Interaction of oxygen supply, oxidative stress, and molecular defence systems during temperature stress in fishes

Beziehungen zwischen Sauerstoffversorgung, oxidativem Stress und molekularen Schutzmechanismen bei Temperaturstress in Fischen

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Frequently used abbreviations

aa	amino acid
AOX	antioxidant
bp	base pairs
CBP	CREB binding protein
Cm	*Chionodraco myersi*
EMSA	electromobility shift assay
EPR	electron paramagnetic spin resonance spectroscopy
GPX	glutathione peroxidase
GSSG	oxidised glutathione
GSH	reduced glutathione
H_2DCFDA	dichlorodihydrofluorescein diacetate
HIF	hypoxia inducible factor
MDA	malondialdehyde-(bis)-acetate
NO	nitric oxide
ODD	oxygen-dependent degradation domain
Om	*Oncorhynchus mykiss*; rainbow trout
Pb	*Pachycara brachycephalum*
PHD	prolyl hydroxylase
POBN	α-(4-pyridyl 1-oxide)-N-t-butyl nitrone
PUFA	poly-unsaturated fatty acid
pVHL	von Hippel Lindau Tumor suppressor protein
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SOD	superoxide dismutase
TAD	transactivation domain
TBARS	thiobarbituric reactive substances
tBOOH	*tert*-butylhydroperoxide
Tc	critical temperature
Th	*Trematomus hansoni*
Tp	*Trematomus pennellii*
Tp	pejus temperature (pejus = getting worse)
XOD	xanthine oxidase
Zv	*Zoarces viviparus*

Summary

Oxygen is the essential substrate for oxidative energy production, but oxygen exposure has to be limited because of the damaging effects of reactive oxygen specie (ROS). Thus, the regulation of oxygen homeostasis within a narrow physiological range is crucial for all aerobic life. In marine ectotherms, temperatures outside the species specific optimum range, which is enclosed by the pejus temperatures (Tp), are supposed to cause progressively decreasing oxygen levels in body fluids and tissues, i.e. functional hypoxia. When critical temperatures (Tc) are reached, transition to anaerobic energy production can be observed. In my doctoral study I tested the hypothesis that temperature induced hypoxia entails oxidative stress, i.e. unbalanced ROS production. Moreover, temperature-induced hypoxia was suggested to induce physiological adjustments mediated by the hypoxia inducible transcription factor (HIF-1), i.e. the master regulator of oxygen homeostasis.

I investigated the long-term influence of environmental temperature and the short-term effect of graded temperature stress on oxidative stress markers and the HIF-1 response in the liver of marine fish from different latitudes under *in vivo* conditions.

The Antarctic zoarcid *Pachycara brachycephalum,* kept at control temperature (0°C) was compared to winter acclimatised (6°C) con-familial temperate *Zoarces viviparus*. A highly oxidised glutathione redox ratio and elevated microsomal lipid radical formation rates in *P. brachycephalum* reflected the increased susceptibility of polar animals for oxidative stress and lipid peroxidation. However, high glutathione levels appeared to buffer elevated lipid radical formation in *P. brachycephalum* and to charge the liver tissue with a high antioxidant capacity. Consequently, oxidative damage markers were low under control conditions (0°C) as well as during warm acclimation to 5°C, when compared to the temperate species. In line with cold enhanced oxidative stress, seasonal temperature changes within the natural temperature range of the temperate *Z. viviparus* caused higher levels of oxidative stress in cold acclimated specimens (6°C) collected in winter than in animals collected in summer (12°C). Thus, in the zoarcids, both cold adaptation and cold acclimatisation were associated with elevated oxidative stress levels.

Sequence determination of the hypoxia-inducible subunit HIF-1α from the temperate *Zoarces viviparus* and four cold-adapted Antarctic fishes (Zoarcidae: *Pachycara brachycephalum*, Notothenioidei: *Trematomus hansoni*, *T. pennellii*, and *Chionodraco myersi*) demonstrated remarkable differences in the deduced peptide sequences compared with mammals and non-polar fishes. In *P. brachycephalum* HIF-1α the N-terminal

functional proline of the oxygen-dependent degradation domain was substituted by leucine, which is so far the first report of this phenotype. As the HIF-1α sequences from the three notothenioids contained both functional proline residues, Pro-Leu substitution cannot be considered a specific polar adaptation. Lack of one functional proline may cause higher resistance of HIF-1α to prolyl hydroxylases and proteasomal degradation. Thus, it can only be speculated that HIF-1α is regulated mainly via transactivation and not so much via degradation / stabilisation in the Antarctic zoarcid.

Increased HIF-1 DNA binding in 5°C acclimated *P. brachycephalum versus* control fish kept at 0°C indicated that at least at the level of dimer formation HIF-1 was still functional, despite Pro-Leu substitution and despite the highly oxidised redox environment. Whereas in *P. brachycephalum* HIF-1 DNA binding was higher at the respective warmer water temperature (5°C *versus*. 0°C), in the temperate *Z. viviparus* it was higher in cold winter (6°C) *versus* summer animals (12°C). In both species, HIF-1 may play a physiological role to adjust tissue oxygen supply to the tolerated temperature range. Moreover, HIF-1 DNA binding occurred at a highly oxidised cellular redox environment in both, *P. brachycephalum* and winter animals of *Z. viviparus*.

In contrast to seasonal cold acclimatisation, acute cold exposure of *Z. viviparus* (2 h) to 1 and 5°C led to a more reduced cellular redox environment, which was accompanied by increased HIF-1 DNA binding. Oxidative damage was increased following 24 h recovery at control temperature. Thus, effects of acute cold exposure and recovery, i.e. cold-induced hypoxia and reoxygenation are reminiscent of ischemia / reperfusion events well described in mammals. Acute heat exposure and recovery caused similar biochemical effects. However, oxidative stress markers were elevated only during early recovery (8 - 12 h). Thus, repair of oxidative damage may be faster following heat than following cold stress.

Moreover, acute cold and heat stress had opposing impacts on the cellular redox balance with cold stress causing a more reduced, and critical heat stress a more oxidised cellular redox environment. The more oxidised conditions during critical heat stress seemed to interfere with the HIF response as reflected in weak HIF-1 signals in EMSA assays. HIF-1 may thus have different functions during long-term (seasonal) and short-term (acute stress) changes of environmental temperatures in the temperate eelpout *Z. viviparus,* and the redox potential may be the modulating factor.

Zusammenfassung

Sauerstoff bildet die Grundlage für den Prozess der oxidativen Phosphorylierung. Eine übermäßige Sauerstoffzufuhr hat jedoch aufgrund der Bildung von reaktiven Sauerstoffkomponenten eine schädigende Wirkung auf Organismen. Aerobe Lebensweise erfordert daher die Einstellung eines empfindlichen Sauerstoffgleichgewichtes innerhalb eines physiologisch eng umgrenzten Bereichs. Der artspezifische optimale Temperaturbereich mariner ektothermer Tiere ist durch die pejus-Temperaturen (Tp) markiert. Außerhalb dieses Bereichs kann es zu fortschreitendem Sauerstoffmangel in Körperflüssigkeiten und Geweben kommen (funktionelle Hypoxie), der im kritischen Temperaturbereich (Tc) schließlich in anaeroben Metabolismus übergeht. In meiner Doktorarbeit habe ich die Hypothese untersucht, dass Temperatur-induzierte Hypoxie zum einen von oxidativem Stress begleitet wird. Zum anderen könnte der Transkriptionsfaktor HIF-1 (Hypoxie-induzierbarer Faktor) eine physiologische Anpassung der Sauerstoff-zufuhr ermöglichen und damit dem Temperatureffekt entgegenwirken.

In dieser Arbeit wurden Langzeiteffekte der Umgebungstemperatur sowie Effekte einer durch akuten Temperaturstress induzierten Hypoxie mit anschließender Erholungsphase auf Sauerstoffstressparameter und Hypoxie-Signalgebung in der Leber mariner Fische aus verschiedenen Breitengraden untersucht.

Aus der Familie der Zoarcidae wurden die Antarktische Aalmutter *Pachycara brachycephalum*, bei 0°C Kontrolltemperatur, und die aus der Nordsee stammende *Zoarces viviparus*, Winter-angepasst bei 6°C, verglichen. Ein stark oxidiertes Glutathion-Redox-Verhältnis und erhöhte mikrosomale Lipidradikal-Bildungsraten in *P. brachycephalum* könnten eine höhere Empfindlichkeit der polaren Art für Sauerstoffstress und Lipidperoxidation anzeigen. Trotzdem schien der hohe Gehalt an Glutathion die erhöhten Lipidradikal-Bildungsraten effektiv zu puffern und die Leber mit einer so hohen antioxidativen Kapazität zu versorgen, dass sowohl unter Kontrollbedingungen als auch nach Wärmeanpassung (5°C) die Sauerstoffschädigungs-parameter in *P. brachycephalum* im Vergleich zur temperierten Art niedrig waren. In *Z. viviparus* zeigte sich im Vergleich von Winter- und Sommertieren erhöhter oxidativer Stress in Wintertieren (6°C). Demnach sind nach den Ergebnissen bei den untersuchten zoarciden Fischen sowohl Kälteadaptation (*P. brachycephalum*) als auch saisonale Kälteanpassung (*Z. viviparus*) mit der Erfordernis verbunden, erhöhten oxidativen Stress auszugleichen.

Eine Sequenzbestimmung der O_2-regulierten Untereinheit HIF-1α bei *Z. viviparus* aus der Nordsee und vier kalt-angepassten Antarktischen Fischen (Zoarcidae: *Pachycara brachycephalum*; Nothenioidei: *Trematomus hansoni, T. pennellii, Chionodraco myersi*) zeigte beachtliche Unterschiede in der abgeleiteten Aminosäuresequenz im Vergleich zu Säugern oder anderen nicht-polaren Fischen. Bei der Antarktischen *P. brachycephalum* war der N-terminale funktionelle Prolin-Rest der HIF-1α Sauerstoffregulierungsdomäne gegen Leucin ausgetauscht. Da in den Sequenzen der drei Notothenioiden beide funktionellen Proline konserviert waren, kann dieser Pro-Leu Austausch aber nicht als eine spezifische polare Anpassung betrachtet werden. Das Fehlen eines funktionellen Prolin-Restes könnte HIF-1α resistent gegenüber Prolylhydroxylasen und dem damit verbundenen proteasomalen Abbau machen. Weitere Untersuchungen müssen zeigen, ob die Funktion von HIF-1α in *P. brachycephalum* weniger über den Abbau / Stabilisierungsmechanismus und mehr über den Transaktivierungsmechanismus reguliert wird. Erhöhte HIF-1 DNA-Bindung bei 5°C gehälterten *P. brachycephalum* im Vergleich zu Kontrollfischen bei 0°C, zeigte, dass HIF-1 trotz Pro-Leu Austausch und stark oxidiertem Redox-Milieu zumindest auf der Ebene der Dimerbildung und DNA-Bindung noch funktionsfähig war.

Während die HIF-1 DNA-Bindungsaktivität in *P. brachycephalum* bei der wärmeren Temperatur höher war (5°C *versus* 0°C), wurden in *Z. viviparus* höhere Werte in Winter- (6°) als in Sommertieren (12°C) gefunden. In beiden Spezies könnte HIF-1 eine Rolle bei der Anpassung der Gewebesauerstoffversorgung an den tolerierten Temperaturbereich spielen. Darüber hinaus wurde sowohl bei *P. brachycephalum* als auch bei *Z. viviparus* Wintertieren HIF-1 DNA-Bindungsaktivität unter gleichzeitig stark oxidiertem Redox-Milieu beobachtet.

Im Gegensatz zu saisonaler Kälteanpassung führte akuter Kältestress (2 h bei 1 und 5°C) zu einem eher reduzierten Redox-Milieu, sowie zu erhöhter HIF-1 DNA-Bindung. Ein Anstieg in Sauerstoffschädigungsparametern wurde nicht nach Kältestress, sondern nach 24 h Erholung bei Kontrolltemperatur gefunden. Das legt nahe, akuten Kältestress und Erholung als Kälte-induzierte Hypoxie und Reoxygenierung zu beschreiben, vergleichbar dem Stressmuster der Ischämie / Reperfusion bei Säugern. Akuter Hitzestress und Erholung hatte prinzipiell dieselben Folgen. Jedoch konnte eine erhöhte Sauerstoffschädigung nur bei kürzeren Erholungsphasen von 8 - 12 h festgestellt werden. Vermutlich findet bei Erholung nach Erwärmung eine schnellere Reparatur der induzierten Sauerstoffschäden statt als bei Erholung nach Abkühlung.

Darüber hinaus hatten akuter Kälte- und Hitzestress gegensätzliche Effekte auf das zelluläre Redox-Milieu, das nach Kältestress eher reduziert und nach Hitzestress eher oxidiert war. Die stärker oxidierten Bedingungen bei Hitzestress scheinen die HIF Antwort zu beeinträchtigen, erkennbar an schwächeren HIF-1 Signalen im EMSA. Es könnte demnach sein, dass der Transkriptionsfaktor HIF-1 in *Z. viviparus* bei längerfristigen (saisonalen) und kurzzeitigen (akuter Stress) Änderungen der Umgebungstemperaturen jeweils unterschiedliche Regulationsmechanismen auslöst und dass das Redox-Milieu eine steuernde Größe darstellt.

1. Introduction

1.1. Oxygen

The adjustment of oxygen delivery and demand for maintenance of oxygen homeostasis within a narrow physiological range is crucial for all aerobic life (Haddad 2002, Massabuau 2003). Oxygen is the essential substrate for oxidative phosphorylation, a process generating most of the ATP required for cellular metabolism. That need for oxygen obscures the fact that oxygen exposure has to be limited because of the hazardous effects of oxygen free radicals (Semenza 2004).

1.2. Oxygen free radicals and reactive oxygen species

Free radicals are chemical species capable of independent existence ("free") with an unpaired electron in the outer valence orbital. This makes them paramagnetic and relatively reactive due to the strong tendency to acquire a second electron in the orbital (Boveris 1998, Halliwell & Gutteridge 1999).

The oxygen molecule contains two unpaired electrons located in different π-antibonding orbitals and with parallel spins (↑↑), thus represents a di-radical. The spin restriction rule makes it difficult for the O_2 molecule to receive a pair of electrons with parallel spins when oxidising another compound. Thus, oxygen must receive one electron at a time (Hermes-Lima 2004). Four successive transfers of single electrons reduce the oxygen molecule to water (fig. 1.1). Some of the intermediates of oxygen reduction are free radicals, such as the superoxide anion and hydroxyl radical, whereas hydrogen peroxide is a non-radical species. In the present work, the term reactive oxygen species (ROS) will be used for the collective description of oxygen free radicals as well as non-radical oxygen derivatives, such as H_2O_2, and also singlet oxygen.

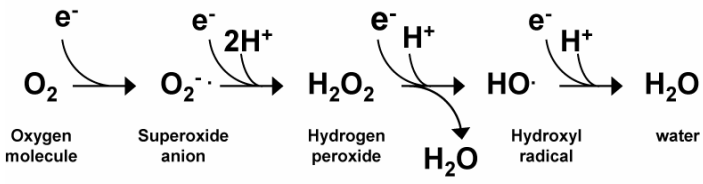

Fig. 1.1: The univalent reduction of the oxygen molecule taken from Boveris (1998).

Mitochondria consume > 90% of the overall oxygen uptake in higher animals (Boveris 1998). They are considered the main cellular site of free radical formation due to electron leakage from the respiratory chain, primarily generating superoxide (Hermes-Lima 2004). Several components of complex I, II and III exhibit thermodynamic properties suitable for the reduction of oxygen to superoxide (Boveris & Cadenas 1997). Among them, ubisemiquinone is considered the principal physiological source of mitochondrial superoxide radicals (Hermes-Lima 2004). Calculations suggest that mitochondrial ROS production may account for 1 - 3% of the mitochondrial oxygen consumption in mammalian species (Boveris 1998).

In hepatocytes, the well developed endoplasmatic reticulum represents an equally important source for superoxide as the mitochondria. Moreover, several enzymes, e.g. NADPH-cytochrome P-450 reductase and cytochrome P-450, cytosolic xanthine oxidase and peroxisomal flavin oxidases are further sources for cellular superoxide and hydrogen peroxide formation (Hermes-Lima 2004).

In biological systems, the Fenton-Haber-Weiss reaction (equations 1.1 and 1.2) with transition metals functioning as catalysts converts the primary product superoxide to the hydroxyl radical, which is one of the most reactive chemical species:

$$O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad (1.1)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + Fe^{3+} + OH^{-} \qquad (1.2)$$

The hydroxyl radical in turn is able to abstract a hydrogen atom from unsaturated fatty acids and, thus, initiate free radical chain reactions. The complex reactions undergone during the lipid peroxidation process lead to a variety of intermediates and products, such as lipid radicals, peroxyl radicals and alkoxyl radicals. Moreover, conjugated dienic hydroperoxides further decompose leading finally to several aldehydes, alkanes, isoprostanes or conjugated dienes (fig. 1.2).

Membrane phospholipids are present where radicals are formed, rendering those easily accessible endogenous targets. Especially the poly-unsaturated fatty acids (PUFAS) are highly susceptible to reactions with free radicals. Lipid peroxidation processes lead to decreased membrane fluidity, disruption of membrane integrity, resulting in the break-down of transmembrane ion gradients. Moreover, inactivation of membrane-bound enzymes can drastically alter cell functioning (Halliwell & Gutteridge 1999). The aldehydic products of lipid peroxidation, such as malondialdehyde (MDA), are highly toxic, leading to cross-links between membrane-proteins and the formation of stable

adducts with DNA. Consequently, lipid peroxidation is considered to be a major cause of cell injury and death (Hermes-Lima 2004).

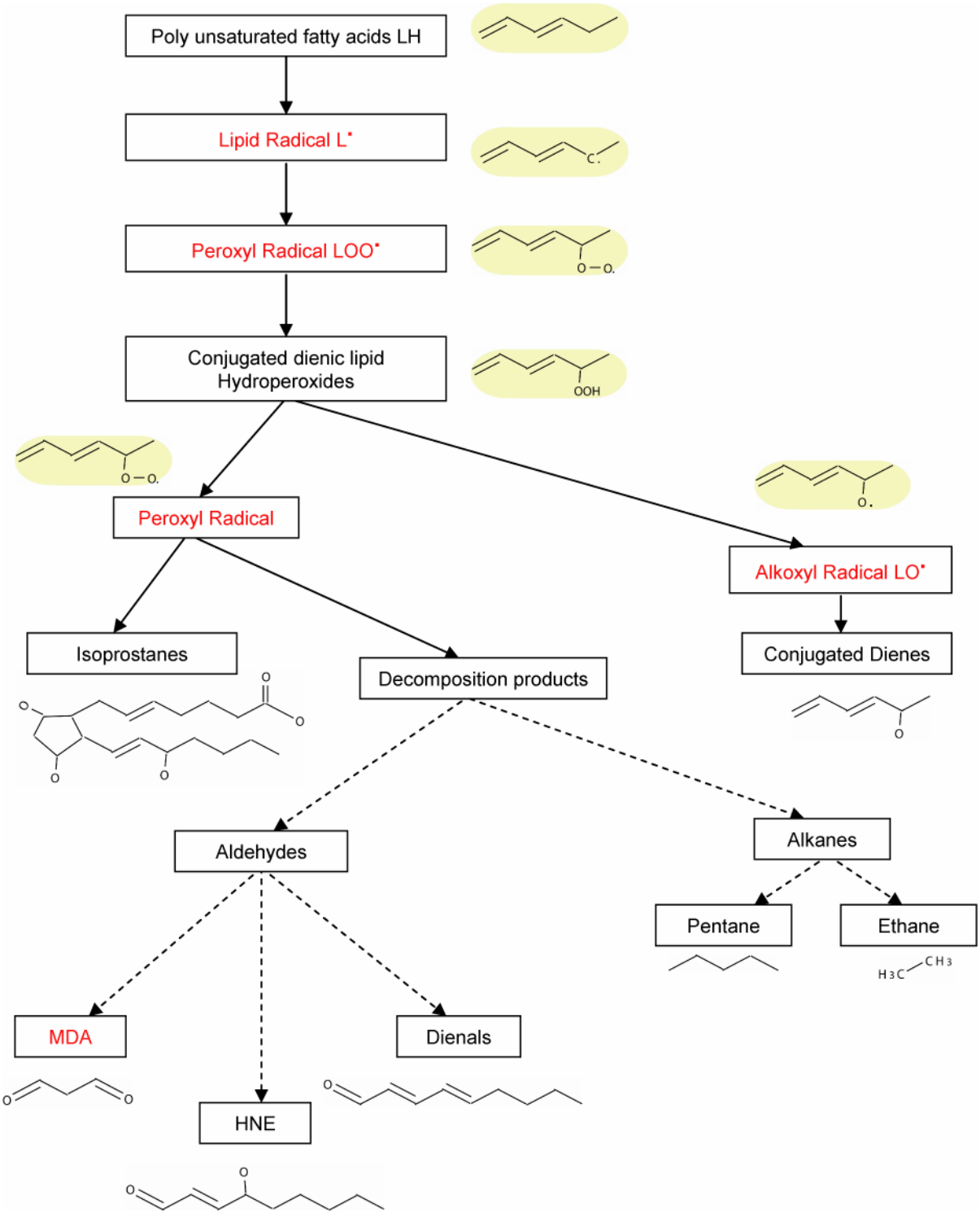

Fig 1.2: Lipid radical chain reaction and lipid peroxidation products modified after Dotan et al. (2004). Radical species and compounds detected in the present thesis are marked by red colour.

Besides, ROS, particularly the hydroxyl radical, can attack almost any cellular structure or molecule. They may cause DNA cross-links and DNA strand-breaks as well as specific modifications of the nucleotides (de Zwart et al. 1999). Protein oxidation processes involve modifications of amino acid site chains, reactive oxygen-mediated peptide cleavage and reactions with lipid and carbohydrate oxidation products. The presence of carbonyl groups in proteins is used as an indicator for oxidative damage (de Zwart et al. 1999). Oxidative protein modification may lead to functional changes, chemical fragmentation, or increased susceptibility to proteolytic attack (Dröge 2002). Altogether, cell function may be altered by free radical generation in many different ways.

1.3. Oxidative stress

Cells have developed sophisticated antioxidant (AOX) mechanisms to prevent free radical formation and to limit their damaging effects (de Zwart et al. 1999). These include AOX enzymes like superoxide dismutase (SOD), catalysing the decomposition of superoxide to hydrogen peroxide as well as catalase and glutathione peroxidase (GPX), mediating the further conversion of H_2O_2 to water. Additionally, proteins exist to sequester transition metals and an array of compounds to scavenge free radicals. The most commonly monitored low molecular weight antioxidants are vitamin E, vitamin C, uric acid, carotenoids, ubiquinol and glutathione (Dotan et al. 2004). Glutathione (GSH), the tripeptide L-γ-glutamyl-L-cysteinyl-glycine, can act as an antioxidant on its own or as a co-substrate of GPX, in both cases yielding oxidised glutathione (GSSG) (Hermes-Lima 2004). Moreover, the glutathione redox couple (GSSG / 2 GSH) due to its high concentration (millimolar range) in the cell can be used as an indicator for changes in the cellular redox environment (Schafer & Buettner 2001). The interaction of glutathione with protein thiol groups (protein-S-glutathiolation) leads to protein mixed disulfide formation, which can both, stabilise / activate or inactivate enzymes. Transcription factors can be similarly affected by this process (Rebrin et al. 2003). Thus, maintenance of an optimal redox status is of prime physiological importance.

Under certain conditions, ROS production is increased to a point where the AOX response may not be sufficient to reset the system to the original level of oxygen homeostasis (Dröge 2002). Such a disturbance in the prooxidant / antioxidant balance has been described as oxidative stress (Sies 1985) and is reflected in changes of the cellular redox state (Klatt & Lamas 2000, Rebrin et al. 2003).

Hypoxia or ischemia, i.e. an arrest of blood flow to a tissue, and subsequent reoxygenation on reperfusion has been extensively studied in mammalian liver, brain, kidney and heart as a major cause for oxidative stress (Jones 1986, McCord 1988, Taniai et al. 2004, reviewed in Halliwell & Gutteridge 1999). During ischemic events there is no oxygen that can be converted to H_2O by cytochrome c oxidase, and consequently the mitochondrial respiratory chain becomes highly reduced. During subsequent reperfusion the quick influx of oxygen interacts with accumulated electrons, inducing a burst of mitochondrial ROS production (Turrens et al. 1991). Additionally, excessive ATP consumption during ischemia leads to the accumulation of hypoxanthine, which, upon reperfusion is rapidly metabolised by xanthine oxidase (XOD) to superoxide (Dröge 2002, Dykens & Shick 1988 for marine ectotherms).

In contrast to the events occurring during reoxygenation, no definitive picture exists with respect to ROS levels during hypoxia itself. Members of the family of NADPH-dependent oxidases have been shown to generate hydrogen peroxide in an oxygen-dependent manner, resulting in decreased cellular ROS levels during hypoxia (Fandrey et al. 1994, Zhu & Bunn 1999, Kietzmann et al. 2000). In striking contrast to these findings, Chandel and Schumacker (Chandel et al. 1997, 1998, 2000, reviewed in Chandel & Schumacker 2000) reported increased ROS levels during oxygen limitation originating from the mitochondria. Until now, there is no common consensus about whether ROS concentration decrease or increase under low oxygen conditions (Wenger 2000, Liu et al. 2004).

1.4. Oxygen-limited thermal tolerance and oxidative stress

Marine ectotherms also undergo frequent episodes of environmental and physiological hypoxia and, thus, are likely to experience elevated levels of ROS formation during recovery from physiological stress (Abele & Puntarulo 2004). Particularly, functional hypoxia accompanying temperature stress according to the concept of oxygen-limited thermal tolerance is thought to contribute to oxidative stress in marine ectotherms (Pörtner 2002). Comparative studies on marine invertebrates and fish (Frederich & Pörtner 2000, Lannig et al. 2004) have demonstrated maximal haemolymph or blood PO_2, indicating balanced oxygen supply and demand and full aerobic scope for activity, in an optimum temperature range. Upon warming or cooling beyond the pejus temperatures (Tp; pejus = getting worse), which enclose the species specific thermal optimum range, decreasing blood PO_2 and whole animal aerobic scope indicate limited oxygen supply to central tissues even in fully oxygenated water (fig. 1.3). Below the optimum range (cold stress) the

greater impact of cooling on the circulatory system than on mitochondrial oxygen demand accounts for a developing functional hypoxia (Lannig et al. 2004). At temperatures above the optimum range (heat stress), the temperature-induced increase in oxygen demand can no longer be compensated for by an increase in ventilation and circulation. In fish, a decline in cardiac performance mainly accounts for limited oxygen supply to tissues during heat stress (Farrell 2002, Lannig et al. 2004).

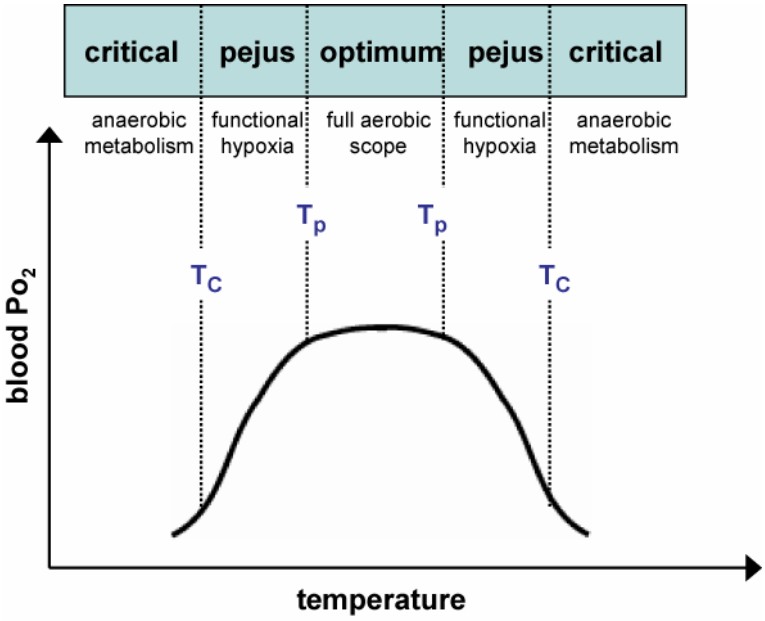

Fig. 1.3: The model of oxygen-limited thermal tolerance modified after Pörtner (2002).
Tc = critical temperature; Tp = pejus temperature (pejus = getting worse).

Upon further warming or cooling beyond the critical temperature limit (Tc), functional hypoxia is enhanced and finally leads to onset of anaerobic metabolism, first affecting tissues with high oxygen demand, such as active aerobic muscle or the liver (Sommer et al. 1997, Van Dijk et al. 1999). Under critical temperature stress, aerobic scope for activity disappears and survival becomes a question of time. It has been suggested by Pörtner (2002) that oxidative stress may already increase when oxygen limitation sets in at pejus, and even more so during extreme hypoxia beyond critically low or high temperatures. Temperature-induced hypoxia may not absolutely resemble ischemia, as blood flow is not interrupted (Mark et al. 2002, Lannig et al. 2004). Yet oxygen limitation and its relief after thermal stress may cause a situation similar to reperfusion after ischemia, favouring the overproduction of ROS when temperature returns to the optimum range and the tissue is reoxygenated.

Moreover, under heat stress, the temperature-dependent rise of mitochondrial respiration may go hand in hand with elevated mitochondria-derived ROS formation, which has been shown for marine invertebrates by *in vitro* measurements (Abele et al. 2002, Heise et al. 2003, Keller et al. 2004). Consequently, passive survival under temperature stress may be supported by maintenance of an efficient AOX system (Pörtner 2002). However, in heat stressed marine molluscs pro- and antioxidant processes appeared balanced below Tc, whereas above Tc SOD denatured and compensation failed (for review see Abele & Puntarulo 2004).

Apart from the detrimental effects of oxidative injury, elevated ROS levels may cause a general disturbance of the cellular redox balance, i.e. the ratio of reduced to oxidised glutathione and, consequently, interfere with redox-dependent signalling cascades (Czubryt et al. 1996, Abele 2002, Nikinmaa et al. 2004). Marine ectotherms may therefore be more tolerant to oxidative stress in situations reminiscent to ischemia / reperfusion, and may also have achieved adjustments of redox-dependent molecular mechanisms.

1.5. Oxidative stress in polar marine ectotherms

Several aspects of life at permanently cold water temperatures may render polar marine ectotherms more susceptible to oxidative stress. One fact is the higher solubility of oxygen in cold seawater and body fluids of ectothermal animals. Moreover, the solubility of oxygen is higher in lipid droplets than in the cytosol. Thus, in oxidative muscles of polar ectotherms high contents of neutral storage lipids provide both, a reservoir of oxygen as well as an enhanced diffusional pathway for oxygen though the tissue (Egginton & Sidell 1989, Desaulniers et al. 1996, Sidell 1998). These findings together with low oxygen consumption rates in those species are thought to result in high tissue oxygen concentrations (Abele & Puntarulo 2004). Additionally, polar invertebrates and fish have higher degrees of lipid unsaturation, in order to achieve homoeoviscous adaptation of membrane transport and to facilitate oxygen diffusion at low temperatures (Sidell 1998). Consequently, elevated levels of lipid unsaturation at higher cellular oxygen concentrations may represent a pre-requisite for the initiation of lipid radical chain reactions in polar animals. Prolonged half-life of free radicals in the cold may further facilitate the propagation of these reactions and extend the oxidative stress to adjacent tissue areas (Abele & Puntarulo 2004). This hypothesis is further supported by the finding of 5 to 6 times higher contents of vitamin E, the most powerful lipid soluble antioxidant, in Antarctic *versus* temperate fish species (Gieseg et al. 2000). The increased susceptibility of

polar ectotherms to lipid peroxidation may present no problem under unstressed conditions, but could become a major obstacle under any form of physiological challenge, such as temperature stress, leading to enhanced cellular ROS production (Abele & Puntarulo 2004).

1.6. Oxygen-dependent gene expression

At high concentrations free radicals are hazardous for living organisms. However, at moderate levels they play an important role as regulatory mediators in signalling processes, including the hypoxia response, i.e. an important mechanism for the regulation of oxygen homeostasis (Dröge 2002). Higher organisms maintain oxygen homeostasis by tight regulation of red blood cell mass and respiratory ventilation. Under low oxygen conditions, genes involved in erythropoiesis, angiogenesis, vasomotor control as well as iron and glucose metabolism are induced by the transcription factor HIF-1 (hypoxia inducible factor; for review see Semenza 2002, Bracken et al. 2003). HIF-1 has been identified in all metazoan species so far investigated (Semenza 2004) and termed the master regulator of oxygen homeostasis (Semenza 1998).

HIF-1 is a hetero-dimeric basic Helix Loop Helix (bHLH) PAS protein, composed of one of the three α-subunits HIF-1α, HIF-2α or HIF-3α and the HIF-1β subunit. While HIF-1β is readily found in the cell under all oxygen conditions, the α-subunits are the crucial factors for oxygen-dependent gene expression (Jiang et al. 1996), with HIF-1α being the most prominent member (Schmid et al. 2004). Although HIF-1α is constitutively expressed, the protein is virtually undetectable under normoxic conditions because of its very short half-life of less than 5 minutes (Salceda & Caro 1997, Dery et al. 2005). It has been shown that prolyl hydroxylases (PHD), using molecular oxygen for hydroxylation of proline residues 402 and 564 in human HIF-1α target the protein for recognition by von Hippel Lindau Tumor suppressor protein (pVHL; Ivan et al. 2001, Jaakkola et al. 2001, Masson et al. 2001). This promotes poly-ubiquitylation by the E3 ubiquitine ligase complex and subsequent HIF-1α degradation by the 26S proteasome (fig. 1.4). Additionally, acetylation of lysine 532 of human HIF-1α was described as a further protein degradation target for HIF-1α that had escaped proline hydroxylation under normoxia (Jeong et al. 2002). Under oxygen limitation proline hydroxylation is impaired and lysine acetylation down-regulated (Dery et al. 2005). This permits stabilisation of HIF-1α protein, and translocation to the nucleus (Kallio et al. 1998), where it dimerises with HIF-1β (ARNT) to the transcriptionally active HIF-1 complex. The hetero-dimer then can bind to hypoxia

responsive elements (HRE) in the promoter regions of its target genes and enhance their expression (Semenza et al. 1996). Oxygen-dependent hydroxylation plays also a role in HIF-1 transactivation. Under normoxia FIH-1 (factor inhibiting HIF) hydroxylates asparagine 803 in the human HIF-1α sequence, preventing the interaction of HIF-1α with general transcriptional co-activators, such as p300 / CBP (fig. 1.4) (Arany et al. 1996, Lando et al. 2002). There is accumulating evidence suggesting that fish cells have all major components required for the HIF response such as HIF-α and β – subunits, as well as p300 (CK421262) and that the mechanism of HIF induction is basically the same in mammals and fish (Soitamo et al. 2001, Powell & Hahn 2002, Nikinmaa & Rees 2005). Moreover, gene expression studies with the goby fish *Gillichthys mirabilis* have shown the hypoxic induction of genes which in mammals are known as HIF target genes (Gracey et al. 2001).

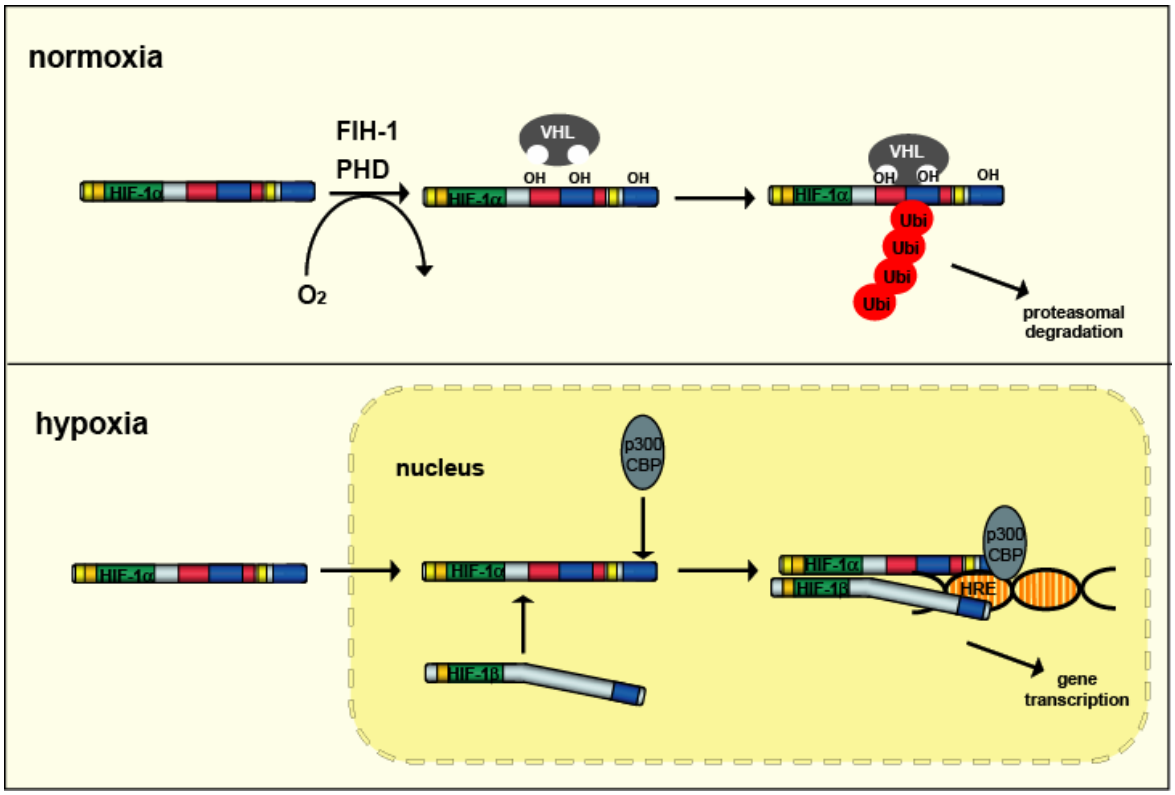

Fig. 1.4: The HIF mechanism modified after Schmid et al. (2004).
The upper part presents oxygen-dependent hydroxylation processes of HIF-1α under normoxic conditions, the lower part HIF-1α stabilisation, nuclear translocation and dimerisation under hypoxia. FIH-1 = factor inhibiting HIF; PHD = prolyl hydroxylase; VHL = von Hippel Lindau Tumor Suppressor protein; Ubi = ubiquitine; HRE = hypoxia responsive element

Whereas the N-terminal half of HIF-1α is characterised by the bHLH and PAS domains, responsible for DNA binding and dimerisation with the β-subunit, the C-terminal half consists of the oxygen-dependent degradation domain (ODD) with the two functional proline and the lysine residues as well as two transactivation domains (fig. 1.5). The

N-terminal transactivation domain (N-TAD) is located within the ODD, whereas the C-terminal transactivation domain (C-TAD), harbouring the functional asparagine residue, is found at the very end of the protein (Lando et al. 2002). Moreover, the HIF-1α sequence contains two nuclear localisation signals (NLS), responsible for the transition of HIF-1α protein from the cytosol into the nucleus (Schmid et al. 2004).

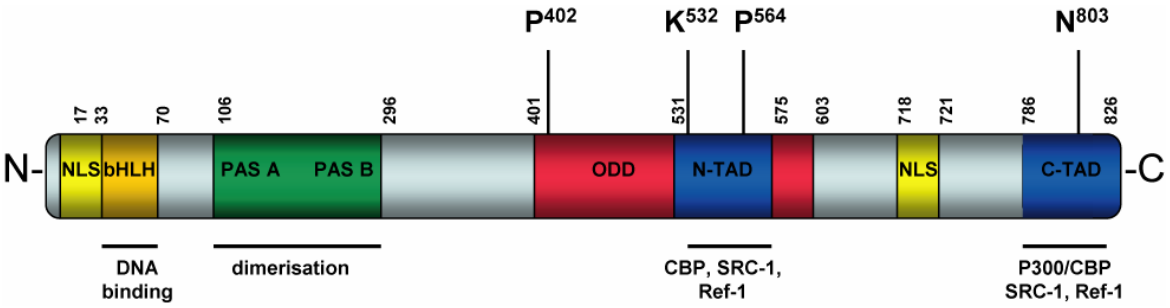

Fig. 1.5: The HIF-1α peptide structure modified after Dery et al. 2005.
NLS = nuclear localisation signal; bHLH basic Helix Loop Helix domain; ODD = oxygen-dependent degradation domain; TAD = transactivation domain; the black lines indicate DNA or protein binding regions.

Although the HIF mechanism is primarily dependent on the availability of molecular oxygen for hydroxylation, reactive oxygen species likely modulate several steps of the hypoxic signalling pathway (for review see Fandrey 2004). On the one hand, prolyl hydroxylases belonging to the family of iron and 2-oxoglutarate-dependent dioxygenases need Fe^{2+} and ascorbate for proper functioning, and thus their enzyme activity may depend on the cellular redox state. Also, the activity of ubiquitine-conjugating enzymes of the proteasomal pathway has been shown to be redox-sensitive (Obin et al. 1998). There is also considerable evidence that the glutathione equilibrium is an important regulator for the HIF response (Haddad et al. 2000). On the other hand, it has been reported that full activity of C-TAD depends on the reducing activity of the redox factor Ref-1 (Lando et al. 2000) which interacts with cysteine 801 in human HIF-1α (Ema et al. 1999). Recent studies using lung vascular cells have demonstrated oxidative modifications of a guanidine in the HRE of the VEGF promoter, which may affect DNA integrity and thus gene expression by influencing DNA-protein interaction (Ziel et al. 2005).

For salmonid fish, further redox-sensitive cysteine residues in bHLH and ODD have been described, influencing the DNA binding activity as well as the protein stability of HIF-1α (Soitamo et al. 2001, Nikinmaa et al. 2004). Consequently, any form of oxidative stress and implicated disturbances of the cellular redox balance may interfere with oxygen-dependent gene expression especially in fish (Nikinmaa et al. 2004, Nikinmaa & Rees

2005). In contrast, one might assume that ectothermal animals frequently exposed to oxidative stress may have developed functional adjustments of their hypoxic signalling response to ensure oxygen-dependent gene expression even under stressful conditions. Similarly, polar fish, living in an oxygen-rich environment, could have reduced capacities for oxygen-dependent gene expression or could have evolved physiological adjustments of the HIF mechanism to guarantee regulation of cellular oxygen homeostasis. Moreover, the question arises whether the HIF-1 mediated hypoxic response could play a role under functional hypoxia and ameliorate progressive oxygen limitation under temperature stress in marine ectotherms.

1.7. Objectives of the study

The aim of the present work was to investigate the effect of temperature stress, anticipated to induce functional hypoxia, on a wide array of oxidative stress parameters and on molecular defence systems, especially the hypoxic response, in marine fish from different latitudes. Different time scales of temperature exposure were studied, from temperature adaptation (evolutionary effects), seasonal acclimatisation and laboratory acclimation (long-term temperature effects of several weeks) to few hours of experimental temperature exposure (short-term effects). Moreover, the investigated temperature range, starting from optimal control conditions increasing to pejus, critical and finally extreme temperatures, allowed for distinguishing various degrees of functional hypoxia.

Particularly, the following questions were addressed:

1. Does the Antarctic eelpout (*Pachycara brachycephalum*) display higher oxidative stress levels than the con-familial temperate eelpout (*Zoarces viviparus*) under normal physiological conditions or at elevated temperatures?

2. Do Antarctic fish, which are adapted to the oxygen-rich polar environment, display functional adjustments of the HIF response to fine-tune oxygen-dependent gene expression to different Co_2 and oxidative stress levels in their tissues?

3. Do variable degrees of short-term temperature stress and the implied functional hypoxia carry a risk of oxidative stress and cause adjustments by hypoxic signalling or does oxidative stress upon temperature induced-hypoxia interfere with the hypoxic response?

2. Material & Methods

2.1. Investigated animals

All investigated species belong to the order of perciform teleosts (fishbase, http://filaman.uni-kiel.de/search.cfm). An overview is given in tab. 2.1. Maps demonstrating the global distribution of all studied species are presented in the appendix (fig. 8.2 – 8.6).

Tab. 2.1: Overview of the investigated species, their max. body size, aquatic environment, climate and their naturally experienced temperature range. Data are taken from fishbase, http://filaman.uni-kiel.de/search.cfm.

species		family	max size (cm)	environment	climate	temp. - range
Zoarces viviparus (common eelpout)	Linnaeus, 1758	Zoarcidae (eelpouts)	52.0	demersal; brackish; marine; 0 – 40 m depth	temperate; 72°N – 49°N, 11°W – 42°E	3 – 18°C
Pachycara brachycephalum	Pappenheim, 1912	Zoarcidae (eelpouts)	35.0	bathydemersal; marine; 200 – 1810 m depth	deep-water; 60°S – 78°S	0 – 2°C
Trematomus hansoni (striped rockcod)	Boulenger, 1902	Nototheniidae (cod icefishes)	41.0	demersal; marine; 6 – 549 m depth	polar; 53°S – 78°S	0°C
Trematomus pennellii (sharp-spined notothen)	Regan, 1914	Nototheniidae (cod icefishes)	24.0	demersal; marine; 0 – 732 m depth	polar; 60°S – 78°S	0°C
Chionodraco myersi	DeWit & Typer, 1960	Channichthyidae (crocodile icefishes)	38.0	bathydemersal; marine; 200 – 800 m depth	deep-water; 61°S – 78°S	0°C

The main focus of the present study was on the family of Zoarcidae (eelpout), which are thought to be a monophyletic group comprising 220 species from Artic to Antarctic (Eastman 1993). Generally, zoarcids are described to be sluggish, benthic fishes living at temperatures of less than 8°C (Anderson 1984).

The common eelpout (*Zoarces viviparus*) from the Northern hemisphere (fig. 2.1A) is ovoviviparous (Götting 1976) and inhabits rocky shores under stones, among algae in tide pools and coastal areas down to 40 m depth (fishbase, http://filaman.uni-kiel.de/search.cfm). *Z. viviparus* can be found from the White Sea, Barents Sea southwards on the Norwegian coast to the English channel and the Irish Sea, as well as on the coasts of the Baltic and the North Sea (72°N - 49°N, 11°W - 42°E; Christiansen et al. 1981; fishbase, http://filaman.uni-kiel.de/search.cfm) with the Wadden Sea being its most southern area of distribution (Knijn et al. 1993). Due to its benthic lifestyle, *Z. viviparus* feeds on gastropods, chironomids, crustaceans, eggs and fry of fishes (fishbase, http://filaman.uni-kiel.de/search.cfm). Since the eelpout is behaviourally a very sedentary

fish, it shows the tendency to form local races (Christiansen et al. 1981, Schmidt 1916-1917). The thermal physiology of this species has been very well studied (Van Dijk et al. 1999, Zakhartsev et al. 2003, Pörtner et al. 2004). Moreover, the common eelpout is highly tolerant to short-term hypoxia, surviving one hour of anoxia (Fischer et al. 1992), and is even able to breathe air when out of the water (fishbase, http://filaman.uni-kiel.de/search.cfm). Together, *Z. viviparus* represents a perfect model-organism for studying temperature-induced hypoxia effects on oxidative stress and hypoxic signalling.

All *Z. viviparus* used for this thesis work were caught with bottom traps in shallow waters near Helgoland, German North Sea (54°11'N, 7°52'E; fig. 2.3A). The sea surface temperature varies between 3°C in winter and 18°C in summer (see fig. 8.1 in the appendix), thus *Z. viviparus* can be called eurythermal.

Fig 2.1A: *Zoarces viviparus*

Fig. 2.1B: *Pachycara brachycephalum*

For comparison of oxidative stress parameters in a latitudinal cline the con-familial, bathydemersal, Antarctic eelpout *Pachycara brachycephalum* was studied (fig. 2.1B). This species is endemic, shows a circum-Antarctic distribution (60°S - 78°S) and can be found in 200 - 1810 m depth (fishbase, http://filaman.uni-kiel.de/search.cfm). Although *P. brachycephalum* is found between 0.76 and -1.8°C, around the Antarctic peninsula a hot spot of occurrence can be found in water layers at 350 m (0.5°C) and 860 m (0.2°C) (Brodte et al., ms submitted). It feeds on mussels, gastropods, amphipods and polychaetes and is oviparous. *P. brachycephalum* for the present work were sampled with releaser traps near King George Island (Maxwell Bay; 62°14'S, 58°40'W; fig 2.3B) at 400 m depth upon an Antarctic expedition of RV Polarstern (ANT XIX/5) in April 2002. Water temperatures in this area were between 0.5 - 0.8°C (K. Mintenbeck, pers. com.).

For HIF-1α sequencing studies presented in publication II two Antarctic members of the family of Nototheniidae (cod icefishes) and one Antarctic member of the family of Channichthyidae (crocodile icefishes) were studied. Both families belong to the suborder of Notothenioidei, the nototheniids being the most diverse family. Most nototheniid species are benthic and Antarctic, only 15 of the 49 species display non-Antarctic distribution (Eastman 1993). The absence of a swim bladder is compensated for by lipids and low mineral content of bones, leading to near neutral buoyancy.

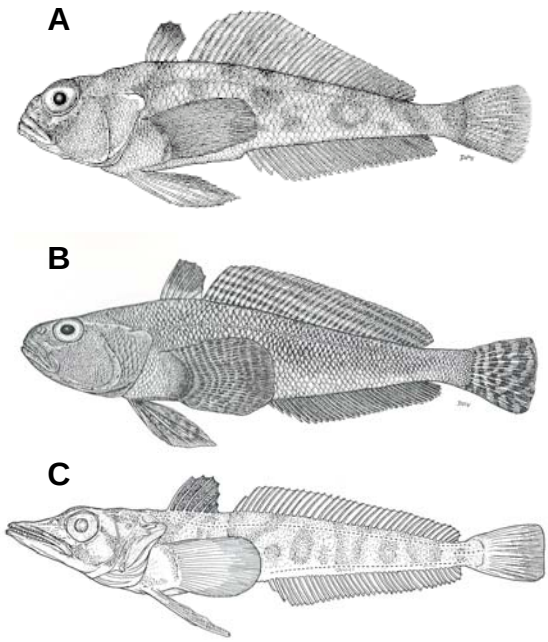

Fig. 2.2: *Trematomus pennellii* (A), *Trematomus hansoni* (B), and *Chionodraco myersi* (C); taken from Gon & Heemstra (1990)

The demersal *Trematomus pennellii* (fig. 2.2A) can be found in the depth range of 0 - 732 m of Scotia Arc, the South Orkney Islands, Davis and Ross Sea in the Southern Ocean (60°S - 78°S; fishbase, http://filaman.uni-kiel.de/search.cfm). Maintained in aquaria, this species has been observed to be more inactive than its congener *T. hansoni* (fig. 2.2B), which displayed considerable spontaneous activity (Eastman 1993). *T. hansoni* is also demersal, occurring in a depth range of 6 - 549 m of South Orkneys, South Georgia and South Shetland islands, the Ross, Davis and Weddell Seas, South Victoria, Adelie, Queen Mary and Princess Ragnhild Coasts in the Southern Ocean (53°S - 78°S; fishbase, http://filaman.uni-kiel.de/search.cfm). Benthic trematids are feeding generalists with variable diets depending on availability (polychaetes, small fishes, fish eggs, amphipods, crustaceans, zooplankton; Eastman 1993).

Channichthyids are fusiform fishes with a very weakly ossified skeleton feeding mainly on krill. They are also known as "white-blooded fishes" due to the lack of haemoglobin, although some non-pigmented erythrocytes are present (Eastman 1993). The complete lack of red blood cells has been interpreted as an extreme adaptation character of Antarctic fish that display reduced haematocrits and haemoglobin concentrations combined with low oxygen affinity (Feller & Gerday 1997). However, in channichthyids, the lack of haem pigments is compensated for not only by the high oxygen content of cold water, but also by a large increase of blood volume and cardiac output (Acierno et al. 1997). *Chionodraco myersi* (fig. 2.2.C) is a bathydemersal channichthyid found between 200 and 800 m and with a circum-Antarctic distribution on the continental shelf (61°S - 78°S; fishbase, http://filaman.uni-kiel.de/search.cfm).

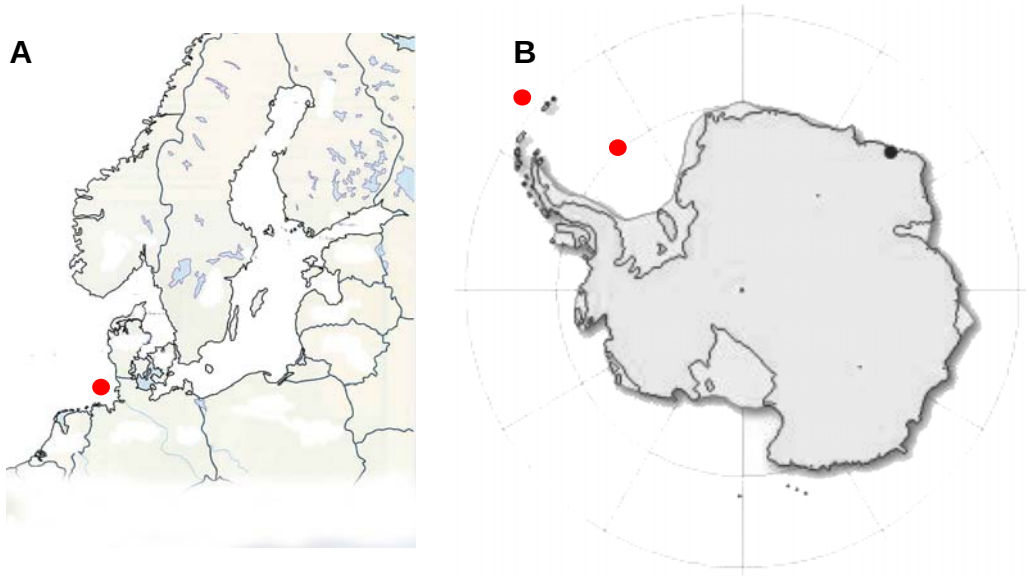

Fig. 2.3: Sampling areas of fish species, indicated by red dots. A: Northern Europe; *Zoarces viviparus* was sampled in the German North Sea. B: Antarctic; *Pachycara brachycephalum* was sampled near King George Island; *Trematomus pennellii*, *Trematomus hansoni*, and *Chionodraco myersi* were sampled in the Weddell Sea.

All samples from notothenioids were taken onboard RV Polarstern on cruise ANT XXI/2 in December 2003 and frozen transported for later analysis at the Alfred-Wegener Institute (Bremerhaven, Germany). The fish were caught in the Weddell Sea (70°56.57S, 10°33.02W; fig. 2.3B) at 300 m depth with a bottom trawl. Water temperatures in this area were around -1.5°C (K. Mintenbeck, pers. com.).

2.2. Experimental conditions

A first set of studies on *Zoarces viviparus* focusing on long-term and short-term temperature effects on oxidative stress and hypoxic signalling (Publication I, III, IV) was

carried out using animals caught from May - July 2002, thus representing summer animals, and kept at Biologische Anstalt Helgoland, Germany in aquaria with a flow-through system of natural sea water and 12°C until the beginning of the experiment in July 2002. For studying acute temperature stress, animals were directly transferred to a separate thermostatted incubation basin and exposed to 1, 5, 18, 22 or 26°C, representing pejus, critical and sub-lethal conditions for this species (Zakhartsev et al. 2003). After 2 h, half of the animals were sacrificed immediately, whereas the other half was returned to control temperatures (12°C) to allow for 24 h of recovery.

A second batch of *Z. viviparus* was caught in September 2003 and transported to the Alfred-Wegener Institute (Bremerhaven, Germany) in November 2003, where the animals were kept in natural sea water at 10°C until the beginning of the experiment in December 2003. To study the effect of different recovery periods after heat stress (publication IV), fish were transferred to the same incubation basin as described above and exposed to 18°C, followed by 0, 6, 8 or 12 h of recovery at 10°C.

For comparison of animals collected in various seasons, as well as for intra-familiar comparison to *P. brachycephalum* (publication I), specimens of *Z. viviparus* were caught in October 2002 and kept at temperatures similar to the outside water at the Biologische Anstalt Helgoland until sampling in February 2003, when water temperatures in the aquaria were 6°C. In the same month temperatures around Helgoland were occasionally down to 4°C (fig. 8.1B in the appendix). These latter animals are called winter animals throughout.

P. brachycephalum for the comparison to *Z. viviparus* were sampled in April 2002 and transported onboard to the Alfred-Wegener Institute (Bremerhaven, Germany), where they were kept at 0°C until start of the experiment (publication I). In December 2002 half of the animals remained at 0°C (controls), whereas the other half was transferred to a separate aquaria system and acclimated to 5°C over 11 weeks until sampled in March 2003.

Fish at Biologische Anstalt Helgoland were fed pieces of frozen white fish, fishes kept at Alfred-Wegener Institute headquarters were fed live common shrimp once a week. All experiments as well as animal maintenance were carried out at a natural salinity of 32 - 33 $^{o}/_{oo}$ and with well-aerated water. Oxygen contents in the water calculated according to Boutelier et al. (1984) ranged between 357 µM (1°C), 275 µM (12°C), and 211 µM (26°C). According to the critical PO_2 values reported by Zakhartsev et al. (2003) for different water temperatures (around 80 µM O_2), all temperature experiments can be considered as normoxia conditions. Prior to experimentation, all animals were maintained

for at least 10 days in the aquaria system to support full recovery from sampling or transportation stress.

After experimental treatments, fish were narcotised with 0.5 g MS 222 * L^{-1} seawater, weighted and killed by cutting the spine. Different tissues were rapidly removed, starting with the most metabolically active, the liver. Tissue aliquots of 100 - 200 mg were frozen in liquid nitrogen and stored at -80°C prior to analysis. Length and sex of the fishes were recorded after tissue sampling. Information about the sampling, size and age of the fishes used for the presented studies is summarized in tab. 8.1 in the appendix.

All biochemical and molecular analyses were carried out in liver tissue, which has been shown to be highly sensitive to temperature-dependent oxygen limitation (Van Dijk et al. 1999). As no sex specific differences were detected for glutathione, SOD, carbonyls and chemiluminescence in samples from both zoarcids within the studies of publication I, samples from male and female individuals could be pooled for all analyses.

2.3. Biochemical assays

2.3.1. Determination of thiobarbituric reactive substances (TBARS) according to Uchiyama & Mihara (1978)

The TBARS content was determined as a marker of lipid peroxidation.

A minimum of 160 mg of deep frozen tissue was ground in liquid nitrogen and homogenised in 1.1% phosphoric acid (1:10 to 1:20; w:v). To 400 µl of homogenate the same amount 1% thiobarbituric acid (TBA, dissolved in 50 mM NaOH, containing 10 mM butylated hydroxytoluene) was added. For blanks TBA was replaced by 3 mM HCl. The solutions were heated for 1 h at 100°C and subsequently extracted with 2 ml butanol by vortexing for 40 sec and subsequent centrifugation (5 min at 1 000 g). For further purification, the resulting supernatant was centrifuged for 5 min at 14 000 g and the TBARS content determined spectrophotometrically as the difference of absorbance A_{532nm} - A_{600nm}. After blank subtraction, the TBARS content was calculated using a 5-point calibration curve, obtained with malondialdehyde-(bis)-acetate standard (MDA; Merck, Darmstadt, Germany) processed in the same way as the samples.

2.3.2 *Tert*-butyl hydroperoxide-initiated chemiluminescence according to Gonzalez Flecha et al. (1991)

Artificial hydroperoxides such as *tert*-butyl hydroperoxide (tBOOH) are known to decompose to alkoxyl or peroxyl radicals and hence stimulate chain reactions of lipid

peroxidation accompanied by increased light emission (Halliwell & Gutteridge 1999). The main sources of chemiluminescence detected by this assay are the dimol emission of singlet oxygen and the photon emission from excited carbonyl groups formed during lipid peroxidation (Gonzalez Flecha et al. 1991).

Approximately 50 mg of liver tissue were homogenised in the 10-fold volume of 30 mM KP_i buffer (pH 7.4) containing 120 mM KCl and centrifuged at 600 g for 10 min. The supernatants were diluted 1:2 to 1:5 in buffer yielding a final protein concentration of 1-2 mg * ml^{-1}. After measurement of blank chemiluminescence, tBOOH was added to a final concentration of 3 mM. The assay was carried out in the dark at room temperature in a liquid scintillation counter (Wallac, Ramsey, USA) in the out-of-coincidence mode, using potassium glass vials kept in the dark for at least 48 h to avoid vial phosphorescence activation by fluorescent light. The obtained chemiluminescence data were determined as counts per minute (cpm). Cpm of each sample were recorded every 260 sec over an entire time period of 70 min. Data corrected for blank chemiluminescence were plotted in a tissue characteristic curve with y = chemiluminescence and t = time. An example is given in fig. 8.7 in the appendix. The area under the curve could be calculated for a time period of 4 000 sec (= 66.67 min) using MatLab 7.0 program (x = min (t) : 1 max (4 000); C = polyfit (t, y, 4); Y = polyval (C, X); area = sum (Y); plot (t, Y, 'ro', X, Y). The resulting arbitrary units were related to the protein content of the sample. Samples obtained from the recovery-time series were analysed by Packard (Zavantem, Belgium) liquid scintillation counter, where cpm recording was over an entire period of 62 min. The area was therefore calculated for the time period of 3 600 sec.

2.3.3. Determination of carbonyl groups according to Levine et al. (1990)

Carbonyl groups in amino acid residues are a measure for oxidative modifications of proteins. Detection of these groups was done after reaction with the carbonyl-specific reagent 2,4-dinitrophenylhydrazine (DNTP).

Samples (100 mg) were homogenised in 10 ml of 50 mM HEPES buffer, pH 7.4, containing 125 mM KCl, 1.1 mM EDTA, 0.6 mM $MgSO_4$ and protease inhibitors (0.5 mg * ml^{-1} leupeptine, 0.7 mg * ml^{-1} pepstatine, 40 mg * ml^{-1} phenylmethylsulfonyl fluoride, 0.5 mg * ml^{-1} aprotinin,) and centrifuged at 100 000 g for 15 min. 0.4 ml of supernatants were incubated at room temperature for 1 h with 1.4 ml 10 mM DNTP in 2 M HCl. Blanks were run without DNTP. During incubation samples were vortexed every 15 min. After 1 h, 0.2 ml of 100% TCA were added to precipitate the protein and

centrifuged for 10 min at 10 000 g. The protein pellet was washed three times with 1 ml ethanol : ethylacetate (1:1), resuspended in 0.6 ml 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH = 2.3) and incubated at 37°C until complete resuspension. The carbonyl content could be measured spectrophotometrically at 360 nm (molar extinction coefficient $\varepsilon = 22\ 000\ M^{-1} * cm^{-1}$).

2.3.4. Determination of reduced (GSH) and oxidised (GSSG) glutathione according to Fariss & Reed (1987)

Glutathione represents a non-enzymatic low molecular weight antioxidant and is, moreover, the most important constituent of the cellular redox milieu (Schafer & Buettner 2001).

Frozen tissue was ground in liquid nitrogen and the resulting powder homogenised in 1:10 (w:v) pre-cooled PCA (10% containing 2 mM bathophenantroline-disulphonic acid). After centrifugation at 15 000 g for 5 min at 4°C, 500 µl of the supernatant were mixed with 10 µl pH-indicator (1 mM m-cresol purple sodium salt containing 0.5 M iodoacetic acid (IAA)) and 50 µl 1 mM γ-glutamyl-glutamate (in 0.3% PCA), added as internal standard. The pH was adjusted to 8.5 with 5 M KOH (containing 0.3 M N-morpholine-propanesulfonic acid). The mixture was incubated at room temperature for 45 min to allow IAA to bind to GSH. Subsequently, samples were centrifuged for 5 min at 15 000 g at 4°C. 300 µl of the supernatant were added to the double amount of 1% 1-fluor-2,4-dinitrobenzene (FDNB, diluted in 100% ethanol, HPLC grade) and derivatised in dark vials at room temperature over 24 h. After centrifugation at 7 500 g for 1 min at 4°C and purification through 0.2 µm nylon membrane filters, samples were stored in dark HPLC vials at -20°C.

HPLC determination was done on a Beckmann Coulter HPLC System using a NH_2-spherisorp column, 5 µm 240 * 4 mm (Waters, Eschborn, Germany). Solvent A: 80% methanol and solvent B: sodium acetate stock in 80% methanol (20:80). Sodium-acetate stock was prepared by dissolving 500 g Na-acetate in 224 ml Milli-Q water and 695 ml of concentrated HPLC-grade acetic acid. The gradient programme was as follows: 10 min hold at 90% A followed by a 25 min linear gradient to 25% A at a flow rate of $1\ ml * min^{-1}$ and a backpressure of 2.3 to 2.8 psi. Peaks were recorded with a photodiode array detector at 365 nm. Concentrations were calculated using 5-point calibration curves for GSH and GSSG standards processed in the same manner as described for the samples.

As GSSG is formed by oxidation of 2 molecules GSH, total glutathione content was calculated as the sum (2 GSSG + GSH). Accordingly, the redox ratio of oxidised to reduced glutathione was expressed as 2 GSSG / GSH.

The *in situ* redox potential was calculated by using the Nernst equation (2.1):

$$\Delta E = E^{\circ pH} - (RT * 2.303 / nF) \log ([GSH]^2 / [GSSG]) \text{ mV} \qquad (2.1)$$

as described by Schafer & Buettner (2001) and by taking into account the different experimental temperatures of the fishes as well as temperature-induced changes of intracellular pH (pH_i, data taken from Sartoris et al. 2003 and adopted to fish liver, Larsen et al. 1997).

2.3.5. Detection of α-tocopherol (vitamin E) according to Desai (1984)

α-Tocopherol represents the most active compound of the lipid soluble non-enzymatic antioxidant vitamin E (Dunlap et al. 2002).

Tissue samples were homogenised in 3- to 5-fold volume of sodium dodecyl sulfate (SDS). To 300 µl homogenate, or 100 µl of microsomal suspension (see below), 1 ml of ethanol was added and vortexed for 1 min. Afterwards, 4 ml of hexane were aggregated and the mixture vortexed again for 1 min. After 10 min centrifugation at 600 g, 300 µl of the lipid phase were dried with nitrogen gas. The remaining lipid soluble compounds were dissolved in 150 µl of methanol : ethanol (1:1) and filtered through 0.22 µm nylon membranes. Hereafter, samples were analysed by reverse phase high performance liquid chromatography (HPLC) and electrochemical detection with an oxidation potential of 0.6 V and a flow rate of 1 ml * min^{-1}. The isocratic system consisted of a column (Supelcosil LC-8, 3 µm 15 cm * 4.6 mm; Supelco, Sigma, Seelze, Germany) and a solvent containing 20 mM $LiClO_4$ in 99% methanol. The α-tocopherol content of the resulting peaks was calculated by relation to the peak area of α-tocopherol standard (67.65 µM). The concentration of the standard was determined spectrophotometrically at 292 nm, $\varepsilon = 3.06\ mM^{-1} * cm^{-1}$.

2.3.6. EPR determination of the intracellular "free" iron according to Woodmansee & Imlay (2002) and Linares et al. (2003)

Iron(II) can act as catalyst for Fenton reactions, leading to enhanced formation of hydroxyl radicals and subsequent initiation of lipid radical chain reactions (Halliwell & Gutteridge 1999). EPR detection of "free" iron by EPR includes all iron (Fe(II) and Fe(III)) that is not firmly integrated into enzymes or iron storage proteins. All chemicals have to be prepared

in iron-free water obtained from Milli-Q filtered trough a sephadex column. After filtration water pH is 9 - 10.

Liver samples pooled from 2 animals were ground in liquid nitrogen and the resulting powder homogenised (1:2, w:v) in 10 mM Tris-HCl buffer pH 7.4 containing desferoxamine mesylate (final concentration 2 mM). 250 µl of each homogenate were frozen (-20°C) for the determination of total tissue iron content as described below. The remaining homogenate was incubated for at least 5 min, transferred to plastic tubes and frozen in liquid nitrogen. EPR detections were carried out with a Bruker (Buenos Aires, Argentina) spectrometer ECS 106 at 77K under permanent cooling of the sample in liquid nitrogen. The EPR settings were centre-field, 1600 G; sweep width, 800 G; time constant 82 ms; modulation amplitude, 4.75 G; power, 10 mW. A standard curve was obtained from $Fe_2(SO_4)_3x7H_2O$ in 200 µM HCl quantified spectrophotometrically at 420 nm; $\varepsilon = 2.865\ mM^{-1} * cm^{-1}$ for the Fe^{3+}:desferal complex.

2.3.7. Determination of the total iron content in tissues according to Brumby & Massey (1967) and Lawrie et al. (1991)

Homogenates were incubated at 50 - 60°C for at least 48 h until complete dryness. Afterwards, dried samples were mineralised in HNO_3 : $HClO_4$ (1:1) and heated to dryness. The remaining inorganic residues were diluted in 4 ml 5% HCl. After addition of 10% thioglycolic acid to convert Fe(III) to Fe(II), the concentration of Fe(II) was measured spectrophotometrically after reaction with 4 mM bathophenanthroline at 535 nm. Quantification was done using a 5-point calibration curve obtained from iron standard (Sigma, Seelze, Germany).

2.3.8. Measurement of iron reduction rate according to Vegh et al. (1988)

The reduction rate of Fe(III) to Fe(II) was measured as a further indicator for the presence of Fenton-reactive Fe(II).

Samples were homogenised in 100 mM KP_i buffer (pH 7.4) in 500 µl final volume. To separate cell debris and lipids, homogenates were centrifuged for 8 min at 10 000 g. The supernatant was mixed with 100 mM Tris-HCl buffer (pH 7.4) and the iron reduction rate recorded spectrophotometrically at 520 nm, $\varepsilon = 13.6\ mM^{-1} * cm^{-1}$, after adding 50 µM Fe-EDTA, 11 mM α-α'-bipyridyl and 500 µM NADPH. Assays were carried out at room temperature.

2.3.9. Extraction of liver microsomes according to Klein et al. (1983)

As lipid radical EPR signals in liver homogenates were abolished by high cytosolic antioxidant potential the microsomal fraction had to be extracted from liver tissue samples. Microsomes represent membrane rearrangements after the homogenisation process, originating mainly from the endoplasmic reticulum, but also the plasma membrane, and they are known to undergo lipid peroxidation in the presence of NADPH and Fe(III) salts (Halliwell & Gutteridge 1999).

A minimum of 0.6 g of liver tissue was needed for microsome extraction. Sample aliquots were pooled from 1 - 3 fish and homogenised in 9 ml of 100 mM KP_i buffer at pH 7.4. After centrifugation at 1 000 g for 10 min and 4°C to remove cell debris, the supernatant was again centrifuged for 1 h at 100 000 g and 4°C. The resulting microsomal pellet was resuspended in 1.5 ml of 125 mM KCl, frozen in liquid nitrogen and stored at -20°C.

2.3.10. EPR spin trapping of lipid radicals in liver microsomes according to Buettner (1987)

Lipid radicals reflect the sensitivity of lipid membranes to ROS attack. α-(4-pyridyl 1-oxide)-N-t-butyl nitrone (POBN) adducts resulting in characteristic EPR spectra with hyperfine coupling constants of $a^N = 15.8$ G and $a^N = 2.6$ G, in agreement with computer spectral simulated signals obtained using those parameters can be assigned to lipid radicals. However, spin trapping studies cannot readily distinguish between peroxyl ($ROO^\bullet$), alkoxyl ($RO^\bullet$) and alkyl ($R^\bullet$) adducts, owing to the similarity of the corresponding coupling constants (Buettner 1987).

To initiate the lipid radical production 5.5 mM ADP, 1 mM NADPH, 0.1 mM $Fe(NH_4)(SO_4)_2x12H_2O$ (in 0.1 N HCl) were added to 1 ml of microsomal suspension. For later detection of the lipid radicals 100 mM of the spin trap POBN was aggregated and samples were refrozen in liquid nitrogen. Immediately before detection samples were rapidly thawed and lipid radical generation measured at room temperature using a Bruker (Buenos Aires, Argentina) spectrometer ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. After 30 min at room temperature lipid radical production of each sample was detected again to see the increase over time. EPR instrument settings were microwave power, 20 mW; modulation amplitude, 1.194 G; time constant, 81.92 ms; receiver gain, $2 * 10^4$. Quantification was done using 4-OH TEMPO standard.

2.3.11. Measurement of superoxide dismutase (SOD) activity according to Livingstone et al. (1992)

The SOD (E.C: 1.15.1.1) activity was determined using a xanthine oxidase (XOD) / cytochrome c assay at 20°C as well as at the respective water temperature of each experimental group.

Frozen liver tissue was ground in liquid nitrogen and the resulting powder homogenised in 20 mM Tris-HCl buffer (pH 7.6, containing 1 mM EDTA). After centrifugation for 3 min at 20 800 g, 4°C, the SOD activity was measured in the supernatant. The assay mixture consisted of 43 mM KP_i buffer (pH 7.8, containing 0.1 mM EDTA), 50 µM xanthine, 100 µM cytochrome c, final concentrations. The actual volume of added 0.3 mU * μl^{-1} XOD was adjusted to yield an extinction increase of 0.02 E * min^{-1} without sample. Likewise, the added sample volume was adjusted to an extinction increase of 0.01 E * min^{-1}. 1 Unit SOD reduces the increase in extinction of superoxide-mediated reduction of oxidised cytochrome c by 50% (measured at 550 nm).

2.3.12. Determination of Glutathione peroxidase activity (GPX) according to Günzler & Flohe (1985)

GPX activity was measured by coupled optical test. Oxidised glutathione GSSG is formed by the reaction of GPX and continuously reduced by an excess of glutathione reductase (GR) activity, providing a constant level of GSH. The rate of NADPH oxidation by GR can be recorded spectrophotometrically at 340 nm.

Tissues were homogenised in 20 mM Tris-HCl buffer (pH 7.6, containing 1 mM EDTA, and 1 mM DTT) (w:v / 1:3). The assay system consisted of 100 mM KP_i reaction buffer (pH 7.0, containing 1 mM EDTA), 10 mM NaN_3 for inhibition of catalase, 10 U * ml^{-1} glutathione reductase and 10 mM GSH. This mixture was incubated with 10 - 100 µl of sample for 10 min at 37°C to reactivate GPX. After addition of 1.5 mM NADPH (in 0.1% $NaHCO_3$), hydroxide-independent NADPH consumption was determined. After 3 min 1.5 mM H_2O_2 was added and the reaction recorded over 5 min. Reagent blanks were run by replacing sample with reaction buffer to control chemical oxidation.

Reactivation of GPX at 37°C led to similar activities as incubation at 20°C or on ice in samples from the polar species, indicating that this incubation has no adverse effect on GPX activity. Although incubation at 37°C had no stimulating effect on GPX activity in the polar species, all samples were treated in the same way as described above in order to achieve the same treatment for all samples.

2.3.13. Griess colorimetric assay according to Misko et al. (1993) and Verdon et al. (1995)

The total content of nitrite and nitrate was measured as an indicator for NO production.

Frozen tissue was homogenised in the 10-fold volume of 30 mM KP_i buffer (pH 7.1 - 7.2). The homogenate was applied to filter tubes (30 000 MWCO) and centrifuged for 1.5 h at 7 650 g, 4°C to remove all organic compounds. To convert all nitrate to nitrite, the inorganic filtrate was incubated for 2 h at room temperature with 5 µM NADPH, 6 M glucose-6-phosphate, 0.06 U * ml^{-1} glucose dehydrogenase, 0.3 U * ml^{-1} nitrate reductase. For the Griess reaction the incubated sample was mixed with 1% sulphanilamide and 0.1% naphthalene-ethylene diamine dihydrochloride in the ratio (1:1:1). Determination of the nitrite content was done spectrophotometrically at 540 nm and quantified using a 3-point calibration curve obtained from KNO_2^- standards incubated in the same way as described for the samples. A second standard curve was generated using $NaNO_3^-$ as control for completely conversion of nitrate to nitrite.

2.3.14. Protein content according to Bradford (1976)

The protein content of the samples was determined spectrophotometrically at 590 nm by the Bradford method using bovine serum albumin (Sigma, Seelze, Germany) as a standard.

2.4. Molecular Studies:

2.4.1. Cloning and Sequencing of the transcription factor HIF-1α

Total RNA was extracted from frozen liver tissues using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Protocol for *P. brachycephalum* as well as notothenioids (*Trematomus hansoni*, *Trematomus pennellii, Chionodraco myersi*) was modified by proteinase K digestion of the tissue as described by Lucassen et al. (2003). Oligotex kit (Qiagen, Hilden, Germany) was used to isolate mRNA from total RNA. The RNA quantification was done spectrophotometrically. A_{260} / A_{280} ratios were > 1.8. Three different primer pairs (tab. 2.2) for hypoxia inducible factor (HIF) -1α were constructed from conserved regions in previously published HIF-1α cDNA sequences (rainbow trout, AF304864; zebrafish, AY326951; grass carp, AY450269, human 31077212). Thus, three overlapping fragments were obtained and identified as partial HIF-1α sequences by BLAST search in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/; translated query *vs*. protein database, blastx). The obtained cDNA fragments were used to

construct gene specific primers (tab. 2.2) for RACE (rapid amplification of cDNA ends) by First Choice RLM-RACE kit (Ambion, Austen, USA).

For reverse transcription 0.2 µg mRNA were incubated 65 min at 42°C and 20 min at 70°C with 1 U * μl^{-1} Superscript RT (Invitrogen, Karlsruhe, Germany), 6 pmol of respective gene specific reverse primer, 0.625 mM of each dNTP, 10 mM DTT, 2.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4).

PCR reactions to amplify RT-products was carried out in a gradient cycler (Tgradient, Biometra) with 0.5 U * μl^{-1} Taq-Polymerase (Eppendorf, Hamburg, Germany) or SuperTaq Plus polymerase (Ambion, Austen, USA) for sequences with an expected length > 1 000 bp, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP, 1 pmol * μl^{-1} primer pair in 20 mM Tris-HCl (pH 8.4), 50 mM KCl. The concentration of $MgCl_2$ was reduced to 1.5 mM if higher concentrations were not successful.

Tab. 2.2: List of all primers used.
Zv: only used for amplification of partial HIF-1α cDNA of *Z. viviparus*, *Pb*: only used for amplification of partial HIF-1α cDNA of *P. brachycephalum*, N: used for amplification of partial HIF-1α cDNA of notothenioids and zoarcids, 2: only used for amplification of partial HIF-2α cDNA of *T. hansoni* and *C. myersi*, * position of partial *Cm*HIF-2α, bp = base pairs.

	Primer	Sequence (5` – 3`)	Position of *Zv*HIF-1α	Product size of *Zv*HIF-1α fragment (bp)	
I	HIF-F1	CCAAGGAACMAAACACAGAARCG	677 – 698		
	HIF-B5	TGAGTCTCMACCCACACAAARCC	1157 – 1135	435	
II	HIF-F1L	TCCAACATCCCTCCAACATCG	878 – 898		
	HIF-B7L	TTCTTCTCGTCCCCCAGCAGGAAC	1376 – 1353	453	
III	HIF-F13	TGTGYGTCAACTAYGTKCTCCAGTGG	1206 – 1229		N
	HIF-B31	TGACTTGGTCCAGRGCACKCAG	2437 – 2416	1175	N
	HIF-5`-B1	GATGTTGGATGGGGTCACAGAC	886 – 865	865	
	HIF-5`-B4Z	TGTATGGCGGCTGAGAAAAGTC	939 – 918	918	*Zv*
	HIF-5`-B6	GTAGATGACAGTGGCTTGTGTTTCC	1173 – 1149	1149	
	HIF-3`-F6	TTGATACAGAGCCCAAAACCC	1805 – 1825	1558	*Pb*
	HIF-3`-F10	AGTCCAGTCCCTTCAGAGTTCTC	1944 – 1966	1417	
IV	HIF2-F1	CTCTGTTCTGCATCAGGTGAA	(438 – 463)*		2
	HIF2-B1	CCAGCGTCCAGGAGATTACTACAC	(1075 – 1053)*	(637)	2

RNA processing for 5`RACE-PCR was modified using 1 µg mRNA for 2 h incubation at 37°C with 1 U CIAP (calf intestine alkaline phosphatase; Gibco, Karlsruhe, Germany). Termination of the CIAP reaction and precipitation with acid phenol : chloroform was carried out according to the manufacturer's protocol. Isopropanol precipitation was performed at -80°C for 1 h. After precipitation with 70% ethanol, the dried pellet was resuspended in 20 µl nuclease-free water, and the RNA concentration determined spectrophotometrically. 250 ng mRNA were used for the following steps as described by the manufacturer.

A standard PCR protocol was used with 35 cycles of 45 sec denaturation at 94°C, annealing 80 sec with a 12°C gradient (58 ± 6°C) and 45 sec elongation at 72°C, followed by a final elongation step of 8 min at 72°C. 3`RACE-PCR and 5`RACE-PCR reactions with *P. brachycephalum* samples were carried out at higher annealing temperatures (61 ± 6°C and 62 ± 6°C, respectively). The 1 175 bp fragments (see primer pair III) of *P. brachycephalum* and notothenioid samples were amplified using a touch-down program with 2 min annealing temperatures from 60 ± 6 to 51 ± 6°C (-1°C / cycle), followed by 25 cycles with 1 min annealing at 57 ± 6°C.

PCR-products were separated in a 1.3% agarose gel. Fragments of appropriate size were purified by QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned by TOPO TA cloning kit (plasmid vector: pCR4-TOPO; chemically competent TOP10 *E. coli*; Invitrogen, Karlsruhe, Germany) as described in the manufacturer's instruction. 6 to 8 clones were separated for overnight liquid culture in LB medium. Cloned plasmids were isolated by Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany). The presence of inserts was checked by restriction digestion with EcoRI (Invitrogen, Karlsruhe, Germany) before sequence determination with T3 or T7 primers by MWG-Biotech (Ebersberg, Germany).

All inserts from positive clones of one species were assembled to obtain full length cDNA sequences of HIF-1α of both zoarcid species and partial HIF-1α sequences of notothenioids. Using the primer pair III additional fragments comprising 1 497 nucleotides were obtained from *T. hansoni* and *C. myersi* and identified by BLAST search as HIF-2α. As after sequence determination with T3 and T7, the centre part of the HIF-2α fragment was still unclear, a specific primer pair (IV) amplifying a 637 bp product at position 438 - 1075 on partial *Cm*HIF-2α cDNA was constructed.

MacVector 7.2 was used for primer design, sequence alignments and analysis, AssemblyLign 1.0.9c for assemblage of overlapping fragments.

2.4.2. Whole-cell extracts from liver tissue for Western blotting analysis and EMSA

Nuclear extraction protocols, developed for rainbow trout tissues (Soitamo et al. 2001) were not readily applicable to eelpout samples: Western blotting analysis with antibodies against HIF-1α resulted in much weaker signal intensities in nuclear extracts compared to whole-cell extracts although the majority of the HIF-1α protein would be expected in the nucleus (Kallio et al. 1998). It might be that the protocol developed for the freshwater fish cannot be used for marine species, probably leading to osmolysis of the nuclei in samples

from marine fish. With regard to the limited amount of eelpout samples, analysis had to be carried out with whole-cell extracts, prepared according to Vuori et al. (2004).

50 mg of liver tissue were homogenised in 200 µl buffer C (20 mM HEPES, pH 7.8, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol, 2 µg * ml^{-1} leupeptine, 2 µg * ml^{-1} antipaine, 2 µg * ml^{-1} pepstatine, 2 µg * ml^{-1} aprotinin, 1 mM Na_3VO_4) and centrifuged at 16 100 g, 30 min, 4°C to remove cell debris.

2.4.3. Western blotting analysis of HIF-1α protein and HSP70

20 µg protein of whole-cell extracts per well were run on 7.5% LiDS-PAGE at 40 mA and transferred to 3 MM nitrocellulose membrane (semi-dry blotting; 1 h; 0.4 A; 13 V). Membranes were blocked for 1 h at room temperature with 3% non-fat dry milk in PBS 0.3% Tween20, rinsed 3 times for 10 min with PBS 0.3% Tween20 and incubated with the primary antibody over night at 4°C followed by 1 h at room temperature. Polyclonal antibodies directed against the N-terminus of rainbow trout HIF-1α were used as described by Soitamo et al. (2001), dilution 1:2 000 in 1% BSA PBS + 0.02% NaN_3. The antibody recognised HIF-1α only in liver samples from *Z. viviparus*, but not from *P. brachycephalum*.

After the incubation, the membranes were washed and incubated for 3 h at room temperature with horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham Biosciences, Freiburg, Germany), dilution 1:7 500 in 3% non-fat dry milk in PBS 0.3% Tween20. After washing the membranes, the signals were detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Freiburg, Germany). Signal intensities of HIF-1α protein bands were calculated from autoradiographed film, using Chemi-Imager digital camera and software (Alpha Innotech Co., San Leandro, USA). Calculation of the %-intensity was based on densitometry of gel images, with the sum of the bands from all experimental groups taken as 100% value.

Analysed membranes were washed three times as described above, blocked again for 30 min at room temperature, washed three times and incubated for 1 h at room temperature with HSP70 antibody (Affinity Bioreagents, Golden, USA), dilution 1:10 000 in 0.5% BSA PBS + 0.02% NaN_3. The membranes were washed again, incubated with anti-mouse secondary antibody (Amersham Biosciences, Freiburg, Germany), diluted 1:20 000 in 3% non-fat dry milk in PBS 0.3% Tween20, at room temperature. HSP70 signals were detected in the same way as described for HIF-1α.

2.4.4. Electromobility shift assay (EMSA) according to Kvietikova et al. (1995) and Soitamo et al. (2001)

EMSA was carried out to study the DNA binding activity of the transcription factor HIF-1 which is supposed to indicate the initiation of hypoxic signalling. The protocol which was used for sample analysis is given in publication III. Subsequently the protocol was slightly modified to obtain stronger EMSA signals:

According to Soitamo et al. (2001), the promoter region of the human erythropoietin (EPO) gene was used as a HIF-sensitive DNA probe (5`-GCCCTACGTGCTGTCTCA-3`) in fish samples. 18 pmol of sense strand and antisense strand were incubated separately with 5 mM $MgCl_2$ for 3 min at 95°C in a total volume of 10 µl. For annealing the heated strands were mixed and incubated for another 3 min at 95°C. The annealed strands were cooled down to room temperature within 3 hours. 5`-endlabeling of the annealed strands (1 pmol * μl^{-1} DNA) was done with 10 U * μl^{-1} T4 polynucleotide kinase and ^{32}P-γ-dATP (10%; v:v) over 30 min at 37°C. After removing unincorporated nucleotides by gelfiltration (sephadex G-25), probes were diluted in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final concentration of 5 fmol * μl^{-1}.

EMSA DNA-protein binding reactions were carried out for 30 min on ice in a total volume of 20 µl, containing 30 µg cell extract, 0.1 µg * μl^{-1} carrier DNA (poly dI-dC), DNA binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT), 1 µl ^{32}P-endlabeled DNA probe, 1 µl bromphenol blue (1.5%). Samples were run on 4% non-denaturating glycerolpolyacrylamide-gel (4% acrylamide, 1% glycerol, 0.33x TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA)). Electrophoresis was performed at 150 V, room temperature, 2 h in 0.33x TBE buffer. Radioactivity was detected and quantified in dried gels with a phosphor storage image system (FLA-5000; Fuji, Tokyo, Japan) and the AIDA software package (raytest, Straubenhardt, Germany).

For testing the specificity of the human EPO, the signal was erased by previous 15 min incubation of the reaction mixture with a 2 500-fold excess of unlabeled probe prior to addition of the labelled EPO probe (fig. 3.C in publ. III). Moreover, specificity was tested by super-shift experiments: 1 µl of undiluted polyclonal antibody against HIF-1α (see above) was added to the EMSA reaction mixture with liver extracts from *Z. viviparus*. In the presence of the antibody, the EMSA signal was markedly reduced, indicating that antibody interaction with the HIF-1α subunit minimised binding of the HIF-1 dimer to the EPO probe (Fig. 3.B in publ. III). This can be explained by the DNA binding function also

being located in the N-terminus of the HIF-1α protein (e.g. Chi & Karliner 2004). To date, there is no antibody available which recognises the HIF-1α protein in liver samples from *P. brachycephalum*, therefore the super-shift assay could not be carried out for the Antarctic eelpout. However, the specificity of the observed band has been determined in three fish species in the laboratory of M. Nikinmaa (pers. com.; see also Soitamo et al 2001). Moreover, the migrating distance was the same in *P. brachycephalum* as in *Z. viviparus*, indicating the same molecular size of the HIF-1-EPO enhancer complex in both zoarcids.

2.5. Studies on isolated hepatocytes

2.5.1. Preparation of hepatocytes according to Mommsen et al. (1994)

Hepatocyte preparation was modified after Mommsen et al. (1994). The liver was rapidly removed from narcotised (0.5 g MS 222 * L^{-1} seawater) *Z. viviparus*, rinsed and perfused in solution I (5.6 mM glucose, 5 mM KCl, 4 mM $NaHCO_3$, 0.3 mM Na_2PO_4, 240 mM NaCl, 0.4 mM KH_2PO_4, 10 mM HEPES, pH 7.4 = Mg^{2+}-free Hanks medium) to remove blood. Subsequently, liver perfusion was carried out using 750 U * ml^{-1} collagenase type IV (Sigma, Seelze, Germany) in solution II (= solution I + 1% BSA) until complete tissue digestion. Separated hepatocytes were filtered through 250 µm gauze and centrifuged for 4 min at 60 g, 0°C. The fatty top layer as well as the remaining supernatant were carefully removed and discarded. The cellular pellet was resuspended in solution II and again centrifuged for 2 min at 60 g and 0°C. Resuspension and centrifugation was repeated until a clear, colourless supernatant was observed. The purified cell pellet was resuspended in a final volume of 2 – 3 ml solution II supplemented with 5 mM glucose, 2 mM MgSO4 (=solution III). After 1 h, cell vitality was controlled by Trypan-blue staining (> 95%) using a Fuchs-Rosenthal-haemocytometer (0.2 mm). A density of 3 - million cells was adjusted.

2.5.2. MitoTracker Green FM staining of hepatic mitochondria

MitoTracker Green FM (Molecular Probes, Eugene, USA), passing trough membranes and accumulating in active mitochondria (Molecular probes; product information) was added to the hepatocyte suspension (4 - 10 µM final concentration) and incubated for 25 min in the darkness at room temperature. Afterwards, stained hepatocytes were washed twice by centrifugation for 2 min at 60 g and 0°C and subsequent resuspension in solution III. Finally, a density of 6 - million cells per ml was adjusted. 40 µl of this suspension was

transferred into a thermostatted microscopy chamber (10°C). A TCS-NT confocal system with an inverted DM IRBE microscope (Leica, Solms, Germany) equipped with an argon-krypton laser was used for confocal imaging of mitochondrial density in *Z. viviparus* hepatocytes. Cell imaging was carried out with 100x / 1.40 - 0.70 oil immersion lens with the following properties: excitation wavelength 488 nm, laser power 40 - 60, confocal pinhole 0.7, emission filter 580 nm, photomultiplier voltage 600 - 800, offset -3. Cells were scanned in glow mode with slow scan speed and an accumulation of 8 scans per picture. A slice of 18 - 30 µm, depending on cell size was scanned in 2 µm sections in *z*-direction to image mitochondria density throughout the cells. A total of 227 hepatocytes obtained from 6 liver preparations were recorded.

2.5.3. Visualisation of ROS production by fish hepatocytes using the fluorescent dye H_2DCFDA

The fluorescent dye H_2DCFDA (dichlorodihydrofluorescein diacetate) was used to detect cellular ROS production.

40 µl hepatocytes were placed in the thermostatted (10°C) chamber. Pre-incubation of cells with H_2DCFDA (D-399; Molecular probes, Eugene, USA), as proposed by the manufacturer proved inconvenient, because fluorescence of hepatocytes had already passed its maximum after a few minutes. Thus, no further increase in fluorescence intensity could be observed upon confocal microscopy. In some samples actually a decrease of fluorescence intensity occurred, probably due to resuspension of the fluorescent dye in the medium (data not shown). Consequently, imaging of fluorescence by confocal microscopy was carried out immediately after adding 50 µM H_2DCFDA to the cells and followed over an entire period of 40 min by using the same Leica TCS-NT confocal system as described above. Images with the size 100 µm² were recorded every 2 min. Properties were 100x / 1.40 - 0.70 oil immersion lens, excitation wavelength 488 nm, laser power 29 (without POBN) or 39 (with POBN), confocal pinhole 0.7, emission filter 505 nm, photomultiplier voltage 721, offset -8, glow-over mode, slow scan speed, accumulation of 4 scans per picture.

To test the ROS-specificity of fluorescent signals, the radical quencher POBN (Janzen 1971) was added together with H_2DCFDA to the hepatocyte suspension.

2.6. Statistics

All values are given as means ± standard deviation. Differences between experimental groups were analysed by student's t-test using Statview 5.0. Significances were evaluated based on the p-level < 0.05.

3. Publications

List of publications and declaration of my contribution towards them.

Publication I

Katja Heise, Maria Susana Estevez, Susana Puntarulo, Monica Galleano, Mikko Nikinmaa, Hans O. Pörtner, Doris Abele

Changes in oxidative stress parameters and activation of hypoxia inducible factor (HIF-1) at different environmental temperatures in polar and temperate zoarcid fish

Physiological and Biochemical Zoology, submitted

I developed the concept together with the six and seventh author. I carried out the practical work at the Alfred-Wegener Institute in Bremerhaven, Germany, at the University of Buenos Aires in Argentina and at the University of Turku in Finland. The practical work in Buenos Aires was supported by the second and the fourth author. Data were analysed by myself and discussed with the third, the fifth, the six and the seventh author. I wrote the first draft of the manuscript, which was revised together with all co-authors.

Publication II

Katja Heise, Magnus Lucassen, Doris Abele

The hypoxia inducible factor (HIF-1α) in cold-adapted Antarctic fishes

Biochimica et Biophysica Acta – Gene structure and expression, submitted

The scientific idea of this study was developed by the second and the third author and myself. The laboratory work was carried out by myself. I analysed and discussed the data together with the second author and wrote the first manuscript, which was improved in cooperation with both co-authors.

Publication III

Katja Heise, Susana Puntarulo, Mikko Nikinmaa, Magnus Lucassen, Hans O. Pörtner, Doris Abele

Oxidative stress and HIF-1 DNA binding during stressful temperatures and recovery in the North Sea eelpout (*Zoarces viviparus*)

Part A: cold stress

Journal of Experimental Biology, submitted

I planned the concept of this study together with the fifth and the sixth author. I carried out the experimental work at the Biologische Anstalt Helgoland and the laboratory and data analysis at the Alfred-Wegener Institute in Bremerhaven, Germany, at the University of Buenos Aires in Argentina and at the University of Turku in Finland. The practical work at the Alfred-Wegener Institute was supported by the second author. The first manuscript was written by myself and revised together with all co-authors.

Publication IV

Katja Heise, Susana Puntarulo, Mikko Nikinmaa, Doris Abele, Hans O. Pörtner

Oxidative stress and HIF-1 DNA binding during stressful temperatures and recovery in the North Sea eelpout (*Zoarces viviparus*)

Part B: heat stress

Journal of Experimental Biology, submitted

The ideas for the experiments were elaborated by the fourth and the fifth author and myself. The practical work and data analysis were conducted by myself at Biologische Anstalt Helgoland, at the Alfred-Wegener Institute in Bremerhaven, Germany, at the University of Buenos Aires in Argentina and at the University of Turku in Finland. The data and my first manuscript draft were discussed together with all co-authors.

Publication I:
PHYSIOLOGICAL AND BIOCHEMICAL ZOOLOGY

Changes in oxidative stress parameters and activation of hypoxia inducible factor (HIF-1) at different environmental temperatures in polar and temperate zoarcid fish

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Abstract

The rate of reactive oxygen species (ROS) formation in marine ectotherms is modified by changing environmental temperatures. We investigated oxidative stress parameters in polar and temperate zoarcid fish at the border of their thermal optimum range, in order to study a possible interference of elevated ROS production with metabolic reorganisation. As exposure to high and low temperature extremes has been shown to cause a state of metabolic hypoxia in marine ectotherms, onset of hypoxic signalling was studied as DNA binding activity of the hypoxia inducible factor (HIF-1) in the liver. The Antarctic eelpout *Pachycara brachycephalum* was maintained at 0 (controls) and acclimated to 5°C. North Sea eelpouts, *Zoarces viviparus*, were maintained at 12°C in summer and at 6°C in winter. Compared at the lower water temperatures (0°C for the polar and 6°C for the temperate eelpout) the polar fish showed lower levels of oxidative stress parameters, but higher susceptibility to liver microsomal lipid radical formation. Moreover, glutathione content was significantly higher in the polar eelpout and oxidised (GSSG) to over 50%. Oxidative damage parameters indicating elevated ROS production were higher in the warm in polar (TBARS) and in the cold in temperate fish (protein carbonyls and TBARS). This higher ROS levels went along with higher DNA binding activity of HIF-1. The hypothetical role of this response in the optimisation of tissue oxygenation at the borders of the thermal optimum range is discussed.

Keywords:
Oxidative stress, lipid peroxidation, hypoxia inducible factor, glutathione, polar fish

Introduction

Eurythermal marine fish can adjust their metabolic performance to seasonal fluctuations of the environmental temperature and are, moreover, tolerant of periodic and non-periodic short term temperature fluctuations. The physiological mechanisms that allow for seasonal adaptation include functional adjustments of the number of mitochondria, enzyme activities and membrane composition to improve resistance to short term fluctuations of summer and winter temperature extremes (Guderley & St-Pierre 2002). Seasonal cold acclimatisation was also found to cause a cold-induced increase in muscle capillary density (Johnston 1982; Egginton & Sidell 1989). According to a recent concept of thermal adaptation, a mismatch between oxygen demand and the capacity of oxygen uptake and distribution by ventilation and circulation sets the limits of thermal tolerance in aquatic ectotherms when exposed to warm and cold temperature extremes (Pörtner 2002). This means that onset of thermal stress initially leads to loss of aerobic scope and a state of metabolic hypoxia in central organs. At more extreme temperatures accumulation of anaerobic metabolites marks the critical temperature limit as seen in several invertebrates and marine fish (Pörtner 2001; 2002). Our hypothesis is, therefore, that the capacity of a metabolic reorganisation during hypoxic events may signify tolerance also of (subcritical) temperature fluctuations in marine ectotherms.

Oxidative stress parameters are frequently measured as stress indicators, because various forms of physiological stress propagate reactive oxygen species (ROS) formation from mitochondria and cytosolic ROS generating oxido-reductase enzymes. Alternatively, insufficient enzymatic antioxidant protection can be the reason for radical mediated damage. In many eurythermal ectotherms, low winter temperatures cause metabolic slow down, and low antioxidant enzyme activities speak for an increased susceptibility of winter animals to oxidative stress (Abele et al. 1998; Buchner et al. 2001; Keller et al. 2004). In general, elevated antioxidant functions are thought to confer higher stress resistance in an organism.

The hypoxia inducible factor (HIF-1) is known to enable hypoxic survival in mammalian tissues and to be functionally controlled by po_2 sensitive prolyl hydroxylases (Ivan et al. 2001). Stabilisation of the HIF-1α subunit enables nuclear translocation and formation of the HIF-1 heterodimer, necessary for the expression of target genes, involved in the tissue specific and the organismal hypoxia response. This implies improved oxygen binding and blood supply to the tissues and enhanced anaerobic capacity (Pugh 2003). All in all, this

system coordinates survival during transient episodes of hypoxia, also characteristic of different environmental stress situations experienced by marine animals (temperature and po_2 fluctuations, H_2S exposure, oxygen shortage during fight and flight response). Among mammalian target genes activated by HIF-1 are the genes for erythropoietin (EPO), the vascular endothelial growth factor (VEGF), nitric oxide synthase and around 11 glycolytic enzymes (Semenza 2002). Transient hypoxia may occur rapidly in ectotherms, because these animals adjust arterial po_2 to a minimum required level in accordance with lifestyle requirements (Massabuau 2003). Exhaustive exercise, sudden temperature or salinity stress that require regulatory efforts or exposure to hydrogen sulphide or xenobiotics may then lead to fluctuations of steady state po_2, causing transient tissue hypoxia. Moreover, the other extreme: hyperoxia can occur in intertidal pools (Abele-Oeschger et al. 1998). Fluctuations between oxygen deficiency and reoxygenation or oxygen overload of a tissue are thought to be accompanied by elevated ROS production causing oxidative stress when unbalanced by antioxidants. As several steps of the oxygen sensitive signalling cascade (stabilisation of HIF-1α, DNA binding activity of HIF-1) are also redox controlled (Haddad et al. 2000; Nikinmaa et al. 2004) and, thus, functionally modulated by ROS, elevated production of ROS may interfere with hypoxic signalling and in so doing may prevent a tissue specific regulatory response (Czubryt et al. 1996; Nikinmaa 2002). This is one reason, why cellular ROS levels are buffered by ROS quenching antioxidants like vitamins E and C, and by the cellular glutathione (GSH) system. Metal exposure and several xenobiotics have been shown to alter the GSH status in fish (Maracine & Segner 1998; Pena et al. 2000; Sayeed et al. 2003), but data on seasonal and regional differences of the glutathione status in marine fish are missing. However, it is known that polar fish carry high levels of the lipid-soluble radical scavenging α-tocopherol (Gieseg et al. 2000), which is thought to be the most active compound of vitamin E (Dunlap et al. 2002).

In the present study we compare two eelpout species from Antarctic (*Pachycara brachycephalum*) and temperate North Sea (*Zoarces viviparus*) environments with respect to cellular oxidative stress parameters including α-tocopherol levels and glutathione redox rate, antioxidant enzyme activities and ROS damage parameters, as well as induction of metabolic reorganisation in response to the onset of functional hypoxia at the borders of their thermal envelope. Both fishes belong to the family of zoarcids, have a similar behaviour (mainly inactive, carnivorous sit-and-wait predators, Van Dijk et al. 1999). Temperature exposure was kept subcritical because the objective was to study the potential for metabolic regulation to compensate for changing environmental temperatures when

approaching the critical limit. In the North Sea species acclimatisation to natural seasonal temperatures was studied at 12°C in summer and 6°C in winter. By contrast, a warming experiment was carried out with the polar species (preference temperature of 1-2°C, E. Brodte, pers. com.), to investigate its regulatory flexibility although it does not experience seasonal temperature fluctuations in nature. Therefore, *P. brachycephalum* were acclimated to their upper tolerance temperature (5°C) close to the border to thermal stress (7°C; Mark et al. 2002).

Interspecies comparison was done at both respective low water temperatures, which are natural for both animals: the polar fish at 0°C and winter acclimatised North Sea eelpout at 6°C, thus, presenting the comparison of cold adaptation *vs.* cold acclimatisation.

Material & Methods

Animal collection and experimental conditions

Antarctic eelpout (*Pachycara brachycephalum*) were caught with bottom traps near King George Island (Maxwell Bay) at 400 m depth during Antarctic expedition with RV Polarstern (ANT XIX/5) in April 2002. At the Alfred-Wegener Institute, a total of 30 animals were kept at 0°C in natural sea water from the North Sea with constant aeration and a day-night cycle of 12 h until the beginning of the acclimation experiment in December 2002. A batch of 15 polar eelpout was acclimated at 5°C over an entire period of 11 weeks. Fish were fed live mud shrimp once a week.

After 11 weeks fish were narcotised with 0.5 g MS222 l^{-1} seawater, weighed and killed by a cut through the spine. Tissue samples of the liver were quickly taken and aliquots of 100 mg frozen in liquid nitrogen. Samples were stored at –80°C. After sampling, sex and length of the eelpout were determined. Length varied between 18 and 34 cm.

14 temperate eelpout, *Zoarces viviparus,* were caught near the island of Helgoland in the German North Sea in December 2002 and were thus treated as winter animals. Lowest habitat water temperatures in winter 2002/03 were about 3°C (sea surface temperature taken from MURSYS, Bundesamt für Seeschiffahrt und Hydrografie). Fish were fed pellets of white fish (*Merlangius merlangus*) once a week and kept in an aquarium with a flow through system of natural filtered sea water, which had the same temperature as the outside water in the natural habitat. In February, tissue samples of the fish were taken as described above, when water temperature was 6°C. For comparison to summer

acclimatisation, samples were taken from another batch of 16 eelpout caught in May and June 2002 and maintained at constant 12°C for one month.

All parameters were determined in liver tissue, as the liver is very sensitive to temperature stress (Van Dijk et al. 1999). The determination of the carbonyl-content, chemiluminescence, α-tocopherol, iron content as well as the EPR measurements, were carried out in the department of Physical Chemistry at the School of Pharmacy and Biochemistry of the University of Buenos Aires, Argentina. Samples were maintained frozen on dry ice during transport.

TBARS (thiobarbituric reactive substances)

were determined as a marker of lipid peroxidation, indicating oxidative damage in the lipid fraction according to Uchiyama & Mihara (1978).

Tert-butyl hydroperoxide-initiated chemiluminescence according to Gonzalez Flecha et al. (1991)

Chemiluminescence indicates an imbalance between pro- and antioxidant processes resulting from depletion of antioxidant compounds like glutathione, vitamin E and vitamin C. Tissue samples were homogenised in 30 mM KP_i buffer (pH 7.4) containing 120 mM KCl and centrifuged at 600 g for 10 min. The supernatants were diluted in buffer, containing a final concentration of 3 mM tert-butyl hydroperoxide and assayed in the dark at room temperature for chemiluminescence (counts per minute) in a liquid scintillation counter in the out-of-coincidence mode, using potassium glass vials kept in the dark for at least 48 h to avoid vial phosphorescence activation by fluorescent light. Data were expressed in a tissue characteristic curve with y = chemiluminescence and x = time and the area under this curve integrated for a time period of 4000 sec (= 66.67 min) using MatLab program and related to the protein content of the sample.

Determination of carbonyl groups according to Levine et al. (1990)

Carbonyl groups were measured as indication for oxidative damage to proteins. Samples were homogenised in 50 mM HEPES buffer containing 125 mM KCl and protease inhibitors (0.5 mg * ml^{-1} leupeptine, 0.7 µg * ml^{-1} pepstatine, 40 µg * ml^{-1} phenylmethylsulfonyl fluoride, 0.5 µg * ml^{-1} aprotinin, 1.1 mM EDTA, 0.6 mM $MgSO_4$) and centrifuged at 100 000 g for 15 min. Supernatants were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2 M HCl. Blanks were run

without DNTP. Afterwards, proteins were precipitated with TCA and centrifuged for 10 min at 10000 g. The protein pellet was washed 3 times with ethanol : ethylacetate (1:1), resuspended in 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH = 2.3) and incubated at 37°C until complete resuspension. The carbonyl content could be measured spectrophotometrically at 360 nm (molar extinction coefficient ε = 22 000 M^{-1} cm^{-1}). The protein content of the samples was determined by the Bradford (1976) method using bovine serum albumin as standard.

Determination of (nitrite and nitrate)

The total content of nitrite and nitrate (Σ NO_2+ NO_3) was measured as indicator for NO production by the Griess colorimetric assay as described in Misko et al. (1993) and Verdon et al. (1995).

Determination of reduced (GSH) and oxidised (GSSG) glutathione according to Fariss & Reed (1987).

The glutathione status represents the most important determinant for the cellular redox environment (Schafer & Buettner 2001). Frozen tissue was ground in liquid nitrogen and the resulting powder homogenised in 1/10 (w/v) pre-cooled PCA (10% containing 2 mM bathophenantroline-disulphonic acid). After centrifugation at 15 000 g for 5 min at 4°C, 500 µl of the supernatant were mixed with 10 µl pH-indicator (1 mM m-cresol purple sodium salt containing 0.5 M iodoacetic acid (IAA)) and 50 µl 1 mM γ-glutamyl-glutamate (in 0.3% PCA) was added as internal standard. The pH was adjusted to 8.5 with 5 M KOH (containing 0.3 M N-morpholine-propanesulfonic acid). The mixture was incubated at room temperature for 45 min, to allow IAA to bind to GSH. Subsequently samples were centrifuged for 5 min at 15 000 g at 4°C. 300 µl of the supernatant were added to the double amount of 1% 1-fluor-2,4-dinitrobenzene (diluted in 100% ethanol, HPLC grade) and derivatised in dark vials at room temperature over 24 h. After centrifugation at 7 500 g for 1 min at 4°C and filtration through 0.2 µm nylon membrane filters, samples were stored in dark HPLC vials at –20°C.

HPLC determination was carried out on a Beckmann Coulter HPLC System using a NH_2-spherisorp column, 5 µm 240 x 4 mm (Waters, Germany). Solvent A: 80% methanol and solvent B: sodium acetate stock in 80% methanol (20:80). Sodium acetate stock was prepared by dissolving 500 g Na-acetate in 224 ml Milli-Q water and 695 ml of concentrated HPLC-grade acetic acid. The gradient programme was as follows: 10 min

hold at 90% A followed by a 25 min linear gradient to 25% A at a flow rate of 1 ml * min^{-1}, and 2.3 to 2.8 psi backpressure. Peaks were recorded with a photodiode array detector at 365 nm. Concentrations were calculated using 5-point calibration curves for GSH and GSSG standards processed in the same manner as described for the samples.

As GSSG is formed by oxidation of 2 molecules GSH, total glutathione content was calculated as the sum (2 GSSG + GSH). Accordingly, the redox ratio of oxidised to reduced glutathione was expressed as 2 GSSG / GSH.

The redox potential was calculated with the Nernst equation, as given by Schafer & Buettner (2001), for the different temperatures of maintenance, also considering the temperature induced changes of intracellular pH (pH_i) in fish liver (data taken from Larsen et al. 1997 and Sartoris et al. 2003).

Detection of α-tocopherol according to Desai (1984)

α-tocopherol represents the most active compound of the lipid-soluble non-enzymatic antioxidant vitamin E (Dunlap et al. 2002). Tissue samples were homogenised with sodium dodecylsulfate (SDS). To 300 µl homogenate, or 100 µl of microsome suspension, 1 ml of ethanol was added and vortexed for 1 min. Then, 4 ml of hexane were added and the mixture vortexed again for 1 min. After 10 min centrifugation at 600 g, the lipid phase was dried with nitrogen, resuspended in methanol:ethanol (1:1) and filtered through nylon membranes, 0.22 µm. Hereafter, samples were analysed by reverse phase high performance liquid chromatography (HPLC) and electrochemical detection with an oxidation potential of 0.6 V and a flow rate of 1 ml * min^{-1}. The isocratic system consisted of a column (Supelcosil LC-8, 3 µm 15 cm x 4.6 mm, Supelco 58983) and a solvent containing 20 mM $LiClO_4$ in 99% methanol.

Determination of the intracellular "free" iron by EPR

Iron contents were measured as possible catalysts for Fenton reactions leading to enhanced formation of $OH^{\cdot}$ and subsequent lipid radical formation (Halliwell & Gutteridge 1985). Tissue samples frozen in liquid nitrogen and pooled from two animals were weighed without thawing and ground to powder in a porcelain mortar. The powder obtained was mixed at ratio 1:2 (w:v) with 10 mM Tris/HCl buffer, pH 7.4, containing deferoxamine mesylate (final concentration 2 mM). After 5 minutes incubation, samples were transferred to cylindrical plastic tubes and frozen in liquid nitrogen (Yegorov et al. 1993; Woodmansee & Imlay 2002). EPR measurements were carried out at 77K; microwave

power, 10 mW; time constant, 82 ms; modulation amplitude, 5 G; centerfield 1600 G; sweep width, 800 G (Linares et al. 2003). A standard curve was generated using solutions of $Fe_2(SO_4)_3x7H_2O$ in 200 µM HCl quantified spectrophotometrically (ε_{420} = 2.865 $mM^{-1} * cm^{-1}$ for the Fe^{3+}:desferal complex). All chemicals were prepared in iron free water, obtained from Milli-Q treated with chelex 100.

Determination of the total iron content in tissues after Brumby & Massey (1967) and Lawrie et al. (1991)

Homogenates were heated at 50 - 60°C for at least 48 h until complete dryness. Afterwards samples were mineralised in HNO_3:$HClO_4$ (1:1) and heated to dryness. The remaining inorganic residues were diluted in 4 ml 5% HCl. After addition of 10% thioglycolic acid to convert Fe(III) to Fe(II), the concentration of Fe(II) was measured spectrophotometrically after reaction with 4 mM bathophenanthroline at 535 nm.

Measurement of iron reduction according Vegh et al. (1988)

Samples were homogenised in 100 mM KP_i buffer pH 7.4 in 500 µl final volume. To separate cell debris and lipids, homogenates were centrifuged for 8 min at 10000g. The supernatant was mixed with 100 mM Tris/HCl buffer pH 7.4 and the iron reduction rate recorded spectrophotometrically at 520 nm, ε = 13.6 $mM^{-1} * cm^{-1}$, after adding 50 µM Fe-EDTA, 11 mM α-α'-bipyridyl and 500 µM NADPH. Assays were carried out at room temperature.

Extraction of liver microsomes according to Klein et al. (1983)

A minimum of 0.6 g of liver tissue was needed for microsome extraction. Sample aliquots were pooled from 2 - 3 fish and homogenised in 9 ml of 100 mM KP_i buffer at pH 7.4. After centrifugation at 1 000 g for 10 min and 4°C to remove cell debris, the supernatant was again centrifuged for 1 h at 10 0000 g and 4°C to precipitate the microsomal fraction. The resulting microsomal pellet was resuspended in 1.5 ml of 125 mM KCl, frozen in liquid nitrogen and stored at –20°C.

EPR- spin trapping of lipid radicals after Buettner (1987) in liver microsomes

Lipid radicals reflect the sensitivity of lipid membranes to ROS attack. Of each microsome solution 1 ml was used. To initiate the lipid radical production the following reactants were added: 5.5 mM ADP, 1 mM NADPH, 0.1 mM $Fe(NH_4)(SO_4)_2x12H_2O$ (in 0.1 N HCl). For

later detection of the lipid radicals 100 mM of the spin trap α-(4-pyridyl 1-oxide)-N-t-butyl nitrone (POBN) was added. After aggregation samples were frozen again in liquid nitrogen. Immediately before detection samples were thawed and lipid radical generation measured at room temperature using a Bruker spectrometer ECS 106, operating at 9.81 GHz with 50 kHz modulation frequency. After 30 min at room temperature lipid radical production of each sample was detected again to see the increase over time. EPR instrument settings were microwave power, 20 mW; modulation amplitude, 1.194 G; time constant, 81.92 ms; receiver gain, $2x10^4$. Quantification was done using 4-OH TEMPO standard.

Superoxide dismutase activity (SOD E.C: 1.15.1.1), being the prime antioxidant enzyme, was determined according to Livingstone et al. (1992) using a xanthine oxidase/cytochrome c assay at 20°C as well as at the respective water temperature of each experimental group. 1 Unit SOD reduces the increase in extinction of superoxide-mediated reduction of oxidised cytochrome c by 50% (measured at 550 nm).

Glutathione peroxidase activity (GPX) was determined as further antioxidant enzyme by a coupled optical test after Günzler & Flohe (1985). Tissues were homogenised in 20 mM Tris/HCl buffer, pH 7.6, containing 1 mM EDTA and 1 mM DTT (1,4-dithiothreitol, w:v/1:3). The assay system consisted of 100 mM KP_i, 1 mM EDTA reaction buffer at pH 7.0, 10 mM sodium azide for inhibition of catalase, 10 U * ml^{-1} glutathione-reductase and 10 mM GSH. This mixture was incubated with 10 - 100 µl of sample for 10 min at 37°C to reactivate GPX. After addition of 1.5 mM NADPH (in 0.1% $NaHCO_3$), hydroxide-independent NADPH consumption was determined at 340 nm. After 3 min, 1.5 mM H_2O_2 was added and the reaction recorded over 5 min. Reagent blanks were run by replacing sample with reaction buffer to control chemical oxidation.

Protein content after Bradford (1976)

The protein content of the samples was determined by the Bradford method using bovine serum albumin as standard.

Determination of the DNA-binding activity of the hypoxia inducible factor (HIF-1)

The DNA binding activity of HIF-1 was determined as indicator for hypoxic signalling. Measurements were carried out at the Department of Biology, University of Turku,

Finland. Samples were transported frozen in liquid nitrogen. Nuclear extraction protocols developed for rainbow trout (Soitamo et al. 2001), were not applicable to small tissue samples. Thus, EMSA and Western blotting were carried out with whole cell extracts, prepared as described in Vuori et al. (2004).

50 mg of liver tissues were homogenised in 200 µl buffer C (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM Hepes, 0.5 mM PMSF, 0.5 mM DTT, 2 µg * ml^{-1} leupeptine, 2 µg * ml^{-1} antipaine, 2 µg * ml^{-1} pepstatine, 2 µg * ml^{-1} aprotinin, 1 mM Na_3VO_4) and centrifuged at 16 100 g, 30 min, 4°C. The supernatant was transferred to a new tube and the protein content determined by the Bio-Rad assay in a microplate reader.

For Western blotting (20 µg protein of whole cell extracts per well) polyclonal antibodies directed against the N-terminus of rainbow trout HIF-1α were used (Soitamo et al. 2001). The antibody recognised HIF-1α in *Z. viviparus*, but not in *P. brachycephalum*.

Following the fish protocol of Soitamo et al. (2001), we used the promoter region of the human erythropoietin (EPO) gene as HIF-sensitive DNA probe (5`-GCCCTACGTGCTGTCTCA-3`). 5´ - endlabeling of the sense strand (2 pmol * μl^{-1} DNA) was done with 10 U * μl^{-1} T4 polynucleotide kinase and ^{32}P-γ-dATP (10 % v/v) over 15 min at 37°C. After removing unincorporated nucleotides by purifying through a sephadex G-25 column, probes were annealed with 2 pmol * μl^{-1} antisense strand, 5 mM $MgCl_2$ and TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

EMSA DNA-protein binding reactions were carried out for 30 min on ice in a total volume of 20 µl, containing 10 µg cell extract, 0.1 µg * μl^{-1} carrier DNA (poly dI-dC), DNA binding buffer (10 mM Tris/HCl, pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol), 1 µl ^{32}P-endlabeled DNA probe, 1 µl bromphenol blue (1.5%). Samples were run on 4% non-denaturating glycerolpolyacrylamide-gel (4% acrylamide, 1% glycerol, TBE buffer). Electrophoresis was performed at 150 V, room temperature, 2 h in TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA). Dried gels were autoradiographed (Kvietikova et al. 1995). Calculation of the % intensity was based on densitometry of gel images, with the sum of all 4 bands taken as 100% value.

For testing the specificity of the human EPO enhancer by supershift experiments, 1 µl of polyclonal antibody (against the N-terminus of rainbow trout HIF-1α, see above) was added to the EMSA reaction mixture with liver extracts from *Z. viviparus*. In the presence of the antibody, the EMSA signal was markedly reduced, indicating that antibody interaction with the HIF-1α subunit minimised binding of the HIF-1 dimer to the EPO probe (Fig. 2.D). This is explained by the DNA binding function also being located in the

N-terminus of the HIF-1α protein (e.g. Chi & Karliner 2004). To date, there is no antibody available which recognises the HIF-1α protein in liver samples from *P. brachycephalum*, therefore the supershift assay could not be carried out for the Antarctic eelpout. The specificity of the observed band has been determined in three fish species in the laboratory of M. Nikinmaa (see also Soitamo et al 2001). Further, the signal of the complex band appeared at the same location as found in North Sea eelpout, indicating the band to have the molecular size of the HIF-1-EPO enhancer complex.

Statistics

All values are given as means ± standard deviation. Differences between experimental groups were analysed by student's t-test using Statview 5.0. Significances were evaluated based on the p-level < 0.05.

Results & Discussion

We investigated the response of oxidative stress parameters in the two zoarcid fish species to the warm and cold exposure at the borders of their optimum temperature range. Although water temperatures in Antarctica never reach 5°C, even a ten months acclimation at this temperature did not lead to reduced growth, nor any differences in physiological condition factors in 5°C acclimated *Pachycara brachycephalum* as reported by Lannig et al. (MS submitted). Consequently, we anticipated metabolic regulation to compensate the temperature change.

Interspecies comparison at low temperature of maintenance

Table 1 depicts measurements of the content of total and of "free" iron in whole tissue homogenates as well as α-tocopherol content and lipid radical generation in liver microsomal isolates. Iron was measured as a transition metal catalyst of free radical formation in liver tissues. The amount of "free" iron, including all iron (Fe(II) and Fe(III)), not bound to enzymes or iron storage proteins, represents catalytically available iron. Binding of iron to ferritin and transferrin is one of the most powerful antioxidant mechanisms in living tissues, as it prevents the iron from acting as Fenton catalyst by generating highly toxic hydroxyl radicals ($^{\cdot}OH$) (Halliwell & Gutteridge 1985). The concentrations of total and of "free" iron were lower in the polar, compared to the

temperate fish. However, a greater proportion of iron in polar eelpout liver was "free" (ratio "free"/total iron 0.59 in *P. brachycephalum* and 0.22 in *Z. viviparus*, tab. 1) and thus available for Fenton kinetics and contributing to lipid radical formation in polar eelpout microsomes (fig. 1B, C). Like other Antarctic fish, *P. brachycephalum* is characterised by low amounts of red blood cells (Mark et al. 2002) and, therefore, may have only a reduced capacity to control "free" iron via ferritin and transferrin binding.

Iron reduction rate, enzymatic and non-enzymatic conversion of Fe(III) to the more powerful oxidising agent Fe(II), was similar in liver homogenates from both fishes. A doubling of iron reduction rate (measured at 20°C) occurred in summer eelpout from the North Sea, as compared to winter activities (24 ± 6 in summer and 11 ± 5 µmol * g fresh weight^{-1} * min^{-1} in winter; p = 0.04). This difference suggests a more intense and presumably enzymatic (and therefore thermally increased) iron metabolism in *Z. viviparus*, to maintain higher turnover of heme proteins in more active summer animals. Contrary to *Z. viviparus*, acclimation of polar eelpout to higher temperatures had no inducing effect on iron reductase activity (10 ± 4 at 0°C and 12 ± 1 µmol * g fresh weight^{-1} * min^{-1} for *P. brachycephalum* at 5°C, p = 0.46), speaking for primarily non-enzymatic catalysis.

As lipid radical EPR signals in liver homogenates were abolished by high cytosolic antioxidant potential, only the microsomal fraction was used in the assay. As individual liver samples did not yield sufficient sample volume, material had to be pooled from 2 or 3 liver samples. Lipid radicals combined with the spin trap POBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of a^N = 15.8 G and a^N = 2.6 G, in agreement with computer spectral simulated signals obtained using the same parameters (fig. 1D). Even though these constants could be assigned to lipid radicals, spin trapping studies cannot readily distinguish between peroxyl (ROO·), alkoxyl (RO·) and alkyl (R·) adducts, owing to the similarity of the corresponding coupling constants (Buettner 1987). EPR signals of lipid radical formation were 4-fold higher in the polar eelpout (fig. 1B, C and tab. 2), indicating elevated susceptibility of polar membrane lipids to ROS attack, although the content of the lipid soluble antioxidant α-tocopherol was also 2-fold higher in the polar eelpout microsomal fraction compared to the temperate species. Elevated sensitivity of polar fish membranes to ROS attack has already been documented by Giardina et al. (1997), Gieseg et al. (2000), Dunlap et al. (2002). This is in agreement with a higher content of unsaturated fatty acids in polar eelpout (E. Brodte, pers. com), which are the main targets of oxidative damage in membranes (Slater 1984), but necessary for homoeoviscous adaptation at low temperatures (Sidell 1998).

Table 2 summarizes the rate of chemiluminescence which results from the imbalance between pro- and antioxidant processes, and the oxidative damage markers TBARS and protein carbonyls, reflecting the level of ROS production in the liver. Moreover, the total content of nitrite and nitrate (Σ NO_2+ NO_3) is given as a measure for NO production. SOD activities in liver tissues, presented in tab. 2, were measured at 20°C and also at the respective water temperature for each group. The table also gives the glutathione peroxidase activity, measured at 20°C, and the α-tocopherol content in liver homogenates.

Comparing both animals at low habitat temperature, the polar *P. brachycephalum* at 0°C and winter acclimatised *Z. viviparus* (6°C), we found less indication for lipid peroxidation (TBARS, $p < 0.01$) and chemiluminescence ($p = 0.04$) in the polar eelpout. SOD activities per g fresh weight were also lower in *P. brachycephalum* at standard assay temperature (20°C, $p = 0.02$), but similar ($p = 0.59$) when assayed at the respective water temperature (6°C for *Z. viviparus* and 0°C for *P. brachycephalum*), indicating the same superoxide scavenging capacity prevailed in both fishes under *in situ* conditions. In line with this, Natoli et al. (1990) found that Cu,ZnSOD enzyme properties in Antarctic icefish (*Chaenocephalus aceratus*) allow for efficient superoxide scavenging at low water temperature.

As already shown for isolated microsomes, liver homogenates of the Antarctic eelpout contained higher α-tocopherol levels compared to the North Sea species ($n = 2$; no statistical evaluation). α-tocopherol concentrations were in the same range as reported from several notothenioid species (30 – 1000 nmol * mg protein^{-1}, Ansaldo et al. 2000).

Interestingly, the polar fish maintained 3-fold higher tissue concentrations of the redox buffer glutathione ($p < 0.01$), which was to over 50 % oxidised to GSSG (tab. 3). Although again, the difference in glutathione redox ratio is not significant between the two species ($p = 0.13$), it seems important in the context that liver tissue of the polar eelpout appeared generally more oxidised. To our knowledge GSSG is usually lower than GSH content (e.g. Ritola et al. 1999; Pena et al. 2000 for other fish species). Moreover, under oxidative stress causing GSH oxidation, GSSG is exported to maintain a constant cellular redox environment (Schafer & Buettner 2001) and avoid adverse side effects of GSSG accumulation (Klatt & Lamas 2000). The intracellular glutathione concentration is much higher than the concentration of other thiols, which makes it the most important determinant of the cellular redox environment (Schafer & Buettner 2001). According to these authors, not only the glutathione redox ratio, but also the quantity of reduced GSH,

which represents the cellular reducing capacity, impacts cellular redox potential because of the square term in the Nernst equation:

$$\Delta E = E^{\circ pH} - (RT*2.303/nF) \log ([GSH]^2/[GSSG]) \text{ mV} \qquad (I)$$

We calculated the redox potential (ΔE) for both eelpout species (tab. 3) according to equation I at *in situ* temperature of each group taking into account the temperature dependent changes of intracellular pH (pH_i). These have been determined for North Sea and Antarctic eelpout white muscle by Sartoris et al. (2003). Liver pH_i was approximated, assuming it to range about 0.28 pH units lower than in the white muscle (Larsen et al. 1997). Calculated redox potentials in tab. 3 are in line with the glutathione redox ratio, indicating a similar redox milieu in both species at low water temperature.

Taken together, our data speak for the need of high antioxidant potential and redox buffering in polar eelpout and are in line with results from Giardina et al. (1997), who demonstrated Antarctic fishes to maintain higher levels of antioxidant defences than Mediterranean species. Similar enzyme activities (SOD and GPX), but higher levels of glutathione and α-tocopherol (n.s.) in Antarctic *vs*. North Sea eelpout, illustrate the importance, especially of the non-enzymatic antioxidant defence for this polar fish. In agreement with this, Dunlap et al. (2002) detected a marine-derived tocopherol (MDT) constituent in Antarctic notothenioids and krill, which confer special antioxidant protection of cellular lipids at low temperatures.

Σ NO_2+ NO_3 (tab. 1) was about 5-times higher ($p < 0.01$) in North Sea than in polar eelpout. This finding provides a first hint that higher NO production rates may occur in temperate than polar fish, whereas the physiological consequences this may have on metabolic regulation in fish cells still wait to be investigated.

Response to warmer water temperatures

Acclimation of *P. brachycephalum* to 5°C and acclimatisation of *Z. viviparus* to summer temperatures (12°C) had distinct effects in both fishes. North Sea eelpout caught during summer had lower SOD (measured at 12 and 20°C), but higher glutathione peroxidase (GPX) activities (measured at 20°C), than winter animals. Earlier work by Ronisz et al. (1999) also reported slightly elevated GPX activities in *Z. viviparus* caught in June or August *vs.* February. α-tocopherol levels in summer animals (n = 4) were more than two times higher as levels in only two winter *Z. viviparus* individuals (no statistical evaluation), and chemiluminescence rates ($p = 0.42$, $n = 5$), as well as lipid ($p = 0.03$, $n = 4$) and protein ($p = 0.04$, $n = 4$) oxidation markers were correspondingly decreased. Moreover, due to

significantly lower GSSG concentrations ($p < 0.01$) at unchanged GSH levels ($p = 0.69$), the glutathione redox ratio was lower ($p < 0.01$) and the Nernst potential (ΔE) more negative ($p = 0.02$) in summer fish. This indicates more reduced redox milieu at warmer water temperature in North Sea eelpout liver.

A different picture was found in polar eelpout, acclimated to 5°C. Oxidative damage parameters increased on warming however, significantly only for TBARS (TBARS $p = 0.03$, $n = 5$; chemiluminescence $p = 0.34$, $n = 5$-7, protein carbonyls $p = 0.11$, $n = 4$-5). This again underlines the high ROS damage sensitivity of polar fish fatty acids. Low molecular antioxidants (α-tocopherol and glutathione-related parameters, tab. 2, 3) were not significantly different in warm acclimated fish, compared to animals maintained at 0°C. Together with constant SOD and elevated GPX activity ($p = 0.03$) at 5°C, this supports the view that 5°C is still not critical for the animals, because they maintain or even induce antioxidant functions. In contrast, exposure of stenothermal Antarctic invertebrates to critically high temperatures caused impairment of SOD activity and onset of oxidative stress (Abele et al. 1998: *Nacella concinna*; Abele et al. 2001: *Yoldia eightsii*).

Taken together, higher levels of oxidative damage (tab. 2) and a more oxidised glutathione redox ratio (tab. 3) are indicative of elevated levels of ROS production in winter acclimatised North Sea eelpout compared to summer animals. In contrast, warm acclimation of the polar eelpout had less clear effects on oxidative stress parameters, with the exception of significantly increased TBARS concentrations again demonstrating elevated susceptibility of polar fish for lipid peroxidation.

Western blotting with an antibody against the N-terminus of rainbow trout HIF-1α confirmed the presence of this protein in North Sea eelpout, but did not reveal seasonal changes in protein amount (fig. 2.A). Therefore, our prime finding is that the HIF-1α protein is permanently present in unstressed North Sea eelpout. As the antibody did not recognise *P. brachycephalum* HIF-1α, protein quantification could not be performed for the polar eelpout. Although preliminary results of the HIF-1α sequence indicate high conservation (~98% on the protein level) between the two eelpout species (Heise, Lucassen, Abele, in prep.), low environmental temperatures may cause different HIF-1α protein folding (see Fields & Somero 1998) in the Antarctic eelpout, preventing antibody-HIF-1α interaction in *P. brachycephalum*. Results from the electromobility shift assay (EMSA) for DNA binding activity of hypoxia inducible transcription factor (HIF-1) with

the human EPO (erythropoietin) enhancer are presented in fig. 2 (fig. 2B: mean values ± standard deviation, n = 5; fig. 2C: EMSA example).

Higher HIF-1 DNA binding activity was found at the higher temperature in the polar and at the lower temperature in the North Sea eelpout (fig. 2.B and 2.C) and is interpreted as a tissue entering a state of beginning oxygen deficiency. Obviously, in eelpout, HIF-1 function is not regulated on the level of protein expression and stabilisation of the α-subunit, but during later steps of the signalling pathway. Besides the cellular oxygenation status, cellular redox state can also affect both, the stability and the DNA binding of HIF-1 (Ema et al. 1999, Haddad et al. 2000, Lando et al. 2000 for mammals, Nikinmaa et al. 2004 for rainbow trout). Thus, also ROS formation is likely to be an important HIF-1α modulating signal (Fandrey 2004).

HIF–1 DNA binding correlates with increased oxidative damage, especially in Z. viviparus.

Our data set represents a first approach to study HIF-1 DNA-binding in the liver of whole animal ectotherms, exposed to temperatures at the border of their thermal optimum range, and directly relate this to tissue oxidation and redox potential in the liver of the living fish. Thus, our approach reflects a co-ordinated physiological response at the whole animal level and, importantly, allows tissue oxygenation to change on a natural time scale. This provides new insight into HIF-1 regulation at above cellular level. Elevated mitochondrial ROS production, elicited by subcritical warming to temperatures above natural, is presumably responsible for the accumulation of TBARS in polar eelpout at 5°C (tab. 2). Likewise, accumulation of oxidative damage markers and enhanced glutathione oxidation in North Sea eelpout at 6°C (tab. 2, 3) indicate higher ROS levels at decreased cellular antioxidant capacity (GPX and α-tocopherol).

Induction of HIF-1, going together with an increase of cellular ROS producing activity, appears like an absolute paradox. Previous models described HIF-1α as controlled by ROS produced by membrane bound NADPH oxido-reductases (Fandrey et al. 1994). Rapid progress in the field of cellular oxygen sensing demonstrated that HIF-1α destabilization involves prolyl hydroxylases controlled by po_2 and not by ROS (Jaakkola et al. 2001). However, until to date, reactive oxygen species involvement in HIF-1 redox regulation is subject to controversial discussions (Fandrey 2004). A second model sees cellular ROS production promoted under hypoxic conditions (Chandel & Schumacker 1999; Chandel et al. 2000; reviewed in Chandel & Schumacker 2000). According to this view, mitochondria

become ROS producers under hypoxic conditions that cause redox changes of electron transport components especially complex III (ubiquinone), and a reduction of cytochrome c oxidase activity. Chandel et al. (1998) found hypoxic transcription of HIF-1 target genes (Northern Blot) to be sensitive to antioxidants. Conflicting views on HIF-1 regulation may result from the use of different cell models from oxygen sensing carotid body cells to hepatoma cells.

Soitamo et al. (2001) carried out Western blotting of HIF-1α in isolated fish hepatocytes at different oxygen concentrations. They found HIF-1α to be stabilised at po_2 between 10% (76 torr) and below 5% (38 torr), whereas a severe drop of HIF-1α stability occurred only under severe hypoxia (1.5 torr). They concluded that in chinook salmon and rainbow trout HIF-1 forms the stable dimeric protein already at physiologically relevant (venous po_2) oxygen partial pressure. Consequently, the authors proposed that oxygen dependent gene expression, involving HIF-1α stabilisation, can occur already under "more or less normoxic conditions" in fish, i.e. at a po_2 close to the optimal range of tissue oxygenation. Our data of temperate eelpout heavily support this view (fig. 2.A). This is distinct to mammalian cell models, where HIF-1α stabilises only under strictly hypoxic conditions and the levels decline within minutes upon reoxygenation (Wang et al. 1995, Fandrey 2004). Although the hypoxia responsive element of the oxygen dependent degradation domain (ODD) of rainbow trout HIF-1α was identical to the respective sequences of mammals and amphibians (Soitamo et al. 2001), four additional cysteine residues near the transactivation domain and the ODD in rainbow trout HIF-1α were discussed to alter the oxygen dependent regulation of the fish HIF-1α protein (Nikinmaa 2002).

The hypothetical role of HIF-1 for the response of zoarcid fishes to changing environmental temperatures

For North Sea winter fish, 6°C is no stress temperature, but well within the temperature range experienced in nature (Fonds et al. 1989; Zakhartsev et al. 2003). However, cold winter temperatures induce diffusive slow down, compromising oxygen delivery to central tissue regions as suggested by Pörtner (2002). Increased DNA-binding of the HIF-1 dimer at 6°C, compared to summer fish, may be a common feature of physiological reorganisation in eurythermal fish, to improve oxygen supply via EPO and VEGF induction. This could counterbalance the cold-induced oxygen limitation before onset of severe functional hypoxia, supposed to occur at critical temperatures. Moreover, it may

prepare the animals to meet metabolic requirements during winter. Seasonal cold acclimatisation was, in fact, found to cause cold-induced increase in capillary density of carp muscle (Johnston 1982, Egginton & Sidell 1989), associated with a rise in aerobic capacity via mitochondrial proliferation in several fish species (Johnston 1982; Egginton & Sidell 1989; Guderley & St-Pierre 2002 for review). HIF-1 DNA-binding in winter eelpout, therefore, seems perfectly in line with the observed trends in other fish species, to improve tissue oxygen delivery in the cold, provided it acts on the same array of target genes as in mammalian tissues.

Quite another question is what happens on warming of fish as performed here with the polar *P. brachycephalum* that also caused HIF-1 to bind to the EPO-enhancer fragment? Cellular oxygen consumption in ectotherms increases with temperature. In other fish this causes a decrease in venous oxygen partial pressure, as shown for cod (Lannig et al. 2004). In the polar eelpout HIF-1 DNA-binding occurred at 5°C, close to the temperature where blood flow levels off (7°C, Mark et al. 2002), indicating that the high border (pejus levels) of the optimal temperature range was almost reached. Further increase of oxygen consumption rates at even higher temperatures will then, especially in the liver, cause an imbalance between oxygen supply and demand as modelled by Pörtner (2002), i.e. functional hypoxia. Again HIF-1 likely supports oxygen homeostasis as the fishes approach critical conditions. In so doing it might represent the first step to initiate physiological adjustments in the polar species during acclimation at above habitat temperature. Prolonged acclimation of *P. brachycephalum* over 10 months at 5°C under laboratory conditions by Lannig et al. (MS submitted), caused a reduction of mitochondrial aerobic capacity in the liver thereby economizing mitochondrial respiratory efficiency. As thermal adjustability is typical for eurythermal ectotherms (Pörtner 2001), Lannig et al. (MS submitted) concluded, that the Antarctic eelpout is not as typically cold stenothermal as supposed of a polar animal.

In summary, we assume that changing environmental temperatures cause higher levels of ROS stress, which may go along with functional hypoxia in both eelpout species, indicated by onset of hypoxic signalling. This can be observed under natural winter conditions in North Sea eelpout, while in a related polar eelpout species, a similar response can be detected under experimental warm acclimation.

Further, Treinin et al. (2003) found increased HIF-1α levels on acclimation of *C. elegans* to higher temperatures, which, obviously, conferred resistance to subsequent exposure to heat stress. These authors developed a concept of “cross-tolerance” in which acclimation to

one stress factor (mild warming) confers protection against also other types of stress. Thus heat exposure may render animals (including mammals) less susceptible to cadmium exposure (Treinin et al. 2003 for *C. elegans*) or to ischemia / reperfusion injury (Horowitz et al. 2004), and HIF-1 seems to be an essential modulator in cross-tolerance requirement.

In the light of these findings, one would hypothesise that functional hypoxia could be a more common phenomenon, accompanying many more forms of environmental stress, than we may currently assume. Thus, HIF-1α certainly has a major function in governing re-regulation under severe hypoxia, but may also be a cross-tolerance factor, induced under many forms of stress that may lead to a functional hypoxia.

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Tables

Table 1

compares α-tocopherol content and lipid radical formation in liver microsomes as well as availability of iron (total iron, total "free" iron detected by EPR, ratio "free" iron/ total iron and iron reduction rate Fe(III) to Fe(II)) in liver homogenates of the polar eelpout *Pachycara brachycephalum* (Pb) and the temperate eelpout *Zoarces viviparus* (Zv). # significant difference of the two species compared at the low water temperature, n.s. = not significant, - no statistical evaluation, as values were obtained from pooled samples in microsomal isolates or calculated from mean values (ratio "free" / total iron), $p < 0.05$, n = 3-5, n = 2 for "free" iron of Zv 6°C. FW = fresh weight.

	P. brachycephalum **(0°C)**	*Z. viviparus* **(6°C)**	Significance $p < 0.05$
α-tocopherol in microsomes (μmol * mg protein^{-1})	0.76	0.27	-
lipid radicals in microsomes (pmol *mg protein^{-1} * min^{-1})	0.54	0.11	-
total iron content (nmol * g FW^{-1})	37 ± 10	150 ± 20	#
total intracellular "free" iron (nmol * g FW^{-1})	22 ± 4	34 ± 7	n.s.
ratio of "free" iron / total iron	0.59	0.22	-
iron reduction rate (μmol * g FW^{-1} * min^{-1})	10 ± 4	11 ± 5	n.s.

Table 2

summarizes oxidative damage marker (thiobarbituric reactive substances (TBARS), chemiluminescence, carbonyl content), total nitrite + nitrate content and antioxidant defence (superoxide dismutase (SOD), glutathione peroxidase, α-tocopherol) in liver samples from the polar eelpout *Pachycara brachycephalum* (Pb) acclimated to 0 and 5°C as well as the temperate eelpout *Zoarces viviparus* (Zv) acclimatised to 6 and 12°C. Means ± standard deviation, n = 4 - 7, n = 2 for α-tocopherol of Zv 6°C. Interspecies comparison: # significant difference between the two species at the respective lower water temperature, intraspecies comparison: * significantly different to the values at the higher water temperature, n.s. = not significant, - no statistical evaluation because n = 2, $p < 0.05$. FW = fresh weight.

	P. brachycephalum **(0°C)**	*P. brachycephalum* **(5°C)**	*Z. viviparus* **(6°C)**	*Z. viviparus* **(12°C)**	Sign. difference Pb 0°C *vs.* Zv 6°C
TBARS (µmol * g FW^{-1})	0.17 ± 0.05 (*)	0.26 ± 0.05	0.6 ± 0.1 (*)	0.4 ± 0.1	#
chemiluminescence rate (cpm^2 * mg $protein^{-1}$)	0.3 ± 0.1 (n.s.)	0.4 ± 0.3	1.1 ± 0.8 (*)	0.6 ± 0.3	#
carbonyl content (nmol * mg $protein^{-1}$)	4 ± 2 (n.s.)	6 ± 2	3.2 ± 0.4 (n.s.)	2.2 ± 0.7	n.s.
total (nitrite+nitrate) content (µmol * mg $protein^{-1}$)	0.03 ± 0.01 (n.s.)	0.047 ± 0.007	0.17 ± 0.06 (n.s.)	0.2 ± 0.1	#
SOD activity (20°C) (U * g FW^{-1})	2200 ± 800 (n.s.)	2400 ± 200	3400 ± 500 (*)	2000 ± 700	#
SOD activity (water temperature) (U * g FW^{-1})	2000 ± 400 (n.s.)	2100 ± 400	2000 ± 1000 (n.s.)	1800 ± 500	n.s.
glutathione peroxidase (20°C) (U * g FW^{-1})	1.6 ± 0.5 (*)	2.6 ± 0.7	1.6 ± 0.6 (*)	4 ± 2	n.s.
α-tocopherol (µmol * g FW^{-1})	300 ± 200 (n.s.)	500 ± 100	93 (-)	240 ± 80	-

Table 3

depicts glutathione redox parameters: the contents of reduced (GSH) and oxidised glutathione (GSSG), total glutathione content and the ratio of oxidised to reduced glutathione. The redox potential (ΔE) was calculated at respective water temperature and *in situ* intracellular pH (pH_i) calculated by the Nernst equation as described in Schafer & Buettner (2001). Changes of pH_i with temperature were elaborated for muscle tissue by Sartoris et al. (2003) and corrected for lower liver pH_i values by 0.28 pH units (Larsen et al. 1997). Values are given for liver tissue of the polar eelpout *Pachycara brachycephalum* (Pb) acclimated to 0 and 5°C as well as the temperate eelpout *Zoarces viviparus* (Zv) acclimatised to 6 and 12°C. Means ± standard deviation, n = 4 - 7. Interspecies comparison: # significant difference between the two species at the respective lower water temperature, intraspecies comparison: * significantly different to the values at the higher water temperature, n.s. = not significant, - no statistical evaluation, $p < 0.05$. FW = fresh weight.

	P. brachycephalum **(0°C)**	*P. brachycephalum* **(5°C)**	*Z. viviparus* **(6°C)**	*Z. viviparus* **(12°C)**	Sign. difference Pb 0°C *vs.* Zv 6°C
GSH (µmol * g FW^{-1})	2.1 ± 0.5 (n.s.)	2.4 ± 0.3	1.0 ± 0.4 (n.s.)	0.9 ± 0.3	#
GSSG (µmol * g FW^{-1})	3.1 ± 1.5 (n.s.)	2.9 ± 0.8	0.8 ± 0.2 (*)	0.16 ± 0.05	#
total glutathione (GSH + 2GSSG) (µmol * g FW^{-1})	8 ± 2 (n.s.)	9 ± 3	2.5 ± 0.5 (*)	1.2 ± 0.3	#
(2GSSG)/GSH	2.7 ± 0.9 (n.s.)	3 ± 1	1.8 ± 0.9 (*)	0.36 ± 0.041	n.s.
Redox potential ΔE (mV)	-253 ± 5 (n.s.)	-252 ± 8	-246 ± 11 (*)	-258 ± 4	n.s.
pHi	7.140	7.065	7.072	6.970	-

Figures

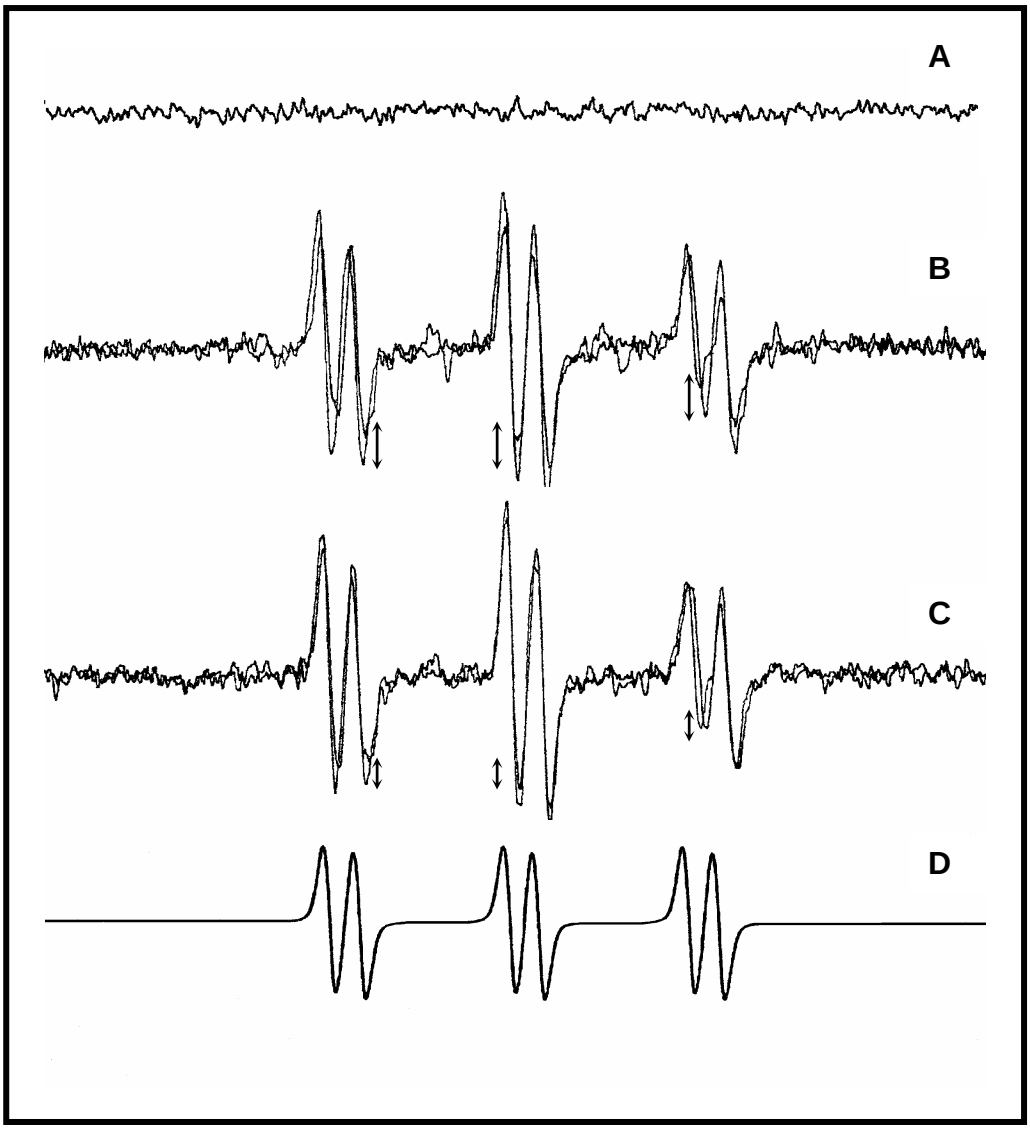

Figure 1

EPR detection of lipid radical formation by liver microsomal extracts. 3.A shows POBN spectra. 3.B Overlay of lipid radical generation at t = 0 min and t = 30 min in the polar eelpout *Pachycara brachycephalum*. 3.C Overlay of lipid radical generation at t = 0 min and t = 30 min in the temperate eelpout *Zoarces viviparus*. 3.D Depicts computer simulated spectra of lipid radicals. Arrows indicate the higher lipid radical generation in the polar eelpout upon 30 min incubation compared to the temperate eelpout.

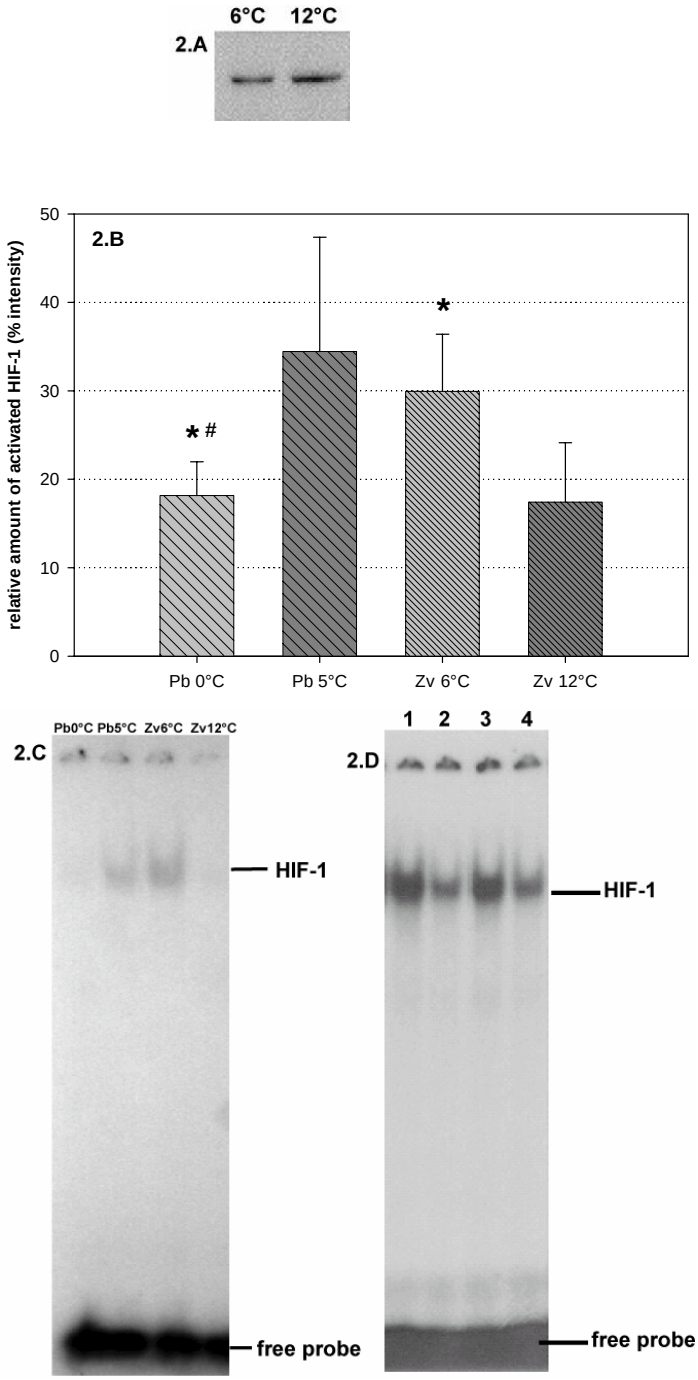

Figure 2

HIF-1: 2.A Western blotting analysis of HIF-1α in liver whole cell extracts from the temperate eelpout *Zoarces viviparus* (Zv) acclimatised to 6 and 12°C. 2.B: HIF-1 DNA binding activity to the human EPO enhancer in electromobility shift assays with liver whole cell extracts from the polar eelpout *Pachycara brachycephalum* (Pb) acclimated to 0 and 5°C as well as the temperate eelpout *Zoarces viviparus* (Zv) acclimatised to 6 and 12°C. Means ± standard deviation, n = 5. % intensity was based on densitometry of gel images, with the sum of all 4 bands taken as 100 % value. Interspecies comparison: # significant difference between the two species at respective lower water temperature, intraspecies comparison: * significantly different from the values at the higher water temperature, $p < 0.05$. 2.C: Example of EMSA. 2.D: Addition of polyclonal antibody directed against the N-terminus of rainbow trout HIF-1α to the reaction mixture in lane 2 and 4 diminished the EMSA signal in liver whole cell extracts from *Z. viviparus* compared to reaction mixtures without antibody (lane 1 and 3) indicating that the fish HIF-1 bound to the human EPO enhancer.

Publication II:
BIOCHIMICA ET BIOPHYSICA ACTA –
GENE STRUCTURE AND EXPRESSION

The hypoxia inducible factor (HIF-1α) in cold-adapted Antarctic fishes

Rapid report

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Summary

Hypoxia inducible factor 1α sequences from one temperate and four cold-adapted Antarctic fishes were determined with remarkable differences in the deduced peptide sequences compared with mammals or other fishes. One functional proline in the oxygen dependent degradation domain of the Antarctic zoarcid was substituted by leucine. Sequences are compared and their implication for cold-adaptation discussed.

Key words:
Hypoxia inducible factor, cold-adaptation, Antarctic fish, prolyl hydroxylation

Antarctic fish have evolved into a highly cold-adapted phenotype (1), including a variety of adaptations of the O_2-transport system (2, 3, 4). High O_2 solubility in aqueous media at low temperatures presumably supports the low hematocrits found in Antarctic notothenioids and zoarcids and the complete absence of red blood cells in icefish (5, 6). We hypothesize that cold adaptation moreover implicates modifications of O_2-dependent gene expression. In mammals the transcription factor HIF-1 (hypoxia inducible factor) activates target genes involved in erythropoiesis, angiogenesis, and glucose metabolism (reviewed in 7, 8) and is seen as the key regulator of O_2 homeostasis (9). The O_2 regulated subunit HIF-1α is constitutively expressed, but rapidly degraded under normoxic conditions (10). Prolyl hydroxylases (PHD) use molecular O_2 for hydroxylation of Pro402 and Pro564 in human HIF-1α, which are subsequently recognized by pVHL (von Hippel Lindau Tumor suppressor protein) enabling HIF-1α proteasomal degradation (11, 12, 13). Under O_2 deprivation, proline hydroxylation is prevented, HIF-1α protein escapes proteolytic degradation, and translocates to the nucleus (10). Following dimerization with HIF-β, the HIF-1 dimer activates target genes containing the hypoxia responsive element (HRE; 14, 15). Both HIF-1 subunits belong to the basic-helix-loop-helix-PAS family of proteins (16). The C-terminal half of HIF-1α contains the oxygen dependent degradation domain (ODD) including the two proline residues and the N-terminal transactivation domain (N-TAD). The C-terminal TAD is regulated mainly by normoxic hydroxylation of Asn803 in human HIF-1α, preventing its interaction with transcriptional co-activators (17).

This study compares the HIF-1α sequences of the common eelpout *Zoarces viviparus* (*Zv*; Zoarcidae), caught in the German North Sea, to the con-familial Antarctic eelpout *Pachycara brachycephalum* (*Pb*), caught during an RV Polarstern cruise near King George Island (Antarctic peninsula). Moreover, zoarcid HIF-1α was compared to three notothenioid species (*Trematomus hansoni, Th; T. pennellii, Tp;* and the icefish species *Chionodraco myersi, Cm*), which were caught in the Antarctic Weddell Sea. Fish sampling, mRNA isolation from liver, RT-PCR, cloning and sequencing of partial HIF-1α cDNA sequences were carried out as described by Lucassen et al. (18). Full-length cDNA sequences for both eelpout were obtained by amplification of overlapping fragments and rapid amplification of cDNA ends (RACE; 19), using the primers listed in table 1. Taq-Polymerase (Eppendorf, Hamburg, Germany) was used for amplification of fragments <1000 bp and SuperTaqPlus-Polymerase (Ambion, Austin, USA) for fragments >1000 bp. A standard PCR protocol was used with 35 cycles of 45 s denaturation at 94°C, annealing 80 s with a 12°C gradient (58±6°C), and 80 s elongation at 72°C, followed by a final

elongation step of 8 min at 72°C. 3`RACE-PCR and 5`RACE-PCR reactions with *P. brachycephalum* samples were carried out with annealing at 61±6°C and 62±6°C, respectively. The 1175 bp fragments (see tab.1) of *P. brachycephalum* and notothenioid samples were amplified using a touch-down program with 2 min annealing at temperatures from 60±6 to 51±6°C (-1°C/cycle), followed by 25 cycles with 1 min annealing at 57±6°C. MacVector 7.2 was used for primer design, assemblage and sequence analysis.

We identified full length HIF-1α nucleotide sequences of the zoarcids with sizes of 3383 (*Z. viviparus*, fig.1) and 3283 (*P. brachycephalum*) base pairs and an open reading frame encoding 747 amino acids for both fishes (Genbank submission in progress). The nucleotide sequences showed 96% identity between the two eelpout species, 41% to human (*Hs*) and 63% identity to rainbow trout (*Om*) HIF-1α. The deduced amino acid sequences had 96% identity between the eelpout species, 50% identity to human, 71% to rainbow trout sequences. Overall, the N-terminal half of the protein containing the bHLH-PAS domains involved in dimerization and DNA binding proved highly conserved between eelpout species (99% identity; not shown), whereas the C-terminal half containing ODD and C-TAD was less conserved (94% identity, fig.2).

For comparison of different cold-adapted species we characterized partial cDNA sequences covering the C-terminal half of HIF-1α from Antarctic notothenioids (Genbank submission in progress). High conservation of the deduced amino acid sequences (amino acids 343-737 of *Zv*HIF-1α) could be found between zoarcids and notothenioids with identities of 75-77% and 82-84% similarities (fig.2). The derived partial amino acid sequences of the marine fishes clustered together with the partial rainbow trout sequence (58-60% identity, 76% similarity), with all six species belonging to the order of perciformes (fig.3). Lower conservation was found when comparing to the cyprinids, order cypriniformes (44-45% identity, 58-60% similarity).

Characterization of rainbow trout HIF-1α demonstrated that the degradation/stabilization mechanism is essentially the same in fish and mammals, but stabilization occurred at much higher O_2 levels in fish (20). This was ascribed to four redox-sensitive cysteine residues in the ODD of rainbow trout HIF-1α in contrast to only one cysteine in the human sequence (21). Four cysteine residues were also identified in the ODD of both zoarcid sequences (position of *Zv*HIF-1α: 442, 496, 512, 578; fig.2). Similarly, the *Trematomus* species (*Tp, Th*) exhibited two and the icefish (*Cm*) three cysteine residues in the ODD (fig.2). Moreover, the notothenioids displayed two additional cysteines in or next to the C-TAD, possibly leading to changes in the redox-sensitivity of the transactivation mechanism (22).

In conjunction with the detection of normoxic HIF-1α protein levels in the liver of *Zoarces viviparus* (23), this supports the idea that O_2-regulated gene expression might play a role in the normal fish physiology accomplished by different redox characteristics of HIF-1α (21, 24, 20). Further, for rainbow trout HIF-1 redox sensitivity of DNA binding conferred by Cys25 (Ser28 in human HIF-1α) has been reported (21). Although we found increased HIF-1 DNA binding in the liver of temperature-stressed *Z. viviparus* occurring at more reduced cellular redox environment (25), both zoarcid sequences have serine at the respective position (not shown).

Surprisingly, the N-terminal (Pro402 in *Hs*HIF-1α) of the two functional proline residues (7) was substituted by leucine in the Antarctic eelpout (*Pb,* fig.2), which is to our knowledge, the first report of this phenotype. Both prolines can be independently recognized by the PHDs (13, 26). Moreover, mutation of both proline residues and the asparagine residue was required to render HIF-1α fully active at normoxia (17). It is conceivable that substitution of one functional proline in *Pb*HIF-1α still allows for hydroxylation of the other conserved residue, and subsequent pVHL mediated proteasomal degradation. On the other hand, mutation of Pro564Ala abrogated pVHL binding activity, and full length *Hs*HIF-1α Pro564Ala proved more stable than wildtype HIF-1α in degradation assays (11). Hence, *Pb*HIF-1α can be expected to be even more inert to PHD action than *Zv*HIF-1α, which conserved both functional prolines. Multiplicity of the hydroxylation sites of HIF-1α is supposed to allow a more complex response to oxygen availability (13). Taken together, these considerations suggest a reduced flexibility of the hypoxic response via the degradation/ stabilization mechanism of HIF-1α in the Antarctic zoarcid. However, we found increased HIF-1 DNA binding in *P. brachycephalum* acclimated to 5°C, supposed to induce temperature dependent O_2 limitation in this species (6, 27), compared to control fish kept at 0°C (23). Thus, despite the proline substitution, HIF-1 might still be functional, at least on the level of dimerization and DNA binding. It has been suggested that the regulation of transcriptional activity is likely the crucial step under most physiological conditions (7) and Asn803 hydroxylation should abolish transactivation of HIF-1 in normoxia, if HIF-1α escapes the degradation process (26). Thus, *Pb*HIF-1 function may be regulated mainly via transactivation. In this context, the Pro-Leu conversion within that anyway leucine-rich site LXLLXL (fig.2) could improve the interaction of HIF-1α with transcriptional co-activators, as leucine-rich regions are important for the interactions of p300 with several transcription factors including HIF-2α (28).

Reduced numbers of proline residues represent one possibility of protein adaptation to cold temperatures in that they increase the structural flexibility of the molecule at low temperature (29). However, amino acid substitutions for cold adaptation of a protein are not expected in functional domains but in between those protein regions (30, 31). To clarify this point, we sequenced the C-terminal part of the HIF-1α gene from two red-blooded notothenioids, *T. hansoni*, *T. pennellii* and also from the hemoglobinless icefish, *C. myersi*, and detected both functional prolines in all three notothenioid HIF-1α fragments (fig.2). Hence, the substitution found in *P. brachycephalum* is not primarily related to cold adaptation of O_2-dependent gene expression in polar fish. In addition to higher structural flexibility by a reduced number of proline residues, amino acid substitutions favouring solvent interactions with a hydrophilic surface were observed in cold adapted proteins (32, 29, 33). Of course, the location of the substituted residue in the folded protein is highly important for its influence on protein structural properties. As no data for the HIF-1α tertiary structure are available, only preliminary assumptions on the impact of amino acid substitutions on HIF-1α cold adaptation are possible. When comparing the deduced amino acid sequences only of the two zoarcids, differences supporting the general trend for cold adaptation of *Pb*HIF-1α can be found as well as others opposed to this trend. However, outlining all differences between *Zv* and *Pb*HIF-1α which are conserved in all cold-adapted proteins, revealed that all substitutions found in the ODD and C-TAD (Ala445Ser, Trp495Arg/Thr, Ile543Thr, Gly702Ser/Asn; positions on *Zv*HIF-1α; indicated by green stars in fig.2) may support higher hydrophilicity and, therewith, cold adaptation. Few substitutions supposed to reduce protein flexibility (Ser606Pro, His609Arg; indicated by orange stars in fig.2), were not located within the functional sites.

Moreover, the alignment (fig.2) revealed another interesting feature. Whereas human HIF-1α protein showed a single glutamine residue at position 365, the respective region of the rainbow trout sequence contained 3 glutamines. Both zoarcids show an accumulation of overall 8 glutamine and glutamate residues. An even larger extension of this Gln-Glu cluster was detected in the notothenioid HIF-1αs with >10 in the *Trematomus* species and a total of 21 residues in the icefish. Especially the glutamate residues of this cluster will render this protein region highly acidic.

We have reported HIF-1α sequences from several cold adapted Antarctic fish species deviating from the known mammal and fish sequences within regulatory ODD. The functional consequences of additional cysteines, Gln-Glu cluster and Pro-Leu substitution in the ODD of *Pb*HIF-1α have to be established in future studies. Moreover, the question

arises what might be the primary function of a HIF protein in icefishes, without red blood cells but endowed with outstanding cardio-vascular adaptations to accommodate this lack (34). Nevertheless, detection of HIF-1α by RT-PCR from normoxic fish samples confirms constitutive expression of this gene in the liver of all four Antarctic species. This might support the suggestion of Semenza (8) that HIF-1 was an essential factor in metazoan evolution.

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Tables

Table 1

List of all primers used in this study.

Zv: only used for amplification of partial HIF-1α cDNA of *Z. viviparus*, *Pb*: only used for amplification of partial HIF-1α cDNA of *P. brachycephalum*, N: used for amplification of partial HIF-1α cDNA of notothenioids and zoarcids.

Primer	Sequence (5` – 3`)	Position of *Z. viviparus* HIF-1α	Product size of *Z. viviparus* HIF-1α fragment (bp)	
HIF-F1	CCAAGGAACMAAACACAGAARCG	677 – 698		
HIF-B5	TGAGTCTCMACCCACACAAARCC	1157 – 1135	435	
HIF-F1L	TCCAACATCCCTCCAACATCG	878 – 898		
HIF-B7L	TTCTTCTCGTCCCCCAGCAGGAAC	1376 – 1353	453	
HIF-F13	TGTGYGTCAACTAYGTKCTCCAGTGG	1206 – 1229		N
HIF-B31	TGACTTGGTCCAGRGCACKCAG	2437 – 2416	1175	N
HIF-5`-B1	GATGTTGGATGGGGTCACAGAC	886 – 8656	865	
HIF-5`-B4Z	TGTATGGCGGCTGAGAAAAGTC	939 – 918	918	*Zv*
HIF-5`-B6	GTAGATGACAGTGGCTTGTGTTTCC	1173 – 1149	1149	
HIF-3`-F6	TTGATACAGAGCCCAAAACCC	1805 – 1825	1558	*Pb*
HIF-3`-F10	AGTCCAGTCCCTTCAGAGTTCTC	1944 – 1966	1417	

Figures

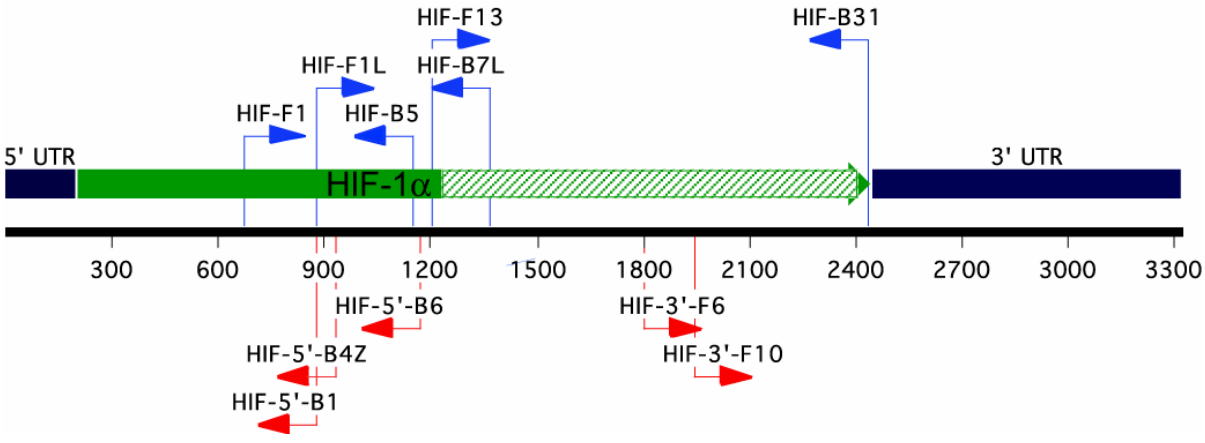

Figure 1

Schematic overview of *Z. viviparus* HIF-1α cDNA: The 5` and 3` untranslated regions (UTR) are indicated by blue bars, the open reading frame by the green bar, the striped part indicating partial HIF-1α used for the alignment in fig.2 and the phylogenetic analysis in fig.3. The locations of RT-PCR primers are indicated by blue, locations of RACE primers by red arrows.

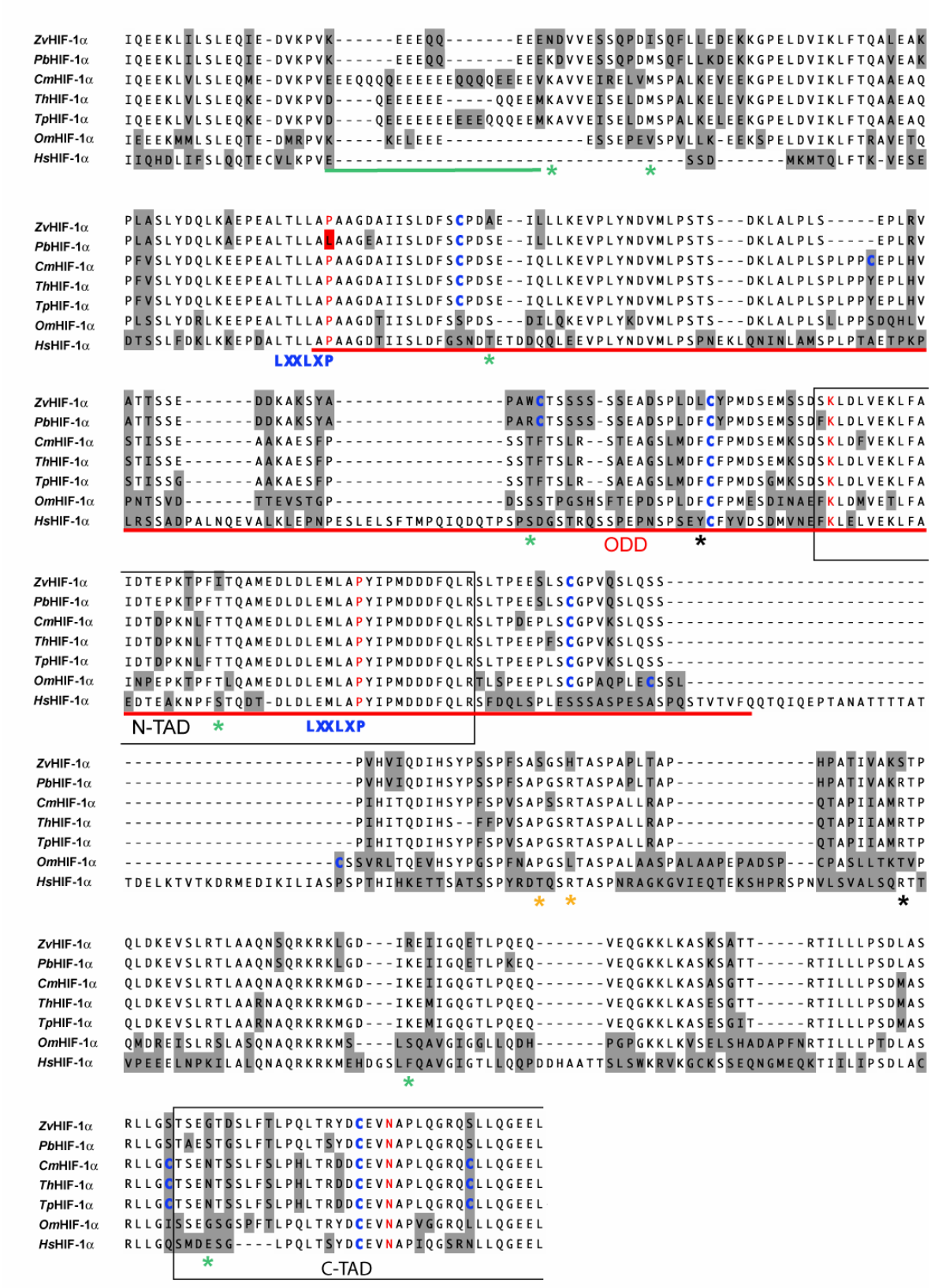

Figure 2

Alignment of partial HIF-1α amino acid sequences (according to fig.1, representing amino acids 343-737 of *Zv*HIF-1α) of the common eelpout *Zoarces viviparus* (*Zv*), the Antarctic eelpout *Pachycara brachycephalum* (*Pb*), the icefish *Chionodraco myersi* (*Cm*) and the red-blooded Antarctic notothenioids *Trematomus hansoni* (*Th*) and *T. pennellii* (*Tp*) as

well as of the rainbow trout *Oncorhynchus mykiss* (*Om*; AF304864) and human (*Hs*; 31077212). The oxygen-dependent degradation domain (ODD) is indicated by the red line below the sequence. The N-terminal (N-TAD) and the C-terminal (C-TAD) transactivation domains are framed by black boxes. LXXLXP represents the binding motif for PHDs (12). Regulatory proline, lysine and asparagine residues are highlighted red, cysteine residues blue. The functional Pro402 of *Hs*HIF-1α appeared substituted to leucine in *Pb*HIF-1α. The green line indicates a Gln-Glu cluster in the fish sequences, prevailing especially in the Antarctic species. Stars mark differences between *Zv* and *Pb*, which in *Pb* are identical or similar in notothenioids; green stars representing differences in line with general trends observed upon protein cold adaptation, orange stars representing differences opposing those trends, black stars representing conserved substitutions. Substitutions supposed to support cold adaptation of HIF-1α are observed mainly in the ODD and the C-TAD.

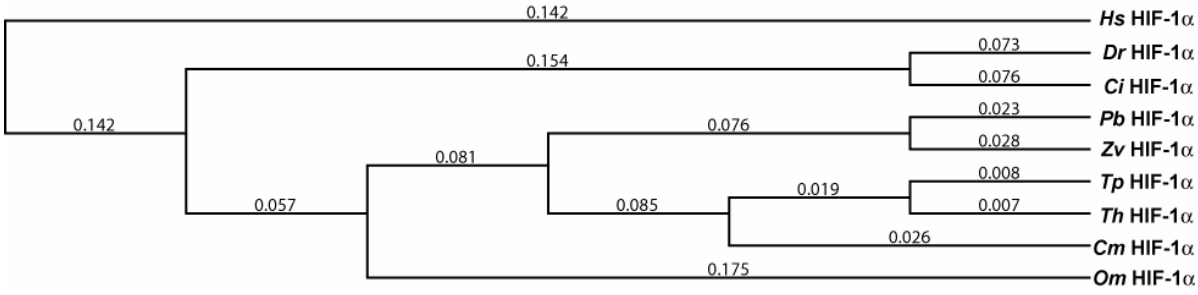

Figure 3

Phylogenetic analysis of the C-terminal half of fish HIF-1α amino acid sequences (according to fig.2) of the zoarcids *Zoarces viviparus* (*Zv*) and *Pachycara brachycephalum* (*Pb*), the notothenioids *Chionodraco myersi* (*Cm*), *Trematomus hansoni* (*Th*) and *T. pennellii* (*Tp*), the cyprinids *Danio rerio* (*Dr*; zebrafish; AY326951)) and *Ctenopharyngodon idella* (*Ci*; grass carp; AY450269) as well as of the salmonid *Oncorhynchus mykiss* (*Om*; rainbow trout; AF304864). The tree was calculated by the Neighbour Joining method (best tree; tie breaking = Systematic; Distance: Uncorrected "p"; Gaps distributed proportionally) with the human sequence (*Hs*; 31077212) as outgroup. Tree calculation by bootstrap (1000 repeats) resulted in the same cluster with all bootstrap values being 100% (not shown).

Publication III:

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Oxidative stress and HIF-1 DNA binding during stressful temperatures and recovery in the North Sea eelpout (*Zoarces viviparus*)

Part A: cold stress

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Abstract

Effects of acute cold exposure (at 1°C and 5°C) on tissue redox state and oxidative stress parameters, as well as the onset of hypoxic signaling were investigated in the North Sea eelpout, *Zoarces viviparus*. Activation of the transcription factor HIF-1 (hypoxia inducible factor) was detected in liver samples after acute cold exposure. HIF-1 activation occurred at more reduced cellular redox milieu. Mildly increased levels of oxidative stress parameters were observed mainly during recovery at control temperature (12°C). This increase in oxidative stress parameters, in spite of maintained antioxidant capacity, indicates that acute cold stress and recovery mimic ischemia / reperfusion events as found in mammals. Notably the non-enzymatic antioxidant defense (e.g. glutathione) may play an important role for eelpout ROS scavenging capacity under cold stress.

Key words:
Cold stress, hypoxia inducible factor, redox state, antioxidants, temperate fish

Introduction

Tissue PO_2 in higher organisms and fish is usually regulated in a very narrow range (Massabuau 2003). Critically low (hypoxic) or high (hyperoxic) PO_2 values are associated with many forms of physiological stress. Generally, these situations are accompanied by oxidative stress, i.e. the unbalanced production of reactive oxygen species (ROS) like $O_2^{-\cdot}$ H_2O_2, $OH^{\cdot}$, $ROO^{\cdot}$ (Sies 1985). Decreasing ROS levels under hypoxia and excess ROS formation when the tissue is flooded with oxygen (reoxygenation) have frequently been described and termed ischemia / reperfusion injury (Chi & Karliner 2004, Erecińska & Silver 2001, Hermes-Lima & Zenteno-Savin 2002).

In addition to their detrimental effects on cellular integrity and function, ROS are involved in cellular signaling (Czubryt et al. 1996, Ehleben et al. 1998, Genius & Fandrey 2000), e.g. in the redox regulation of the hypoxia inducible transcription factor (HIF-1) (Huang et al. 1996, Ema et al. 1999, Lando et al. 2000, Liu et al. 2004, Acker & Acker 2004). The target genes of HIF-1 support cellular and systemic survival during oxygen shortage (for review see Semenza 2002). HIF-1 is a heterodimer with the β-subunit (ARNT) being constitutively present, whereas the α-subunit is regulated in an oxygen-sensitive manner (Jiang et al. 1996). In mammalian cells HIF-1α is permanently expressed and rapidly degraded under normoxia by the ubiquitine-proteasome system resulting in low steady state protein levels (Kallio et al. 1999, Cockman et al. 2000). Under hypoxia the HIF-1α protein stabilizes and accumulates, translocates into the nucleus and dimerizes with the β-subunit to the transcriptionally active HIF-1 dimer.

Oxygen dependent prolyl hydroxylases (PHDs) control normoxic HIF-1α destabilization, which depends on PO_2 (Ivan et al. 2001, Jaakkola et al. 2001, and Masson et al. 2001). However, the cellular redox state is the quintessential regulator of the PHD/HIF interaction, indicated by the fact that the central iron atom of PHDs undergoes redox cycling. Many different redox effectors (ascorbate, ferrous iron, thioredoxin etc.) can influence PHD activity (Acker & Acker 2004, Fandrey 2004). Further, ROS may affect redox chemistry of thiol groups in regulatory proteins, e.g. the transcriptional co-activators CBP/p300, Ref-1, and in HIF-subunits themselves (Ema et al. 1999, Haddad et al. 2000, Lando et al. 2000, Liu et al. 2004), and may therefore be involved in intracellular redox sensitive signaling pathways. In addition, ROS, produced by NADP(H) oxidase in response to extracellular signals (Görlach et al. 2000) can induce HIF-1α stabilization under normoxic conditions (reviewed by Acker & Acker 2004, see papers cited like Haddad & Land 2001).

In marine ectotherms, changing environmental temperatures outside the optimal temperature range cause a decrease of whole animal aerobic scope upon both warming and cooling and can induce a state of functional hypoxia in fully oxygenated water (Pörtner 2002). In line with findings in several marine invertebrates, Lannig et al. (2004) suggested for Atlantic cod that below the optimum temperature range a larger reduction of blood circulation and oxygen transport than of tissue oxygen demand might be responsible for the significant lowering of venous PO_2. In fish, cardiovascular performance likely sets the temperature range for optimal aerobic scope (Mark et al. 2002, Lannig et al. 2004, and Pörtner et al. 2004). The progressive mismatch between oxygen supply and demand in the cold thus causes cold-induced hypoxia in fish tissues. Hypoxia and subsequent reperfusion in central tissues might then form a basis for cold-induced oxidative stress. Survival in habitats where temperatures may transiently fluctuate beyond the thermal optimum range will then require protection of molecular functions by heat shock proteins and increased antioxidant defenses. Thus, our approach was to investigate the consequences of cold stress in fish and to analyze whether hypoxia inducible transcription factor HIF-1 in combination with a signal for cellular redox state respond to and thus indicate hypoxia induced by cold temperatures in fish. Furthermore, we used oxidative stress parameters *in vivo* as another signal for a more oxidized cellular redox environment, hypothesized to be involved in the induction of HIF-1 signaling during acute cold stress and recovery.

The investigation was carried out with a North Sea population of the eurythermal eelpout, *Zoarces viviparus* from Helgoland Island, which experiences temperatures as low as 3-4°C in winter. Also the fishes were shown to be of considerable tolerance to short-term hypoxia (Fischer et al. 1992) and therefore likely endowed with a well functioning hypoxic signaling system, possibly involved in cellular signaling not only during hypoxia *per se* but also during acute cold stress.

Material & Methods

Experimental design

Summer acclimatized eelpout (*Zoarces viviparus*) were caught with bottom traps in shallow waters near the island Helgoland in the German North Sea in June and July 2002. Animals were kept in flow-through aquaria with filtered North Sea water, under constant aeration and a natural day-night cycle at 12°C. Prior to experimental treatment the fish

were kept for at least 10 days in the aquaria to allow full recovery from handling stress. Eelpouts were fed pieces of fresh white fish once a week.

To mimic acute cold stress, the animals were transferred to 5°C (at the low limit of habitat temperatures) and to 1°C (extremely cold) over 2 h. Whereas 50% of the exposed animals were sacrificed directly following cold exposure, the other 50% were returned to 12°C and allowed to recover for 24 h.

For sampling, fish were narcotized with 0.5 g MS222/L seawater, weighed and killed by cutting through the spine. Tissue samples were rapidly removed with sterilized scissors, knifes and forceps, starting with the most metabolically active (liver). Tissue aliquots of about 100 mg were immediately frozen in liquid nitrogen. Samples were stored at -80°C prior to analysis. After sampling sex and length of each fish were determined.

Each experimental group comprised 9 - 16 fish. Fish length varied between 18 and 32 cm. Average fish mass was 47.3 ± 28.3 g (max 159.8 g; min 16.1 g).

Determination of thiobarbituric reactive substances (TBARS)

TBARS were determined as a marker of lipid peroxidation by the TBA (thiobarbituric acid) assay using malondialdehyde-(bis)-acetate (MDA, Merck) as standard (Uchiyama & Mihara 1978).

Tert-butyl hydroperoxide-initiated chemiluminescence

was measured according to Gonzalez Flecha et al. (1991) as indicator for an imbalance between pro- and antioxidant processes resulting from depletion of antioxidant compounds like glutathione, vitamin E and vitamin C. Tissue samples were homogenised in 30 mM KP_i buffer (pH 7.4) containing 120 mM KCl and centrifuged at 600 g for 10 min. The supernatants were diluted in buffer, containing a final concentration of 3 mM tert-butyl hydroperoxide and assayed in the dark at room temperature for chemiluminescence in a liquid scintillation counter (Wallac) in the out-of-coincidence mode, using potassium glass vials kept in the dark for at least 48 h to avoid vial phosphorescence activation by fluorescent light. The chemiluminescence data were determined as counts per minute and were expressed in a tissue specific curve with y = chemiluminescence and x = time. The area under this curve could be calculated for a time period of 3600 sec using the Mat Lab program. The results are expressed as arbitrary area units per mg protein over the studied period.

Determination of protein carbonyl content

Carbonyl groups were measured as indication for oxidative damage to proteins according to Levine et al. (1990). Samples were homogenised in 50 mM HEPES buffer, pH 7.4, containing 125 mM KCl, 0.6 mM $MgSO_4$, 1.1 mM EDTA, and protease inhibitors (0.5 mg * ml^{-1} leupeptine, 0.7 µg * ml^{-1} pepstatine, 40 µg * ml^{-1} phenylmethylsulfonyl fluoride, 0.5 µg * ml^{-1} aprotinin) and centrifuged at 100,000 g for 15 min. Supernatants were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2 M HCl. Blanks were run without DNTP. Afterwards, proteins were precipitated with TCA and centrifuged for 10 min at 10,000 g. The protein pellet was washed 3 times with ethanol:ethylacetate (1:1), resuspended in 6 M guanidine hydrochloride, 20 mM potassium phosphate (pH = 2.3) and incubated at 37°C until complete resuspension. The carbonyl content could be measured spectrophotometrically at 360 nm (molar extinction coefficient $\varepsilon = 22{,}000\ M^{-1}\ cm^{-1}$).

Determination of reduced (GSH) and oxidized (GSSG) glutathione

The glutathione status represents the most important determinant for the cellular redox environment (Schafer & Buettner 2001). The content of GSH and GSSG was determined according to Fariss & Reed (1987). Frozen tissue was ground in liquid nitrogen, and the resulting powder was homogenised in 1:10 (w:v) pre-cooled PCA (10% containing 2 mM bathophenantroline-disulphonic acid). After centrifugation at 15,000 g for 5 min at 4°C, 500 µl of the supernatant were mixed with 10 µl pH-indicator (1 mM m-cresol purple sodium salt containing 0.5 M iodoacetic acid (IAA)) and 50 µl 1 mM γ-glutamyl-glutamate (in 0.3% PCA) was added as internal standard. The pH was adjusted to 8.5 with 5 M KOH (containing 0.3 M N-morpholine-propanesulfonic acid). The mixture was incubated at room temperature for 45 min, to allow binding of IAA to GSH. Subsequently samples were centrifuged for 5 min at 15,000 g and 4°C. 300 µl of the supernatant were added to the double amount of 1% 1-fluor-2,4-dinitrobenzene (diluted in 100% ethanol, HPLC grade) and derivatised in dark vials at room temperature over 24 h. After centrifugation at 7,500 g for 1 min at 4°C and filtration through 0.2 µm nylon membrane filters, samples were stored in dark HPLC vials at -20°C.

HPLC determination was carried out on a Beckmann Coulter HPLC System using an NH_2-spherisorp column, 5 µm 240 x 4 mm (Waters, Germany). Solvent A: 80% methanol and solvent B: sodium acetate stock in 80% methanol (20:80). Sodium acetate stock was prepared by dissolving 500 g Na-acetate in 224 ml H_2O and 695 ml of concentrated HPLC-

grade acetic acid. The gradient programme was as follows: 10 min hold at 90% A followed by a 25 min linear gradient to 25% A at a flow rate of 1 ml * min^{-1}, and 2.3 to 2.8 psi backpressure. Peaks were recorded with a photodiode array detector at 365 nm. Concentrations were calculated using 5-point calibration curves for GSH and GSSG standards processed in the same manner as described for the samples.

As GSSG is formed by oxidation of 2 molecules GSH, total glutathione content was calculated as the sum (2 GSSG + GSH). Accordingly, the redox ratio of oxidised to reduced glutathione was expressed as 2 GSSG / GSH.

The redox potential was calculated with the Nernst equation, as given by Schafer & Buettner (2001), for the different temperatures of maintenance, also considering the temperature induced changes of intracellular pH (pH_i) in fish liver (data taken from Larsen et al. 1997 and Sartoris et al. 2003).

Determination of enzymatic antioxidants

Superoxide dismutase activity (SOD E.C: 1.15.1.1) was determined according to Livingstone et al. (1992), using a xanthine oxidase/cytochrome c assay at 20°C and at the respective stress temperature of each experimental group. 1 Unit SOD reduces the increase in extinction of superoxide-mediated reduction of oxidized cytochrome c by 50% (measured at 550 nm).

Glutathione peroxidase activity (GPX) was determined at 20°C in a coupled optical test according to Günzler & Flohe (1985).

Protein content

The protein content of the samples was determined according to Bradford (1976) using bovine serum albumin as a standard.

Western blot analysis

Nuclear extraction protocols, developed for rainbow trout cells (Soitamo et al. 2001), are not applicable to small tissue samples. Thus, EMSA and Western blotting were carried out with whole cell extracts, prepared as described by Vuori et al. (2004).

50 mg of liver tissues were homogenized in 200 µl buffer C (20 mM HEPES, pH 7.8, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol, 2 µg * ml^{-1} leupeptine, 2 µg * ml^{-1} antipaine, 2 µg * ml^{-1} pepstatine, 2 µg * ml^{-1} aprotinin, 1 mM Na_3VO_4) and centrifuged at 16,100 g, 30 min, 4°C to remove cell debris.

20 µg protein of whole cell extracts per well were run on 7.5% LiDS-PAGE at 40 mA and transferred to 3 MM nitrocellulose membrane (semi-dry blotting; 1 h; 0.4 A; 13 V). Membranes were blocked for 1 h at room temperature with 3% non-fat dry milk in PBS 0.3% Tween20, rinsed 3 times for 10 min with PBS 0.3% Tween20 and incubated with the primary antibody over night at 4°C followed by 1 h at room temperature. Polyclonal antibodies directed against the N-terminus of rainbow trout HIF-1α as described in Soitamo et al. (2001) were used in a dilution of 1:2000 in 1% BSA PBS + 0.02% NaN_3. Afterwards, the membranes were washed and incubated for 3 h at room temperature with horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech), dilution 1:7500 in 3% non-fat dry milk in PBS 0.3% Tween20. After washing the membranes, the signals were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Signal intensities of HIF-1α protein bands were calculated from autoradiographed film, using Chemi-Imager digital camera and software (Alpha Innotech Co.). Calculation of the % intensity was based on densitometry of gel images, with the sum of the bands from all experimental groups taken as 100% value.

Electromobility shift assay (EMSA)

Following the fish protocol of Soitamo et al. (2001), the promoter region of the human erythropoietin (EPO) gene as HIF-sensitive DNA probe (5`-GCCCTACGTGCTGTCTCA-3`) was used. 5`-endlabeling of the sense strand (2 pmol * μl^{-1} DNA) was done with 10 U * μl^{-1} T4 polynucleotide kinase and ^{32}P-γ-dATP (10% v:v) over 15 min at 37°C. After removing unincorporated nucleotides by gelfiltration (sephadex G-25), probes were annealed with 2 pmol * μl^{-1} antisense strand in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$.

EMSA DNA-protein binding reactions were carried out for 30 min on ice in a total volume of 20 µl, containing 10 µg cell extract, 0.1 µg * μl^{-1} carrier DNA (poly dI-dC), DNA binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol), 1 µl ^{32}P-endlabeled DNA probe, 1 µl bromphenol blue (1.5%). Samples were run on 4% non-denaturating glycerolpolyacrylamide-gel (4% acrylamide, 1% glycerol, 0.33x TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA)). Electrophoresis was performed at 150 V and room temperature, for 2 h in 0.33x TBE buffer. Dried gels were autoradiographed (Kvietikova et al. 1995). Calculation of the % intensity was based on densitometry of gel images, with the sum of all bands taken as 100% value.

For testing the specificity of the human EPO enhancer by supershift experiments, 1 µl of polyclonal antibody (against the N-terminus of rainbow trout HIF-1α, see above) was added to the EMSA reaction mixture with liver extracts from *Z. viviparus*. In the presence of the antibody, the EMSA signal was markedly reduced, indicating that antibody interaction with the HIF-1α subunit minimized specific binding of the HIF-1 dimer to the EPO probe (Fig. 3.B). Moreover, the signal was erased by previous 15 min incubation of the reaction mixture with a 2500-fold excess of unlabeled probe prior to addition of the labeled EPO probe. An excess of mutated EPO probe M18 (5`-TTGCCCTAAAAGCTGTCTCAG-3`; Gorr et al. 2004) was added to minimize non-specific (but not HIF) binding (fig. 2.C). In the latter experiment radioactivity was detected quantified in dried gels with a phosphor storage image system (FLA-5000; Fuji, Tokyo, Japan) and the AIDA software package (raytest, Straubenhardt, Germany).

Statistics

All values are given as means ± standard deviation. Differences between experimental groups were analyzed by student's t-test using Statview 5.0. Significances were evaluated based on the p-level < 0.05.

Results

Visual behavior recordings during cold stress and recovery

Visual records of swimming behavior included mobility level (active swimming per time interval) and swim speed when exposed to acute temperature changes. At control temperature 12°C the animals were continuously active. At 5°C swimming speed was maintained at the same level, but spontaneous mobility was observed less often. By contrast, at 1°C, spontaneous mobility was completely abolished and much slower induced swimming speed was observed than at warmer temperatures. However, directly after returning cold stressed fishes to 12°C for recovery, swimming performance was not any different from unstressed control fishes. No fish died during cold exposure or during recovery from cold exposure.

Antioxidant capacity

The entire set of oxidative stress parameters and cellular HIF-1 signaling was determined in the liver tissue.

Antioxidant enzyme activities (SOD, GPX) in the liver during cold exposure and recovery are given in tab. 1. Superoxide dismutase (SOD) activity was measured at a standard temperature of 20°C, and at the respective exposure temperature, to determine the *in vivo* superoxide scavenging capacity. Although acute cold exposure to 1°C led to a slight increase in SOD activity ($p = 0.13$; measured at 20°C), no significant change in SOD activity occurred in any group compared to controls. *In vivo* SOD activity was the same under all experimental conditions. Due to the limited amount of sample material, glutathione peroxidase (GPX) activity could only be measured at 20°C. Slightly lower GPX activities after cold exposure and recovery were not significantly different from unstressed controls ($p > 0.25$).

The ratio of oxidized (GSSG) to reduced (GSH) glutathione (fig. 1.A) is an indicator of a tissue redox environment (Schafer & Buettner 2001). This ratio was significantly decreased after cold stress at 1°C compared to unstressed controls ($p < 0.01$). Accordingly, the redox potential ΔE (fig. 1.B) was more negative ($p < 0.01$), indicating the tissue to be more reduced at the low water temperature. Following recovery from 1°C, both, the glutathione redox ratio and the redox potential returned to control levels. Exposure to 5°C did not provoke changes of the glutathione redox ratio ($p = 0.66$), but ΔE still indicates a more reduced redox environment ($p = 0.03$) compared to controls, although less negative than under more severe cold stress (1°C). The total glutathione content was significantly higher after exposure to 1°C, when compared to 5°C ($p = 0.03$) and decreased during recovery from cold stress at 1°C to below control levels (fig. 1.C). These changes in the total glutathione content and glutathione redox ratio after exposure to 1°C can be explained by concomitant changes of GSH (fig. 1.D), because the amount of oxidized GSSG (fig. 1.E) remained unchanged.

Oxidative stress parameters

Fig. 2 presents the changes of oxidative stress parameters in the liver. The tert-butyl hydroperoxide initiated chemiluminescence rate (fig. 2.A) is an indicator for the imbalance between pro- and antioxidant processes in a tissue (Gonzalez Flecha et al. 1991) and allows for the evaluation of the *ex vivo* susceptibility of lipids to oxidation (Dotan et al. 2004). TBARS (thiobarbituric reactive substances; fig. 2.B) accumulate during enhanced lipid peroxidation processes, whereas carbonyls (fig. 2.C) indicate oxidative modifications of

proteins. All these parameters indicate unbalanced oxidative stress and are indirect measures of increased ROS action in a tissue.

Cold stress led to only slightly higher chemiluminescence rates after exposure to 1°C ($p = 0.06$), mildly elevated TBARS levels after exposure to 1°C ($p = 0.08$) and 5°C ($p = 0.02$), and unchanged carbonyl contents. By contrast, recovery from both cold stress treatments caused a pronounced increase in TBARS content ($p = 0.05$ for recovery from 1°C; $p < 0.01$ for recovery from 5°C). Oxidative protein damage after recovery from both cold stress situations ($p = 0.24$ for 1°C, $p = 0.19$; fig. 2.C) as well as chemiluminescence rates after recovery following 5°C cold stress ($p = 0.09$; fig. 2.A) showed the same trend, but were not significant due to high inter-individual variability.

Taken together the oxidative stress parameters indicate elevated oxidative stress to occur after recovery from severe (1°C), as well as mild cold stress (5°C).

Hypoxic signaling

Western blot analysis with antibodies raised against rainbow trout HIF-1α confirmed the expression of the protein in all experimental groups, including unstressed control eelpout (fig. 3. A). Further, the level of HIF-1α protein did not show any clear temperature dependence.

The electromobility shift assay (EMSA) detects the DNA binding activity of the HIF-1 dimer to the (human) erythropoietin enhancer. The specificity of the human EPO enhancer was tested by supershift experiments (Fig. 3.B) and by adding an excess unlabeled probe (fig. 3.C).

HIF-1 DNA binding was significantly higher after 1°C cold stress ($p < 0.01$) and returned to control levels during recovery ($p = 0.64$). By contrast, 5°C cold stress caused only a minor increase of HIF-1 DNA binding activity ($p = 0.08$) compared to control fish, but the EMSA signal was significantly higher after 24 h recovery from 5°C cold stress ($p < 0.01$). Further, HIF-1 DNA binding was generally higher at more reduced glutathione redox ratios (fig. 4, linear regression, $p = 0.03$).

Discussion

According to the model of oxygen limited thermal tolerance, temperatures outside the thermal optimum range cause functional hypoxia in ectothermal animals including fish (Pörtner 2002). In the present study of hypoxia tolerant eelpout, the hypothesis was tested that short-term cold stress induces hypoxic signaling and that oxidative stress during recovery indicates tissue reoxygenation after the hypoxic period, mimicking ischemia / reperfusion events in mammals. We chose a short period (2 h) of acute cold exposure, to avoid any adaptive shifts in thermal tolerance thresholds. Some adaptive responses to cold can occur very fast, like changes in the fluidity of the plasma membrane, observed within 6 h in trout hepatocytes after cooling from 20 to 5°C (Williams & Hazel 1994).

Oxidative stress during cold stress and recovery

Similar SOD activities measured at 20°C confirm the absence of any acclimatory processes within 2 hours of temperature exposure (tab. 1). Unchanged SOD activities measured in cold exposed fishes at the respective temperature indicate maintenance of *in vivo* superoxide scavenging capacities during experimental cold exposure and during recovery. The compensated *in vivo* SOD activities underline the general importance of SOD as a primary enzymatic ROS quencher during stressful cold exposure. Moreover, GPX activity also remained unchanged in our experiment (tab. 1) and, taken that also winter acclimatization of *Z. viviparus* resulted in only minor changes of AOX enzyme activities (Heise et al., in prep), it seems that the enzymatic antioxidant defense is not be mobilized to any further extent at low temperatures. ROS scavenging potential is further sustained by an increase in reduced glutathione levels resulting in a more reduced redox potential under severe cold stress (1°C; fig. 1.D and B). It may be quicker and less costly for the fish to increase the production of the low molecular weight antioxidant glutathione during cold exposure, when energy generating processes are slowed-down, than to newly synthesize enzymes. Elevated glutathione levels (or increased activities of key antioxidant enzymes) have been observed in many anoxia/hypoxia tolerant animals in natural situations reminiscent to ischemia / reperfusion such as anoxia, desiccation or freezing. These patterns were interpreted as an “anticipatory response” during periods of decreased tissue PO_2, to meet oxidative stress arising during the subsequent tissue reoxygenation (Hermes-Lima & Zenteno-Savin 2002).

In *Z. viviparus* the more reduced redox state and the increased HIF-1 DNA binding upon exposure to 1° and 5°C (here HIF-1 enhancer binding increased only insignificantly) speaks for the onset of temperature induced hypoxia during cold stress (fig. 1. B, 3.D). During such hypoxic or ischemic events, the mitochondrial respiratory chain, notably complex III, becomes highly reduced (for review see Acker & Acker 2004, Hermes-Lima 2004). The accumulated electrons than evoke increased ROS formation as the tissue is reoxygenated (McCord 1988, Turrens et al. 1991, Chi & Karliner 2004). The higher oxidative stress levels (TBARS, carbonyls) seen in the eelpout during recovery and the return of the redox potential to more oxidized control values may reflect this "reoxygenation stress" in *Z. viviparus* (fig. 2.B, C,1.B). Whereas ischemia / reperfusion is highly damaging for most mammals, it is not for many anoxia tolerant animals, which display the "anticipatory AOX response" (Abele 2002, Hermes-Lima & Zenteno-Savin 2002, and Storey & Storey 2004). In these animals induction of antioxidants occurs during the metabolically depressed and in some cases "torpid" (low body temperature) state. Maintenance but not anticipatory increase of antioxidant enzyme activities in cold exposed *Z. viviparus*, and only mildly elevated oxidative stress parameters during recovery show that hypoxic stress was not severe in the fishes, consistent with undiminished swimming performance directly after return to control temperature (12°C). This may be due to the fact that in this bottom dwelling fish, respiratory slow down at low temperature may minimize oxygen supply to demand gradients during short-term cold exposure. In this case, HIF-1 DNA binding in an EMSA assay and tissue redox state qualify as very sensitive indicators for the onset of tissue hypoxia in fish.

HIF-1 during cold stress in fish

The mechanism of degradation and stabilization of HIF-1α seems basically to be the same in fish and mammals. However, stabilization occurred at much higher oxygen levels in rainbow trout, i.e. at normoxic venous PO_2 (Soitamo et al. 2001). Several redox-sensitive cysteine residues in the oxygen dependent degradation domain of trout HIF-1α protein may be involved in this stabilization (Nikinmaa et al. 2004), which are also present in the *Z. viviparus* HIF-1α protein (Heise et al., ms submitted). This may also explain our findings of constitutive HIF-1α protein levels in unstressed, i.e. normoxic *Z. viviparus*.

Coming to the low temperature response, significant increase of HIF-1 DNA binding was found upon severe short-term cold exposure to 1°C (this study) as well as upon winter acclimatization of *Z. viviparus* (Heise et al., in prep.). Apparently, this represents a

common physiological response in this species when exposed to low water temperatures. This might improve blood flow and oxygen supply to the liver and therewith counterbalance acute and seasonal temperature effects.

Interestingly, 2 h exposure to mild (5°C) and to severe cold stress (1°C) with or without 24 h recovery at the control temperature 12°C led to different response patterns: HIF-1 DNA binding activity was significantly higher at 1°C and only insignificantly enhanced at 5°C (fig. 3.D). Instead, HIF-1 DNA binding was strongly enhanced after recovery from 5°C. Seemingly, during severe cold stress of 1°C internal oxygen stores will be more rapidly depleted than at 5°C and, therewith, directly cause a HIF response. By contrast, upon mild cold stress the HIF response was found delayed to the recovery phase. Clearly, a mechanistic explanation has to be established.

The cellular oxygen concentration may not be the only factor shaping the HIF response. Several studies have documented the importance of redox regulation for HIF-1α stabilization and HIF-1 DNA binding (Huang et al. 1996, Lando et al. 2000, Acker & Acker 2004 for review). Also for fish (rainbow trout), *in vitro* studies have demonstrated increased HIF-1 DNA binding activity as redox conditions become more reduced (Nikinmaa et al. 2004). Similarly, our data indicate higher HIF-1 DNA binding during cold exposure, with a more negative cellular redox value (fig. 2.B, 4). This clearly underlines the suggestion of Nikinmaa & Rees (2005) that any form of oxidative stress may affect the function of HIF-1.

Temperature itself may also or alternatively act as a HIF regulating factor. Mammalian studies suggest that upon interaction with HSP90 HIF-1α might be stabilized under normoxic conditions (Minet et al. 1999, Katschinski et al. 2002). In Crucian carp an increase of HIF-1 DNA binding on acclimation to reduced temperature was observed, and was associated with increased levels of HSP70 and 90 (E. Rissanen, H. Numminen, J. Sollid, G. Nilsson and M. Nikinmaa, unpublished results). Thus, in fish normoxic HIF-1 functions, mediated by interaction with heat shock proteins might be favoured in the cold. Heat shock proteins, however, are also induced by hypoxia (review by Feder & Hofmann 1999) such that hypoxia and temperature effects may be intertwined. Similar to the results obtained with eelpout under control conditions, considerable HIF-1α protein levels were observed in Crucian carp under unstressed, and therefore presumably normoxic conditions. Nikinmaa & Rees (2005) suggest that HIF-1 may play oxygen-independent roles in fish physiology. The cross-tolerance concept by Horowitz et al. (2004) also implies that

temperature stress and perturbation of oxygen supply and demand, and probably other environmental stressors may share similar protective pathways.

Conclusions

This study shows the *in vivo* activation of the transcription factor HIF-1 upon acute cold exposure as well as during subsequent recovery. It demonstrates the induction of hypoxic signaling, possibly involved in metabolic reorganization at extremely cold temperatures for this fish. We further report a more reduced redox environment after cold exposure and an increase of oxidative stress parameters during recovery from acute cold stress. Thus, this situation may resemble ischemia / reperfusion events. Moreover, HIF-1 DNA binding in combination with changes in the cellular redox state may be sensitive markers for cold-induced hypoxia in tissues.

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Tables

Table 1

Antioxidant enzyme activities in *Z. viviparus* liver after 2 h cold exposure to 1 and 5°C as well as after 24 h recovery from cold exposure.

Superoxide dismutase (SOD) activities were measured at 20°C and at respective stress temperature; glutathione peroxidase (GPX) activities were measured at 20°C. Means ± standard deviation, n = 3-5, $p < 0.05$. ww = wet weight.

	unstressed	**1°C**	**1°C recovery.**	**5°C**	**5°C recovery**
SOD activity at 20°C (U * g ww^{-1})	2000 ± 700	3100 ± 1200	2200 ± 1000	1700 ± 500	1700 ± 500
SOD activity at stress temperature (U * g ww^{-1})	1800 ± 500	1500 ± 200	1500 ± 500	2000 ± 800	1500 ± 300
GPX activity at 20°C (U * g ww^{-1})	4.3 ± 2.2	2.4 ± 2.4	2.5 ± 1.9	3.2 ± 1.1	3.1 ± 1.4

Figures

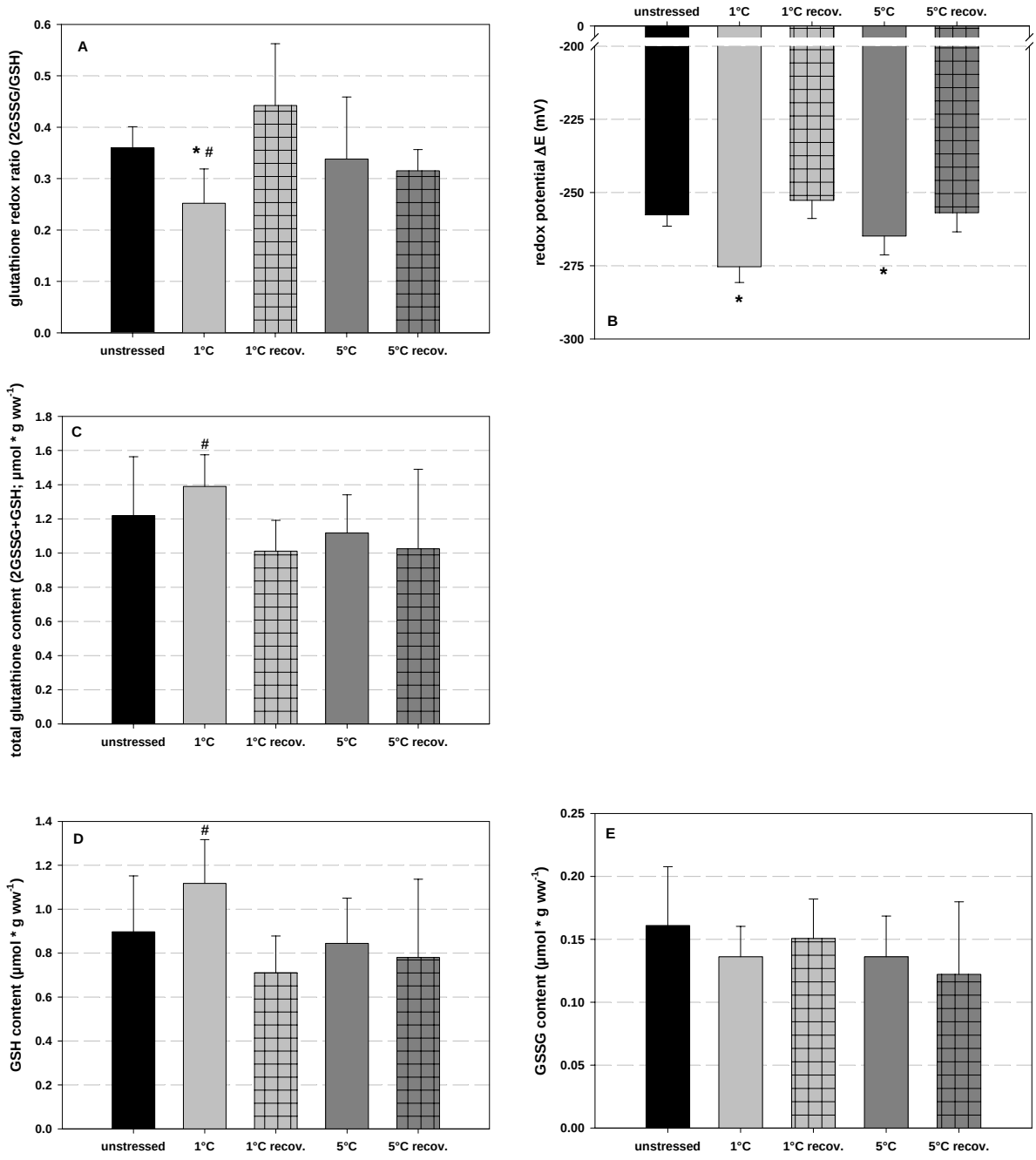

Figure 1

Glutathione redox chemistry in *Z. viviparus* liver samples after 2 h cold exposure to 1 and 5°C as well as after 24 h recovery (recov.) from cold exposure.

A: Glutathione redox ratio (2GSSG/GSH), B: redox potential ΔE at *in situ* temperature and *in situ* intracellular pH (adopted from Sartoris et al. (2003), Larsen et al. (1997)) calculated after Schaffer & Buettner (2001), C: total glutathione content (2GSSG+GSH), D: content of reduced glutathione (GSH), E: content of oxidized glutathione (GSSG), Means ± standard deviation, n = 4-7, * = significantly different from the unstressed group, # = significant difference to the respective recovery group, $p < 0.05$. ww = wet weight.

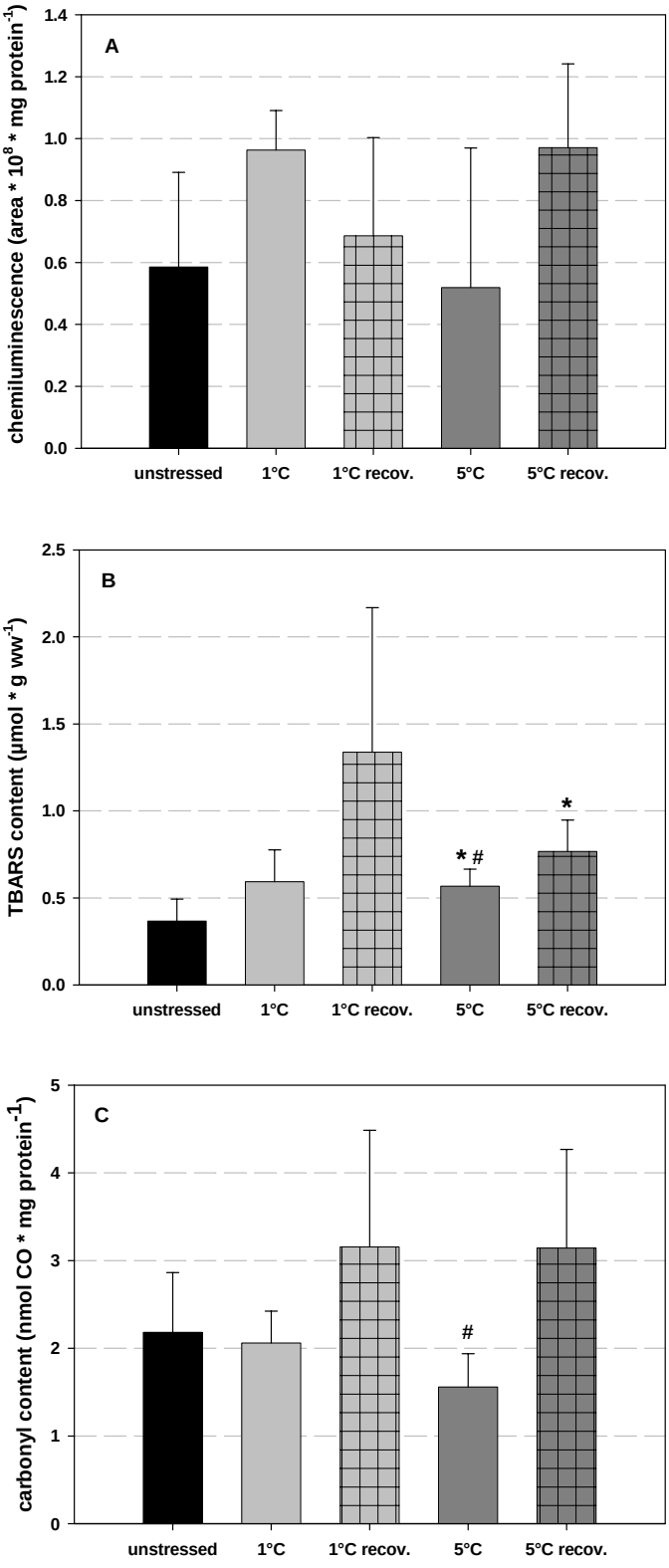

Figure 2

Oxidative stress markers in *Z. viviparus* liver samples after 2 h cold exposure to 1 and 5°C as well as after 24 h recovery (recov.) from cold exposure.

A: tert-butyl hydroperoxide initiated chemiluminescence indicating imbalance between pro- and antioxidant processes, B: content of thiobarbituric reactive substances (TBARS) indicating lipid peroxidation, C: carbonyl content indicating protein oxidation. Means ± standard deviation, n = 3-6, * = significantly different from the unstressed group, # = significant difference to the respective recovery group, $p < 0.05$. ww = wet weight.

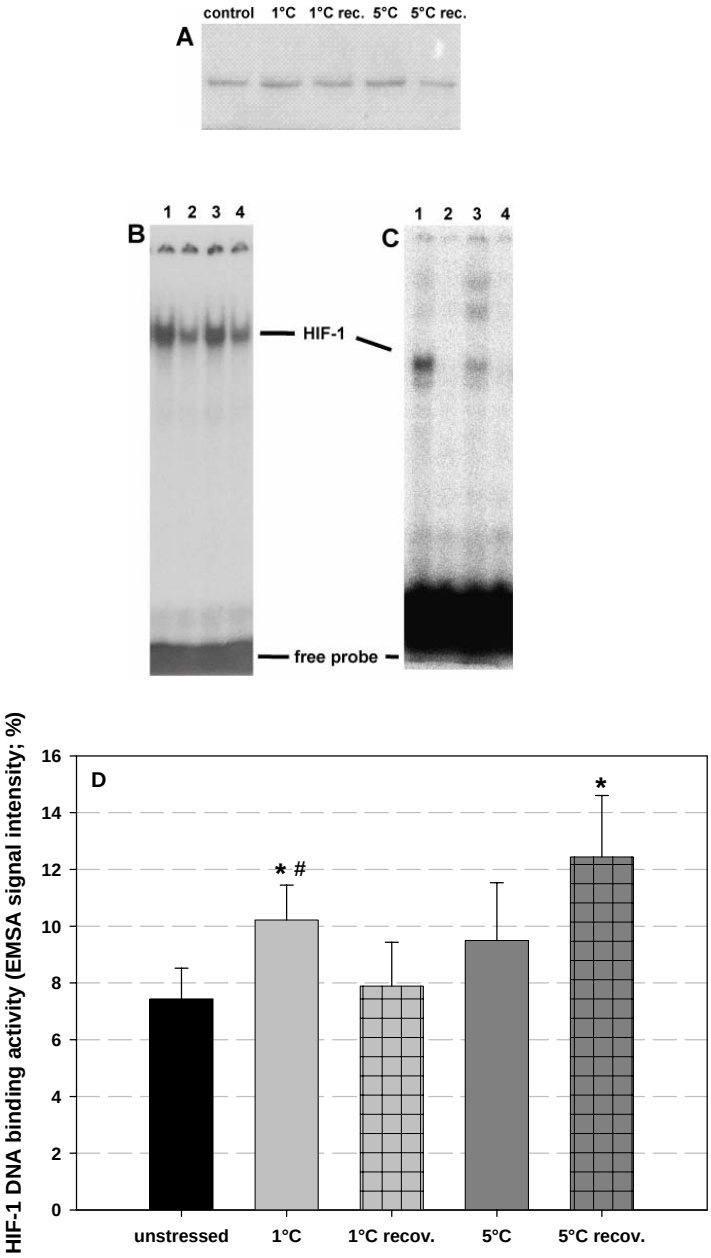

Figure 3

HIF-1 in *Z. viviparus* whole cell extracts from the liver after 2 h cold exposure to 1 and 5°C as well as after 24 h recovery (recov.) from cold exposure.

A: Representative Western blot with antibodies against HIF-1α protein, B: electromobility shift assay (EMSA) demonstrating HIF-1 DNA binding to the human erythropoietin enhancer: Addition of polyclonal antibody directed against the N-terminus of rainbow trout HIF-1α to the reaction mixture in lane 2 and 4 diminished the EMSA signal in liver whole cell extracts from *Z. viviparus* compared to reaction mixtures without antibody (lane 1 and 3) indicating that the fish HIF-1 bound to the human EPO enhancer, C: addition of an excess of unlabeled EPO probe completely erased the signal, addition of a mutated EPO probe M18 reduced the unspecific signal. Lane 1: control signal with labeled EPO probe, lane 2: labeled EPO probe + 2500-fold excess of unlabeled EPO probe, lane 3 labeled EPO probe + mutated EPO probe M18, lane 4: labeled EPO probe + 2500-fold excess of unlabeled EPO probe + mutated EPO probe M18, D: signal intensity of HIF-1 DNA binding in electromobility shift assays in unstressed, 1°C, 1°C recovery, 5°C and 5°C recovery exposed *Z. viviparus*. Means ± standard deviation, n = 4-5, * = significantly different from the unstressed group, # = significant difference to the respective recovery group, $p < 0.05$.

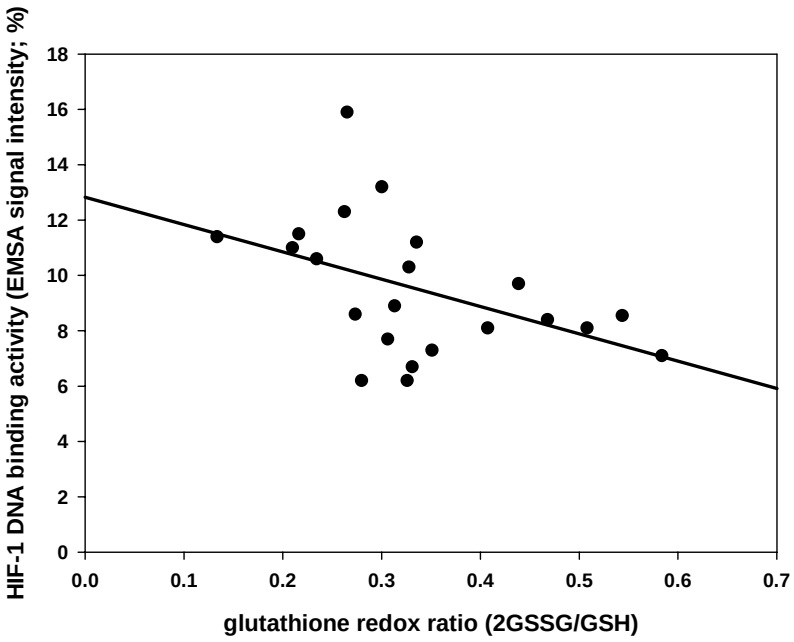

Figure 4

Higher HIF-1 DNA binding activity occurred at a more reduced glutathione redox ratio in liver samples from *Z. viviparus* (linear regression, $y = -9.9\ x + 12.8$, $R^2 = 0.2$, $n = 22$, $p < 0.05$, Statview 5.0).

Publication IV:
JOURNAL OF EXPERIMENTAL BIOLOGY

Oxidative stress and HIF-1 DNA binding during stressful temperatures and recovery in the North Sea eelpout (*Zoarces viviparus*)

Part B: heat stress

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Abstract

The interplay between antioxidants, heat shock proteins and hypoxic signaling is supposed to be important for passive survival of critical temperature stress, e.g. during unfavorable conditions in hot summers. We investigated the effect of mild (18°C), critical (22°C) and severe (26°C) experimental heat stress, assumed to induce different degrees of functional hypoxia, as well as the effect of recovery following heat stress on these parameters in liver samples of the common eelpout *Zoarces viviparus*.

Upon heat exposure to critical and higher temperatures we found an increase in oxidative damage markers such as TBARS (thiobarbituric reactive substances) and a more oxidized glutathione redox milieu, combined with reduced activities of the antioxidant enzyme SOD at 26°C. Together, this points to higher oxidative stress levels during hyperthermia. Moreover, heat-induced hypoxia and subsequent reoxygenation upon recovery had comparable effects as ischemia / reperfusion events in mammals, as indicated by increased protein oxidation and chemiluminescence rates within the first 12 hours upon recovery. A recovery-time series experiment revealed that the onset of oxidative damage repair occurred faster during recovery from heat than recently reported for recovery from cold stress.

HSP70 levels were found only slightly elevated after recovery from sub-lethal heat stress, indicating minor importance of the heat shock response in this species. The DNA binding activity of the hypoxia inducible transcription factor (HIF-1) was elevated only during mild heat exposure (18°C), but appeared impaired at more severe heat stress. We suppose that the more oxidized redox state during extreme heat may interfere with the hypoxic signaling response.

Key words:
Hyperthermia, heat shock proteins, oxidative stress, redox state, hypoxia inducible factor, temperate fish

Introduction

Exposure of cold blooded marine animals to elevated temperatures accelerates mitochondrial respiration and has been shown to increase mitochondrial reactive oxygen species (ROS) formation (Heise et al. 2003, Keller et al. 2004). Excess ROS production by intensively respiring mitochondria is held responsible for cellular damage expected upon exposure of an ectotherm to warming (hyperthermia) and heat stress (Abele et al. 1998, 2002).

On the other hand, as more oxygen is consumed in peripheral tissues under severe thermal stress, limitation of oxygen supply to central tissues can occur, and provoke a state of thermally induced transient hypoxia. Onset of heat-induced hypoxia in body fluids and tissues has been found as animals were warmed beyond their pejus temperatures (Tp), indicating the limits of the species specific thermal optimum range. These findings have led to the concept of oxygen and capacity limited thermal tolerance (for review see Pörtner 2002). In fish, capacity limits of cardiac performance account for progressive mismatch between oxygen supply and demand in the whole animal during warming (Mark et al. 2002, Farrell 2002, Lannig et al. 2004). The decline in cardiac performance could reflect problems with the hearts own oxygen supply (Farrell 2002). Oxygen extraction by working skeletal muscle during exercise would further increase oxygen demand at high temperatures. When a critical temperature (Tc) is reached, aerobic scope for activity disappears and mitochondria progressively switch to anaerobic energy production, which allows only time limited survival (Zielinski & Pörtner 1996, Sommer et al. 1997, reviewed in Pörtner 2002).

Complications can result, when oxygen deprived tissues are re-oxygenated, a situation which resembles ischemia / reperfusion in mammalian tissues (Jones 1986, McCord 1988, Erecinska & Silver 2001, Chi & Karliner 2004). Elevated ROS formation may thus not only result as a consequence of stressful hyperthermia, but also as a consequence of reoxygenation during recovery (Halliwell & Gutteridge 1999).

There is likely considerable overlap between hypoxic signaling, the heat shock response, and oxidative stress during stressful warming (cf. Pörtner 2002). Ischemia / reperfusion and ROS production are known to induce heat shock protein (HSP) transcription and synthesis in mammalian cells and to play a common denominator role for both pro- and anti-apoptotic processes under hyperthermia (Flanagan et al. 1998, Katschinski et al. 2000, Skulachev 2001, King et al. 2002, Kregel 2002). Therefore, short-term tolerance to

oxidative stress provided by an effective antioxidant system (Abele & Puntarulo 2004), as well as up-regulation of HSPs, mediating the refolding of heat-damaged proteins, may support survival under heating (Moseley 1997, Pörtner 2002).

Moreover, the transcription factor HIF-1 (hypoxia inducible factor), which activates genes involved in angiogenesis, erythropoiesis and glucose metabolism (for review see Wenger 2000, Semenza 2004) has been shown to be functional for heat acclimation of *C. elegans* (Treinin et al. 2003). Loss of the HIF-1 gene abolished heat resistance despite of an up-regulation of HSP72 in the nematode model and metabolic reorganization mediated by HIF-1 could thus contribute to ameliorate the temperature induced oxygen limitation upon (sub)-critical warming. Although the causal link between heat and hypoxia resistance may relate to the onset of functional hypoxia during stressful heating as demonstrated for marine fishes, clear evidence supporting this mechanistic link has still to be elaborated.

The common eelpout is a eurythermal marine fish which offers an appropriate model-system to study the interdependence of oxidative stress, hypoxic signaling and HSP expression under high temperature stress. Acclimation to and stress response at high temperatures and heat-induced hypoxia is well studied in this animal (Zakhartsev et al. 2003, Pörtner et al. 2004). The critical temperature, where anaerobic energy production sets in was found to range between 22 and 24°C (Van Dijk et al. 1999). Graded short-term exposure to temperatures, mildly and severely elevated over the control temperature should, therefore, allow to distinguish between the effect of hyperthermia and the combination of heat and functional hypoxia beyond the critical temperature limit. All parameters were determined in the liver tissue, which has been reported to be the most sensitive organ to synthesize HSP70 in response to hyperthermia (King et al. 2002) and also to suffer from temperature induced oxygen limitation (Van Dijk et al. 1999).

Material & Methods

Experimental design:

A) Graded heat stress and 24 h recovery

Summer acclimatized eelpout (*Zoarces viviparus*) were caught with bottom traps in shallow waters near the island Helgoland in the German North Sea in June and July 2002. Animals were kept in flow-through aquaria with filtered North Sea water under constant

aeration and a natural day-night cycle at 12°C. Before the experimental phase, the fishes were kept at least for 10 days in the aquaria to recover from handling stress. Eelpouts were fed pieces of fresh white fish once a week.

To mimic acute heat stress, the animals were transferred to 18°C, the high temperature limit in the habitat, which is above Tp (Zakhartsev et al. 2003), as well as to 22°C, which according to Van Dijk et al. (1999) is the critical temperature (Tc) and eventually to 26°C which represents extreme heat stress, where the animals are close to the onset of loss of equilibrium at 27-28°C (Zakhartsev et al. 2003). Exposure time was limited to 2 h. Following heat exposure, 50% of the animals were directly sacrificed, whereas the other half was returned to 12°C and allowed to recover for 24 h.

For tissue sampling, fishes were narcotized with 0.5 g MS222/L seawater, weighed and killed by cutting through the spine. Tissues were rapidly removed, starting with the most metabolically active (liver) and aliquots of 100 mg immediately frozen in liquid nitrogen. Samples were stored at -80°C prior to analysis. After sampling sex and length of each fish were determined.

Each experimental group comprised 12 - 16 fish. Fish length varied between 18 and 26 cm (21.5 ± 2.6). Average fish mass was 40.2 ± 20.8 g (max 132 g; min 16 g).

B) Recovery time series

For a second experimental series we tested the effects of different recovery times in order to assess a potentially time dependent maximum of reoxygenation stress. *Zoarces viviparus* were caught near the island Helgoland in September 2003 and transported to the Alfred-Wegener Institute two weeks prior to experimentation, where the fish were kept at 10°C in natural sea water with constant aeration and a day-night cycle of 12 h. Fish were fed live shrimp once a week. Animals were exposed only to 18°C over 2 h to induce thermal stress followed by 0 h, 2 h, 8 h or 12 h recovery at control temperature (10°C). Samples were taken as described above. Each experimental group comprised 6 - 8 fish. Fish length varied between 20 and 27 cm (23.6 ± 2.7). Average fish mass was 50.4 ± 22.2 g (113 g max; 17 g min.).

Determination of thiobarbituric reactive substances (TBARS)

TBARS were determined as a marker of lipid peroxidation by the TBA (thiobarbituric acid) assay using malondialdehyde-(bis)-acetate (MDA, Merck) as standard (Uchiyama & Mihara 1978).

Tert-butyl hydroperoxide (tBOOH)-initiated chemiluminescence

was measured according to Gonzalez Flecha et al. (1991) as indicator for an imbalance between pro- and antioxidant processes resulting from depletion of antioxidant compounds like glutathione, vitamin E and vitamin C. Tissue samples were homogenised in 30 mM KP_i buffer (pH 7.4) containing 120 mM KCl and centrifuged at 600 g for 10 min. The supernatants were diluted in buffer, containing a final concentration of 3 mM tert-butyl hydroperoxide and assayed in the dark at room temperature for chemiluminescence (counts per minute) in a liquid scintillation counter (Wallac) in the out-of-coincidence mode, using potassium glass vials kept in the dark for at least 48 h to avoid vial phosphorescence activation by fluorescent light. The chemiluminescence data were determined as counts per minute and were expressed in a tissue specific curve with y = chemiluminescence and x = time. The area under this curve could be calculated for a time period of 3600 sec using the Mat Lab program. The results are expressed as arbitrary area units per mg protein over the studied period.

Determination of protein carbonyl content

Carbonyl groups were measured as indication for oxidative damage to proteins according to Levine et al. (1990). Samples were homogenised in 50 mM HEPES buffer, pH 7.4, containing 125 mM KCl, 1.1 mM EDTA, 0.6 mM $MgSO_4$ and protease inhibitors (0.5 mg * ml^{-1} leupeptine, 0.7 µg * ml^{-1} pepstatine, 40 µg * ml^{-1} phenylmethylsulfonyl fluoride, 0.5 µg * ml^{-1} aprotinin) and centrifuged at 100,000 g for 15 min. Supernatants were incubated at room temperature for 1 h with 10 mM 2,4-dinitrophenylhydrazine (DNTP) in 2 M HCl. Blanks were run without DNTP. Afterwards, proteins were precipitated with TCA and centrifuged for 10 min at 10,000 g. The protein pellet was washed 3 times with ethanol:ethylacetate (1:1), resuspended in 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH = 2.3) and incubated at 37°C until complete resuspension. The carbonyl content could be measured spectrophotometrically at 360 nm (molar extinction coefficient ε = 22,000 M^{-1} cm^{-1}).

Determination of reduced (GSH) and oxidized (GSSG) glutathione

The glutathione status represents the most important determinant for the cellular redox environment (Schafer & Buettner 2001). The content of GSH and GSSG was determined according to Fariss & Reed (1987). Frozen tissue was ground in liquid nitrogen and the resulting powder homogenised in 1:10 (w:v) pre-cooled PCA (10% containing 2 mM

bathophenantroline-disulphonic acid). After centrifugation at 15,000 g for 5 min at 4°C, 500 µl of the supernatant were mixed with 10 µl pH-indicator (1 mM m-cresol purple sodium salt containing 0.5 M iodoacetic acid (IAA)). 50 µl 1 mM γ-glutamyl-glutamate (in 0.3% PCA) was added as internal standard. The pH was adjusted to 8.5 with 5 M KOH (containing 0.3 M N-morpholine-propanesulfonic acid). The mixture was incubated at room temperature for 45 min, to allow IAA to bind to GSH. Subsequently samples were centrifuged for 5 min at 15,000 g at 4°C. 300 µl of the supernatant were added to the double amount of 1% 1-fluor-2,4-dinitrobenzene (diluted in 100% ethanol, HPLC grade) and derivatised in dark vials at room temperature over 24 h. After centrifugation at 7,500 g for 1 min at 4°C and filtration through 0.2 µm nylon membrane filters, samples were stored in dark HPLC vials at -20°C.

HPLC determination was carried out on a Beckmann Coulter HPLC System using a NH_2-spherisorp column, 5 µm 240 x 4 mm (Waters, Germany). Solvent A: 80% methanol and solvent B: sodium acetate stock in 80% methanol (20:80). Sodium acetate stock was prepared by dissolving 500 g Na-acetate in 224 ml Milli-Q water and 695 ml of concentrated HPLC-grade acetic acid. The gradient programme was as follows: 10 min hold at 90% A followed by a 25 min linear gradient to 25% A at a flow rate of 1 ml * min^{-1}, and 2.3 to 2.8 psi backpressure. Peaks were recorded with a photodiode array detector at 365 nm. Concentrations were calculated using 5-point calibration curves for GSH and GSSG standards processed in the same manner as described for the samples.

As GSSG is formed by oxidation of 2 molecules GSH, total glutathione content was calculated as the sum (2 GSSG + GSH). Accordingly, the redox ratio of oxidised to reduced glutathione was expressed as 2 GSSG / GSH.

The redox potential was calculated with the Nernst equation, as given by Schafer & Buettner (2001), for the different temperatures of maintenance, also considering the temperature induced changes of intracellular pH (pH_i) in fish liver (data taken from Larsen et al. 1997 and Sartoris et al. 2003).

Determination of enzymatic antioxidants

Superoxide dismutase activity (SOD E.C: 1.15.1.1) was determined according to Livingstone et al. (1992), using a xanthine oxidase/cytochrome c assay at 20°C as well as at the respective stress temperature of each experimental group. 1 Unit SOD reduces the increase in extinction of superoxide-mediated reduction of oxidized cytochrome c by 50% (measured at 550 nm).

Glutathione peroxidase activity (GPX) was determined at 20°C in a coupled optical test according to Günzler & Flohe (1985).

Protein content

The protein content of the samples was determined according to Bradford (1976) using bovine serum albumin as a standard.

Western blot analysis

Nuclear extraction protocols, developed for rainbow trout cells (Soitamo et al. 2001), are not applicable to small tissue samples. Thus, EMSA and Western blotting were carried out with whole cell extracts, prepared as described by Vuori et al. (2004).

50 mg of liver tissues were homogenized in 200 µl buffer C (20 mM HEPES, pH 7.8, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol, 2 µg * ml^{-1} leupeptine, 2 µg * ml^{-1} antipaine, 2 µg * ml^{-1} pepstatine, 2 µg * ml^{-1} aprotinin, 1 mM Na_3VO_4) and centrifuged at 16,100 g, 30 min, 4°C.

20 µg protein of whole cell extracts per well were run on 7.5% LiDS-PAGE at 40 mA and transferred to 3 MM nitrocellulose membrane (semi-dry blotting; 1 h; 0. 4A; 13 V). Membranes were blocked for 1 h at room temperature with 3% non-fat dry milk in PBS 0.3% Tween20, rinsed 3 times for 10 min with PBS 0.3% Tween20 and incubated with the primary antibody over night at 4°C followed by 1 h at room temperature. Polyclonal antibodies directed against the N-terminus of rainbow trout HIF-1α as described in Soitamo et al. (2001) were used in a dilution of 1:2000 in 1% BSA PBS + 0.02% NaN_3. Afterwards, the membranes were washed and incubated for 3 h at room temperature with horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech), dilution 1:7500 in 3 % non-fat dry milk in PBS 0.3% Tween20. After washing the membranes, the signals were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). Signal intensities of HIF-1α protein bands were calculated from autoradiographed film, using Chemi-Imager digital camera and software (Alpha Innotech Co.). Calculation of the % intensity was based on densitometry of gel images, with the sum of the bands from all experimental groups taken as 100% value.

Electromobility shift assay (EMSA)

Following the fish protocol of Soitamo et al. (2001), the promoter region of the human erythropoietin (EPO) gene as HIF-sensitive DNA probe (5`-GCCCTACGTGCTGTCTCA-

3`) was used. 5`-endlabeling of the sense strand (2 pmol * μl^{-1} DNA) was done with 10 U * μl^{-1} T4 polynucleotide kinase and ^{32}P-γ-dATP (10% v:v) over 15 min at 37°C. After removing unincorporated nucleotides by gelfiltration (sephadex G-25), probes were annealed with 2 pmol * μl^{-1} antisense strand in 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$)

EMSA DNA-protein binding reactions were carried out for 30 min on ice in a total volume of 20 µl, containing 10 µg cell extract, 0.1 µg * μl^{-1} carrier DNA (poly dI-dC), DNA binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol), 1 µl ^{32}P-endlabeled DNA probe, 1 µl bromphenol blue (1.5%). Samples were run on 4% non-denaturating glycerolpolyacrylamide-gel (4% acrylamide, 1% glycerol, 0.33x TBE buffer (89 mM Tris, 89 mM boric acid, 5 mM EDTA). Electrophoresis was performed at 150 V and room temperature, for 2 h in 0.33x TBE buffer. Dried gels were autoradiographed (Kvietikova et al. 1995). Calculation of the % intensity was based on densitometry of gel images, with the sum of all bands taken as 100% value.

Testing the specificity of the human EPO enhancer by supershift experiments and by addition of an excess of unlabeled probe are depicted in Heise et al. (part A).

Statistics

All values are given as means ± standard deviation. Differences between experimental groups were analyzed by student's t-test using Statview 5.0. Significances were evaluated based on the p-level < 0.05.

Results

A) Graded heat stress and 24 h recovery

Behavior during heat stress and recovery

Observations of behavior included mobility level (frequency of spontaneous swimming) and swimming speed observed during 2 h exposure to heat stress. Eelpouts at 18°C maintained mobility and swimming speed at control level or even higher. At 22°C fishes showed spontaneous mobility but at a reduced speed compared to 12°C. Exposure to 26°C led to an even more obvious slow-down of swimming and to severely reduced spontaneous mobility. However, directly after returning the fish to 12°C for recovery, there was no

difference in swimming speed and mobility between unstressed controls and all heat exposed groups. No fish died during heat exposure or recovery.

Antioxidant capacity in the liver

Fig. 1 shows antioxidant enzyme activities in the liver in unstressed, heat stressed and recovery groups. Superoxide dismutase (SOD) activity, measured at standardized temperature of 20°C (fig. 1.A), was unchanged from control levels after heat exposure to 18 and 22°C, but significantly reduced after 2 h at 26°C ($p = 0.04$). Partial recovery of the reduced SOD activity was achieved by 24 h maintenance at 12°C ($p = 0.3$ compared to control, $p = 0.06$ compared to 26°C). When SOD was assayed at stress temperature (fig. 1.B) there was no significant difference in SOD activities between any of the heat stressed and the control groups ($p > 0.05$). However, a mild activity increase at 22°C and a decrease at 26°C were mirrored in a significantly lower *in situ* SOD activity at 26°C compared to 18 or 22°C ($p \leq 0.03$).

Glutathione peroxidase (GPX) activities assayed at 20°C (fig. 1.C) did not show significant changes ($p > 0.16$) compared to control levels. A minor increase at 22°C and a slight decrease at 26°C were in line with the trend already observed for SOD activities.

Fig. 2 gives a differentiated overview of glutathione-related parameters in all experimental groups. The total glutathione content (controls: 1.2 ± 0.3 µmol * g ww^{-1}) as well as the contents of reduced (GSH, control: 0.9 ± 0.6 µmol * g ww^{-1}) and oxidized (GSSG, fig. 2.A) glutathione did not show significant changes following heat exposure or recovery from heat stress (total glutathione: $p > 0.35$, GSH: $p > 0.18$, data for these parameters are not included in Fig. 2). However, a trend for GSSG to rise upon recovery from 26°C was close to being significant ($p = 0.05$, fig. 2.A). Glutathione redox ratio (2GSSG/GSH) was significantly reduced ($p < 0.01$, fig. 2.B) in fishes exposed to 18°C compared to controls (12°C). Calculation of the redox environment ΔE according to Schafer & Buettner (2001) are based on GSH and GSSG concentrations, the water temperature, and the temperature-dependent change of intracellular pH (adopted from Sartoris et al. 2003, Larsen et al. 19997). The resulting ΔE values (fig. 2.C) remained close to control levels at 18°C ($p = 0.28$) and during recovery from 18°C ($p = 0.77$). By contrast, warming to 22°C ($p < 0.01$) as well as 26°C ($p = 0.03$) led to a significantly more oxidized cellular redox environment. Following recovery from each high temperature exposure ΔE values returned to control level within 24 h of recovery ($p > 0.33$).

Oxidative damage parameters in the liver

The exposure to a source of free radicals such as tBOOH allows for the evaluation of the *ex vivo* susceptibility of lipids to oxidation (Dotan et al. 2004). The main reactive species detected by the assay of tBOOH- initiated chemiluminescence according to Gonzalez Flecha et al. (1991) are the dimol emission of singlet oxygen and the photon emission from excited carbonyl groups formed during lipid peroxidation. It has been shown that this assay evaluates the imbalance between pro- and antioxidant processes resulting from depletion of low-molecular weight antioxidant compounds like glutathione, ascorbate and α-tocopherol.

Accordingly, unaltered tBOOH initiated chemiluminescence rates in all experimental groups (fig. 3.A), indicate that prooxidant processes were well balanced by antioxidant compounds (Gonzalez Flecha et al. 1991). However, thiobarbituric reactive substances (TBARS) as markers of lipid peroxidation were elevated over control levels after all heat treatments and remained elevated throughout recovery (p-values: 0.06 / 18°C, 0.04 / 22°C, < 0.01 / 26°C, 0.02 / 18°C recovery, 0.03 / 22°C recovery, 0.04 / 26°C recovery, fig. 3.B). Differences between stressed groups were insignificant, with TBARS content at 18°C being higher than at 22 or 26°C. After recovery from 18 and 22°C, TBARS content was reduced in comparison to the respective heat stress group, but after 24 h levels were still somewhat higher than in controls.

The carbonyl content of the liver reflects oxidative modifications of proteins (fig. 3.C) and was similar in all groups with the exception of higher carbonyl levels after recovery from 18°C ($p = 0.03$ compared to controls).

Hypoxic signaling and molecular defense in the liver

Higher levels of heat shock protein (HSP70) were detected only after recovery from 26°C (1.5 ± 0.2 % signal intensity) compared to control levels (1.0 ± 0.3 %, $p = 0.05$) and to the 26°C heat stress group (1.0 ± 0.3 %, $p = 0.02$).

The HIF-1α protein could be detected in all experimental groups including unstressed controls and recovering specimens (data not shown) and did not show temperature dependent concentration changes. Electromobility shift assays (EMSA), demonstrating DNA binding of HIF-1 to the human erythropoietin enhancer, reached only weak signal intensities following heat stress (fig. 4.A). DNA binding of HIF-1 significantly above control levels was found only after recovery from exposure to 18°C ($p = 0.04$; fig. 4.B). Taking the data from this and from our parallel study on cold stress together (Heise et al. part A), HIF-1 DNA binding in *Z. viviparus* was generally enhanced at more reduced

cellular redox environment ΔE (fig. 4.C, linear regression, Statview 5.0, $p = 0.03$; $R^2 = 0.3$).

B) Recovery from exposure to 18°C

Recovery over time was analyzed in a different batch of animals collected in September 2003. Liver SOD activities of unstressed controls were measured at 20°C (3470 ± 750 U * g ww^{-1}) and at 10°C (2600 ± 480 U * g ww^{-1}) and remained unchanged after exposure to 18°C and subsequent recovery ($p > 0.08$; data not shown).

Glutathione parameters of the recovery time series are presented in fig 5. The content of GSH was significantly increased following exposure to 18°C ($p < 0.01$, fig. 5.A). Throughout recovery we also found GSH significantly elevated over controls ($p = 0.04$ / 2 h rec., $p = 0.01$ / 8 h rec., $p = 0.01$ / 12 h rec.) but lower than in the heat stressed group. Due to high variability in the control group, GSSG content was only insignificantly increased above 10°C controls upon warming and in the recovery groups (fig. 5.B). However, due to an increase of both GSH and GSSG, the total glutathione content was significantly higher in the 18°C group and also in all recovery groups (p-levels: < 0.01 / 18°C, 0.04 / 2 h rec., 0.01 / 8 h rec., 0.01 / 12 h rec., fig. 5.C). Moreover, constant glutathione redox ratio and redox potential ΔE indicates an unchanged liver redox environment under all exposure conditions (fig. 5.D, E). Liver oxidative stress parameters of the recovery time series are given in fig. 6. A significant increase in tBOOH initiated chemiluminescence rates was found after 12 h recovery from 18°C ($p < 0.01$; fig 6.A). The content of protein carbonyls was significantly increased during the initial phase (2 and 8 h) of recovery at control temperature ($p = 0.08$ / 2 h rec., $p = 0.04$ / 8 h rec.) and returned to control levels within 12 h of recovery from warming ($p = 0.41$; fig. 6.B). TBARS were not measured in this experiment.

Interestingly, some parameters showed significant differences between both experimental series with respect to controls and also with respect to fish exposed to 18°C (tab. 1). The carbonyl content was significantly higher in control and 18°C fish of experiment A (graded heat stress) compared to B (recovery time series). This went along with significantly lower SOD activities in experiment A. Further, the increase in GSH and total glutathione content upon exposure to 18°C (fig. 5.A, C) in experiment B, not observed in experiment A (see above), caused a significant difference of the glutathione tissue concentration between both experiments (tab. 1). However, the redox potential was similar and only insignificantly lower in experiment B in both, controls and fish at 18°C.

Discussion

The present study investigates the physiological response of the common eelpout *Zoarces viviparus* to short-term experimental hyperthermia and heat stress, suggested to cause functional hypoxia in central tissues (Pörtner 2002). Specifically we addressed the question, whether oxidative stress occurred during hyperthermia and / or during recovery at control temperature and whether antioxidants (AOX), heat shock proteins (HSP) and hypoxic signaling were induced. The interplay between these defense systems is supposed to be important for passive survival of critical temperature stress, e.g. during unfavorable conditions in hot summers.

Based on the previous evaluation of the thermal biology of *Z. viviparus*, as summarized in Zakhartsev et al. (2003), we chose 18, 22 and 26°C as stress temperatures, covering the range from sub-critical values (18°C) beyond pejus temperatures, where the animals started to lose their aerobic scope, to the critical heat stress limit, where heat-induced hypoxia becomes more severe and anaerobic metabolism sets in (22°C). Finally, animals were exposed to 26°C slightly below the temperature where Zakhartsev and co-workers found an onset of muscular spasms and loss of equilibrium.

Response to graded heat stress and recovery

Induction of antioxidant enzymes is an important line of defense against oxidative stress in biological systems (Storey 1996, Parihar et al. 1997) but can be compromised under temperature stress due to thermal impairment of protein function (reviewed by Abele & Puntarulo 2004). In the present study, only slightly higher SOD and GPX activities after exposure to 22°C were recorded, whereas particularly SOD was reduced under extreme hyperthermia (26°C, fig. 1). Similarly, impairment especially of SOD above Tc has been reported for different marine invertebrates (Abele et al. 1998, 2001, 2002). This loss of enzymatic antioxidant activity beyond critical temperatures might relate to heat-induced protein denaturation or disturbances of protein synthesis (Pörtner 2002, Kregel 2002). Interestingly, although GPX activity was not significantly elevated, the cellular redox environment, which is mainly determined by the ratio of oxidized to reduced glutathione (Schafer & Buettner 2001), was more oxidized under critical hyperthermia at 22°C and 26°C (fig. 2.C). Presumably this relates to spontaneous, non-enzymatic GSH oxidation by emerging ROS under hyperthermia. It may also be due to loss of function of the enzyme glutathione reductase, which re-converts oxidized to reduced glutathione. At all events, a

more oxidized glutathione redox ratio under heat stress stands against our findings of a more reduced redox environment under severe cold stress in eelpout (Heise et al., part A). Thus, heat and cold stress outside the thermal tolerance range had opposite effects on the cellular redox milieu, which indicates that oxidative stress presented a greater problem during acute warming than during cooling of this temperate fish species. However, the glutathione antioxidant capacity seemed sufficient to counterbalance an assumed temperature induced acceleration of ROS formation rates also upon severe heat stress, as indicated by constant chemiluminescence levels on tBOOH-induction (Gonzalez Flecha et al. 1991). By contrast, either ROS production itself, or the oxidizing reaction with GSH appeared slower in the cold.

The glutathione based antioxidant effect is thought to be protective in the hydrophilic protein fraction and, indeed, protein oxidation was not elevated at critically high temperatures (≤ 22°C). In the hydrophobic lipid fraction the antioxidant glutathione is much less soluble and therefore less effective, and TBARS levels rose significantly over controls at 22°C and higher. In agreement with these findings, glutathione depletion in heat stressed marine sponges resulted in a loss of antioxidant protection (Bachinski et al. 1997) and the same effect has been observed in mammalian systems (Freeman et al. 1990). It has to be considered that higher TBARS levels in heat stressed eelpout could also be due to temperature induced changes in the rates of their degradation with the rates of ROS formation being unaffected upon heating. However, recent *in vitro* studies on isolated mitochondria obtained from marine invertebrates have clearly shown increasing ROS rates with rising temperature (Abele et al. 2002, Heise et al. 2003, Keller et al. 2004) and similar effects can be expected in marine fish.

A heat shock response has been demonstrated in a number of studies using fish cells (Arai et al. 1994, Airaksinen et al. 1998, 2003) and also in whole animal experiments with marine (Dietz & Somero 1992) and freshwater fish (Molina et al. 2000) exposed to a variety of stressors including heat (for review see Iwama et al. 1998). We studied HSP70 as it represents the most temperature sensitive HSP family (Krebs & Bettencourt 1999, Kregel 2002) also in fish (Iwama et al. 1998). Insignificantly higher HSP70 levels were found only after recovery from 26°C, which supports the view that major inactivation of native proteins has not yet occurred at this temperature. This conclusion is in line with the finding that protein oxidation remained close to control levels. It is commonly observed, that the synthesis of HSPs is activated near the upper temperatures that ectothermal species encounter in their habitat (Tomanek & Somero 2000). After a relatively short heat stress

period of 2 h, HSP are supposed to start increasing directly after returning the fishes to control temperatures. It seems possible, that HSP levels were higher earlier during the recovery phase. A shorter recovery phase may therefore have detected HSP levels also following milder heat stress of 18 or 22°C. Thus, in the present eelpout case HSPs were either not induced although Tc was reached or measurements after 24 h were too late to detect the heat shock response. In addition, it is conceivable that a preconditioning exposure to sub-critical heating may be required to confer enhanced heat shock protein expression, leading to transiently improved tolerance of severe heat stress (Moseley 1997, King et al. 2002, reviewed in Kregel 2002).

Recent results by Treinin et al. (2003) demonstrated that the transcription factor HIF-1 is essential for heat acclimation in *C. elegans* and probably also in rat and mouse. Stabilization or activation of HIF-1 occurring within 1 h of heat exposure in liver and kidney from mice appeared to be mediated by increased HSP90 levels (Katschinski et al. 2002). Furthermore, increased HIF-1 DNA binding was observed after long-term warm acclimation (to 5°C) of the Antarctic eelpout *Pachycara brachycephalum* (Heise et al. ms in prep.). Therefore, we hypothesized that heat stress could activate hypoxic signaling also in the North Sea eelpout *Z. viviparus*. However, only sub-critical warming in the pejus temperature range (18°C) caused a significant induction of HIF-1 activity (fig. 4.A, B), whereas higher temperatures produced only weak EMSA signals. We therefore conclude that metabolic reorganization to improve oxygen supply can only be functional in the pejus range of this fish species (15-22°C, Zakhartsev et al. 2003) as aerobic scope diminishes. This interpretation would also apply to the findings obtained in the Antarctic eelpout acclimated to 5°C, which is close to pejus conditions (6-7°C, Mark et al. 2002). The observed transition of *Z. viviparus* liver tissue to anaerobic metabolism observed on longer warming beyond the Tc by Van Dijk et al. (1999) is a consequence of extreme hypoxia and probably regulated via reversible enzyme phosphorylation and leading to accumulation of glycolytic substrates, while not controlled by HIF-1 induced up-regulation of glycolytic enzymes (Semenza et al. 1994). Also, other transcription factors such as Sp1 and Sp3 could be responsible for the temperature mediated induction of glycolytic enzymes as shown for β-enolase and pyruvate kinase M in mammalian muscle cells subjected to hypoxia (Discher et al. 1998).

Moreover, HIF-1 DNA binding was generally higher at a more reduced redox environment. Thus the more oxidized conditions at 22 and 26°C (fig. 2.C) might "switch-off" the hypoxic signal (Abele 2002) and therewith prevent the more complex HIF-1

induced physiological response. By contrast, cold stress led to a more reduced cellular redox milieu and increased HIF-1 DNA binding (Heise et al., part A). All in all, the response to cold stress might much more allow for a HIF regulated hypoxia response than the response to heat stress in fish.

In any event, redox sensitivity and the presence of the protein in normoxic samples suggest that HIF-1 could have significant normoxic functions, which we presently know nothing about (Nikinmaa & Rees 2005).

Recovery time series

In contrast to cold stress (Heise et al. part A), we did not find increased oxidative stress markers (chemiluminescence and TBARS, fig 3. A. B.) upon 24 h recovery compared to the respective heat stress group, except for the higher carbonyl content after recovery from 18°C (fig. 3.C). Thus, on first sight, heat-induced hypoxia did not so clearly follow the ischemia / reperfusion pattern of oxidative stress in mammals (e.g. Halliwell & Gutteridge 1999). To see whether oxidative damage repair might have been faster and already finished within 24 h of recovery from high temperature stress, we performed a recovery-time series with another batch of eelpout caught in autumn.

An unaltered redox potential (ΔE) in all groups (fig. 5.E) confirmed our first experimental finding that neither sub-critical temperature stress (18°C) itself, nor the subsequent recovery phase disturbed the cellular redox milieu. Increased chemiluminescence rates and carbonyl contents during recovery intervals of up to 12 hrs (fig. 6.A, B) confirm our prediction that repair of oxidative damage could be faster following heat than following cold stress. The distinct time patterns of both parameters can be explained by the different underlying processes. Chemiluminescence depends on the progressive exploitation of small molecule antioxidants under ongoing oxidative stress, obviously still active after 12 h. Protein oxidation depends on oxidative stress and induction of repair mechanisms, i.e. proteasomal degradation and new synthesis (Dröge 2002), which set in after about 10 h of recovery, so that the 12 h values were already back to control levels in the recovery time series with autumn animals. Taking into account that exactly the same protein carbonyl parameters were still elevated after 24 hrs in summer animals illustrates the complex interaction of various repair systems and the unknown importance of seasonal preconditioning. Thus, it is possible that seasonal changes in the protein turnover between the experimental fishes, the first batch (A) caught in summer

2002 (kept at 12°C), and the second one caught in autumn 2003 (kept at 10°C), account for the detected variations.

Conclusions

The data we presented here support the hypothesis that heat-induced hypoxia and reoxygenation upon recovery in the North Sea eelpout have effects comparable to ischemia / reperfusion events in mammals. However, onset of oxidative damage repair occurred faster during recovery from heat than from cold stress (Heise et al., part A). Hypoxic signaling and subsequent metabolic reorganization to counterbalance thermal oxygen limitation could play a role only in the pejus temperature range, but appears impaired at critical and higher temperatures (22 and 26°C), presumably because of the more oxidized cellular redox state.

The fishes, most probably, do not die from oxidative damage, although SOD was impaired and lipid peroxidation was significantly increased upon critical heating. However, oxidative stress effects leading to a more oxidized cellular redox state under critical heat exposure (-245 mV *vs.* -260 mV in controls) can exacerbate the hypoxic deficit, by impairment of a more active HIF-1 signal. In contrast, we found a more reduced tissue redox state under stressful cooling (-275 mV *vs*. -260 in controls) and more significant HIF-1 DNA binding, which likely enabled a regulatory response and, thereby, stabilization of physiological functions in the cold.

Acknowledgements

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Tables

Table 1

Statistical comparison of oxidative stress markers in liver samples from unstressed controls and 18°C exposed *Z. viviparus* obtained during the graded heat stress experiment (A) and during the recovery time series (B). * significant difference between both experiments.

Parameter in summer (A) *vs.* fall (B)	**unstressed controls**		**18°C exposed fish**	
SOD activity at 20°C	(A) < (B)	p = 0.01 (*)	(A) < (B)	p < 0.01 (*)
GSH content	(A) > (B)	p = 0.72	(A) < (B)	p < 0.03 (*)
GSSG content	(A) < (B)	p = 0.14	(A) < (B)	p < 0.01 (*)
total glutathione	(A) < (B)	p = 0.49	(A) < (B)	p < 0.01 (*)
2GSSG/GSH	(A) < (B)	p = 0.06	(A) < (B)	p = 0.03 (*)
redox potential ΔE	(A) < (B)	p = 0.40	(A) < (B)	p = 0.34
carbonyl content	(A) > (B)	p < 0.01 (*)	(A) > (B)	p < 0.01

Figures

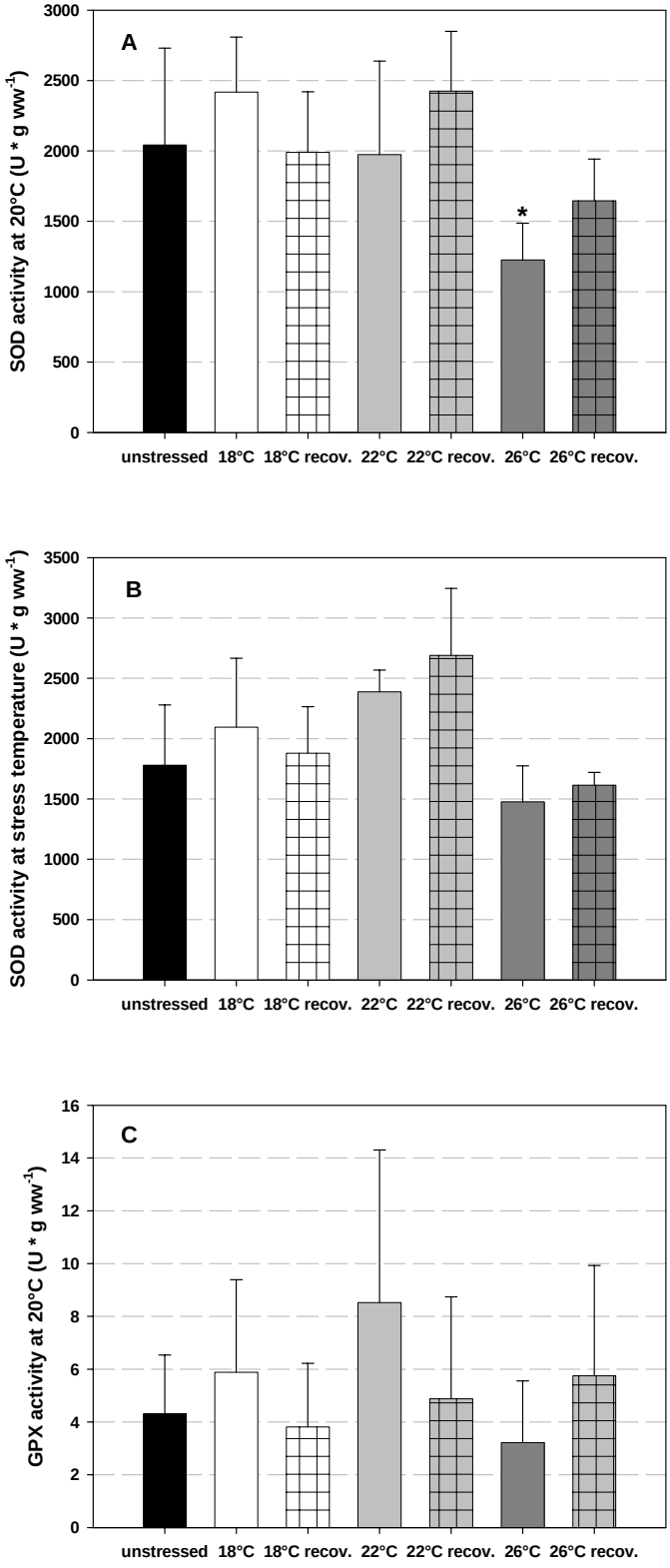


Figure 1

Antioxidant enzyme activity in *Z. viviparus* liver samples after 2 h of heat exposure to 18, 22 and 26°C as well as after 24 h recovery (recov.) from the respective temperature. A: superoxide dismutase (SOD) activity measured at 20°C. B: SOD activity measured at the respective stress temperature. C: glutathione peroxidase (GPX) activity measured at 20°C. Means ± standard deviation, n = 3-5, * = significantly different from the unstressed group, $p < 0.05$, ww = wet weight.

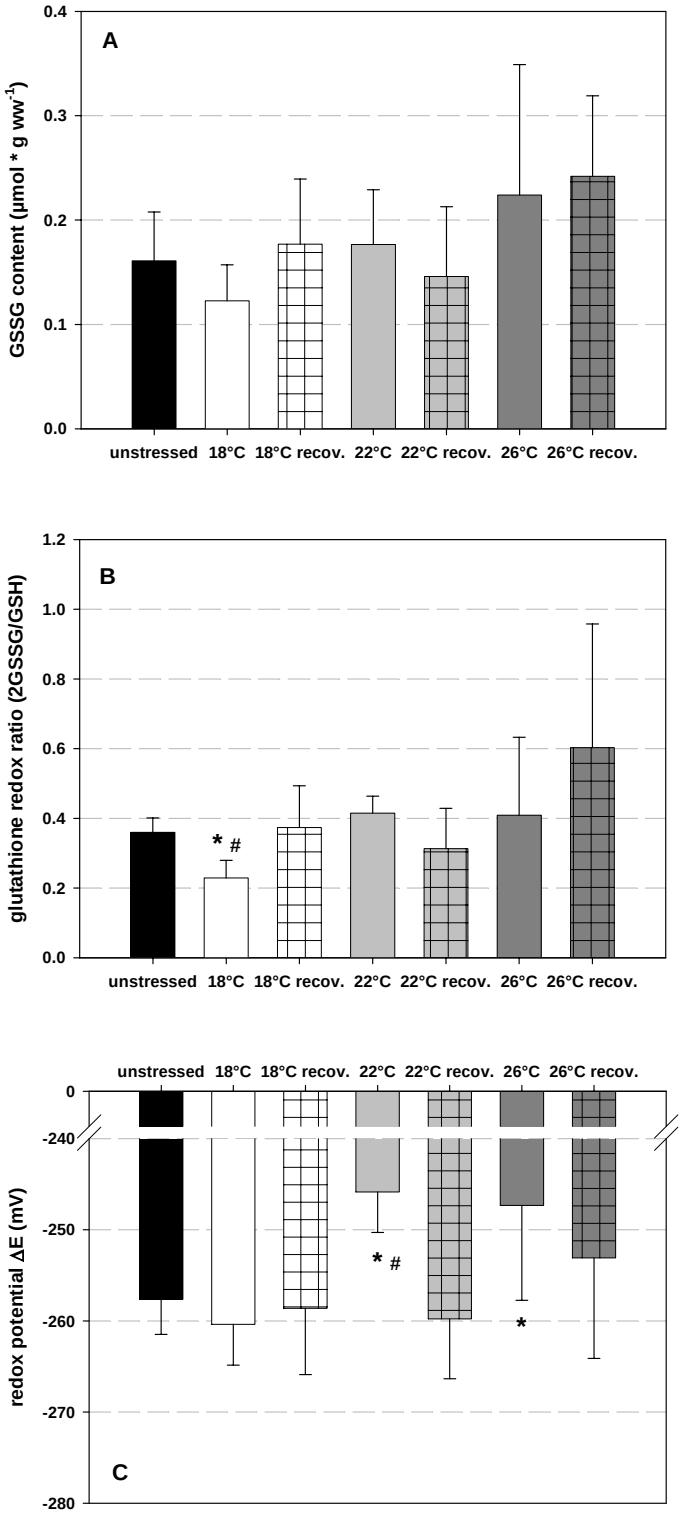


Figure 2

Glutathione redox chemistry in *Z. viviparus* liver samples after 2 h of heat exposure to 18, 22 and 26°C as well as after 24 h recovery (recov.) from these temperatures. A: content of oxidized glutathione (GSSG), B: Glutathione redox ratio (2GSSG/GSH), C: redox potential ΔE at *in situ* temperature and *in situ* intracellular pH (adopted from Sartoris et al. 2003, Larsen et al. 1997) calculated after Schaffer & Buettner (2001). Means ± standard deviation, n = 4-7, * = significantly different from the unstressed group, # = significant difference to the respective recovery group, $p < 0.05$, ww = wet weight.

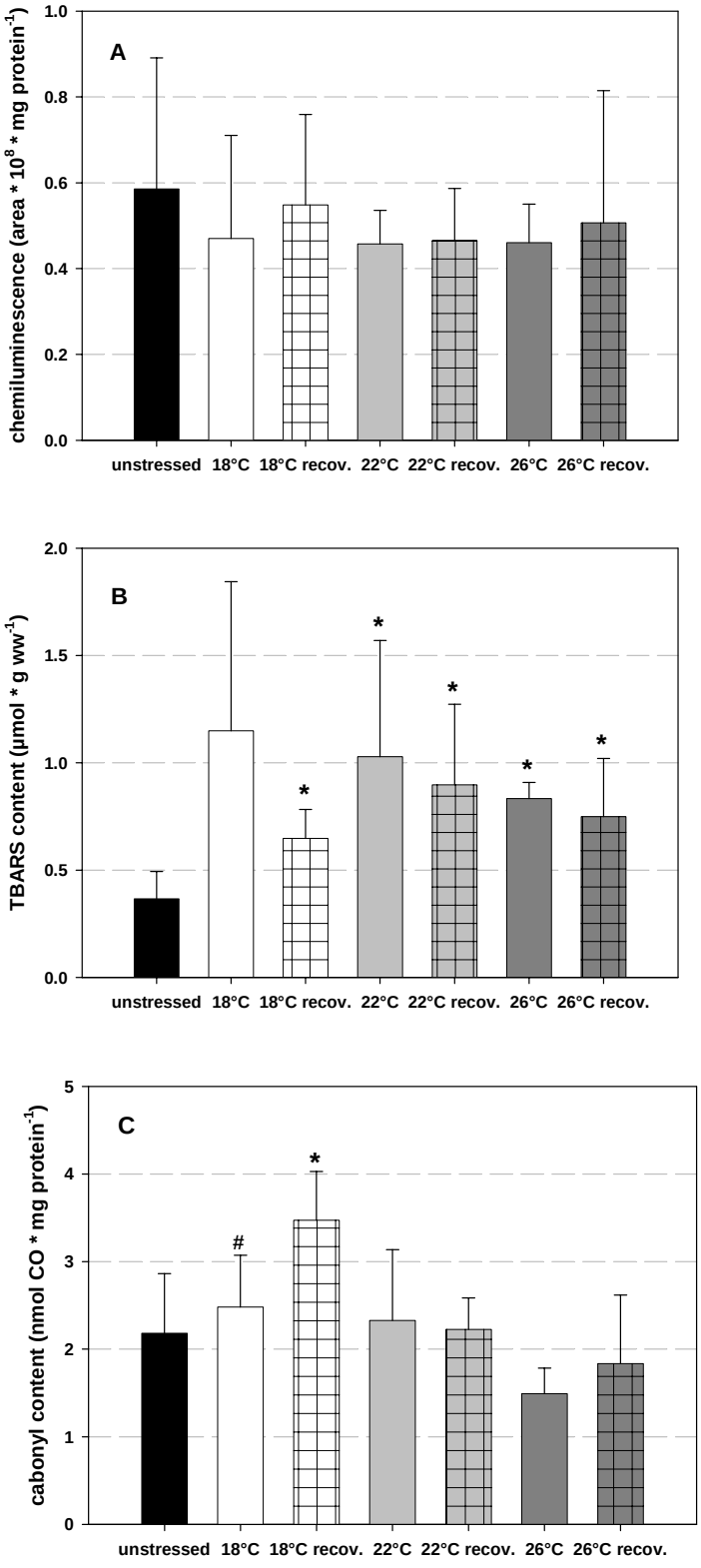


Figure 3

Oxidative stress markers in *Z. viviparus* liver samples after 2 h of heat exposure to 18, 22 and 26°C as well as after 24 h recovery (recov.) from these temperatures. A: tert-butyl hydroperoxide initiated chemiluminescence indicating imbalance between pro- and antioxidant processes, B: content of thiobarbituric reactive substances (TBARS) indicating lipid peroxidation, C: carbonyl content indicating protein oxidation. Means ± standard deviation, n = 4-6, * = significantly different from the unstressed group, # = significant difference to the respective recovery group, $p < 0.05$, ww = wet weight.

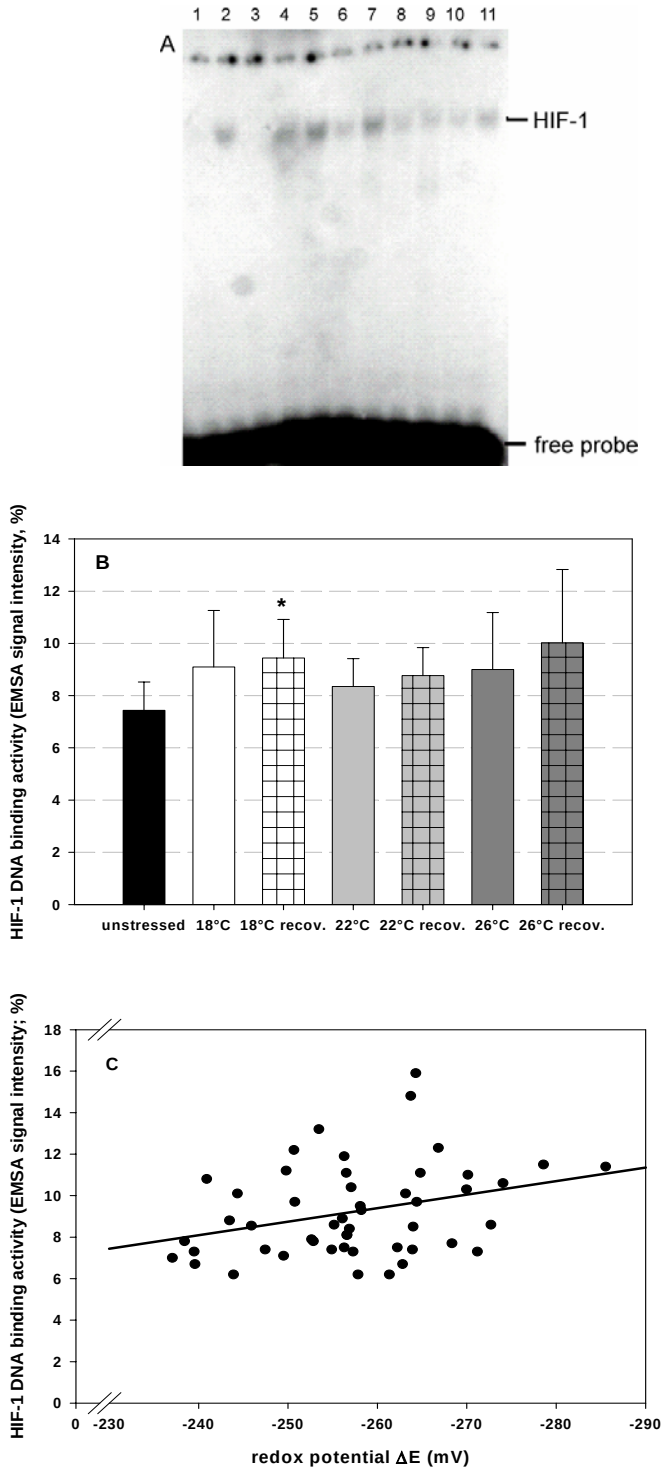


Figure 4

DNA binding activity of HIF-1 in *Z. viviparus* whole cell extracts from the liver after 2 h of heat exposure to 18, 22 and 26°C as well as after 24 h recovery (recov.) from these temperatures.

A: signal intensity of HIF-1 DNA binding to the human erythropoietin enhancer. Lanes 1: unstressed, 2: 1°C, 3: 1°C recovery, 4: 5°C, 5: 5°C recovery, 6: 18°C, 7: 18°C recovery, 8: 22°C, 9: 22°C recovery, 10: 26°C, 11: 26°C recovery; data taken from the heat stress experiment (this study) and from cold stress (Heise et al. part A). B: HIF-1 DNA binding. Means ± standard deviation, n = 4-5, * = significantly different from the unstressed group, $p < 0.05$. C: Linear regression demonstrating increased HIF-1 DNA binding at a more reduced redox environment ($R^2 = 0.3$, n = 50, $p < 0.05$, Statview 5.0). Data taken from the heat stress experiment (this study) and from cold stress (Heise et al. part A).

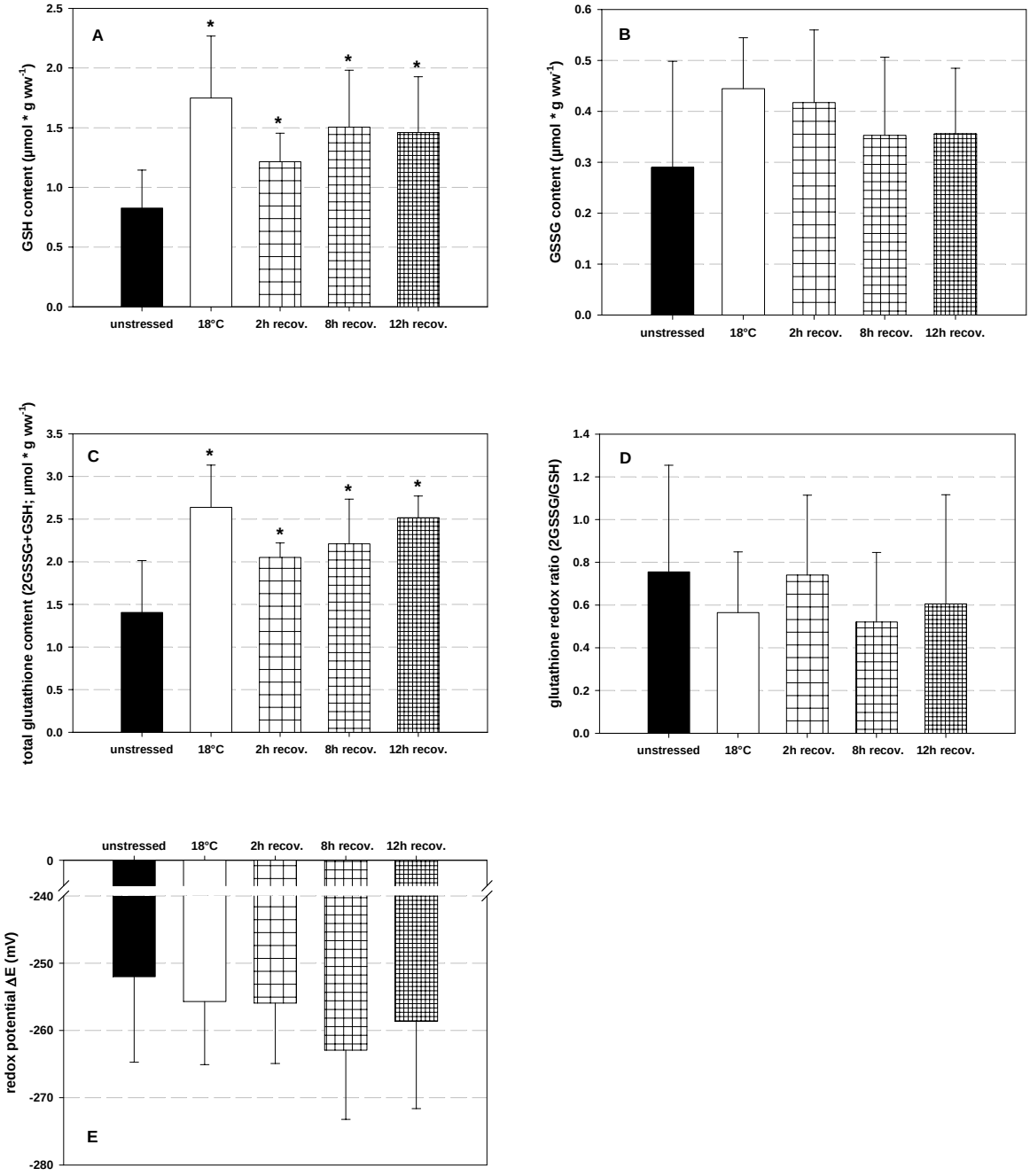


Figure 5

Glutathione redox chemistry in *Z. viviparus* liver samples after 2 h of exposure to 18°C as well as after 2, 8 or 12 h of recovery (recov.). A: content of reduced glutathione (GSH), B: content of oxidized glutathione (GSSG), C: total glutathione content (2GSSG+GSH), D: glutathione redox ratio (2GSSG/GSH), E: redox potential ΔE at *in situ* temperature and *in situ* intracellular pH (adopted from Sartoris et al. 2003, Larsen et al. 1997) calculated after Schaffer & Buettner (2001). Means ± standard deviation, n = 4-5, * = significantly different from the unstressed group, $p < 0.05$, ww = wet weight.

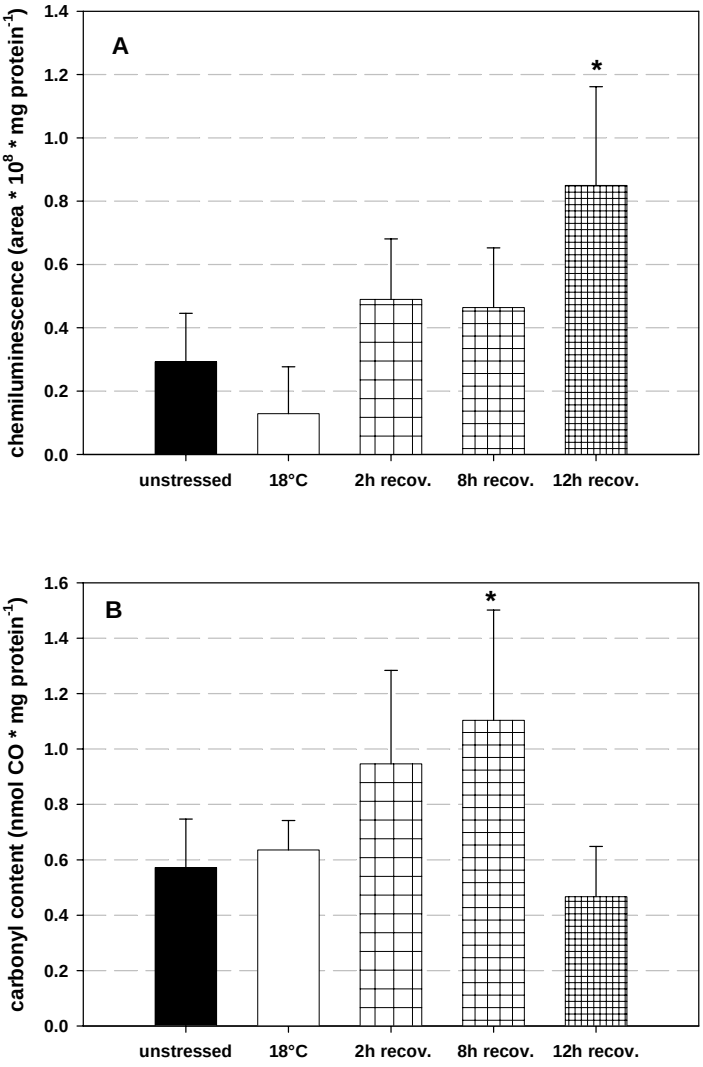


Figure 6
Oxidative stress markers in *Z. viviparus* liver samples after 2 h of heat exposure to 18°C as well as after 2, 8 or 12 h of recovery (recov.). A: tert-butyl hydroperoxide initiated chemiluminescence indicating imbalance between pro- and antioxidant processes, B: carbonyl content indicating protein oxidation. Means ± standard deviation, n = 3-5, * = significantly different from the unstressed group, $p < 0.05$.

4. Additional results

In the following section I will present additional results which are not included in any of the publications I - IV.

4.1. Unknown thiol peak in liver samples from *P. brachycephalum*

In publication I data of glutathione-related parameters (GSH, GSSG, total glutathione, glutathione redox ratio) determined in the liver tissue of *P. brachycephalum*, acclimated to 0 and 5°C are presented. The HPLC spectrum of FDNB-derivatised liver samples from *P. brachycephalum* revealed the presence of an additional thiol peak (termed: "unknown peak") eluting before the peak of the internal standard γ-glutamyl-glutamate. The unknown compound displayed an extremely large peak area; hence the compound may be present in very high concentrations. (For comparison: peak area of the unknown compound was about 42 000 000 arbitrary units *vs.* about 700 000 units for GSH obtained from liver samples of both eelpout species.) However, it has to be admitted that a large peak area may not always be linked to a high concentration of a compound, because different substances may display different reactivities towards FDNB (M. Köck, pers. com.).

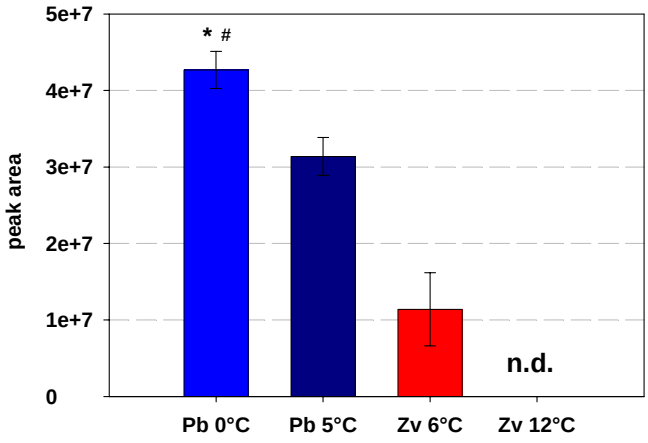


Fig. 4.1: Peak area of unknown thiol peak detected in FDNB-derivatised liver samples of *P. brachycephalum* (Pb) and *Z. viviparus* (Zv), maintained at different environmental temperatures. * intra-species comparison, significantly different to the respective warmer water temperature. n.d = not determined, # inter-species comparison, significant difference between Pb 0°C and Zv 6°C, n = 4-7, $p < 0.05$.

The peak area, thus the concentration of the thiol compound was dependent on acclimation temperature (fig. 4.1). A similar peak could be detected in liver samples from *Z. viviparus*, however, at much lower concentrations, with peak area also depending on environmental temperature. In 12°C *Z. viviparus* the peak area of the unknown compound

could not be determined, as it was not sufficiently separated from another peak. However, peak height in 12°C *Z. viviparus* was about 30 mAU *vs*. 300 mAU in 6°C *Z. viviparus* and about 2 000 mAU in 0°C acclimated *P. brachycephalum*. Thus, in both species, the peak area was larger at the respective colder water temperature (6°C for *Z. viviparus*; 0°C for *P. brachycephalum*).

Assuming that this unknown thiol occurred at putatively high levels, as deduced from the large peak size, it might have an important role for intracellular redox balance in the liver of *P. brachycephalum*. Therefore, I tried to identify the compound, by using commercially available thiol standards, processed in the same manner as the glutathione standards (fig. 4.2). However, the unknown peak did not overlap with any of the tested standards such as cysteine, cysteine-cysteine, or cysteine-glutathione.

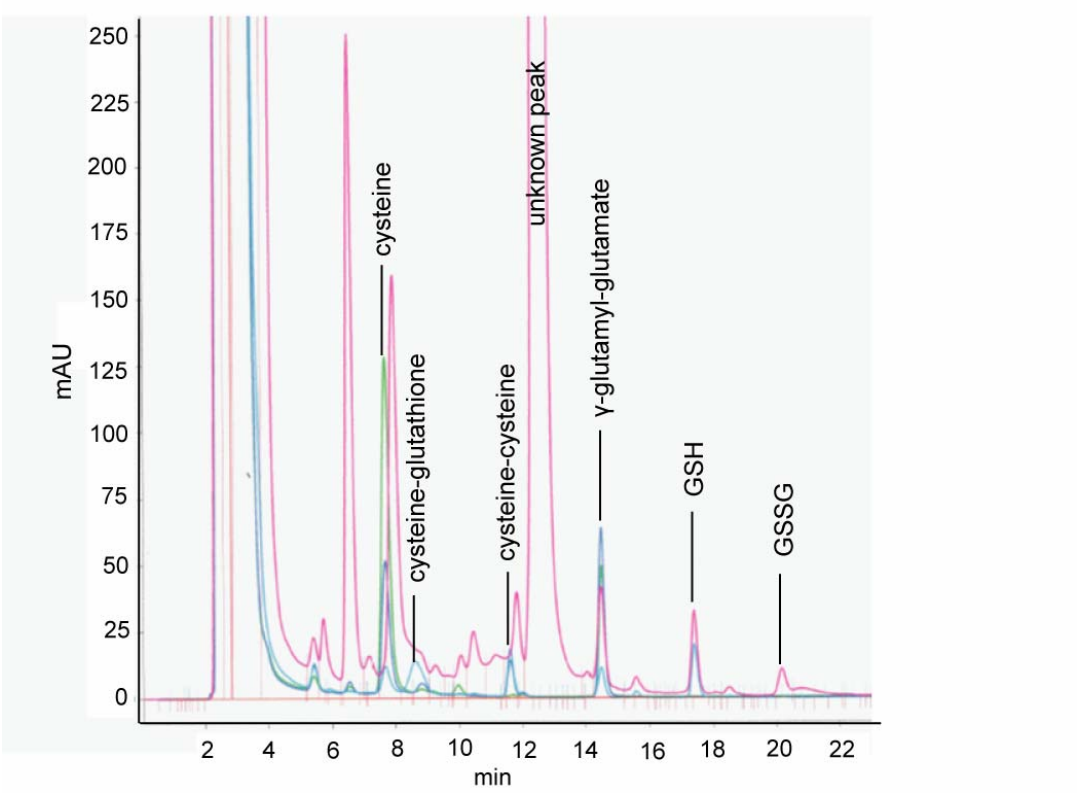


Fig. 4.2: overlay of HPLC spectra of FDNB-derivatised liver samples from *P. brachycephalum* (pink) as well as the thiol standards cysteine (green), cysteine-glutathione (light blue), and cysteine-cysteine (dark blue).

The unknown compound might represent glutamate-cysteine, which is formed by the γ-glutamyl-cycle as a precursor of glutathione (Kidd 1997). It may be speculated that in the polar *P. brachycephalum* a high content of glutathione precursor compounds serve as a pool to meet a rapid glutathione turn-over a low water temperatures. Within the scope of

this thesis it was not possible to further clarify the identity and function of the unknown thiol compound.

Additional standards could be tested in future studies. Also, methods such as HPLC mass spectroscopy could be applied for chemical characterisation of the compound.

4.2. Cloning and sequencing of the Hypoxia-inducible factor

4.2.1 HIF-1α

Zoarcid full length HIF-1α cDNA sequences were identified with sizes of 3 383 (*Z. viviparus*) and 3 283 (*P. brachycephalum*) base pairs (fig. 4.3) with an open reading frame of 2 244 base pairs, coding for 747 amino acids (fig. 4.4). The zoarcid nucleotide sequences displayed 96% identity. The identity level was 40 - 41% to the human (*Hs*) sequence and 47 - 63% to different fish sequences (tab. 4.1).

Tab. 4.1: Identities within full length HIF-1α cDNA sequences of *Zoarces viviparus* (*Zv*; DQ089695) and *Pachycara brachycephalum* (*Pb*; DQ089691) to the sequences of rainbow trout *Oncorhynchus mykiss* (*Om*; AF304864), grass carp *Ctenopharyngodon idella* (*Ci*; AY326951) and zebrafish *Danio rerio* (*Dr*; AY450269) as well as human *Homo sapiens* (*Hs*; 31077212). bp = base pairs.

	Identity to *Zv*	Identity to *Pb*
Om	2299 bp = 63%	2293 bp = 63%
Ci	1936 bp = 50%	1925 bp = 50%
Dr	1924 bp = 47%	1917 bp = 47%
Hs	1628 bp = 41%	1622 bp = 40%

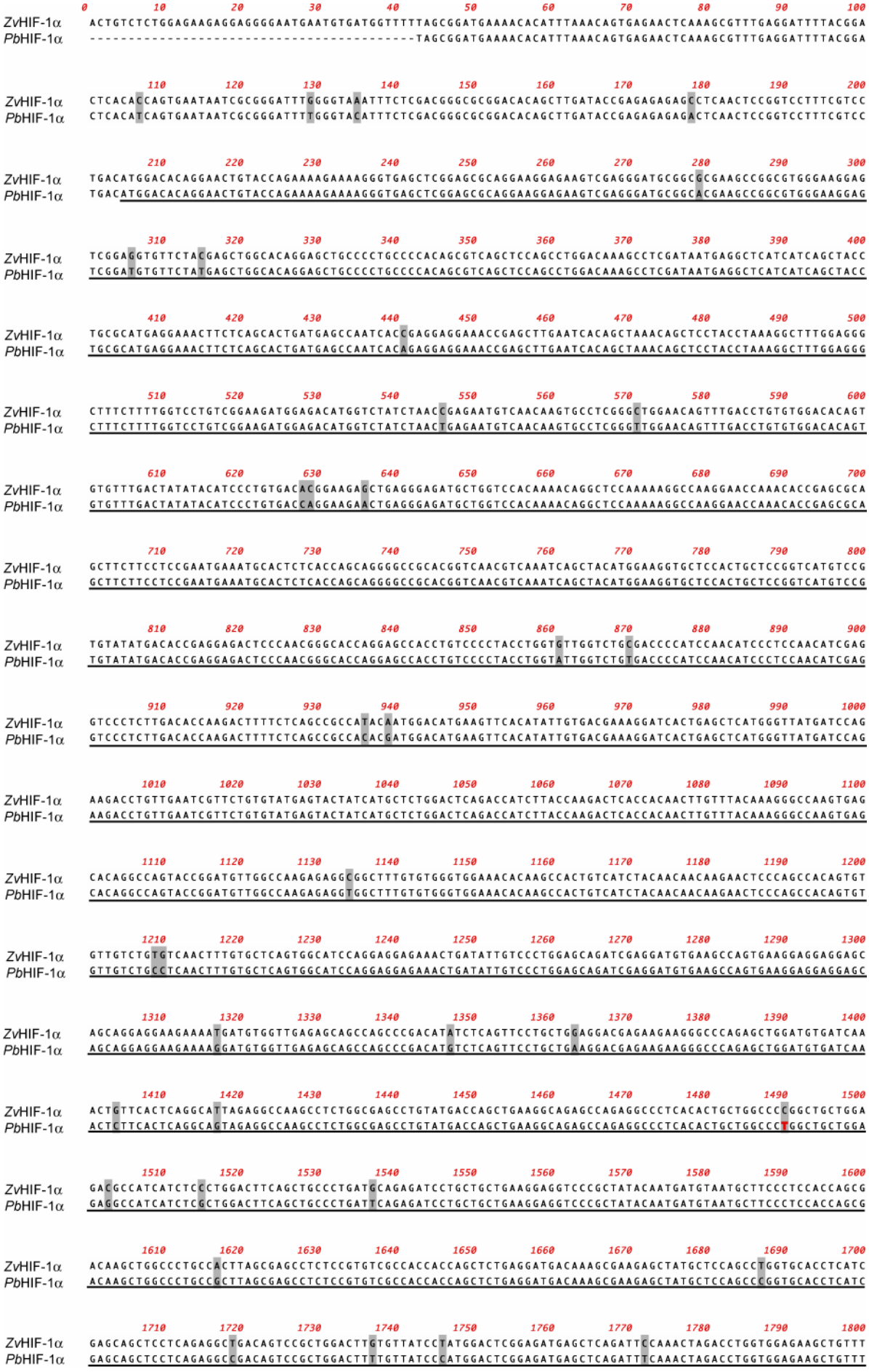
The deduced amino acid sequences of HIF-1α showed 96% identity (97% similarity) between both zoarcids, 61 - 71% identity (72 - 83% similarity) to other fishes and 50% identity (64% similarity) to the human sequence (tab. 4.2).

Tab. 4.2: Identities within deduced full length HIF-1α amino acid sequences of *Zoarces viviparus* (*Zv*; DQ089695) and *Pachycara brachycephalum* (*Pb*; DQ089691) to the sequences of rainbow trout *Oncorhynchus mykiss* (*Om*; AF304864), grass carp *Ctenopharyngodon idella* (*Ci*; AY326951) and zebrafish *Danio rerio* (*Dr*; AY450269) as well as human *Homo sapiens* (*Hs*; 31077212). aa = amino acid.

	identity		similarity	
	Zv	*Pb*	*Zv*	*Pb*
Om	548 aa = 71%	548 aa = 71%	93 aa = 12%	96 aa = 12%
Ci	481 aa = 62%	479 aa = 61%	96 aa = 12%	100 aa = 12%
Dr	485 aa = 62%	481 aa = 61%	88 aa = 11%	92 aa = 11%
Hs	421 aa = 50%	421 aa = 50%	117 aa = 14%	123 aa = 14%

Functional sites of HIF-1α such as the basic Helix-Loop-Helix (bHLH) domain, PAS A and PAS B domains, the oxygen-dependent degradation domain (ODD), as well as the two transactivation domains N-TAD and C-TAD were identified in the deduced amino acid sequences of zoarcid HIF-1α (fig. 4.4). Moreover, the hydroxyl-accepting asparagine residue of the C-TAD (Asn803 in *Hs*HIF-1α) as well as the functional lysine residue of the ODD (Lys532 in *Hs*HIF-1α) were conserved in both zoarcid sequences. By contrast, only *Zv*HIF-1α displayed both hydroxyl-accepting proline residues within the ODD, whereas in

*Pb*HIF-1α the N-terminal proline (Pro402 in *Hs*HIF-1α) was substituted by leucine (fig. 4.4).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PbHIF-1α ----------TAGCGGATGAAAACACATTTAAACAGTGAGAACTCAAAGCGTTTGAGGATTTTACGGA
110 120 130 140 150 160 170 180 190 200
ZvHIF-1α CTCACACCAGTGAATAATCGCGGGATTTGGGGTAAATTTCTCGACGGGCGCGGACACAGCTTGATACCGAGAGAGAGCCTCAACTCCGGTCCTTTCGTCC
PbHIF-1α CTCACATCAGTGAATAATCGCGGGATTTTGGGTACATTTCTCGACGGGCGCGGACACAGCTTGATACCGAGAGAGAGACTCAACTCCGGTCCTTTCGTCC
210 220 230 240 250 260 270 280 290 300
ZvHIF-1α TGACATGGACACAGGAACTGTACCAGAAAAGAAAAGGGTGAGCTCGGAGCGCAGGAAGGAGAAGTCGAGGGATGCGGCGCGAAGCCGGCGTGGGAAGGAG
PbHIF-1α TGACATGGACACAGGAACTGTACCAGAAAAGAAAAGGGTGAGCTCGGAGCGCAGGAAGGAGAAGTCGAGGGATGCGGCACGAAGCCGGCGTGGGAAGGAG
310 320 330 340 350 360 370 380 390 400
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PbHIF-1α TCGGATGTGTTCTATGAGCTGGCACAGGAGCTGCCCCTGCCCCACAGCGTCAGCTCCAGCCTGGACAAAGCCTCGATAATGAGGCTCATCATCAGCTACC
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ZvHIF-1α TGCGCATGAGGAAACTTCTCAGCACTGATGAGCCAATCACCGAGGAGGAAACCGAGCTTGAATCACAGCTAAACAGCTCCTACCTAAAGGCTTTGGAGGG
PbHIF-1α TGCGCATGAGGAAACTTCTCAGCACTGATGAGCCAATCACAGAGGAGGAAACCGAGCTTGAATCACAGCTAAACAGCTCCTACCTAAAGGCTTTGGAGGG
510 520 530 540 550 560 570 580 590 600
ZvHIF-1α CTTTCTTTTGGTCCTGTCGGAAGATGGAGACATGGTCTATCTAACCGAGAATGTCAACAAGTGCCTCGGGCTGGAACAGTTTGACCTGTGTGGACACAGT
PbHIF-1α CTTTCTTTTGGTCCTGTCGGAAGATGGAGACATGGTCTATCTAACTGAGAATGTCAACAAGTGCCTCGGGTTGGAACAGTTTGACCTGTGTGGACACAGT
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PbHIF-1α GTGTTTGACTATATACATCCCTGTGACCAGGAAGAACTGAGGGAGATGCTGGTCCACAAAACAGGCTCCAAAAAGGCCAAGGAACCAAACACCGAGCGCA
710 720 730 740 750 760 770 780 790 800
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PbHIF-1α GCTTCTTCCTCCGAATGAAATGCACTCTCACCAGCAGGGGCCGCACGGTCAACGTCAAATCAGCTACATGGAAGGTGCTCCACTGCTCCGGTCATGTCCG
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PbHIF-1α TGTATATGACACCGAGGAGACTCCCAACGGGCACCAGGAGCCACCTGTCCCCTACCTGGTATTGGTCTGTGACCCCATCCAACATCCCTCCAACATCGAG
910 920 930 940 950 960 970 980 990 1000
ZvHIF-1α GTCCCTCTTGACACCAAGACTTTTCTCAGCCGCCATACAATGGACATGAAGTTCACATATTGTGACGAAAGGATCACTGAGCTCATGGGTTATGATCCAG
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ZvHIF-1α CACAGGCCAGTACCGGATGTTGGCCAAGAGAGGCGGCTTTGTGTGGGTGGAAACACAAGCCACTGTCATCTACAACAACAAGAACTCCCAGCCACAGTGT
PbHIF-1α CACAGGCCAGTACCGGATGTTGGCCAAGAGAGGTGGCTTTGTGTGGGTGGAAACACAAGCCACTGTCATCTACAACAACAAGAACTCCCAGCCACAGTGT
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
ZvHIF-1α GTTGTCTGTGTCAACTTTGTGCTCAGTGGCATCCAGGAGGAGAAACTGATATTGTCCCTGGAGCAGATCGAGGATGTGAAGCCAGTGAAGGAGGAGGAGC
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PbHIF-1α ACTCTTCACTCAGGCAGTAGAGGCCAAGCCTCTGGCGAGCCTGTATGACCAGCTGAAGGCAGAGCCAGAGGCCCTCACACTGCTGGCCCTGGCTGCTGGA
1510 1520 1530 1540 1550 1560 1570 1580 1590 1600
ZvHIF-1α GACGCCATCATCTCCCTGGACTTCAGCTGCCCTGATGCAGAGATCCTGCTGCTGAAGGAGGTCCCGCTATACAATGATGTAATGCTTCCCTCCACCAGCG
PbHIF-1α GAGGCCATCATCTCGCTGGACTTCAGCTGCCCTGATTCAGAGATCCTGCTGCTGAAGGAGGTCCCGCTATACAATGATGTAATGCTTCCCTCCACCAGCG
1610 1620 1630 1640 1650 1660 1670 1680 1690 1700
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PbHIF-1α ACAAGCTGGCCCTGCCGCTTAGCGAGCCTCTCCGTGTCGCCACCACCAGCTCTGAGGATGACAAAGCGAAGAGCTATGCTCCAGCCCGGTGCACCTCATC
1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
ZvHIF-1α GAGCAGCTCCTCAGAGGCTGACAGTCCGCTGGACTTGTGTTATCCTATGGACTCGGAGATGAGCTCAGATTCCAAACTAGACCTGGTGGAGAAGCTGTTT
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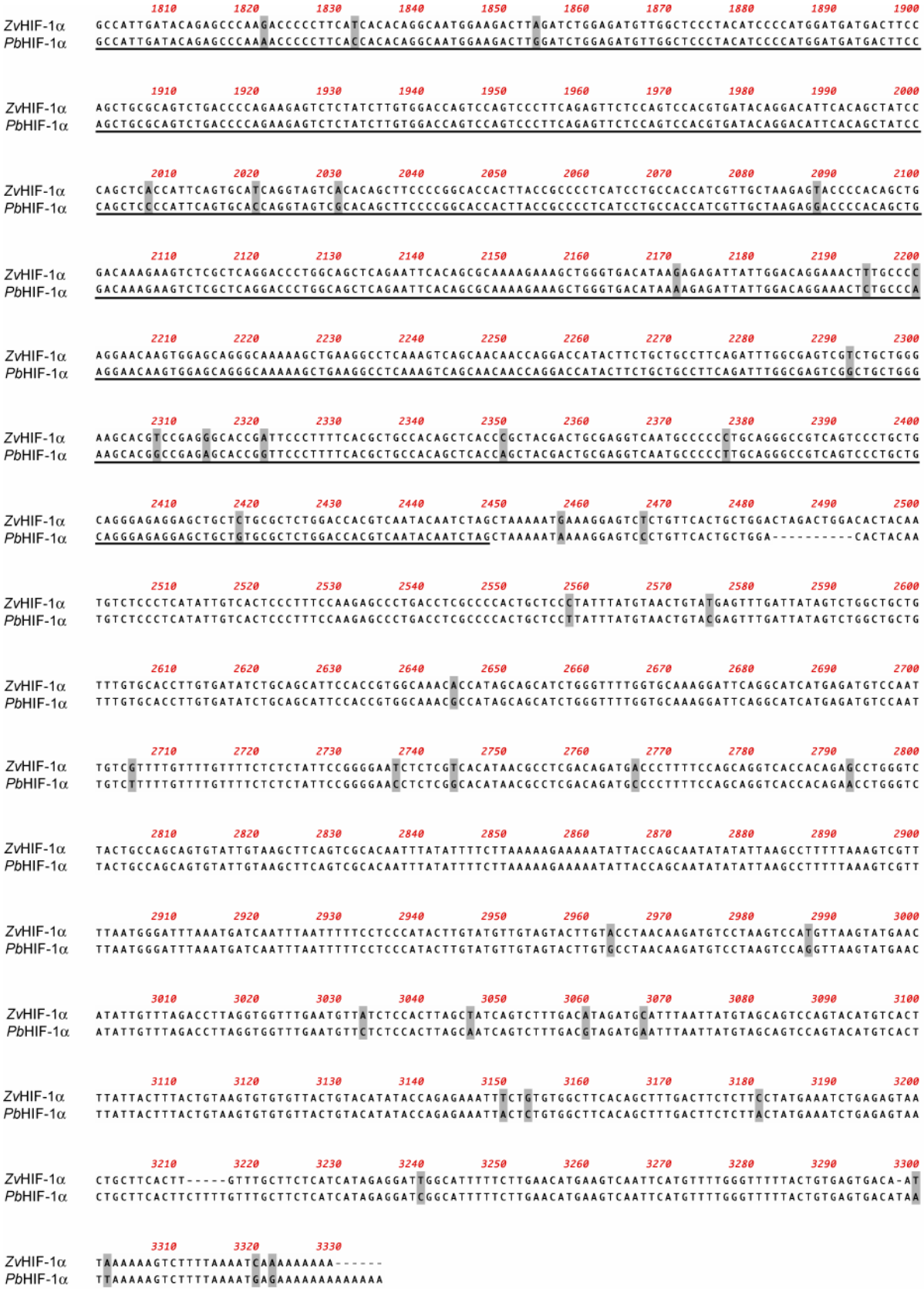


Fig. 4.3: Full length HIF-1α cDNA sequences of *P. brachycephalum* (*Pb*; DQ089690) and *Z. viviparus* (*Zv*; DQ089695) in *5`-3`* direction. The black line below the sequences indicates the open reading frames. The C-T nucleotide conversion leading to Pro-Leu substitution in the deduced amino acid sequence of *Pb*HIF-1α is highlighted in red.

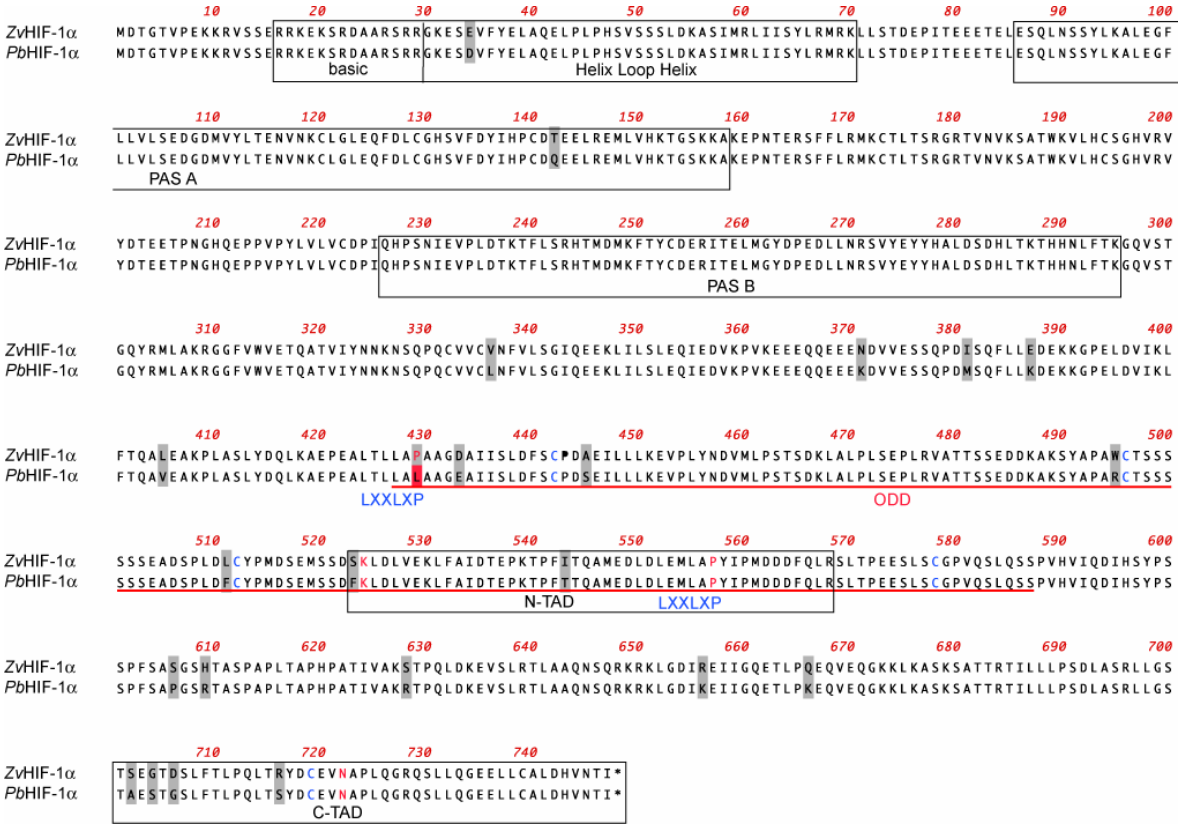


Fig. 4.4: Deduced full length amino acid sequences of HIF-1α of *P. brachycephalum* (*Pb*; DQ089691) and *Z. viviparus* (*Zv*; DQ089695) in C-N direction. Black boxes indicate functional domains, the red line below the sequence mark the oxygen-dependent degradation domain (ODD) adopted from Soitamo et al. (2001). N-TAD = N-terminal transactivation domain, C-TAD = C-terminal transactivation domain. LXXLXP represents the binding motif for prolyl hydroxylases (Huang et al. 2002). Functional amino acids are highlighted in red, cysteines in ODD and C-TAD blue. The Pro-Leu substitution of the N-terminal proline residue (Pro402 in human HIF-1α) in *P. brachycephalum* is marked red.

4.2.2 HIF-2α

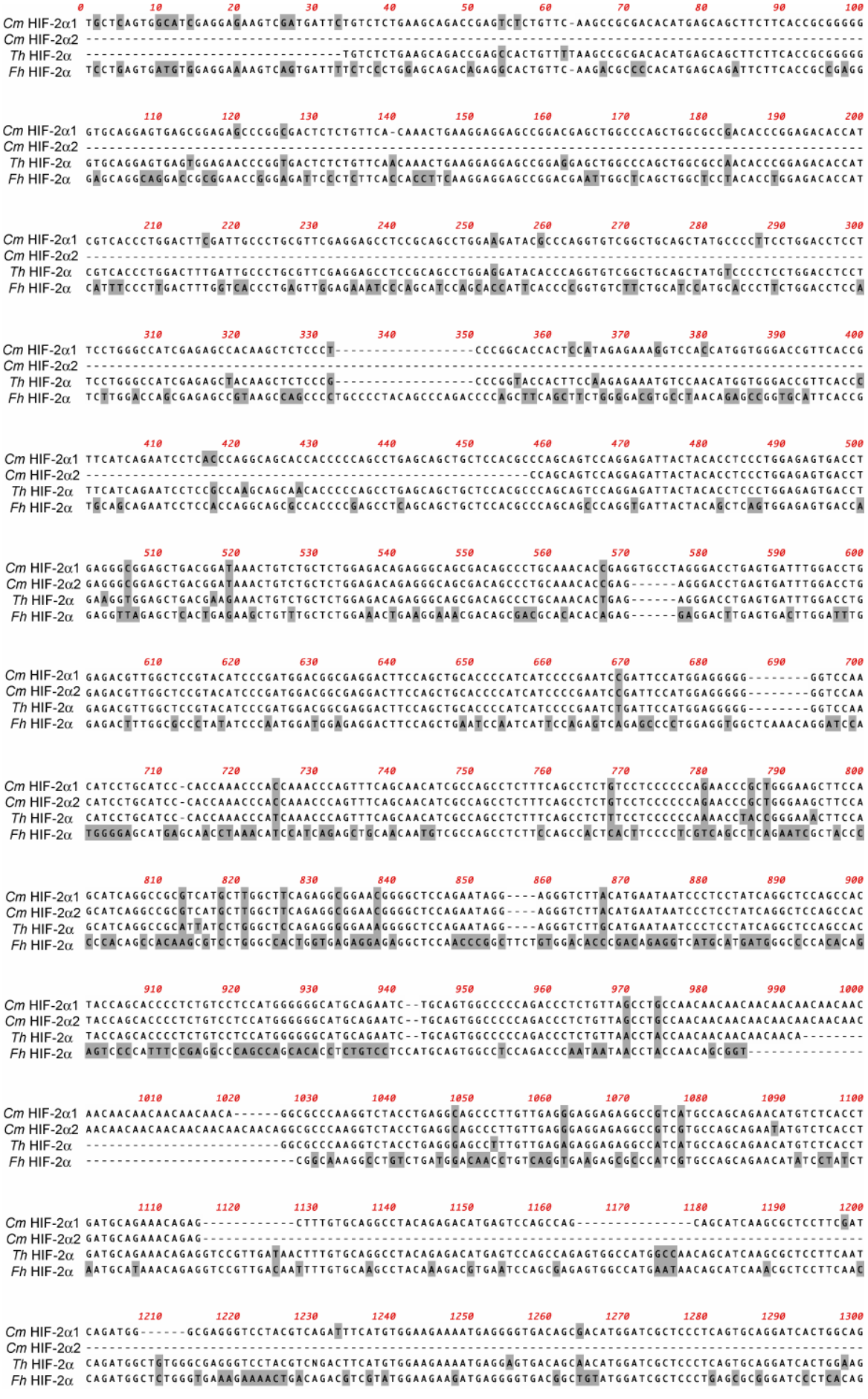
Upon amplification of a HIF-1α fragment using primer pair III (see "Material & Methods") an additional fragment of 1 497 nucleotides was detected in PCR samples of the notothenioids *T. hansoni* and *C. myersi* and identified as HIF-2α. Fig. 4.5 depicts the determined cDNA sequences, aligned with HIF-2α of the killifish *Fundulus heteroclitus* (*Fh*) and fig. 4.7 shows the deduced amino acid sequences, aligned with the sequences of *F. heteroclitus (Fh)* and the grass carp *Ctenopharyngodon idella* (*Ci*). The partial HIF-2α nucleotide sequences displayed 91% identity between the notothenioids, and 65 - 66% to the killifish. The deduced amino acid sequences had 80% identity (82% similarity) between notothenioids and had 50 - 57% identity (61 - 67%% similarity) to the partial killifish sequence (*Fh*). Less conservation was found compared to the partial grass carp sequence (*Ci*) with 42 - 48% identity (55 - 62% similarity) (tab. 4.3 and phylogenetic tree in fig. 4.6).

Tab. 4.3: Identities and similarities within deduced amino acid of partial HIF-2α sequences (C-terminal half) of *Chionodraco myersi* (*Cm*; DQ089690), *Trematomus hansoni* (*Th*; DQ089693), *Ctenopharyngodon idella* (*Ci*, AY577524), *Fundulus heteroclitus* (*Fh*, AF402782) and *Homo sapiens* (*Hs*, BC051338).

Identity / similarity	*Cm*	*Th*	*Ci*	*Fh*	*Hs*
Cm	-	80% / 82%	42% / 55%	50% / 61%	34% / 44%
Th	80% / 82%	-	48% / 62%	57% / 67%	36% / 47%
Ci	42% / 55%	48% / 62%	-	38% / 51%	38% / 51%
Fh	50% / 61%	57% / 67%	43% / 59%	-	34% / 51%
Hs	34% / 44%	36% / 47%	38% / 51%	34% / 49%	-

Despite low identities (similarities) of 42 - 80% (55 - 82%) of the C-terminal half of HIF-2α sequences between fishes, as well as between fishes and human with 34 - 38% identity (44 - 51% similarity), functional proline residues and the LXXLXP motif for PHD binding in the ODD as well as the functional asparagine residue and di-leucine repeats in the C-TAD were conserved in all sequences (fig. 4.7). Additionally, a Glu cluster, comprising 6 residues in *Th*HIF-2α and 15 residues in *Cm*HIF-2α, located C-terminal of the functional proline residues, was identified.

To clarify the centre part of the 1 497 bp fragments of notothenioid HIF-2α, an additional primer pair (IV; see Material & Methods) was constructed yielding a 637 bp product at position 438-1075 on partial *Cm*HIF-2α cDNA. Sequencing of plasmids obtained from different clones revealed an alternative HIF-2α variant in *C. myersi* (fig. 4.5). The deduced amino acid sequence missed the proline and arginine residue at position 191 - 192 of the earlier sequence, but displayed two additional glutamate residues in the Glu cluster.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Cm HIF-2α2 ----------
Th HIF-2α ----------TGTCTCTGAAGCAGACCGAGCCACTGTTTTAAGCCGCGACACATGAGCAGCTTCTTCACCGCGGGGG
Fh HIF-2α TCCTGAGTGATGTGGAGGAAAAGTCAGTGATTTTCTCCCTGGAGCAGACAGAGGCACTGTTC-AAGACGCCCCACATGAGCAGATTCTTCACCGCCGAGG
110 120 130 140 150 160 170 180 190 200
Cm HIF-2α1 GTGCAGGAGTGAGCGGAGAGCCCGGCGACTCTCTGTTCA-CAAACTGAAGGAGGAGCCGGACGAGCTGGCCCAGCTGGCGCCGACACCCGGAGACACCAT
Cm HIF-2α2 ----------
Th HIF-2α GTGCAGGAGTGAGTGGAGAACCCGGTGACTCTCTGTTCAACAAACTGAAGGAGGAGCCGGAGGAGCTGGCCCAGCTGGCGCCAACACCCGGAGACACCAT
Fh HIF-2α GAGCAGGCAGGACCGCGGAACCGGGAGATTCCCTCTTCACCACCTTCAAGGAGGAGCCGGACGAATTGGCTCAGCTGGCTCCTACACCTGGAGACACCAT
210 220 230 240 250 260 270 280 290 300
Cm HIF-2α1 CGTCACCCTGGACTTCGATTGCCCTGCGTTCGAGGAGCCTCCGCAGCCTGGAAGATACGCCCAGGTGTCGGCTGCAGCTATGCCCCTTCCTGGACCTCCT
Cm HIF-2α2 ----------
Th HIF-2α CGTCACCCTGGACTTTGATTGCCCTGCGTTCGAGGAGCCTCCGCAGCCTGGAGGATACACCCAGGTGTCGGCTGCAGCTATGTCCCCTCCTGGACCTCCT
Fh HIF-2α CATTTCCCTTGACTTTGGTCACCCTGAGTTGGAGAAATCCCAGCATCCAGCACCATTCACCCCGGTGTCTTCTGCATCCATGCACCCTTCTGGACCTCCA
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Cm HIF-2α2 ----------
Th HIF-2α TCCTGGGCCATCGAGAGCTACAAGCTCTCCCG----------CCCGGTACCACTTCCAAGAGAAATGTCCAACATGGTGGGACCGTTCACCC
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Th HIF-2α TTCATCAGAATCCTCCGCCAAGCAGCAACACCCCCAGCCTGAGCAGCTGCTCCACGCCCAGCAGTCCAGGAGATTACTACACCTCCCTGGAGAGTGACCT
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810 820 830 840 850 860 870 880 890 900
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Th HIF-2α GCATCAGGCCGCATTATCCTGGGCTCCAGAGGGGGAAAGGGGCTCCAGAATAGG----AGGGTCTTGCATGAATAATCCCTCCTATCAGGCTCCAGCCAC
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910 920 930 940 950 960 970 980 990 1000
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Cm HIF-2α2 TACCAGCACCCCTCTGTCCTCCATGGGGGGCATGCAGAATC--TGCAGTGGCCCCCAGACCCTCTGTTAGCCTGCCAACAACAACAACAACAACAACAAC
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Fh HIF-2α AGTCCCCATTTCCGAGGCCCAGCCAGCACACCTCTGTCCTCCATGCAGTGGCCTCCAGACCCAATAATAACCTACCAACAGCGGT----------
1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
Cm HIF-2α1 AACAACAACAACAACAACA------GGCGCCCAAGGTCTACCTGAGGCAGCCCTTGTTGAGGGAGGAGAGGCCGTCATGCCAGCAGAACATGTCTCACCT
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Th HIF-2α GATGCAGAAACAGAGGTCCGTTGATAACTTTGTGCAGGCCTACAGAGACATGAGTCCAGCCAGAGTGGCCATGGCCAACAGCATCAAGCGCTCCTTCAAT
Fh HIF-2α AATGCATAAACAGAGGTCCGTTGACAATTTTGTGCAAGCCTACAAAGACGTGAATCCAGCGAGAGTGGCCATGAATAACAGCATCAAACGCTCCTTCAAC
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300
Cm HIF-2α1 CAGATGG------GCGAGGGTCCTACGTCAGATTTCATGTGGAAGAAAATGAGGGGTGACAGCGACATGGATCGCTCCCTCAGTGCAGGATCACTGGCAG
Cm HIF-2α2 ----------
Th HIF-2α CAGATGGCTGTGGGCGAGGGTCCTACGTCNGACTTCATGTGGAAGAAAATGAGGAGTGACAGCAACATGGATCGCTCCCTCAGTGCAGGATCACTGGAAG
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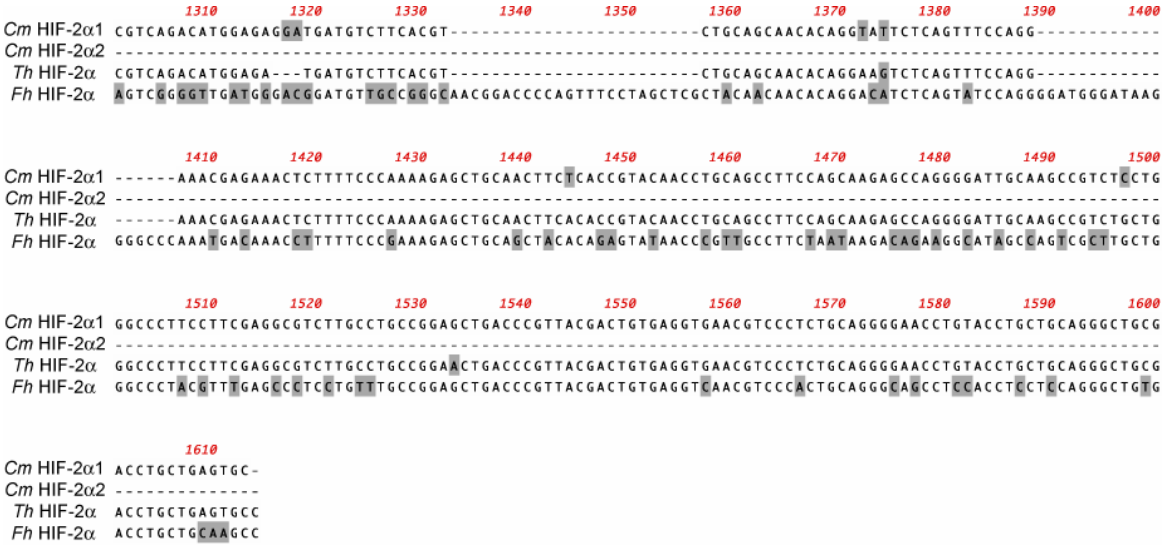

Fig. 4.5: Partial HIF-2α cDNA sequences (C-terminal half) of *Chionodraco myersi* (*Cm*; DQ089690), *Trematomus hansoni* (*Th*; DQ089693) and *Fundulus heteroclitus* (*Fh*, AF402782) in 5`-3` direction. Moreover, a different variant of partial *Cm*HIF-2α cDNA obtained with primer pair IV (HIF2-F1, HIF2-B1; see tab. 2.2 in Material & Methods; unpublished).

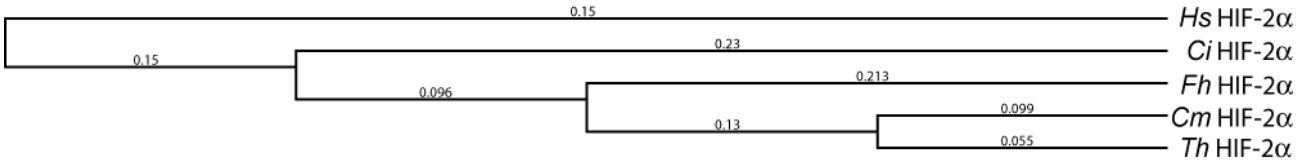

Fig. 4.6: Phylogenetic analysis of the C-terminal half of fish HIF-2α amino acid sequences of the notothenioids *Chionodraco myersi* (*Cm*; DQ0896690) and *Trematomus hansoni* (*Th*; DQ0896693), the cyprinid *Ctenopharyngodon idella* (*Ci*, AY577524) and the cyprinidontid *Fundulus heteroclitus* (*Fh*, AF402782). The tree was calculated by the Neighbour Joining method (best tree; tie breaking = Systematic; Distance: Uncorrected "p"; Gaps distributed proportionally with the human sequence (*Hs*; BC051338) as out-group. Tree calculation by bootstrap (1000 repeats) resulted in the same cluster with all bootstrap values being 100% (not shown).

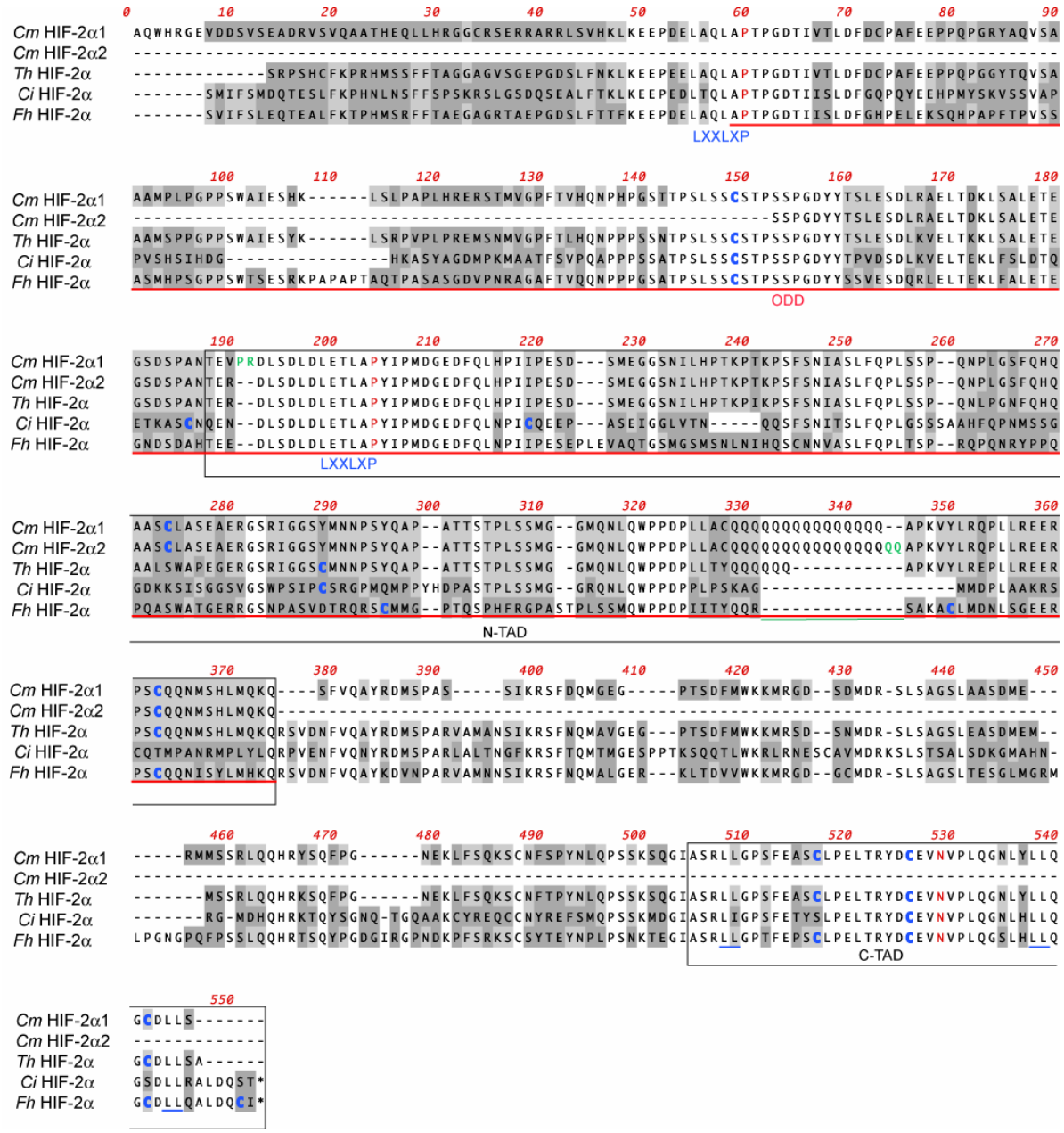

Fig. 4.7: Deduced amino acid sequences of HIF-2α (C-terminal half) of *Chionodraco myersi* (*Cm*; DQ0896690), *Trematomus hansoni* (*Th*; DQ089693), *Ctenopharyngodon idella* (*Ci*, AY577524) and *Fundulus heteroclitus* (*Fh*, AF402782) in C-N direction. Moreover, the deduced amino acid sequence of a different variant of partial *Cm*HIF-2α obtained with primer pair IV (HIF2-F1, HIF2-B1; see tab. 2.2 in Material & Methods) is included in the alignment. Black boxes indicate functional domains, the red line below the sequence mark the oxygen dependent degradation domain (ODD) adopted from Powell & Hahn (2002). The green line indicates the Gln cluster, the blue lines di-leucine repeats (O'Rourke et al. 1999). Functional amino acids are highlighted in red, cysteines in ODD and C-TAD blue, differences between the two *Cm*HIF-2α forms green. N-TAD = N-terminal transactivation domain, C-TAD = C-terminal transactivation domain. LXXLXP represents the binding motif for prolyl hydroxylases (Huang et al. 2002).

4.3. Total nitrite + nitrate content in the liver of *Z. viviparus* after 2 h temperature stress

The total content of nitrite and nitrate, depicted in fig. 4.8, was measured as an indicator for NO production in the liver upon short-term temperature stress and subsequent recovery of *Z. viviparus*. The rationale of this test is based on the fast reaction of NO with molecular oxygen to form nitrite and the subsequent reaction of nitrite and oxy-haemoglobin yielding nitrate.

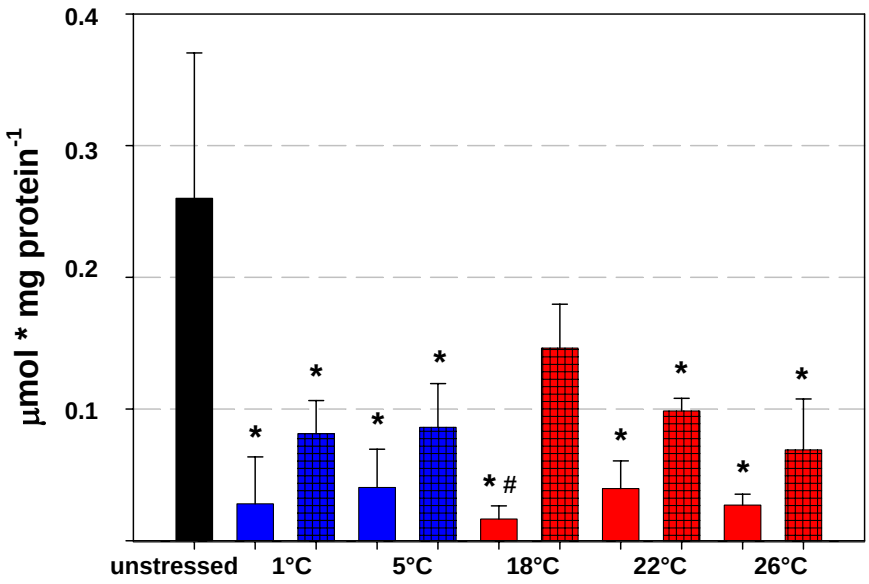

Fig. 4.8: Total nitrite + nitrate content in liver samples from *Z. viviparus* exposed to 2 h of temperature stress and subsequent recovery. Black columns indicate unstressed controls (summer acclimatised and kept at 12°C), blue columns indicate cold stress, red columns indicate heat stress, and columns with checked pattern indicate recovery. * significantly different to unstressed controls, # significantly different to the respective recovery group, n = 3 - 6, $p < 0.05$.

The total nitrite + nitrate content was highest in unstressed controls and significantly reduced upon acute exposure to both, cold or heat. Upon 24 h at control temperature (12°C), the total nitrite + nitrate content recovered, but did not reach control levels within 24 h.

4.4. Studies on isolated fish hepatocytes

4.4.1. MitoTracker Green FM staining of *Z. viviparus* hepatocytes

Mitochondrial density in *Z. viviparus* hepatocytes was visualised by staining the cells with the fluorophor MitoTracker Green FM and subsequent confocal imaging. Fig. 4.9 depicts a typical set of scans throughout hepatocytes with 2 µm sections.

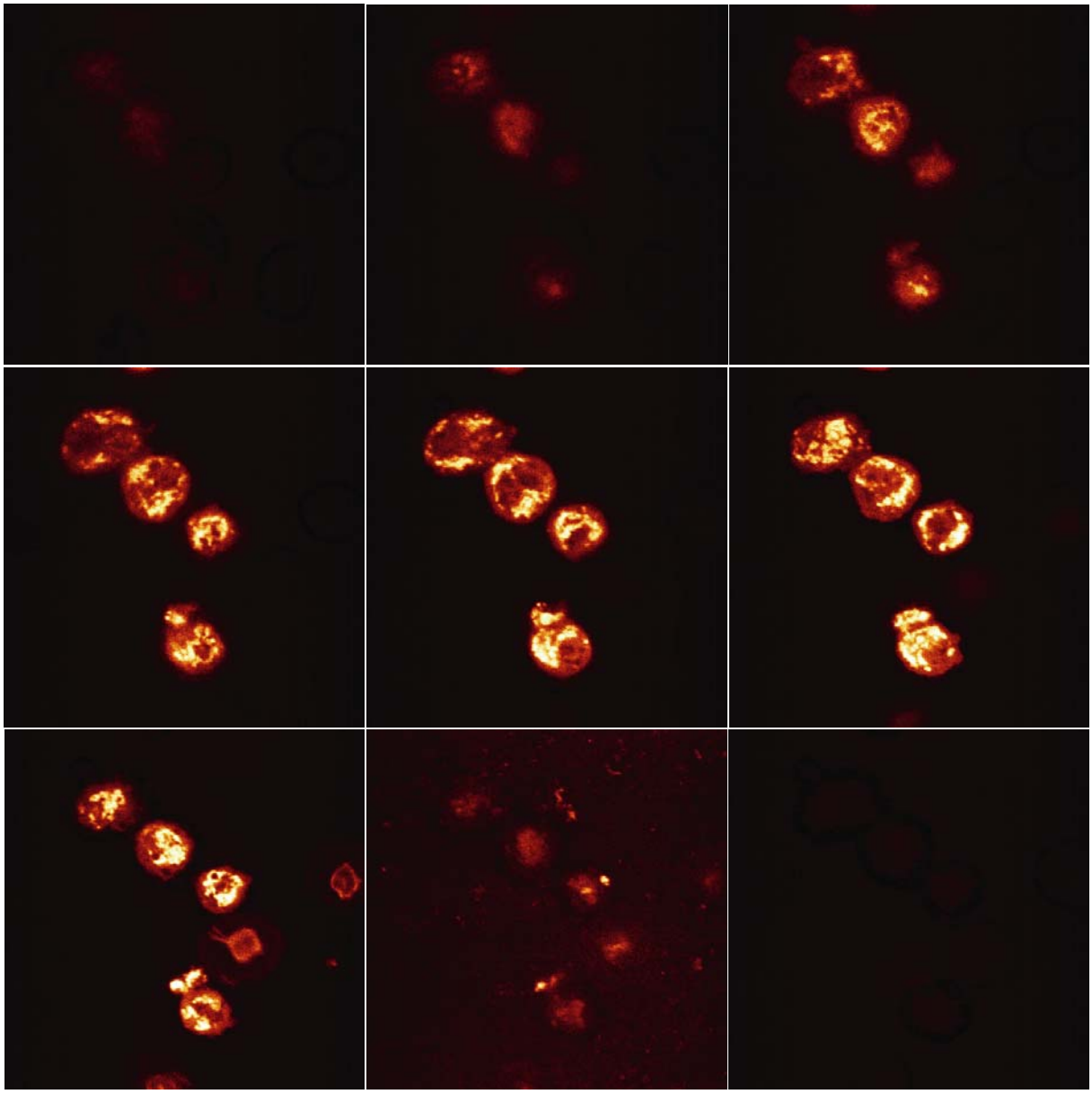

Fig. 4.9: Typical example of confocal imaging of mitochondrial density in *Z. viviparus* hepatocytes.
Image size (*xy*-*z*): 50 µm² * 18 µm; 2 µm sections; zoom 2.0. Properties: 100x / 1.40 - 0.70 oil immersion lens, excitation wavelength 488nm, laser power 46, confocal pinhole 0.7, emission filter 580 nm, photomultiplier voltage 732, offset -3, glow mode, slow scan speed, accumulation of 8 scans / picture.

Mean hepatocyte volume was 2 100 ± 800 µm³, although the complete range comprised sizes from 100 to 6 000 µm³. In contrast to invertebrate cells containing only 30 - 40

mitochondria per cell (Abele et al. 2002), which can easily be counted, quantification of mitochondrial numbers cannot be assessed in *Z. viviparus* hepatocytes with this technique, due to the far higher mitochondrial densities.

4.4.2. Measurement of ROS production in fish hepatocytes using H_2DCFDA fluorescence

According to the product information of Molecular Probes (Eugene, USA) H_2DCFDA (dichlorodihydrofluorescein diacetate) is oxidised by ROS such as H_2O_2, peroxyl radicals and peroxinitrite anion to the fluorescent DCF (fluorescin) and thus, can be used for intracellular reactive oxygen species detection. However, control assays using radical scavengers have to be carried out to prove ROS-specificity of the fluorescent oxidation. Horse-radish peroxidase (HRP), increasing H_2O_2 formation as well as catalase, decreasing H_2O_2 formation cannot be used to prove for H_2DCFDA fluorescence specific for intracellular ROS formation, as both substances react with this dye leading to an increase of ROS-unspecific fluorescence (E. Philipp, unpubl. observ., Hempel et al. 1999). Therefore, the spin trap POBN to quench any radical species (Janzen 1971) was used to test the ROS-specificity of fluorescent signals.

Comparison of confocal images with transmission images, depicted in fig. 4.10, reveals two types of cells, circular cells being hepatocytes, as well as cylindrical cells being erythrocytes, which were not completely removed during the isolation process. Following aggregation of H_2DCFDA fluorescence developed only in the hepatocytes (fig. 4.10).

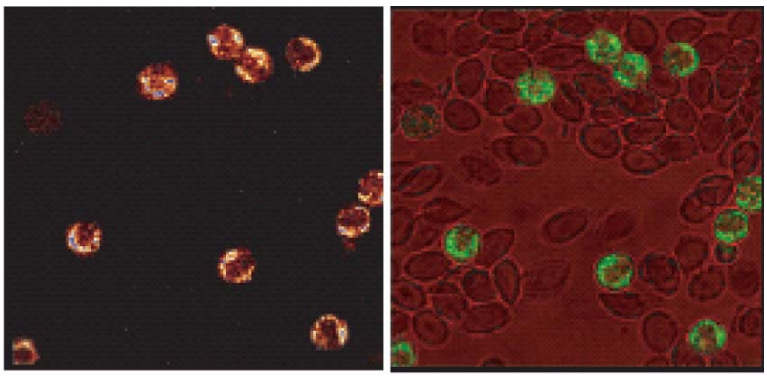

Fig: 4.10: Confocal scans of fluorescence of *Z. viviparus* hepatocytes 22 min after aggregation of 50 µM H_2DCFDA. Image on the left side: confocal scan; image on the right side: associated transmission image. Fluorescence can be observed only in circular cells being hepatocytes, but not in cylindrical cells being erythrocytes.

Fluorescence of hepatocyte solutions of *Z. viviparus* was imaged once every 2 min over a 40 min time interval by confocal microscopy. The image series in the upper part of fig.

4.11 shows progressively increasing fluorescence over a 40 min time interval following addition of 50 µM H_2DCFDA. By contrast, upon concomitant aggregation of 50 µM H_2DCFDA and 5 mM POBN, fluorescence appeared depressed within the same time period (image series in the lower part of fig. 4.11). Thus, increasing fluorescence, indicating accumulation of the fluorescent dye DCF may be attributed to oxidation by ROS generated from *Z. viviparus* hepatocytes.

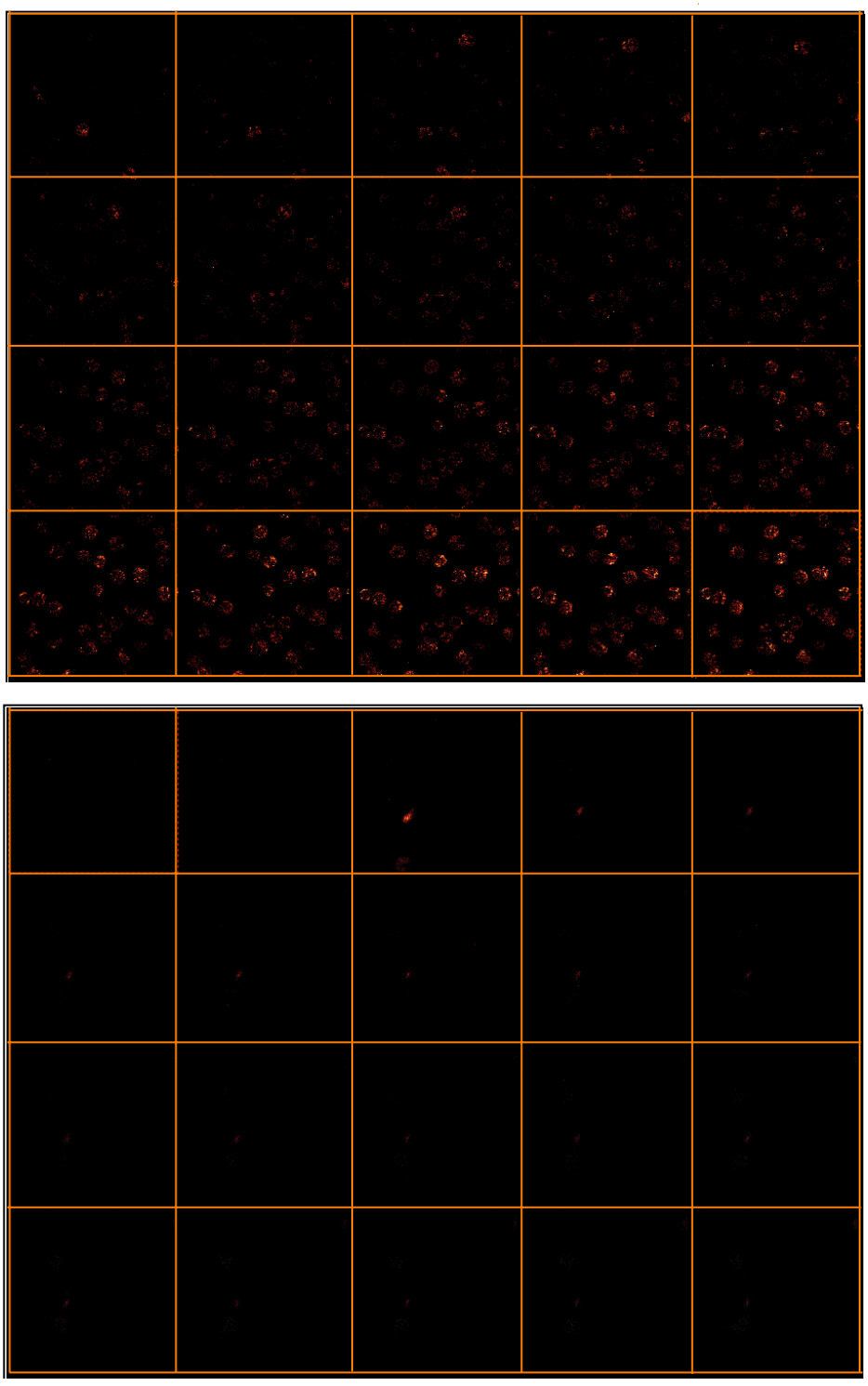

Fig. 4.11: Confocal scans of fluorescence of *Z. viviparus* hepatocytes, recorded every 2 min over a 40 min time period. The image series in the upper part shows progressively increasing fluorescence of hepatocytes after addition of 50 µM H_2DCFDA. By contrast, the image series in the lower part shows depression of fluorescence in hepatocytes after concomitant aggregation of 50 µM H_2DCFDA and 5 mM of the spin trap POBN. Image size (*xy*): 100 µm²; Properties: 100x / 1.40 - 0.70 oil immersion lens, excitation wavelength 488 nm, laser power 29 (without POBN) or 39 (with POBN), confocal pinhole 0.7, emission filter 505 nm, photomultiplier voltage 721, offset -8, glowover mode, slow scan speed, accumulation of 4 scans / picture.

5. Discussion

The presented thesis investigated long-term and short-term *in vivo* impacts of environmental temperature and graded temperature stress, supposed to induce various degrees of functional hypoxia, on oxidative stress and hypoxic signalling in marine fish from different latitudes. In the following section, I will discuss the data presented in publications I - IV and in the section "additional results".

5.1. Oxidative stress and ROS generation in the liver of zoarcids

To answer the questions addressed in this work, whole animal experiments were carried out, where fishes were maintained at different temperatures, with subsequent freezing of sampled tissues. This set-up does, however, not allow for direct measurements of ROS generation in the living fish. Thus, oxidative stress levels were determined in frozen samples of experimental animals by analysing oxidative damage markers. It has to be considered that altered concentrations of, e.g. TBARS or protein carbonyls might not always be indicative of unbalanced pro- and antioxidant processes. Although this is hypothetical, they may also result from different velocities of repair at varying experimental temperatures, while the net ROS generation rates may be unchanged due to parallel changes of ROS producing and scavenging processes (M. Nikinmaa, pers. com.). Therefore, I used a combination of several oxidative damage and AOX markers to assess oxidative stress levels. Moreover, exposure to a source of free radicals allows for the evaluation of the *ex vivo* susceptibility of lipids to oxidation e.g. by tBOOH-induced chemiluminescence (Dotan et al. 2004). By this assay, occurrence of oxidative stress under experimental conditions in the fish can be delineated from the *ex vivo* ROS generation (mainly singlet oxygen and excited carbonyl groups), which is higher in a tissue where antioxidants have been depleted during experimental exposure. Chemiluminescence rates in this assay detect oxidative stress resulting from an imbalance of pro-oxidant processes and low molecular weight antioxidants, but are not affected by enzymatic AOX defence (Gonzalez Flecha et al. 1991).

In vitro studies with isolated liver mitochondria of *Zoarces viviparus* were unable to detect any H_2O_2 release to the medium when using the fluorescent dye homovanillic acid (Abele et al. 2004). Therefore, confocal microscopy and the fluorescent dye H_2DCFDA were used in an initial approach to visualise the ROS generation of isolated *Z. viviparus* hepatocytes. The obtained results must be considered preliminary. However, the increasing

fluorescence over time can be interpreted as continuous basal generation of reactive oxygen species in *Z. viviparus* hepatocytes, as addition of the spin trap POBN depressed fluorescence within the experimental time span (fig. 4.11 in "additional results). Future studies have to evaluate the type and also the main cellular sources of ROS generated in *Z. viviparus* hepatocytes.

5.2. Higher oxidative stress levels in polar fish living at permanently low temperatures?

- comparison of the Antarctic (*P. brachycephalum*) and the temperate eelpout (*Z. viviparus*) -

Polar marine ectotherms are characterised by a higher content of unsaturated fatty acids in order to maintain membrane fluidity at low water temperature (Sidell 1998). In line with this common observation, higher concentration of unsaturated fatty acids were found in Antarctic eelpout, *Pachycara brachycephalum*, compared to the con-familial common eelpout *Zoarces viviparus* from the German North Sea (E. Brodte, pers. com). It has been suggested that higher levels of lipid unsaturation in combination with the enhanced aqueous oxygen solubility at cold temperatures render polar ectotherms vastly more sensitive to oxidative damage from lipid peroxidation processes (for review Abele & Puntarulo 2004). To test this hypothesis, I studied a wide array of oxidative stress parameters in liver samples of the zoarcids, *P. brachycephalum* kept at its habitat temperature of 0°C, and *Z. viviparus* winter acclimatised to 6°C. Lipid radical generation rates measured by EPR spin trapping (fig. 1 and tab. 1 in publ. I) were indeed higher in liver microsomes of *P. brachycephalum*. However, TBARS content, which is an indicator for lipid peroxidation products, was only one half of that found in *Z. viviparus* (tab. 2 in publ. I). It might be that other lipid peroxidation products prevail in the polar zoarcid, which are not detected by the TBARS assay. In mammalian tissues, TBARS such as MDA, which are highly toxic, causing protein cross-links and interacting with DNA bases, are rapidly metabolised (Halliwell & Gutteridge 1999). Therefore, a lower TBARS content despite higher lipid radical formation rates may reflect successful cleavage of such products in *P. brachycephalum*. This suggestion is supported by the finding that lipid radicals were only detectable in liver microsomes void of antioxidants, but not in liver homogenates, which contain all cellular antioxidants. Lower tBOOH-induced chemiluminescence rates (tab. 2 in publ. I) in *P. brachycephalum* than in *Z. viviparus*, reflect high AOX capacity due to elevated levels of low molecular weight antioxidants

(Gonzalez Flecha et al. 1991). Indeed, both α-tocopherol and total glutathione were two to three times higher in the polar compared to the temperate eelpout (tab. 3 in publ. I).

Interestingly, glutathione was oxidised to 50%, or even higher in some specimens of *P. brachycephalum,* resulting in a significantly more oxidised redox potential (ΔE). As far as I know, this is the highest GSSG / GSH ratio reported for fish (tab. 5.1).

Tab. 5.1: Glutathione-related parameters in the liver tissue of several fish species.
Contents are given in µmol * g wet weight^{-1}. n.d. = not determined.

fish species	origin	climate / water temperature	GSH	GSSG	total glutathione	2GSSG/ GSH	reference
Pachycara brachycephalum	Antarctic, marine	polar 0°C	2.1	3.1	8	2.7	this study
Zoarces viviparus	Germany, marine	temperate, 6°C (winter)	1.0	0.8	2.5	1.8	this study
Zoarces viviparus	Germany, marine	temperate, 10°C (autumn)	0.8	0.3	1.4	0.7	this study
Zoarces viviparus	Germany, marine	temperate, 12°C (summer)	0.9	0.16	1.2	0.36	this study
Sparus aurata (gilthead seabreams)	Spain, marine	subtropical 20°C	4.4	0.04	4.48	0.02	Pascual et al. 2003
Salvelinus alpinus (Arctic charr)	Finland, freshwater	temperate, 10°C 14°C	1.44 1.08	0.05 0.05	1.55 1.19	0.07 0.09	Ritola et al. 2000
Oncorhynchus mykiss (rainbow trout)	Finland, freshwater	temperate, autumn	1.3	0.12	1.54	0.19	Ritola et al. 1999
Oncorhynchus mykiss (rainbow trout)	Sweden, freshwater	temperate, 10°C	1.36 1.17 1.45 1.14	0.09 0.04 0.10 0.03	1.54 1.25 1.65 1.20	0.13 0.07 0.14 0.05	Stephensen et al. 2002
Ameriurus nebulosus (brown bullhead) *Ictalurus punctatus* (channel catfish)	USA, freshwater	temperate, 20°C	n.d.	n.d.	1.3 1.4	n.d	Ploch et al. 1999
Anguilla anguilla (european eel)	Spain, freshwater	20°C	0.21	0.03	0.27	0.29	Pena et al. 2000

Such extremely high GSSG concentrations might indicate excessive oxidative stress in the liver of this species. They also underline the importance of glutathione as an antioxidant effectively protecting cellular constituents from oxidative damage by forming lipid radicals, as reflected in low TBARS contents as well as low protein oxidation and chemiluminescence rates compared to the temperate eelpout. Moreover, the high degree of glutathione oxidation is quite surprising, as GSSG rarely exceeds 10% of total glutathione in healthy cells (Kidd 1997). Generally, over-produced GSSG molecules are rapidly exported from hepatocytes because they can have deleterious effects on cell integrity and physiological processes (Reed 1990). Through protein-S-thiolation, GSSG can modify the activity of important metabolic enzymes (Klatt & Lamas 2000). However, the fact that such high degrees of glutathione oxidation were found in unstressed animals illustrates that they are not harmful for *P. brachycephalum*. It might be that in this polar fish, metabolic

processes adapted to function at low temperatures are also adapted to operate in a different, more oxidised redox environment. As glutathione concentrations are not available for other Antarctic fish is not clear, if a highly oxidised glutathione redox ratio represents a specific polar characteristic. It may also be a consequence of 2 to 6 times higher total glutathione levels in *P. brachycephalum* compared to other fishes (tab. 5.1).

It has been suggested that the high susceptibility of polar ectotherms to oxidative damage may become deleterious under any form of physiological stress (Abele & Puntarulo 2004). To test this hypothesis, I investigated the response of oxidative stress parameters in the liver of *P. brachycephalum* acclimated to 5°C. This temperature, although being outside the habitat temperature range of this species (0.5 - 0.8°C, K. Mintenbeck, pers. com.), is not supposed to be critical, as a ten months acclimation of *P. brachycephalum* to 5°C did not cause reduced physiological condition factors compared to animals kept at 0°C (Lannig et al. 2005). However, 5°C is close to the upper pejus temperature of this species (6 - 7°C; Mark et al. 2002) when pre-acclimated to 0°C. Thus, beginning oxygen limitation together with elevated mitochondrial respiration at 5 *versus* 0°C (Lannig et al. 2005) was originally expected to induce higher oxidative stress levels. However, oxidative damage parameters were only slightly elevated in warm acclimated *versus* control *P. brachycephalum*. Only TBARS content was significantly higher compared to control fish kept at 0°C, but temperature induced changes in TBARS levels were much lower than changes observed in *Z. viviparus* upon seasonal acclimatisation (tab. 2 in publ. I). Also, the redox environment remained constant during warm acclimation of *P. brachycephalum*. It appears that particularly the redox couple glutathione charges the liver tissue of this polar fish with such a high AOX capacity that oxidative damage is not augmented upon warm acclimation. Recently, Mark et al. (in revision) reported higher UCP2 expression in 5°C *versus* 0°C acclimated *P. brachycephalum*. UCP2 might increase mitochondrial proton leak and thus, reduce the mitochondrial membrane potential and superoxide generation (Skulachev 1996). Thus, increased UCP2 levels might represent a means for *P. brachycephalum* to control mitochondrial ROS production upon warming. Moreover, Lannig et al. (2005) have shown reduced liver aerobic capacities in *P. brachycephalum* upon 5°C acclimation, which is seen as a physiological response typical for cold-eurythermal animals (Pörtner 2002). Probably, *P. brachycephalum* has to be considered more eurythermal than other Antarctic animals. Thus, the presented findings support previous conclusions that *P. brachycephalum* is able to warm acclimate and adjust life functions including positive growth to higher temperatures (Storch et al. 2005, Lannig et al. 2005, Brodte et al. ms submitted). This

assumption is further supported by the observation that in contrast to the Antarctic zoarcid, several benthic Antarctic notothenioids survived warm acclimation to 4°C only for a few weeks (Weinstein & Somero 1998). However, recent findings in the more active pelagic *Pagothenia borchgrevinki* have shown an unexpected capacity to warm acclimate (Lowe et al. 2005).

Altogether the presented data demonstrate only a minor impact of sub-critical alterations of ambient water temperature on oxidative stress levels in the liver of the Antarctic eelpout *P. brachycephalum*, probably because the species is able to warm acclimate and because the AOX capacity provided by the glutathione redox couple is highly efficient, to counteract the high risk for oxidative damage resulting from lipid peroxidation processes.

By contrast, different oxidative stress levels were observed in the temperate eelpout *Z. viviparus* upon seasonal temperature changes within the natural temperature range (for ambient water temperatures at Helgoland see fig. 8.1 in the appendix). Comparison of summer acclimatised *Z. viviparus* kept at 12°C with winter animals kept at 6°C revealed lower overall AOX capacities (lower α-tocopherol contents and lower GPX activities at similar SOD activities) and higher levels of oxidative damage markers (TBARS, protein carbonyl and chemiluminescence rates) in 6°C acclimated winter fish (tab. 2 in publ. I). The apparent increase in oxidative stress was also reflected in a more oxidised glutathione redox ratio (tab. 3 in publ. I).

It can be concluded that both, the development of cold adaptation features of the polar eelpout *P. brachycephalum,* as well as the expression of seasonal cold acclimatisation characteristics of the temperate eelpout *Z. viviparus* were associated with higher levels of oxidative stress, mainly visible in a highly oxidised cellular redox environment.

5.3. Influence of high oxygen contents and oxidative stress levels on the HIF mechanism?

- comparison of HIF sequences and DNA binding activity in polar and temperate fish -

Low environmental temperatures in combination with high oxygen solubility may be the cause for a variety of adaptations in the oxygen-transport systems of Antarctic fish (Wells et al. 1980, Acierno et al. 1997). Very low haematocrit values are characteristic for Antarctic notothenioids and zoarcids, with an extreme effectuation of this adaptation in icefishes completely void of red blood cells (Davison et al. 1997, Mark et al. 2002). Such modifications together with an extremely oxidised cellular redox environment could cause

disturbances of the HIF signalling mechanism or might produce a need for functional adjustments of this system to ensure oxygen-dependent gene expression in polar fishes.

In an initial step to test this hypothesis, I characterised full length HIF-1α cDNA sequences of the zoarcids *P. brachycephalum* and *Z. viviparus* as well as partial cDNA sequences comprising the C-terminal half of HIF-1α of two red-blooded notothenioids (*Trematomus hansoni* and *T. pennellii*) and an icefish *Chionodraco myersi* (publ. II). Moreover, I identified a partial cDNA sequence of HIF-2α in *T. hansoni* and *C. myersi* (fig. 4.5 in "additional results"). The detection of HIF-α sequences by RT-PCR in all four polar species, confirms constitutive expression of these genes. This emphasises a ubiquitous physiological importance of the transcription factor HIF in fish, as suggested by Nikinmaa & Rees (2005). It may also underlie the suggestion of Semenza (2004) that the HIF-1 signalling mechanism was essential to metazoan evolution.

Within the full length cDNA sequences of zoarcid HIF-1α an open reading frame encoding for 747 amino acids was identified (fig. 4.3 and 4.4 in "additional results"). Several substitutions located in the ODD and the C-TAD were identified as conserved in all Antarctic species and different from the *Z. viviparus* sequence (indicated by green stars in fig. 2, publ. II), which might support a more hydrophilic character and, thus, favour protein cold adaptation (Feller et al. 1997, D'Amico et al. 2002, Pörtner et al. in press).

Moreover, additional cysteines were noticed in the ODD of zoarcid and notothenioid HIF-1α. This could have similar implications for the function of HIF as reported in rainbow trout, where four cysteines in the ODD confer elevated redox-sensitivity to HIF-1α stabilisation (Nikinmaa et al. 2004). Future studies should address the question whether increased redox-sensitivity mediated by these cysteine residues is a special feature for oxygen-dependent gene expression in fish. A further interesting finding was the substitution of the single Glu365 in human HIF-1α by a Gln-Glu cluster in marine fish HIF-1α, comprising 8 residues in the zoarcids, over 10 in *Trematomus* and 21 residues in the icefish. Although the function of this cluster remains obscure, especially the glutamate residue of the cluster will cause a highly acidic site in the respective protein region.

Most astonishing was the substitution of the N-terminal functional proline residue (Pro402 in human HIF-1α) in *P. brachycephalum* by leucine (fig. 2 in publ. II, fig. 4.4 in "additional results"). This is, so far, the only wild-type animal of this phenotype. It can be suggested that HIF-1α might still be functional as pVHL mediated proteasomal degradation may occur via hydroxylation of the other conserved proline residue (Masson et al. 2001, Mazure et al. 2004).

However, it has been shown that mutation in any amino acid of the LXXLXP sequence, which is the recognition motif for PHDs, can be tolerated, except in the hydroxyl-accepting proline residues (Huang et al. 2002, Min et al. 2002). Thus, the Pro-Leu substitution in *Pb*HIF-1α protein could render it more stable than in other species (Ivan et al. 2001). Consequently, the flexibility of the HIF response via the degradation / stabilisation mechanism might be reduced in *P. brachycephalum* (Masson et al. 2001). By contrast, Pro-Leu conversion within that anyway leucine-rich region may facilitate the interaction of HIF-1 with the transcriptional co-activator p300, as leucine-rich regions have been demonstrated to be crucial for p300 interaction with transcription factors (O'Rourke et al. 1999). It is intriguing to speculate from these structural differences that *Pb*HIF-1 regulation may function mainly via transactivation and not so much via the degradation / stabilisation mechanism. In the three notothenioid HIF-1α sequences both functional prolines appeared conserved (fig. 2 in publ. II), thus the Pro-Leu substitution in *Pb*HIF-1α cannot be considered an essential polar adaptation.

Yet it is conceivable that different polar fishes have evolved various modifications of their HIF mechanism. Thus, the findings of additional cysteines in functional domains, inserts of Gln-Glu cluster and Pro-Leu substitution in HIF-1α sequences, might represent diverse adjustments to ensure HIF mediated oxygen-dependent gene expression in cold adapted fish, despite high oxygen solubility in the water and potentially more oxidised cellular redox environment.

Phylogenetic comparisons suggest that several rounds of gene duplication of an ancestral HIF-α gene led to the α-forms (Powell & Hahn 2002). HIF-1α and HIF-2α both seem to regulate oxygen-dependent gene regulation via the same HRE element (Rose et al. 2002). Whereas HIF-1α is ubiquitously expressed, HIF-2α is restricted to endothelial cells in mammals (Powell & Hahn 2002). Despite apparently diverse effects of both α-subunits during embryogenesis it is unknown, whether they play different roles during hypoxia (Gu et al. 2000). Analysis of the C-terminal half of HIF-2α in liver samples of the notothenioids *T. hansoni* and *C. myersi* revealed lower conservation of the deduced amino acid sequences between both species compared to the partial HIF-1α peptides of the notothenioids (80 *versus* 92% identity). However, functional proline and asparagine residues as well as di-leucine repeats proved conserved in notothenioid HIF-2α. Similarly to the Gln-Glu cluster in HIF-1α, both notothenioid HIF-2α sequences contained a Glu cluster. Future studies will clarify whether the regulation of oxygen homeostasis by the HIF response could be supported by the action of HIF-2α in Antarctic notothenioids.

In a second step, the HIF function was analysed at the protein level in warm acclimated (5°C) *versus* control (0°C) *P. brachycephalum*, as well as in winter (6°C) and summer (12°C) *Z. viviparus* (fig. 2.B, C in publ. I). Western blotting measuring the abundance of HIF-1α protein could be carried out only for *Z. viviparus*. The available antibody, originally produced against rainbow trout HIF-1α, did not recognise the protein in *P. brachycephalum* although both eelpout HIF-1α sequences displayed high identity, whereas the identity of *Zv* was considerably lower to *Om*HIF-1α (see above). However, already minor differences in primary structures are sufficient for changing protein conformation in cold adapted animals (Fields & Somero 1997, for review see Somero 2004), which might explain the failure of the rainbow trout antibody. Significant HIF-1α protein levels could be detected with similar quantities in liver samples of both winter and summer *Z. viviparus*. This result was quite surprising, as both batches of *Z. viviparus*, acclimatised to temperatures well within their naturally experienced temperature regime, have to be considered as unstressed, normoxic animals. In contrast, the HIF-1α protein cannot be detected in the liver of normoxic mammals (Stroka et al. 2001). It is reasonable to assume that, like in rainbow trout, HIF-1α stabilisation in unstressed eelpout may be linked to the abundance of surplus cysteine residues in the ODD (Soitamo et al. 2001, Nikinmaa et al. 2004). Even though HIF-1α protein levels were unchanged in both groups of *Z. viviparus*, electromobility shift assays (EMSA) demonstrated higher DNA binding activity in winter (6°C) compared to summer (12°C) animals. Moreover, water temperature had opposite effects on HIF-1 DNA binding activity in both species, being enhanced in cold winter *Z. viviparus*, and in warm acclimated *P. brachycephalum* as compared to summer *Z. viviparus* and 0°C acclimated *P. brachycephalum*. Higher HIF-1 DNA binding activity can be interpreted as induction of hypoxic signalling. Thus, in 6°C winter *Z. viviparus*, the onset of hypoxic signalling could indicate physiological reorganisation to improve oxygen supply at low temperatures. This might counterbalance the cold-induced slow-down of oxygen delivery to central tissue regions (Pörtner 2002), and thus, prevent severe functional hypoxia. Likewise, in warm acclimated *P. brachycephalum*, close to upper pejus temperature (6 - 7°C, Mark et al. 2002), the HIF response might support oxygen homeostasis before onset of heat-induced functional hypoxia. Thus, it appears that HIF-1 could be a common regulatory tool to adjust tissue oxygenation upon seasonal acclimatisation within the benevolent temperature regime (*Z. viviparus*) and at the border of the thermal optimum range (*P. brachycephalum*).

Additionally, higher DNA binding activity upon warm acclimation demonstrates that HIF-1 is still functional in *P. brachycephalum*, despite the Pro-Leu substitution (see above) and despite the highly oxidised cellular redox environment.

Elevated HIF-1 DNA binding in winter *versus* summer *Z. viviparus* and constitutive, unchanged levels of HIF-1α protein in both acclimatisation groups suggests that as proposed for *P. brachycephalum*, the HIF-1 response could be regulated mainly during later steps of the signalling cascade also in the common eelpout: Translocation, dimer formation or transactivation could be more important than degradation / stabilisation. Future studies at the cellular level are needed to clarify this issue.

It has to be mentioned that increased HIF-1 DNA binding occurred together with higher TBARS levels (but unchanged redox environment) in warm acclimated *P. brachycephalum* and with increased levels of oxidative damage markers together with a more oxidised redox environment in cold winter *Z. viviparus*. Thus, despite the general observation that HIF signalling is favoured at a more reduced redox environment in fish (Nikinmaa et al. 2004), higher oxidative stress levels does not seem to impair HIF induced regulation of oxygen homeostasis in the zoarcids, upon long-term temperature changes within the thermal optimum range. Future studies have to determine the target genes of HIF-1 in zoarcids and notothenioids and have to show whether HIF is actually able to enhance their expression, particularly in Antarctic species, including icefish.

5.4. Oxidative stress and hypoxic signalling induced by short-term temperature stress?

- acute cold or heat stress and subsequent recovery studied in the temperate eelpout -

In marine ectotherms, temperatures outside the optimum range, above or below the pejus threshold (Tp) are supposed to cause progressively decreasing oxygen levels in body fluids and tissues, i.e. functional hypoxia (Pörtner 2002). When critical temperatures (Tc) are reached, transition to anaerobic metabolism can be observed (Sommer et al. 1997, Van Dijk et al. 1999). It has been suggested that onset of functional hypoxia in the pejus range may be linked to increasing oxidative stress, becoming worse during critical temperature stress (Pörtner 2002, Abele 2002). The following text will discuss the short-term *in vivo* exposure to graded cold and heat stress ranging from pejus to critical and sub-lethal temperatures.

Interestingly, effects of short-term (2 h) cold exposure to 1 or 5°C on liver oxidative stress parameters were different from those of winter acclimatisation. When compared to

unstressed controls, protein carbonyl contents were unchanged following acute cold stress, chemiluminescence rates in 1°C exposed *Z. viviparus* were only slightly higher and TBARS in 1 and 5°C exposed fish only mildly elevated (fig. 2 in publ. III). Calculation of the cellular redox potential at *in situ* pHi and *in situ* temperature with the Nernst equation, using the concentrations of the glutathione redox couple, even revealed more reduced redox conditions in both cold exposed groups compared to unstressed control fish (fig. 1.B in publ. III). However, TBARS and carbonyl contents were higher after 24 h recovery following cold stress, with the redox potential having returned to control levels. When taking the increased HIF-1 DNA binding activity into account, especially after exposure to 1°C (fig. 3.D in publ. III), the sum of these results indicates temperature-induced hypoxia during acute cold stress and reoxygenation accompanied by unbalanced oxidative stress upon recovery. Thus, cold stress and subsequent recovery can have similar biochemical effects in these ectotherms as the ischemia / reperfusion event described in mammals (Jones 1986, McCord 1988, Chi & Karliner 2004). Whereas this situation is highly pathological in mammalian tissues, the observed elevations in oxidative stress parameters do not seem dramatic in *Z. viviparus*. Actually, many anoxia tolerant animals such as goldfish, garter snakes and wood frogs have been found to deal well with oxidative stress arising from natural situations reminiscent to ischemia / reperfusion, such as anoxia, desiccation or freezing (Hermes-Lima & Zenteno-Savin 2002).

At first sight, short-term (2 h) heat stress and subsequent 24 h recovery did not show a comparable ischemia / reperfusion effect on oxidative stress parameters, as chemiluminescence rates were similar in unstressed, heat stressed, and heat recovered *Z. viviparus* (fig. 3 in publ. IV). Protein carbonyls were elevated only after recovery following 18°C, but not after recovery following more severe heat stress (22 or 26°C). However, it seemed possible that repair of oxidative damage was simply faster following heat than following cold stress, such that after 24 h all parameters had already returned to control levels. Therefore, a second heat stress experiment was carried out, where *Z. viviparus* were exposed to 18°C heat stress followed by shorter recovery periods of 0, 2, 8, or 12 h at acclimation temperature. In fact, chemiluminescence rates and protein carbonyls indicated increased oxidative stress within the first 12 h of recovery, supporting the idea of faster repair of oxidative damage during recovery from heat than from cold stress (fig. 6 in publ. IV). Furthermore, these results confirmed that the biochemical effects of heat stress and recovery resemble ischemia / reperfusion. Again, *Z. viviparus* appeared tolerant to oxidative stress arising from short-term acute warming and recovery, as no

animal died during experimentation. Moreover, swimming performance of all recovery groups was observed to be similar to unstressed fishes immediately after they were returned to control temperature. It is, however, conceivable that frequent warming / recooling events would exacerbate the situation for the animals.

Nitric oxide (NO) is another radical species, which has been ascribed regulatory function during low tissue PO_2 due to its vasodilatory effect (Palmer et al. 1987). Moreover, nanomolar concentrations of NO inhibit cytochrome c oxidase activity and thus mitochondrial O_2 consumption in peripheral cells, which may add to improve oxygen availability to central organs (Monacada & Erusalimsky 2002, Hagen et al. 2003). Further, NO treatment of isolated mitochondria has been shown to increase superoxide production, leading to enhanced formation of highly toxic peroxinitrite (Poderoso et al. 1996, Abe et al. 1999). Upon hypoxia, NO levels may either increase or decrease, depending on exposure time and tissue studied (Malyshev et al. 1999, Sandau et al. 2001). It is difficult to plausibly predict the role and effect of NO in temperature-induced hypoxia in eelpout, as the Griess assay, detecting the oxidation products of NO (total nitrite + nitrate), does not measure the actual *in situ* NO concentrations (fig. 4.8 in "additional results"). However, reduced levels of total nitrite + nitrate might reflect temperature-induced disturbances of the NO producing enzyme NO-synthase. Similarly, slightly higher total nitrite + nitrate levels after recovery could indicate partial recovery of the enzyme. If this were the case, NO would not be expected to improve oxygen availability in the central liver of *Z. viviparus* during stressful cold or heat exposure.

An important difference between cold and heat stress was discovered through calculations of the glutathione based redox potential (ΔE). Whereas ΔE was generally more reduced upon cold exposure, it was more oxidised after critical (22°C) or extreme (26°C) heat stress and unchanged after exposure to 18°C, i.e. within the pejus range. This was consistent with significantly increased HIF-1 DNA binding upon cold and 18°C exposure, but only weak EMSA HIF-1 signals upon higher heat stress of 22 or 26°C (fig. 4.A, B in publ. IV). It has been shown, that although the HIF response is mainly controlled by oxygen-dependent hydroxylation of prolines and asparagine, some steps of the HIF response can be modulated by ROS and are thus sensitive to redox changes (Ema et al. 1999, Haddad et al. 2000, Lando et al. 2000, Liu et al. 2004). Statistical analysis indeed confirmed the general trend for higher HIF-1 DNA binding activity in *Z. viviparus* liver tissue at a more reduced cellular redox potential leading to the conjecture that ROS may interfere with HIF-1 signalling during stressful warming. Nikinmaa et al. (2004) have also

shown enhanced DNA binding activity of rainbow trout HIF-1 under reduced redox conditions, which they attributed to a cysteine residue at position 25 in rainbow trout HIF-1α, which is Ser28 in the human sequence conferring redox-independence to DNA binding of human HIF-1 (Lando et al. 2000). Similar to the human sequence, serine was identified at the respective position in *Zv*HIF-1α (fig. 4.4 in "additional results"). It might be that increased HIF-1 DNA binding together with more reduced conditions represents a secondary event and that the primary redox regulated step of the HIF response is brought forth during transactivation (Lando et al. 2000, Evans et al. 2000).

Taken together, short-term cold and heat stress had opposing impact on the cellular redox balance, with cold stress causing a more reduced, and heat stress a more oxidised cellular redox environment. The more oxidised conditions seemed to interfere with the HIF response and might thus prevent the expression of hypoxia inducible genes under heat stress. In other words, functional hypoxia under critical heat stress will not be counterbalanced by adjustments of the oxygen delivery system. The observed accumulation of anaerobic metabolites in *Z. viviparus* liver and muscle tissue beyond the Tc (Van Dijk et al. 1999) may not require HIF-1 induced up-regulation of glycolytic enzymes (Semenza et al. 1994, 1996). Also, other transcription factors such as Sp1 and Sp3 could be responsible for the temperature mediated expression of glycolytic enzymes as shown for β-enolase and pyruvate kinase M in mammalian muscle cells subjected to hypoxia (Discher et al. 1998).

By contrast, cold stress as well as sub-critical heat exposure (18°C) in the pejus range probably allow for hypoxic signalling. Thus, HIF-1 might be a physiological regulator counterbalancing early onset of functional hypoxia at the border of the optimum temperature range and in the warm pejus range, but appears impaired at critically high temperatures presumably because the redox sensitive signalling pathway is compromised by enhanced oxidative stress.

5.5. What is the inducing effect for the HIF response?

-thermal oxygen limitation or temperature itself or both-

In the previous section, temperature stress / recovery was interpreted to be reminiscent of temperature-induced hypoxia / reoxygenation. HIF-1 was seen as a physiological regulator during functional hypoxia in the cold and in the warm pejus temperature range. Along that line of argumentation cold stress may resemble much more the "normal" hypoxia response than the response to heat stress in fish. However, it has to be taken in mind that Atlantic

cod so far is the only fish species where a cold-induced decrease in blood PO_2 indicating functional hypoxia has directly been demonstrated (Lannig et al. 2004). Although similar effects are likely for *Z. viviparus*, so far only heat-induced functional hypoxia has been confirmed (Zakhartsev et al. 2003, Pörtner et al. 2004). Therefore, I will investigate a different explanation which may act independently or in addition to temperature-induced hypoxia, and considers temperature itself as factor influencing HIF regulation. Few authors have investigated the possible relationship of HIF and changing temperatures. HIF-1 proved necessary for heat acclimation of the nematode *C. elegans* (Treinin et al. 2003). Moreover, *in vivo* experiments with mice demonstrated that an increase in HSP90 protein upon heating might stabilise HIF-1α even under normoxic conditions (Minet et al. 1999, Katschinski et al. 2002). Likewise, increased HIF-1 DNA binding was observed with a decrease in acclimation temperature from 26 to 18 and 8°C in crucian carp, which was associated with increased levels of HSP70 and 90 (E. Rissanen, H. Numminen, M. Nikinmaa; unpublished results). It seems that normoxic HIF-1 functions might be mediated by its interactions with heat shock proteins. In fish, this might be favoured in the cold. Whether this applies to *Z. viviparus* remains unclear, as HSP90 protein levels were not determined and HSP70 protein levels did not show any temperature-dependent changes (own unpublished data). Similarly to *Z. viviparus*, considerable normoxic HIF-1α protein levels were observed in crucian carp with high inter-individual differences (E. Rissanen, H. Numminen, M. Nikinmaa; unpublished results). It has been argued by Nikinmaa & Rees (2005) that those findings together with the apparent high importance of the cellular redox state suggest that HIF-1α may have oxygen-independent roles in the physiology of fishes, with the temperature effect being one of those normoxic roles. Thus, the investigation of HSP-HIF interaction in normoxic fish warrants further efforts.

A recent hypothesis proposed a cross-tolerance effect of heat acclimation and perturbation of oxygen supply and demand, and probably other environmental stressors by shared similar protective pathways where HIF-1 seemed to be an essential modulator (Horowitz 2001, Horowitz et al. 2004). Thus, at least in the cold, onset of thermal oxygen limitation and the temperature itself may go hand in hand to induce the HIF response in liver of temperature stressed *Z. viviparus*.

6. Conclusions & Perspectives

The aim of my thesis was to evaluate the *in vivo* importance of oxidative stress and the hypoxic response induced by the transcription factor HIF-1 in liver of polar and temperate marine fish. In a second approach oxidative stress and HIF-1 were investigated in liver of the temperate species (*Zoarces viviparus*) exposed to acute heat and cold stress ranging from pejus to critical and above critical conditions, suspected to induce various degrees of functional hypoxia. I will now answer the questions addressed in the introduction based on the findings from my doctoral studies.

1. The Antarctic eelpout (*Pachycara brachycephalum*) appeared more prone to oxidative stress under regular unstressed field conditions compared to the temperate con-familial *Zoarces viviparus*. This may be the reason why this polar fish has such a high glutathione redox buffering capacity.

Higher lipid radical formation in liver microsomes from the Antarctic eelpout *Pachycara brachycephalum* compared to the temperate eelpout *Zoarces viviparus* (German North Sea), speak for a higher susceptibility of polar ectotherms to lipid peroxidation and may relate to the higher content of unsaturated fatty acids in their cellular lipid fraction. However, in *P. brachycephalum*, this high risk for oxidative damage seems effectively counterbalanced, as deduced from oxidative stress parameters. Particularly, the redox buffer glutathione appeared to charge the liver tissue of the polar *P. brachycephalum* with a very high antioxidant capacity. Interestingly, glutathione was found oxidised to over 50% in both, control (0°C) and warm acclimated (5°C) *P. brachycephalum*. On the one hand, such a high degree of glutathione oxidation underlines the elevated risk for oxidative stress and the importance of glutathione as antioxidant. On the other hand, this observation may also suggest that physiological processes adapted to function at low temperatures operate in a more oxidised cellular redox environment in this polar fish. Comparative studies will demonstrate whether a redox shift applies also for other cold-adapted species.

Whereas warm acclimation to 5°C led to only slight changes of oxidative damage markers indicating a minor impact of varying water temperatures on oxidative stress levels in the polar *P. brachycephalum,* seasonal temperature changes within the natural temperature range caused different oxidative stress levels in the temperate species: Oxidative damage marker were increased in cold winter *Z. viviparus* (6°C) compared to

summer animals (12°C). Thus, both, cold adaptation of the polar eelpout *P. brachycephalum,* as well as seasonal cold acclimatisation of the temperate eelpout *Z. viviparus,* was associated with enhanced potential for elevated tissue oxidation, i.e. higher oxidative stress levels.

2. The higher levels of tissue oxidation together with the high oxygen solubility at low environmental temperatures may be the reason for differences in HIF-α sequences and HIF responses compared with mammals, to ensure oxygen-dependent gene expression in polar fishes.

The constitutive expression of HIF-α sequences coding for the hypoxia-inducible subunit of HIF, in all four investigated polar species (*P. brachycephalum, T. hansoni, T. pennellii, C. myersi*) illustrates that the transcription factor HIF may have a basic physiological function in fish. Whether the physiological function is the same as in mammals remains to be shown. Future studies will show whether notothenioids exhibit a hypoxic HIF response and what target genes will be induced in polar fishes, which commonly do not encounter oxygen limitation or heat stress within their natural environment. However, constitutive HIF-1α protein levels in *Z. viviparus* support the idea that the function of the protein or the regulatory mechanism has undergone an evolutionary change from fish to mammals.

Generally, protein cold adaptation may be supported by several amino acid substitutions located in the ODD and C-TAD of the deduced HIF-1α peptide sequences which might confer higher hydrophilicity. Moreover, several features in the deduced amino acid sequence, such as a Glu-Gln cluster in HIF-1α (in all five studied species) and a Glu cluster in HIF-2α (in *T. hansoni, C. myersi*) may be indicative of special adaptations of the HIF response in the investigated fishes. Additional cysteine residues may enhance redox-sensitivity to HIF-1α stabilisation or transactivation, similar the rainbow trout HIF-1α protein (Soitamo et al. 2001, Nikinmaa et al. 2004). Future studies should scrutinise the possible modifications of redox-sensitivity and also investigate whether this is a special feature of oxygen-dependent gene expression in fish and especially in polar species. The highly surprising finding of the substitution of the N-terminal functional proline by leucine in *P. brachycephalum* HIF-1α is so far the only wild-type sequence, where this phenotype has been described. The three notothenioid HIF-1α sequences demonstrated both proline residues, so that this Pro-Leu substitution cannot plausibly be considered a specific polar adaptation. It is intriguing to think that *Pb*HIF-1 function may be regulated mainly via

transactivation and not so much via degradation / stabilisation. Comparative studies of *Zv* and *Pb*HIF-1α functionality could reveal the effect of this substitution on HIF-1α protein stability and address the question what might be the important regulatory step of the HIF response in polar eelpout.

Higher HIF-1 DNA binding activity in *P. brachycephalum* was observed in warm acclimated to 5°C compared to control fish kept at 0°C, and also in winter acclimatised (6°C) *Z. viviparus versus* summer animals (12°C). In both species HIF-mediated metabolic reorganisation may counterbalance the temperature-induced onset of tissue hypoxia. Thus, it may be speculated that HIF-1 plays a common regulatory role to adjust tissue oxygenation upon seasonal acclimatisation within the natural temperature range (*Z. viviparus*), and at the warm border of the thermal optimum range (*P. brachycephalum*), respectively. Again, it remains to be shown, whether commonly known target genes such as VEGF or EPO are induced as part of the response to long-term (seasonal and acclimatory) changes of ambient water temperatures or whether HIF may have a different function in this context. Moreover, increased HIF-1 binding occurred at a more oxidised redox environment in winter *Z. viviparus* and an unchanged, but basically highly oxidised redox environment in warm acclimated *P. brachycephalum*. Thus, higher basal oxidative stress levels during the colder winter season did apparently not interfere with hypoxic signalling in *Z. viviparus*. Respectively, in *P. brachycephalum*, HIF-1 signalling may indeed be adapted to function in a more oxidised redox environment.

3. Short-term temperature stress / recovery resembled ischemia / reperfusion in mammals. Cold and heat stress had opposing effects on oxidative stress levels, the cellular redox potential and the HIF response in the temperate eelpout *Zoarces viviparus*.

In contrast to seasonal acclimatisation to tolerated winter cold (6°C), short-term (2 h) cold stress of 1 or 5°C had different effects on oxidative stress parameters in *Z. viviparus*. Oxidative damage markers were increased following 24 h recovery from cold stress, but not directly after cold stress itself which, moreover, was characterised by a more reduced cellular redox environment. Higher HIF-1 DNA binding activity indicated onset of hypoxic signalling following exposure to 1°C. Together, cold stress and subsequent recovery at control temperature represent a similar biochemical situation as ischemia / reperfusion in mammals.

Elevated levels of oxidative damage markers following recovery from heat stress were observed only when exposing the heat stressed *Z. viviparus* to shorter recovery periods of 8 - 12 h, indicating more rapid repair of oxidative damage following heat than following cold stress. Whereas acute cold exposure led to a more reduced cellular redox environment and higher HIF-1 DNA binding, heat stress of 22 or 26°C caused more oxidised redox conditions and only weak HIF-1 signals in EMSA assays (fig. 6.1). After less stressful warming to 18°C stronger HIF-1 signals were observed with the redox environment unchanged.

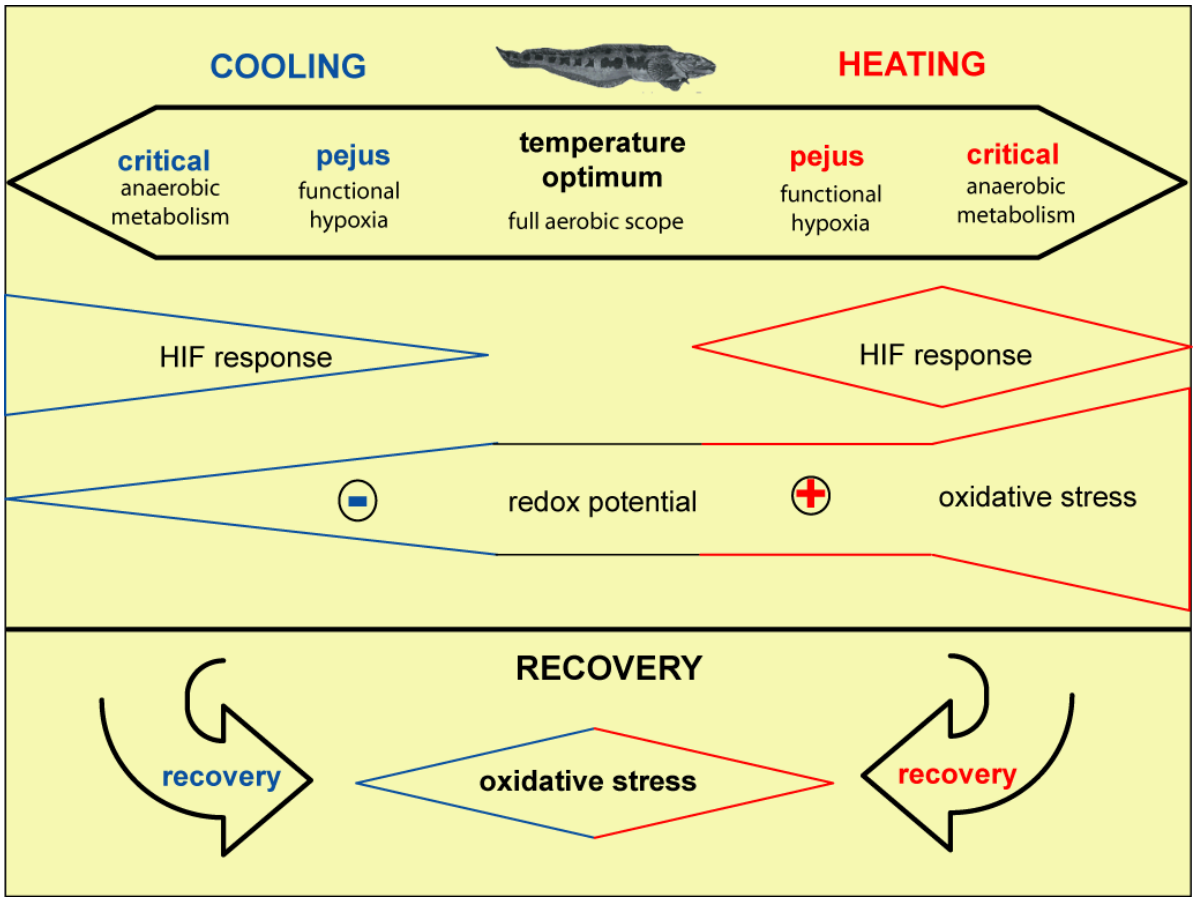

Fig. 6.1: Response of *Zoarces viviparus* to acute temperature stress (2 h). HIF-1 might have a modulating function in the liver during acute cold exposure and during warming within the pejus temperature range, but appeared impaired at critically high temperatures. The cellular redox potential in the liver was more reduced during cold stress and more oxidised above critical temperatures. Moreover, heat stress was accompanied by increasing oxidative stress.
The lower part of the graphic shows increasing oxidative stress in the liver during recovery following cold or following heat stress. Together, these results support the hypothesis of temperature induced hypoxia and recovery in the eelpout *Z. viviparus* being reminiscent to ischemia / reperfusion events in endothermal mammals.

Likewise, a general trend for higher HIF-1 DNA binding at more reduced cellular redox environment was observed in *Z. viviparus*. Together, HIF may be a physiological regulator in fish counterbalancing early functional hypoxia during acute cooling and in the warm

pejus range, but appears impaired at critically high temperatures due to the adverse effects of increasing oxidative stress (fig. 6.1). Furthermore, HIF-1 in combination with the cellular redox environment appeared to be sensitive markers for temperature-induced hypoxia upon acute stress in tissues.

The discrepancy between the cellular redox potentials during long-term (seasonal) and short-term (acute stress) cold exposure of *Z. viviparus*, in both cases associated with higher HIF-1 DNA binding stays open to speculation. It may indicate a different physiological function for HIF-1 during seasonal acclimatisation within the benevolent temperature range and during acute temperature stress. Thus, different target genes may be induced during long- and short-term changes of environmental temperatures, and the redox potential could be the regulating factor for that.

Continuous ROS formation by isolated *Zoarces viviparus* hepatocytes could be visualised by confocal microscopy using the fluorescent dye H_2DCFDA. However, the type of the generated reactive oxygen species and also the cellular source remain obscure. Therefore, detailed studies have to be carried out using different radical quencher and inhibitors of various ROS sources to determine the origin of ROS, e.g. mitochondria or cytosolic enzymes, under temperature-induced hypoxia. Moreover, those studies require further evaluation of the method for quantitative measurements of cellular ROS production, e.g. under different experimental temperatures and oxygen partial pressures. Using inhibitors of the NO synthase and artificial NO donors, the role of NO could be investigated in fish hepatocytes. Together, cellular studies should analyse the clear interdependence of HIF-1 and ROS (redox potential) as well as NO during temperature-induced *versus* environmental hypoxia.

Collectively, the proposed future studies could extend the presented *in vivo* findings and scrutinise the molecular mechanisms controlling the counterbalancing response during temperature-induced hypoxia.

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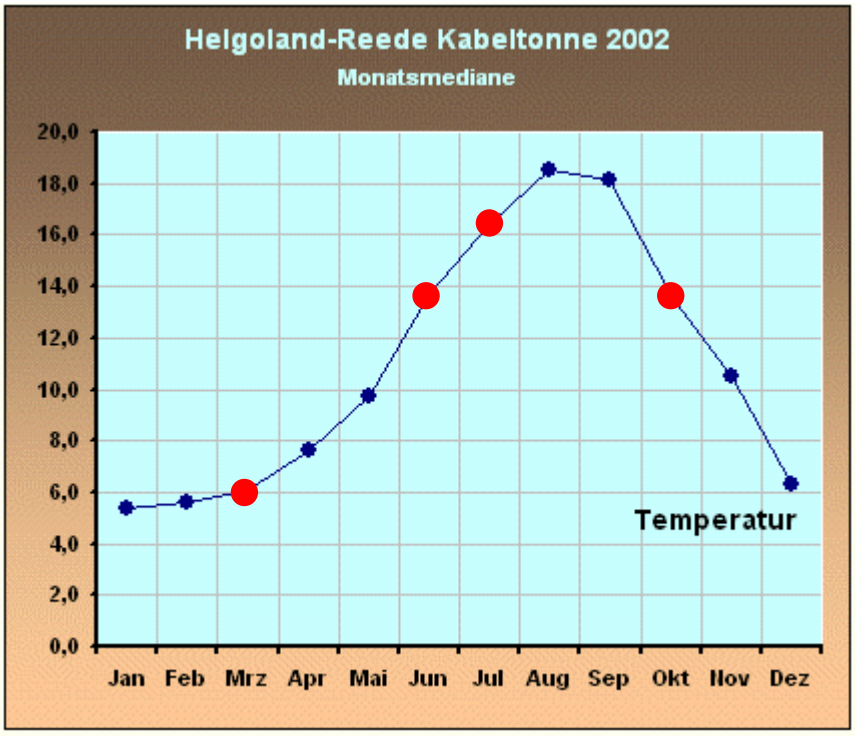
8. Appendix

Temperature regime Helgoland

A



B

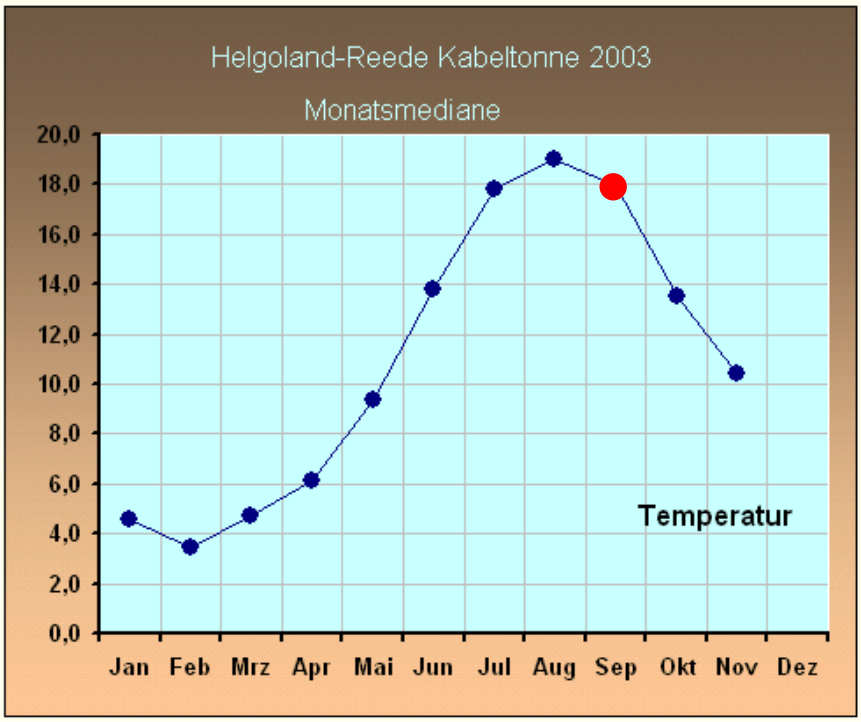


Fig 8.1: Median monthly sea surface temperatures next to Helgoland, sampling area "Helgoland-Reede Kabeltonne" of the years 2002 (A) and 2003 (B). Red dots indicate sampling of *Z. viviparus* used for the present work. Figures are taken from MURSYS environmental reports of the BSH (Bundesamt für Seeschiffahrt und Hydrographie; http://www.bsh.de/de/Meeresdaten/Beobachtungen/MURSYS-Umweltreportsystem/).

Global distribution of investigated fish species

Distribution of the respective species is indicated by red dots. All maps are taken from OBIS, http://www.iobis.org/Welcome.htm.

Zoarcids

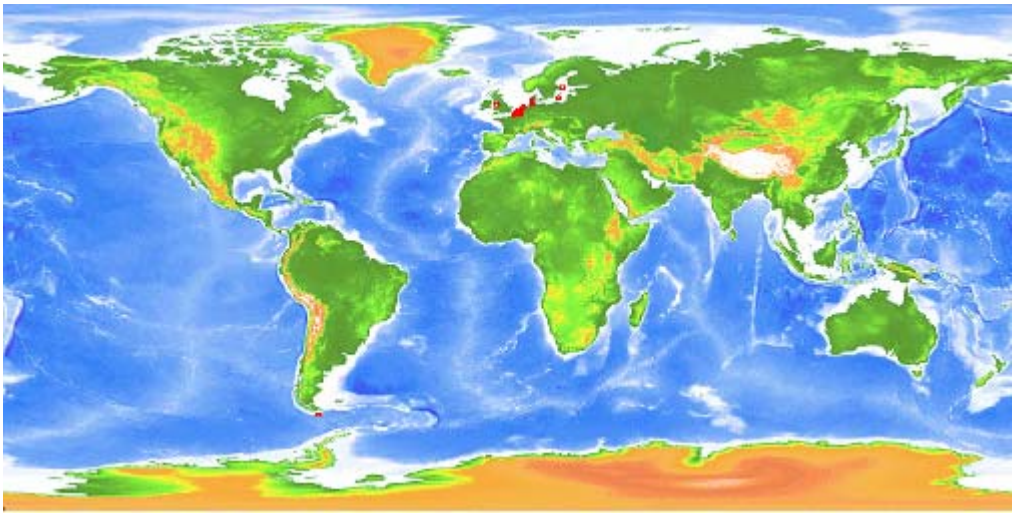


Fig. 8.2: Global distribution of *Zoarces viviparus*

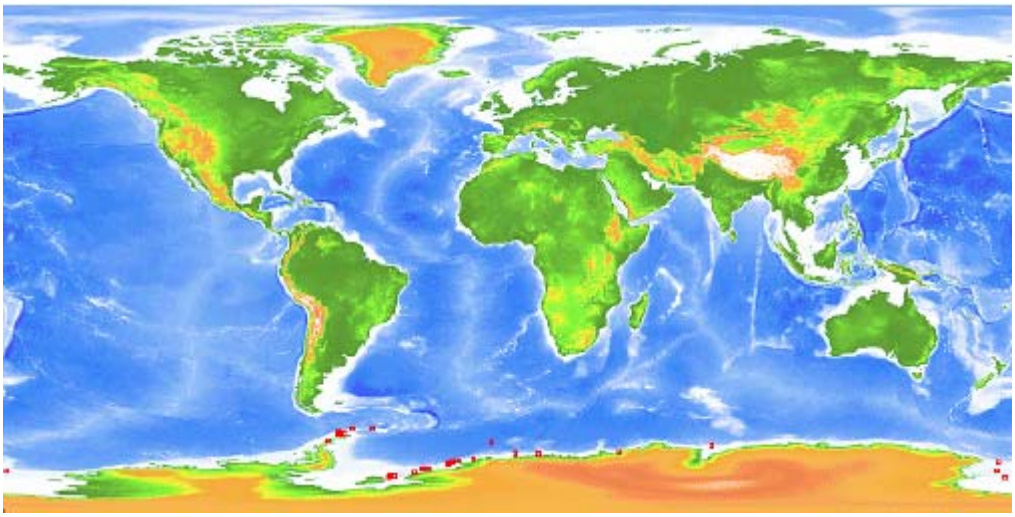


Fig 8.3: Global distribution of *Pachycara brachycephalum*

Notothenioids

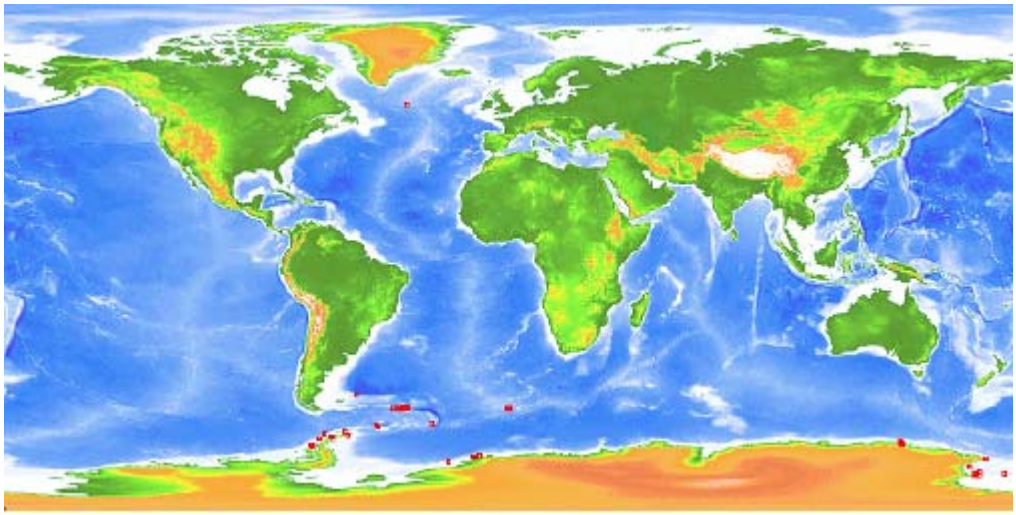


Fig. 8.4: Global distribution of *Trematomus hansoni*

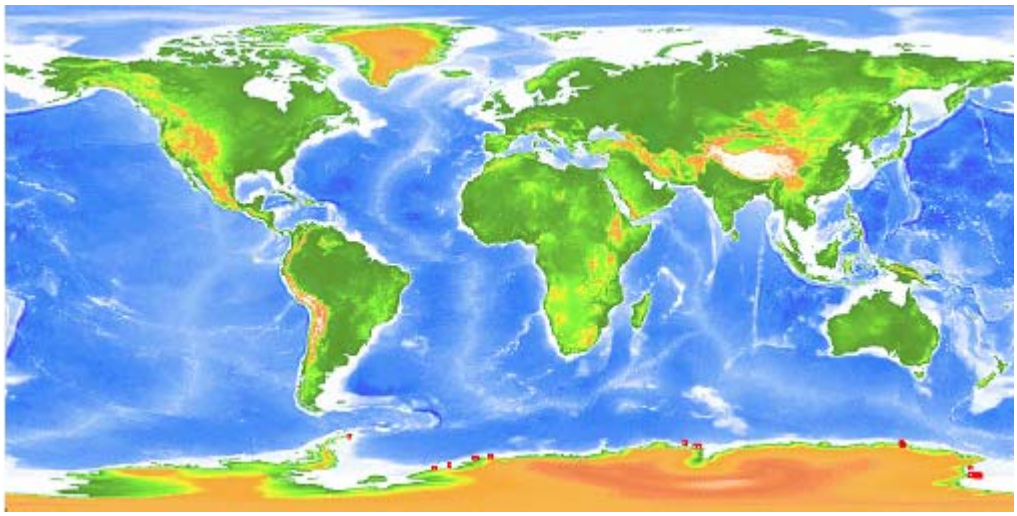


Fig. 8.5: Global distribution of *Trematomus pennellii*

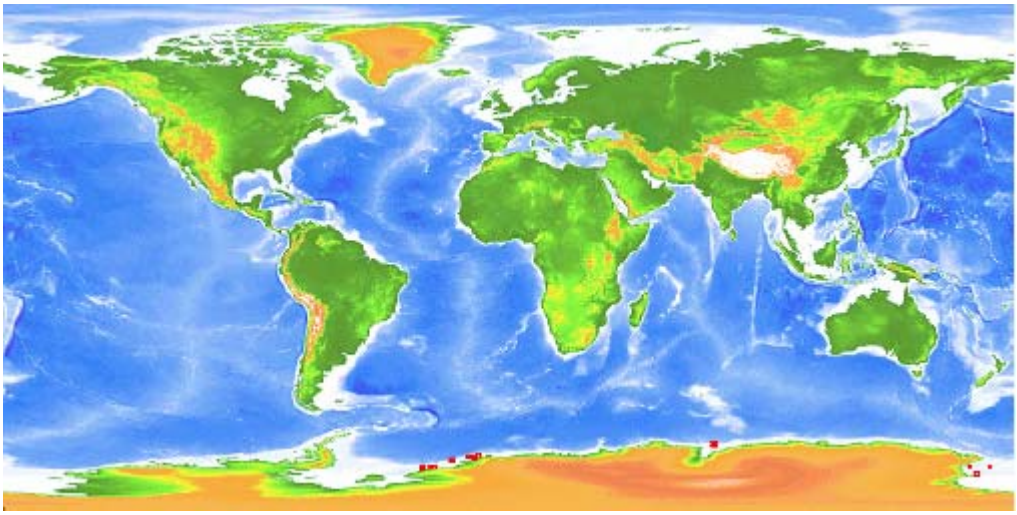


Fig. 8.6: Global distribution of *Chionodraco myersi*

tBOOH-initiated chemiluminescence

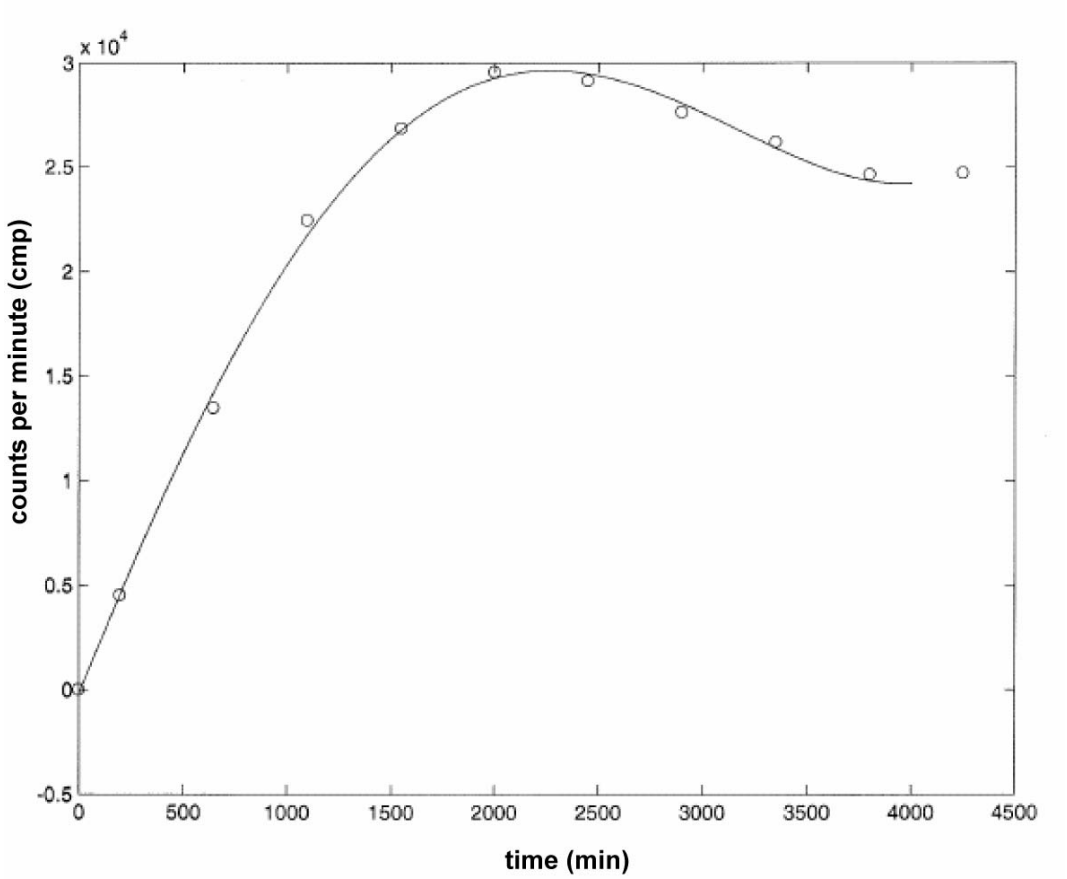


Fig. 8.7: Characteristic curve obtained from tBOOH-initiated chemiluminescence. The area under the curve could be calculated for a time period of 4 000 sec (= 66.67 min) using MatLab 7.0 program (x = min (t) : 1 max (4 000); C = polyfit (t, y, 4); Y = polyval (C, X); area = sum (Y); plot (t, Y, 'ro', X, Y).

Tab. 8.1: Information on the fishes used in this thesis work. * Age determination of *P. brachycephalum* (*Pb*) was done according to Brodte et al. (ms submitted); age determination of *Z. viviparus* (*Zv*) according to E. Brodte (unpublished results).

species	location	coordinates	depth (m)	sampling date	trap	start of acclimation	experimentation / tissue sampling date	body length (cm)	body weight (g)	age* (years)	experiment	publi-cation
Zoarces viviparus	Helgoland; North Sea	54°11'N 7°52' E	~ 10	Mar. / April 2002	bottom trap	02.05.2002 (10°C)	14.05-15.06.2002 15.09.-14.10.2002	25.4 ± 1.8	52.7 ± 12.6	means: >4.5 min: 3 max: > 4.5	hepatocyte studies	-
				May / June 2002		03.06.02 (12°C)	04.07.- 05.07.2002	22.8 ± 3.5	54.4 ± 31.1	means: 3-4 min: 1 max: > 4.5	seasonal comp; comparison to *Pb*	I
							04.07.- -26.07.2002	23.3±3.6	60.5±34.7	means: 3-4 min: 1 max: > 4.5	acute cold stress, acute heat stress	III IV
				09.-17.07.02		July 2002 (12°C)	29.07 – 09-08.2002	21.5±2.6	40.2±20.8	means: 2-3 min: 1 max: > 4.5	acute heat stress	IV
				Oct. 2002		Oct. 2002 (6°C)	11.02. – 12.02.2002	23.7±1.6	49.9±15.4	means: 4-5 min: 2 max: > 4.5	seasonal comp; comparison to *Pb*	I
				Sep. 2003		21.11.03 (10°C)	03.12.-07.12.2003	23.6 ± 2.7	504. ± 22.2	means: 4 min: 1.5 max: > 4.5	recovery time series	III
Pachycara brachycephalum	King George Island (Maxwell Bay)	62°14' S 58° 40' W	400	ANT XIX/5; April 2002	releaser trap	June 2002 (0°C)	03.03.-04.03.2003	23.4±3.7	43.4±12.4	means: 7 min: 4 max: 11	control acclimation; comparison to *Zv*	I
						18.12.02 (5°C)		24.5±2.2	48.4±10.6	means: 8-9 min: 6-7 max: 11	warm acclimation to 5°C; comparison to *Zv*	I
				ANT XVII/3 May 2000		-	19.09./16.10.01	-	-	-	HIF-1α sequencing	II
Trematomus hansoni	Weddell Sea	70°56.57 S 10°33.02 W	300 – 333	ANT XXI/2; Dec. 2003	bottom trawl	-	25.12.03	-	-	-	HIF-1α sequencing	II
Trematomus pennellii	Weddell Sea	70°56.57 S 10°33.02 W	300 – 333	ANT XXI/2; Dec. 2003	bottom trawl-	-	25.12.03	-	-	-	HIF-1α sequencing	II
Chionodraco myersi	Weddell Sea	70°56.57 S 10°33.02 W	300 – 333	ANT XXI/2; Dec. 2003	bottom trawl	-	25.12.03				HIF-1α sequencing	II

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Bremerhaven, den 14. September 2005

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Erklärung gemäß § 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.

_____

Katja Heise