most suitable to maintain the physiological functioning of eelpout hepatocytes, hence it was chosen as the basic medium for primary culture of eelpout hepatocytes and attention was focused on the enhancement of cellular survival rates by adjusting media composition. Improvement of specific culture conditions was carried out in North Sea eelpout hepatocyte suspensions under the same culture conditions as before. To reduce the high variability in cell counting caused by cellular reaggregation during primary culture, survival of the cells was monitored by CS activities, which was shown to correlate with the number of viable cells in L-15 (figure 9A).

Buffer concentration

Initially, the concentration of added HEPES buffer was revisited. Therefore, hepatocytes were incubated in L-15 medium containing 0, 10 or 20 mM HEPES.

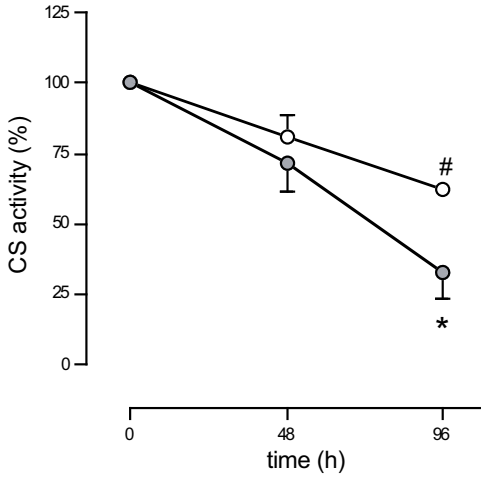

Figure 10: Effect of buffer concentration on the maintenance of hepatocytes from *Z. viviparus*. CS activities were determined in cells incubated in L-15 medium containing 10 (open circles) or 20 mM HEPES (shaded circles). * Significant difference from initial value; # significant difference from incubation with 20 mM HEPES. Data are given in percent of the initial value as means ± range (n = 2).

Hepatocytes maintained in L-15 without addition of HEPES buffer were in a bad visual condition. Cells floated near the surface and pellet formation by centrifugation was inhibited. Accordingly, determination of CS activities was not possible under these

conditions. Cells incubated in culture media supplied with 10 mM HEPES evenly settled on the ground. In hepatocytes incubated with highest buffer concentrations of 20 mM HEPES, cells also settled on the ground, but partly drifted to the surface during incubation. The visual condition of the cells supports the picture obtained by measuring CS activities (figure 10). For cells cultured with 10 mM HEPES, regression analysis revealed a significant linear decrease of CS activity by approximately 9% per day ($P = 0.016$). With the higher buffer concentration, cells displayed a significant loss of CS activities over time, resulting in $68 \pm 13\%$ lower activities after 96 h of incubation than at the beginning. According to this observation, the buffer concentration was reduced to 10 mM HEPES in subsequent incubations, which is in line with the buffer concentration in solutions used for isolation of eelpout hepatocytes (cf. chapter 2.2.1).

Serum-free cell culture additives

For further improvement of cellular viability in primary culture, the effects of some serum-free cell culture additives were investigated.

In a first attempt, surface coating of culture dishes with Matrigel™ (BD Biosciences, Bedford, Massachusetts), a commercially available matrix extracted from mouse sarcoma cells, was tested. Therefore, the surface of the culture vials was coated with a thin layer of the matrix according to the manufacturers manual. A matrix solution containing 20 or 80 μg of Matrigel™ per cm^2 of vial surface was added and incubated for 1 h at room temperature. After aspiration of unbound material, culture plates were used for incubations as in previous experiments. A simultaneous control was carried out without the addition of matrix components.

Matrigel™ had no effect on the maintenance of eelpout hepatocytes. CS activities were very similar in cells incubated in uncoated and matrix coated plates. Under all conditions CS activities decreased significantly by approximately 40% within the first 48 h of incubation and remained on this level until the end of incubation (figure 11).

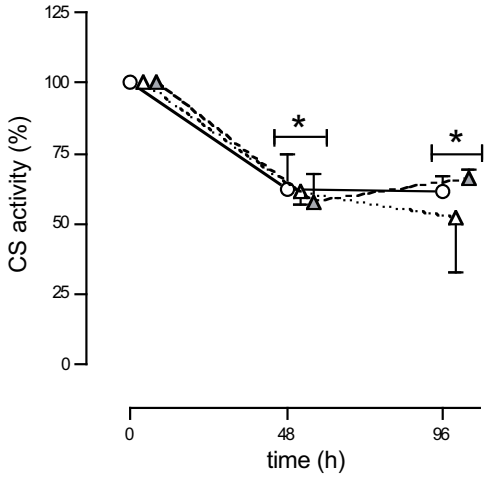

Figure 11: Influence of Matrigel™ on the survival of eelpout hepatocytes. CS activities were determined in cells incubated on uncoated culture plates (open circles) and on plates coated with 20 (open triangles) or 80 μg Matrigel™ (shaded triangles) per cm^2 surface. * Significant difference from initial value. Data are given in percent of the initial value as means ± range (n = 2). To improve clarity, curves were shifted along the time axis.

Since Matrigel™ failed to enhance long-term survival of eelpout hepatocytes, the impact of bovine serum albumin (BSA) on cellular viability was investigated. Therefore, hepatocytes were cultured in L-15 medium without BSA and in L-15 supplemented with 1% BSA as used in the isolation buffer (cf. chapter 2.2.1). In contrast to previous incubations, medium exchange was omitted, but an additional incubation was performed with 1% BSA, with half of the medium being exchanged after 48 h.

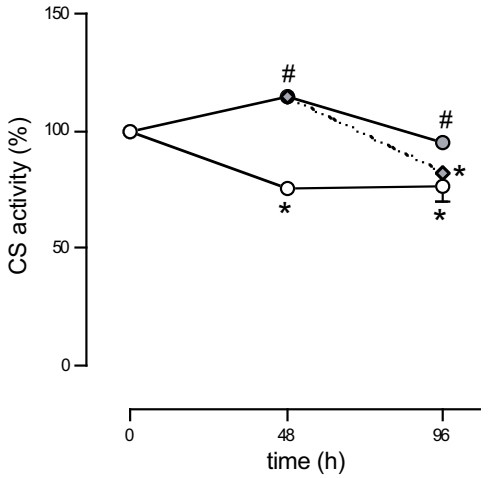

Figure 12: Impact of BSA on the maintenance of hepatocytes from *Z. viviparus*. CS activities were determined in cells incubated without BSA (open circles), with 1% BSA (shaded circles) or with 1% BSA after medium exchange (shaded diamonds). * Significant difference from initial value; # significant difference from other incubations. Data are given in percent of the initial value as means ± range (n = 2).

In hepatocytes cultured without the addition of BSA, CS activities decreased significantly over time by almost 25% (figure 12). During incubation with culture medium supplemented with 1% BSA no significant correlation between enzyme activities and cell culture period occurred and CS activities were significantly increased over the simultaneous incubation without BSA by 24 – 51%. In the semi-static approach carried out with BSA-enriched medium, CS activities were significantly reduced by ~15% after the exchange of half of the medium at the second day.

According to this observation, subsequent incubations were performed with Leibovitz L-15 medium containing 240 mM NaCl, 10 mM HEPES and 1% BSA. To prevent energy limitation in hepatocytes during incubation, 5 μmol glucose were supplemented per $1 \cdot 10^6$ cells on a daily basis.

3.1.3 Verification of the culture conditions

To test the suitability of primary liver cell culture for studies of the mechanisms of temperature adaptation and to gain information about the time period required for metabolic adjustments in isolated cells, a long-term incubation was carried out with hepatocytes from warm acclimated *Z. viviparus*, applying two different temperatures and a couple of potential effectors (adenosine, cortisol and epinephrine). Changes of energy metabolism were monitored by the activities of CS and of cytochrome *c* oxidase (COX).

Hepatocytes were responsive to several of the applied incubation conditions, with the main changes observed for COX activities. Cold exposure and epinephrine treatment significantly increased COX activities after 48 – 120 h of incubation, while the application of adenosine decreased the activity of this enzyme compared to the warm incubated control (figure 13B/D). COX activities in cortisol treated cells exhibited a high variability, but did not significantly differ from the control incubation (figure 13D). CS activities were solely affected by epinephrine treatment, which elevated activity levels over the 11°C control (figure 13B). In all treatments adjustments of enzyme activities and the establishment of a new steady state level occurred within the first 48 h of incubation, which allowed for a reduction of the incubation period for the intended mechanistic studies to 72 h, thereby increasing the multiplicity of simultaneous treatments.

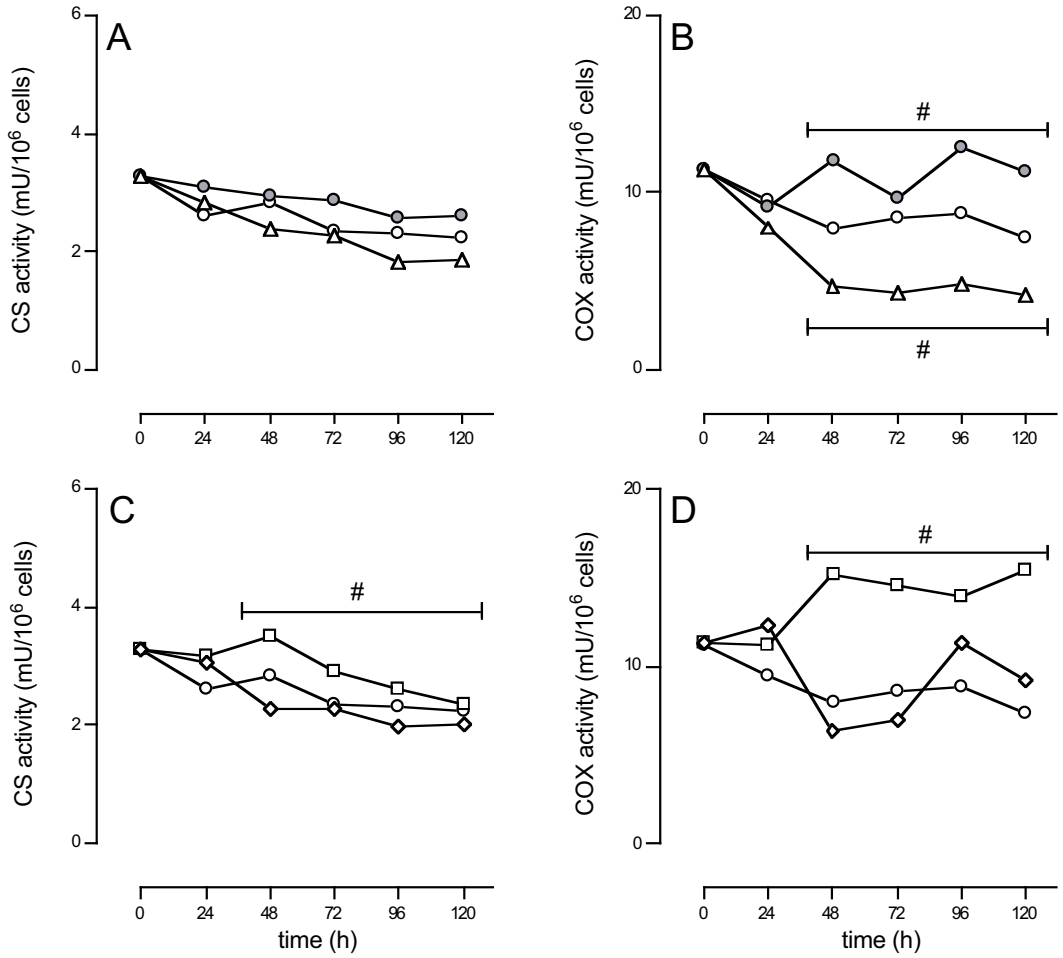

Figure 13: Time course of mitochondrial enzyme activities in eelpout hepatocytes during primary culture under varying conditions. CS (A/C) and COX activities (B/D) were determined in hepatocytes from warm acclimated *Z. viviparus* directly after preparation (t = 0) or after 24 – 120 h of incubation at 11°C (open symbols) or 4°C (shaded symbols). Cells were incubated under control conditions (circles) or treated with adenosine (triangles), cortisol (diamonds) or epinephrine (squares). # Significant difference from control incubation (Repeated measures ANOVA). Data were derived from one preparation.

The viability of eelpout hepatocytes under the established culture conditions was further verified by stereological investigation of the cells. Hepatocytes from 11°C acclimated eelpouts were incubated at either 4 or 11°C for up to 72 h, according to the experimental set-up of the intended studies. Cells were sampled after 48 and 72 h of incubation and counted in a Fuchs-Rosenthal haemocytometer dish. Viability was monitored by trypan-blue exclusion.

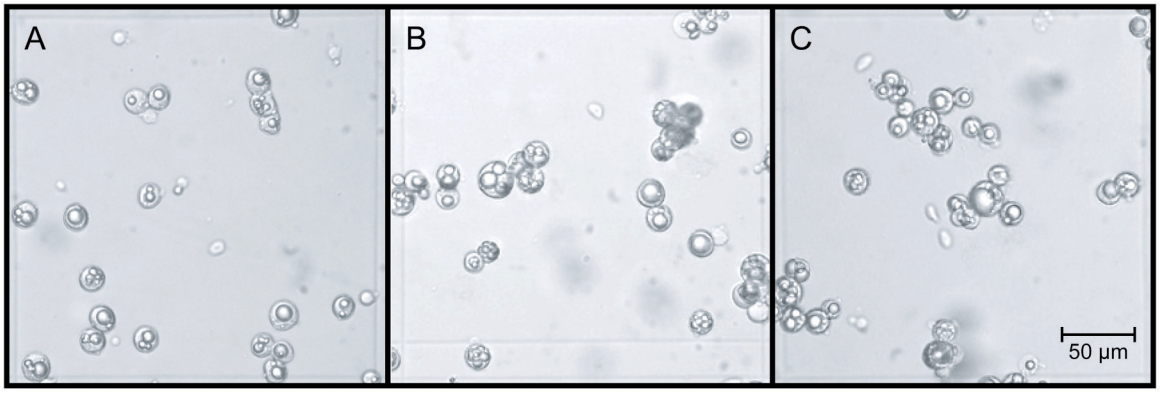

Figure 14: Appearance of hepatocytes under the microscope during primary cell culture. Cells of a representative preparation directly after isolation (A) and after 72 h of incubation at either 4°C (B) or 11°C (C) were photographed in a Fuchs-Rosenthal haemocytometer under the addition of trypan-blue dye.

Appearance of hepatocytes under the microscope was conserved during both tested incubation temperatures under the applied cell culture conditions (figure 14). Cells showed only little uptake of trypan-blue dye. The fraction of cells that excluded the dye was always > 83% and did not significantly differ from the initial value or between incubation temperatures. The total number of viable hepatocytes was maintained at initial levels at both temperatures during the whole incubation period (figure 15A).

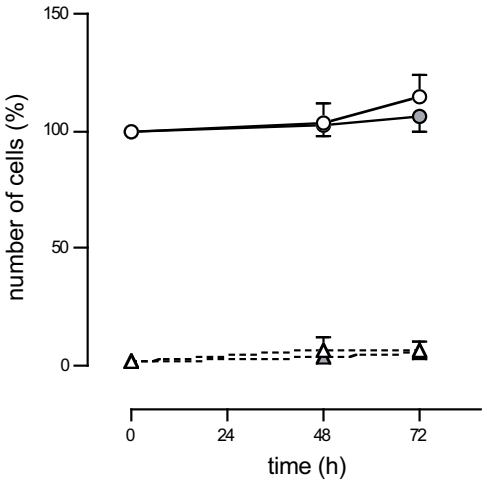

Figure 15: Viability and fraction of dead cells during primary culture of hepatocytes prepared from common eelpout (*Z. viviparus*). The number of viable cells, referred to the initial value (circles) and the fraction of dead cells in total cell counts (triangles) was determined during primary culture carried out at either 11°C (shaded symbols) or 4°C (open symbols). Cell counting was performed as triplicates for each sample. Data are given as means ± SE (n = 3).

3.2 Temperature effects on hepatocytes

To elaborate the ability of eelpout liver cells to thermally adapt the capacity of aerobic metabolism, the effects of temperature acclimation *in vivo* were compared to the adjustments induced by temperature incubation of isolated hepatocytes *in vitro*. Changes in mitochondrial capacities were monitored by analysing the activities of the mitochondrial key-enzymes citrate synthase (CS) and cytochrome *c* oxidase (COX) and the expression of the respective genes.

3.2.1 In vivo temperature acclimation

Acclimation of North Sea eelpout to either 4 or 11°C in the aquarium in summer did not affect body weight or condition factors of the animals. In contrast, hepatosomatic indices increased significantly by 80 ± 14% during cold acclimation (table 4).

Table 4: Body weight, hepatosomatic indices (HSI) and condition factors of long-term acclimated *Z. viviparus* in winter and summer. HSI were determined as percentages of liver to body mass. Condition factors were calculated as body mass (g) x total length $(cm)^{-3}$ x 1000. * Significant difference from animals sampled in summer and acclimated at 11°C. Values are means ± SE (n = 3 – 8).

	summer 4°C	summer 11°C	winter 11°C
body weight (g)	73.2 ± 10.2	56.4 ± 5.7	75.3 ± 37.1
HSI (%)	2.31 ± 0.18*	1.28 ± 0.14	1.38 ± 0.25
condition factor	3.94 ± 0.18	3.40 ± 0.20	3.22 ± 0.14

Baseline data on the effect of temperature acclimation on enzyme activities and RNA expression *in vivo* were determined in freshly isolated hepatocytes of *Z. viviparus* acclimated at 4 and 11°C. In cold acclimated fish, CS activities per 10^6 cells were significantly, by 44 ± 10% increased over those in warm acclimated animals (figure 16A), while activities of COX remained unaffected by whole animal acclimation (figure 16D). Total RNA and protein content did not significantly differ between acclimation temperatures, albeit almost 55% higher RNA levels were noticed after cold acclimation (figure 17A/D). In contrast to enzyme activities, the mRNA expression of CS was left unchanged by *in vivo* temperature acclimation (figure 18A). The mRNA expression of COX was determined by use of the mitochondrial encoded COX2 and the nuclear encoded COX4 subunits. For both subunits, mRNA levels remained unaffected by long-term temperature acclimation of *Z. viviparus in vivo* (figure 18D/G), in line with unchanged COX activities.

3.2.2 Temperature effects on enzyme activities in isolated hepatocytes

To test for effects induced by thermal acclimation of isolated hepatocytes *in vitro*, cells prepared from cold and warm acclimated eelpout were cultured at both, 4 and 11°C. Temperature acclimation *in vivo* influenced the response of both mitochondrial enzymes to the incubation of isolated hepatocytes. Warm incubation of hepatocytes prepared from 4°C acclimated eelpout caused a significant decrease of CS and COX activities by 26 ± 4 and 30 ± 4%, respectively, compared to their cold incubated counterparts (figure 16B/E). In contrast, enzyme activities in cells isolated from warm acclimated fish were not altered by cold or warm incubation (figure 16C/F).

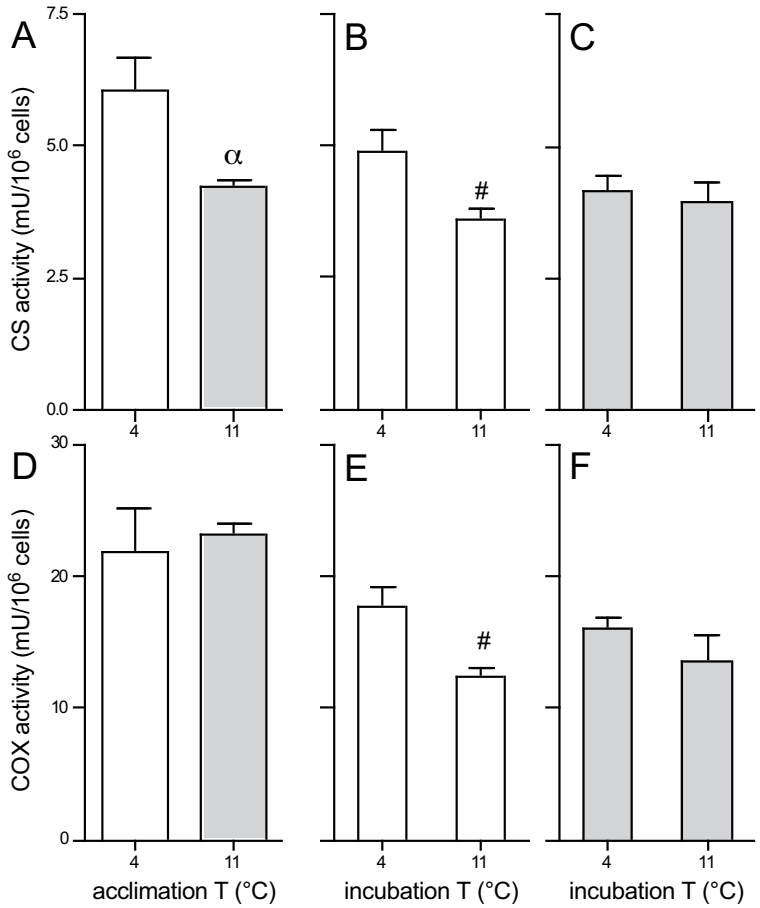

Figure 16: Activities of CS (A – C) and COX (D – F) in hepatocytes prepared from warm and cold acclimated eelpout (*Z. viviparus*). Activities were determined in cells from cold (white bars) and warm acclimated (grey bars) specimens directly after preparation (A/D; n = 4) and after 48 – 72 h of incubation at either 4 or 11°C (B/C/E/F; n = 8). α Significant difference between acclimation temperatures; # significant difference from the corresponding group incubated at 4°C. Values are means ± SE.

3.2.3 Temperature effects on RNA and protein contents of isolated cells

Total RNA and protein contents of liver cells isolated from cold-acclimated *Z. viviparus* decreased significantly by 34 ± 11% and 30 ± 4%, respectively when cultured at 11°C, compared to the cold incubated control (figure 17B/E). In cells from warm acclimated specimens, *in vitro* cold incubation increased RNA contents significantly by 53 ± 15% (figure 17C). Protein contents in these cells displayed an initial decrease at both incubation temperatures, reaching steady state levels after 48 h, with a small but significant elevation of protein contents by 13 ± 5% in cells maintained at 4°C above those incubated at 11°C. (figure 17F/embedded graph).

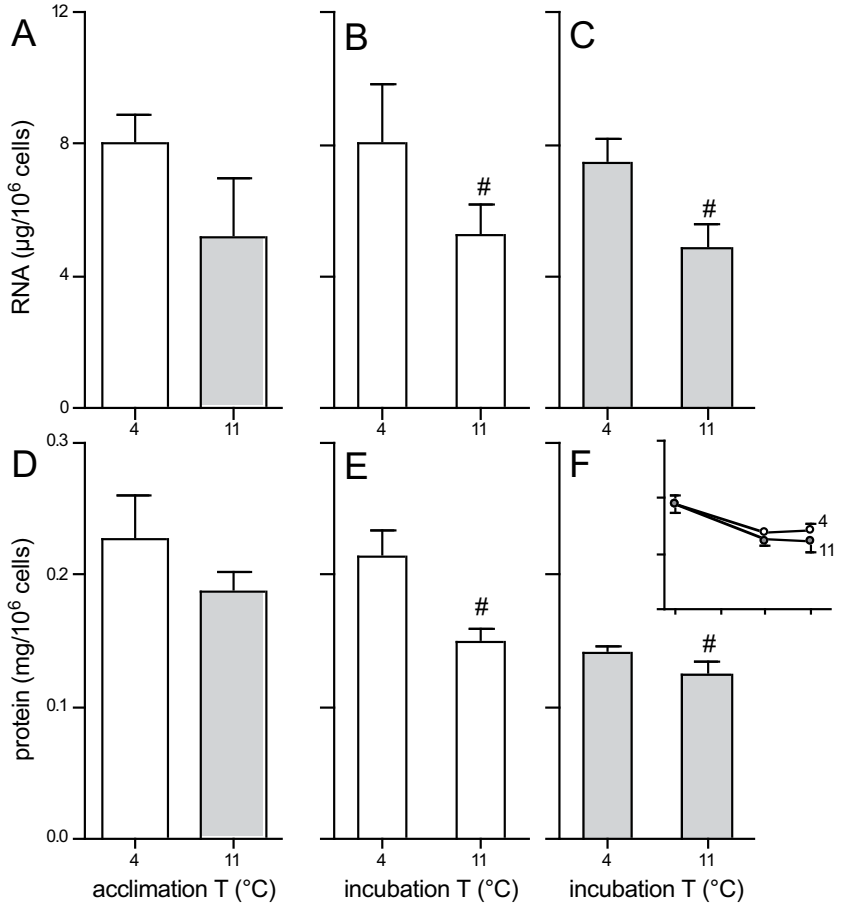

Figure 17: Total RNA (A – C) and protein (D – E) content of hepatocytes prepared from warm and cold acclimated eelpout (*Z. viviparus*). Amounts were determined in cells prepared from cold (white bars) and warm acclimated (grey bars) specimens directly after preparation (A/D; n = 4) and after 48 – 72 h of incubation at either 4 or 11°C (B/C/E/F; n = 8). The embedded graph shows the changes of protein content over time in cell from warm acclimated animals. # Significant difference from the corresponding group incubated at 4°C. Values are means ± SE.

3.2.4 Effects of in vitro incubation temperature on transcript levels

The expression of CS mRNA displayed a similar pattern for both, cells from cold and warm acclimated eelpout, and remained on a constant level, independent of the *in vivo* acclimation temperature (figure 18B/C).

Although *in vivo* acclimation temperature did not influence COX mRNA expression in freshly isolated hepatocytes, it significantly affected the response of hepatocytes to different incubation temperatures. In cells from cold acclimated *Z. viviparus*, warm incubation induced a significant reduction of COX2 and COX4 mRNA levels by 34 ± 6% and 37 ± 6%, respectively (figure 18E/H). In hepatocytes isolated from warm acclimated eelpout, COX4 mRNA levels were maintained at significantly, by 34 ± 10% higher levels in cold than in warm incubated cells (figure 18I), while COX2 expression was left unchanged.

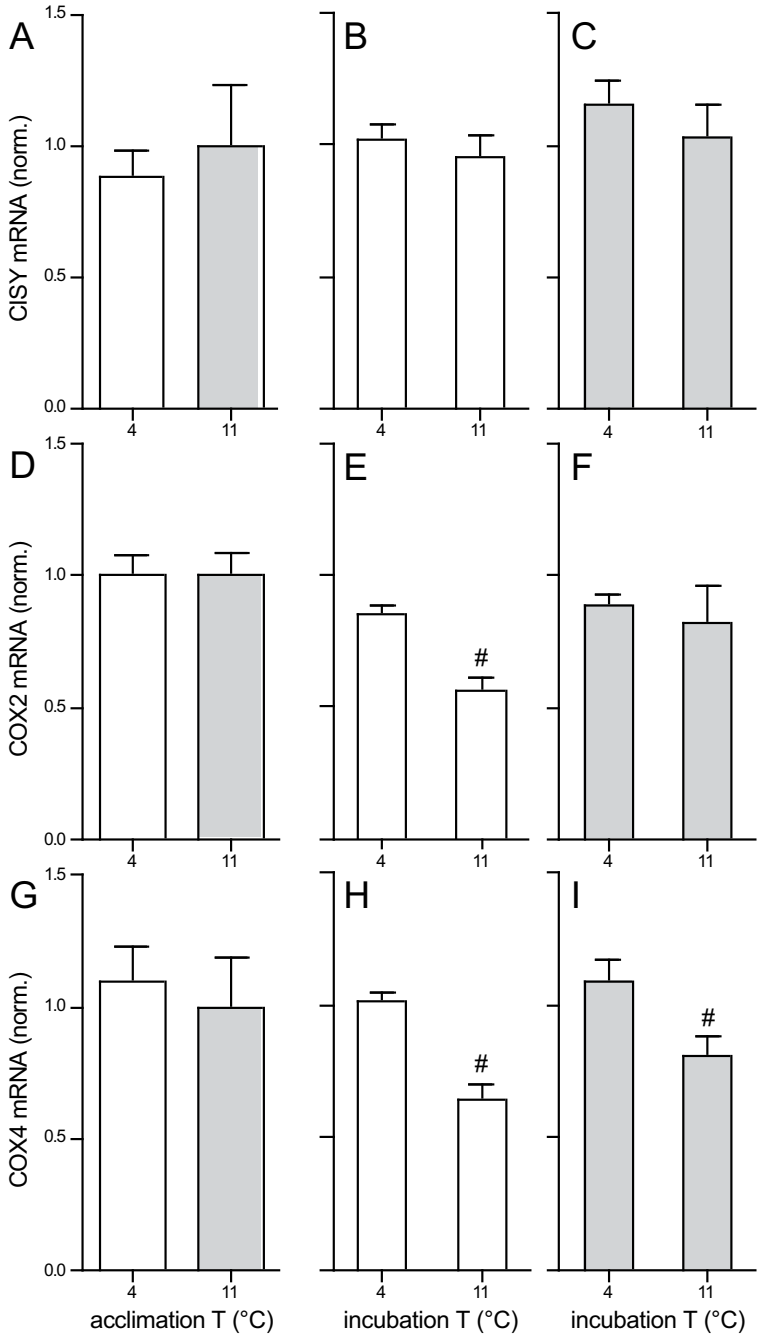

Figure 18: Relative mRNA expression of CS and different COX subunits in hepatocytes of warm and cold acclimated eelpout (*Z. viviparus*). Relative amounts of CS (A – C), COX2 (D – F) and COX4 (G – I) mRNA were determined in cells from cold (white bars) and warm acclimated (grey bars) fish directly after preparation (A/D/G; n = 4) and after 48 – 72 h of incubation at either 4 or 11°C (B/C/E/F/H/I; n = 8). mRNA levels were determined by RPA in equal amounts of total RNA and expressed relative to the mean of freshly isolated hepatocytes from warm acclimated fish. # Significant difference from corresponding group incubated at 4°C. Values are means ± SE.

3.3 Effects of extracellular pH in isolated hepatocytes

The potential role of extracellular pH as a signal for temperature acclimation was tested in the early phase of the experiments. Therefore, the influence of decreased medium pH values of 7.4, 7.2 and 6.5 on the activities of mitochondrial enzymes was monitored in hepatocytes prepared from cold acclimated eelpout in winter, incubated at 4°C and compared to the effect induced by temperature incubation at either 4 or 11°C under control condition with a pH of 7.8.

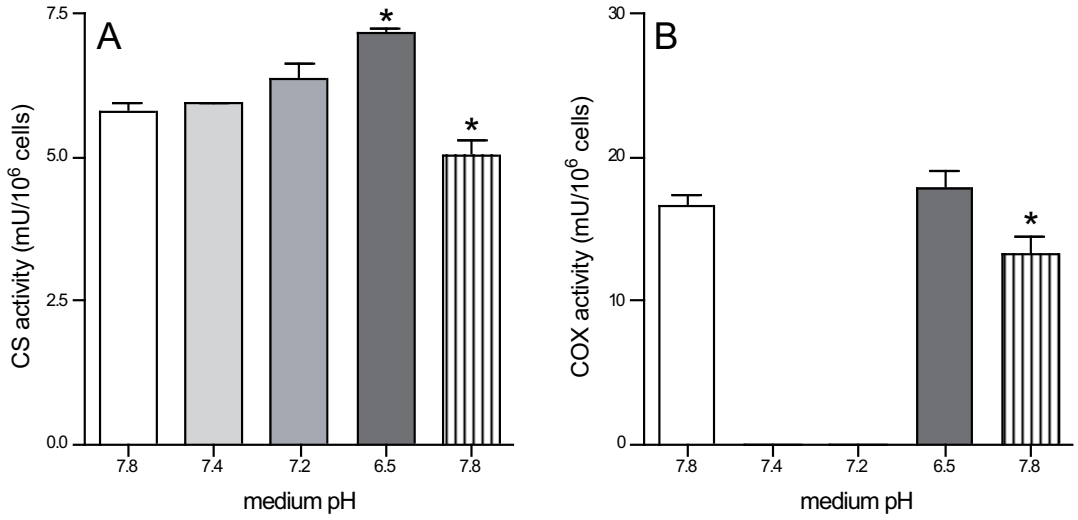

Figure 19: The effect of extracellular pH on enzyme activities in hepatocytes from cold acclimated *Z. viviparus*. CS activities (A) were measured in cells incubated for 48 – 96 h at 4°C with a medium pH of 7.8 (control conditions), 7.4, 7.2 or 6.5, respectively, or at 11°C under control conditions (striped bars). COX activities (B) were determined for the groups exposed to the lowest and the highest pH. All medium pH values were adjusted at 4°C. * Significant difference from cells incubated at 4°C at pH 7.8. Data are given as means ± SE (pH 7.8; n = 4 – 8) or as means ± range (n = 2)

CS activities in hepatocytes incubated with extracellular pH values of 7.4 and 7.2 within the physiological range did not significantly differ from the parallel control cultured at the same temperature with a pH of 7.8 (COX activities were not determined for these groups). A further decrease of medium pH to 6.5 induced a significant increase of CS activities by 23 ± 3% (mean ± range) (figure 19A), while COX activities remained unchanged (figure 19B). In contrast, warm incubation of the cells at the control pH, adjusted at 4°C, resulting in a pH of 7.6 at 11°C, caused a significant decrease of CS and COX activities by 14 ± 5% and 20 ± 7% (means ± SE), respectively (figure 19A/B).

3.4 Impact of adenosine on thermal acclimation

3.4.1 Adenosine levels during cold exposure in vivo

To elaborate a potential for adenosine as a signal for temperature adaptation, the concentrations of the metabolite were monitored in the early phase of cold acclimation *in vivo*.

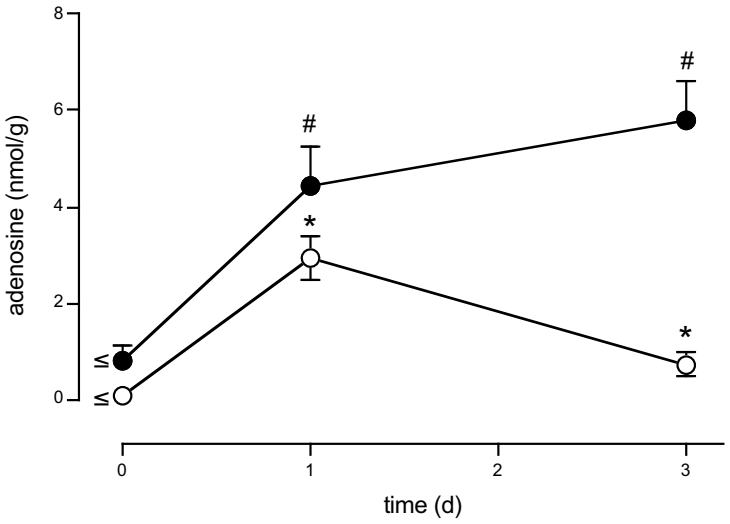

Figure 20: Adenosine levels during 1 or 3 days of cold exposure in blood serum (open circles) and liver (filled circles) of eelpout (*Z. viviparus*). The fish were acclimated to 11°C (t = 0) and transferred to 4°C. ≤ Data point includes samples with adenosine concentration below detection limit. Here, the detection limit of the method was used for calculations. * Significant difference from other time points; # significant difference from control (t = 0). Values are means ± SE (n = 9 – 10).

Adenosine concentrations in blood serum and liver of unstressed, long-term warm-acclimated *Z. viviparus* were found to be below or close to the detection limit (figure 20; 0 days). In serum, adenosine levels were significantly increased after 24 h of acute cold exposure. After three days serum adenosine concentrations were found significantly lower than on the first day, but they were still elevated compared to the warm acclimated group. In the liver of *Z. viviparus* adenosine levels were also significantly increased after 24 h of cold exposure, but concentrations remained high during three days of cold exposure (figure 20).

3.4.2 Adenosine effects on enzyme activities

The impact of adenosine on energy metabolism was investigated by application of adenosine to isolated hepatocytes in primary culture. Adenosine treatment of the cells had no impact on CS activities (figure 21A/B), but significantly affected COX activities. In cells from cold-acclimated eelpout, adenosine treatment induced a reduction of COX activities by 16 ± 8% at 4°C and by 18 ± 8% at 11°C compared to their respective control incubation (figure 21C). In cells from warm-acclimated *Z. viviparus* COX activities remained more or less unaffected at 4°C incubation temperature, but adenosine treatment during warm incubation significantly reduced COX activities by 25 ± 12% compared to the untreated control at 11°C (figure 21D).

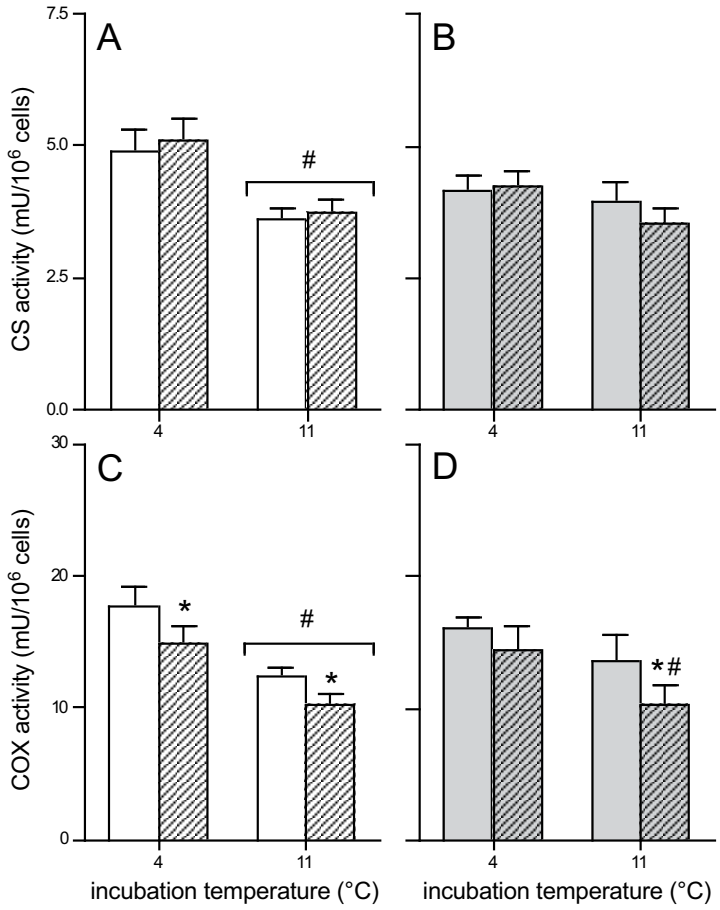

Figure 21: Effects of adenosine on the activities of CS (A/B) and COX (C/D) in hepatocytes of *Z. viviparus*. Data were determined in cells from cold (A/C; white bars) and warm acclimated (B/D; grey bars) eelpout after 48 – 72 h of incubation at 4 or 11°C under control conditions (empty bars) or treated with adenosine (hatched bars). * Significant difference from control group incubated at the same temperature; # significant difference from corresponding group incubated at 4°C. Values are means ± SE (n = 5 – 8).

3.4.3 Adenosine effects on total RNA and protein contents

Total RNA in cells prepared from cold acclimated *Z. viviparus* remained unaffected by adenosine treatment (figure 22A). In contrast, in hepatocytes from warm acclimated eelpout, RNA content was significantly reduced during incubation with adenosine in the cold, by 29 ± 9% at 4°C. Reduction by 22 ± 11% at 11°C remained non-significant (P = 0.081) (figure 22B). Cellular protein content was unaltered during adenosine treatment, independent of the original acclimation temperature of the animals. Similar to the parallel controls, protein concentrations in adenosine treated cells were significantly lower in the cold than in the warmth, by 32 ± 7% and 24 ± 7% in cells derived from cold versus warm incubated specimens, respectively (figure 22C/D)

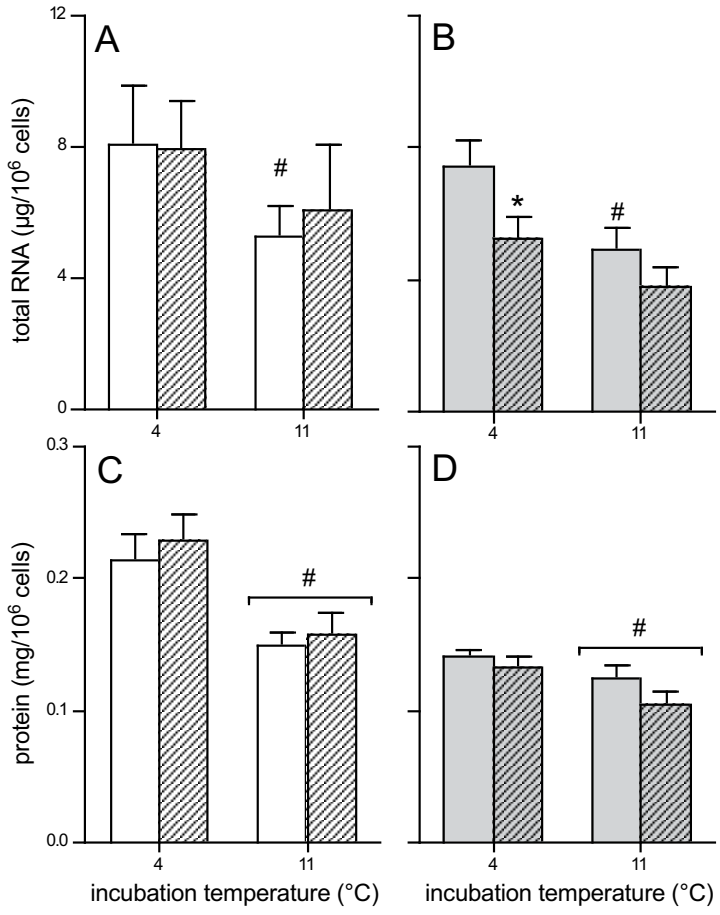

Figure 22: Effects of adenosine on total RNA (A/B) and protein contents (C/D) in hepatocytes of *Z. viviparus*. Data were determined in cells from cold (A/C; white bars) and warm acclimated (B/D; grey bars) eelpout after 48 – 72 h of incubation at 4 or 11°C under control conditions (empty bars) or treated with adenosine (hatched bars). * Significant difference from control group incubated at the same temperature; # significant difference from the corresponding group incubated at 4°C. Values are means ± SE (n = 5 – 8).

3.4.4 Adenosine effects on specific mRNA expression

In accordance with CS activities, the expression of CS mRNA was not affected by adenosine treatment of eelpout liver cells *in vitro* (figure 23A/B).

Although adenosine treatment had caused a drop in COX activities (figure 21C/D), it significantly increased the mRNA expression of the enzyme. In cells from cold acclimated fish COX2 mRNA levels were elevated by 27 ± 7% at 4°C and by 95 ± 7% at 11°C (figure 23C), and the expression of COX4 was increased by 81 ± 14% at 11°C (figure 23E). The increase in COX2 and COX4 expression was less pronounced in hepatocytes from warm acclimated eelpout, but followed the same pattern. Here, adenosine treatment at 11°C resulted in 66 ± 20% higher COX2 mRNA levels than in control cells (figure 23D) and caused an increase of COX4 mRNA close to the control levels seen at 4°C (figure 23F).

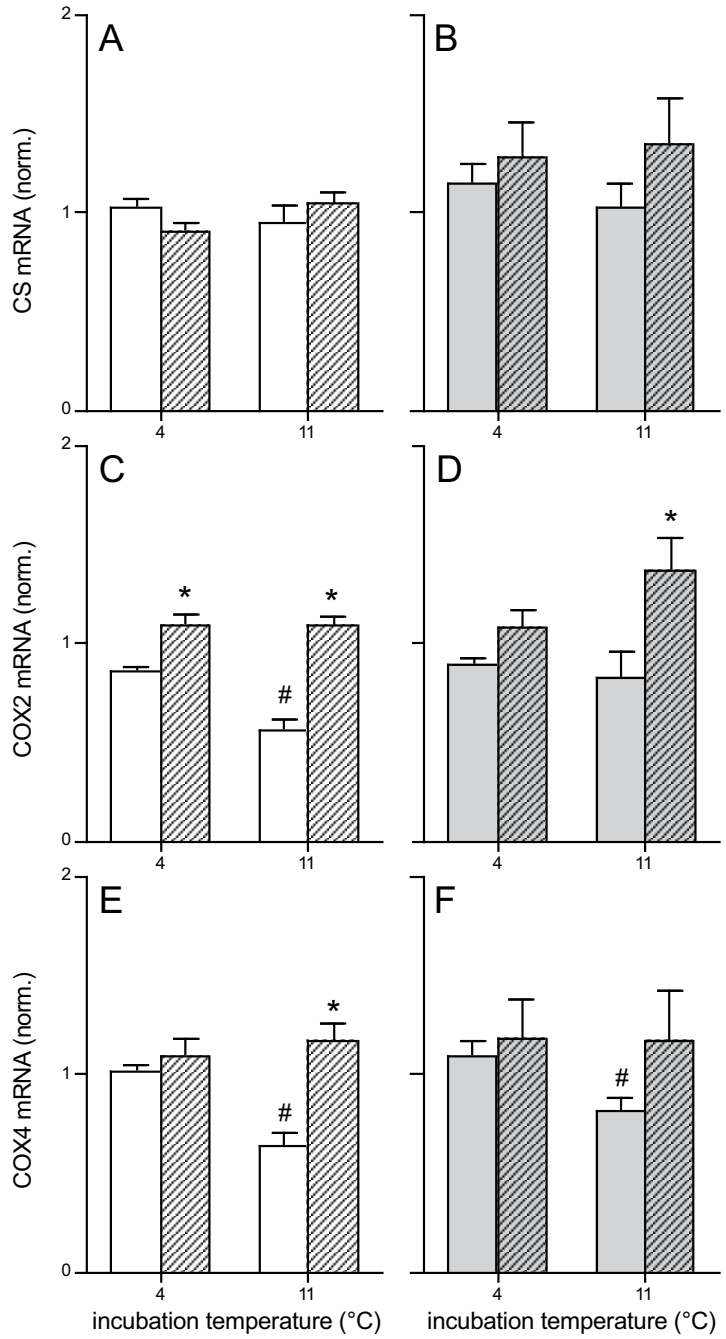

Figure 23: Effects of adenosine on relative mRNA expression of CS and of different COX subunits in hepatocytes of *Z. viviparus*. Relative amounts of CS (A/B), COX2 (C/D) and COX4 (E/F) were determined in cells from cold (white bars) and warm acclimated (grey bars) eelpout after 48 – 72 h of incubation at 4 or 11°C under control conditions (empty bars) or treated with adenosine (hatched bars). mRNA levels were determined by RPA in equal amounts of total RNA and expressed relative to the mean of freshly isolated hepatocytes from warm acclimated fish. * Significant difference from control group incubated at the same temperature; # significant difference from corresponding group incubated at 4°C. Values are means ± SE (n = 5 – 8).

3.4.5 Impact of adenosine receptor antagonists and agonists

A potential role for adenosine-receptor interactions in eliciting adenosine effects was tested by the addition of 8-phenyltheophylline (8-PT), an antagonist for adenosine A_1-receptors, and 5'-(N-ethylcarboxamido)adenosine (NECA), a potent non-selective agonist for adenosine receptors (Ralevic and Burnstock, 1998). 8-PT and NECA treated samples were compared to the analogous control and adenosine treated groups of the respective preparations.

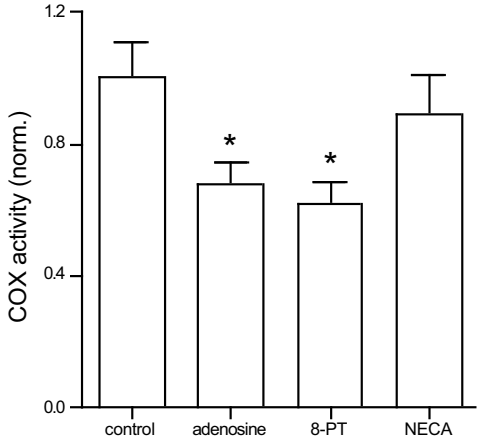

Figure 24: Influence of adenosine receptor antagonists/agonists on adenosine action. COX activities in hepatocytes were determined after 48 – 72 h of incubation with adenosine combined with adenosine receptor antagonist 8-PT or after incubation with adenosine agonist NECA and compared to their analogous control and adenosine treated groups. Cells were prepared from warm-acclimated *Z. viviparus* and incubated at 11°C. * Significant difference from control. Values are means ± SE (n = 4 – 6), expressed relative to the mean of the control group.

Hepatocytes treated with the receptor blocker 8-PT in addition to adenosine exhibited COX activities identical to the ones measured in cells treated with adenosine alone, both significantly lower than the activities determined for the control group (figure 24). This suggests that adenosine A_1 receptors are not involved in the adenosine effects described above. The assumption is supported by the observation that the adenosine agonist had no effect either. COX activities in cells treated with NECA instead of adenosine were almost identical to those of the control group and were higher, albeit not significantly ($P = 0.139$), than in adenosine treated cells (figure 24). Thus, a receptor-mediated hormone-like action of adenosine on COX activity can be excluded as unlikely.

3.4.6 Cellular adenosine consumption

For further insight into its mode of action, adenosine levels were determined during cell culture. Since adenosine was stable in culture medium at room temperature for at least three days, the amount of adenosine metabolized or taken up by the cells can be calculated from surplus adenosine in hepatocyte medium and the added adenosine.

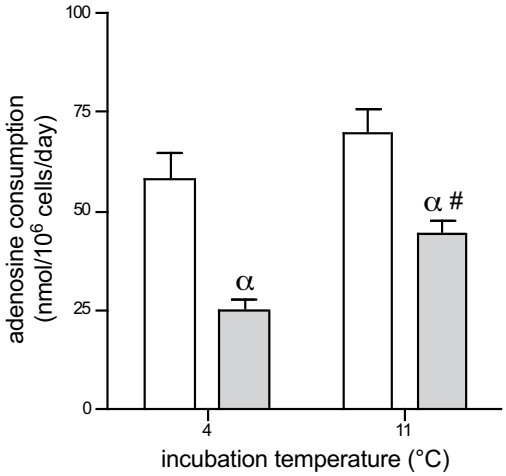

Figure 25: Daily adenosine consumption of hepatocytes. Cells from cold (white bars) and warm acclimated *Z. viviparus* were incubated with adenosine at 4 and 11°C for 48 – 72 h. Consumption was calculated from adenosine surplus in culture medium and the added adenosine amounts. α Significant difference between acclimations; # significant difference from the corresponding group incubated at 4°C. Values are means ± SE (n = 6).

Eelpout hepatocytes continuously consumed adenosine throughout the whole culture period, with a constant rate between 48 – 72 h of incubation. The consumption rate significantly depended on initial acclimation temperature *in vivo* and was increased in cells prepared from cold-acclimated *Z. viviparus* over their warm-acclimated counterparts, by 131 ± 27% and 57 ± 13% during incubation at 4 and 11°C, respectively. In hepatocytes from warm-acclimated fish, the consumption rate was further affected by the applied temperature *in vitro*, resulting in 76 ± 12% higher rates during warm than during cold incubation (figure 25).

3.5 Influence of stress hormones on cultured hepatocytes

To test for the potential role of the major stress hormones of fish in thermal adaptation, epinephrine and cortisol were applied during incubation of hepatocytes at either 4 or 11°C.

3.5.1 Hormone concentrations

According to the results of a preliminary experiment (cf. chapter 3.5.5), initial incubations with stress hormones were carried out with a concentration of 0.1 μM of either epinephrine or cortisol. However, since this low dose induced only minor effects in the present incubation series, the impact of an increased concentration of 10.0 μM was also tested.

The response of all measured cellular parameters was found to be independent of the applied concentration for both epinephrine (figure 26A) and cortisol (figure 26B). For the latter, a small disparity occurred for COX4 expression, which was significantly different from the control only when incubated with the low, but not with the high dose of cortisol (figure 26B). However, statistical analysis revealed no differences between both concentrations ($P > 0.200$). Accordingly, data obtained under all tested concentrations were pooled and taken as one cortisol and epinephrine treatment, respectively.

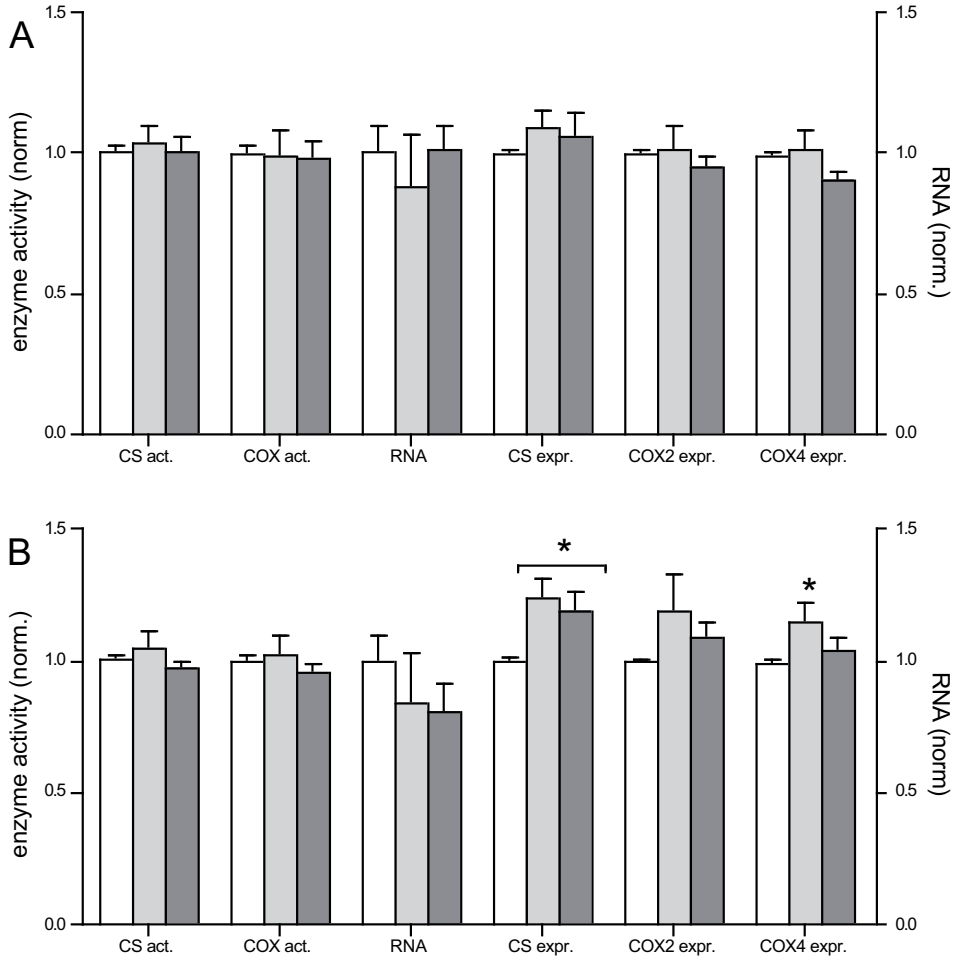

Figure 26: Impact of hormonal concentrations on effects in isolated eelpout hepatocytes. CS and COX activities (act.), total RNA contents and the mRNA expression (expr.) of CS and two COX subunits were determined in cells treated with a low (0.1 μM; light grey bars) or high dose (10.0 μM; dark grey bars) of either epinephrine (A) or cortisol (B). All data were normalized to the mean of the respective control (white bars set to 1). * Significant difference from control. Values are means ± SE (n = 8 – 11).

3.5.2 Effects of epinephrine and cortisol on enzyme activities

Treatment of isolated hepatocytes with either epinephrine or cortisol revealed no effects on enzyme activities. Both CS and COX activities were maintained at similar levels as in the corresponding control group (figure 27).

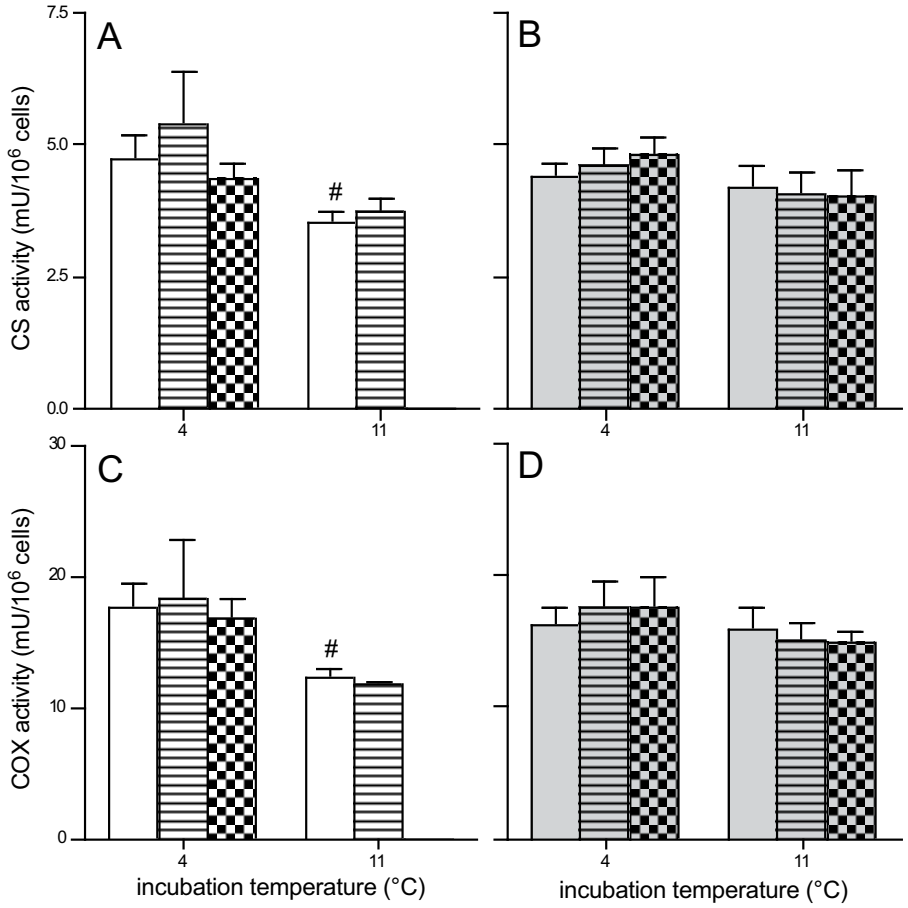

Figure 27: Effects of stress hormones on mitochondrial enzyme activities in eelpout hepatocytes at different incubation temperatures. Activities of CS (A/B) and COX (C/D) were determined in cells from cold (white bars) and warm acclimated (grey bars) eelpout after 48 – 72 h of incubation at 4 or 11°C under control conditions (empty bars) or treated with epinephrine (striped bars) or cortisol (chequered bars). # Significant difference from corresponding group incubated at 4°C. Values are means ± SE (n = 3 – 6).

3.5.3 Effects of epinephrine and cortisol on total RNA and protein contents

The application of epinephrine as well as cortisol to isolated hepatocytes revealed no effect on total RNA amounts or protein contents. Independent of the original *in vivo* acclimation temperature, no significant differences occurred compared to the respective temperature controls (figure 28).

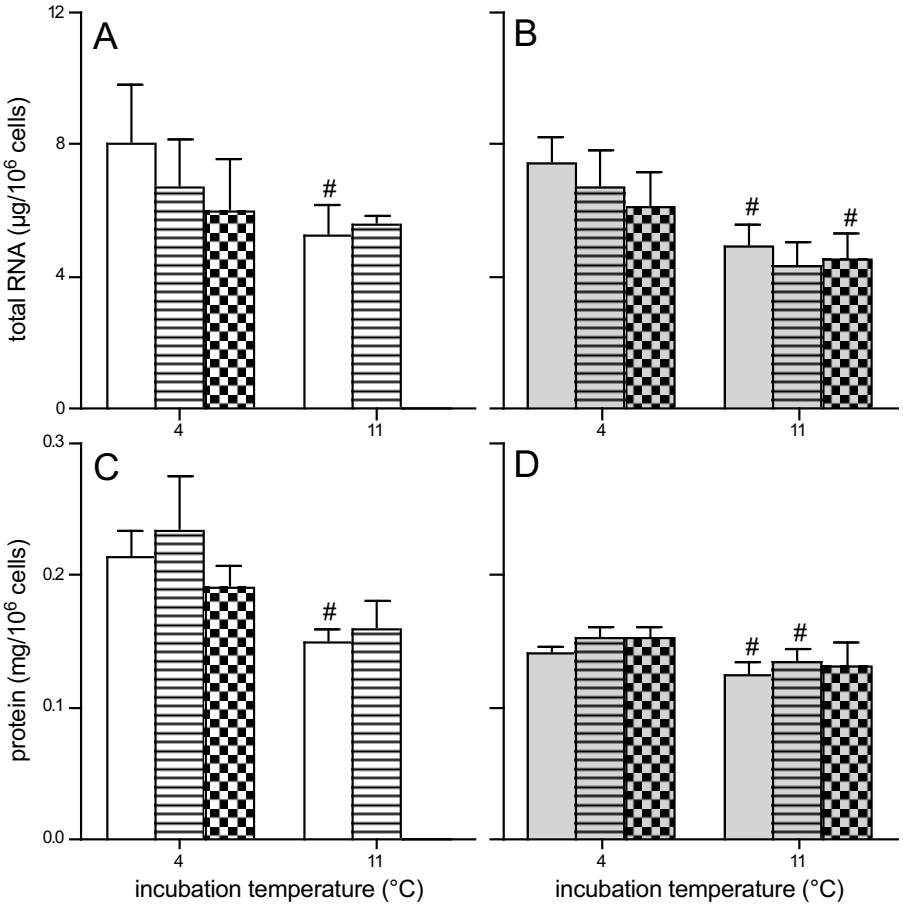

Figure 28: Effects of stress hormones on total RNA (A/B) and protein contents (C/D) in eelpout hepatocytes at different incubation temperatures. Data were determined in cells from cold (white bars) and warm acclimated (grey bars) eelpout after 48 – 72 h of incubation at 4 or 11°C under control conditions (empty bars) or treated with epinephrine (striped bars) or cortisol (chequered bars). # Significant difference from corresponding group incubated at 4°C. Values are means ± SE (n = 3 – 6).

3.5.4 Effects of epinephrine and cortisol on mRNA expression

In contrast to enzyme activities, the mRNA expression of the respective genes was partly affected by the addition of stress hormones. The addition of cortisol led to a significant rise of CS mRNA levels by 26 ± 8% in hepatocytes from cold and by 19 ± 3% in cells from warm-acclimated eelpout, when incubated at their previous *in vivo* temperature (figure 29A/B). In cells from warm-acclimated eelpout, the corticosteroid induced a significant increase of COX4 mRNA expression by 19 ± 5% (figure 29F) while COX2 mRNA levels remained unchanged. Epinephrine treatment revealed no significant effects on specific CS or COX2 mRNA levels (figure 29A-D) and only resulted in a small, but significant decrease of COX4 mRNA levels by 11 ± 5% in cold incubated cells from 4°C acclimated fish (figure 29E).

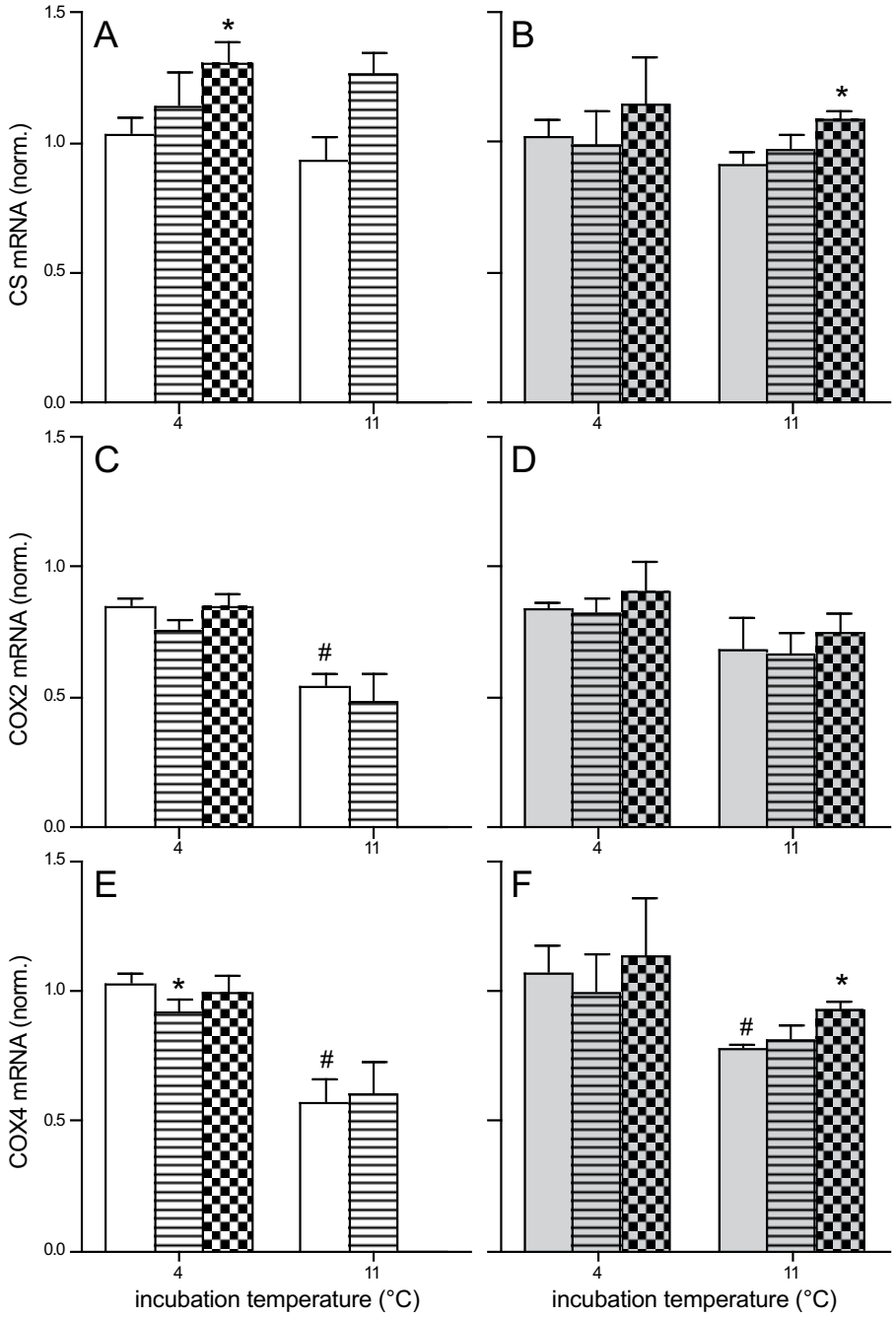

Figure 29: Relative expression of CS and two COX subunits in eelpout hepatocytes at different incubation temperatures under the influence of stress hormones. Relative amounts of CS (A/B), COX2 (B/C) and COX4 (E/F) were determined in cells from cold (white bars) and warm acclimated (grey bars) eelpout after 48 – 72 h of incubation at 4 or 11°C under control conditions (empty bars) or treated with epinephrine (striped bars) or cortisol (chequered bars). mRNA levels were determined by RPA in equal amounts of total RNA and expressed relative to the mean of freshly isolated hepatocytes from warm acclimated fish. * Significant difference from control group incubated at the same temperature; # significant difference from corresponding group incubated at 4°C. Values are means ± SE (n = 3 – 6).

3.5.5 Seasonal variation of hormone response

In contrast to the above-described data obtained during experiments carried out in late summer, a preliminary incubation series carried out during start-up of the project in the winter before resulted in a different picture for the effects of epinephrine treatment. Animals used for the winter experiments were kept under conditions identical to those for the summer incubations and both groups displayed similar body sizes, condition factors and hepatosomatic indices (table 4). In the following enzyme activities, total RNA concentrations and protein content of winter and summer incubations (as reported above) are compared under the same conditions, i.e. cells were isolated from warm acclimated fish, incubated at 11°C and treated with 0.1 μM epinephrine or cortisol per day. All winter cells were derived from one preparation. Other effectors tested during this and a further incubation (low temperatures and adenosine), were all studied in the same preparation and included in the seasonal comparison (figure 30).

When compared to the respective control, cold incubation of hepatocytes had no significant effect on CS or COX activities (figure 30A/B), but resulted in a significant increase of total RNA levels by 53 ± 17 in summer and 55 ± 24% (figure 30C) in winter experiments. Cellular protein contents were also increased in both, summer and winter cold incubations, by 13 ± 5 and 15 ± 6%, respectively (figure 30D). The daily addition of adenosine during cell culture significantly reduced COX activities by 30 ± 11% in summer and by 45 ± 7% in winter (figure 30B). Cellular RNA concentrations showed a similar, albeit not significant decrease in both summer and winter incubations (figure 30C), whereas CS activities or cellular protein contents remained unaffected (figure 30A/D). For both, cold incubation and adenosine treatment, the differences between summer and winter experiments were not significant. Incubation with cortisol also revealed no difference in the effect on enzyme activities or RNA contents in summer and winter incubations. In contrast to all other effectors, however, the response of eelpout hepatocytes to epinephrine was found to vary between seasons. Activities of COX remained unaltered in summer but were significantly, 79 ± 13% elevated over controls after epinephrine treatment in winter (figure 30B). Cellular total RNA concentrations were significantly, 355 ± 27% elevated in response to epinephrine in cells obtained during winter experiments, but remained unchanged in hepatocytes during summer experiments (figure 30C). Protein contents were also increased by 25 ± 14% in winter but not in summer (figure 30D). Trends were also

visible in CS activities, with a 20% increase in the winter incubation only, albeit not quite significant (p = 0.0531; figure 30A). Overall, both the changes in COX activities and in total RNA concentrations induced by epinephrine were found to vary significantly between seasons, with a greater response seen in cells during winter.

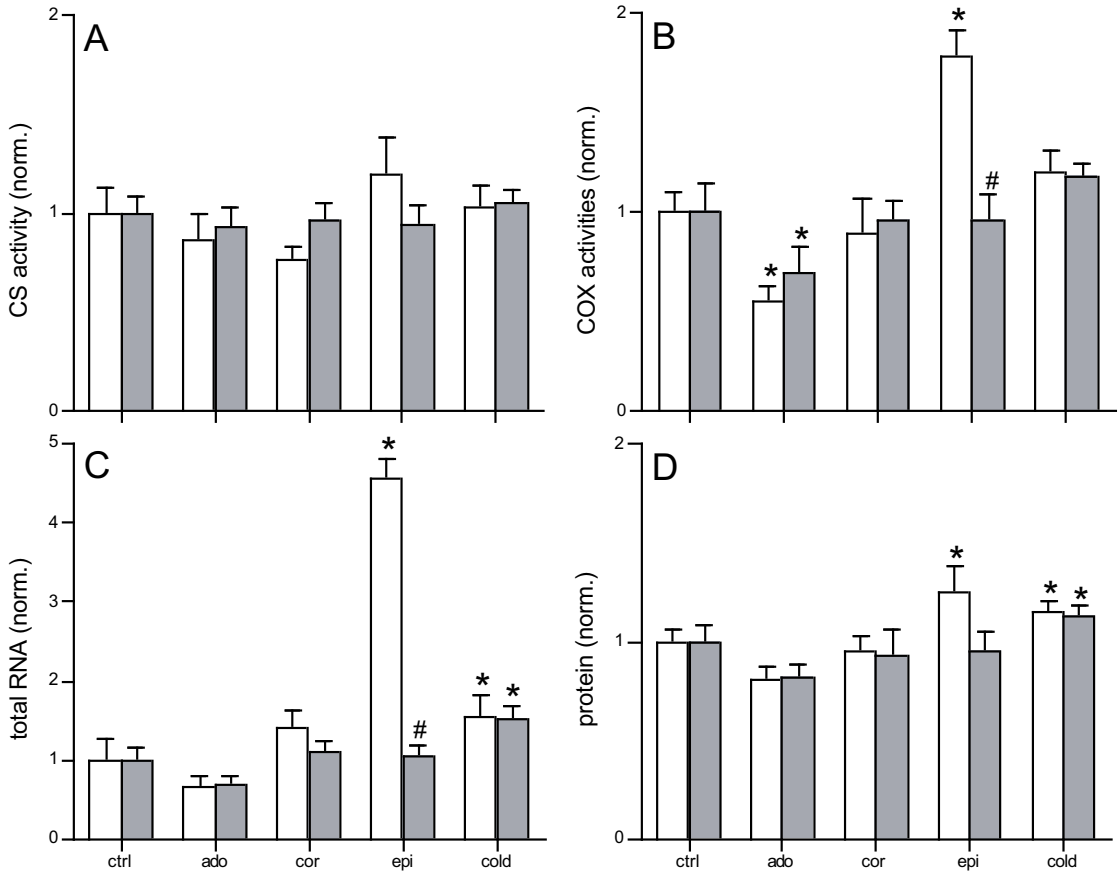

Figure 30: Impact of season on the response of isolated eelpout hepatocytes to various effectors. CS activities (A), COX activities (B) and total RNA concentrations (C) were determined in cells isolated from warm acclimated *Z. viviparus* in summer (grey bars) or winter (empty bars). Cells were incubated for 48 – 120 h under control conditions (ctrl), at 4°C (cold) or under treatment with adenosine (ado), cortisol (cor) or epinephrine (epi). * Significant difference from the corresponding control of the same season; # significant difference from the corresponding incubation in winter. Values are means ± SE (n = 3 – 6), expressed relative to the mean of the respective control.

4 Discussion

Cold exposure of ectothermic animals is well known to induce a compensatory adjustment of mitochondrial capacities and functions. This thesis addresses the signals and molecular mechanisms that coordinate the process of thermal adaptation at the cellular level. Based on the establishment of a primary liver cell culture for the model organism *Z. viviparus,* I investigated the basic ability of isolated hepatocytes to acclimate to temperature changes and elaborated the impact of some potential effectors that are suggested to induce or modulate adjustment of the energy metabolism.

4.1 Primary liver cell culture for *Z. viviparus*

The investigation of mechanisms and potential signals that coordinate the process of thermal adaptation requires an experimental system that allows comparing the effects induced by individual impact factors within a time period sufficient for the induction of metabolic adjustments. Primary cell culture represents a suitable system to meet these demands. Since no protocol for the long-term maintenance of hepatocytes from *Z. viviparus* was available in the literature, the first objective of the present thesis was to establish a primary liver cell culture for this model organism. For a successful realisation of the intended mechanistic studies, a number of basic requirements have to be met by primary culture. First, cellular viability has to be supported throughout the whole culture period, independent of the applied incubation temperature. Second, the physiological integrity of the cells and responsiveness to external stimuli has to be maintained during cell culture. And finally, the experimental design should provide identical ambient conditions for each cell in the culture and allow the extraction of consistently sized samples.

Eelpout hepatocytes, as isolated fish hepatocytes in general (Segner, 1998), tend to re-aggregate to larger cell clusters within a few hours after preparation, which prevents the sampling of definite cell numbers. This problem was efficiently avoided by a portioning of cells prior to primary culture. Nonetheless, cellular aggregation complicates the application of consistent conditions for each cell, since oxygen availability as well as the access to nutrients or cellular effectors (e.g. hormones) likely decrease towards the centre of those clusters. The incubation of hepatocytes at lowered densities, in culture dishes that provided a greater surface area per volume, reduced the formation of cell aggregates, thus improving the homogeneity of culture conditions. However, the effects of cellular density on the

survival (figure 7) indicate that a minimum of intercellular contact is necessary to maintain viability of eelpout hepatocytes. This is in line with the important role of cell-cell interactions reported for fish hepatocytes (Segner, 1998).

The main improvements in conserving cellular viability and functions were achieved by adjustments of the culture medium. Three different serum-free media were tested on eelpout liver cells: Dulbecco's Modified Eagle Medium (DMEM), Leibovitz L-15 medium (L-15) and HepatoZYME™. The latter, especially designed to maintain the phenotypic expression of enzymes linked to the biotransformation system in mammalian hepatocytes, distinctly improved survival of the cells, compared to both of the other media (figure 8A). However, total RNA content per cell decreased steeply during culture in HepatoZYME™ (figure 8B), which in combination with conserved viability, may indicate a metabolic suppression in the cells. DMEM proved to be unsuitable for both, the maintenance of cellular viability and metabolism, since it induced a similar steep decrease of cellular RNA levels as HepatoZYME™ and a considerable loss of viable cells (figure 8). In eelpout hepatocytes cultured in L-15, the decrease of total RNA quantities and CS activities in the entire sub-sample corresponded to the loss of viable cells during cell culture (figure 9), indicating a conservation of the metabolic capacity of each cell.

L-15 differs from DMEM in the composition of inorganic salts, with a higher concentration of NaCl (due to its proprietary unknown formulation HepatoZYME™ can not be included in this comparison). Both media were originally developed for mammalian cells, and the salt concentration conforms to the extracellular osmolality of terrestrial vertebrates. In contrast, marine teleosts possess a higher osmolality in the extracellular fluids, which can mainly be ascribed to elevated NaCl concentration (Eckert, 1993). Fish hepatocytes, as animal cells in general, exposed to hyposmotic conditions experience an initial swelling, which is subsequently compensated by an active ion release (Ollivier et al., 2006). This regulatory volume decrease is associated with an increase of cellular energy demand and may thereby affect long-term survival of the cells. In eelpout hepatocytes, an increase of medium osmolality by the addition of NaCl, investigated during the start-up of cell culture development with DMEM, was found to support cellular survival (figure 6), in line with the findings for primary culture of fin cells from deep-sea eels (Koyama et al., 2003) and the increased salt concentration recommended for isolation of hepatocytes from marine fish (Mommsen et al., 1994). However, DMEM and L-15 posses a similar

osmolality of ~312 and 318 mosm/l, respectively (according to the product specifications), and both media were supplemented with equal amounts of NaCl, thus osmolality cannot account for the difference in metabolic capacities observed during the media comparison experiment (figure 8). A further difference in the composition of both media is the larger assortment of amino acids at higher concentrations in L-15 than in DMEM. In rat hepatocytes elevated amino acids levels were found to increase the synthesis of various proteins that are involved for example in translation initiation, signal transduction, transport and oxidative phosphorylation (Jaleel and Nair, 2004). Thus, high amino acid concentrations may prevent metabolic suppression in isolated liver cells and, therefore, contribute to the improved physiological maintenance of eelpout hepatocytes in L-15.

The maintenance of cellular metabolism, achieved by incubation with Leibovitz L-15 medium, provided the basis for primary culture, however, the viability of eelpout hepatocytes still decreased over time. A major improvement of cellular survival was attained by the addition of bovine serum albumin (BSA) to the medium (figure 12). The positive effects of albumin in cell culture are primarily ascribed to its capacity to bind fatty acids, which are insoluble in aqueous solutions, and deliver them to cells in culture (Yamane et al., 1975). Furthermore, the protein can bind various other molecules, for example bivalent cations or amino acids, thereby protecting them from oxidation and improving their availability for the cell. However, the high binding capacity of albumin might inhibit the application of some potent cellular effectors. For example the thyroid hormone triiodothyronine is bound and thereby inactivated by the addition of BSA (Wilson and McMurray, 1981). In previous studies on fish liver cell culture other serum-free culture additives, like collagen or laminin were successfully applied to improve survival (Mommsen et al., 1994). Matrigel™, a commercially available membrane matrix, mainly consisting of laminin and collagen, was tested in the present study. In contrast to other fish species (Ferraris et al., 2002), Matrigel™ had no effect on the viability of eelpout hepatocytes (figure 11), thus the addition of BSA remained indispensable.

With the final culture conditions viability of eelpout hepatocytes was completely conserved for at least three days (figure 15). Cells were in excellent visual condition and no signs of swelling or shrinkage occurred (figure 14). Enzyme activities as well as RNA contents were maintained on a high level under these conditions and cells were responsive to temperature changes, bioenergetic disturbances or hormonal treatment (cf. chapter 3.3 –

3.5). This indicates a good conservation of the physiological integrity of the cells. Metabolic adjustments to different treatments in cultured hepatocytes were visible after 2 days and no further changes occurred thereafter, at least until the 5th day of incubation (figure 13). In contrast, in the time course of *in vivo* cold acclimation carried out on North Sea eelpout (Lucassen et al., 2003), the first adjustment occurred on day 4 day after a reduction within 12 h of water temperature from 10 to 3.5°C. The decreased response time in isolated hepatocytes may arise from the acute change of cellular microenvironment applied *in vitro*, while a shift of environmental condition *in vivo* likely requires some time depending on circulatory perfusion and associated delay of when the signal reaches the cellular level. Due to the faster response in isolated cells, reaching steady state levels after 48 h (figure 13), incubation period was reduced to 72 h, thereby increasing the variety of simultaneous treatments. Thus, apart from the constraint caused by the use of BSA, primary culture of eelpout hepatocytes established in this thesis is a suitable tool to investigate the cellular response to temperature changes and the involvement of potential effector(s).

4.2 Temperature acclimation in isolated cells

To test for the requirement of systemic effector(s) in the induction or modulation of thermal acclimation, the adjustments of energy metabolism induced by whole animal temperature acclimation (*in vivo*) were compared to the effects observed during temperature incubation of cells detached from any systemic input (*in vitro).* Changes of mitochondrial capacities and functions, monitored by the activity and expression of the mitochondrial key-enzymes citrate synthase (CS) and cytochrome *c* oxidase (COX), were observed in isolated eelpout hepatocytes after temperature acclimation *in vivo* and during cold or warm exposure in primary culture *in vitro*.

In fish red and white muscle cold acclimation typically induces a parallel increase of both mitochondrial enzymes (Battersby and Moyes, 1998; Lannig et al., 2003; Lucassen et al., 2006). In contrast, the cold acclimation response in the liver of North Sea eelpout is typified by elevated activities (referred to gram fresh weight) of the mitochondrial matrix enzyme CS in conjunction with conserved capacities of membrane bound COX (Lucassen et al., 2003). This situation is conserved in freshly isolated hepatocytes. Cold acclimation of *Z. viviparus in vivo* had no impact on COX activities (figure 16D), but resulted in an increase of CS activities given per 10^6 cells (figure 16A). The cold induced increase of the

matrix (CS) over the membrane enzyme (COX) is assumed to support anabolic processes that involve intermediate products of the citrate cycle, like for example lipid synthesis, which is found generally enhanced in the cold (Pörtner, 2002b). This indicates a functional change of liver mitochondria in the cold.

Despite elevated CS activities in eelpout hepatocytes after *in vivo* cold exposure, the relative mRNA levels of the respective gene remained unchanged (figure 18A). This indicates a loose relationship between message levels and enzyme functional capacities and suggests an important role for posttranscriptional mechanisms (Lucassen et al., 2003). Similar to the respective enzyme activities, the transcript levels of two COX subunits, the mitochondrial-encoded subunit COX2 (figure 18D) and the nuclear-encoded subunit COX4 (figure 18G) were not affected by *in vivo* temperature acclimation. The acclimation profile of freshly isolated hepatocytes is thus in line with the situation in liver of thermally acclimated specimens (Lucassen et al., 2003).

The consecutive response of isolated hepatocytes to temperature clearly depended on the previous acclimation temperature of the cells *in vivo*. Warm incubation of cells from cold acclimated fish induced a reduction of the activities of both mitochondrial enzymes similar to observations in muscle (Lannig et al. 2003; Lucassen et al., 2006) (figure 16B/E) and a decrease of mRNA expression of both COX subunits (figure 18E/H). Furthermore, warming resulted in a concomitant reduction of total RNA amounts and protein contents (figure 17B/E). The main fraction of total RNA is provided by ribosomal (rRNA) and transfer RNA (tRNA), both of which are involved in protein synthesis (Millward et al., 1973). Thus a decrease of total RNA in combination with decreased cellular protein contents may indicate a reduction of protein synthesis rates. A decrease of mitochondrial enzyme activities and protein synthesis rates during warm incubation of isolated hepatocytes is in line with an earlier study carried out with channel catfish (Koban, 1986). Together with the excellent viability of eelpout hepatocytes during the whole incubation period (figure 15), this suggests that fish liver cells without any systemic input may display basic adjustment to warming. However, the difference to the *in vivo* effects of warm exposure in the liver indicates the involvement of further systemic effector(s) in the modulation of warm acclimation.

In contrast, cold incubation of hepatocytes from warm acclimated eelpout left enzyme activities unchanged (figure 16C/F) and solely increased the mRNA levels of COX4

(figure 18I) and total RNA amounts (figure 17C). Protein contents in this group decreased at both acclimation temperatures compared to the initial value, reaching a significantly higher steady state level at 4 than at 11°C (figure 17F). As mentioned above, the main part of total RNA consists of rRNA and tRNA, thus the increased total RNA levels suggest a compensation of protein synthesis capacity during cold incubation of isolated cells. The changes of cellular protein content, an indicator for the actual protein synthesis rate, were less noticeable than the ones observed for total RNA levels. This mismatch between the protein synthesis capacities and rates suggests that this process may be impaired during *in vitro* cold acclimation of hepatocytes. The lack of metabolic cold compensation in isolated hepatocytes is in line with the concept that a cold-induced mismatch of energy demand and supply becomes effective at a high organisational level, the intact animal, as a consequence of limitations in oxygen supply (Pörtner, 2001; 2002a). In primary culture, the decrease in the metabolic rate of isolated cells in the cold occurs at sufficient oxygen supply and parallels the reduction of energy demand. Hence, this experimental situation reduces the pressure to acclimate, an observation, which indicates that cold acclimation *in vivo* occurs in response to systemic signal(s).

4.3 Extracellular pH as a signal for thermal adaptation

The literature proposes a number of potential effector(s) that might be involved in thermal adaptation. Since mitochondrial capacities change unidirectionally with temperature, at least within the thermal tolerance window, it was postulated that the potential effector to coordinate these adjustment also has be unidirectional. Extracellular pH (pH_e), which decreases with increasing temperature by approximately -0.018 pH units per °C (Hochochka and Somero, 2002) would be a suitable candidate to act as such a signal. During short-term incubation (60 min) of hepatocytes from polar eelpout *P. brachycephalum* oxygen consumption was found to change linearly with medium pH, resulting in an almost 40% lower respiration rate at pH 6.5 than under control conditions (pH 7.9) and a concomitant inhibition of protein synthesis rate by 80% (Langenbuch and Pörtner, 2003). Long-term incubation of liver cells from common eelpout at medium pH values within the physiological range (7.8 – 7.2) left CS activities unchanged (figure 19A). This insensitivity to pH_e changes is in line with a high capacity for active regulation of intracellular acid-base status as observed for North Sea eelpout and suggested for eurythermal animals in general (Van Dijk et al., 1997; Sartoris et al., 2003). A further

reduction of medium pH to 6.5, far below the decrement achieved by warming, resulted in an increase of CS activities in eelpout hepatocytes, while COX activities remained unchanged (figure 19). This indicates a functional change of mitochondria during prolonged acidosis, possibly to provide precursors for amino acids, which might occur as a contribution to compensate for reduced protein synthesis rate (Langenbuch and Pörtner, 2003). Due to the low sensitivity of isolated cells to pH_e changes within the physiological range and the contrasting effects of low pH_e, resembling high temperatures, and *in vivo* warm acclimation on CS activities, the involvement of extracellular pH as a direct signal for thermal adaptation appears unlikely.

4.4 The role of adenosine in thermal adaptation

One attractive hypothesis suggests the contribution of bioenergetic factors to adjustments of mitochondrial capacities or functions during thermal acclimation (Leary and Moyes, 2000). Such a factor may be released during mismatch of energy supply and demand *in vivo*. A potential candidate to act as such a bioenergetic signal is adenosine. This metabolite is produced during degradation of intra- and extracellular AMP, following a breakdown of ATP, and is often accumulated when energy demand exceeds energy supply. Adenosine can be released from the cell by specialized nucleoside transporters and can be distributed to the whole organism (Buck, 2004). Therefore, adenosine might act as a hormone-like effector in response to bioenergetic challenges as during temperature changes.

4.4.1 Adenosine levels during in vivo cold exposure

Elevated adenosine levels have frequently been reported following anoxic or hypoxic conditions. In the brain of epaulette shark, adenosine levels increased during acute anoxia, whereas the levels of phosphorylated adenylates (ATP + ADP + AMP) remained virtually unchanged (Renshaw et al., 2002). However, there is no literature available concerning the effect of temperature. To elucidate a potential role of the metabolite in thermal acclimation, adenosine concentrations were determined in North Sea eelpout during cold exposure. In blood serum and liver, adenosine concentrations were found to increase significantly during 24 h after a temperature shift from 11 to 4°C (figure 20). This is in line with a prolonged response (> 12 h) to temperature changes observed in eurythermal North Sea eelpout during adjustments of intracellular pH (Sartoris et al. 2003). Furthermore, the

study reported that tissue ATP concentrations were conserved during temperature incubations within the thermal tolerance window. This indicates that minor decrements in ATP levels elicit small but significant changes in adenosine concentrations. These observations suggest that adenosine might be a suitable signal to support metabolic adaptation in response to such bioenergetic disturbances.

Adenosine concentrations observed in eelpout after cold exposure were similar to those previously reported for the heart of short-horned sculpin during acute anoxia (MacCormack and Driedzic, 2004), however, the response of adenosine levels occurred during considerably different time periods. Hypoxic conditions usually elicit an increase in adenosine levels for minutes or hours (Renshaw et al., 2002; Lutz and Kabler, 1997). In contrast, cold induced adenosine accumulation in *Z. viviparus* persisted for at least three days (figure 20), possibly as long as the hypoxic challenge remained uncompensated for. After three days of cold exposure liver adenosine concentrations remained high, whereas serum adenosine had already significantly decreased (figure 20). In a study of the time course of temperature acclimation in North Sea eelpout, cold compensation of energy metabolism in liver, monitored through analysis of mitochondrial enzymes, was found to become visible after 4 days (Lucassen et al., 2003). In line with thermally induced hypoxia and functional insufficiency extended exposure to temperature change may result in prolonged elevation of adenosine levels until the seasonal acclimation process is well underway. This would allow adenosine to contribute to the cold acclimation process.

4.4.2 Adenosine effects at the cellular level

When applied to isolated hepatocytes, adenosine had no effect on CS, but distinctly affected COX activities. Although *in vivo* acclimation of *Z. viviparus* neither changed initial activities nor the mRNA expression of COX, the cellular response to adenosine was found to depend on the thermal origin of the cells. Responsiveness to adenosine was enhanced in hepatocytes from cold acclimated fish, however, the magnitude of effects increased at higher incubation temperatures. Overall, adenosine likely participates as a modulator of thermal acclimation.

The adenosine effect comprised two major components, a decrease of COX activity (figure 21C/D) and a concomitant increase of COX mRNA expression (figure 23C – F). The activity of COX, the terminal oxygen-consuming step of the respiratory chain, is often

used as an approximation for the aerobic capacity of the cells (Kadenbach et al., 2000). Thus, it can be assumed that adenosine reduces the capacity of aerobic energy production. This is in line with observations by Krumschnabel et al. (2000), who found reduced oxygen consumption rates under the acute effect (10 – 30 min) of adenosine in trout hepatocytes in parallel to a decrease of protein synthesis rate. They assumed that the deceleration of oxygen uptake was due to diminished cellular ATP demand caused by adenosine. Such an effect may be paralleled or followed by the reduction of COX capacities as observed in eelpout hepatocytes. However, although total RNA amounts were slightly decreased by adenosine treatment in cells from warm acclimated animals, protein contents were maintained at a similar level as in concomitant controls (figure 22B/D). Therefore, an effect of the metabolite on protein synthesis rate cannot be confirmed for the present study, but the findings suggest a slowed degradation of proteins.

The suppressing effect of adenosine on aerobic capacities is contrasted by the stimulating effect on the expression of COX. Since this effect was more pronounced in cells incubated at 11°C, adenosine treatment of isolated hepatocytes abolished the warming induced reduction of COX expression. As a result, mRNA remained at similar levels at both incubation temperatures (figure 23C/E/F), resembling the expression pattern obtained during *in vivo* temperature acclimation (figure 18D/G). Adenosine treatment thus results in a discrepancy between the levels of transcription (increased) and the capacity of the enzyme (decreased). Similarly, loose coordination between message levels and enzyme functional capacity was found in the time-course of whole animal acclimation (Lucassen et al., 2003). Adenosine may thus influence the coordination of transcriptional and translational activities or cause posttranslational modification of the enzyme proteins.

The question arises how adenosine exerts these effects. Most effects of adenosine concerning energy metabolism have been ascribed to the hormone-like interaction of the metabolite with specialized adenosine receptors (Buck, 2004). In eelpout hepatocytes, the application of the adenosine A_1 receptor antagonist 8-PT in addition to adenosine and of the non-selective receptor agonist NECA resulted in a similar pattern as incubations with adenosine alone and under control conditions, respectively (figure 24). Both ligands have efficiently been used to block and stimulate adenosine receptors in other fish species (Krumschnabel et al., 2000; Rosati et al., 1995). Thus, a receptor-mediated action of adenosine can be excluded for the present experiments. Furthermore, hepatocytes

continuously consumed adenosine in a temperature dependent degree. In line with the intensity of the adenosine-induced effects, adenosine consumption was increased after *in vivo* cold acclimation, possibly due to an involvement of the metabolite in some cold compensated energy metabolism related processes, and was stimulated at elevated *in vitro* incubation temperatures (figure 25). These observations suggest diffusive entry and intracellular action of adenosine.

Although no data exist for fish, adenosine may act through different mechanisms in fish hepatocytes. The metabolite can be reconverted to AMP by adenosine kinase and give rise to subsequent ATP synthesis (Bontemps et al., 1983). ATP is known as an allosteric inhibitor of COX but also of CS (Lehninger, 1998), thus adenosine treatment might result in reduced activities of both enzymes which has however, not been observed here. A possible reason may be the "second mechanism of respiratory control", whereby the allosteric ATP inhibition of COX is switched on by cAMP-dependent phosphorylation and switched off by calcium-induced dephosphorylation (Ludwig et al., 2001). Adenosine has the potential to alter both, the intracellular cAMP as well as the calcium level. However, a rise of intracellular cAMP levels was only demonstrated in association with hormone-like adenosine action (Buck, 2004), whereas an elevation of intracellular calcium concentration was also observed, when adenosine receptors were not involved (Tinton et al., 1996; Krumschnabel et al., 2000). Since a receptor-mediated action of the metabolite on COX activities can be excluded and calcium switches of the allosteric inhibition, an adenosine action mediated by an increased sensitivity of COX to ATP inhibition appears unlikely. High intracellular adenosine concentrations were further found to prevent the hydrolysis of S-adenosylhomocysteine (SAH), a competitive inhibitor of most S-adenosylmethionine (SAM) dependent methyltransferases (Kloor and Oswald, 2004), which are involved in the methylation of many molecules, e.g. proteins, DNA and RNA (Chiang et al., 1996). In knockout mice, deficient for the synthesis of hepatic SAM, the protein levels of COX1 and COX2 were found to be only half of those in wild type mice, whereas the mRNA levels of COX2 remained unaltered, indicating a translational down-regulation of COX (Santamaria et al., 2003). Although the underlying mechanisms still need to be investigated, the inhibition of SAM-dependent methyltransferases by adenosine may account for the mismatch between the expression and activity levels of COX, observed in adenosine treated eelpout hepatocytes. The adenosine-related increase in the expression of both, the nuclear and the mitochondrial encoded COX subunits is most remarkable and suggests

coordinated regulation of nuclear and mitochondrial genes, thereby substantiating the observed effects. The simplest explanation would be transcriptional compensation of the suppressed translation of COX. However, with the data at hand and the sparse literature available, the mechanism of how adenosine affects COX transcription remains to be elaborated.

4.5 Hormonal contributions to thermal adaptation

Besides bioenergetic effectors, hormones are frequently suggested to participate in the induction of metabolic adjustments to bioenergetic disturbances (Umminger, 1978). Various physiological functions are under endocrine control, for example growth, maturation and reproduction. Thyroid hormones are usually the first class of hormones that comes to mind concerning the regulation of aerobic capacity in general. Thyroxine (T4) and its active form triiodothyronine (T3) are well known to increase metabolic rate in mammals (Goglia et al., 1999; Weitzel et al., 2003), but were also found to affect mitochondrial enzyme activities in fish (Tripathi and Verma, 2003b; LeRay et al., 1970). However, the active form of the thyroid hormone is bound and thereby inactivated by bovine serum albumin (Wilson and McMurray, 1981), which was found to be indispensable for long-term maintenance of eelpout hepatocytes. Therefore, an investigation of this class of hormones was not feasible under the prevailing circumstances.

Another class of hormones that has early been hypothesized to be involved in thermal adaptation are stress-hormones (Umminger, 1978). For ectothermic organisms, acute temperature changes may represent a form of stress and provoke the release of stress hormones. Two major classes of hormones mediate the stress response in teleost fish: corticosteroids with cortisol as the single representative occurring in fish, and catecholamines with epinephrine as one of the primary hormones in this class (Wendelaar Bonga, 1997). A response of the respective hormone levels to temperature has been shown in a study on *Oreochromis aureus*, a subtropical freshwater inhabitant, where acute cold exposure was found to increase the plasma concentration of epinephrine and cortisol. Clear difference occurred between the time periods in which these hormones were present. Epinephrine was released into the circulation almost immediately following the stress impulse and was rapidly removed from the plasma, whereas the release of cortisol was slightly delayed, but the elevation of plasma levels was more prolonged (Chen et al., 2002). Therefore the potential role of the two major stress hormones in the induction or

regulation of thermal adaptation was investigated in isolated eelpout hepatocytes. Since the effects induced by each of these hormones clearly differed, each hormone is discussed separately.

4.5.1 The impact of cortisol on metabolic adjustments

The main effects of cortisol on eelpout hepatocytes occurred at the transcript level, with an increase in specific mRNA levels of CS in cells incubated at the animal's acclimation temperature (figure 29A/B). A small increment became visible for the nuclear encoded COX subunit (COX4), but only in the warmth (figure 29F). These findings are in line with the generalized observation that steroid hormone action on energy metabolism in general is mainly mediated by alterations at the transcript level (Scheller and Sekeris, 2003).

Due to its lipophilic nature, the corticosteroid can easily pass the cell membrane and bind to intracellular corticosteroid receptors (CR). Two types of receptors, a cytosolic and a nuclear CR, have been determined for fish (Chakraborti and Weisbart, 1987; McLeese et al., 1994), in line with the pathways established for cortisol action. In the nucleus, the cortisol-receptor complex acts as a ligand-activated transcription factor by binding to corticoid responsive elements (CRE) of the DNA and hence inducing the transcription of individual hormonal controlled genes (Beato et al., 1995). Various genes encoding for enzymes of oxidative phosphorylation, including several COX subunits, but also nuclear and mitochondrial transcription factors like nuclear respiratory factors (NRF-1, NRF-2) and the mitochondrial transcription factor A (mtTFA) are suspected to be under control of corticosteroids (Scheller and Sekeris, 2003). In the cytosol, the cortisol-receptor complex was shown to affect transcript levels by altering the stability of individual mRNAs. The degradation rate of mRNA in the cytosol is regulated by sequences and structures (*cis* elements) in the untranslating regions at the 3' and 5' end and by proteins binding to these structures (*trans* acting factors) (Staton et al., 2000). Similar to other steroid hormones, the cytosolic cortisol-receptor complex was shown to operate as a *trans* acting factor, stabilizing or destabilizing individual mRNAs (Ing, 2005). Both pathways might account for the elevated CS and COX4 mRNA levels in eelpout hepatocytes under cortisol treatment. A nuclear action appears to be more likely, since the corticosteroid mainly affected the expression of nuclear encoded genes. Furthermore, cortisol effects on cytosolic mRNA stability are usually reported to support decay (Kracht and Saklatvala, 2002). However, the data collected in the present study do not allow distinguishing

between the contributions of increased mRNA transcription or stability to the observed effect.

Although the mRNA levels of CS and COX4 were significantly elevated in response to cortisol treatment, the capacities of both mitochondrial enzymes did not change. In a time series of cold acclimation in the liver of North Sea eelpout (Lucassen et al., 2003), CS mRNA levels were found to increase significantly between the 4th and 6th day of cold exposure, whereas at that stage, CS activities were not yet significantly different from warm acclimated controls. Within this time period mRNA levels of COX2 were also increased, albeit not significantly, at unchanged enzymatic capacities. Thus, cortisol treatment of isolated eelpout hepatocytes resembles the picture observed *in vivo*, supporting a potential role for the stress hormone in the beginning of the acclimation process.

However, cold acclimation of common eelpout *in vivo* induced a constant rise of CS activities in the liver, reaching the significance level at day 9 (Lucassen et al., 2003). Similarly, *in vivo* application of cortisol for 7 – 10 days was accompanied by an increase of CS activities in liver, muscle and brain of catfish and in the liver of American eels (Tripathi and Verma, 2003a; Foster and Moon, 1986). Within the time period of up to 5 days observed in the present study, no effect of cortisol on CS capacities became visible (figure 13, 27A/B). This suggests that further effector(s) might be involved in whole animal adaptation of energy metabolism that help converting increased transcript level into elevated activities or that extended time periods are required for the onset of translation.

4.5.2 Epinephrine effects on energy metabolism

Epinephrine treatment of hepatocytes isolated from eelpout during summer had only a minor effect on mitochondrial enzyme expression and activities. The latter remained completely unaltered (figure 27) and only the expression of COX4 was slightly, but significantly, decreased after epinephrine treatment of cells from cold acclimated eelpout at 4°C (figure 29E). These findings contrasted those obtained during the initial trials in eelpout investigated during winter.

In contrast to summer experiments, epinephrine treatment of hepatocytes in winter induced an increase of CS and COX activities (figure 30A/B), resembling the cold-induced adjustments typical for fish muscle (Lannig et al., 2003; Lucassen et al., 2006).

Furthermore, elevated levels of total RNA and protein, indicative for an increased protein synthesis rate, occurred during winter incubations (figure 30C/D), in line with the effects observed in white muscle of North Sea eelpout after *in vivo* cold acclimation (Storch et al., 2005). This supports a potential role for epinephrine in the early phase of cold adaptation. Only little information is available in the literature about the mode of epinephrine action on mitochondrial energy metabolism, however, the mechanisms involved in adjustments of glucose levels in the liver have frequently been studied. Cellular epinephrine action is mediated by a divergent class of adrenoreceptors (AR), distinguished according to their signalling pathways. In fish liver, evidence has been provided for the existence of at least two classes, β-AR and recently also α_1-AR (Fabbri et al., 1998). A potential pathway, that might result in elevation of protein synthesis rates and enzyme activities as observed in the present study, involves the β-AR. Epinephrine binding to β-AR activates adenylate cyclase, giving rise to high intracellular cAMP levels, which activate protein kinase A (PKA) (Fabbri et al., 1998). PKA directly phosphorylates transcription factors like the cAMP response element binding protein (CREB) and is the initial step of a signalling cascade, which results in the activation of several further transcription factors (Horbinski and Chu, 2005; Hunzicker-Dunn and Maizels. 2006). However, since mRNA levels were not determined in winter experiments and epinephrine effects during the later incubation series in summer were only minor, the mode of action remains speculative.

The striking differences between epinephrine effects in summer and winter might indicate a seasonal variation of sensitivity to epinephrine. Further study is needed to support this conclusion. The results for all other tested potential effectors were no different in summer and winter experiments, supporting the reliability of these conclusions (figure 30). Furthermore, the impact of seasonality on the epinephrine response suggests that this effect cannot be ascribed to general metabolic changes, but might result from a higher sensitivity of liver cells to the catecholamine in winter. Furthermore, fish used in both winter and summer experimental series were of comparable size and fitness (table 4) and maintained at the same temperature under equal conditions in the aquaria further supporting an effect of seasonality.

Some evidence is available from the literature for a dependence on acclimation temperature of other responses to epinephrine. In rainbow trout, cold acclimation significantly increased the stimulating effect of epinephrine on heart rate (Keen et al.,

1993; Aho and Vornanen, 2001) and on the rate of glucose release from perfused livers (McKinley and Hazel, 1993). The increase in epinephrine sensitivity is supported by a higher number of β-adrenoreceptors in hepatocytes from 5°C versus 20°C acclimated trout (McKinley and Hazel, 2000). However, in eelpout hepatocytes no differences were observed regardless of initial acclimation temperature of animals during summer arguing again for a seasonal pattern.

The density of β-AR in erythrocytes of rainbow trout was also shown to exhibit seasonal variations, with the lowest number of receptors per cell found in spring and the highest in autumn (Koldkjær et al., 2004). Furthermore, artificial elevation of plasma catecholamine levels in rainbow trout resulted in a down-regulation of β-AR densities of red blood cells (Gilmour et al., 1994). In two catfish species (*C. batrachus* and *H. fossilis*) circulating catecholamine levels were found to depend on reproductive stage, with high plasma concentrations during prespawning and spawning phases (gonadal recrudescence) and low levels during postspawning and resting phases (Manickam and Joy, 1990; Senthilkumaran and Joy, 1995). The common eelpout is viviparous and these observations in catfish may not apply. However, changes of testosterone and estradiol levels during the reproduction cycle of *Z. viviparus,* with high levels in the phase of gonadal recrudescence (August/September) and low levels during pregnancy and the release of the juveniles (January/February) (Larsson et al., 2002) exhibit a similar pattern as obtained for non-viviparous fish (Wingfield and Grimm, 1977). This suggests that circulating epinephrine levels may also be higher in eelpout during summer and explain the observed differences between summer and winter experiments.

4.6 Conclusions and perspectives

The lack of cold acclimation in isolated fish hepatocytes *in vitro,* maintained at ample oxygen and nutrient supply, supports the hypothesis that a cold-induced mismatch of energy demand and supply only becomes effective at a higher organisation level, as a consequence of limitations in oxygen supply. This strongly indicates the involvement of (a) systemic signal(s) in the induction of cold acclimation. Furthermore, the differences observed between warm acclimation patterns in whole animals and isolated cells also suggest that systemic effector(s) contribute to the modulation of thermal adjustments *in vivo* to match tissue specific demands. Several potential effectors that are suspected to act as such a signal were investigated in the present thesis and some emerged as promising candidates: adenosine, cortisol and epinephrine. Their effects are summarized in figure 31.

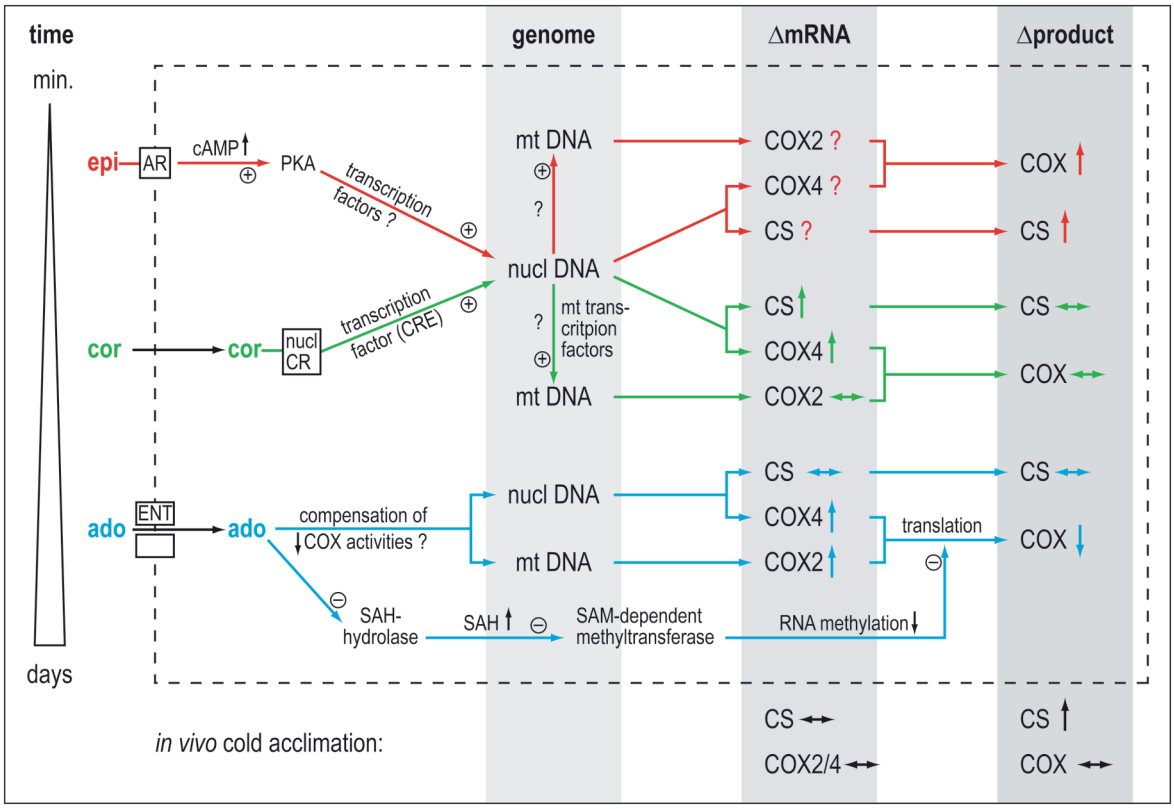

Figure 31: Action of potential signals for temperature adaptation in eelpout hepatocytes. Depicted are the changes in mRNA expression (ΔmRNA) and activity levels (Δproduct) of CS and COX induced by epinephrine (epi; red), cortisol (cor; green) and adenosine (ado; blue), compared to the respective control incubation, and the supposed mode of action for each signal (⊕: activation; ⊖:inhibition). mRNA and activity levels in eelpout hepatocytes after long-term *in vivo* cold acclimation are given for comparison. The time scale gives an estimation for the appearance of epi, cor (Chen et al., 2002) and ado during *in vivo* cold exposure. AR: adrenoreceptor; CR: corticosteroid receptor; ENT: equilibrative nucleoside transporter; PKA: protein kinase A; SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; mt: mitochondrial; nucl: nuclear.

The accumulation of adenosine observed during cold exposure *in vivo* would allow for a role for the metabolite in thermal acclimation, supporting the hypothesis that bioenergetic effectors participate in the regulation of metabolic adjustments. Application of adenosine to isolated hepatocytes reduced COX activity, but induced a compensatory increase of COX mRNA levels, resulting in an expression pattern in isolated hepatocytes similar to the one found during whole animal temperature acclimation. The contrasting effect on COX activities and mRNA expression suggests that adenosine blocks the translation of COX mRNA. This modulating role may become important during warm acclimation when excess COX activity is removed, or during cold acclimation when build-up of excess COX activity is prevented. Receptor-mediated adenosine action was not involved in the observed effects, suggesting diffusive entry and intracellular action of the metabolite, likely mediated by an inhibition of RNA methylation through high SAH levels. Nonetheless, the lack of an adenosine effects on CS activity and expression levels indicate that further signals of thermal acclimation remain to be identified. The similarity of cortisol-induced effects on the transcription of CS and COX to changes observed in the beginning of *in vivo* cold acclimation (Lucassen et al., 2003) supports a potential role for the corticosteroid in the early phase of thermal acclimation. In contrast to whole animal cold acclimation as well as to *in vivo* cortisol application (Tripathi and Verma, 2003a; Foster and Moon, 1986) the hormone failed to increase CS activities in isolated hepatocytes. This indicates that further processes or additional time is necessary to transfer increased transcript levels into elevated protein levels. The release of both epinephrine and cortisol during cold stress (Chen et al., 2002) suggests that the catecholamine may operate as an additional signal, supported by the strong adjustments of enzyme activities in hepatocytes observed in winter experiments. The higher cellular sensitivity for this hormone in early winter supports its role during seasonal acclimatisation.

Since cold exposure induces the sequenced release of both, stress hormones and adenosine, a progressive interaction appears possible, at least during early winter. As a possible scenario, the epinephrine-induced increase of protein synthesis rate would support the translation of elevated CS and COX transcript levels induced by cortisol, while adenosine accumulation, which occurred for a prolonged period in liver, would inhibit the production of excess COX activities. As a consequence, CS activities would increase over COX activities, resembling the typical *in vivo* cold acclimation pattern in eelpout liver.

The findings of the present thesis confirm the necessity of systemic signals for the induction and the control of thermal adaptation and suggest a role for adenosine and stress hormones in this process. Nonetheless, further efforts are required to substantiate the effects observed in eelpout hepatocytes. The detailed mechanism of adenosine action including the effect on translation awaits further investigation. A combined incubation with L-homocysteine which was found to enhance the inhibitory effect of adenosine on protein synthesis (Tinton and Buc-Calderon, 1995) and the determination of intracellular adenosine levels during cell culture might help to clarify the picture. Additional incubation series, including a determination of adrenoreceptor densities are also required to confirm the impact of season on epinephrine response, and prolonged incubations should be performed with cortisol to elaborate the potential long-term effect of the hormone on enzyme activities. Furthermore, a measurement of the consecutive release of both hormones during cold acclimatisation in summer and winter could elucidate the role of stress hormones in whole animal acclimation. Finally, an investigation of a potential interaction of the observed effectors could boost the understanding of the mechanisms involved in thermal adaptation. With the primary culture for eelpout hepatocytes, a suitable tool to process these observations has been established during the present thesis. However, a further improvement of the cell system would be desirable. In particularly the development of culture conditions that support a proliferation of eelpout hepatocytes and the establishment of a cell line for the this model organism would be very promising. Such a model system allows for a high number of simultaneous incubations at equal terms and for longer time periods. This will give chance to test the hypotheses concerning a progressive interaction of all signals identified in this thesis, thereby simulating a complete thermal acclimation in a cellular system.

5 References

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Publications

The following publications were prepared with data collected during this thesis:

Publication I:

Eckerle, L.G., Lucassen, M., Hirse, T. and Pörtner, H.O. (2007). Cold induced changes of adenosine levels in common eelpout (*Zoarces viviparus*): A role in modulating cytochrome-c-oxidase.
Journal of Experimental Biology (submitted)

Publication II:

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Lars Gregor Eckerle
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Bremerhaven, den 20. November 2007

Erklärung gem. § 5 (1) Nr. 3 PromO

Ich erkläre hiermit,

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

und

2. dass ich außer dem jetzt laufenden Promotionsverfahren auch kein anderes beantragt habe.